INTRODUCTION

Nanocarrier technology has been applied to numerous processes in the pharmaceutical, food, and cosmetics industries. The nanocarriers can be divided into polymer-based nanoparticles and lipid-based nanoparticles [1-3]. For cosmetic and pharmaceutical products, nanoparticles are advantageous for dermal application that improve the release profile and increase skin penetration of drugs or active ingredients into the skin [1, 3-7]. Nanomulsions (also referred to as mini-emulsions, ultra fine emulsions, and submicron emulsions) are lipid-based nanoparticles with small droplet size, in the range of 20-200 nm, which appeared to be transparency or translucence [4-5, 8-10]. They are widely used in cosmetics because of their stability against sedimentation, creaming, flocculation and coalescence and can be prepared using lower surfactant concentrations compared with microemulsions [5, 11-13]. Nanomulsions are non-equilibrium systems that cannot be formed spontaneously but required an energy input [4-6, 13-14]. Two main processes have been suggested to prepare the nanoemulsions. First, the high-energy emulsification method that required the mechanical energy such as high-shear stirring, ultrasonic emulsification and high-pressure homogenization [4-6]. Second, the low-energy emulsification method that involved with chemical energy stored in the components of the surfactant system to be the emulsifiers. The low energy method can be divided into phase inversion temperature method (PIT) that during the process, the temperature changed suddenly at a constant composition and phase inversion composition method (PIC) that the composition was changed during emulsification while unchanging the temperature [4, 9, 12, 15]. Both of these situations that leading to phase inversion could produce a tiny oil droplet size. This study focused on the PIC method in which the nanoemulsions were produced by the titration of water into the mixture containing oils and surfactants leading to the changes of a water-in-oil to an oil-in-water emulsion or vice versa. As an increasing the amount of water in the system, the water-in-oil (W/O) emulsion becomes a multiple emulsion (O/W/O) and finally to oil-in-water (O/W) emulsion with a smaller droplet size [16-18].

Essential oils are complex mixtures of volatile compounds such as terpenoids, phenol-derived aromatic components, and aliphatic components that can be physically separated from plant organs or membranous tissues [19-21]. Essential oils have been used for many biological properties including bactericidal, fungicidal, insecticidal, antioxidant, anti-tyrosinase and other medicinal properties [20-25]. Furthermore, they are widely used in pharmaceutical, cosmetic, agricultural, and food industries.

However, the essential oils have a high volatility and some can be easily decomposed by heat, humidity, light, or oxygen [26-27]. Therefore, many studies have investigated for their encapsulation in various colloidal systems such as microcapsules, microspheres, nanoemulsions as well as liposomes in order to decrease the volatility, improve an absorption mechanism and increase their bio efficacy [25, 27-29]. The present study aimed to develop nanoemulsions loaded with essential oils blend as actives using the PIC technique and evaluate their physicochemical characteristics. The in vitro anti-tyrosinase activity and their stability under various storage conditions was also determined.

MATERIALS AND METHODS

Materials

Mushroom tyrosinase and L-tyrosine, were purchased from Sigma-Aldrich (St. Louis, MO, USA), virgin coconut oil (HLE=8.0) and various essential oils, purchased from United Chemical and Trading Co., Ltd. Essential oils blend are composed of Cape jasmine absolute, various essential oils, purchased from United Chemical and Trading Co., Ltd. Essential oils blend are composed of Cape jasmine absolute, Lemongrass oil and Basil oil. PEG 40 hydrogenated castor oil (PHC) and sorbitan monooleate (SMO) as surfactant system. Initially, an organic phase containing carrier oil and surfactant mixture was stirred using a magnetic stirrer for a period of time to produce nanoemulsion or vice versa. As an increasing the amount of water in the system, the water-in-oil (W/O) emulsion becomes a multiple emulsion (O/W/O) and finally to oil-in-water (O/W) emulsion with a smaller droplet size [16-18].

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METHODS

Development of nanoemulsions by PIC technique

Phase inversion composition (PIC) technique was used as a low-energy method [16-18] to prepare the nanoemulsions consisting of virgin coconut oil (V) as an carrier oil, PEG 40 hydrogenated castor oil (PHC) and sorbitan monooleate (SMO) as surfactant system. Initially, an organic phase containing carrier oil and surfactant mixture was stirred using a magnetic stirrer for a period of time.
The essential oils blend (EB) has been tested for anti-tyrosinase activity and presented good activity with IC50 value of 307.73µg/mL, compared with the freshly prepared. The formulas based on good correlation spectroscopy (zetasizer® version 5.00, Malvern Instruments Ltd, Malvern, UK). Each preparation was diluted with water (1:100). The measurements were done in triplicate [30].

Zeta potential measurements
The surface charge of each formulation was also determined by measuring the zeta potential (ZP) at 25°C using photon correlation spectroscopy (zetasizer® version 5.00, Malvern Instruments Ltd, Malvern, UK). Each preparation was diluted with water (1:100). The measurements were done in triplicate [30].

Physical stability testing of the nanoemulsions
All the nanoemulsions were also characterized after 3 months storage at room temperature (30°C), 4 °C, 45 °C and heating-cooling cycling. For the standard solutions, five concentrations (0.0125-0.2 v/v) of limonene, linalool or estragole solution in ethanol were prepared and analyzed in triplicate using GC-FID. The peak area was plotted against the concentration of each standard for calibration curve.

In vitro anti-tyrosinase activity of nanoemulsions loaded with essential oils blend
The anti-tyrosinase activity of EB loaded nanoemulsions was determined using the modified dopachrome method with L-tyrosine as substrate that modified from Watcharee K. et al. with slightly modified [33]. The experiment was conducted in a 96-well microtiter plate. Briefly, each 70 µL of sample was mixed with 70 µL of phosphate buffer solution (20 mM, pH 6.8) and 70 µL of tyrosinase (240 Unit/mL). The mixture was then incubated at 25°C for 10 min before adding 70 µL of L-tyrosine. Finally, after 15 min of incubation at 25°C the generated dopachrome was determined by the absorbance measurement at 450 nm using multimode detector. The results were compared to a control consisting of nanoemulsions without essential oils blend in place of the sample. The anti-tyrosinase activity of each sample expressed as percentage tyrosinase inhibition was calculated using the following equation.

\[
\% \text{ inhibition} = \left( \frac{A_a - A_b}{A_a} \right) \times 100
\]

Aa=absorbance at 450 nm without test sample
Ab=absorbance at 450 nm with test sample

RESULTS AND DISCUSSION
Development of nanoemulsions by PIC technique
In the experiment, virgin coconut oil (V) was used as an oil phase. Effects of surfactant concentration on the droplet size and zeta potential were investigated by preparing a series of formula with a fixed oil phase composition. In addition, the surfactant to oil ratio (SOR) was also varied as mentioned in table 1.

To compare the effect of the surfactant system on droplet size, the combination of two non-ionic surfactants with different HLB were investigated. PEG 40 hydrogenated castor oil and sorbitan monolaurate were used in volume ratios of 1:1, 2:1 and 1:2 as shown in table 1. The results revealed that the surfactant mixtures (PHC:SMO) at volume ratio of 2:1 with HLB 10.76 (V2, V5, V8) showed the smaller droplet size than at 1:1 ratio with HLB 9.15 (V1, V4, V7) whereas at volume ratio 1:2 with HLB 5.52 (V3, V6, V9), the nanoemulsions were unstable due to phase separation (table 2). According to the effect of SOR, the result presented that the mean

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Surfactant to Oil Ratio (SOR)</th>
<th>Surfactant mixtures PHC:SMO</th>
<th>Hydrophilic-lipophilic balance (HLB) of surfactant system</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>1:1</td>
<td>1:1</td>
<td>9.15</td>
</tr>
<tr>
<td>V2</td>
<td>1:1</td>
<td>2:1</td>
<td>10.76</td>
</tr>
<tr>
<td>V3</td>
<td>1:1</td>
<td>1:2</td>
<td>5.52</td>
</tr>
<tr>
<td>V4</td>
<td>1.5:1</td>
<td>1:1</td>
<td>9.15</td>
</tr>
<tr>
<td>V5</td>
<td>1.5:1</td>
<td>2:1</td>
<td>10.76</td>
</tr>
<tr>
<td>V6</td>
<td>1.5:1</td>
<td>1:2</td>
<td>5.52</td>
</tr>
<tr>
<td>V7</td>
<td>2:1</td>
<td>1:1</td>
<td>9.15</td>
</tr>
<tr>
<td>V8</td>
<td>2:1</td>
<td>2:1</td>
<td>10.76</td>
</tr>
<tr>
<td>V9</td>
<td>2:1</td>
<td>1:2</td>
<td>5.52</td>
</tr>
</tbody>
</table>

Characterization of nanoemulsions
Droplet size measurements
The droplet size of the nanoemulsions was measured using photon correlation spectroscopy (zetasizer® version 5.00, Malvern Instruments Ltd, Malvern, UK). Each formulation was diluted with water (1:100) at 25°C. All size measurements were done in triplicate. The average results were reported and size distribution was presented by the polydispersity index values (PDI) [17].

Table 1: Compositions of different nanoemulsions

<table>
<thead>
<tr>
<th>Surfactant to Oil Ratio (SOR)</th>
<th>Surfactant mixtures PHC:SMO</th>
<th>Hydrophilic-lipophilic balance (HLB) of surfactant system</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>1:1</td>
<td>9.15</td>
</tr>
<tr>
<td>V2</td>
<td>1:1</td>
<td>10.76</td>
</tr>
<tr>
<td>V3</td>
<td>1:1</td>
<td>5.52</td>
</tr>
<tr>
<td>V4</td>
<td>1.5:1</td>
<td>9.15</td>
</tr>
<tr>
<td>V5</td>
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</tr>
<tr>
<td>V6</td>
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<tr>
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</tr>
<tr>
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<td>2:1</td>
<td>10.76</td>
</tr>
<tr>
<td>V9</td>
<td>2:1</td>
<td>5.52</td>
</tr>
</tbody>
</table>

In the experiment, virgin coconut oil (V) was used as an oil phase. Effects of surfactant concentration on the droplet size and zeta potential were investigated by preparing a series of formula with a fixed oil phase composition. In addition, the surfactant to oil ratio (SOR) was also varied as mentioned in table 1.

Development of nanoemulsions by PIC technique
This analysis was carried out to investigate the morphology and size of the produced nanoemulsions [31-32]. To perform TEM analysis, each formulation was dropped to a 300 mesh copper grid and air dried for 24 h. The samples were then viewed using a transmission electron microscope (Japan) operated at 80 kV at 40000X magnification.

Morphology of nanoemulsions by transmission electron microscopic (TEM)
This analysis was carried out to investigate the morphology and size of the produced nanoemulsions [31-32]. To perform TEM analysis, each formulation was dropped to a 300 mesh copper grid and air dried for 15 min. Then a drop of 2% phosphotungstic acid was applied to the grid for 1 min. Finally, the grid was analyzed by a JEOL JEM-1200 EXII electron microscope (Japan) operated at 80 kV at 40000X magnification.

Chemical stability of essential oils blend loaded nanoemulsions by gas chromatography (GC-FID) analysis
The chemical stability of EB loaded nanoemulsions at before and after storage conditions were analyzed by GC-FID using DB-1 silicone capillary column 30 m×0.53 mm. (1.5 μm film thickness). The injector temperature was set at 250 °C and an initial temperature at 50 °C. The column oven temperature was programmed from 50 to 120°C at 10 °C/min; to 120 -150°C, at 2°C/min; and finally up to 280°C, at 10°C/min (3 min hold). The carrier gas was nitrogen, at a flow rate of 1 ml/min. Detector temperature was set at 300 °C. The injection volume was 1 µL and the running time was 29.00 min. The samples were evaluated at 0 and 90 days after various storage conditions and at the end of heating-cooling cycling. For the standard solutions, five concentrations (0.0125-0.2 v/v) of limonene, linalool or estragole solution in ethanol were prepared and analyzed in triplicate using GC-FID. The peak area was plotted against the concentration of each standard for calibration curve.

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particle diameter decreased when the SOR was increased from 1:1 to 2:1 (table 2). The smallest droplet size around 40 nm was observed for the system with the highest surfactant concentration (SOR=2:1) in V8 with zeta potential of -25.87 mV.

The polydispersity index (PDI) value of all formulas (except the unstable formulas) ranging from 0.10 - 0.16 represented the homogeneity of particles. Their zeta potential ranged between -19.40 to -26.70 mV (table 2) indicated a good stability. Some theory explained that the good stability of the system should have zeta potential more than |30 mV|. However, in this study the non-ionic surfactants were used that the system may be stabilized by a steric stabilization and showed low charges. The stabilization was depended on the attachment of ethylene oxide group and long hydrocarbon chain to the surfaces of the particles. Therefore, the zeta potential can be low positive or negative charges.

After long term storage at various temperatures and heating-cooling for 6 cycles, no physical change in appearance was observed in all formulations. The formula V8 with SOR at 2:1 and surfactant system (PHC: SMO) at 2:1 ratio was then selected for loading with the essential oils blend due to the smallest droplet size with quite translucent in appearance and lowest PDI among all the formulations.

### Development and characterization of EB loaded nanoemulsions

The physical stability of the EB loaded nanoemulsions (EBV8) exhibited an average droplet size between 29.55 to 37.12 nm with PDI 0.06-0.18, as shown in table 3. The average droplet size of all formulations after stored at 4°C, 45°C, room temperature with and without light protection for 3 months and after heating-cooling cycling for 6 cycles showed a slightly changes, whereas the phase separation has not occurred. The polydispersity index (PDI) values of all formulations were less than 0.2 that indicated the relative homogeneity. Their zeta potentials were changed after storage at various conditions ranging between -14.51 to -20.40 mV but these was not affected the physical changes of the formulations.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Surfactant to oil ratio (SOR)</th>
<th>droplet size (nm)</th>
<th>Polydispersity index (PDI)</th>
<th>zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V1</td>
<td>1:1</td>
<td>100.11 ± 0.85</td>
<td>0.15 ± 0.01</td>
<td>-26.70 ± 2.80</td>
</tr>
<tr>
<td>V2</td>
<td>1:1</td>
<td>76.02 ± 0.11</td>
<td>0.12 ± 0.00</td>
<td>-20.23 ± 2.22</td>
</tr>
<tr>
<td>V3</td>
<td>1:1</td>
<td></td>
<td>phase separation</td>
<td></td>
</tr>
<tr>
<td>V4</td>
<td>1.5:1</td>
<td>96.48 ± 0.84</td>
<td>0.16 ± 0.01</td>
<td>-19.40 ± 4.10</td>
</tr>
<tr>
<td>V5</td>
<td>1.5:1</td>
<td>58.33 ± 0.33</td>
<td>0.10 ± 0.01</td>
<td>-19.50 ± 0.66</td>
</tr>
<tr>
<td>V6</td>
<td>1.5:1</td>
<td></td>
<td>phase separation</td>
<td></td>
</tr>
<tr>
<td>V7</td>
<td>2:1</td>
<td>47.69 ± 0.28</td>
<td>0.16 ± 0.00</td>
<td>-24.17 ± 1.97</td>
</tr>
<tr>
<td>V8</td>
<td>2:1</td>
<td>39.34 ± 0.20</td>
<td>0.10 ± 0.02</td>
<td>-25.87 ± 3.42</td>
</tr>
<tr>
<td>V9</td>
<td>2:1</td>
<td></td>
<td>phase separation</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2: The average droplet size, PDI and zeta potential of all nanoemulsions**

Transmission electron microscopic (TEM) analysis of the produced nanoemulsions

The morphology of nanoemulsions (V8 and EBV8) determined by TEM as shown in fig. 1 presented mostly spherical shapes of the droplets surrounding by the adsorbed surfactants. Besides, their droplets size was less than 50 nm that corresponding with the results obtained from the Zetasizer®.

Chemical stability determination of EB loaded nanoemulsions by Gas chromatography with Flame Ionization Detector (GC-FID)

According to GC-FID analysis, a good chemical stability of all formulations was observed. The GC chromatogram of EBV8 showed three major peaks of volatile compounds; limonene, linalool and estragole which were used as markers (fig. 2). After storage at 4°C, 45°C, room temperature with and without light protection for 3 months and after heating-cooling cycling, the amount of limonene in all formulations did not significantly change (p<0.05) as same as linalool and estragole. These observations confirmed chemical stability of the volatile compounds consisting in the nanoemulsions. The results indicated that temperature and light are not affected to the stability of EB loaded in nanoemulsions.

**Table 3: The average droplet size, PDI and zeta potential of nanoemulsions loaded with essential oils blend at various storage conditions after 3 month**

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>droplet size(nm)</th>
<th>polydispersity index (PDI)</th>
<th>zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>29.55 ± 0.21</td>
<td>0.12 ± 0.01</td>
<td>-19.20 ± 3.66</td>
</tr>
<tr>
<td>Day 90</td>
<td>33.28 ± 0.13</td>
<td>0.15 ± 0.00</td>
<td>-16.13 ± 2.48</td>
</tr>
<tr>
<td>RT-L</td>
<td>33.41 ± 0.38</td>
<td>0.13 ± 0.01</td>
<td>-14.51 ± 1.59</td>
</tr>
<tr>
<td>RT-LP</td>
<td>36.37 ± 0.20</td>
<td>0.18 ± 0.00</td>
<td>-15.60 ± 1.57</td>
</tr>
<tr>
<td>4°C</td>
<td>37.12 ± 0.09</td>
<td>0.11 ± 0.02</td>
<td>-15.77 ± 2.11</td>
</tr>
<tr>
<td>45°C</td>
<td>33.09 ± 0.31</td>
<td>0.06 ± 0.01</td>
<td>-20.40 ± 3.87</td>
</tr>
</tbody>
</table>

**In vitro anti-tyrosinase activity of EB loaded nanoemulsions**

The anti-tyrosinase activity of freshly prepared nanoemulsions loaded with essential oils blend was compared with the formulations kept in various storage conditions after 90 days. The results were shown in fig. 3. Interestingly was found that all the formulations exhibited a good stability in anti-tyrosinase activity which was not significantly different from the starting date (p<0.05). These results confirmed their stability and are corresponding with the chemical stability mentioned above.

![Fig. 1: The nanoemulsions structure (A), TEM of the nanoemulsions; V8 (B), EBV8 (C)](image-url)
CONCLUSION

In this study, the nanoemulsions loaded with essential oils blend were successfully prepared by phase inversion composition technique (PIC) which is a low-energy method. The results showed that the selected formulations with SOR at 2:1 and a nonionic surfactant system consisting of PEG 40 hydrogenated castor oil and sorbitan monooleate at 2:1 volume ratio presented good physicochemical characteristics after storage at 4°C, 45°C, room temperature with and without light protection for 3 months and after heating-cooling cycling. The incorporation of essential oils blend into the nanoemulsions confirmed the chemical stability of volatile compounds consisting in the nanoemulsions. Additionally, from the in vitro anti-tyrosinase activity test, the nanoemulsions exhibited a good inhibitory effect on mushroom tyrosinase. Therefore, the nanoemulsions loaded with essential oils blend could be a promising delivery system for skin brightening application.

ACKNOWLEDGEMENT

The authors thank the Thailand Research Fund – Master Research Grants (TRF-MAG) and Welltech Biotechnology Co., Ltd., Bangkok, Thailand for financial support. We also thank the Faculty of Pharmacy Chiang Mai University for all facilities.

CONFLICT OF INTERESTS

Declared None

REFERENCES


