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Depigmenting action of a nanoemulsion containing heartwood extract of *Artocarpus incisus* on UVB-induced hyperpigmentation in C57BL/6 mice

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Synopsis

Melasma hyperpigmentation is an acquired disorder predominantly affecting the female population. The present study was conducted to determine the potential of a botanical extract to reduce observable hyperpigmentation. The extract from heartwood of *Artocarpus incisus* was formulated into nanoemulsions, and the depigmenting efficacy of the formulated nanoemulsion was determined *in vivo.* HPLC analysis showed that the extract contained artocarpin in an amount of 44.5 ± 0.1% w/w. The extract exhibited melanogenesis inhibition with an IC_{50} value of 30.2 ± 2.4 mg/ml, while kojic acid, a well known lightening agent, exhibited an IC_{50} of 51.4 ± 5.1 mg/ml. The nanoemulsion containing the extract was then formulated and prepared by the phase inversion technique. The concentration of the extract used was about six times its IC_{50}. The optimal formula containing 0.02% w/w extract, 41.6% w/w isopropyl myristate, 0.03% w/w α-tocopherol, 5% w/w glyceryl monostearate (co-emulsifier), 8% w/w ceteareth-10 (emulsifier), 0.05% triethanolamine, 0.03% w/w carbopol 940, and water adjusted to 100% w/w provided a homogeneous o/w emulsion with a droplet size of 325 ± 15 nm and a polydispersity of 0.31 ± 0.02. The depigmenting efficacy was then observed following topical application of the formulated nanoemulsion to UVB-stimulated hyperpigmented dorsal skin of C57BL/6 mice. A strongly visible decrease in hyperpigmentation was observed after six weeks of treatment with the formulated nanoemulsion. The degree of pigmentation decreased after the application was 84 ± 4 units, while that after the application of the extracted prepared into solution was 51 ± 3 units. The applied areas would return to their original color after treatment was stopped for four weeks.

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INTRODUCTION

Melasma is a common acquired hyperpigmentary disorder characterized by dark patches or macules located on the cheeks, forehead, upper lip, chin, and neck. Pigmentation is predominantly found in females, accounting for 90% of all cases (1,2). It appears in all racial types, but it occurs more frequently in persons with darker complexions (Fitzpatrick's skin type IV) (3,4), especially those living in high-ultraviolet-radiation areas, including Thailand. Although melasma is a disfiguring skin condition that worsens with sunlight, it is considered to be a cosmetic problem, as there is no pain or other associated symptoms.

Nowadays, safe and effective components extracted from natural sources have been reported for their potential application in improving hyperpigmentation disorders. These extracts are mostly free from harmful side effects. For this reason, there is an increasing interest in finding natural extracts for application in personal care products and cosmetics. Our previous study revealed that the extract from heartwood of *Artocarpus incisus* (breadfruit or Sa-kae in Thai) provided tyrosinase inhibitory and anti-oxidation activities (5). Additionally, it was found that artocarpin, the major component of *A. incisus* extract, exhibited skin lightening effects on the UVB-induced hyperpigmented dorsal skin of brownish guinea pigs (6,7). These findings indicated the extract from *A. incisus* heartwood to be a potential source of depigmenting agents and that its site of action is in the substratum, in particular within the epidermis and hair follicles. Therefore, to increase the efficacy of the extract, the tools for increasing penetration of the extract through skin are needed.

As we know, delivery systems play an important role in the fields of cosmetics and dermopharmaceuticals. The key aspects of the delivery systems are increasing and/or improving penetration and efficacy, controlling delivery, separating incompatible actives, prolonging shelf life, and/or decreasing the degradation of active compounds. Nanoemulsions have increasingly become one of the most popular delivery systems. Nanoemulsions can be defined as oil-in-water (o/w) emulsions, with mean droplet diameters ranging from 50 to 1,000 nm (8). They can be "transparent" (mean droplet size <200 nm) or "milky" (mean droplet size ≈ 500 nm) (9,10). Nanoemulsions would influence the transport properties of the active ingredients since their compositions such as emulsifiers and co-emulsifiers can enhance the skin penetration of active ingredients and increase their concentrations in the skin. Additionally, due to smaller particle sizes, they offer higher stability against creaming or sedimentation as compared to macroemulsions (10). Furthermore, nanoemulsions are more suitable for the transportation of lipophilic compounds, according to the lipophilic interior of nanoemulsions.

Therefore, in the present study, the natural extract from heartwood of *A. incisus* was formulated into various nanoemulsion formulations. An *in vivo* study of UVB-induced hyperpigmentation in C57BL/6 mice was performed to investigate visible results of the formulated nanoemulsion in application for the purpose of depigmenting. The results from this study showed that a nanoemulsion containing heartwood extract from *A. incisus* could remarkably improve the hyperpigmented lesion on the dorsal skin of C57BL/6 mice.

MATERIALS AND METHODS

PREPARATION OF HEARTWOOD EXTRACT OF *A. INCISUS*

The heartwood of *A. incisus* was collected in July 2007 from Phitsanulok Province, Thailand. To prepare the extract, the heartwood portion was chipped, exposed to the sun, and

dried at 50°C by using a hot-air oven. Then the dried chipped heartwood was milled into powder. After that, 500 grams of the powder was extracted with 800 ml of diethyl ether (analytical grade, LabScan Asia, Co. Ltd., Bangkok, Thailand) at room temperature for two days, according to previous studies (5,6) with minor modification. The obtained mixture was filtered through a cloth to remove particulates and the diethyl ether was then removed by evaporation with a vacuum evaporator set at 33°C. The resultant powder was stored in a tight amber glass at -20°C for further studies.

QUANTIFICATION OF ARTOCARPIN IN THE EXTRACT

The quantity of artocarpin, the major component of *A. incisus*'s heartwood extract, was determined by using isocratic high-performance liquid chromatography (HPLC) according to our previous study (5) with minor modification. The artocarpin standard was provided by Assist. Prof. Atawit Somsiri of the Faculty of Pharmaceutical Sciences, Naresuan University (11). The HPLC instrument consisted of an SPD-10M10AVP diode array detector and an SCL-10A central unit (Shimadzu Co., Ltd., Kyoto, Japan). An Alltima C18 column (5 μm), 250 × 4.60 mm in diameter (Alltech Associates Inc., Illinois), was applied as stationary phase. The effluent consisted of a mixture of methanol (HPLC grade, LabScan Asia Co. Ltd):water (80:20). The flow rate of the effluent was 1 ml/min and the injection volume was 20 μ1. The quantification of artocarpin was based on the peak area at 282 nm. Determinations were performed in triplicate and the results were the average of three independent determinations.

IC_{50} VALUE OF MELANOGENESIS INHIBITORY ACTIVITY OF THE EXTRACT IN MOUSE MELANOCYTE CELLS

The IC_{50} value, the equivalent concentration to provide 50% melanogenesis inhibition, was determined by log prohibit analysis using six different final concentrations of the extract. In the present study, the extract concentration used for nanoemulsion formulation was based on this value.

CELLS AND TREATMENT

B16-F1 mouse melanoma cells (Lot No. 300122-43) were purchased from Cell Lines Services, Eppelheim, Germany. First, B16F1 melanoma cells were initially cultured in a 25-cm^2 flask (3.2×10^6 cells/cm^2) in Dulbecco's Modified Eagle's Medium (low glucose, Sigma-Aldrich Co., St. Louis, Missouri), supplemented with 10% fetal bovine serum (FBS, GIBCO, California) at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed every two days. The passage numbers (the number of times the cell has been replated and allowed to grow back to confluency) of 5 to 8 were used in this study. Before being tested, the cell suspension was transferred from a 25-cm^2 flask into a 24-well plate (1×10^5 cells/well) and kept in an incubator (37°C, 5% CO_2) overnight for complete adherence of the cells on the culture plate. After 24 h of cultivation, the old medium was replaced with 1.0 ml of new DMEM medium containing various concentrations (10, 15, 25, 40, 80, and 100 μg/ml) of *A. incisus* extracts dissolved in dimethyl sulfoxide (DMSO,

99.5% GC plant cell culture tested, Merck, Darmstadt, Germany). At the final concentration, the amount of DMSO used was not more than 0.1% v/v. The control cells were treated with 0.1% v/v DMSO. Kojic acid (Sigma-Aldrich) of various concentrations (10, 15, 25, 40, 80, and 100 μg/ml) was used as a positive marker.

MELANIN CONTENT ASSAY

After treatment for four days, the treated cells were trypsinized with trypsin EDTA (GIBCO, Ontario, Canada) and washed twice with phosphate buffer saline (PBS). Then the collected cells were lysed in 1 N NaOH containing 10% v/v of DMSO and heated at 80°C for 1 h (12). Finally, the amount of melanin was determined from the absorbance at the wavelength of 490 nm by using a microplate spectrophotometer (model Spectra Count®, Perkin Elmer, Connecticut, USA). The percentage of inhibition was calculated from the reduction in the absorbance value of the treated cells compared with that of the control adjusted to 100%. All experiments were performed in triplicate.

CELL VIABILITY MEASUREMENT

A hemocytometer was used for counting viable cells that were not stained with the blue dye of a trypan blue solution (R&D grade, Sigma-Aldrich). A microscopic technique was also used to investigate the phenotypic appearance of the melanocyte cells before and after treatment with the extract.

NANOEMULSION FORMULATION

Nanoemulsions containing *A. incisus* extract were prepared by the phase inversion temperature (PTT) method with some modifications (13,14). The water phase was composed of ceteareth-10 (Brij 56®, nonionic emulsifier, Sigma-Aldrich), 0.05% w/w triethanolamine (TEA, Riedel-de Haen, RdH Laborchemikalien GmbH & Co. KG, Seelze, Germany), 0.03% w/w carbopol 940 (BASF, Ludwigshafen, Germany), and deionized water. The oil phase contained the self-bodying agent glyceryl monostearate (GMS, co-emulsifier, Huls AG, Witten, Germany), 0.03% w/w α-tocopherol (Cognis, Düsseldorf, Germany), 41.6% w/w isopropyl myristate (IPM, Cogins), and 0.02% w/w *A. incisus* extract. The concentration of the extract used was about six times the IC_{50} value of the melanogenesis-inhibitory activity of the extract, according to the above-mentioned study.

For preparation processes, ceteareth-10 was dissolved in the required amount of deionized water and preheated to 75°C before being added to the oil phase. In the oil phase, the *A. incisus* extract was first dissolved in IPM. The obtained solution was then mixed with other compositions of the oil phase, followed by heating to 70°C. Afterwards, the two phases were homogeneously mixed using an Ultra-Turrax T25 homogenizer (Janke & Kunkel IKA Labortechnik, Staufen, Germany) for 5 min at a rate of 8,000 rpm to form coarse o/w emulsions. The resultant emulsions were heated to a specific phase-inversion temperature (90°C), according to our preliminary study, at which point w/o emulsions were formed. Then, the hot emulsions were cooled rapidly in an ice bath without a high input of mechanical energy, resulting in finely dispersed o/w emulsions. Carbopol 940

was added to the finely dispersed emulsion. Finally, TEA was added to neutralize the carbopol polymer.

The effect of the contents of the emulsifier and co-emulsifier on the droplet size was also investigated in this study. The contents of the emulsifier (4–16% w/w) and co-emulsifier (1–8% w/w) were varied, and the formula that provided the smallest droplet size would be selected for stability studies and *in vivo* studies of UVB-induced hyperpigmentation in C57BL/6 mice.

CHARACTERIZATION OF THE FORMULATED NANOEMULSIONS

Morphology. The morphology of the nanoemulsions was observed using transmission electron microscopy (TEM, Technai F20, Philips, Eindhoven, The Netherlands). To perform the TEM observations, the nanoemulsions were diluted with deionized water. A drop of the diluted nanoemulsions was then applied to carbon-coated grids. Two minutes later, the excess was drawn off with filter paper. A saturated uranyl acetate aqueous solution was used as a staining agent. After air drying, the morphology of the sample was observed by TEM at a magnification of 370 kx.

Droplet size analyses. The mean droplet size and polydispersity value of the nanoemulsions were analyzed by photon correlation spectroscopy (PCS), employing a Zetasizer (Model Nano ZS90, Malvern instruments Ltd., Malvern, Worcestershire, UK). An aliquot of the nanoemulsions was re-suspended in water. Measurements were performed at a fixed angle of 90° to the incident light, and data were collected over a period of 3 min. Three measurements were performed separately on each prepared sample.

Viscosity determination. The viscosity of the nanoemulsions was determined by a viscometer (model DV-III, Brookfield, Arizona) equipped with a cone and plate (plate diameter 40 mm, cone angle 4°). Three measurements were carried out at a temperature of 30°C with a torque of 10 rpm and were performed separately on each prepared sample.

pH measurement. The pH of the nanoemulsions was measured using a pH meter (Model Delta 320, Mettler Toledo, Guangzhou, China). Three measurements were made separately on each prepared sample.

STABILITY OF THE SELECTED NANOEMULSION FORMULA CONTAINING *A. INCISUS* EXTRACT

The stability of the nanoemulsions containing *A. incisus* extract was studied under normal and accelerated conditions. For the normal condition, the selected formula was stored at room temperature (35° ± 3°C, during summer at Phitsanulok Province, Thailand) for three months. For the accelerated condition, the nanoemulsion was subjected to seven heat-cool cycles (4°C for 24 h and alternated to 45°C for another 24 h). The quantity of artocarpin was determined by the HPLC method described above. Moreover, the physical appearances of the nanoemulsions were examined in terms of viscosity, droplet size, and pH value.

IN VIVO STUDY ON THE DEPIGMENTING EFFICACY OF THE SELECTED NANOEMULSION FORMULA IN UVB-INDUCED HYPERPIGMENTATION OF C57BL/6 MICE

Three male C57BL/6 mice (National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand), aged five weeks and weighing 20–25 g, were used. The mice were

housed on sawdust, three per cage, with free access to food and tap water. Room illumination was on an automated cycle of 12 h darkness, and room temperature was maintained at 25° ± 2°C. They were acclimatized to the laboratory conditions for at least one week prior to the experiments.

UVB-induced hyperpigmentation was induced on the backs of the C57BL/6 mice by following previous studies (15,16) with modification. The mice were anesthetized with 5.47 g/ml solution of pentobarbital sodium (0.8 ml/kg). The back hair of the animal was shaved with a razor in the tail-to-head direction without damaging the skin. A bank of five fluorescent sunlamps (Toshiba FL8BLB, Japan) emitting rays between 275 and 305 nm, with a peak at 305 nm, was used as a UVB source. The lamp-to-skin distance was 30 cm. Two separate areas (2 × 2 cm) on the back of each animal were irradiated with 91.4 J/cm^2 per exposure from the UVB lamps for ten days. The formulated nanoemulsion was topically applied to one of the irradiated areas twice a day for six weeks, while the aqueous solution of the extract (0.02% w/w) was applied to the other one.

The skin color of the treated area was evaluated by a Mexameter MX® 18 (Courage and Khazaka Electronic GmbH, Cologne, Germany). Once every week from the beginning of sample applications, the degree of depigmentation was evaluated as the melanin value (M), estimated as follows:

$$\Delta M = M\,(\text{at day } 0) - M\,(\text{at each day measured})$$

The M value expresses the melanin value or degree of hyperpigmentation and the high value of ΔM indicates the depigmenting efficacy of the formula.

STATISTICAL ANALYSIS

A Student's *t*-test was used for comparison between the averages of two independent groups. A *p*-value of equal to or less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

QUANTITY OF ARTOCARPIN IN THE EXTRACT

The appearance of *A. incisus* extract was a yellow powder. According to the HPLC method, the amount of artocarpin in the extract was determined to be 44.5 ± 0.1% w/w. The obtained result corresponded with that of our previous study, indicating 45.2 ± 0.5% w/w of artocarpin in the extract (5).

IC_{50} VALUE OF MELANOGENESIS-INHIBITORY ACTIVITY OF THE EXTRACT IN MOUSE MELANOCYTE CELLS

The *A. incisus* extract exhibited a dose-dependent inhibition of melanin production in B16F1 cells, as shown in Figure 1. At concentrations of 10, 15, 25, 40, 80, and 100 μg/ml of *A. incisus* extract, the percentage of melanin reduction compared to the control was

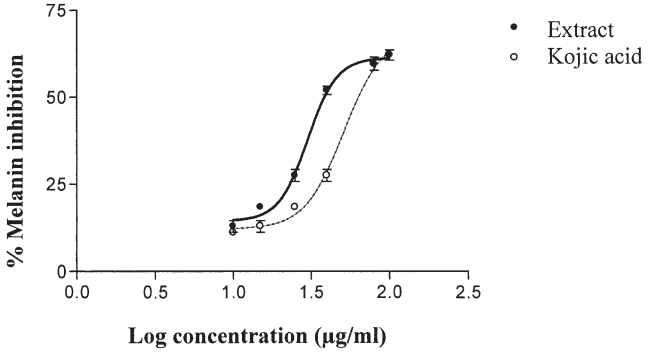

Figure 1. Inhibitory activities of *A. incisus* extract and kojic acid on melanin synthesis in melanocyte B16F1 melanoma cells.

12.9 ± 2.9%, 18.4 ± 1.6%, 27.5 ± 3.0%, 51.9 ± 2.1%, 59.6 ± 3.3%, and 62.1 ± 2.5%, respectively. The IC_{50} for melanogenesis inhibition was 30.2 ± 2.4 µg/ml, while kojic acid exhibited melanogenesis-inhibitory activity with an IC_{50} of 51.4 ± 5.1 µg/ml.

The number of viable cells treated with various concentrations (10, 40, and 100 µg/ml) of *A. incisus* extract was also evaluated by staining cells with blue dye, as shown in Figure 2. When compared to the control (cell treated with 0.01% DMSO), the 100 µg/ml extract showed a significant inhibitory ($p < 0.01$) effect on the growth of B16F1 cells after incubation for three days. The effect of *A. incisus* extract or kojic acid, at a concentration of 40 µg/ml, on the morphology of melanocyte cells is shown in Figure 3. The results of the microscopic observations revealed that, after treatment for four days, kojic acid induced changes in the morphology of the melanocytes by losing dendrites (Figure 3C), whereas changes in cell morphology were not observed in cells treated with the extract (Figure 3B). The obtained results indicated that the extract at low concentration (40 µg/ml) can suppress melanin production without affecting cell morphology or cell growth, but that at a higher concentration (100 µg/ml), melanogenesis inhibition resulted from decreasing cell numbers. We theorize that several mechanisms, including tyrosinase enzyme activity, tyrosine transportation, and/or melanosome transportation, were possibly

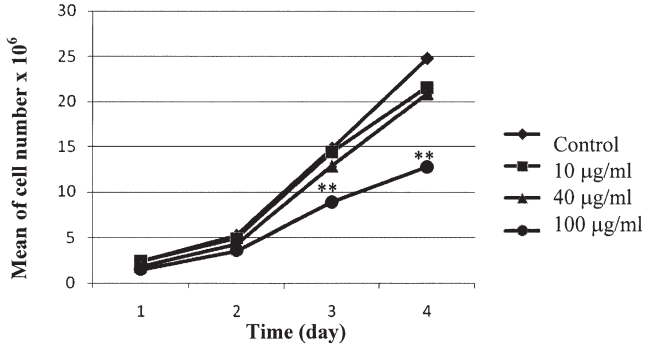

Figure 2. Effect of *A. incisus* extract on the viability of melanocyte B16F1 melanoma cells. Each point represents mean ± S.D. of triplicate study. Student's *t*-test showed a significant difference from the control group, ** $p < 0.01$.

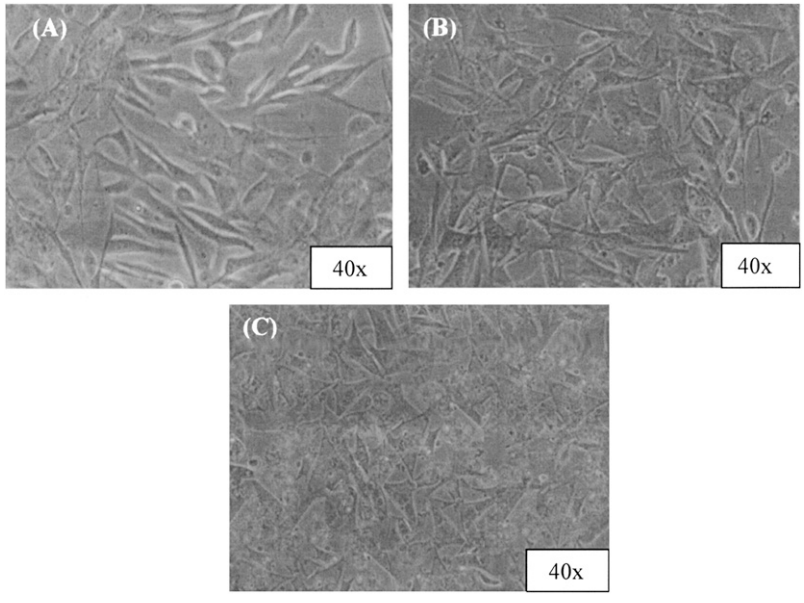

Figure 3. Morphology of melanocyte B16F1 melanoma cells treated with (A) 0.1% DMSO (control), (B) 40 μg/ml of *A. incius* extract, and (c) 40 μg/ml of kojic acid for four days (at magnification of 40×).

involved with the melanogenesis-inhibitory activity of the extract at the low concentration used. Further studies are needed to prove our theory.

THE FORMULATED NANOEMULSIONS

Effect of ceteareth-10 (emulsifier) on mean droplet size. The effect of ceteareth-10 on the mean droplet size was observed by varying its concentration of 4%, 8%, 10%, 12%, and 16% w/w while maintaining the amount of GMS (co-emulsifier) at 5% w/w. As shown in Figure 4, the optimal droplet size of 325 ± 15 nm with a polydispersity of 0.31 ± 0.02 was obtained when formulated with 8% w/w of ceteareth-10. In most cases, increasing the concentration of surfactant resulted in smaller droplet sizes due to the increase in the interfacial area and the decrease in the interfacial tension (17–19). However, these obtained results were unexpected. According to analysis of mean droplet size by PCS, the larger droplet size was observed when the amount of ceteareth-10 was increased. One possible explanation was the presence of aggregation resulting from the thermodynamic instability of the nanoemulsions. The small particle size possesses a high interfacial area that is energetically in the suboptimal state, consequently leading to agglomeration of small droplets in order to decrease the free energy of the system (20).

Effect of GMS (co-emulsifier) on mean droplet size. The effect of the amount of co-emulsifier, GMS mixed with a fixed concentration of emulsifier, ceteareth-10 (8% w/w), on the mean droplet size is shown in Figure 5. In the condition without GMS, the mean droplet size of the prepared nanoemulsion was 412 nm (data not shown). When its concentration was increased from 1% to 5% w/w, the mean droplet size was decreased from 413 to 325 nm. The detection of small droplet size by PCS indicated non-aggregation of the small droplets.

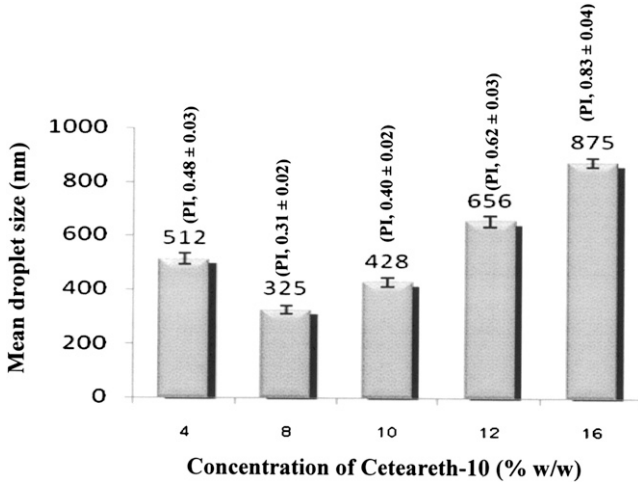

Figure 4. Effect of emulsifier ceteareth-10 (Brij 56®) concentration on the mean droplet size of the formulated nanoemulsions. The concentration of ceteareth-10 varied from 4% to 16% w/w, while that of GMS was fixed at 5% w/w. Each bar represents mean ± S.D. of three separately prepared batches.

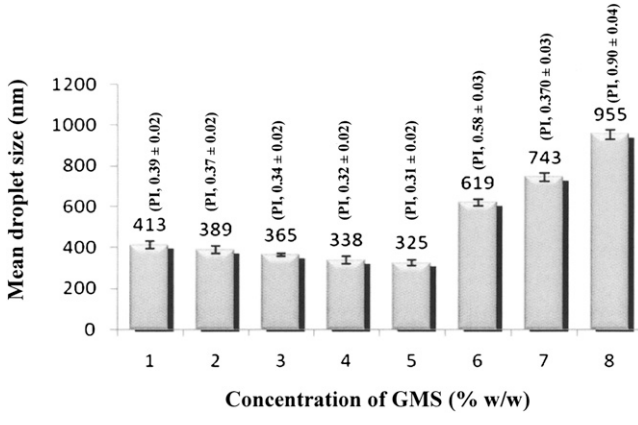

Figure 5. Effect of co-emulsifier GMS concentration on the mean droplet size of the formulated nanoemulsions. The concentration of GMS varied from 1% to 8% w/w, while that of ceteareth-10 (Brij 56®) was fixed at 8% w/w. Each bar represents mean ± S.D. of three separately prepared batches.

In general, GMS at an optimal concentration cannot only decrease the droplet size but also may stabilize the nano-droplet through steric hindrance of the monoester group (21,22) and/or the adsorbed co-emulsifier layer surrounding the droplet (10). In contrast, when the concentration of GMS was increased (more than 5% w/w), the mean droplet sizes and polydispersity values were obviously increased. The obtained results coincided with those of another study that indicated that increasing the GMS content above 5–10% resulted in larger particles and a broader particle size distribution (23). As GMS is a solid lipid, its high content in the formulation possibly leads to a decrease in homogenization efficiency and an increase in aggregation of the droplets.

From the above-mentioned study, the formulation consisting of 8% w/w ceteareth-10 and 5% w/w GMS provided the smallest droplet size (325 ± 15 nm), with low polydispersity values (0.31 ± 0.02). This combination was thus selected for nanoemulsion

preparation, and the prepared nanoemulsion was further studied for stability and *in vivo* depigmenting efficacy. The physical appearance and the transmission electron photomicrograph of the prepared nanoemulsion are shown in Figure 6.

STABILITY OF THE SELECTED NANOEMULSION FORMULA

Characteristics of the selected formula including pH, viscosity, mean droplet size, and the amount of artocarpin before and after stability testing are summarized in Table I. There was no significant difference in mean droplet size, viscosity, and pH values after three months of storage at room temperature (35° ± 3°C). The artocarpin remaining in the formula was about 93% w/w. However, after storing under accelerated conditions, employing seven heat-cool cycles, the percentage of artocarpin remaining was lower (about 86% w/w). Moreover, the mean droplet size of the nanoemulsion was increased. This phenomenon corresponded with its decreasing viscosity. Unlike microemulsions, which are thermodynamically stable, nanoemulsions are only kinetically stable. Being stored under stress possibly accelerated thinning or disruption of the layer of adsorbed co-emulsifier surrounding the droplet, consequently leading to flocculation or coalescence of the small droplets.

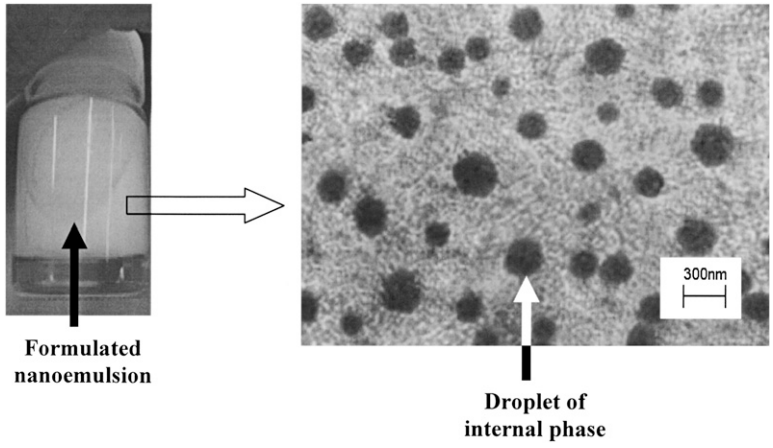

Figure 6. Physical appearance and transmission electron photomicrograph of the nanoemulsion formula consisting of 8% w/w ceteareth-10 (emulsifier), 5% w/w GMS (co-emulsifier), 41.6% w/w IPM, 0.05% w/w TEA, 0.03% w/w α-tocopherol, 0.03% w/w carbopol 940, 0.02% w/w *A. incisus* extract, and water adjusted to 100% w/w.

Table I
Characteristics of the Selected Nanoemulsion Formula Before and After Stability Testing

		After	
Characteristic	Before	Normal condition	Accelerated condition
Artocarpin content (% w/w)	0.014 ± 0.007	0.013 ± 0.003	0.012 ± 0.002
Mean droplet size (nm)	325 ± 15	347 ± 19	453 ± 27*
Viscosity (cps)	749 ± 17	726 ± 19	673 ± 20*
pH	5.52 ± 0.01	5.52 ± 0.01	5.53 ± 0.01

Student's *t*-test showed a significant difference of mean droplet size and viscosity between before-and-after stability testing, * $p < 0.05$.

DEPIGMENTING EFFICACY OF THE SELECTED NANOEMULSION FORMULA IN UVB-INDUCED HYPERPIGMENTATION OF C57BL/6 MICE

Figure 7 shows photographs of the depigmenting effect on the dorsal skin of the C57BL/6 mouse. As shown in Figure 7C, after treatment with the nanoemulsion containing the extract for four weeks, a greater decrease in hyperpigmentation was observed when compared with the extract prepared as a solution. Furthermore, a strongly visible decrease in hyperpigmentation was observed at week 6, as shown in Figure 7D. The melanin values measured by a Mexameter MX® 18 at the UV-induced hyperpigmented dorsal skin areas from the day after tanning through the six weeks of application are shown in Figure 8A. The nanoemulsion containing the extract decreased the degree of pigmentation (ΔM-value) by 84 ± 4 units while the extract solution decreased by 51 ± 3 units (Figure 8B). This indicated that the extract formulated into a nanoemulsion provided a higher skin-depigmenting potential than that prepared in solution form. Higher efficacy of the nanoemulsion is probably due to destabilization of the bilayer structure of the stratum corneum by permeation enhancers like IPM, ceteareth-10, and the GMS contained in the formula.

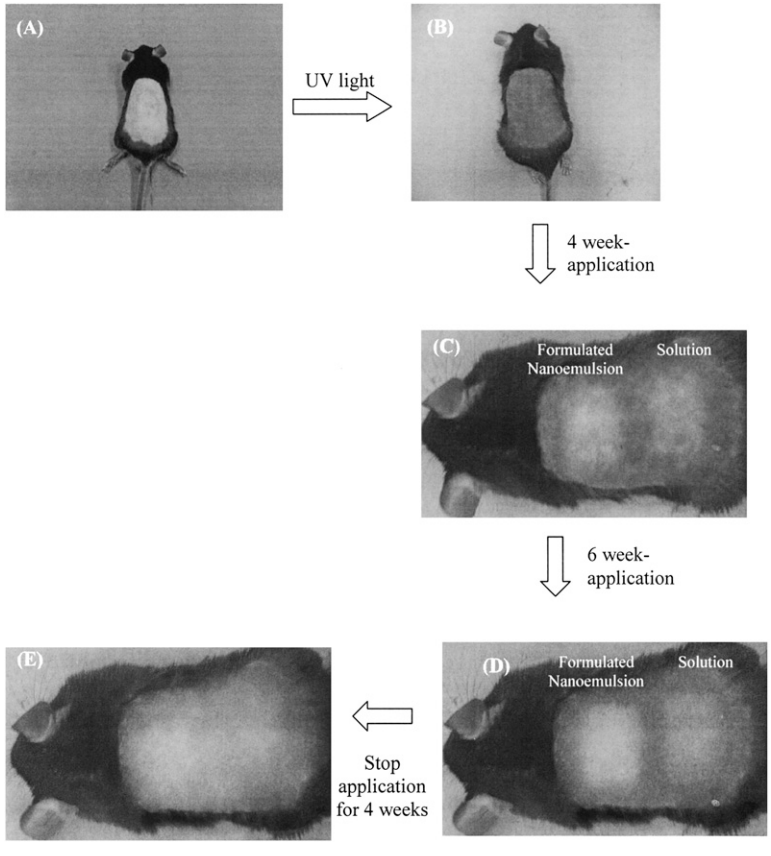

Figure 7. Effect of the selected nanoemulsion formula containing 0.02% w/w extract or the solution containing 0.02% w/w extract on improvement of UVB-induced hyperpigmentation in C57BL/6 mouse skin. All photographs were from the same animal: (A) before UV radiation; (B) initial pigmentation after UV irradiation; (C,D) after topical application for four weeks and six weeks, respectively; and (E) after application had been stopped for four weeks.

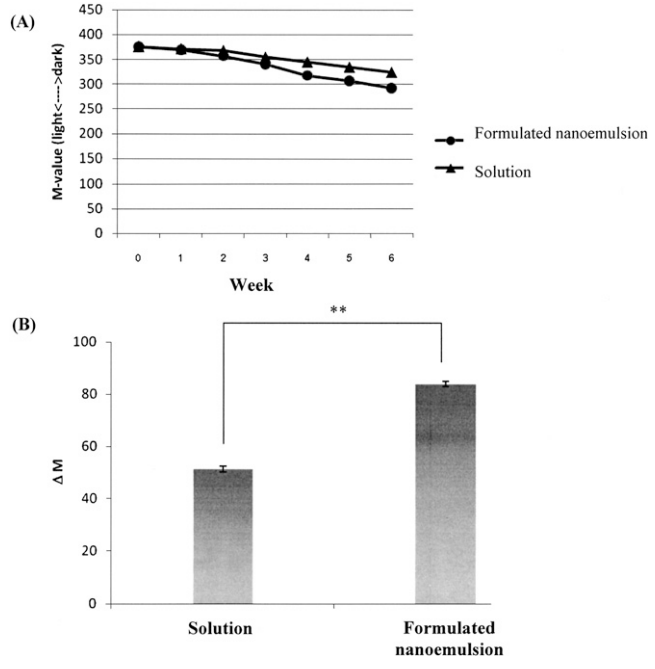

Figure 8. (A) Averaged melanin values measured by a Mexameter MX® 18 at the UV-induced hyperpigmented dorsal skin areas from the day after tanning (week 0) through six weeks of application. (B) The degree of depigmentation (ΔM-value) after application of the prepared nanoemulsion containing 0.02% w/w extract or the solution containing 0.02% w/w extract for six weeks. Each point or bar represents mean ± S.D. (n = 3). Student's *t*-test showed a significant difference between the two formulations, ** $p<0.01$.

Generally, most of the lipophilic agents pass the stratum corneum through an intercellular lipid bilayer pathway (24). The destabilization of such a lipid bilayer will enhance the lipid pathway's permeability to lipophilic agents such as artocarpin, the major component of the extract. Moreover, the small droplet size and low viscosity of the nanoemulsion make it an excellent carrier for enhancing percutaneous uptake of lipophilic compounds through the stratum corneum and/or hair follicles. This is because the number of internal droplets that can interact on a fixed area of the targeted sites will increase when the droplet size and viscosity decrease (25). Therefore, the higher efficacy of the nanoemulsion formulation could be the result of the combined effects of the enhancers of skin permeation as well as the small droplet size of the nanoemulsions.

As mentioned previously, artocarpin has depigmenting effects on UVB-induced hyperpigmentation on the dorsal skin of brownish guinea pigs (7). In the present study, therefore, it is possible to conclude that the melanogenesis-inhibitory activity of *A. incisus* extract is also involved with artocarpin. Generally, the ideal of depigmenting agents should have a potent, rapid, and selective bleaching effect on hyperactivated melanocyte cells and carry no short- or long-term side effects. The present *in vivo* study showed that reduced hyperpigmentation was observed following topical applications of the extract for four weeks and that the treated areas (UVB-induced hypigmentation) could return to close to their original color after stopping treatment for four weeks (Figure 7E). This finding implies that no permanent dysfunction of skin cells is caused by the extract of *A. incisus* when the optimal concentration is used. Furthermore, visible edema or scaling was

not observed at any dorsal skin sites treated with either the nanoemulsion containing the extract or the extract solution during any of the experimental days.

CONCLUSIONS

Nowadays, botanical extracts are playing an increasingly important role in cosmetics. Together with being used in suitable formulations, the efficacy of such extracts indicates their role as enhancers. Nanoemulsions are one of the attractive formula options for application in cosmetics, as their small droplet size can promote penetration of active ingredients into the skin.

Our study indicates that a formulated nanoemulsion containing *A. incisus* extract has the potential for application in skin depigmentation. It could act as a remedy for hyperpigmentation in UVB-induced hyperpigmentation. Side effects such as permanent depigmentation and edema were not found during application for six weeks. However, further clinical studies of the formulated product should be performed to ensure its efficacy and safety before marketing in the future.

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