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Inhibition of melanogenesis by *Erigeron canadensis* via down-regulating melanogenic enzymes in B16F10 melanoma cells

Eun-Suk Hong, Duc Thi Minh Nguyen, Dung Hoang Nguyen, and Eun-Ki Kim†

Department of Biological Engineering, Inha University, Incheon 402-751, Korea
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Abstract−The effects of *Erigeron canadensis* extract on melanogenesis and cell toxicity in cultured B16F10 mouse melanoma cells were investigated. *E. canadensis* extract down regulated melanin synthesis effectively at a non-toxic concentration. Its extract was fractionated by using a recycling HPLC with GS310 column (21.5×500 mm, 10-15 μM) into five fractions. The fraction 1 showed melanin inhibition by 48.0% at 100 mg/ml which was 2.5 times more efficient than the depigmenting effect of commercial arbutin (17.5%) and also did not show cell toxicity. To elucidate the depigmenting mechanism of fraction 1, *in vitro* and cellular tyrosinase activity, antioxidant activity, and protein level of the main melanogenic enzymes, such as tyrosinase, TRP-1 and TRP-2 were evaluated. Fraction 1 inhibited melanin synthesis in B16F10 melanoma cells by decreasing protein levels of melanogenic enzymes, especially tyrosinase. In conclusion, we suggest that this fraction may be a safe and effective depigmentation agent.

Key words: *Erigeron canadensis*, Melanogenesis, Depigmenting, Tyrosinase

INTRODUCTION

Skin pigmentation, resulting from the production and redistribution of melanin in the epidermis, is the major physiologic defense mechanism against UV radiation. However, increased production and accumulation of melanin induces many skin problems such as melasma, postinflammatory melanoderma and solar lentigo [1,2]. Melanogenesis is composed of complex pathways restricted to a specialized cell, the melanocyte, that leads to the formation of colored melanin polymers from the amino acid L-tyrosine. Melanin synthesis is catalyzed by at least three enzymatic proteins, tyrosinase, tyrosinase-related proteins-1 (TRP-1) and dopachrome tautomerase (DCT, also known as tyrosinse related protein-2, TRP-2). Tyrosinase is the rate-limiting enzyme of the generation of L-dopaquinone from L-tyrosine and is also able to oxidize L-3,4-dihydroxyphenylalanin (L-DOPA) to L-dopaquinone [3,4]. Depigmentation can be achieved by regulating the transcription and activity of tyrosinase, TRP-1, tyrosinase TRP-2, peroxidase, the uptake and distribution of melanosomes in recipient keratinocytes, melanin and melanosome degradation and turnover of 'pigmented' keratinocytes [5-7].

Some chemicals such as hydroquinone, Arbutin, Kojic Acid, and Ascorbic Acid are well-known to inhibit melanogenesis by inhibition of tyrosinase activity [8]. Even though these chemicals have some inhibitory effects in melanoma cells and are widely used as whitening agents in many cosmetic formulations, there is some controversy about their whitening effects in normal melanocytes [9]. In addition, these agents are unstable, have a bad smell, are weakly active and may be unsafe in human beings. Kojic acid inhibits tyrosinase activity by absorption of copper ion. However its instability and side effects such as skin irritation and induction of liver cancer leads to its noncommercial use [10]. Ascorbic acid and its derivatives are easily oxidized. Hydroquinone has a good whiting effect but its application is restricted due to allergic reactions and melanocyte-toxicity [11]. Thus, applications of these compounds are restricted in the cosmeceutical industry.

Recently, there were some reports on the isolation of active depigmenting materials from natural herbs such as the mulberry tree, safflower seeds, *Amberboa ramose* and esculetin [12-15]. In this study, *Erigeron canadensis*, which is used as medicinal herb for the removal of fever, stopping of bleeding, evacuation and as an antiparasitic, was investigated. The effective fraction was isolated and its effects on melanogenesis as well as the melanin inhibition mechanism in B16F10 were examined.

MATERIALS AND METHODS

1. Materials

Tibetan herbs were imported from Tibet and filtered by using filter paper (PTFE 0.2 μm, Advantenc MFS Inc.). Special grade organic solvents were purchased from Duksan Pure Chemical Co, Ltd (Korea). Mushroom tyrosinase, L-3,4-dihydroxyphenylalanin (L-DOPA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Dimethylsulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (USA). All tissue culture media and components were purchased from GIBCO BRL.

2. Separation of Samples

The pulverized *E. canadensis* was extracted three times with 99.5% methanol (MeOH) for 24 hrs. The mixture was filtered and evaporated. The methanol extract was chromatographed by using preparative HPLC on a GS310 column (21.5×500 mm, 10-15 μM) to give five fractions. The eluant was 70% MeOH. The flow rate was from 1 to 3 ml/min, and the injection volume was from 1 to 3 mL.

3. Mushroom Tyrosinase, DPPH, Melanin and Cell Toxicity

Dry extract was dissolved in 10% MeOH. Forty μL of 0.1 M phosphate buffer solution, 100 μL sample, 110 unit mushroom tyrosinase, 5 mM L-DOPA were mixed. The mixture was incubated at

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

37 ºC for 2 min and the reaction was monitored at 475 nm by using an ELISA microplate reader [16]. Antioxidant activity was performed by the method of Bolis. 100 μL of sample, 100 μL of 0.3 mM DPPH solution were mixed. The mixture was then incubated at 37 ºC for 30 min and the reaction was monitored at 540 nm by using an ELISA microplate reader [17]. To measure melanin inhibition, B16F10 murine melanoma cells obtained from Korea cell line bank were used. The B16F10 cells were grown in a humidified incubator at 37 ºC under 5% CO_2. Cells were routinely incubated in DMEM supplemented with 5% fetal bovine serum. B16 cells were seeded into 6-well plate at density of 1.5×10^3 cells/well. After 24 hr, triplicate cultures were fed with fresh media and samples. After 48 hr, 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 ºC for 1 hr and transferred to 96-well plate. Relative melanin content was determined by absorbance at 405 nm in ELISA reader [18]. MTT assay was used to determine cell toxicity. After treatment and incubation of the samples for 48 hrs, the medium was removed and 100 μL of 0.5 mg/mL MTT was added to each well and incubated at 37 ºC for 4 hrs. The MTT solution was then removed and 100 μL DMSO was added to each well. The formazan formation was measured by absorbance at 570 nm in ELISA reader [18].

4. Cellular Tyrosinase and Western Blotting

After the treatment and extraction as described above, cell extracts were centrifuged for 5 min at 5,000 rpm. Cell pellet was sonicated in a lysis buffer containing 1% Triton X-100 and cocktail. After 1 hr incubation at 4 ºC, the resulting extract was centrifuged at 15,000 rpm for 10 min at 4 ºC to get the supernatants. Then, 50 μL of supernatant and 100 μL of 0.1 M phosphate buffer were combined and pre-incubated at 37 ºC for 10 min. After that, 50 μL of 0.15% L-DOPA was added. After 20 min incubation at 37 ºC, the reaction was determined by absorbance at 405 nm in ELISA reader [19]. Cells were treated as described above. After incubation with samples at 37 ºC for 48 hr, the cells were washed in PBS and lyzed in extraction buffer containing 1% Nonidet P-40, 0.01% SDS, and the protease inhibitor cocktail. Protein contents were determined by BCA assay kit. An equal amount of each protein extract was resolved by using 10% SDS polyacrylamide gel, transblotted onto PVDF membrane and the membranes were blocked with 5% non-fat milk in a TBS buffer. Following the blocking, the membranes were incubated with tyrosinase or TRP-1 or TRP-2. The membranes were then incubated with HRP-conjugated anti-rabbit IgG at a dilution of 1 : 2,000. Immunoreactive bands were detected with enhanced chemiluminescence by using an ECL kit according to the manufacturer's instructions [19]. All the measurements were performed in triplicate. The accuracy of experimental results was reported as means of ±standard deviation (SD).

RESULTS

1. Effect of *Erigeron canadensis* and its Fraction on Melanin Content and Cell Toxicity

Tibetan herbs were screened for finding novel depigmenting agents by using a commercial agent, Arbutin, as a positive control. Among them, *E. canadensis* showed a remarkable depigmenting effect on B16F10 cells (Fig. 1). Melanin content decreased 25.3% after treating them by 50 mg/ml *E. canadensis*. These results showed a much higher efficiency than the depigmenting effect of the Arbutin (17.5%). This plant also showed low cells toxicity (4.1%). To isolate the active components, this MeOH extract was chromatographed on a GS310 column by using 70% MeOH as the eluant to yield five fractions (Fig. 2). Melanin inhibition rate and cell toxicity, mushroom tyrosinase, cellular tyrosinase, and DPPP assays were performed to test the effect of each fraction on the melanogenesis process (Table 1). In order to know the direct inhibitory effect of each fraction on tyrosinase activity, *in vitro* tyrosinase activity testing was done. Compared with the control, fractions 2, 4 and 5 reduced tyrosinase activity by 40%, but fractions 1 and 3 did not. This suggested that fractions

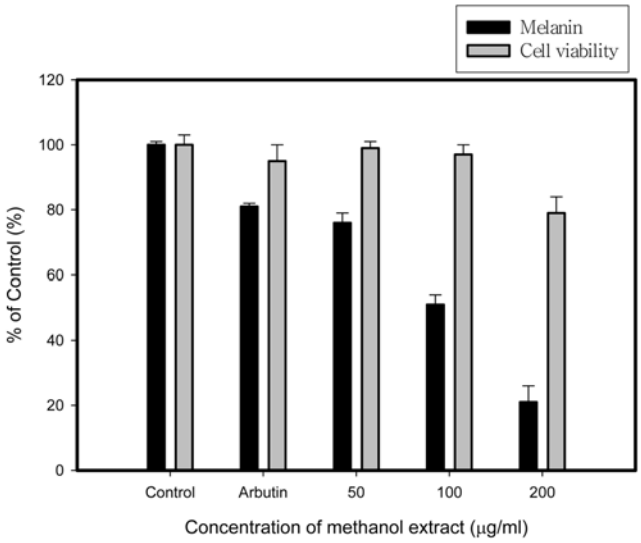

Fig. 1. The effect of E. canadensis methanol extract on melanin and cell toxicity. B16F10 cells were treated with E. canadensis methanol extract for 2 days and the cells were then harvested. Melanin contents and cell viability were measured as described in Materials and Methods. Data were expressed as the change of the melanin content level and cell viability relative to the untreated control. Each determination was made in triplicate and data shown are means±S.D.

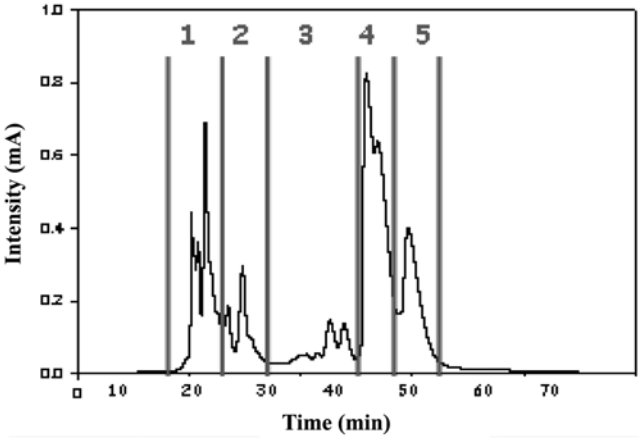

Fig. 2. The chromatogram of E. canadensis methanol extract after being fractionated by preparative HPLC.

Table 1. The effect of *E. canadensis* on mushroom tyrosinase, cellular tyrosinase, antioxidant, melanin and cell toxicity (unit; %)

Fraction	Mushroom tyrosinase activity	Cellular tyrosinase activity	Antioxidant activity	Melanin contents	Cell toxicity
1	98.04±1.02	43.23±0.38	25.19±0.31	22.23±3.31	22.17±1.22
2	60.23±1.20	45.26±1.14	19.31±0.22	89±2.45	5.45±4.32
3	98.04±1.02	44.53±2.21	21.65±0.18	50.56±4.41	56.32±5.42
4	59.85±1.14	36.24±1.76	20.23±0.55	60.44±1.78	0.18±0.12
5	57.46±0.95	39.46±1.38	3.49±0.46	81.23±2.56	0.32±1.36

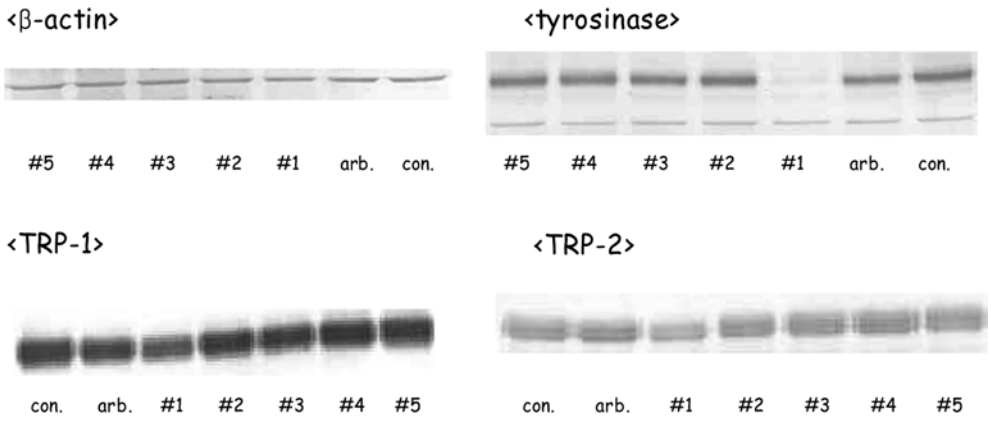

Fig. 3. Protein level of melanogenic enzymes (tyrosinase, TRP-1, TRP-2) after treatment with E. canadensis fractions. B16F10 melanoma cells were treated with five fractions at concentration 200 μg/ml for two days and then harvested. Total protein was extracted and subjected to Western blotting as described in Materials and Methods.

2, 4, and 5 inhibited tyrosinase activity directly. However, fractions 1 and 3 may inhibit melanogenesis by another mechanism. To determine the effect of these fractions on cellular tyrosinase activity, cellular tyrosinase activity testing was performed. Compared with the untreated control, all fractions reduced tyrosinase activity by 50% to 70%. To examine the antioxidant activity, a DPPH assay was done. All of E. *Canadensis* showed low antioxidant activity. Compared with vitamin C, *E. canadensis*'s role as an antioxidant was thought to be minimal. To check the effect of these fractions on melanin inhibition and toxicity, a melanin assay and MTT assay were performed. Fractions 1, 3 and 4 had higher melanin inhibition rates than that of Arbutin. Especially, fraction 1 exhibited 2.5 times stronger depigmenting activity than that of Arbutin without any toxicity.

2. Effect of Fractions of *Erigeron canadensis* on Melanogenesis

To explore the mechanism responsible for the decreased pigmentation, the changes in the protein levels of three important melanogenic enzymes (tyrosinase, TRP-1 and TRP-2) were investigated (Fig. 3). Fraction 1 inhibited tyrosinase protein expression dramatically. It also inhibited TRP-1 and TRP-2. These results suggested that the depigmenting activity of fraction 1 was due to inhibition of protein levels of the main melanogenic enzymes especially tyrosinase. The remaining fractions did not show inhibitory effects on tyrosinase, TRP-1, TRP-2. Due to it being the most effective on depigmenting activity, fraction 1 was further tested in a range of concentrations for its effect on melanin inhibition and cell toxicity (Table 2).

The melanogenesis of B16F10 cells was significantly decreased in a dose-dependent manner after treating by fraction 1. At 100 μg/ml, it inhibited 2.5 times more than Arbutin at the same concentra-

Table 2. The effect of fraction 1 on activity of melanin, cellular tyrosinase, and cell toxicity

Concentration (ppm)	Melanin content (%)	Cellular tyrosinase activity (%)	Cell toxicity (%)
50	54.32±3.56	63.21±3.17	0.19±2.32
100	53.41±2.21	68.34±4.46	0.47±2.11
200	42.71±2.03	29.37±4.19	50.43±12.34

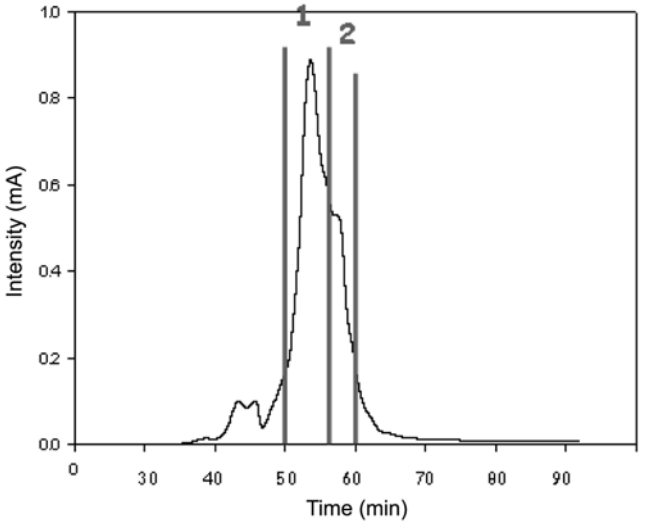

Fig. 4. The chromatogram of fraction 1 after separated by preparative HPLC.

tion. In addition, fraction 1 showed a very low IC_{50} (50 μg/ml). This fraction did not exhibit cell toxicity until 100 μg/ml was used. We also examined the inhibitory effect of fraction 1 on tyrosinase activity. After two days, fraction 1 clearly showed tyrosinase inhibitory activity in a concentration-dependent manner. At 100 μg/ml, the cellular tyrosinase activity was decreased by 30% compared with that in the control cells.

In order to isolate the active compound from fraction 1, this fraction was separated by using preparative HPLC (Fig. 4). In the chromatogram, two peaks were identified. Each of them was repeatedly collected. Now, this study will endeavor to determine the compound structures of each of them.

DISCUSSION

Some skin-depigmenting compounds have been used for the treatment of hyperpigmentary disorders in humans; however, none are completely satisfactory. There still remains a need for novel skin depigmenting agents. In this study, *E. canadensis* was investigated for its effect on melanin inhibition. *E. canadensis* showed a remarkable depigmenting effect and a low toxicity on B16 cells. At a concentration of 50 μg/ml, the melanin content was decreased by 25.3%, which was more efficient than the depigmenting effect of the well-known commercial agent, Arbutin (17.5% inhibition at 100 μg/ml). To isolate the purified effective components, preparative HPLC was used to produce five fractions. Then the effect of five fractions on melanin contents, cell viability, tyrosinase activity and antioxidant activity were examined. Fraction 1 was found to be the most effective for reducing melanin content and not toxic. This fraction did not exhibit a direct inhibitory effect on mushroom tyrosinase and low antioxidant activity. However, it significantly reduced the protein level of melanogenic enzymes (tyrosinase, TRP-1 and 2) especially tyrosinase. These results suggest that the depigmenting effect of this fraction works through inhibiting the key melanogenic enzymes at the expression level. In future work, the structure and the effect on MITF of this fraction is to be studied.

CONCLUSION

We have shown that *E. canadensis* exhibited low cytotoxicity and high depigmenting activity. After fractionation, fraction 1 showed a significant inhibitory effect on melanin synthesis at non-toxic concentration. The possible mechanism for depigmenting activity of this is inhibition of the expression of key melanogenic enzymes. Therefore, we suggest that *E. canadensis* can be useful and safe as a new skin whitening material in cosmetics.

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