Korean J. Chem. Eng., **28**(4), 1070-1073 (2011)
DOI: 10.1007/s11814-010-0457-3

Identification of depigmenting components from *Nigella glandulifera* Freyn

Dung Nguyen Hoang, Ji-Ean Lee, and Eun-Ki Kim†

Department of Biological Engineering, Inha University, National Research Lab of Skinbioactive Materials, 253 Yonghyun-dong, Nam-gu, Incheon 402-751, Korea
(*Received 2 June 2010 • accepted 14 October 2010*)

Abstract−To develop a novel skin depigmenting agent from natural sources, the inhibition of melanogenesis by Chinese plants, *N. glandulifrea*, was evaluated. The methanol extract of this plant showed significantly down-regulated melanin synthesis in a dose-dependent manner at a non-toxic concentration in cultured B16F10 mouse melanoma cells. This extract was further fractionated by using solvent-solvent partition and silica open column chromatography to identify the active components. From GC-MS data, oleic acid methyl ester was found as one of the depigmenting agents. In conclusion, we suggest that this fraction may be a safe and effective depigmentation agent.

Key words: Melanogenesis, Depigmenting, Identification

INTRODUCTION

Melanin is the phenolic biopolymer that is responsible for pigmentation [1]. Various skin colors for individuals are mostly determined by types, amount, and arrangement of melanin, which is synthesized by epidermal melanocytes. Melanin plays an important role in preventing ultraviolet (UV) light-induced skin damage. However, increased levels of epidermal melanin synthesis can darken the skin. Various dermatologic disorders result in the accumulation of excessive levels of epidermal pigmentation. Because of the visible nature of dermatologic diseases, they have a considerable psychological effect on affected patients [2]. A number of melanogenesis inhibitors such as arbutin, kojic acid, hydroquinone have already been reported and used in cosmetics. However, the clinical effects of those chemicals are unsatisfactory because of their low activity and cell toxicity [3-5]. Therefore, it is clearly necessary to find safer and more effective skin-whitening agents. Much effort has been devoted toward developing novel agents, especially from traditional herbs because of their safety and various bioactive components such as sugars, fatty acid, flavonoids, tannins, saponins [6-10].

Nigella glandulifera (*Ranunculaceae*) is an annual erect herbaceous plant that is widely found in the southwest and west of China. The whole herb has been used as a folk remedy for treatment of colds, cough and insomnia. *N. glandulifera* seeds are commonly eaten in many food preparations by the Uigur people in South West China. The seeds are believed to have diuretic, analgesic, spasmolytic, galactagogue, bronchodilator properties, and to cure edema, urinary calculus, and bronchial asthma [11,12]. However, little work has been done regarding the effects of active compounds isolated from *N. glandulifera* against melanin synthesis activity, despite its excellent pharmacological action.

In our previous study, dioctyl phthalate isolated in *N. glandulifera* was found to inhibit melanin synthesis by inhibiting the cellulose tyrosinase activity in B16F10 melanoma cells [13]. In an effort to find another skin whitening active compound, activity-guided isolation was performed.

MATERIALS AND METHODS

1. Materials

Dulbecco's modified eagle medium, fetal bovine serum, trypsin-EDTA, Phosphate buffered saline (PBS), Penicillin/streptomycin were purchased from Invitrogen Corp. (CA, U.S.A). Arbutin, DMSO (dimethyl sulfoxide), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, U.S.A). Methanol, hexane, chloroform (99.5% purity) was purchased from Samchun Chemical Co. (South Korea). Analytical grade HPLC solvents were purchased from J.T Baker U.S.A. Silica gel 60 (0.063-0.2 mm) was purchased from Merck.

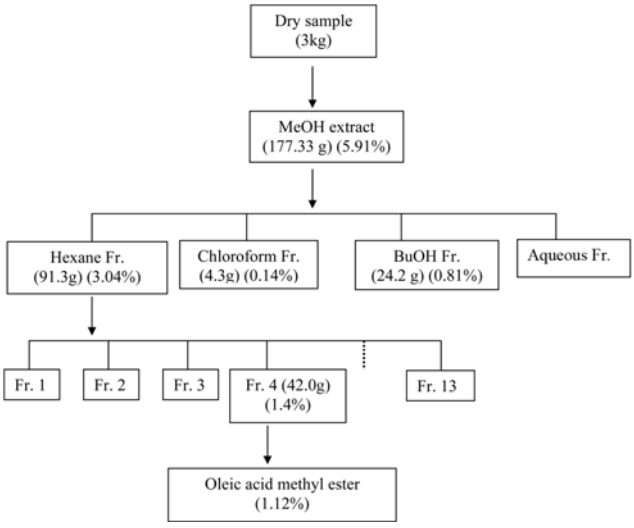

Fig. 1. The fractionation scheme of the methanol extract of *N. glandulifera*.

†To whom correspondence should be addressed.
E-mail: ekkim@inha.ac.kr

2. Extraction Methods

The *Nigella glandulifera* was minced and extracted three times with 99.5% Methanol (MeOH) for 24 h at room temperature. The resulting mixture (177.3 g) was filtered and evaporated at 35 °C under vacuum condition. Subsequently, the methanol extract was successfully partitioned with hexane, chloroform, butanol to obtain the hexane (91.3 g), chloroform (4.3 g) and butanol fraction (24.2 g). The hexane fraction was further chromatographed on a silica gel column with hexane-ethylacetate as mobile phases to obtain thirteen fractions. The fraction number four (42.0 g) was analyzed by GC-MS and oleic acid methyl ester (33.67 g) was identified as the main component (Fig. 1).

3. GC-MS Analysis

The component in fraction four was determined by using a GC model fitted with DB-23 fused silica capillary column (30 m×0.25 mm i.d.: 0.2 μM) and equipped with an FID. The temperatures of the injection port and of the detector were set at 250 °C and 275 °C. The oven temperature was increased from 150 to 250 °C at a rate of 10 °C/min, maintained for 1 min at 150 °C, then increased at 10 °C/min to 250 °C and maintained for 10 min at 250 °C. The carrier gas was helium at a flow rate of 20 mL/min.

The mass spectrometer was fitted with EI source operated 70 eV, and mass spectra were recorded in the m/z range of 35-400 a.m.u. in the full-scan acquisition mode. Compounds were identified by comparison of the obtained mass spectra of the relevant chromatographic peaks with spectra of the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) libraries. The main active compound in fraction 4, oleic acid methyl ester, was confirmed by comparing its retention times with authentic standard.

4. 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 subcultured every 3 days until a maximal passage number of 20 was achieved.

5. Melanin Assay

To measure melanin inhibition, B16F10 murine melanoma cells were used. B16 cells were seeded into 12-well plates at density of 1.5×10^3 cells/well. After 24 h, triplicate cultures were fed with fresh media and samples. After 48 h, cells were detached by trypsin/EDTA and harvested. Cell suspensions were then centrifuged for 5 min at 5,000 rpm, washed with PBS and then solubilized in 200 μl of extraction buffer (1 N NaOH, 10% DMSO), heated at 80 °C for 1 h and transferred to 96-well plates. Relative melanin content was determined by absorbance at 405 nm in ELISA reader [14].

6. Cell Toxicity Assay

MTT assay was used to determine cell toxicity. After treatment and incubation of the samples for 48 hr, the medium was removed and 100 μl of 0.5 mg/mL MTT solution was added to each well and incubated at 37 °C for 4 h. The MTT solution was then removed and 200 μl DMSO was added to each well. The formazan formation was measured by absorbance at 570 nm in ELISA reader [14].

7. Statistical Analysis

All determinations were run in triplicate on at least two different experiments, and the results were reported as the mean and standard deviation. The statistically significant differences from the control were analyzed by the Student's *t*-test.

RESULTS AND DISCUSSION

1. Effect of Methanol Extract on Melanin and Cell Viability

Desirable skin-whitening agents should inhibit the synthesis of

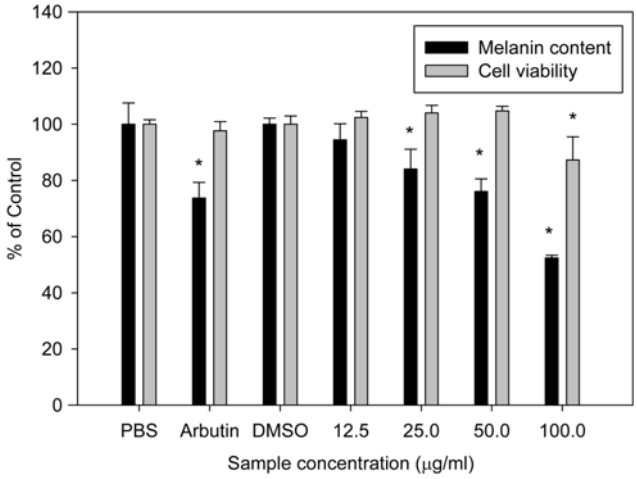

Fig. 2. Effect of methanol extract of *N. glanduliferea* on melanin synthesis in B16F10 melanoma cells. B16F10 cells were treated with Methanol extract of *N. glandulifera* (0 to 100 μg/ml) for 2 days and the cells were then harvested. Melanin content and cell viability were measured as described in Materials and Methods. Data were expressed as the change of the melanin content level and cell viability relative to untreated control. Each determination was made in triplicate and data shown are means±S.D. *P<0.05: statistically significant vs. value of control group.

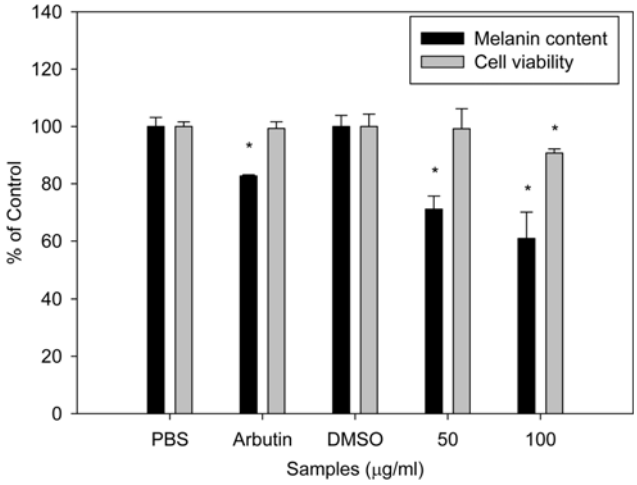

Fig. 3. Effect of hexane fraction on melanin synthesis in B16F10 melanoma cells. B16F10 cells were treated with Hexane fraction (0 to 100 μg/ml) for 2 days and the cells were then harvested. Melanin content and cell viability were measured as described in Materials and Methods. Data were expressed as the change of the melanin content level and cell viability relative to untreated control. Each determination was made in triplicate and data shown are means±S.D. *P<0.05: statistically significant vs. value of control group.

melanin in melanosomes by acting specifically to reduce the synthesis. They should also exhibit low cytotoxicity and be non-mutagenic. To investigate the effect of *N. glandulifera* in melanin synthesis, the melanin content in B16F10 melanoma cells was measured after being treated with different concentrations of sample. The results showed that the methanol extract of this plant can significantly inhibit melanogenesis in a dose-dependent manner. To confirm that the melanin inhibition activity is not caused by cell toxicity, an MTT assay was performed. The results showed that the methanol extract did not show any toxicity up to 100 μg/ml (Fig. 2).

2. Effect of Hexane Fraction on Melanin Synthesis and Cell Viability

To find the active fraction having melanin inhibition, solvent-solvent partition was done. After successful partition by using hexane, chloroform, butanol, these fractions were examined for their effect on melanin and cell viability. Among these fractions, hexane fraction showed significantly effect on melanin inhibition in a dose-dependent manner. This fraction also exhibited low toxicity up to the concentration 100 μg/ml (Fig. 3).

3. Effect of Fraction Four on Melanin Synthesis and Cell Viability

Because of high inhibition on melanin synthesis, the hexane fraction was further separated to find the main active components. By using silica open column chromatography with hexane-ethylacetate as the eluents, thirteen fractions were obtained. The fraction four showed significant inhibition on melanin synthesis with low toxicity up to 12.5 μg/ml (Fig. 4). By using GC-MS analysis and comparing the retention time with authentic standards, oleic acid methyl ester; 2,5-Cyclohexadien-1,4-dione,2-methyl-5-(1-methylethyl); phenol, 2-methyl-5-(1-methylethyl); 11,14-Eicosadienoic acid methyl ester; ether, p-menth-6-en-2-yl methyl were identified (Fig. 5 and

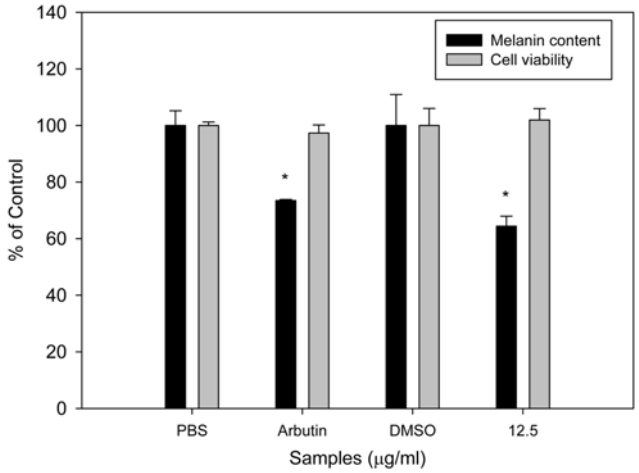

Fig. 4. Effect of fraction 4 on melanin synthesis in B16F10 melanoma cells. B16F10 cells were treated with fraction 4 (0 to 25 μg/ml) for 2 days and the cells were then harvested. Melanin content and cell viability were measured as described in Materials and Methods. Data were expressed as the change of the melanin content level and cell viability relative to untreated control. Each determination was made in triplicate and data shown are means±S.D. *P<0.05: statistically significant vs. value of control group.

Table 1). Oleic acid is known as a melanin inhibitor. This unsaturated fatty acid inhibits melanin synthesis in B16F10 melanoma cell and inhibits UVB-induced hyperpigmentation in brownish guinea pigs by inhibiting the effect of tyrosinase activity. It also stimulates the rate of turnover of the stratum corneum [15,16]. Since oleic acid methyl ester is the ester form of oleic acid, we also investigated the

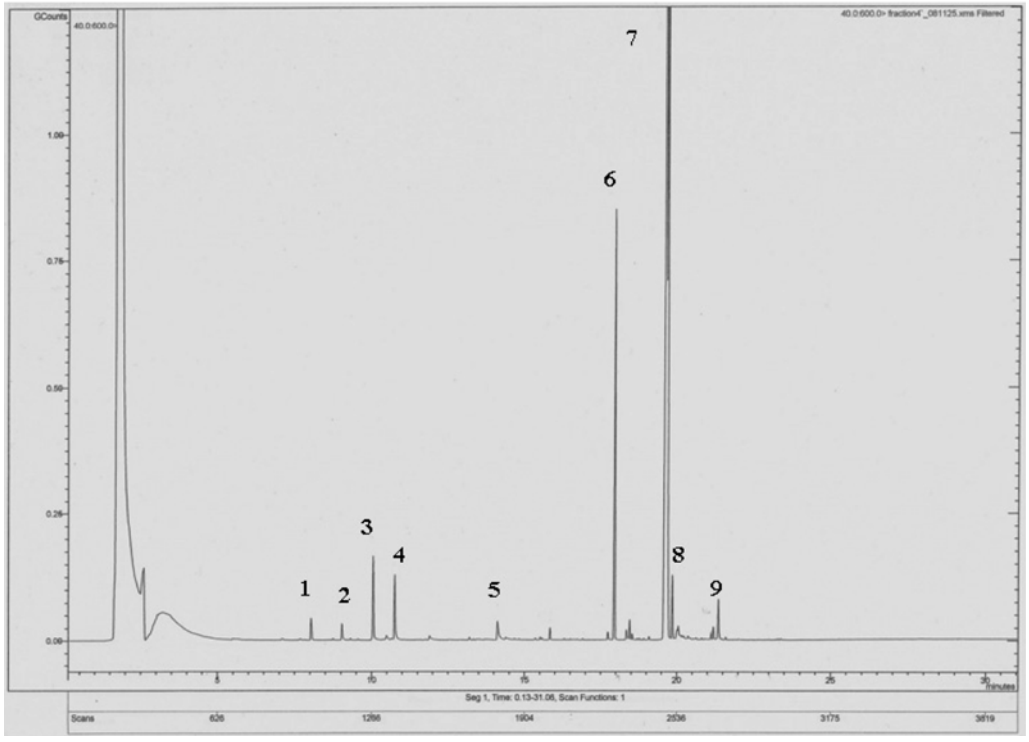

Fig. 5. GC spectrum of fraction four extracted from *N. glandulifera*.

Table 1. Chemical composition of fraction four extracted from *N. glandulifera*

Peak No	Compounds	Retention time (min)	Composition* (%)
1	Ether, p-menth-6-en-2-yl methyl	8.020	0.93
2	3-Cyclohexane-1-ol, 4-methyl-1(1-methylethyl)	9.054	<0.001
3	2,5-Cyclohexadien-1,4-dione, 2-methyl-5-(1-methylethyl)	10.061	2.59
4	Phenol, 2-methyl-5-(1-methylethyl)	10.767	2.24
5	2-Methoxy-4-ethyl-6methylphenol	14.142	<0.001
6	Palmitic acid methyl ester	17.949	12.50
7	Oleic acid methyl ester	19.637	80.16
8	Heptadecanoic acid, 16-methyl-, methyl ester	19.837	<0.001
9	9-Octadecenoic acid (Z)	20.021	1.59

*Relative area percentage (peak area relative to total peak area %)

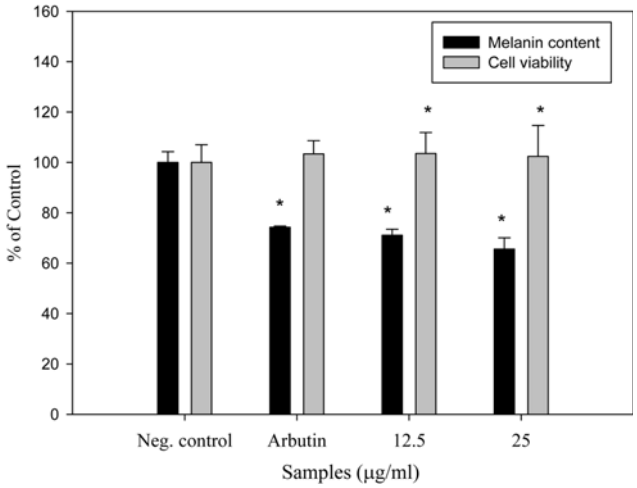

Fig. 6. Effect of oleic acid methyl ester on melanin synthesis in B16F10 melanoma cells. B16F10 cells were treated with oleic acid methyl ester (0 to 25 μg/ml) for 2 days and the cells were then harvested. Melanin content and cell viability were measured as described in Materials and Methods. Data were expressed as the change of the melanin content level and cell viability relative to untreated control. Each determination was made in triplicate and data shown are means±S.D. *P<0.05: statistically significant vs. value of control group.

effect of this compound in B16F10 melanoma cells (Fig. 6). The results showed that oleic acid methyl ester also inhibited melanin synthesis in the dose dependent manner without toxicity. In *N. glandulifera*, the yield of isolated oleic acid methyl ester is 1.12% (w/w) and 80.16% in fraction four (w/w). Because oleic acid methyl ester is the main active component in *N. glandulifera*, it is expected that the melanin inhibition activity is due to the activity of oleic acid methyl ester

CONCLUSION

The methanol extract of *N. glandulifera* seeds showed a dose-dependent inhibitory effect on the melanin synthesis of melanoma B16F10 cells without toxicity. To identify the compounds involved in the melanin inhibiting effect of the methanol extract, solvent-solvent partition, silica gel open column chromatography and GC-MS analysis were used. Oleic acid methyl ester was found as the main component that has depigmenting activity in this plant. In conclusion, *N. glandulifera* can be used as skin whitening agent.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the Notional Research Foundation of Korea (NRF) funded by the Ministry of Education Science and Technology (ROA-2007-00-10015-0).

REFERENCES

1. P. A. Riley, *Pigment Cell Res.*, **16**, 548 (2003).
2. B. Halioua, M. G. Beumont and F. Lune, *Int. J. Dermatology*, **39**, 801 (2000).
3. L. Petit and G. E. Piérard, *Int. J. Cosmetic Sci.*, **25**, 169 (2003).
4. S. C. Taylor, *Skin Pharm. Appl. Skin Phys.* **12**, 139 (1999).
5. V. Zawa and S. T. Mhaskar, *J. Cosmet Dermatol*, **3**, 234 (2004).
6. S. Briganti, E. Camera and M. Picardo, *Pigment Cell Res.*, **16**, 101 (2003).
7. F. Solano, S. Briganti, M. Picardo and G. Ghanem, *Pigment Cell Res.*, **19**, 550 (2006).
8. W. Zhu and J. Gao, *J. Investig. Dermatol. Symp. Proc.*, **13**, 20 (2008).
9. K. H. Wang, R. D. Lin, F. L. Hsu, Y. H. Huang, H. C. Chang, C. Y. Huang and M. H. Lee, *J. Ethnopharmacol.*, **106**, 353 (2006).
10. H. I. Lee, B. S. Yoo, M. A. Yoo and S. Y. Byun, *Korean J. Chem. Eng.*, **24**, 655 (2007).
11. Y. M. Liu, J. S. Yang and Q. H. Liu, *Chem. Pharm. Bull.*, **52**, 454 (2004).
12. Z. Tian, Y. M. Liu, S. B. Chen, J. S. Yang, P. G. Xiao, L. Wang and E. Wu, *Molecular*, **11**, 693 (2206).
13. D. T. M. Nguyen, D. H. Nguyen, H. L. Lyun, H. B. Lee, J. H. Shin and E. K. Kim, *J. Microbiol. Biotechnol.*, **17**, 1585 (2007).
14. J. S. Roh, J. Y. Han, J. H. Kim and J. K. Hwang, *Biol. Pharm. Bull.*, **27**, 1976 (2004).
15. H. Ando, Z. M. Wen, H. Y. Kim, J. C. Valencia, G. E. Costin, H. Watabe, K. I. Yasumoto, Y. Niki, H. Kondoh, M. Ichihashi and V. J. Hearing, *Biochem. J.*, **290**, 375 (2006).
16. H. Ando, A. Ryu, A. Hashimoto, M. Oka and M. Ichihashi, *Arch. Dermatol. Res.*, **290**, 375 (1998).