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Inhibitory effects of ethanol extract of *Atractylodis Rhizoma* on melanogenesis in B16/F10 melanoma cells

Hyun-Ja Chun[1,*], Jeong-Ho Lee[2], Eun-Young Choi[3] and Seung-Hwa Baek[2]

[1]*Division of Natural Sciences and* [2]*Department of Herbal Resources, Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan 570-749, Korea;* [3]*Department of Skin and Care, Kimcheon Science College, Kimcheon 740-703, Korea*

SUMMARY

The inhibitory effect of *Atractylodis Rhizoma alba* extract(AM) on melanogenesis was studied by using B16/F10 melanoma in culture. Cells were cultured in the presence of various concentrations of AM for 48 hrs, and the experiment of total melanin content as a final product and activity of tyrosinase, a key enzyme, in melanogenesis. AM significantly inhibited tyrosinase activity, and melanin content in a dose-dependent manner. These results show that *Atractylodes macrocephala* extract could be developed as skin whitening components of cosmetics.

Key words: *Atractylodes macrocephala*(AM); Melanin biogenesis; Tyrosinase

INTRODUCTION

The rhizome of *Atractylodes macrocephala* Koidz. is known as a tonic in China. It is reported as a nutrient for energy and stomach complaints and for treatment of dyspepsia and anorexia in pharmacopeia of People's Republic of China (Ernst and Ding, 1987). The main constituent of the rhizome of *Atractylodes macrocephala* Koidz. is atractylon (Endo *et al.*, 1979; Lin *et al.*, 1997).

Some Atractylodes plants is empirically classified into two groups depending on the medicinal plant, such as *A. japonika and A. lancea.* These two relatively crude drugs are said to possess different therapeutic effects, the former having antisudorific activity and the latter diaphoretic activity. The chemical constitutions of both plants also show differences in sesquiterpenes and acetylene compounds (Chen, 1987; Chen *et al.*, 1997).

The purpose of the present study was to compare and investigate the role of *Atractylodes macrocephala* Koidz. on the regulation of melanogenesis in B16/F10 melanoma.

In this paper, we demonstrate that the cells exhibit a dose-dependent inhibition in their proliferation without apoptosis after treatment of cells with ethanol extract of *Atractylodes macrocephala* Koidz. We also investigate that ethanol extract of *Atractylodes macrocephala* Koidz. inhibited melanization of B16/F10 melanoma in dose-dependent manners.

MATERIALS AND METHODS

Preparation of AM

The plant sample (11.55 g) was obtained from the oriental drug store, Bohwa-dang (Iksan, Korea). The dried AM was chopped and repeatedly extracted with ethanol at room temperature. The ethanol solution was filtered and evaporated under vacuum in order to get the ethanol extract, and kept at -70°C. The yield of ethanol extract from AM was about 17.3% (2.0 g).

Cell culture

The murine B16/F10 melanoma cells were acquired from the American Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 g/ml streptomycin (Biofluids, Rockville, MD) in a humidified atmosphere containing 5% CO_2 in air at 37°C.

*Correspondence: Dr Hyun-Ja Chun, Division of Natural Sciences, Wonkwang University, Iksan, 570-749, Korea. Tel: 82-63-850-6918; E-mail: hjchun@wonkwang.ac.kr

MTT assay

Growth of B16/F10 melanoma cells in the presence of AM was determined by MTT assay. This assay was performed as described in the modified method of Mosmann (Mosmann 1983). Cells were seeded in 96 well micro-culture plate at 2×10^3 cells per well and allowed to adhere overnight. Cells were exposed to 1,0 to 50 μg/ml of AM for 48 hrs. MTT was prepared at a concentration of 5 mg/ml in sterile PBS. A 20 aliquot of the stock solution of MTT was added to each well. After 3 hrs of incubation at 37°C, 150of DMSO was added to each well in order to dissolve the formazan crystals. Optical densities were measured at 570 nm using a 96 well multi-scanner (Dynatech Instruments, Toronto, CA). A well containing DMEM medium, MTT, and extraction buffer in the absence of B16/F10 melanoma cells was used as the blank. The results obtained were calculated from three experiments and presented as a percentage of control value.

Trypan blue exclusion test

Cells were seeded at a density of 1×10^5 cells/well. After treating with AM for 48 h, detached and adhering cells were harvested and suspended in PBS. One volume of 0.4% trypan blue solution (Sigma Chemical Co., St. Louis, MO., USA) was added to two volumes of the cell suspension, and the dye-excluding cells were counted using a light microscope in a hemocytometer.

Assay of melanin content

Melanin content was determined according to modification of the procedure described by Hosoi (Hosoi *et al.*, 1985). Cells were harvested with 0.25% trypsin-EDTA, washed twice with cold-phosphate buffered saline (PBS), and solved in 1 ml of 1 N NaOH for 1 h at 80°C. Lysates were centrifuged at 12,000×g for 10 min. The absorbance of the supernatant was measured at 405 nm. Melanin content was calculated by comparison with a standard curve using a synthetic melanin (Sigma Chemical Co., St. Louis, MO., USA) and was expressed as a percentage of control value.

Determination of tyrosinase activity

Tyrosinase activity was determined by the method of Matinez-Esparza (Matinez-Esparza *et al.*, 1998). For this experiment, cells were incubated with different concentrations of for 48 hrs. The cells were harvested, washed with cold-PBS, and lysed in 100of lysis buffer (0.1 M phosphate buffer, pH 6.8, containing 1% (w/v) Triton X-100) supplemented with protease inhibitors (0.1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.1 mM PMSF) for 30 min on ice. Cellular extracts were clarified by centrifugation at 9,000×g for 20 min. Protein concentration was determined by Bradford's method (Bio-Rad) using BSA as a standard. A 50 μl of each extract was mixed with 100 μl of 0.1 M sodium phosphate (pH 7.0) at 37°C for 5 min, and then added 50 μl of 10 mM catechol. Activity was assayed at 405 nm for 1 h using ELISA reader.

Statistical analysis

Data were expressed as means±standard deviation. Student's t-test was used to determine the statistical differences between mean values. Differences were considered significant when the P value was less than 0.05.

RESULTS AND DISCUSSION

Effect of AM on cell viability of B16/F10 melanoma cells

This assay is a method to examine the level of cytotoxicity by surveying mitochondria activity in cells. The established B16/F10 melanoma cell line offers a model system with readily quantifiable markers of melanogenesis. The effect of the exposure to increasing concentrations of AM on the cell viability of B16/F10 melanoma cells, as assessed by the MTT assay, is shown in Fig. 1. The cells were incubated with the concentrations of 1, 5, 10, 20, and 50 μg/ml AM for 48 h. AM had no significant effect on the cell viability in all treatment groups as well as morphological change by itself. We further investigated the number of viable cells by means of the trypan blue exclusion test to examine if there was any difference between control group and AM treated groups. The percentage of cells excluding trypan blue did not show any significant decrease after AM treatment for 48 h (Fig. 2). Together all data, the results showed that the cells treated with AM in the range of 1-50 μg/ml for 48 h did not exhibit cytotoxicity and therefore,

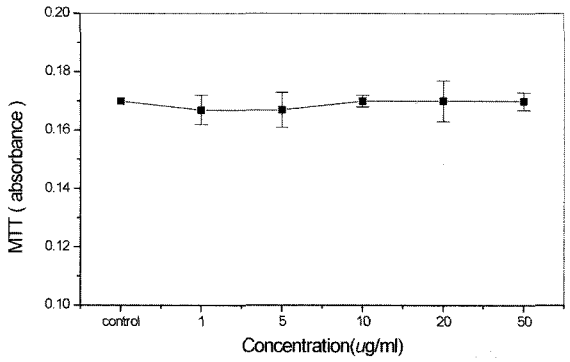

Fig. 1. Effect of AM on the viability of B16 melanoma cells. The cells were treated with various concentration of AM for 48 h. The viability of the cells was measured by MTT assay. Data mean±SD. This experiment was repeated eight times.

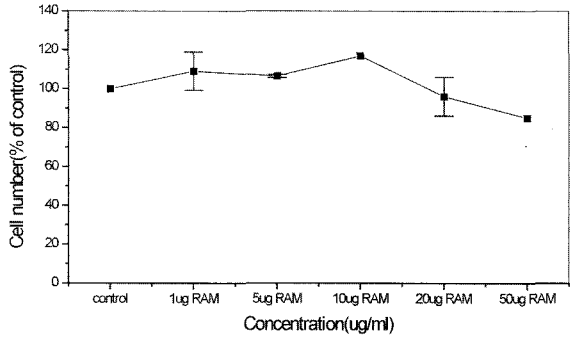

Fig. 2. Effect of AM on the viability if B16 melanoma cells. The cells were treated for 48 h with various concentration of AM. The viability of the cells was measured by trypan blue test.

further studies in this paper were estimated within this range of AM.

Effect of AM on tyrosinase activity

Tyrosinase is the key enzyme in melanin biosynthesis (Hearing and Jimenez, 1987), and a hallmark of differentiation in melanocytes and melanoma cells. Tyrosinase catalyzes the hydroxylation of tyrosine to L-DOPA, the initial and rate-limiting step in melanin synthesis, and further, the oxidation of L-DOPA to DOPAquinone (Korner and Pawelek, 1980). In the absence of thiolic compounds, DOPAquinone evolves spontaneously to L-DOPAchrome, and then to melanin. We investigated whether the effect AM showed significantly tyrosinase activity on the tyrosinase activity in B16/F10 melanoma cells. Cells were incubated with AM in range of 1-50 μg/ml for 48 h and then tyrosinase activity was estimated.

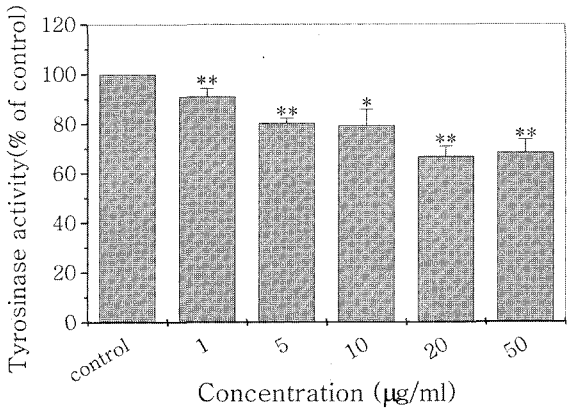

Fig. 3. Inhibitory effect of AM concentration on tyrosinase activity of B16 melanoma cells. Cells were seeded at 1×10^5 cells/well. After the treatment of AM for 48 h, tyrosinase activity were measured at 405 nm. Results were expressed as % of control and data are mean±S.D. of at least five determinations.
Significantly different from control group ($p<0.05$, **$p<0.01$).

In AM-treated B16/F10 melanoma cells, the tyrosinase activity was decreased in a dose-dependent manner (Fig. 3).

Effect of AM on melanin content

Melanocytes are specialized cells located at the basal layer of the epidermis and melanin pigment is the most important characteristic of differentiation in melanocytes or melanoma cells (Ranson *et al.*, 1988). In melanocytes and melanoma cells, melanin biosynthesis or melanogenesis is regulated by tyrosinase, which is a bifunctional copper-containing enzyme. Melanin biosynthesis consists in a cascade of enzymatic and spontaneous reactions that converts tyrosine to melanin pigments.

To clarify the influence of AM on melanogenesis, the content of the final product melanin content was also measured. Cells were incubated with AM in range of 1-50 μg/ml for 48 h and then melanin content was estimated. In AM-treated B16/F10 melanoma cells, the melanin content was decreased in a dose-dependent manner (Fig. 4). These data indicate that AM is sufficient to decrease the melanin content in the cells.

Comparing the tyrosinase activity and melanin contents of the treatment group with AM, tyrosinase activity was greater than melanin content when treated with several concentrations of AM. It is considered that the role of tyrosinase is related to

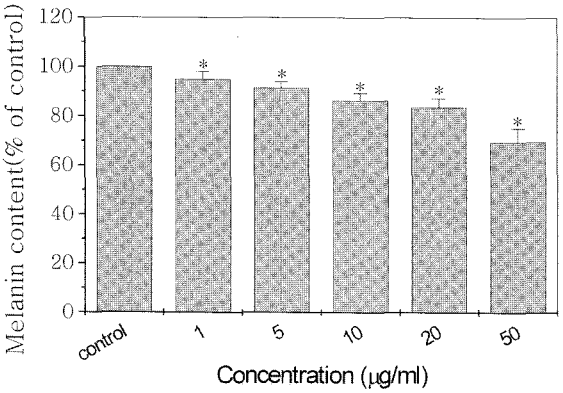

Fig. 4. Inhibitory effect of AM concentration on melanin content of B16 melanoma cells. Cells were seeded at 1×10^5 cells/well. After the treatment of AM for 48h, melanin content were measured at 405 nm. Results were expressed as control and data were mean±S.D. of at least five determinations.
Significantly different from control group ($p<0.05$)

the initial phase, the hydroxylation of tyrosine to L-DOPA, in melanogenesis, whereas melanin formation is the final step passing through other numberous (complex) pathways and several stages. These data support that AM inhibited the activity of tyrosinase and down-regulated the melanin content.

These results clearly indicate that AM regulates melanogenesis by inhibiting the activity of tyrosinase, and is sufficient to decrease the melanin formation in the B16/F10 melanoma cells.

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