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Inhibition of Melanogenesis by Dioctyl Phthalate Isolated from *Nigella glandulifera* Freyn

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Abstract Although a number of melanogenesis inhibitors have recently been reported and used as cosmetic additives, none is completely satisfactory, leaving a need for novel skin-depigmenting agents. Thus, to develop a novel skin-depigmenting agent from natural sources, the inhibition of melanogenesis by Chinese plants was evaluated. A methanolic extract of *Nigella glandulifera* Freyn was found to inhibit the melanin synthesis of murine B16F10 melanoma cells by 43.7% and exhibited a low cytotoxicity (8.1%) at a concentration of 100 μg/ml. Thus, to identify the melanogenesis-inhibiting mechanism, the inhibitory activity towards tyrosinase, the key enzyme of melanogenesis, was further evaluated, and the results showed inhibitory effects on the activity of intracellular tyrosinase yet not on mushroom tyrosinase. Finally, to isolate the compounds with a hypopigmenting capability, activity-guided isolation was performed, and Dioctyl phthalate identified as inhibiting melanogenesis.

Keywords: Dioctyl phthalate, depigmenting, *Nigella glandulifera* Freyn

Melanin, a ubiquitous class of biological pigments that are responsible for the color of human skin, eyes, and hair, is produced by specialized pigment cells known as melanocytes and deposited within discrete membrane-bound organelles called melanosomes [15]. Melanogenesis is started by the hydroxylation of tyrosine into DOPA, which is oxidized to dopaquinone. This first and rate-limiting step of melanin formation is mediated by tyrosinase, the key enzyme required for melanin production [5, 14]. Dopaquinone then undergoes a further multistep pathway to produce two types of melanin pigment: black-brown eumelanins and yellow to reddish pheomelanins. Human skin color then depends on the size, number, shape, and distribution of melanosomes, along with the chemical nature of their melanin content [6, 9, 13].

Although melanin plays an important role in protecting skin from photo damage, the increased production and accumulation of melanin in the skin characterize a large number of skin diseases, including acquired hyperpigmentation, such as melasma, postinflammatory melanoderma, and solar lentigo [19]. Therefore, various skin-whitening agents have been developed to control melanogenesis. Melanin biosynthesis can be inhibited by avoiding UV exposure, inhibiting melanocyte metabolism, and regulating melanogenic enzymes or the uptake and distribution of melanosomes in recipient melanocytes [2, 8, 12, 16].

A number of melanogenesis inhibitors have already been reported and since used as cosmetic additives. However, many are of limited effectiveness, difficult to formulate, and even cause reactions or side effects after long-term usage. Thus, attention has recently been focused on the use of natural products in cosmetics [1, 7, 10]. Therefore, on the basis of its longstanding safety record, the evaluation of Chinese herbal medicine as regards the treatment of skin pigmentation abnormalities may be beneficial for the development of new and more efficient remedies. Accordingly, this study examined the effect of *Nigella glandulifera* Freyn on melanogenesis using cultured B16F10 mouse melanoma cells. *Nigella glandulifera* (Ranunculaceae) is an annual erect herbaceous plant that is widely found in the southwest and west of China. *N. glandulifera* seeds are commonly eaten in many food preparations by Uigur, plus the seeds are believed to have diuretic, analgesic, spasmolytic, galactagogue, and bronchodilator properties, and to cure edema, urinary calculus, and bronchial asthma [11]. In the current study, the skin-whitening efficacy of *N. glandulifera* seeds was examined and the active compounds further separated to identify the mechanism of depigmentation.

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MATERIALS AND METHODS

Materials and Reagents

The L-DOPA (3,4-dihydroxy-L-phenylalanine), DMSO (dimethyl sulfoxide), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and mushroom tyrosinase (50KU) were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and the DMEM media, fetal bovine serum, 0.5% trypsin-EDTA, phosphate-buffered saline (PBS), and penicillin/streptomycin were from Invitrogen Corp. (CA, U.S.A.). The analytical grade HPLC solvents were from J. T Baker U.S.A.

Extraction and Isolation

The dried seeds were minced using a grinder (Mill Powder Tech Solution, Taiwan), and the sample powder (1 kg) was extracted three times with four volumes of 95% MeOH for 24 h. The resulting mixtures were then filtrated and concentrated to dryness at 40°C under a vacuum to produce the MeOH extract (140 g). Thereafter, the methanol extract (20 g) was separated using silica gel column chromatography with a hexane:chloroform system (5:5→0:10) or chloroform: methanol system (10:0→0:10) to give further fractions. Fractions showing good inhibitory effects on melanogenesis were further purified by silica gel column chromatography using a hexane: chloroform gradient (1:0→0:1) to yield purified compounds.

Cell Culture

The B16F10 murine melanoma cells were purchased from the ATCC (American Type Culture Collection) and cultured in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (100 units/ml) at 37°C in a humidified atmosphere with 5% CO_2. The cells were passed every 3 days until a maximal passage number of 30 was achieved.

Cell Viability Assay

The cell viability was determined using an MTT assay. The cells were seeded into a 96-well plate at a density of 2.5×10^3 cells per well. After 24 h of incubation, the culture medium was removed and a new medium containing the test substance added to each well in quintuplicate. After 2 days of incubation, the cell viability was assayed using an MTT solution. The percentages of viable cells in each well were calculated relative to the optical density (OD) value of living cells in the control group (100%).

Measurement of Melanin

The cells were seeded into a 6-well plate (Falcon, U.S.A.) at a density of 6×10^4 cells per well. After 24 h, the medium was replaced with a fresh medium containing various concentrations of the compounds. After 2 days of incubation, the adherent cells were washed with phosphate-buffered saline (PBS) and detached from the plate using 0.05% trypsin-EDTA. The cells were collected in a test tube, washed twice with PBS, and the cell number determined using tryptan blue. The melanin from 5×10^5 cells was then extracted using a mixture of 1 N NaOH: 10% DMSO at 80°C for 1 h. After centrifugation at 3,000 rpm for 5 min, the melanin content was determined at 475 nm using an ELISA (enzyme-linked immunosorbent assay) microplate reader.

Assay of Tyrosinase Activity in Cultured B16F10 Melanoma Cells

The tyrosinase activity was assayed as the dopa oxidase activity. The B16F10 cells were seeded in 6-well plates at a density of 6×10^4 cells per well and cultured for 24 h. After being treated with the samples for 48 h, the cells were washed with PBS and lysed with a lysis buffer (0.1 M phosphate buffer, pH 6.8, containing 1% Triton X-100). The cells were then disrupted by sonication for 1 h at 4°C, and the lysates clarified by centrifugation at 13,000 rpm for 20 min. After quantifying the protein content using a protein assay kit (Bio-Rad, U.S.A.), the cell lysates were adjusted to the same amount of protein with a lysis buffer. Reaction mixtures consisting of 40 μg of protein, 40 μl of 5 mM L-DOPA, and 0.1 M PBS (pH 6.8) were assayed on a 96-well plate at 37°C, and the absorbance measured at 475 nm using an ELISA reader every 10 min for 1 h.

Assay of *In Vitro* Tyrosinase Activity

To assay the mushroom tyrosinase activity, reaction mixtures consisting of 100 μl of a sample, 22 units of mushroom tyrosinase, 40 μl of 5 mM L-DOPA, and 0.1 M PBS (pH 6.8) were assayed on a 96-well plate at 37°C. After 20 min, the absorbance was measured as described above. Kojic acid was used as a positive control and the inhibitory percentage of mushroom tyrosinase was calculated as follows:

$$\% \text{ inhibition} = \{[(A-B)-(C-D)]/(A-B)\}\times100$$

A: OD at 475 nm without test substance and with mushroom tyrosinase
B: OD at 475 nm without test substance and mushroom tyrosinase
C: OD at 475 nm with test substance and mushroom tyrosinase
D: OD at 475 nm with test substance and without mushroom tyrosinase

For the experiments measuring the direct inhibitory effect on murine tyrosinase, the total protein was isolated from the B16F10 melanoma cells and used as the tyrosinase enzyme source. In a 96-well plate, the same amounts of protein (40 μg) were reacted with 5 mM L-DOPA and 0.1 M PBS (pH 6.8) on the samples at 37°C. After 30 min, the absorbance was measured at 475 nm. The inhibitory percentage of murine tyrosinase was then calculated as described above.

Statistical Analysis

The data were expressed as mean values ±SD, and statistically significant differences from the control analyzed using a Student's *t*-test.

RESULTS

Methanol Crude Extract of *N. glandulifera* Inhibited Melanin Synthesis in B16F10 Melanoma Cells

The effect of *N. glandulifera* on melanogenesis in B16F10 melanoma cells was evaluated after 48 h of incubation (Fig. 1). Concentrations of more than 25 μg/ml significantly inhibited melanin synthesis, and the IC_{50} of the depigmenting effect was calculated as 118.5 μg/ml. A toxicity related to the crude extract was observed at a concentration of more than 100 μg/ml (Fig. 2).

Inhibitory Effect of Crude Extract on Tyrosinase Activity

Two kinds of tyrosinase, intracellular tyrosinase extracted from the B16F10 melanoma cells and mushroom tyrosinase, were used in this study. From the results of the *in vitro* tyrosinase assay, the direct effect on the catalytic activities of murine tyrosinase is shown in Fig. 3. The cellular tyrosinase activity was not significantly affected when varying the concentration of the crude extract, whereas the mushroom tyrosinase activity remained unaffected with concentrations of up to 150 μg/ml (data not shown). However, as shown in Fig. 4, in the cell-based assay, the reaction of the cellular tyrosinase extracted from the treated cells showed a decrease in absorbance at 475 nm. Therefore, when compared with the nontreated cells, treatment with the crude extract at a concentration of

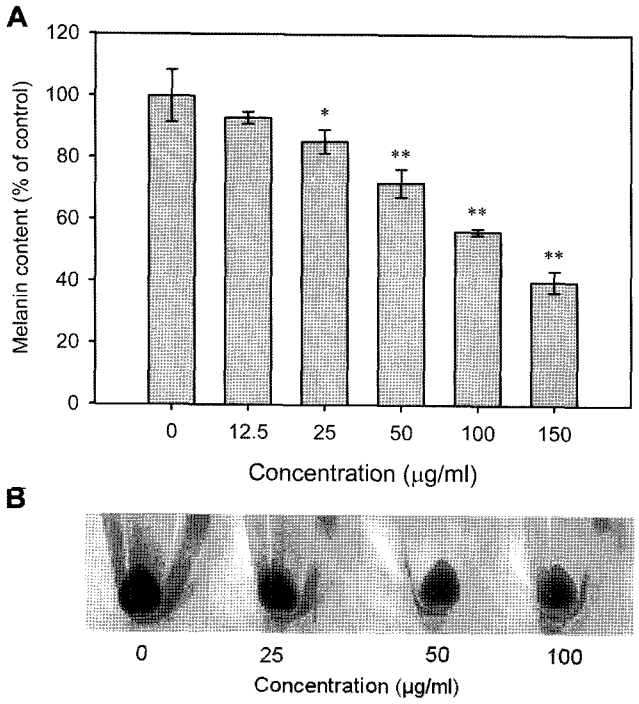

Fig. 1. Effect of crude extract of *N. glandulifera* seeds on melanin synthesis in cultured B16F10 melanoma cells. **P*<0.05, ***P*<0.01: statistically significant *vs.* value of control group.
A. B16F10 cells were treated with the crude extract (12.5 to 150 μg/ml) for 2 days, and then harvested, pelleted, and the melanin contents assayed, as described in Materials and Methods. Each bar represents the means±SD of three separate experiments. **B.** Photograph of effects of *N. glandulifera* on melanin synthesis of B16F10 melanoma cells.

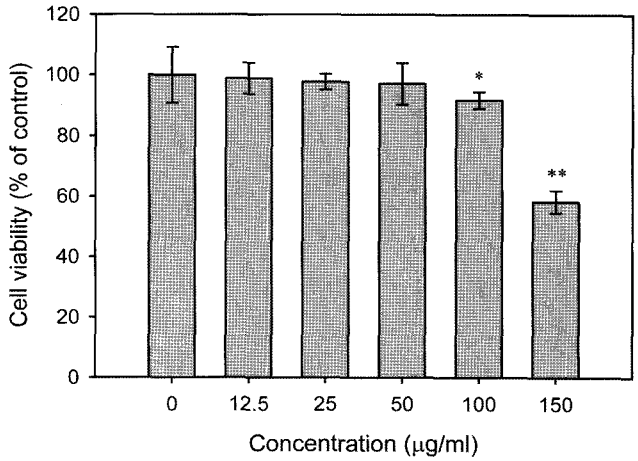

Fig. 2. Cell viability of B16F10 melanoma cells treated with crude extract of *N. glandulifera* seeds.
B16F10 cells were treated with the crude extract (0 to 150 μg/ml) for 2 days. The formazan precipitates were then quantitated based on the absorbance at 540 nm, and the viabilities expressed as a percent of the control. Each bar represents the means±SD of five separate experiments. **P*<0.05, ***P*<0.01: statistically significant *vs.* value of control group.

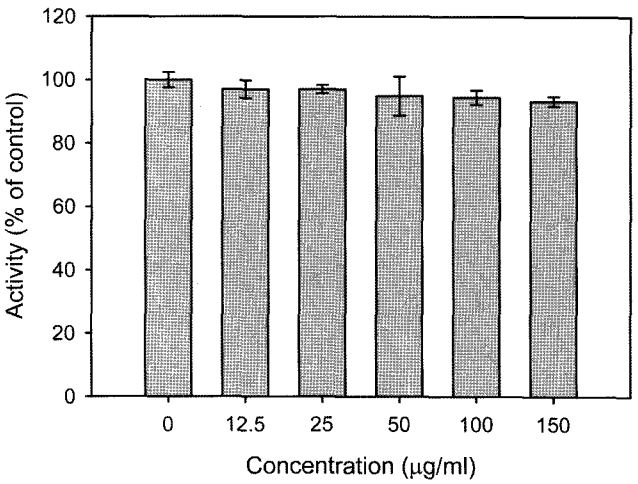

Fig. 3. Effect of various concentrations of crude extract of *N. glandulifera* seeds on catalytic activity of cellular tyrosinase.
B16F10 cells were treated with the crude extract (0 to 150 μg/ml) for 2 days, harvested, and tyrosinase extracted from the treated cells. The cellular tyrosinase was then incubated with 40 μl of 5 mM L-DOPA at 37°C for 20 min. Each bar represents the means±SD of three separate experiments.

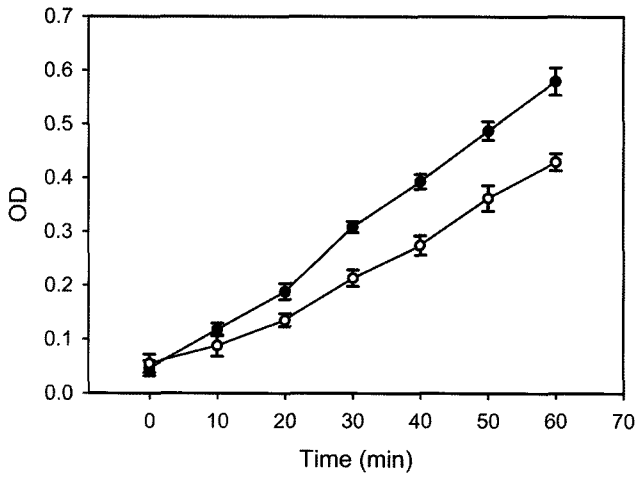

Fig. 4. Effect of crude extract of *N. glandulifera* seeds on cellular tyrosinase.
B16F10 cells were treated with the crude extract at 100 μg/ml for 2 days, harvested, and tyrosinase was extracted from the treated cells. The cellular tyrosinase was then incubated with 40 μl of 5 mM L-DOPA at 37°C, and then the dopachrome contents were measured at 475 nm. Each bar represents the means±SD of three separate experiments. (●): Untreated, (○): 100 mM of crude extract.

100 μg/ml resulted in a 34.7% inhibition of intracellular tyrosinase activity in the B16F10 melanoma cells.

Extraction and Isolation

The extraction and isolation were performed as described in Materials and Methods. Among the six fractions finally collected, fraction 2 (4.5 g) and fraction 4 (3.1 g) showed

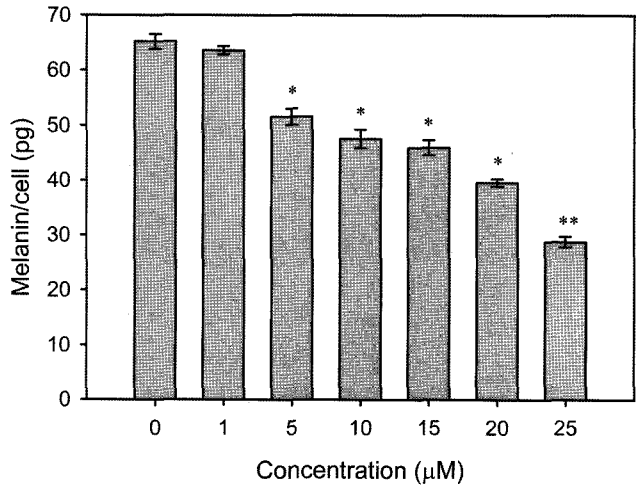

Fig. 5. Inhibitory effect of DOP on melaninogenesis of cultured B16F10 melanoma cells.
B16F10 cells were treated with DOP (0 to 25 μM) for 2 days, and then harvested, pelleted, and the melanin contents assayed, as described in Materials and Methods. The melanin contents were expressed as a percent of the control. Each bar represents the means±SD of three separate experiments. *P<0.05, **P<0.01: statistically significant *vs.* value of control group.

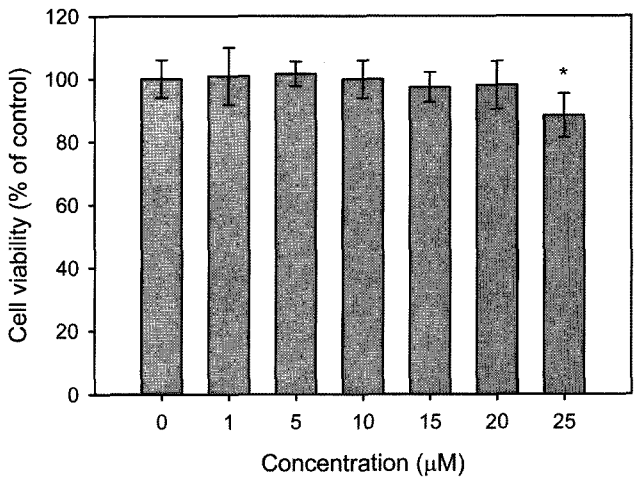

Fig. 6. Effect of DOP on cell viability of cultured B16F10 melanoma cells. *P<0.05.
B16F10 cells were treated with the crude extract (0 to 150 μg/ml) for 2 days. The formazan precipitates were then quantitated based on the absorbance at 540 nm, and the viability expressed as a percent of the control. Each bar represents the means±SD of five separate experiments. *P<0.05: statistically significant *vs.* value of control group.

an inhibitory effect on melanogenesis (data not shown). Fraction 2, the major compounds, was further purified by silica gel column chromatography with a gradient of hexane and chloroform to yield compound 1 (0.62 g). Finally, compound 1 was identified as Dioctyl phthalate (data not shown) by LC-MS, ^{1}H, and ^{13}C NMR.

Effect of Dioctyl Phthalate (DOP) on Melanogenesis of B16F10 Melanoma Cells

Among the active compounds isolated from *N. glandulifera*, Dioctyl phthalate (DOP) significantly inhibited the

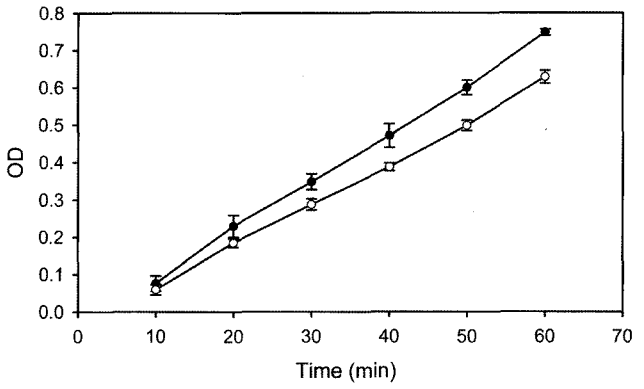

Fig. 7. Effect of DOP on cellular tyrosinase activity of B16F10 melanoma cells.
B16F10 cells were treated with DOP (0 and 10 μM) for 2 days, harvested, and tyrosinase extracted from the treated cells. The cellular tyrosinase was then incubated with 40 μl of 5 mM L-DOPA at 37°C and the dopachrome contents measured at 475 nm. Each bar represents the means±SD of three separate experiments. (●): Untreated, (○): 10 mM of DOP.

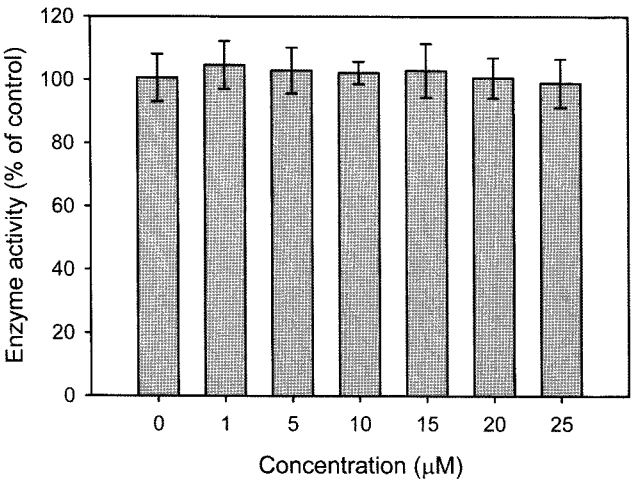

Fig. 8. Effect of DOP on catalytic activity of B16F10 murine tyrosinase.
B16F10 cells were treated with the crude extract (0 to 25 μM) for 2 days, harvested, and tyrosinase extracted from the treated cells. The cellular tyrosinase was then reacted with 40 μl of 5 mM L-DOPA at 37°C for 20 min and the dopachrome contents measured at 475 nm. Each bar represents the means±SD of three separate experiments.

melanogenesis of the cultured B16F10 cells (IC_{50}=24 μM) (Fig. 5), whereas the cell viabilities were not reduced at effective concentrations (Fig. 6). Finally, DOP inhibited 25.8% of the cellular tyrosinase activity in the treated B16F10 melanoma cells at a concentration of 10 μM (Fig. 7), yet did not directly inhibit the catalytic activity of cellular tyrosinase (Fig. 8). These results showed very similar trends to those for the crude seed extract.

DISCUSSION

In the current study, the crude extract of *N. glandulifera* seeds showed a dose-dependent inhibitory effect on the melanin synthesis of melanoma B16F10 cells (IC_{50}= 118.5 μg/ml). In *in vitro* assays, the L-DOPA oxidation activities of murine tyrosinase and mushroom tyrosinase were not directly affected. However, a significant inhibitory effect (34.7%) on the cellular tyrosinase activity of treated cells was found at a concentration of 100 μg/ml. Therefore, the present results suggest that the depigmenting effect of the sample worked through suppressing the synthesis, a posttranslational process, or stimulation of the degradation of tyrosinase. However, the effective dose of the crude extract exhibited some toxicity towards the B16F10 melanoma cells.

To identify the compounds involved in the melanin-inhibiting effect of the crude extract, further guided isolation was performed to yield DOP. Additional investigation of DOP in relation to melanogenesis, toxicity, and tyrosinase activity revealed that this compound had a potent inhibitory effect on the melanogenesis of B16F10 melanoma cells (IC_{50}=24 μM). Moreover, the compound also showed the same action mode as the crude extract of *N. glandulifera* seeds. DOP inhibited the murine tyrosinase activity in B16F10 melanoma cells by 25.8% at a concentration of 10 μM without directly inhibiting the catalytic activity of murine tyrosinase.

DOP (also known as di-2-ethylhexyl phthalate) is a plasticizer that is primarily used in polyvinyl chloride resins. In isolation experiments, the presence of DOP can be related to the extraction solvent, as DOP is usually found in alkylogen solvents [4]. However, this amount is negligible when compared with the DOP isolated from the dried seeds of *N. glandulifera* (0.62 g/kg) in the present study. Usually, the presence of DOP in plants is from environmental pollution, such as the release of DOP from plastic films. DOP has already been found in many vegetables, such as Chinese tomato fruit and cabbage, yet the cumulative content is less than 0.01 g/kg fresh weight [3]. Therefore, the higher DOP content in the dried seeds of *N. glandulifera* may indicate that this plant possesses a stronger ability to accumulate DOP. As such, the exact depigmentation mechanism of DOP and the effects of the other compounds isolated from the *N. glandulifera* seeds are being further investigated. However, for other pigmentation studies, researchers should consider that DOP may influence the results as a contaminant of solvents or plants. Furthermore, many functional cosmetic products have recently been sold in the market without any confirmation of their exact components. Therefore, the present study suggests that plants extracts with a depigmenting effect should be evaluated as regards the components used in the cosmetic formulation, as DOP was recently classified as an endocrine disrupter and its use prohibited [17, 18].

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