Anti-melanogenic activity of methanolic extract from leaves of *Sorbaria sorbifolia* var. *stellipila* Max. on α-MSH-stimulated B16 melanoma 4A5 cells

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**Abstract**

**Background:** Melanin is a pigment, which is widely distributed in organisms. In humans, melanin pigments determine eye, hair, and skin color and protect the skin from damage by ultraviolet radiation; however, immoderate distribution of melanin in the skin causes discoloration. In the present study, we screened methanolic extracts from leaves of 47 plant species, most of which are native to East Asia, for the inhibitory activity against melanogenesis.

**Methods:** B16 melanoma 4A5 cells were used in all assays conducted in this study. Melanin content assay was performed using methanolic extracts from leaves of 47 plant species. Cytotoxicity of the extract from leaves of *Sorbaria sorbifolia* var. *stellipila* Max. (SME) was evaluated by WST-8 assay. Tyrosinase activity was determined using the lysate of α-MSH-stimulated B16 melanoma 4A5 cells and L-dopa as a colorimetric substrate. Melanogenic gene expression was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

**Results:** SME showed the highest inhibitory activity among tested samples without cytotoxicity. SME exhibited the inhibition potency for the enzymatic activity of tyrosinase. In addition, qRT-PCR result displayed that SME downregulates the expression of melanogenic genes including tyrosinase, TRP-1, TRP-2, and the transcription factor MITF.

**Conclusion:** Overall results revealed that the extract from leaves of *S. sorbifolia* var. *stellipila* Max. has a potential to be used as a skin whitening agent.

**Keywords:** B16 melanoma 4A5 cells, *Sorbaria sorbifolia*, Melanin, Tyrosinase, MITF, Tyrosinase-related protein

**Background**

Melanogenesis is the production of melanin pigments in melanosomes of melanocytes (D’Mello et al. 2016). Melanin pigments are widely distributed in organisms ranging from bacteria, plants, to animals (Solano 2014). In humans, melanin pigments determine eye, hair, and skin color and protect the skin from damage by ultraviolet (UV) radiation (Costin and Hearing 2007); however, immoderate distribution of melanin in the skin causes discoloration (Skoczynska et al. 2017). UV radiation stimulates the production of α-melanocyte-stimulating hormone (α-MSH) from keratinocytes and gene expression of melanocortin 1 receptor (MC1R), one of whose endogenous ligands is α-MSH (Bologna et al. 1989; Chakraborty et al. 1995; Yang et al. 1997; Corre et al. 2004; Barnetson et al. 2006). The binding of α-MSH to MC1R stimulates adenyl cyclase to increase the cAMP level, leading to enhanced activity of cAMP-dependent protein kinase A (Buscà and Ballotti 2000). The enzyme subsequently leads to phosphorylation of cAMP response element-binding protein that expresses microphthalmia-associated transcription factor (MITF) gene (Rodriguez and Setaluri 2014). MITF, a major transcription factor for melanogenesis,
induces the expression of melanogenic enzymes including tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2 (Vachtenheim and Borovansky 2010). Tyrosinase mediates hydroxylation of L-tyrosine to 3-hydroxy-L-tyrosine (L-dopa) and oxidizes L-dopa into L-dopaquinone. TRP-2 oxidizes dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), whereas TRP-1 catalyzes the conversion of DHICA to carboxylated indole-quinone via oxidation reaction (Barber et al. 1984; Kobayashi et al. 1994). In the presence of thiol materials, dopaquinone reacts with them, leading to the formation of pheomelanin (Lee et al. 2016). On the other hand, in the absence of thiol materials, dopaquinone is converted to eumelanin (Burchill et al. 1986). The proportion and amount of pheomelanin and eumelanin mainly determine the phenotype of human skin color (Ito and Wakamatsu 2003).

Natural products are well recognized as important sources for developing biologically active substances. Natural products with diverse structures exhibit various biological functions such as antifungal, anticancer, and pesticidal activities. Several extracts from natural resources have been reported to exhibit anti-melanogenic activity (Bourhim et al. 2018; Seo et al. 2019; Wang et al. 2019). Extracts with the anti-melanogenic activity can be used as skin whitening cosmetics agents (Smit et al. 2009); thus, investigations on natural products possessing an inhibition potency for melanogenesis were performed in the present study. After screening methanolic extracts from leaves of various plant species, most of which are native to East Asia, for the inhibitory activity on melanogenesis, we found the anti-melanogenesis activity in the extract from Sorbaria sorbifolia var. stellipila Max. Our results indicate that the leaves of S. sorbifolia var. stellipila Max. would have a potential to be used as a skin whitening cosmetics agent.

Methods
Reagents
Dulbecco’s modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, L-tyrosine, Triton-X 100, and synthetic melanin were obtained from Sigma-Aldrich (St. Louis, MO, USA). α-MSH was purchased from Abcam (Cambridge, UK). 4-Hydroxyphenyl β-D-glucopyranoside (arbutin) was purchased from Tokyo Chemical Industry (Tokyo, Japan). L-Dopa was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan) or Nacalai Tesque unless otherwise noted.

Plant material and extraction
Fresh leaves of 47 plant species were collected in Republic of Korea and identified by plant taxonomist, Dr. San-Sub Han at Jeonbuk National University, Republic of Korea. The leaves were dried under shade at room temperature, pulverized using a grinder (HMF-3260S, Hanil Electric, Seoul, Republic of Korea) up to 0.6 mm. One kilogram of powdered leaves was extracted twice in 1 L of methanol per each extraction for 2 days and filtered. The combined filtrates were concentrated using a rotary vacuum evaporator (NE-1101, EYELA, Tokyo, Japan) at 35 °C, and the concentrate was extracted in 50 mL of water. The extract was dried using a freeze dryer (FD5505, ilShinBioBase, Dongducheon, Republic of Korea). The dried extract of each sample was dissolved in dimethyl sulfoxide (DMSO) and used for the experiments described below. The herbarium specimen of S. sorbifolia var. stellipila Max. has been deposited at the Department of Biological Environment, College of Agriculture and Life Sciences, Kangwon National University, Republic of Korea, with voucher number KPNS-534.

Cell culture
B16 melanoma 4A5 cells were obtained from Riken Cell Bank (Tsukuba, Japan) and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO2.

Melanin content assay
B16 melanoma 4A5 cells were seeded at 3.0 × 10^4 cells/well in a 24-well culture plate (BD Falcon, Franklin Lakes, NJ, USA) and preincubated at 37 °C for 24 h. After removing culture media, 1 mL of fresh DMEM containing 10% FBS, 100 nM α-MSH, 1.6 mM L-tyrosine, and 1 μL of a plant leaf extract or of 100 mM arbutin dissolved in DMSO was added to each well and cultured at 37 °C for 72 h. One milliliter of fresh DMEM containing 10% FBS, 100 nM α-MSH, 1.6 mM L-tyrosine, and 1 μL of DMSO was added to the positive control wells, whereas 1 mL of fresh DMEM containing 10% FBS, 1.6 mM L-tyrosine, and 1 μL of DMSO to the negative control wells. After removing culture media, each well was washed with phosphate-buffered saline (PBS, pH 7.4) once. After removing PBS, 200 μL of 1 M NaOH containing 10% DMSO were added to each well and incubated at 80 °C for 1 h to lyse cells. After incubation, 100 μL of the cell lysates were transferred to a 96-well microplate, and the absorbance was measured at 415 nm using a microplate reader (Model 680, Bio-Rad Laboratories, Richmond, CA, USA). The amount of melanin was determined according to a standard curve prepared with synthetic melanin standard solutions. Protein concentration of each cell lysate was measured using a DC protein assay kit (Bio-Rad Laboratories) with bovine serum albumin as a protein standard according to the manufacturer’s instructions. Data are shown as the amount of melanin per the protein amount of each cell lysate.
Cell viability assay
A colorimetric cell viability assay was carried out using Cell Count Reagent SF that contains (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8, Nacalai Tesque). B16 melanoma 4A5 cells were seeded at 3.0 × 10^3 cells/well in a 96-well culture plate (Corning, Corning, NY, USA) and preincubated at 37 °C for 24 h. After removing culture media, 100 μL of fresh DMEM containing 10% FBS, 100 nM α-MSH, 1.6 mM L-tyrosine, and 0.1 μL of the methanolic extract from leaves of S. sorbifolia var. stelllipila Max. (SME) dissolved in DMSO or of DMSO alone (control) was added to each well and cultured at 37 °C for 72 h. After removing culture media, 100 μL of fresh DMEM containing 10% WST-8 solution was added to each well and incubated at 37 °C for 1 h. The absorbance was then measured at 450 nm using a microplate reader (Model 680, Bio-Rad Laboratories). Relative cell viability was expressed by comparing the absorbance of each sample well with that of the control well.

Tyrosinase activity assay
Tyrosinase activity was determined according to a previously reported method (Lee et al. 2015) with slight modifications. B16 melanoma 4A5 cells were seeded at 3.0 × 10^6 cells/dish in a 100-mm culture dish (BD Falcon) and precultured at 37 °C for 24 h. After removing culture media, 10 mL of fresh DMEM containing 10% FBS, 100 nM α-MSH, and 1.6 mM L-tyrosine were added to the dish and cultured at 37 °C for 72 h. After removing culture media, cells were washed with PBS once. After removing PBS, cells were suspended in 2 mL of 10 mM sodium phosphate buffer (NaPB, pH 7.4) containing 0.01% Triton-X 100 and 50 nM phenylmethylsulfonyl fluoride and frozen at −35 °C. After thawing the cells, the suspension was centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatant was used as a tyrosinase solution.

Every reaction of tyrosinase activity assay consisted of 50 μL of 2 mM L-dopa and 100 μL of 10 mM NaPB containing 1 μL of SME, 1 μL of 100 mM arbutin, or 1 μL of DMSO alone (control) in each well of a 96-well microplate. After preincubation at 37 °C for 10 min, 50 μL of the tyrosinase solution (0.7 mg protein/mL) was added to each well of the microplate and incubated at 37 °C for 60 min. The produced amount of dopachrome was determined by measuring the absorbance at 450 nm using a microplate reader (Model 680, Bio-Rad Laboratories).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)
B16 melanoma 4A5 cells were seeded at 5.0 × 10^4 cells/well in a 24-well culture plate and preincubated at 37 °C

| Table 1 Primers used for qRT-PCR |
|-----------------|-----------------|
| Gene            | Sequence (5′ to 3′) |
| MITF (sense)    | 5′-TCGCCTGATCTGGTGAATCG-3′ |
| MITF (antisense)| 5′-TCTGCCCTGTGCTGACAAG-3′ |
| Tyrosinase (sense) | 5′-CTGATCTGGCATGGCTTC-3′ |
| Tyrosinase (antisense) | 5′-ATGAGGAGTGCTGCTTTC-3′ |
| TRP1 (sense)    | 5′-GAGCCTTCTTTTCTCCCTCC-3′ |
| TRP1 (antisense) | 5′-TCCCATCAAGTGCTCACTGTC-3′ |
| TRP2 (sense)    | 5′-TTGCCCTACTGGAACCTTG-3′ |
| TRP2 (antisense) | 5′-TGGGTCATCTTGTGTCCTGTG-3′ |
| β-Actin (sense) | 5′-CACCTCTCAGGCTCTTCC-3′ |
| β-Actin (antisense) | 5′-GTGTGGGGCTACAGGCTTGG-3′ |
for 24 h. After removing culture media, 1 mL of fresh DMEM containing 10% FBS, 100 nM α-MSH, 1.6 mM L-tyrosine, and 1 μL of SME was added to each well and cultured at 37 °C for 24 h. One milliliter of fresh DMEM containing 10% FBS, 100 nM α-MSH, 1.6 mM L-tyrosine, and 1 μL of DMSO was added to the positive control wells, whereas 1 mL of fresh DMEM containing 10% FBS, 1.6 mM L-tyrosine, and 1 μL of DMSO to the negative control wells. After removing culture media, 1 mL of Sepasol RNA I Super G (Nacalai Tesque) was added to extract RNA from cells. Following total RNA extraction, cDNA synthesis and real-time PCR were performed according to a previously described method (Nishi et al. 2011). The primer pairs used in this study for amplification were shown in Table 1. β-Actin was used as an internal control to normalize the real-time PCR result.

Statistical analysis

Data obtained are expressed as the mean ± SEM. Statistical analyses were performed using GraphPad Prism version 7.02 (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance followed by Dunnnett’s multiple comparison test or Tukey’s multiple comparison test was used to assess the statistical significance. P values of less than 0.05 were considered statistically significant.

Results

Screening of plant leaf extracts

To find a novel plant species with the inhibitory activity on melanogenesis, methanolic extracts from leaves of 47 plant species were prepared and screened. B16 melanoma 4A5 cells were used as a model of melanocytes in this study. When various methanolic extracts were subjected to the melanin assay at 10 μg/mL, the extract from leaves of S. sorbifolia var. stellipila Max. (SME) showed the highest inhibitory activity among tested samples with a statistical significance against control (Fig. 1).

Effect of SME on melanogenesis

Next, we investigated the characteristics of SME. B16 melanoma 4A5 cells were treated with various concentrations of SME for 72 h, and then, the amount of melanin was determined by measuring the absorbance of cell lysates. As shown in Fig. 2a, melanin was produced in α-MSH-stimulated cells significantly higher than in unstimulated cells. We found that SME suppresses the melanogenesis in a dose-dependent manner. Melanogenesis was significantly suppressed by treating B16 melanoma 4A5 cells with 10 μg/mL of SME compared with DMSO alone. Arbutin, a hydroquinone glycoside traditionally used as a skin whitening agent (Sakuma et al. 1999; Hu et al. 2009), was used for comparison and tended to suppress

![Fig. 2 Effects of SME on melanogenesis and cell viability of B16 melanoma 4A5 cells.](image-url)
melanogenesis of B16 melanoma 4A5 cells. We also evaluated the cytotoxic potential of SME to B16 melanoma 4A5 cells by the WST-8 assay. Cells seeded in a 96-well culture plate were treated with various concentrations of SME or with DMSO alone (control) for 72 h, and then, the WST-8 reagent was added to each well of the culture plate. As shown in Fig. 2b, there was no statistically significant difference in the live cell number between SME-treated and non-treated cells. On the basis of the result, SME was used at 10 μg/mL or lower concentrations in the subsequent experiments.

Effect of SME on tyrosinase activity
We next investigated how SME suppresses melanogenesis. Tyrosinase prepared from B16 melanoma 4A5 cells stimulated with α-MSH was mixed with 2.5, 5.0, or 10 μg/mL of SME. Tyrosinase activity was subsequently measured using L-dopa as a colorimetric substrate. As shown in Fig. 3, SME inhibited tyrosinase activity in a dose-dependent manner. The enzyme activity was significantly inhibited by 5.0 and 10 μg/mL of SME. Arbutin, a well-known tyrosinase inhibitor, also significantly suppressed the enzyme activity. Tyrosinase activity was suppressed by 34% by treating with 0.5 mM arbutin, while that was inhibited by 17% with 10 μg/mL of SME.

Effect of SME on α-MSH-induced melanogenic gene expression
To investigate whether SME affects the expression of melanogenesis genes, qRT-PCR was performed. As shown in Fig. 4, the expression of melanogenic genes, including tyrosinase, TRP-1, TRP-2, and transcription factor MITF, was significantly upregulated by the stimulus with α-MSH. qRT-PCR experiment displayed that SME significantly reduces the transcriptional level of MITF in a dose-dependent manner. The MITF expression level in B16 melanoma 4A5 cells decreased to that in unstimulated cells by treating cells with 10 μg/mL of SME. SME also significantly reduced the transcription of tyrosinase, TRP-1, and TRP-2 genes (Fig. 4).

Discussion
In this study, methanolic extracts from leaves of 47 plant species, most of which are native to East Asia, were screened, and the extract from leaves of *S. sorbifolia* var. *stellipila* Max. (SME) showed the highest inhibitory activity among tested samples with a statistical significance. Among tested species, *S. sorbifolia* var. *stellipila* Max., *Crataegus scabrida* Sarg., *Prunus armeniaca* var. *ansu* Maxim., *Chaenomeles sinensis* Koehne, *Prunus sargentii* Rehder, and *Rosa rugosa* Thunb. belong to Rosaceae family; however, the inhibitory activity on melanogenesis was observed in the extract of *S. sorbifolia* var. *stellipila* Max. only (Fig. 1), suggesting that the bioactive molecule might not be commonly found in the plants of Rosaceae family, but also be specifically produced in *S. sorbifolia* var. *stellipila* Max only.

It has been reported that several cytotoxic constituents are contained in methanolic extract from the aerial parts of *S. sorbifolia* var. *stellipila* Max. (Kim et al. 1997). We thus evaluated the cytotoxic potential of SME to B16 melanoma 4A5 cells by the WST-8 assay and confirmed that SME does not affect the live cell number of B16 melanoma 4A5 cells up to 10 μg/mL (Fig. 2b). The melanogenesis-suppressive activity of SME was thus revealed to be attributed to the biological activity of constituent(s) in SME but not to cytotoxicity.

Melanogenesis is known to be modulated by three melanogenic enzymes: tyrosinase, TRP-1, and TRP-2. Among these three enzymes, tyrosinase (EC 1.14.18.1) is the pivotal enzyme responsible for the conversion of L-tyrosine to dopaquinone via L-dopa and controls the rate of melanin biosynthesis (Hearing and Tsukamoto 1991). For developing anti-melanogenic agents, direct targeting of the component involved in melanin synthesis, such as tyrosinase, is common (Gunia-Krzyżak et al. 2016; Bin et al. 2016). In fact, most of skin whitening agents on the
market are tyrosinase inhibitors. The effect of SME on the enzymatic activity of tyrosinase was thus evaluated. We found that SME inhibits enzymatic activity of tyrosinase in a dose-dependent manner (Fig. 3); however, the inhibition potency of 10 μg/mL of SME on the tyrosinase activity was lower than that of arbutin. Since melanin production in B16 melanoma 4A5 cells was more strongly inhibited with 10 μg/mL of SME than with arbutin (Fig. 2a), it was assumed that the anti-melanogenic effect of SME was mediated in part by the direct inhibition of tyrosinase activity and by an additional manner different from inhibiting tyrosinase activity.

Tyrosinase, TRP-1, and TRP-2 are the major enzymes involved in melanogenesis. Expression of these three proteins is under the control of MITF, a major regulator for melanogenesis. Gene expression of MITF is induced in response to α-MSH stimulation. We found that the expression of melanogenic genes, including tyrosinase, TRP-1, TRP-2, and transcription factor MITF, is significantly downregulated by treating cells with SME (Fig. 4). Zaitsev et al. (1969) have reported that hyperoside (quercetin 3-galactoside) is contained in leaves of S. sorbifolia. Hyperoside has been reported to inhibit tyrosinase activity and expression of TRP-1 and TRP-2 genes (Jung et al. 2014). Thus, the anti-melanogenesis activity of SME might be partially attributed to hyperoside. Further investigations are required to identify the bioactive constituents in SME. Taken together, SME attenuated melanin production through downregulated expression of melanogenesis-related genes.

**Conclusions**

In this study, we screened methanolic extracts from leaves of 47 plant species, most of which are native to East Asia, for the inhibitory activity against melanogenesis using B16 melanoma 4A5 cells and found the anti-melanogenesis...
activity in SME. SME exerted the activity through the direct inhibition of enzymatic activity of tyrosinase and downregulated expression of melanogenesis-related genes, such as tyrosinase, MITF, TRP-1, and TRP-2, as summarized in Fig. 5. Overall results showed that SME has a potential to be used as a skin whitening cosmetics agent.

Abbreviations
Arbutin: 4-Hydroxyphenyl β-D-glucopyranoside; DHICA: 5,6-Dihydroxyindole-2-carboxylic acid; DMEM: Dulbecco’s modified Eagle medium; DMSO: Dimethyl sulfoxide; FBS: Fetal bovine serum; L-Dopa: 3-Hydroxy-L-tyrosine; MC1R: Melanocortin 1 receptor; MITF: Microphthalmia-associated transcription factor; NaPB: Sodium phosphate buffer; PBS: Phosphate-buffered saline; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; SME: Methanolic extract from leaves of S. sorbifolia var. stellipila Max; TRP: Tyrosinase-related protein; WST-8: 2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; α-MSH: α-Melanocyte-stimulating hormone

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Authors’ contributions
KN conceived the research; MM performed the experiments; DN and JS assisted with the experiments; IHK, MK, and SK contributed to the sample acquisition; KN, MM, and TS analyzed the data; TS provided the experimental equipment; KN wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests
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Fig. 5 A scheme showing the mechanism of action of SME in inhibiting melanogenesis
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