

Melanogenesis Inhibitory Activities of Diarylheptanoids from *Alnus hirsuta* Turcz in B16 Mouse Melanoma Cell

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Four diarylheptanoids, (5R)-1,7-bis (3,4-dihydroxyphenyl)-heptane-5-O- β -D-glucoside (**1**), (5R)-1,7-bis (3,4-dihydroxyphenyl)-heptane-5-ol (**2**), oregonin (**3**), hirsutanonol (**4**), were isolated from the bark of *Alnus hirsuta* Turcz and its inhibitory effects on melanogenesis by measuring the melanin level and tyrosinase activity in B16 melanoma cell were examined. Melanin level and tyrosinase activity were reduced to 75 to 85% by addition of diarylheptanoids to incubation medium of the melanoma cell. On the other hand, melanin level and tyrosinase activity were reduced to 13 to 43% by the addition of diarylheptanoids to incubation medium of the melanoma cell treated with melanogenesis stimulator, α -MSH and forskolin. These melanogenesis inhibitory effects were significantly different compared with control.

Key words: Diarylheptanoids, *Alnus hirsuta* Turcz, melanogenesis, tyrosinase activity

INTRODUCTION

Melanin pigmentation in human skin is a major defense mechanism against ultraviolet light of the sun, but abnormal pigmentation such as freckles or chloasma (liver spot and melasma) could be a serious aesthetic problem (Iwata *et al*, 1990). It was reported that some flavonoid (Chun *et al*, 2002) and tannin (Kim *et al*, 2001) showed inhibitory activities of tyrosinase and melanogenesis. Recently, we found that diarylheptanoids from *Alnus* species grown in Korea have several biological activities including inhibitory activity of inducible nitric oxide synthase (Lee *et al*, 2000A), inhibitory activity of cyclooxygenase-2 expression (Lee *et al*, 2000B) and anti-oxidative activity (Lee *et al*, 2000C). In this paper, we examined the inhibitory effect of diarylheptanoid from the bark of *Alnus hirsuta* Turcz on the tyrosinase activity and the melanin biosynthesis in B16 melanoma cells.

MATERIALS AND METHODS

Isolation and identification

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Barks of *A. hirsuta* Turcz were collected in Mt. Chunggei near Seoul, Korea in October of 2000 and identified by Prof. S. H. Toh (College of Pharmacy, Dongduk Womans University, Seoul, Korea). A voucher specimen (AHB0081) is deposited at the herbarium of College of Pharmacy, Chung-Ang University (Korea).

Fresh barks (2.5 kg) of *A. hirsuta* Turcz were extracted with 80% aqueous Me₂CO at room temperature for 3 days. After removal Me₂CO *in vacuo*, the aqueous solution was filtered. The filtrate was concentrated and then applied to a column of Sephadex LH-20 (2 kg, 10 \times 70 cm). Elution with H₂O containing proportions of MeOH afforded 4 fractions, A (40 g), B (30 g), C (20 g) and D (20 g). Fraction A was composed of sugar and minerals.

Repeated column chromatography of fr B on MCI-gel CHP 20P (75-150 μ m, 400 g, 5 \times 60 cm) with a H₂O:MeOH gradient (from H₂O to 100% MeOH) and low pressure liquid column chromatography (YMC-gel ODS-A, 500/400 mesh, 200 g, 4.4 \times 25 cm) with a H₂O:MeOH gradient (from H₂O to 50% MeOH) yielded (5R)-1,7-bis-(3,4-dihydroxyphenyl)-5-heptane-O- β -D-glucoside (**1**, 2 g, 0.08 w/w) and oregonin (**3**, 1.2 g, 0.048 w/w%). Column chromatography of fr. C on MCI-gel (400 g, 5 \times 60 cm) with a H₂O:MeOH gradient (from H₂O to 100% MeOH) and low pressure liquid column chromatography (YMC-gel ODS-A, 200 g, 4.4 \times 25 cm) with a H₂O:MeOH gradient (from 10%MeOH

to 50% MeOH) yielded (5R)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-ol (**2**, 150 mg, 0.006 w/w%). Column chromatography of fr. D on MCI-gel (300 g, 4 × 50 cm) with a H₂O:MeOH gradient (from H₂O to 100% MeOH) and low pressure liquid column chromatography (YMC-gel ODS-A, 100 g, 3.2 × 25 cm) with a H₂O:MeOH gradient (from 40%MeOH to 80% MeOH) yielded hirsutanonol (**4**, 300 mg, 0.012 w/w%).

The structures of compounds **1–4** were identified as (5R)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-O-β-D-glucoside, (5R)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-ol, oregonin and hirsutanonol, respectively, by comparison their spectral data with those reported in the literature (Lee *et al.*, 2000D). Full details of the isolation and characterization are available on request from the author of correspondence.

Cell Culture

B16 murine melanoma cells were cultured in Dulbecco's modified Eagles medium with 10% fetal bovine serum and penicillin/streptomycin (100 IU/50 μg/ml) in a humidified atmosphere containing 5% CO₂ in air at 37°C.

Enzyme Activity Assay and Melanin Determination

Tyrosinase activity was estimated by measuring the rate of oxidation of L-dopa. Cells from a subconfluent monolayer in a 6-well plate well were suspended in 100 μl of phosphate buffer, pH 6.8, containing 1%(w/v) Triton X-100. After vortexing to lyse the cells, the extracts were clarified by centrifugation at 10,000 rpm for 5 min in an Eppendorf tube. The tyrosinase substrate L-dopa (2 mg/ml) was prepared in the same lysis phosphate buffer (without Triton). 40 μl of each extract was put in a 96-well plate, and 100 μl of lysis buffer was added to start the enzymatic assay. Absorbance at 570 nm was read after 1hr incubation at 37°C using a microplate reader.

For melanin determination, after 48 hr treatment with α-MSH or forskolin, cells from a confluent 3.5 cm diameter

plate were solubilized in 100 μl of 1 N NaOH and diluted with 400 μl of distilled water. The samples were incubated at 60°C for 1h and vortexed to solubilized the melanin.

Absorbance at 405 nm was compared with a standard curve of known concentrations of fungal melanin prepared in a final NaOH concentration of 0.2 N.

RESULTS AND DISCUSSION

Fresh barks of *A. hirsuta* Turcz were extracted with aqueous acetone and the extract was subjected to a combination of Sephadex LH-20, MCI-gel CHP 20P and YMC-gel ODS-A chromatography to afford four diarylheptanoids including (5R)-1,7-bis-(3,4-dihydroxyphenyl)-5-heptane-O-β-D-glucoside (**1**), (5R)-1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-ol (**2**), oregonin (**3**) and hirsutanonol (**4**).

MTT assay showed that non-cytotoxic range of these diarylheptanoids on B16 melanoma cell as 0-20 μg/ml and the treatment ranges of compounds were determined as 2.5, 5, 10 and 20 μg/ml.

Melanin level and tyrosinase activity were reduced to 75 to 85% by addition of diarylheptanoids to incubation medium of the melanoma cell. All the compounds showed moderate inhibitory effect on intrinsic tyrosinase activity and melanin level in melanoma cell (Fig. 1 and 2).

On the other hand, melanogenesis could be stimulated by ultraviolet A and B radiation and by a large array of effectors including α-MSH and pharmacological agents such as forskolin in cultured melanocyte or in melanoma cells. It is known that forskolin increases the intracellular cAMP pathway in melanogenesis. The stimulation of melanogenesis by cAMP-elevating agents seems to occur through the induction of tyrosinase expression and stimulation of its intrinsic enzymatic activity ensuing post-translational modifications (Roser *et al.*, 1996). In this experiment,

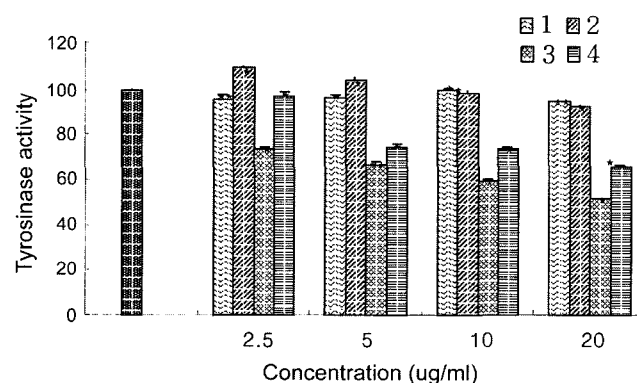
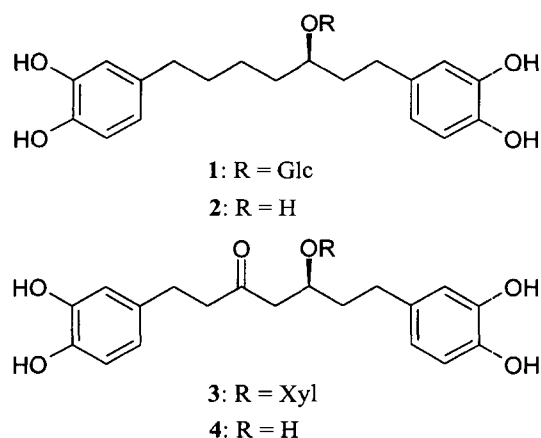


Fig. 1. Inhibitory effect of diarylheptanoids on tyrosinase activity in B16 melanoma cells. Tyrosinase activities were measured at 405 nm. Results were expressed as % control and data mean ± S.D. of at least three determinations. *Significantly different from control group (**p < 0.01, *p < 0.05).

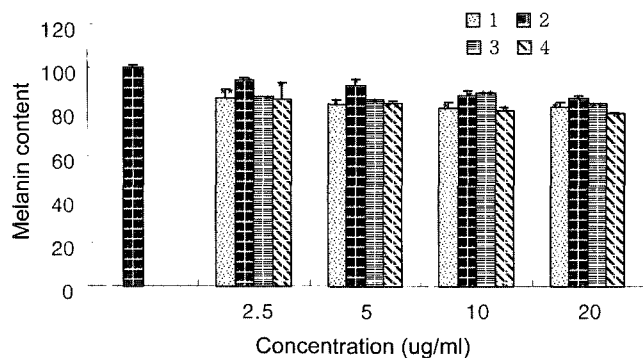


Fig. 2. Inhibitory effect of diarylheptanoids on melanin synthesis in B16 melanoma cells. Melanin contents were measured at 405 nm. Results were expressed as % control and data mean \pm S.D. of at least three determinations. *Significantly different from control group (** $p < 0.01$, * $p < 0.05$).

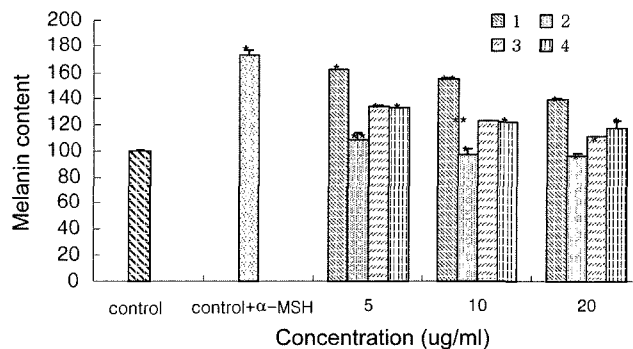


Fig. 5. Inhibitory effect of diarylheptanoids in the presence of treatment of 100 nM α -MSH in B16 melanoma cells. Melanin contents were measured at 405 nm. Results were expressed as % control and data mean \pm S.D. of at least three determinations. *Significantly different from control group (** $p < 0.01$, * $p < 0.05$).

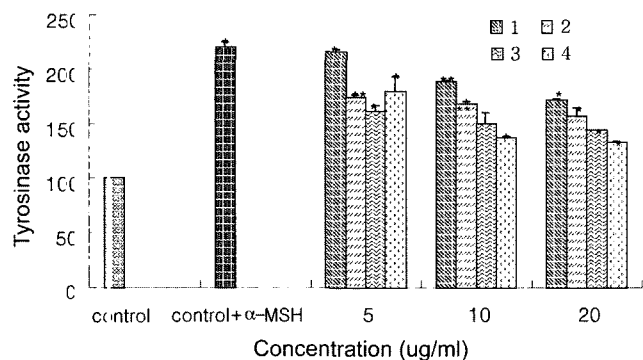


Fig. 3. Inhibitory effect of diarylheptanoids in the presence of treatment of 100 nM α -MSH in B16 melanoma cells. Tyrosinase activities were measured at 405 nm. Results were expressed as % control and data mean \pm S.D. of at least three determinations. *Significantly different from control group (** $p < 0.01$, * $p < 0.05$).

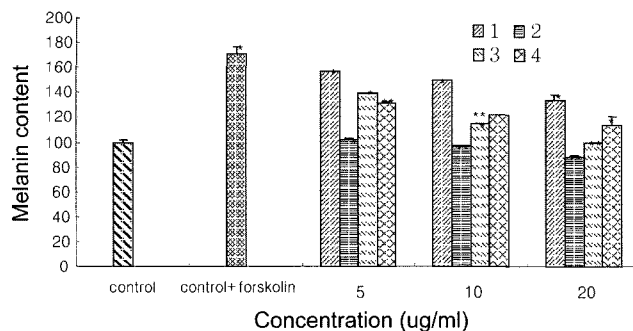


Fig. 6. Inhibitory effect of diarylheptanoids in the presence of treatment of 10 M forskolin in B16 melanoma cells. Melanin contents were measured at 405 nm. Results were expressed as % control and data mean \pm S.D. of at least three determinations. *Significantly different from control group (** $p < 0.01$, * $p < 0.05$).

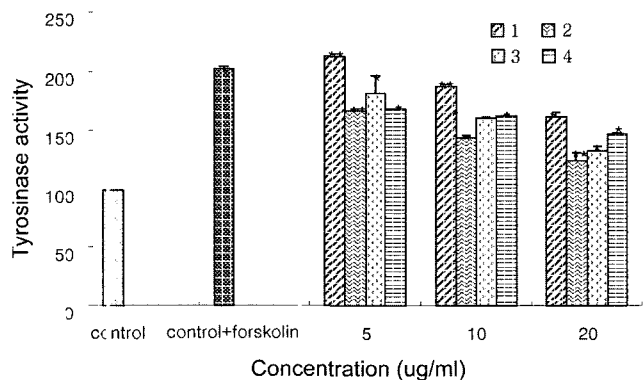


Fig. 4. Inhibitory effect of diarylheptanoids in the presence of treatment of 10 M forskolin in B16 melanoma cells. Tyrosinase activities were measured at 405 nm. Results were expressed as % control and data mean \pm S.D. of at least three determinations. *Significantly different from control group (** $p < 0.01$, * $p < 0.05$).

melanin level and tyrosinase activity was reduced to 13 to 43% by the addition of diarylheptanoids to incubation

medium of the melanoma cell treated with melanogenesis stimulator, α -MSH and forskolin. These effects were significantly different ($p < 0.05$) compared with control (Fig 3, 4, 5 and 6).

These results suggest that diarylheptanoids from the barks of *Alnus hirsuta* Turcz are potential inhibitor against melanogenesis and it could be further developed as skin whitening cosmeceuticals.

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