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Melanogenesis inhibitory effect of dehydroevodiamine isolated from fruits of *Evodia rutaecarpa*

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Abstract−Dehydroevodiamine, an alkaloid, was isolated from the fruit of *Evodia rutaecarpa* and melanin production, and tyrosinase inhibition in B16F10 melanoma cells treated with the isolated dehydroevodiamine was investigated. The compound decreased melanin synthesis significantly without promoting cytotoxicity. The IC_{50} value of dehydroevodiamine for melanogenesis and cell viability were 59.8 μM and 90.0 μM, respectively. The L-dopa oxidase activity of mushroom tyrosinase was reduced after dehydroevodiamine treatment by about 22.4% at a concentration of 33.2 μM. However, there was no effect on cellular tyrosinase activity. These results indicate that the observed decrease in melanin content after treatment with dehydroevodiamine was attributed to the direct inhibition of tyrosinase activity, rather than the suppression of tyrosinase gene expression. Dehydroevodiamine may be a promising new agent for use in cosmeceutical application.

Key words: Melanogenesis, *Evodia rutaecarpa*, Dehydroevodiamine, Skin Whitening, Cosmeceutical

INTRODUCTION

Pigmentation is a widely occurring phenomenon that is mainly determined by melanin, a biopolymer that is synthesized in epidermal melanocytes and transferred into adjacent basal keratinocytes [1]. Melanin pigments can be classified mainly into two types: the alkali-soluble yellow to reddish-brown pheomelanins, and the brown to black eumelanins. Both of these pigment types are derived from the precursor dopaquinone, which is formed via the oxidation of L-tyrosine by tyrosinase [2,3]. Although melanin protects the skin from UV irradiation-induced DNA damage, overproduction and accumulation of melanin in the skin results in abnormal hyperpigmentation such as melasma, freckles and lentigines [4]. Numerous melanogenesis inhibitors from natural sources have been utilized as depigmenting agents including arbutin, ellagic acid, ascorbic acid, *Glycyrrhizae Radix* extract, *Mori Cortex Radicis* extract, *Broussonetia* papyrifera extract etc [5].

The fruit of *Evodia rutaecarpa* (Rutaceae), a well-known traditional herbal medicine, which is also known as *Evodiae fructus*, has been used to treat gastrointestinal disorders, headache, hypertension and post-partum haemorrhages [6]. Many of these effects can be attributed to the main components of the fruit: alkaloids such as evodiamine, dehydroevodiamine and rutaecarpine [7]. Although the whitening activity of the ethanol extract of *Evodiae fructus* has been patented by Amorepacific Corporation (KR 0345225), the chemical composition responsible for the activity has not been identified. Therefore, *E. fructus* was extracted and isolated to investigate the melanogenesis inhibitory ingredients.

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EXPERIMENTAL

1. Materials and Reagents

Dulbecco's modified eagle medium, fetal bovine serum, penicillin/streptomycin, trypsin EDTA, phosphate buffered saline (PBS), were purchased from Invitrogen Corp. (CA, U.S.A). Arbutin, mushroom tyrosinase, L-DOPA (3,4-dihydroxy-L-phenylalanine), 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). 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.

2. Extraction and Isolation

Dried fruits of *Evodia rutaecarpa* (10 g) were pulverized and extracted with water (250 ml) for 6 h at 80 °C. The resulting mixtures were sequentially partitioned in increasing polarity to afford the chloroform, ethyl acetate, butanol and residual extract. According to the depigmenting activity, the butanol fraction (1.04 g) was dried at 40 °C under vacuum and dissolved in methanol. Subsequently, a filtrated fraction was separated by open column chromatography on a silica gel 60 using an elution phase that consisted of chloroform-methanol in the ratios of 9 : 1, 17 : 3, 1 : 1, 0 : 1. A second fraction (17 : 3) was the only one that has melanogenesis inhibitory effect among them (11 mg). Based on previous reports and several spectral data including ^{1}H-NMR, ^{13}C-NMR (VARIAN NEW 300, USA), ESI-MS (FINNIGAM NAVIGATOR, UK), the chemical was structurally identified as dehydroevodiamine (Fig. 2) [8,9].

3. Cell Culture

B16F10 murine melanoma cells, originally derived from C57BL/

6J mice, were purchased from American Type Culture Collection. Cells were cultured in DMEM medium containing 10% (v/v) FBS and 1% (v/v) penicillin/ streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was changed every two days, and the cells were subcultured until 90% growth was achieved on the plates [10].

4. Assays

To measure the melanin content, B16F10 cells (3×10^5/ml) were seeded onto a 6-well plate and pre-incubated at 37 °C for 24 hrs. Culture medium containing the test compounds was then added in triplicate. After 48 hrs incubation, the cells were harvested and washed twice with PBS. 1 N NaOH solution containing 10% DMSO was added and heated at 80 °C for 1 hr. Finally, the lysed cell extract was transferred to a 96 well plate and the relative melanin content was detected at 405 nm using an ELISA micro plate reader. Arbutin (740 μM), a well known melanin inhibitor, was used as a positive control [11].

Cell viability was assayed based on the activity of the mitochondrial dehydrogenase enzyme that reduces yellow MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan, which results in a purple color. Briefly, B16F10 cells were seeded in a 96-well plate (2.5×10^3/well) and fresh medium containing the test samples was added to the cells after 24 hrs pre-incubation. MTT solution supplemented in the media was added to each well when the cell growth achieved 90% confluence on the well surface. The color was quantified spectrophotometrically at 540 nm using an ELISA reader [12].

B16F10 cells were plated on a 6-well plate and treated with several different concentrations of the compound. After 72 hrs incubation, the cells were harvested by trypsinization and lysed with buffer composed of 0.1 M sodium phosphate buffer (pH 6.8), 0.1% Triton-X and protease inhibitor. The cells were disrupted by sonication for 1 hr at 4 °C and separated by centrifugation at 16,000 rpm for 20 minutes. The protein concentration was determined using a protein assay kit (Bio-Rad, U.S.A.) [13].

As a key enzyme, tyrosinase catalyzes DOPA to DOPA quinone in the melanogenesis pathway. To investigate the effects of the isolated compound on mushroom tyrosinase activity, reaction mixtures, including 0.1 M sodium phosphate buffer (pH 6.8), test sample, 5 mM L-DOPA and 22 U/well mushroom tyrosinase, were incubated at 37 °C for 30 minutes. Absorbance was read at 475 nm and kojic acid (704 μM) was used as a positive control.

In cellular tyrosinase activity assay, B16F10 cells were treated with the appropriate sample and the enzyme was extracted using the method described above. Reactions were carried out by mixing cellular tyrosinase, 5 mM L-DOPA and 0.1 M sodium phosphate buffer (pH 6.8) at 37 °C for 60 minutes. Absorbance was measured at 475 nm using an ELISA reader [14-16].

All data were reported as mean values±standard deviation (SD). The statistically significant differences between the control and the sample were analyzed by the Student's t-test. The criterion for statistical significance is expressed as * $p<0.05$ and ** $p<0.01$.

RESULTS AND DISCUSSION

Although a number of melanogenesis inhibitors from plant extracts have been reported and are being utilized as cosmeceutical additives, the active ingredients are nearly unknown. Therefore, water extract of *E. fructus* was partitioned to afford the chloroform, ethyl acetate, butanol, residual extract and the most potent butanol fraction (Fig. 1) yielded dehydroevodiamine (Fig. 2) as the active ingredient based on its ability to inhibit melanin production. Dehydroevodiamine has been reported to play various pharmaceutical roles such as displaying hypotensive and negative chronotropic effects, inhibiting nitric oxide production, selectively increasing cerebral blood flow and possessing neuroprotective effects. It could also protect against learning and memory impairment in both scopolamine- and Ab-induced amnesia animal. The mechanism of the neuroprotective effects of dehyroevodiamine has been examined by Peng et al. [17,18].

A novel biological function of dehydroevodiamine was investigated. Melanin production in B16F10 cells treated with dehydroevodiamine was examined in order to determine its depigmenting activity. As shown in Fig. 3, melanin content was reduced to 70% after treatment with dehydroevodiamine at a concentration of 33 μM, while that of arbutin, the positive control, was 86.4%. Moreover, there was no significant difference in cell proliferation between control and dehydroevodiamine-treated cells, suggesting that the

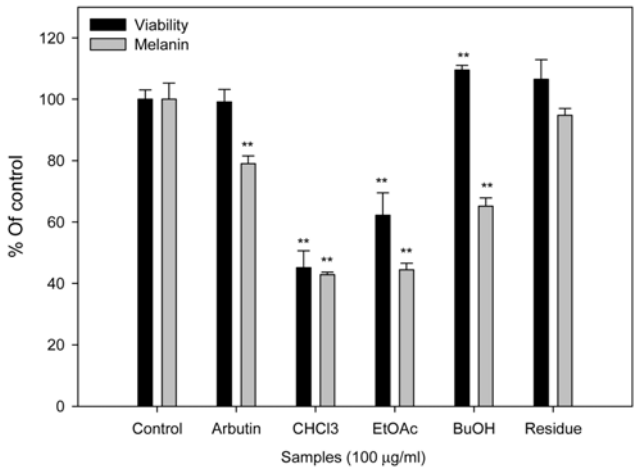

Fig. 1. Effect of partitioned extracts on melanogenesis in B16F10 melanoma cells. B16F10 cells were treated with 100 μg/ml of partitioned extracts and arbutin (740 μM) was used as positive control. Data are expressed as the percent of the control and each column represents the mean±S.D. of three independent measurements. Asterisks indicate a significant difference compared with the control group, * P<0.05, ** P<0.01.

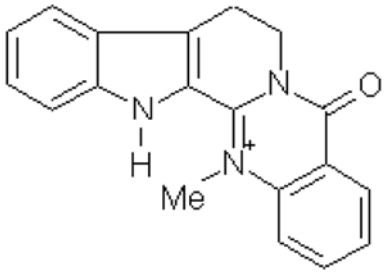

Fig. 2. Chemical structure of dehydroevodiamine isolated from fruits of Evodia rutaecarpa.

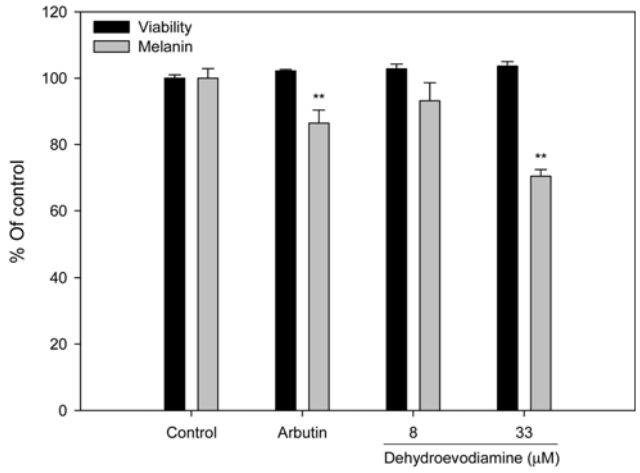

Fig. 3. Effect of dehydroevodiamine on melanogenesis in B16F10 melanoma cells. B16F10 cells were treated with dehydroevodiamine (8 μM and 33 μM) and arbutin (740 μM) for 2 days, harvested, and the melanin contents and viability assayed, as described in Materials and Methods. Data are expressed as the percent of the control and each column represents the mean±S.D. of three independent measurements. Asterisks indicate a significant difference compared with the control group, * P<0.05, ** P<0.01.

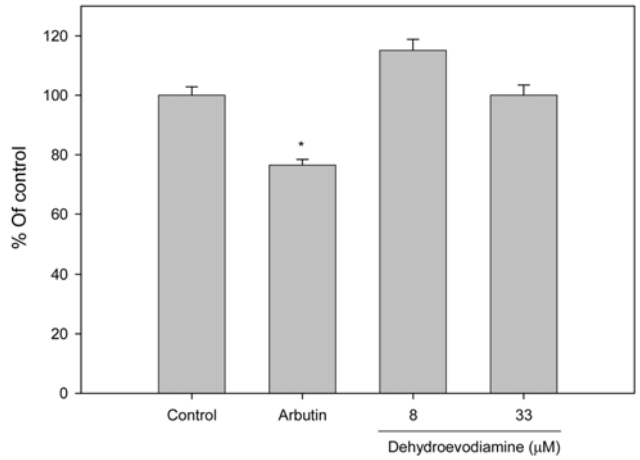

Fig. 4. Cell based tyrosinase assay. B16F10 cells were treated with dehydroevodiamine (8 μM and 33 μM) and arbutin (740 μM) for 2 days, harvested, and tyrosinase extracted from the treated cells as described in Materials and Methods. Data are expressed as the percent of the control, and each column represents the mean±S.D. of three independent measurements. Asterisks indicate a significant difference compared with the control group, * P<0.05, ** P<0.01.

inhibitory activity was not attributed to cytotoxicity. The IC_{50} value for the depigmenting activity of dehydroevodiamine was 59.8 μM and the cell viability was 90.0 μM (data was not shown).

In melanogenesis, the first and rate-limiting step is mediated by tyrosinase, the key enzyme required for melanin formation. Therefore, to investigate the melanogenesis inhibitory function of dehydroevodiamine, the catalytic activity and expression of tyrosinase was evaluated. From these experiments, no effect on tyrosinase expression as well as tyrosinase activity, as assessed in the cell based assays (Western blot data are not shown), was observed after treatment with dehydroevodiamine (Fig. 4). In contrast, the oxidation activity of mushroom tyrosinase decreased to 77.6% (Fig. 5). These results suggest that the reduced melanin content after treatment with dehydroevodiamine was attributed to the direct inhibition of tyrosinase activity, rather than the suppression of tyrosinase expression.

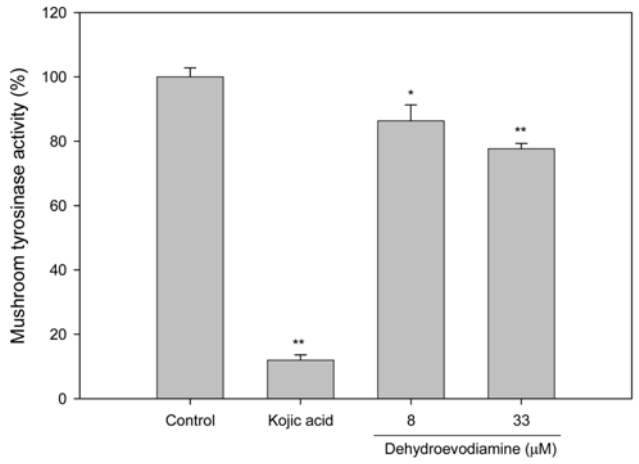

Fig. 5. Effect of dehydroevodiamine on mushroom tyrosinase activity. Dehydroevodiamine (8 μM and 33 μM) was incubated with 40 μl of 5 mM L-DOPA and mushroom tyrosinase at 37 °C for 30 min and the dopachrome contents measured at 475 nm. Kojic acid (704 μM), a potent mushroom tyrosinase inhibitor, was used as positive control. Data are expressed as the percent of the control, and each column represents the mean±S.D. of three independent measurements. Asterisks indicate a significant difference compared with the control group, * P<0.05, ** P<0.01.

Based on these combined findings, dehydroevodiamine isolated from the fruits of *Evodia rutaecarpa* may be a promising compound for use in treating hyperpigmentation.

CONCLUSION

A depigmenting agent, dehydroevodiamine was isolated and identified from the water extract of *E. fructus*. The compound decreased melanogenesis significantly without cytotoxicity and the mechanism was exhibited direct inhibition of tyrosinase based on the enzyme assay. Numerous factors are involved in melanogenesis; thus the possible mechanism of depigmenting caused by dehydroevodiamine should be further studied. Depigmenting activity of dehydroevodiamine was investigated for the first time in the study and could be used for cosmeceutical application.

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