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Inhibitory effects of some medicinal plant extracts on the tyrosinase promoter activity on B16 mouse melanoma cells

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SUMMARY

Melanin is specifically produced in melanocytes. The pathway for melanin biosynthesis is mainly controlled by tyrosinase. To estimate the inhibitory effect of melanin biosynthesis from 31 medicinal plants extracts, we tested the inhibitory effects of the tyrosinase promoter on B16 mouse melanoma cells. The result of this study demonstrated that Mori Radicis Cortex and Castena Fractus extracts only in tested medicinal plant extracts have high inhibitory effects on tyrosinase promoters with very low cytotoxicity on B16 mouse melanoma cells. Therefore, extracts of Mori Radicis Cortex and Castena Fractus were evaluated as very effective negative regulators of tyrosinase gene expression.

INTRODUCTION

Melanin determines the hair, skin, and eye color of animals, and plays a protective role against harmful UV irradiation, oxidants, etc. It synthesizes specific melanocytes of hair follicles, eyes, and the basal layer of epidermis. Synthesis of the melanin pigment is stimulated by an increase of ultraviolet radiation exposure or other factors.

Melanin pigments synthesize from L-tyrosine via several step oxidation reactions if melanocytes are activated by the stimulation of UV light. The melanocyte synthesizes two types of pigments, pheomelanin from red to red brown color and eumelanin from dark brown to black colors. Generally, eumelanin is referred to as melanin and pigmentation produces it. Melanin biosynthesis takes part in several enzymes such as tyrosinase (polyphenol oxidase), DHICA oxidase (TRP1), DOPAchrome tautomerase (TRP2), and catechol-O-methyltransferase (COMT). The rate-limiting steps of melanin biosynthesis are catalyzed by tyrosinase, which catalyzes the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and of L-DOPA to DOPAquinone. Recent studies on the prevention of

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pigmentation focused on the screening of tyrosinase inhibitory materials from natural products. As a result of these studies, arbutin (Maeda *et al.*, 1996; Chakraborty *et al.*, 1998), kojic acid (Chen *et al.*, 1991; Cabanes *et al.*, 1994), vitamin C and theirs derivatives (Ando *et al.*, 1993; Ando *et al.*, 1995; Murata *et al.*, 1995; Egawa *et al.*, 1997), lactic acid and theirs derivatives (Ando *et al.*, 1995), retinoic acid (Ortonne *et al.*, 1992), and many natural products (Ohmori *et al.*, 1995; Maeda *et al.*, 1997; Koide *et al.*, 1997; Okano *et al.*, 1997; Kinoshita *et al.*, 1997; Yokota *et al.*, 1998) were widely used in cosmetic products and drugs as whitening agents.

In this study, in order to estimate the inhibitory effect of melanin biosynthesis, we selected plant such as oriental herbal drugs from previously reported medicinal plants that have a whitening effect, and tested the inhibitory effects of the tyrosinase promoter by using the medicinal plant extracts on B16 mouse melanoma cells.

MATERIALS AND METHODS

Preparation and extraction of medicinal plants

Medicinal plants were purchased from oriental herb drug stores in Gwangju and voucher specimens were deposited in the Biochemistry Lab., Department of Biology, Chonnam National University. Each of the dried medicinal plants were sliced, and then extracted 3 times with 95% methanol at room temperature, and concentrated under reduced pressure below 45℃ using a vacuum evaporator. To test the inhibitory effect of these extracts on the tyrosinase promoter activity on melanogensis B16 mouse melanoma cells, the concentrated extracts were suspended with 100 mg of 1 ㎖ through ethanol : dimethyl sulfuroxide (DMSO) (1:1).

Cell culture

B16 mouse melanoma cells were maintained in a RPMI 1640 medium supplemented with a 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin G (100 IU/㎖), streptomycin (100μg/㎖), and L-glutamine (2mM) in a humidified incubator containing 5% CO_2 and 95% air at 37℃. Transfected B16 mouse melanoma cells were maintained in a selective media (RPMI 1640 medium supplemented with a 10% (v/v) heat-inactivated fetal bovine serum (FBS), Penicillin G (100 IU/㎖), Streptomycin (100μg/㎖), L-glutamine (2mM), and geneticin(200μg/㎖)) in a humidified incubator containing 5% CO_2 and 95% air at 37℃.

Stable transfection experiment

To complete the stable transfection of melanogenesis B16 mouse melanoma cells were seeded with 4×10^5 in 35mm-dishes and transfection was carried out the following day using 6 μℓ of LipofectAMINE (Life Technologies, Inc) and 2μg of total plasmid DNA in a 1ml final volume. A luciferase reporter vector-pGL2 constructed in a 1.0-kilobase pair fragment of a human tyrosinase gene promoter was transfected with the test plasmids to monitor the variability in transfection efficiency. Five hours after transfection, the cells were exchanged with selective media containing-geneticin (600 μg/㎖) to obtain transfected B16 mouse melanoma cells only Thereafter, media exchange was repeated at intervals of 2~3 days until there was a colony formation of transfected cells.

Luciferase assay

Transfected B16 mouse melanoma cells were seeded with 6×10^4 in 24-well dishes and incubated for 24 hours. Thereafter the cells were washed with a phosphate

buffered saline (PBS, pH 7.4) and lysed with a 25mM Tris-phosphate buffer (pH 7.8) containing 1% Triton X-100, 2mM EDTA, and 2mM dithiothreitol. Soluble extracts were harvested and used to measure luciferase activity. Luciferase activity was determined with 100ul of 0.5mM luciferin and peak light emission was recorded on a luminometer (Lumat LB 9507, Berthold).

Inhibitory effects of the tyrosinase promoter

Transfected B16 mouse melanoma cells were seeded with 6 × 10^4 in 24-well dishes. After incubation of 24 hours, the cells exchanged new growth media and were treated for 6 hours with 10μg, 100μg, 1mg each of the medicinal plant extracts in a 1ml final volume, and then washed with a phosphate buffered saline (pH 7.4) and lysed with a luciferase lysis buffer. Soluble extracts were harvested and measured luciferase activity. Inhibitory effects from medicinal plant extracts on the tyrosinase promoter activity were evaluated based on their luciferase activity value.

Concentration at 50% of the inhibition rate (IC_{50}) of the tyrosinase promoter from medicinal plant extracts was also determined using the luciferase assay method as described above.

Cytotoxicity of medicinal plant extracts

Transfected B16 mouse melanoma cells were seeded with 6 × 10^4 in 24-well dishes and incubated for 24 hours. Thereafter the cells exchanged new media and were treated for 6 hours with 1μg, 10μg, 50μg, 100μg, each of the medicinal plant extracts in a 1ml final volume. Lastly, the cytotoxicity of the medicinal plant extracts was evaluated by the MTT assay (Mossman et al., 1983).

RESULTS

Inhibitory effects of the tyrosinase promoter

The inhibitory effects of the tyrosinase promoter was tested using extracts of 31 medicinal plants on B16 mouse melanoma cells by using lucuifease assay. Medicinal plant extracts showed variable activity on the tyrosinase promoter on B16 melanoma cells, as shown in Table 1.

Table 1. Inhibitory effects of some medicinal plant extracts on the tyrosinase promoter in B16 mouse melanoma cells

Pharmaceutical Name	Scientific Name	Luciferase Activity (%)		
		10 μg/mℓ	100 μg/mℓ	1 μg/mℓ
Akebiae Caulis	*Akebia quinata, Lardizabalaceae*	129±6.4	123±21.1	ND
Angelicae Dahuricae Radix	*Angelica dahurica, Umbelliferae*	110±1.4	56±2.1	ND
Angelicae Gigantis Radix	*Angelica sinensis, Umbelliferae*	85±13.8	1±0.8	ND
Arecae Semen	*Areca catechu, Palmae*	105±3.4	62±1.8	50±11.3
Armeniaceae Semen	*Prunus armeniaca, Amygdalaceae*	130±7.0	118±5.4	ND
Castena Cortex Fractus	*Castanea mollissima, Fagaceae*	62±8.5	53±6.9	ND
Castena Fructus	*Castanea mollissima, Fagaceae*	126±3.2	136±14.2	23±5.1
Cimicifugae Rhizoma	*Cimicifuga heracleifolia, Ranunculaceae*	117±4.1	134±16.7	ND
Cnidii Rhizoma	*Cindium officinale, Umbelliferae*	139±12.7	132±5.5	67±6.7

Ephedrae Herba	***Ephedra sinica, Ephedraceae***	**137±4.7**	**120±9.2**	**ND**
Eucommiiae Cortex	***Eucommia ulmoides, Eucommiaceae***	**92±0.9**	**40±4.2**	**ND**
Ginseng Folium	***Panax ginseng, Araliaceae***	**108±4.5**	**81±8.3**	**ND**
Glycyrrhizae Radix	***Glycyrrhiza galbra, Leguminosae***	**138±7.1**	**158±15.5**	**ND**
Houttuyniae Herba	***Houttuynia cordata, Saururaceae***	**127±15.8**	**59±6.6**	**ND**
Junci Coulis Medulla	***Juncus decipiens, Juncaceae***	**143±14.5**	**98±7.9**	**ND**
Lithospermi Radix	***Lithospermum erythrorhizon, Boraginaaceae***	**67±2.0**	**66±9.0**	**ND**
Lycii Cortex Radicis	***Lycium chinenes, Solanaceae***	**132±10.0**	**132±15.5**	**ND**
Lycii Fractus	***Lycium chinenes, Solanaceae***	**101±3.5**	**105±20.1**	**117±10.0**
Mori Radicis Cortex	***Morus alba, Moraceae***	**42±5.4**	**8±0.5**	**ND**
Moutan Cortex Radicis	***Paeonia suffruticosa, Paeoniaceae***	**123±11.9**	**134±16.9**	**58±0.3**
Paeoniae Radix	***Paeonia japonica, Paeoniaceae***	**116±8.0**	**135±0.6**	**83±7.3**
Persicae Semen	***Prunus persica, Amygdalaceae***	**101±4.6**	**97±8.2**	**ND**
Platycodi Radix	***Platycodon grandifiorum, campanulaceae***	**108±4.5**	**108±3.0**	**ND**
Polygoni Multiflori	***Polygonum multiflorum, Polygonaceae***	**129±8.2**	**114±3.9**	**89±8.9**
Raphani Radix	***Raphanus sativus, Cruciferae***	**94±14.9**	**116±11.2**	**ND**
Rehmannia Radix	***Rehmmannia glutinosa, Scrophulariaceae***	**112±7.5**	**119±10.0**	**ND**
Rhei Rhizoma	***Rhem coreanum, Polygonaceae***	**133±3.5**	**119±7.0**	**ND**
Rubus Fractus	***Rubus coreanus, Rosaceae***	**99±7.1**	**92±7.5**	**20±1.5**
Sophorae Radix	***Sophora flavescens, Leguminosae***	**129±2.4**	**37±1.1**	**ND**
Ulmi Cortex	***Ulmus pumila, Ulmaceae***	**100±13.5**	**109±5.0**	**65±12.7**
Zingiberis Rhizoma	***Zingiber officinale, Zingiberaceae***	**82±1.9**	**1±0.4**	**ND**

*ND: Not detected

Especially, the extracts of Mori Radicis Cortex (*Morus alba*), Castena Fractus Cortex (*Castanea mollissima*), and Lithospermi Radix (*Lithospermum erythrorhiz-on)* in tested medicinal plants, showed high inhibitory effects of the tyrosinase promoter on lower concentrations of 10 ㎍/㎖. Extracts of Angelicae Dahuricae Radix (*Angelica dahurica*), Angelicae Gigantis Radix (*Angelica sinensis*), Arecae Semen (*Areca catechu*), Eucommiae Cortex (*Eucommia ulmoides*), Houttuyniae Herba (*Houttuynia cordata*), Ulmi Cortex (*Ulmus pumila*), and Zingiberis Rhizoma (*Zingiber officinale*) exhibited moderate inhibitory effects of the tyrosinase promoter on 100 ㎍/㎖. But most of the extracts in concentrations of 1 ㎎/㎖ could not detected luciferase activity because they led to cell lysis.

Most of the medicinal plant extracts have a higher concentration value of IC_{50}, as shown in Table 2. Mori Radicis Cortex extracts especially, exhibited an IC_{50} value on much lower concentrations than 10 ㎍/㎖. While extracts of Arecae Semen and Lithospermi Radix exhibited an IC_{50} value in high concentrations (300 ㎍/㎖, 500 ㎍/㎖). Others, except those listed in Table 2, exhibited activation effects rather than inhibition effects of the tyrosinase promoter in concentrations of 10 or 100 ㎍/㎖.

Table 2. IC_{50} of some medicinal plant extracts on the tyrosinase promoter in B16 mouse melanoma cells

Pharmaceutical Name	Scientific Name	IC_{50} (㎍/㎖)
Angelicae Dahuricae Radix	*Angelica dahurica, Umbelliferae*	100
Angelicae Gigantis Radix	*Angelica sinensis, Umbelliferae*	30
Arecae Semen	*Areca catechu, Palmae*	300
Castena Cortex Fractus	*Castanea mollissima, Fagaceae*	100
Eucommiiae Cortex	*Eucommia ulmoides, Eucommiaceae*	100
Houttuyniae Herba	*Houttuynia cordata, Saururaceae*	100
Lithospermi Radix	*Lithospermum erythrorhizon, Boraginaaceae*	500
Mori Radicis Cortex	*Morus alba, Moraceae*	10
Sophorae Radix	*Sophora flavescens, Leguminosae*	50
Zingiberis Rhizoma	*Zingiber officinale, Zingiberaceae*	30

Cytotoxicity of medicinal plant extracts

Medicinal plant extracts having an inhibitory effect above 40% of the tyrosinase promoter under the concentrations of 100 ㎍/㎖ were selected, and then tested on their MTT of B16 melanoma cell. Extracts of Castena Fractus Cortex and Mori Radicis Cortex only, exhibited very lower cytotoxicity on IC_{50} on B16 mouse melanoma cells, as shown Table 3. And Lithospermi Radix extracts exhibited no cytotoxicity on cells under 100 ㎍/㎖ concentration but exhibited high cytotoxicity in a 1mg/ml concentration. On the other hand, extracts of Angelicae Dahuricae Radix, Angelicae Gigantis Radix Arecae Semen, Eucommiae Cortex, Houttuyniae Herba, Ulmi Cortex, and Zingiberis Rhizoma exhibited high cytotoxicity in 100 ㎍/㎖ on B16 mouse melanoma cells.

Table 3. Cytotoxicity of some medicinal plant extracts in B16 mouse melanoma cells

Pharmaceutical Name	MTT Assay(%)				MTT_{50}
	1 ㎍/㎖	10 ㎍/㎖	50 ㎍/㎖	100 ㎍/㎖	(㎍/㎖)
Angelicae Dahuricae Radix	123±2.9	125±17.0	28±0.6	23±1.1	39
Angelicae Gigantis Radix	104±4.9	49±3.3	21±1.7	23±3.3	9
Arecae Semen	109±7.7	106±5.9	76±7.6	58±4.0	-
Castena Cortex Fractus	98±8.0	98±2.7	96±3.2	74±0.9	-
Eucommiiae Cortex	100±4.6	102±4.9	82±3.0	35±1.6	84

Houttuyniae Herba	102±2.8	101±3.9	25±4.7	25±4.6	36
Lithospermi Radix	169±9.7	186±22.4	211±20.4	108±8.6	-
Mori Radicis Cortex	104±5.3	85±6.6	25±1.7	ND	33
Sophorae Radix	112±6.2	86±3.2	29±0.8	ND	35
Zingiberis Rhizoma	119±3.3	108±5.6	33±6.3	28±1.1	40

* ND: Not detected.

DISCUSSION

Until present, many cosmetic product researchers, Biochemists, and Biopharmacologists have been using a lot of effort to prevent the pigmentation of melanin. Results reported a number of tyrosinase inhibitors found in natural products such as medicinal plants (Jang *et al.*, 1997;Park *et al.*, 1997; Lee *et al.*, 1997; Shin *et al.*, 1997; Kubo *et al.*, 1998; Shimizu *et al.*, 1998; Shin *et al.*, 1998; Yokota *et al.*, 1998; Seo *et al.*, 1999; Kubo *et al.*, 1999), marine botany (Choi *et al* 1998), and microorganism (Goetghebeur *et al.*, 1996; Sano *et al.*, 1996). But these compounds had the fault of lowering and shortening their effects because they join the tyrosinase enzyme activity level. On the other hand, more recently, many researchers have performed studies about regulation mechanisms and suppressors of melanin biosynthesis on the gene expression level using molecular biology techniques(Lowings *et al.*, 1992; Prota *et al.*, 1993; Ando *et al.*, 1993; Kondoh *et al.*, 1995; Shibahara *et al.*, 1995; Kichina *et al.*, 1996; Ferguson *et al.*, 1997; Maeda *et al.*, 1997; Nakajima *et al.*, 1997) to develop a more effective melanogenic inhibitor.

We tested the inhibitory activity of the tyrosinase promoter and cytotoxicity from 31 medicinal plant extracts on B16 mouse melanoma cells. In this study, methanol extracts of Angelicae Dahuricae Radix, Angelicae Gigantis Radix, Arecae Semen, Castena Fractus Cortex, Eucommiae Cortex, Houttuyniae Herba, Lithospermi Radix, Mori Radicis Cortex, Ulmi Cortex, and Zingiberis Rhizoma only exhibited high inhibition effects on the tyrosinase promoter on B16 mouse melanoma cells. But these tyrosinase promoter inhibitory effects were closely related with the cytotxicity of medicinal plant extracts with the exception of Mori Radicis Cortex, Castena Fractus Cortex, and Lithospermi Radix. Inhibitory effects of the tyrosinase promoter from tested medicinal plants depended on concentration and cytotoxicity. On the other hand, some medicinal plant extracts exhibited more active than inhibition of the tyrosinase promoter under 100 $\mu g/m\ell$ but exhibited inhibitory effects in 1 $\mu g/m\ell$ concentrations. As a result of these studies, these medicinal plants could be possibly utilizated as positive regulators in tyrosinase gene expression.

In conclusion, extracts of Mori Radicis Cortex and Castena Fractus in tested medicinal plants demonstrated to be an effective and the safe negative regulator for the gene expression of tyrosinase.

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