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

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Received: 18 Mar 2014 Revised and Accepted: 18 Apr 2014

MATERIALS AND METHODS

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 minimizes 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 (O2•−), hydroxyl (•OH), peroxyl (RO2•) and alkoxyl (RO•) 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.

INTRODUCTION

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

The average particle size (2-average size), size distribution and zeta potential were evaluated by a Zetasizer 25 (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 predetermined 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 stearic acid, glycerol monostearate and mineral oil in the concentration of 19-26%. Ceteareth 25, Tween, Span or triethanalamine 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 F_9) were also prepared as EA and F_9 creams.

Determination of antioxidant activities

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method

The antioxidant activity of the cream containing ME (EA or F_9) and ME-NLCs was determined by DPPH radical scavenging assay [11]. Briefly, the EA or F_9 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 = \( \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \), Where \( A_{control} \) is the absorbance of the control reaction and \( A_{sample} \) is the absorbance of the sample.

Determination of antioxidant activity with thioarbituric 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 F_9) 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 Chiang Mai 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 = \([A_{initial} - A_{final}] / A_{initial} \times 100\). 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 with 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 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 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.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Initial (day 0)</th>
<th>30</th>
<th>60</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Particle size (nm)</td>
<td>Zeta potential (mV)</td>
<td>Particle size (nm)</td>
<td>Zeta potential (mV)</td>
</tr>
<tr>
<td>1:2-SS-EA</td>
<td>175.3 ± 2.7</td>
<td>0.22 ± 0.039</td>
<td>-35.1</td>
<td>237.8 ± 0.9</td>
</tr>
<tr>
<td>1:2-S7-EA</td>
<td>151.0 ± 2.6</td>
<td>0.18 ± 0.016</td>
<td>-34.5</td>
<td>161.9 ± 1.3</td>
</tr>
<tr>
<td>1:2-S10-EA</td>
<td>157.3 ± 1.7</td>
<td>0.16 ± 0.016</td>
<td>-40.3</td>
<td>156.4 ± 1.6</td>
</tr>
<tr>
<td>1:2-S12-EA</td>
<td>126.3 ± 1.5</td>
<td>0.13 ± 0.016</td>
<td>-43.8</td>
<td>159.5 ± 2.5</td>
</tr>
<tr>
<td>1:2-S7-EA</td>
<td>144.5 ± 4.0</td>
<td>0.12 ± 0.019</td>
<td>-34.9</td>
<td>146.2 ± 0.01</td>
</tr>
<tr>
<td>1:2-S5-EA</td>
<td>155.6 ± 1.5</td>
<td>0.12 ± 0.013</td>
<td>-42.9</td>
<td>157.5 ± 2.4</td>
</tr>
<tr>
<td>1:2-S10-EA</td>
<td>148.5 ± 0.8</td>
<td>0.11 ± 0.004</td>
<td>-36.3</td>
<td>143.4 ± 0.0</td>
</tr>
<tr>
<td>1:2-S12-EA</td>
<td>130.0 ± 0.1</td>
<td>0.117 ± 0.020</td>
<td>-49.0</td>
<td>140.2 ± 0.00</td>
</tr>
<tr>
<td>1:2-S12-F9</td>
<td>146.9 ± 0.6</td>
<td>0.14 ± 0.008</td>
<td>-38.1</td>
<td>154.7 ± 0.2</td>
</tr>
<tr>
<td>1:2-S12-F9</td>
<td>149.8 ± 0.9</td>
<td>0.216 ± 0.01</td>
<td>-41.7</td>
<td>149.4 ± 0.0</td>
</tr>
</tbody>
</table>

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. 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 different. 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 2: The antioxidant activity (percentages of inhibition) of EA creams at 4ºC 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

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% F_9-NLCs cream which contained active fraction loaded in NLCs exhibited significantly difference in the decreasing of Ro and Rz comparing with 0.018% F_9 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 pays 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**

<table>
<thead>
<tr>
<th>Test substances</th>
<th>PII</th>
<th>Classification of skin irritation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5%w/v Sodium lauryl sulfate</td>
<td>3.83</td>
<td>Moderately irritating</td>
</tr>
<tr>
<td>EA extract</td>
<td>0</td>
<td>Non irritating</td>
</tr>
<tr>
<td>F_9 fraction</td>
<td>0</td>
<td>Non irritating</td>
</tr>
<tr>
<td>Cream base</td>
<td>0.17</td>
<td>Non irritating</td>
</tr>
<tr>
<td>EA cream</td>
<td>0</td>
<td>Non irritating</td>
</tr>
<tr>
<td>F_9 cream</td>
<td>0.17</td>
<td>Non irritating</td>
</tr>
<tr>
<td>EA-NLCs cream</td>
<td>0</td>
<td>Non irritating</td>
</tr>
<tr>
<td>F_9-NLCs cream</td>
<td>0</td>
<td>Non irritating</td>
</tr>
</tbody>
</table>

**CONCLUSION**

This study demonstrated that cream containing NLCs loaded with ME (EA or F_9) 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.

**ACKNOWLEDGMENT**

The authors gratefully thank to National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency, Thailand for financial support and the Faculty of Pharmacy Chiang Mai University for all facilities.

**REFERENCES**