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Inhibitory effects of Korean indigenous plants on tyrosinase and melanogenesis

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Synopsis

To search for new depigmenting cosmetic ingredients from Korean herbal extracts of traditional Korean medicines (TKMs), we screened about 17 TKM extracts collected in the Republic of Korea. Samples were prepared from the natural plants, including medicinal plants such as *Chrysanthemum indicum* (flower), using methanol, methylene chloride, ethyl acetate (EtOAc), *n*-butyl alcohol, and water as the extraction and/or the partitioning solvents. We then tested their inhibitory effects on melanogenesis by using *in vitro* tyrosinase inhibition assay, *in vitro* L-3,4-dihydroxy-indole-2-carboxylic acid (L-DOPA) auto-oxidation assay, and B16 melanoma cells. In addition, cytotoxicity testing (NR_{50} and MTT_{50}) was conducted to evaluate safety. From the results of these assays, four fractions with good efficacy and low toxicity were selected among them, including EtOAc fraction of *Smilax china* (rhizome), *Paeonia lactiflora* (root), and *Polyporus umbellatus* (sclerotium), and BuOH fraction of *Evodia officinalis* (fruit). In the inhibition assay of intracellular tyrosinase activity and melanogenesis in B16 melanoma cell line, the four plant fractions showed dose-dependent inhibitory effects, and the EtOAc fraction of *P. lactiflora* showed the highest activity among the four fractions. The EtOAc fraction of *P. lactiflora* was found to be the most effective substrate.

INTRODUCTION

Skin color is determined by the type, amount, and size of melanin formed by melanocytes between the dermis and epidermis (1,2). Therefore, in the development of depigmenting agents, it is important to understand the skin structure, physiological functions, and mechanisms of melanin biosynthesis.

Melanin is formed in the melanosome, a unique intracellular structure of the melanocyte. Melanosomes contain enzymes that are involved in melanin biosynthesis, such as tyrosinase, tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2. It has been reported that TRP-1 stabilizes enzymes in the melanosome. Furthermore, melanosomes contain matrix proteins, which are thought to act as the location of enzyme synthesis.

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The factors involved in melanin biosynthesis inside the melanosome include intracellular regulating factors that are involved in the synthesis and transfer of enzymes such as tyrosinase, dopachrome tautomerase, peroxidase, catalase, and glutathione reductase; metal ions such as copper, zinc, and iron; and interferon (IFN), prostaglandin (PG), and histamine.

In this study, 17 plant species were selected among the traditional Korean medicines (TKMs) mentioned in traditional herbal medicine books. Until now, reports have been published on tyrosinase inhibition and L-3,4-dihydroxy-indole-2-carboxylic acid (L-DOPA) auto-oxidation inhibition by methanol extracts of *Chrysanthemum indicum*, *Evodia officinalis*, *Prunus mume,* and *Poncirus trifoliata* (3); tyrosinase inhibition by *E. officinalis*, *Gentiana scabra*, *Forsythia viridissima*, *Smilax china*, *Lonicera japonica*, *Paeonia lactiflora Pall*, *P. trifoliata*, *Vitex rotundifolia*, and *P. mume* (4); and DOPA auto-oxidation inhibition by *C. indicum*, *E. officinalis*, *G. scabra*, *Houttuynia cordata*, *L. japonica*, *F. viridissima*, *Euonymus alatus*, *P. lactiflora*, *Perilla frutescens*, *P. trifoliata*, and *V. rotundifolia* (5). However, they only described investigation at the screening stage, and later investigation results have not been found yet.

A total of 17 kinds of plants were extracted and fractionated using five different solvents. Eighty-five extracts and fractions were screened to elucidate their depigmenting effects using a tyrosinase inhibition assay and an L-DOPA auto-oxidation inhibition assay, resulting in the identification of 10 kinds of candidate depigmenting fraction. The resulting 10 fractions were tested again at various concentrations for tyrosinase inhibition and L-DOPA auto-oxidation inhibition to determine 50% inhibitory concentration (IC_{50}). Finally, four fractions with good efficacy were selected through the *in vivo* melanin synthesis inhibition and cytotoxicity tests. From these activities, ethyl acetate (EtOAc) fraction of *P. lactiflora* was found to be the most effective constituent and was finally selected to develop a skin-whitening agent.

MATERIALS AND METHODS

MATERIALS

Seventeen plants were obtained from the Oriental Medicinal Market in Seoul, South Korea, and were identified with help from the Korea Pharmaceutical Traders Association (Table I). The B16 melanoma cell line and transformed mouse fibroblast L929 were obtained from the Korean Cell Line Bank (Seoul, Korea).

EXTRACTION AND ISOLATION OF PLANTS

The scheme of the fractionation of plant extracts is shown in Figure 1. About 500 g of each sample was soaked twice in 2000 ml of 80% methanol at room temperature for 24 h twice and filtered with a Whatman No. 5 filter paper to remove the solid material. The filtrates were evaporated in vacua to dryness (methanolic extract).

To isolate the pure compounds, the methanolic extract was mixed with distilled water and partitioned with methylene chloride (MC), EtOAc, and *n*-butanol (*n*-BuOH). About 10–50 g

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Table I
List of Plants and Their TKM Names in Korea

Scientific name	TKM name	Part used
Chrysanthemum indicum	Gamguk	Flower
Lonicera japonica	Geumeunhwa	Flower bud
Vitex rotundifolia	Manhyeongja	Fruit
Forsythia viridissima	Yeongyo	Fruit
Evodia officinalis	Osuyu	Fruit
Gentiana scabra	Yongdam	Root and rhizome
Perilla frutescens (leaf and sprig)	Jasoyeop	Leaf and sprig
P. frutescens (fruit)	Jasoja	Fruit
Polyporus umbellatus	Jeoryeong	Sclerotium
Houttuynia cordata	Jeupchae	Whole plant
Celosia cristata	Cheongsangja	Seed
Smilax china	Tobokryeong	Rhizome
Prunus mume	Omae	Steamed and dried fruit
Poncirus trifoliata	Jisil	Fruit
Euonymus alatus	Gwijeonu	Alated sprigs
Thuja orientalis	Baekjain	Seed
Paeonia lactiflora	Jakyak	Root

of the methanol extract was resuspended in 200 ml of water and was then extracted three times with the same volume of MC. The resulting aqueous layer was sequentially extracted with EtOAc and then *n*-BuOH using the same procedure as that used for MC extraction. The MC, EtOAc, *n*-BuOH, and aqueous layer were evaporated in vacua (VV2000, Heidolph, Schwabach, Germany), and approximately 1–20 g of powder or gel was obtained for testing (6,7).

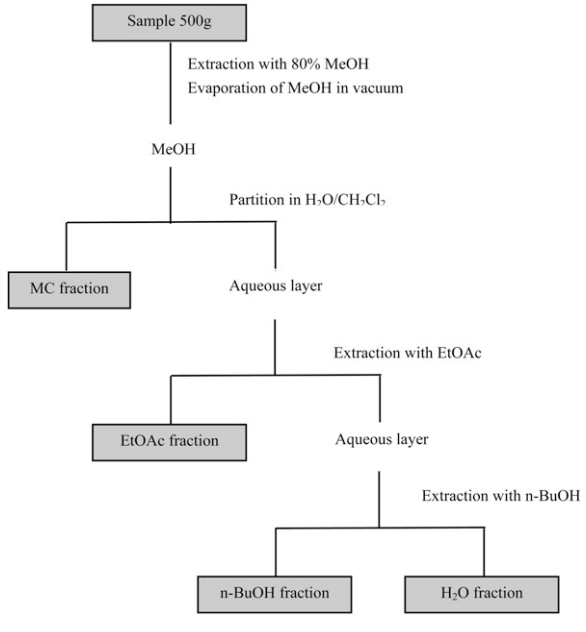

Figure 1. The isolation of active fractions from plant samples.

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IN VITRO MUSHROOM TYROSINASE INHIBITION TEST

Enzyme activity was determined by the method described by Ishihara *et al.* (8), modified according to the results of preliminary testing to search for optimized conditions. Each of the extracts and fractions was dissolved in 0.1 M sodium phosphate buffer (pH 6.5) and diluted to 1% individually. The solution was used as a test sample. Then, 20 µl of test solution, 220 µl of 0.1 M sodium phosphate buffer (pH 6.5), 40 µl of 1.5 mM tyrosine, and 20 µl of 2000 unit/ml mushroom tyrosinase were sequentially transferred to a 96-well plate and incubated at 37°C for 10 min. The absorbance was measured at 490 nm with an ELISA reader (GENios, Tecan, Männedorf, Switzerland). As a blank solution, 0.1 M sodium phosphate buffer (pH 6.5) was added, instead of test solution.

IN VITRO INHIBITION OF L-DOPA AUTO-OXIDATION

Enzyme activity was determined using the method described by Kong *et al.* (9), modified according to the results of preliminary testing for optimized conditions. Each of the extracts and fractions was dissolved in 0.1 M sodium phosphate buffer (pH 6.5) and diluted to 1% individually. The solution was used as a test sample. Then, 20 µl of test solution, 220 µl of 0.1 M sodium phosphate buffer (pH 6.5), 40 µl of 500 µM L-DOPA (Sigma-Aldrich Chemical Co., St. Louis, MO), and 20 µl of 2000 unit/ml mushroom tyrosinase were sequentially transferred to a 96-well plate and incubated at 37°C for 10 min. The absorbance was then measured at 490 nm with an ELISA reader. As a blank solution, 0.1 M sodium phosphate buffer (pH 6.5) was added instead of test solution.

CELL CYTOTOXICITY TEST

Neutral red assay. Transformed mouse fibroblast L929 was inoculated into a 96-well plate containing Dulbecco's modified Eagle's medium (DMEM) with 2% bovine calf serum (BCS) (3×10^3 cells/well) and incubated at 5% CO_2 and 37°C for 48 h. The medium was then replaced with new serum-free medium containing transferrin (10 µg/ml) and ethanolamine (2 µM) and again incubated for 48 h after the addition of sample dilutions. After incubation, 100 µl of neutral red (NR) solution (5 µg/ml) was added into each well and was allowed to react at 37°C for 3 h. After the reaction, the supernatant was removed and the solution was fixed with 1.0% formalin containing 1.0% $CaCl_2$ to immobilize cells for 5–10 min. After removal of the immobilization solution, 100 µl of 50% ethanol with 1.0% acetic acid was added to each well to extract pigments. The absorbance was then measured at 540–630 nm (double wavelength) with an ELISA reader to calculate the NR_{50}.

MTT assay. Transformed mouse fibroblast L929 was inoculated into a 96-well plate containing DMEM with 2% BCS (3×10^3 cells/well) and incubated at 5% CO_2 and 37°C for 48 h. The medium was then replaced with new serum-free medium containing transferrin (10 µg/ml) and ethanolamine (2 µM) and again incubated for 48 h after the addition of sample dilutions. After incubation, 100 µl of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well, and was allowed to react at 37°C for 4 h. After the reaction, the supernatant was removed and 100 µl of isopropanol containing 0.04 M HCl was added to each well to extract formazan from cells. The absorbance was then measured at 570–630 nm (double wavelength) with an ELISA reader to calculate the MTT_{50}.

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INTRACELLULAR TYROSINASE INHIBITION TEST

From tyrosinase inhibition and cell cytotoxicity tests, four samples with a good inhibition effect and no cytotoxicity were selected to conduct intracellular tyrosinase inhibition and melanin synthesis tests. A six-well plate containing DMEM media (Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS; Gibco) (2×10^4 cells/well) was inoculated with 2 ml of murine melanoma (B16) cells and incubated at 5% CO_2 and 37°C (CO_2 air-jacketed incubator; NuAire, Plymouth, MN) for 24 h. The medium was then replaced with fresh DMEM containing 10% FBS, 2 μM α-melanocyte-stimulating hormone (MSH), and 2 mM theophylline. Selected plant extracts and fractions were diluted with fresh medium and incubated with cells at 5% CO_2 and 37°C until more than approximately 80%.

After incubation, the medium was removed, cells were washed with phosphate-buffered saline (PBS), and trypsin was applied for the recovery of cells. The cell pellet was transferred to an eppendorf tube and centrifuged at 10,000 rpm for 10 min to remove the supernatant. The cell pellet was washed two or three more times with PBS and centrifugation was repeated. Then, 100 μl of 0.1% Triton X-100 was added to dissolve cells and obtain intracellular tyrosinase extract, and 220 μl of 0.1 M phosphate buffer (pH 6.5), 40 μl of 1.5 mM tyrosine solution, and 20 μl of tyrosinase extract were added sequentially (10). After reacting at 37°C for 60 min, the absorbance was measured at 490 nm with an ELISA reader. For a blank test, only B16 melanoma cells were incubated without sample and the recovered tyrosinase extract was added.

INTRACELLULAR MELANIN SYNTHESIS INHIBITION TEST

A six-well plate containing DMEM with 10% FBS (2×10^4 cells/well) was inoculated with 2 ml of murine melanoma (B16) and incubated at 5% CO_2 and 37°C for 24 h. The medium was then replaced with fresh DMEM containing 10% FBS, 2 μM α-MSH, and 2 mM theophylline. Murine melanoma B16 cells were grown, incubated, and processed until a pellet was formed as described earlier. The cell pellet was dried at 60°C.

Then, 100 μl of 1 N NaOH was added to the pellet to dissolve intracellular melanin. After dilution of melanin with PBS, the absorbance was measured at 490 nm with an ELISA reader to determine the melanin synthesis inhibition rate. For a blank test, only B16 melanoma cells were incubated without sample and the recovered tyrosinase extract was added.

IN VIVO DEPIGMENTING EFFICACY TEST

Based on the results of the *in vitro* tyrosinase inhibition test, *in vitro* L-DOPA autoxidation inhibition test, intracellular tyrosinase inhibition test, intracellular melanin synthesis inhibition test, and cell cytotoxicity test, the EtOAC fraction of *P. lactifora* and BuOH fraction of *E. officinalis* were proven to have good depigmenting effects and no toxicity, and were then subjected to an *in vivo* efficacy study using guinea pigs to estimate the depigmenting effect in human body.

Four female brown guinea pigs were used for an *in vivo* depigmentation study. To induce pigmentation, the backs of the animals were completely shaved with an electric

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shaver and marked for ultraviolet (UV) irradiation (1.5 × 1.5 cm^2, six points). They were irradiated with 500 mJ/cm^2 UV rays using Waldmann UV 800 (Herbert Waldmann, Villinogen-Schwenningen, Germany, GmbH&E, Philis TL/12 lamp emitting 280–305 nm). This irradiation process was repeated three times at 1-week intervals (total 1500 mJ/cm^2) (11).

The plant extract and fraction samples were dissolved in vehicle (propylene glycol : ethanol : water at 5:3:2) to 2% and were applied on the backs of the guinea pigs after the UV induction pigmentation procedure was completed. Application of the sample was continued twice a day for 6 weeks. Hydroquinone was dissolved in the same vehicle. Hydroquinone and vehicle were used as positive and negative blanks, respectively.

The degree of pigmentation on treated areas was measured according to melanin index with a mexameter (Courage-Khazaka Electronic, Koln, Germany) at 0, 1, 2, 3, 4, 5, and 6 weeks, and potential adverse effects were assessed.

RESULTS AND DISCUSSION

SCREENING OF EFFECTIVE DEPIGMENTING PLANT EXTRACT

In vitro *tyrosinase inhibition*. Methanol, MC, EtOAc, *n*-BuOH, and water fraction of 17 natural plants, including *C. indicum* (Gamguk), were diluted to 0.1% test solutions with ethanol. These were used for *in vitro* tyrosinase (mushroom tyrosinase) inhibition tests (data not shown).

From this test, the *n*-BuOH fraction of *E. officinalis*, EtOAc fraction of *P. frutescens*, *n*-BuOH fraction of *P. frutescens*, EtOAc fraction of *P. umbellatus*, methanol extract of *S. china*, EtOAc fraction of *S. china*, *n*-BuOH fraction of *S. china*, EtOAc fraction of *P. mume*, EtOAc fraction of *P. trifoliata*, *n*-BuOH fraction of *P. trifoliata*, methanol extract of *P. lactiflora*, MC fraction of *P. lactiflora*, EtOAc fraction of *P. lactiflora*, and *n*-BuOH fraction of *P. lactiflora* showed good *in vitro* tyrosinase inhibition results (more than 90%).

The *n*-BuOH fraction of *L. japonica*, *n*-BuOH fraction of *P. frutescens*, methanol extract of *E. alatus*, EtOAc fraction of *E. alatus*, and *n*-BuOH fraction of *E. alatus* also showed good tyrosinase inhibition results (80%–90%). However, *G. scabra*, *H. cordata*, and *Celosia cristata* showed relatively low activity (less than 70%).

In vitro *L-DOPA auto-oxidation inhibition.* Samples showing good results in the *in vitro* tyrosinase inhibition tests were selected for *in vitro* L-DOPA auto-oxidation inhibition tests (data not shown).

The *n*-BuOH fraction of *E. officinalis*, EtOAc fraction of *P. frutescens* (leaf and sprig), *n*-BuOH fraction of *P. frutescens* (leaf and sprig), EtOAc fraction of *P. umbellatus*, methanol extract of *S. china*, EtOAc fraction of *S. china*, and EtOAc fraction of *P. lactiflora* showed good auto-oxidation inhibition effect (more than 60%).

Meanwhile, the EtOAc fraction of *P. frutescens* (leaf and sprig), *n*-BuOH fraction of *P. frutescens* (leaf and sprig), EtOAc fraction of *P. umbellatus*, and EtOAc fraction of *P. mume*, which had good *in vitro* tyrosinase inhibition results, did not show desirable antioxidation inhibition effects (less than 10%). From these results, it was determined that the two tests have to be conducted in parallel to appropriately evaluate new candidate substances.

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IN VITRO TYROSINASE INHIBITION RATE AND L-DOPA AUTO-OXIDATION INHIBITION RATE

For this test, EtOAc fractions of *S. china*, *P. lactiflora*, *P. frutescens*, *E. alatus*, *P. mume*, and *P. trifoliata*; MC fraction of *P. lactiflora*; and *n*-BuOH fractions of *E. officinalis* and *H. cordata* were selected. Samples were dissolved in ethanol and the *in vitro* tyrosinase inhibition test was conducted using mushroom tyrosinase with arbutin, a depigmenting substance widely used in Korea, as a control. The results are shown in Figure 2.

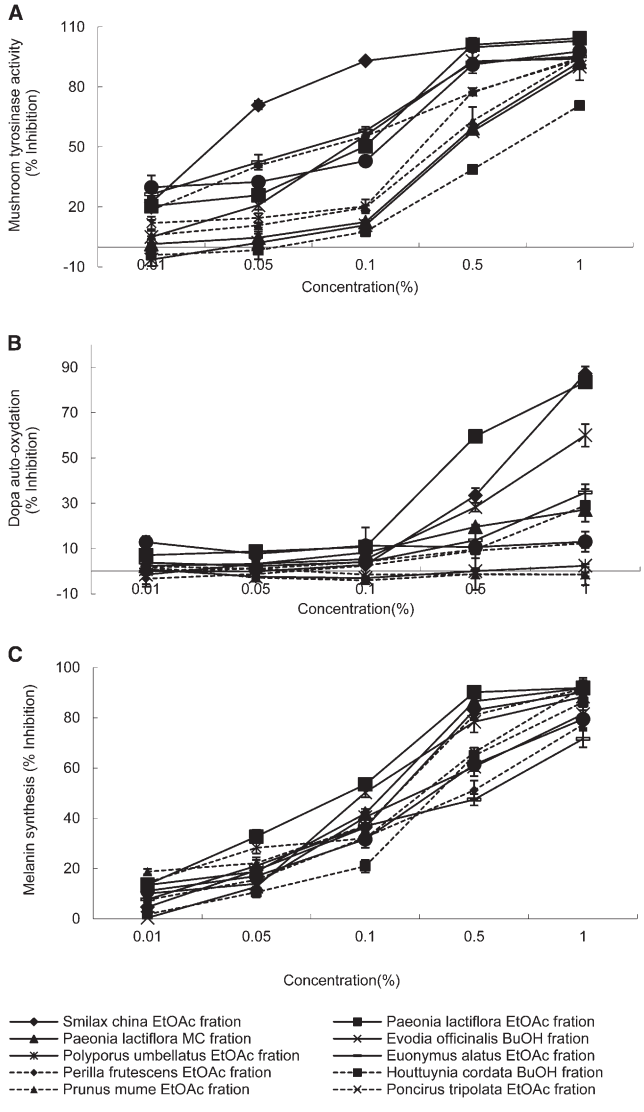

Figure 2. Inhibitory effects of *S. china* EtOAc fraction, *P. lactiflora* EtOAc fraction, *P. lactiflora* MC fraction, *E. officinalis* BuOH fraction, *P. umbellatus* EtOAc fraction, *E. alatus* EtOAc fraction, *P. frutescens* EtOAc fraction, *H. cardata* BuOH fraction, *P. mume* EtOAc fraction, *P. trifoliata* EtOAc fraction, and arbutin on (A) tyrosinase activity, (B) L-DOPA auto-oxidation, and (C) melanin synthesis. Results are shown as the averages ± SD of three independent experiments.

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As shown in Figure 2A, the 10 selected extracts showed a pattern in which an increase in concentration was correlated with an increase in the tyrosinase inhibition effect. In particular, EtOAc fractions of *S. china*, *P. lactiflora*, *E. alatus*, and *P. frutescens* showed better inhibition effects than arbutin (positive control). IC_{50} values obtained from *in vitro* tyrosinase inhibition tests are presented Table II.

As shown in Figure 2B, EtOAc fractions of *S. china*, *P. lactiflora*, and *E. officinalis* showed the pattern in which an increase in concentration was correlated with an increase in the L-DOPA auto-oxidation inhibition effect. However, the inhibitory effect of L-DOPA auto-oxidation was less than that of tyrosinase. The other samples showed an inhibitory effect below 50%, even at a concentration of 1%.

IN VITRO MELANIN SYNTHESIS INHIBITION

For this study, a total of 10 fractions (EtOAc fractions of *S. china*, *P. lactiflora*, *P. umbellatus*, *E. alatus*, *P. frutescens*, *P. mume*, and *P. trifoliata*; MC extract of *P. lactiflora*; and *n*-BuOH fractions of *E. officinalis* and *H. cordata*) were dissolved in ethanol, and *in vitro* melanin synthesis inhibition tests were conducted using mushroom tyrosinase with arbutin as a control. As shown in Figure 2C, the 10 selected fractions showed the pattern in which an increase in concentration was correlated with an increase in the melanin synthesis inhibition effect. In particular, EtOAc fractions of *S. china*, *P. lactiflora*, *P. umbellatus*, *P. mume*, and *P. trifoliata*; MC fraction of *P. lactiflora*; and *n*-BuOH fraction of *E. officinalis* showed better inhibition effects than arbutin (positive control). The IC_{50} values obtained from *in vitro* tyrosinase inhibition tests are presented in Table II.

As a result of the screening test, the 10 selected fractions were used in three *in vitro* depigmenting efficacy tests: tyrosinase inhibition test, L-DOPA auto-oxidation inhibition test, and melanin synthesis inhibition test. EtOAc fractions of *S. china*, *P. lactiflora*, and *P. umbellatus* and BuOH fraction of *E. officinalis* showed better inhibition effects than arbutin for two or more types of efficacy tests.

Table II
IC_{50} on Tyrosinase Activity and Melanin Synthesis

Samples	Fraction	IC_{50} (%)	
		Tyrosinase activity	Melanin synthesis
Smilax china	EtOAc	0.023	0.141
Paeonia lactiflora	EtOAc	0.073	0.085
P. lactiflora	MC	0.266	0.117
Evodia officinalis	BuOH	0.296	0.146
Polyporus umbellatus	EtOAc	0.101	0.209
Euonymus alatus	EtOAc	0.055	0.330
Perilla frutescens	EtOAc	0.077	0.295
Houttuynia cordata	BuOH	0.685	0.239
Prunus mume	EtOAc	0.213	0.119
Poncirus trifoliata	EtOAc	0.167	0.147
Arbutin	—	0.087	0.229

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CELL CYTOTOXICITY TEST

The NR assay and MTT assay were conducted to investigate the sensitivity of transformed mouse fibroblast L929 to test the cytotoxicities of samples.

A total of 10 fractions (EtOAc fractions of *S. china*, *P. lactiflora*, *P. umbellatus*, *E. alatus*, *P. frutescens*, *P. mume*, and *H. cordata*; MC fraction of *P. lactiflora*; and n-BuOH fractions of *E. officinalis* and *H. cordata*) were selected. Samples were dissolved in ethanol and the sensitivity of transformed mouse fibroblast L929 to test materials was tested. The results are shown in Figure 3.

As shown in Figure 3A, 10 fractions, including EtOAc fraction of *S. china*, were found to have cytotoxicity at the concentration of 0.01% or less. However, the fractions showed similar results for NR_{50}, except for the EtOAc fraction of *P. trifoliata*, which were greater than 0.1%.

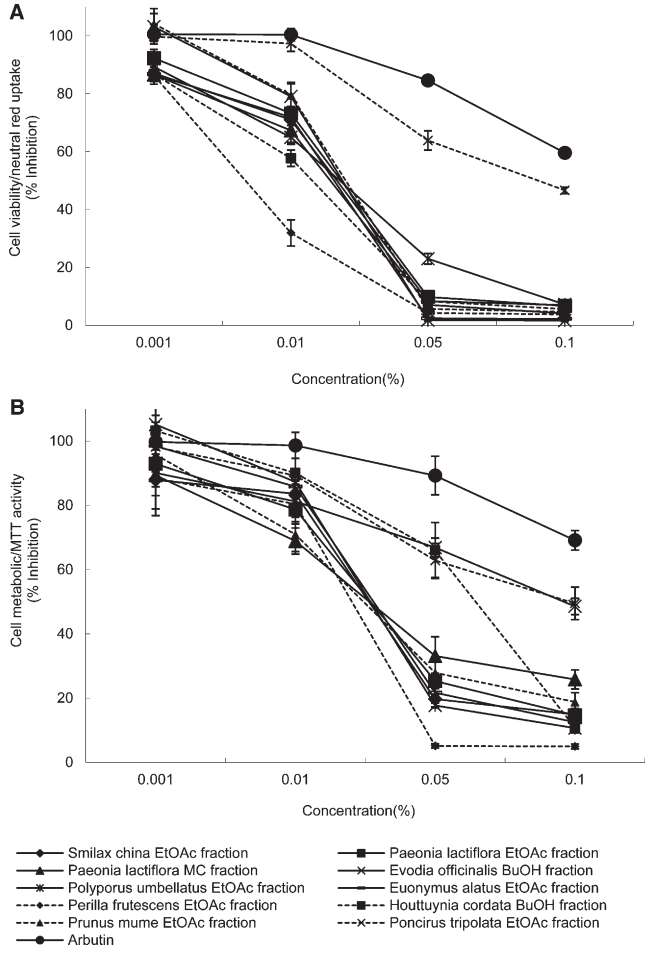

Figure 3. Inhibitory effects of *S. china* EtOAc fraction, *P. lactiflora* EtOAc fraction, *P. lactiflora* MC fraction, *E. officinalis* BuOH fraction, *P. umbellatus* EtOAc fraction, *E. alatus* EtOAc fraction, *P. frutescens* EtOAc fraction, *H. cordata* BuOH fraction, *P. mume* EtOAc fraction, *P. trifoliata* EtOAc fraction, and arbutin by (A) NR assay and (B) MTT assay. Results are shown as the averages ± SD of three independent experiments.

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As shown in Figure 3B, 10 fractions, including EtOAc fraction of *S. china*, were found to have cytotoxicity at the concentration of 0.01% or less. However, the fractions showed similar results for MTT_{50} except for the EtOAc fractions of *E. officinalis* and *P. trifoliata*, which were greater than 0.1%.

As indicated by the results of cytotoxicity testing (NR assay and MTT assay), the proper concentration of test material was determined to be approximately 0.001%, which showed relatively good cell survival results for intracellular assay.

INTRACELLULAR TYROSINASE INHIBITION

The method used for the evaluation of the depigmenting effect using melanocytes has an advantage in that it can be used to analyze the overall effects of sample on melanin biosynthesis. However, since this method is complex and time consuming, it is difficult to perform multiple sample tests simultaneously. Therefore, from the result of the *in vitro* tyrosinase inhibition test, L-DOPA auto-oxidation inhibition test, and melanin synthesis inhibition test, 4 out of 10 plant fractions were selected for the intracellular tyrosinase inhibition test.

The EtOAc fractions of *S. china*, *P. lactiflora*, and *P. umbellatus* and BuOH fraction of *E. officinalis* showed better inhibition effects than arbutin in two or more of the efficacy tests. The appropriate concentrations of test materials were determined to be 0.001%, 0.0025%, and 0.005% from the results of cytotoxicity tests.

Samples were dissolved in DMEM, and the intracellular tyrosinase inhibition test was conducted using B16 melanoma cells with arbutin as a control. The four fractions showed significant increases in intracellular tyrosinase inhibition as their concentrations increased.

The levels of inhibition by EtOAc fractions of *S. china* and *P. umbellatus* and BuOH fraction of *E. officinalis* were similar to that of arbutin. However, the EtOAc fraction of *P. lactiflora* showed a significant, excellent tyrosinase inhibition effect compared to arbutin (Figure 4A). The IC_{50} values were 19.9 μg/ml for *P. lactiflora*, 43.8 μg/ml for *E. officinalis*, 43.5 μg/ml for *P. umbellatus*, and >50 μg/ml of *S. china* and arbutin.

INTRACELLULAR MELANIN SYNTHESIS INHIBITION

The same fractions used in the intracellular tyrosinase inhibition test were dissolved in DMEM, and the intracellular melanin synthesis inhibition test was conducted using B16 melanoma cells with arbutin as a control. The four fractions showed a significant increase in intracellular melanin biosynthesis inhibition as the concentration increased. The levels of inhibition by EtOAc fractions of *S. china* and *P. umbellatus* and BuOH fraction of *E. officinalis* were similar to that of arbutin. However, the EtOAc fraction of *P. lactiflora* showed a significant, excellent melanin biosynthesis inhibition effect compared to arbutin (Figure 4B). The IC_{50} values were 16.6 μg/ml for *P. lactiflora*, 26.7 μg/ml for *E. officinalis*, 32.1 μg/ml for *P. umbellatus*, and 50 μg/ml or more for *S. china* and arbutin.

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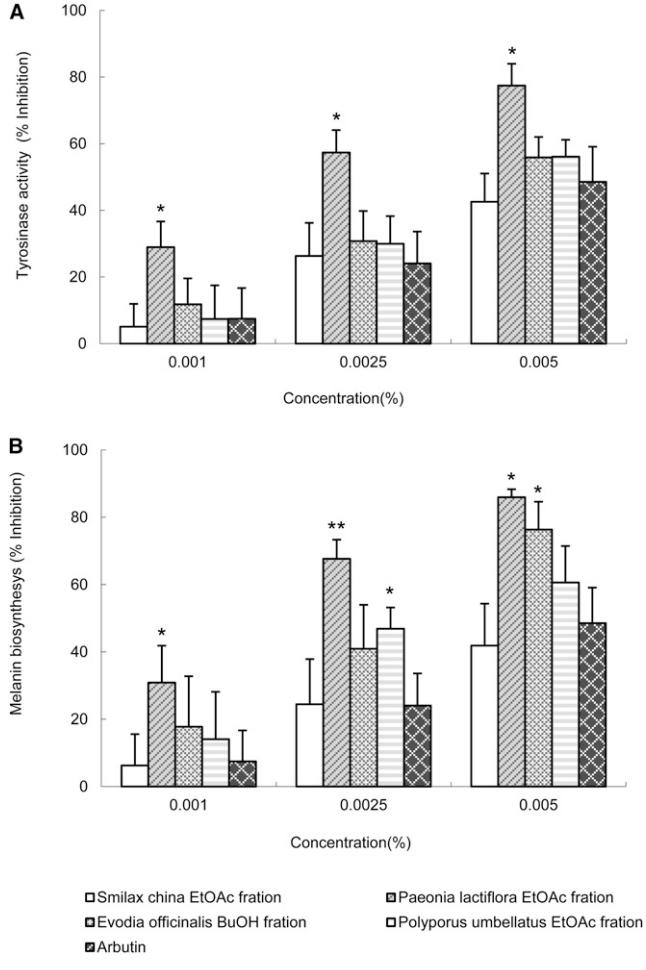

Figure 4. Inhibitory effects on (A) tyrosinase activity and (B) melanin biosynthesis in B16 melanoma cells (% of control). Results are shown as the averages ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$ compared to arbutin using a *t*-test.

IN VIVO DEPIGMENTING EFFICACY TEST

EtOAc fraction of *P. lactiflora* and BuOH fraction of *E. officinalis*, which showed good results for the intracellular depigmenting efficacy test, were selected as samples for the *in vivo* test. The samples and hydroquinone as a positive control dissolved in vehicle to 2% were evaluated for their topical effects on skin pigmentation of a brown guinea pig through daily application for 6 weeks of treatment. Results are shown in Figure 5.

The *P. lactiflora* fraction- and hydroquinone-treated sites showed skin-lightening effects as time increased, but the skin-lightening effect of *E. officinalis* was slight. From the viewpoint of adverse effects, *P. lactiflora*- and *E. officinalis*-treated sites have shown slight skin irritation on one or two animals. In contrast, hydroquinone-treated sites showed signs of irritation after 2 weeks and these signs were more intense at 6 weeks.

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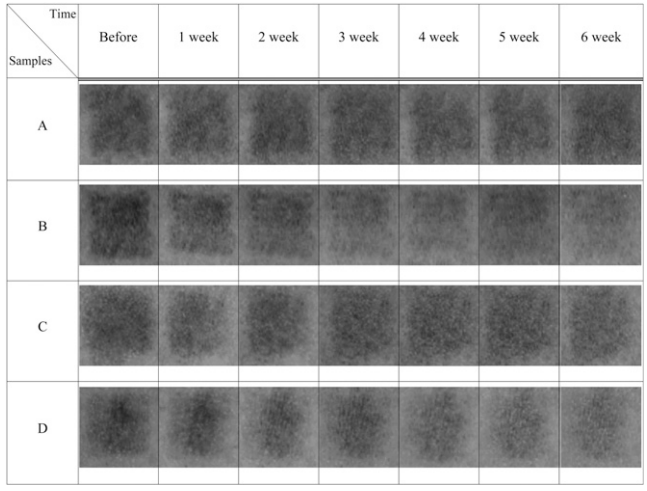

Figure 5. Representative photographs showing the depigmenting effect on UV-induced hyperpigmentation: (A) vehicle, (B) 2% of *P. lactiflora* fraction, (C) 2% of *E. officinalis* fraction, and (D) hydroquinone (positive control).

The degree of pigmentation on treated areas was measured according to the melanin index with a mexameter and is shown in the Figure 6. Both the EtOAc fractions of *P. lactiflora* and hydroquinone resulted in a significant skin-lightening effect compared to vehicle at 6 weeks. In conclusion, as a result of *in vitro* and *in vivo* depigmenting tests, the EtOAc fraction of *P. lactiflora* was found to be the most effective substrate among the natural plant extracts.

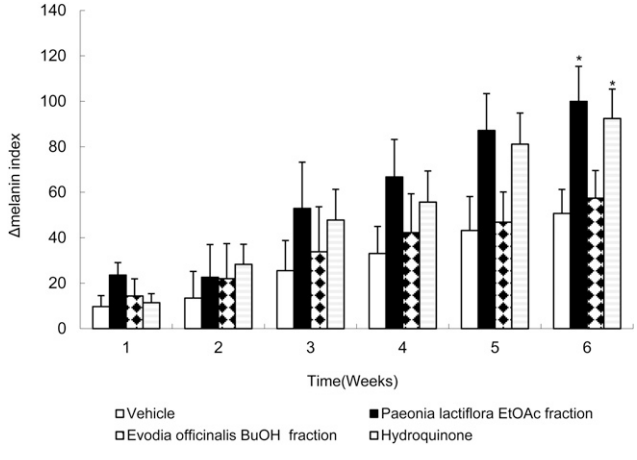

Figure 6. The degree of depigmentation (Δmelanin index) before and 1, 2, 3, 4, 5, and 6 weeks after daily topical application of vehicle, 2% *P. lactiflora* fraction, 2% *E. officinalis* fraction, and 2% hydroquinone. Results are shown as the averages ± SD of three independent experiments. *p <0.05 compared to vehicle using t-test.

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CONCLUSIONS

In this study, we investigated the evaluation methods for screening of natural depigmenting substances and searched for depigmenting substances from 17 natural plants, including *S. china*. To screen potential depigmenting substances, an *in vitro* tyrosinase inhibition test using mushroom tyrosinase, *in vitro* L-DOPA auto-oxidation inhibition test, *in vitro* melanin synthesis inhibition test, intracellular tyrosinase inhibition test, and intracellular melanin synthesis inhibition test were conducted. In addition, a cytotoxicity study was performed. Finally, we conducted an *in vivo* depigmentation study of candidate depigmenting plant extracts with brown guinea pigs. Results from such tests can be summarized as follows:

(1) A total of 85 samples (extraction and fractionation of 17 natural substances with MeOH, MC, EtOAc, *n*-BuOH, and H_2O) were tested for tyrosinase inhibition and *in vitro* L-DOPA auto-oxidation inhibition with mushroom tyrosinase. From these tests, 10 fractions, including the EtOAC fraction of *S. china*, were selected as candidate depigmenting substances.

(2) Ten candidate depigmenting substances at various concentrations were subjected to an *in vitro* tyrosinase inhibition test, *in vitro* L-DOPA auto-oxidation inhibition test, and *in vitro* melanin synthesis inhibition test. In addition, cytotoxicity tests (NR_{50} and MTT_{50}) were conducted to evaluate safety. From these tests, four extracts with good efficacy and low toxicity were selected, and included the EtOAc fraction of *S. china*, EtOAc fraction of *P. lactiflora*, BuOH fraction of *E. officinalis*, and EtOAc fraction of *P. umbellatus*.

(3) The four selected plant fractions showed concentration-dependent inhibitory effects of intracellular tyrosinase activity and intracellular melanin biosynthesis in the B16 melanoma cell line. More specifically, the EtOAc fraction of *P. lactiflora* showed a significant, excellent depigmenting effect in both the intracellular tyrosinase inhibition test and the intracellular melanin biosynthesis inhibition test compared to arbutin, which is a depigmenting substance widely used in Korea. The IC_{50} values were 19.9 μg/ml for *P. lactiflora*, 43.8 μg/ml for *E. officinalis*, 43.5 μg/ml for *P. umbellatus* in the intracellular tyrosinase inhibition test and 16.6 μg/ml for *P. lactiflora*, 26.7 μg/ml for *E. officinalis*, 32.1 μg/ml for *P. umbellatus* in the intracellular melanin inhibition test.

(4) The melanin index was measured with the artificially pigmented skin of brown guinea pigs to measure the final depigmenting effects of the EtOAc fraction of *P. lactiflora* and the BuOH fraction of *E. officinalis*, which showed a good result in the *in vitro* test. The EtOAc fraction of *P. lactiflora* also showed significant skin-lightening effects compared to the vehicle.

In this study, the EtOAc fraction of *P. lactiflora* was found to be the most effective substrate. It was particularly effective depigmenting agent compared to arbutin in the *in vitro* test and showed a similar effect to that of hydroquinone in the *in vivo* test. However, the cytotoxicity of EtOAC fraction of *P. lactiflora* is still higher than arbutin. There needs to be a better search for active components from specific fraction of *P. lactiflora* without any cytotoxicity. For further study, gallic acid, methyl gallate, and pentagalloyl glucose, major components in the *P. lactiflora*, need to be purified through an activity-guided isolation and identified by spectroscopic analysis from the EtOAC fraction of *P. lactiflora*.

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