

Original Article

DEVELOPMENT OF CREAM CONTAINING NANOSTRUCTURED LIPID CARRIERS LOADED
MARIGOLD (*TAGETES ERECTA* LINN) FLOWERS EXTRACT FOR ANTI-WRINKLES
APPLICATION

PIMPORN LEELAPORNPISID*, SUNEE CHANSAKAOW, SUVISA NA-BOONLONG AND PENSACK JANTRAWUT

Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, 50200, Thailand

Email: pim_leela@hotmail.com,

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ABSTRACT

Objective: In this study, Marigold flower extracts were entrapped in nanostructured lipid carriers (NLCs), incorporated into cream base to obtain Marigold nano-cosmeceutical cream and tested for anti-wrinkles capability.

Methods: The ethyl acetate extract (EA) and semi-purified fraction (F₉) with high antioxidant activity and total phenolic content were selected for loading in the stable NLCs. Then their characterization, antioxidant activity and stability test were investigated and also the determination for skin irritation as well as anti-wrinkles capability in healthy volunteers.

Results: The results demonstrated the particle size of the selected ME-NLCs was in range 160 to 220 nm and showed good physical stability at 90 days after preparation. All of ME-NLCs containing creams exhibited no skin irritation in healthy volunteers. The wrinkles parameters evaluated by Skin Visiometer SV600[®] in 25 healthy volunteers after using creams containing ME-NLCs were significantly ($p < 0.05$) reduced when compared with before treatment and cream containing extract unloaded in NLCs.

Conclusion: The developed nano-cosmeceutical creams of Marigold flower extract from this study could be regarded as the effective anti-wrinkles formulation.

Keywords: Nanostructured lipid carriers, Marigold flower extracts, *Tagetes erecta* L., Antioxidant activity, Anti-wrinkles cosmetic.

INTRODUCTION

Nanostructured lipid carriers (NLCs) is the second generation of lipid nanoparticles developed after the first generation; solid lipid nanoparticles (SLNs) [1,2]. NLCs are distinguishable from SLNs by the composition of solid matrix. SLNs consist of only solid lipids while NLCs contained the blend of solid and liquid lipid. NLCs are more advantages than SLNs because they present a less ordered lipid matrix which may provide higher loading capacity and they also minimize or avoids some potential problems associated with SLNs such as drug expulsion during storage, low drug loading and high water content of SLNs dispersion [2,3]. The lipid nanoparticles were found to be advantages in preventing the degradation and improving the skin penetration of the loaded active compounds.

Skin aging is a progressive deterioration of physiological functions of skin resulting in undesirable appearances. There are several theories that related to skin aging and the most mentioned is free radical theory [4,5]. Free radicals or reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydroxyl ($\cdot OH$), peroxy (RO_2^{\cdot}) and alkoxy (RO^{\cdot}) attack our cell membranes and the accumulated radicals slow down cellular function, therefore reducing the body's self-repair capabilities that leading to many skin aging appearances including wrinkles, sagging and age spots. Wrinkles are the important sign of skin aging that everyone does not desire. Antioxidative agents are believed to play a role in the prevention of cells from oxidative stress by scavenging these free radicals and discontinue the lipid peroxidation chain reaction [6,7]. Thus, many anti-wrinkles or anti-aging products with antioxidative agent as active compounds are launched into cosmeceutical markets. Moreover, the natural substances are progressively expanded in cosmeceutical products because they are believed to be safer than synthetic ingredients and environmental friendly. Thai plants are also increasingly interested to be researched and developed for pharmaceutical/cosmetic applications. Marigold (*Tagetes erecta* Linn.), the common well known plants in family Compositae, was selected for determining the active constituents for anti-wrinkles product. Its antioxidative property was reported in many studies

[8,9] as well as our previous study [10]. It is one of the widely and easily cultivate plants in Thailand. Therefore, this research was emphasized to develop the nanostructured lipid carriers (NLCs) containing marigold extracts and formulate as nano-cosmeceutical products with antioxidant and anti-wrinkles properties as well.

MATERIALS AND METHODS

Materials

The ethyl acetate (EA) extract and semi-purified fraction (F₉) from Marigold flowers were supported by Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University, Thailand. Both extracts were selected from all fractions due to their highest antioxidant activity tested by 2, 2-Diphenyl-1-picryl hydrazyl (DPPH) and thiobarbituric acid-reactive substance (TBARS) assays [10].

Formulation, characterization and stability test of marigold extract loaded nanostructured lipid carriers (ME-NLCs)

Formulation of marigold extract loaded nanostructured lipid carriers (ME-NLCs)

The unloaded NLCs formulations with 10% of lipid phase and 5-15% of surfactant were prepared. The formulations consisted of glyceryl monostearate (GMS) and stearic acid (SA) as solid lipid and octyl dodecanol (OD) as liquid lipid. The effect of solid: liquid lipid ratio on characteristic of NLCs was also observed in each group of formulations by varying it into 2 ratios of solid: liquid lipid; 1:2 and 2:1. A lipid phase which contains the blend of solid lipids, liquid lipids and lipophilic surfactant was melted at about 70°C. Then the hot aqueous phase containing hydrophilic surfactant was gradually dispersed into the hot lipid phase using high speed homogenizer (Yellow line DI 25 basic, IKA Werke GmbH & Co.KG Germany) at 8,000 rpm for 5 min. The total surfactants content used in each formulation is between 5-15%w/w. Then the pre-emulsion was processed at 800 bar, 70°C for 5 cycles using a high pressure homogenizer (AVESTIN C3, Malvern, Canada). A certain amount of the marigold extract (EA or F₉) was mixed with the melted lipids

phase. Then, the obtained hot lipid dispersion was cooled down to room temperature while stirring with magnetic stirrer. The lipids recrystallized and obtained the nanostructured lipid carriers (ME-NLCs).

Marigold extracts loaded nanostructured lipid carriers (ME-NLCs) characterization

The average particle size (Z-average size), size distribution and zeta potential were evaluated by a Zetasizer ZS (Malvern Instruments Ltd., Malvern, UK). The experimental measurements were repeated 3 times for each sample. Before the measurement, the NLCs dispersions were diluted with deionized water (initial NLCs dispersion: water = 1:1,000) to obtain the suitable concentration for the measurement.

Stability studies of marigold extract loaded nanostructured lipid carriers (ME-NLCs)

The stability test of ME-NLCs was investigated in four conditions including room temperature, 4°C and 45 °C for 3 months and 6 cycles of heating-cooling cycling method which defined as alternation of storage conditions from 45°C for 48 h to 4°C for another 48 h. At the predetermine times; 30, 60 and 90 days after the test, each sample was investigated for the particles size, polydispersity index (PDI), zeta potential and physical properties.

Formulation, characterization, stability test and antioxidant activity of marigold extract loaded nanostructured lipid carrier cream (ME-NLCs cream)

Formulation of ME-NLCs cream

Cream bases containing various oily materials such as stearyl alcohol, stearic acid, glyceryl monostearate and mineral oil in the concentration of 19-26%, Cetareth 25, Tween, Span or triethanolamine stearate as surfactants in concentration of 1-6% and propylene glycol as humectants were prepared using conventional hot process. After homogenous cream bases were obtained, then characterized for their physical properties and stability. Finally, the most stable cream base was chosen for incorporating with the selected ME-NLCs formulations at 15 and 30% concentrations. The creams containing an equally amount of Marigold extract (EA or F₉) were also prepared as EA and F₉ creams.

Determination of antioxidant activities

2, 2-Diphenyl-1-picryl hydrazyl (DPPH) radical scavenging method

The antioxidant activity of the cream containing ME (EA or F₉) and ME-NLCs was determined by DPPH radical scavenging assay [11]. Briefly, the EA or F₉ was extracted from the formulation by absolute ethanol (99%) and centrifuged at 10,000 rpm for 30 min. The supernatant of the test sample was added into a 96-well microplate with 20 µl and DPPH in ethanol 180 µl was also added. The reaction mixture was kept in dark at 25°C for 30 min. The absorbance was then measured at 520 nm with microplate reader (DTX-880 multimode detector, Beckman Coulter Inc., USA). The experiments were done in triplicates. This was tested to compare between at before and after storage in various conditions for 3 months as mentioned before. The percentage of inhibition was calculated according to the equation: percentage of inhibition = $[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$, Where A_{control} is the absorbance of the control reaction and A_{sample} is the absorbance of the sample.

Determination of antioxidant activity with thiobarbituric acid-reactive substance (TBARS) method

A modified TBARS assay was used to measure the antioxidant activity of the cream containing ME-NLCs as well as ME (EA or F₉) in term of inhibition on lipid peroxidation. Liposome suspension, consisting of cholesterol, phosphatidylcholine and 0.2 M potassium phosphate buffer (pH 7.2) was prepared. The marigold extracts in ethanol was mixed with 0.07M AAPH. The resulting mixture was incubated at 50°C for 24 h. After incubation, the solution was mixed with 0.2% BHT, 3% Triton-X, 20% acetic acid and 0.6% TBA,

respectively. Then the mixture was heated for 30 min at 90°C and cooled to room temperature. The absorbance of the mixture was measured spectrophotometrically at 540 nm with a microplate reader. The percentage inhibition was calculated same as in DPPH assay.

Clinical evaluation in human volunteers

The skin irritation test as well as efficacy testing protocols of this study were approved by the Committee on Human Rights Related to Human Experimentation of ChiangMai University. Before participating in the clinical study, all of volunteers received the information of this study and signed a written informed consent that contained all the basic elements outlined. Twenty-five Thai healthy volunteers aged 30-55 years were selected.

Skin irritation testing

Finn chamber® that contained the samples were attracted on the volunteers' back. After 48 h of application, the patches were removed and the test sites were cleaned suddenly with purified water. The skin irritations as erythema and edema were evaluated at 1, 24 and 48 h based on Draize scoring system [12].

Wrinkles reducing capacity test of marigold extract loaded nanostructured lipid carriers cream (ME-NLCs cream)

The same group of volunteers was also tested for wrinkles reducing capacity by applied the test creams twice daily, morning and evening, for 8 weeks. The study protocol included the evaluation at day 0 for initial value, 4 and 8 weeks after the treatment. Skin-Visiometer SV600 FW was used as device measuring four parameters as Ra, Rz, surface and volume values are refer to the difference of skin condition after the application.

Statistical analysis

Paired t-test was used to examine the changing in Ra, Rz, surface and volume values, before and after each treatment. The percentage efficiency values were evaluated by the following equation: % Efficiency value = $[(V_m - V_o) / V_o] \times 100$. Whereas V_o is the value at initial point (day 0) and V_m is the value at measuring point (4 and 8 weeks). The data were subjected to two way analysis of variance and the significance of the difference between means was determined by Duncan's multiple range test ($P < 0.05$) using SPSS for Windows.

RESULTS AND DISCUSSION

Characterization and stability test of marigold extract loaded nanostructured lipid carrier (ME-NLCs)

After preparation, the formulations inappropriate appearances (aggregation of particles, creaming or gelation) were excluded from the characterization study. The result found that formulations composed of solid lipids (GMS and SA): liquid lipid (OD) at 1:2 and 2:1 ratios showed the optimal particles in range of 125-175 nm, zeta potential in the range of 35-50 mV and acceptable appearance which were considered for further development of ME-NLCs. From our previous antioxidant activity study, the ethyl acetate extract (EA) and fraction 9 (F₉) were chosen to loaded in NLCs as active ingredient. The amounts of EA and F₉ in NLCs were calculated from the IC₅₀ value obtained by antioxidant assay For EA-NLCs, the concentration of surfactant (combining of Tween® and Span®) was varied from 5-12% and PEG400 was added in order to solubilize ME in liquid lipid [13]. Table 1 showed the results of particle size, PDI and zeta potential value of EA-NLCs dispersion after 1, 30, 60 and 90 days of storage at room temperature (RT). The incorporation of EA into NLCs resulted in the larger particle size while PDI and zeta potential did not affect. However the particle size of all EA-NLCs did not exceed 300 nm remained in nano-size. In general, particle aggregation is less likely to occur for charged particles with high zeta potential ($> |30| \text{ mV}$) due to electric repulsion. Though this rule cannot be strictly applied in the presence of Tween and Span; the steric stabilizer, which can decrease the zeta potential [14-16]. All of EA-NLCs were subjected to the stability test at three conditions; 4°C,

room temperature (RT) and H/C cycling for 6 cycles. The results revealed that EA-NLCs from solid: liquid lipid = 1:2 with 12%

surfactant was more stable than other formulations with no creaming, phase separation and particle aggregation.

Table 1: Mean particle size, polydispersity index (PDI) and zeta potential value of marigold extract loaded NLCs dispersion (ME-NLCs) after storage for 30, 60 and 90 days.

Formulations	Storage time (Day)											
	Initial (day 0)			30			60			90		
	Particle size (nm.)	PDI	Zeta potential (mV.)	Particle size (nm.)	PDI	Zeta potential (mV.)	Particle size (nm.)	PDI	Zeta potential (mV.)	Particle size (nm.)	PDI	Zeta potential (mV.)
1:2-S5-EA	175.3±3.7	0.223±0.030	-35.1	237.8±0.9	0.258±0.013	-33.3	282.1±10.8	0.347±0.016	-43.1	NA	NA	NA
1:2-S7-EA	151.0±2.6	0.189±0.16	-34.5	161.9±1.3	0.225±0.004	-42.1	176.3±10.6	0.294±0.067	-45.0	265.0±19.7	0.464±0.016	-45.1
1:2-S10-EA	157.3±1.7	0.167±0.016	-40.3	156.4±1.6	0.164±0.011	-45.1	156.8±0.45	0.183±0.009	-50.3	171.5±15.8	0.196±0.013	-49.5
1:2-S12-EA	126.3±1.5	0.131±0.016	-43.8	159.5±2.5	0.205±0.010	-41.1	177.8±1.0	0.149±0.020	-36.2	220.1±1.9	0.205±0.017	-38.4
1:2-S7-EA	144.5±4.0	0.122±0.019	-34.9	146.2±0.500	0.120±0.018	-41.9	148.1±2.8	0.178±0.006	-50.1	299.4±8.6	0.437±0.014	-46.0
2:1-S5-EA	153.6±1.5	0.129±0.013	-42.9	157.5±2.4	0.176±0.016	-39.0	154.0±2.3	0.202±0.019	-46.3	NA	NA	NA
2:1-S10-EA	146.8±0.8	0.115±0.004	-36.3	143.4±0.9	0.106±0.001	-43.6	162.3±2.7	0.751±0.018	-49.5	192.7±2.6	0.283±0.014	-47.5
2:1-S12-EA	136.0±0.1	0.117±0.020	-49.0	140.2±0.3	0.069±0.004	-42.4	176.5±0.7	0.091±0.011	-42.7	191.0±0.29	0.079±0.020	-42.2
1:2-S12-F_9	146.9±0.6	0.140±0.008	-38.1	154.7±2.6	0.187±0.031	-36.3	153.9±3.3	0.197±0.022	-40.8	160.2±2.3	0.222±0.015	-46.1
2:1-S12-F_9	149.8±0.9	0.216±0.011	-41.7	140.4±1.0	0.140±0.019	-40.2	141.1±1.0	0.177±0.030	-37.7	138.8±1.0	0.166±0.004	-41.7±

Note: EA = the most active ethyl acetate extract; F_9 = the most active fraction; 1:2 and 2:1 = ratios of solid: liquid lipid; S = surfactant concentration (%w/w) and NA = formulation instability

The solid: liquid lipid ratios which were 1:2 and 2:1 together with 12% of surfactant were selected to formulate the F_9-NLCs. The obtained F_9-NLCs had the particle sizes lower than 200 nm after 90 days. (160.2 and 138.8 nm). Both of them were stable after stability test at 4°C and RT conditions. From physical appearance (no creaming, separation or particle aggregation), particle size and stability evaluation were found that the solid: liquid lipid = 1:2 was more suitable for incorporating into cosmetic cream than 2:1 because of its higher viscosity. In conclusion, the 1:2 ratio of solid: liquid lipid and 12% surfactant was the best NLCs formulation for entrapping EA and F_9

Antioxidant activities of ME-NLCs creams

Fig. 1 and 2 showed the antioxidant activity (percentages of inhibition) of all prepared creams at 4°C and room temperature (RT) by DPPH and TBARS assays. The decreasing in percentages of inhibition during storage time at all conditions was observed in all tested creams. For F_9 formulations (Fig.1), the 30% F_9-NLCs cream showed the highest % inhibition among all F_9 tested creams. In addition, it showed the highest %inhibition value at 4°C and RT (with and without light) conditions for both methods and also significantly different from other conditions. At RT with light, %inhibition of ME-NLCs containing creams were higher than ME cream with significantly difference. This might say that the encapsulation of F_9 into NLCs can increase the stability of F_9 to light resulting in the higher %inhibition. For EA formulations (Fig. 2), using DPPH method, 0.045% EA cream showed higher %inhibition than 30% EA-NLCs containing cream at 4°C. For TBARS method at 4°C and RT (with and without light), the 30% EA-NLCs cream also revealed higher %inhibition than 0.045% EA cream. Form physical appearance, stability test and antioxidant activity results can concluded that creams containing 30% F_9-NLCs and 30% EA-NLCs creams were suitable for further experiments as skin irritation test in human volunteers and wrinkles reducing capacity test.

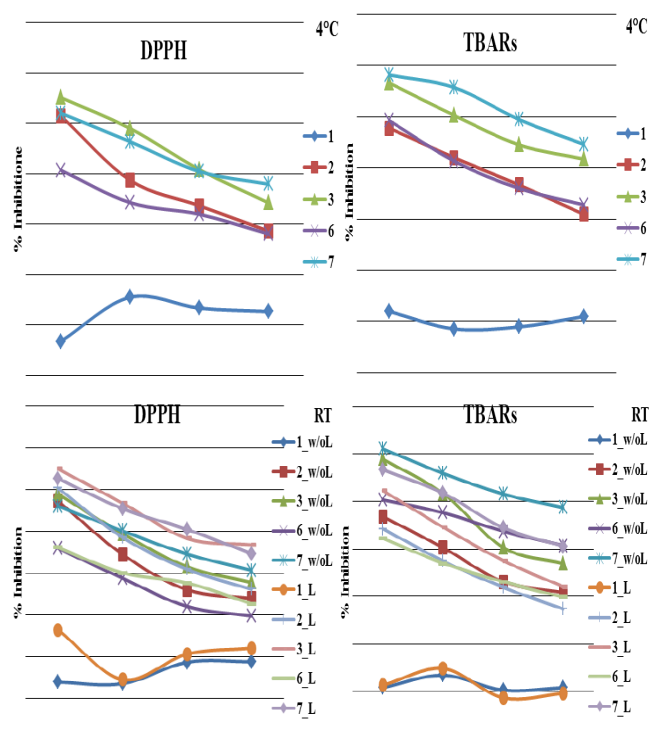


Fig. 1: The antioxidant activity (percentages of inhibition) of F_9 creams at 4°C and room temperature (RT) by DPPH and TBARS assays (1 = cream base, 2 = 0.009% F_9 cream, 3 = 0.018% F_9 cream, 6 = 15% F_9-NLCs cream, 7 = 30% F_9-NLCs cream, L = light and w/oL = without light)

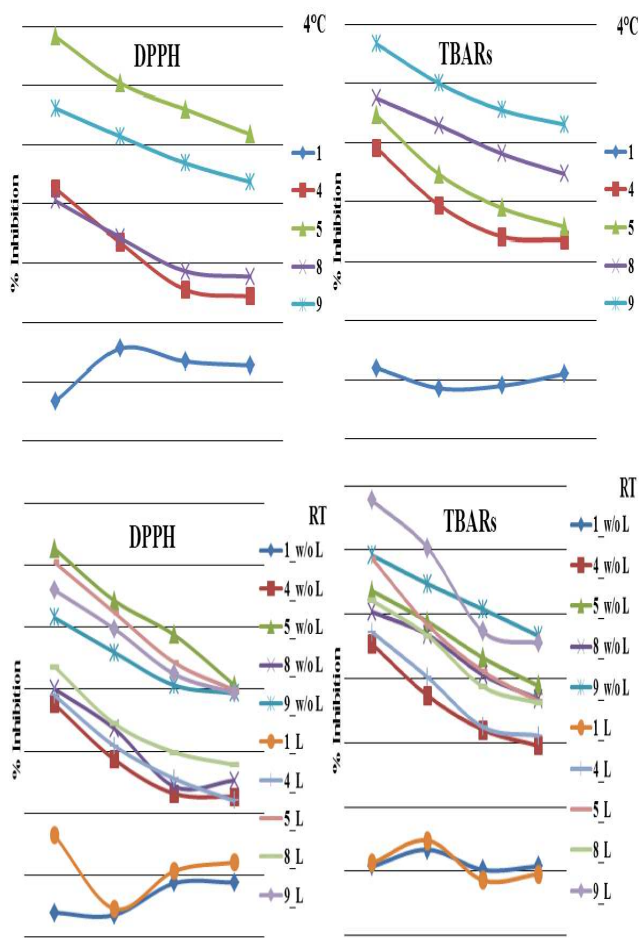


Fig 2: The antioxidant activity (percentages of inhibition) of EA creams at 4°C and room temperature (RT) by DPPH and TBARS assays (1 = cream base, 4 = 0.0225% EA cream, 5 = 0.045% EA cream, 8 = 15% EA-NLCs cream, 9 = 30% EA-NLCs cream, L = light and w/oL = without light)

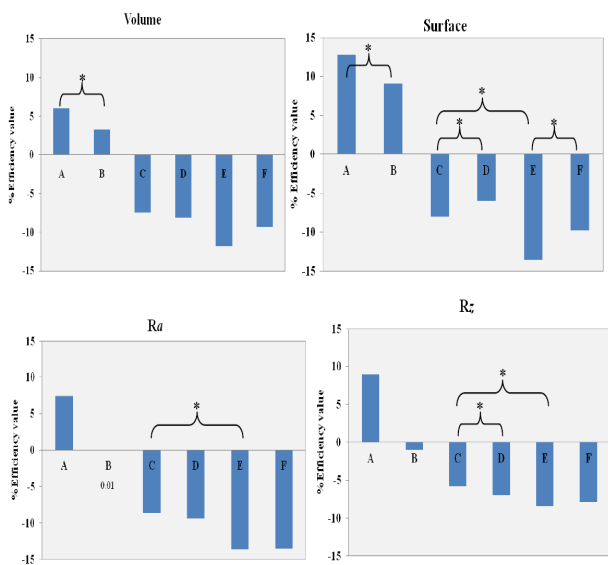


Fig 3: The percentages of efficiency value on volume, surface, Ra and Rz parameters after 8 weeks of treatment at p<0.05; A=untreated area, B=cream base, C= 0.018%F_9 Cream, D= 0.045%EA Cream, E= 30%F_9-NLCs cream and F= 30%EA -NLCs cream

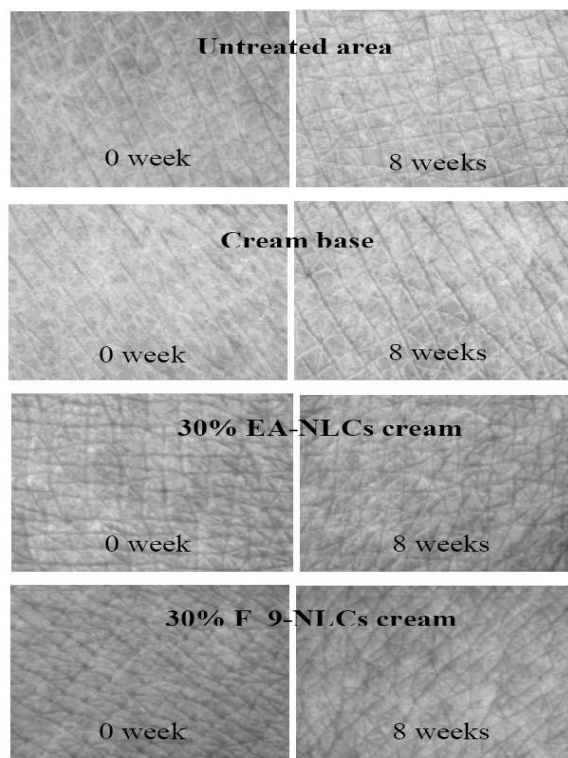


Fig 4: Comparison of the skin roughness before (left) and after application for 8 weeks (right) of various topical cream formulations

Formulation of marigold extract loaded nanostructured lipid carrier cream (ME-NLCs cream)

NLCs dispersions are usually incorporated in a convenient topical dosage form like creams or hydrogel to obtain a desired semisolid consistency [17,18]. At first step, ME-NLCs dispersion was incorporated into the stable cream base resulted in the decreasing of formulation viscosity after stability test. Then 5% Carbopol® was added in order to improve their viscosity. In this study, the cream containing ME-NLCs was prepared by directly mixing into cream base with a gentle stirring. Each ME-NLCs was prepared at 15 and 30% concentrations. All of NLCs containing creams including 15% EA-NLCs, 30% EA-NLCs, 15% F_9-NLCs and 30% F_9-NLCs creams were investigated for their physical stability and antioxidant activity stability compared with cream base and ME (EA or F_9) creams in an equally concentration of the extract. The physical stability results demonstrated that, the particle sizes of nanoparticles were increased during storage times. At day 90, the particles sizes of 15% and 30% of EA and F_9-NLCs creams were in the range of 333.7 to 453.3 and 311.4 to 566.9 nm, respectively. 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 NLCs.

Skin irritation test in human volunteers

The Finn Chamber® occlusive patch test was used to study the skin irritation in human. In this study, twenty five volunteers were test with 8 samples. The results were shown in Table 2. All test samples exhibited no skin irritation (PII<0.5) whereas 1.5%w/v sodium lauryl sulfate which was a positive control revealed moderately irritating (PII=3.83).

Wrinkles reducing capacity test of marigold extract loaded nanostructured lipid carrier cream (ME-NLCs cream)

To compare the difference of all treatments, the percentages of efficiency were calculated and statically analyzed at p <0.05. Fig. 3 and 4 showed the skin surfaces and the percentages of efficiency value on volume, surface, Ra and Rz parameters after 8 weeks of treatment. The applications of 30% F_9-NLCs and 30% EA-NLCs creams

showed significantly difference against untreated and placebo areas for all parameters. Moreover, 30% F₉-NLCs cream which contained active fraction loaded in NLCs exhibited significantly difference in the decreasing of Ra and Rz, comparing with 0.018% F₉ cream (unloaded active fraction).

For 30% EA-NLCs and 0.045% EA cream, they exhibited non-significantly difference in all parameters. It could be concluded that the antioxidant capacity of ME plays an important role in skin wrinkles reducing efficacy and the entrapment of ME into NLCs also showed better effects.

Table 2: Primary Irritation Index (PII) and skin irritation reaction in 25 volunteers of marigold flower extracts and selected creams

Test substances	PII	Classification of skin irritation
1.5%w/v Sodium lauryl sulfate	3.83	Moderately irritating
EA extract	0	Non irritating
F ₉ fraction	0	Non irritating
Cream base	0.17	Non irritating
EA cream	0	Non irritating
F ₉ cream	0	Non irritating
EA-NLCs cream	0.17	Non irritating
F ₉ -NLCs cream	0	Non irritating

CONCLUSION

This study demonstrated that cream containing NLCs loaded with ME (EA or F₉) was stable at 4°C and room temperature conditions as well as could also reduce skin wrinkles compared with untreated and cream base. There are a few studies about marigold extract as active compound in the present cosmetic market. Marigold flower extract presented as another potent antioxidant activity from natural source which can be useful in anti-aging or anti-wrinkles cosmetic products. The results from this study indicated that NLCs were promising delivery system for Marigold flower extract that can be used as anti-wrinkles application and this can be value added to the Marigold which widely grown in northern of Thailand.

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