Depigmenting effect of *Sterculia lynchnophera* on B16F10 melanoma and C57BL/6 melan-a cells


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Abstract—To develop a novel skin depigmenting agent from natural sources, the inhibition of melanogenesis by the Chinese herb, *Sterculia lynchnophera* (SL), was evaluated. Treatment of B16F10 melanoma cells and melan-a cells with SL exhibited a 32.9% and 68.2% inhibition of melanin synthesis without cytotoxicity at a concentration of 200 µg/ml, respectively. This herb possessed a high free radical scavenging activity with IC50=11.02 µM. The methanol extract of SL slightly inhibited in vitro mushroom tyrosinase activity (23.4% at a concentration of 200 µg/ml) and had a significant inhibitory effect on cellular tyrosinase activity (48.65% and 88.56% inhibition at the concentration 200 µg/ml in B16F10 cells and C57BL/6 melan-a cells, respectively). From the western blotting results, SL inhibited the expression of tyrosinase and tyrosinase related protein 1 (TRP-1). Taken together, we suggest that SL may be a safe and effective depigmentation agent.

Key words: Depigmenting, Melanin, Melanogenesis, Tyrosinase

INTRODUCTION

Melanin biosynthesis is a complicated process involving many factors including the key enzymes tyrosinase, tyrosinase related protein-1 (TRP-1), tyrosinase related protein-2 (TRP-2), cytokines from autocrine and paracrine and those related to melanin transportation and decomposition [1-5]. Melanin plays an important role in preventing ultraviolet (UV) light-induced skin damage, but increased levels of epidermal melanin synthesis can darken the skin. Various dermatologic disorders result in the accumulation of excessive levels of epidermal pigmentation [6].

Many efforts have been made to develop new therapeutic agents against pigmentation abnormalities, especially using novel biologically active compounds from natural plants [7-16]. Medicinal plants are most suitable for pharmacological research and drug development, because their constituents can be used not only as therapeutic agents but also as starting materials or models for the synthesis of drugs or pharmacologically active compounds. Evaluation of Chinese herbal medicine in the treatment of skin pigmentation abnormalities may be beneficial for the development of new and more efficient remedies [17].

Pandahai (Boat-fruited Sterculia Seed) is a traditional Chinese drug and specified as the seeds of *S. lynchnophera* Hance in the Chinese pharmacopoeia. This traditional drug is reputed for its prevention of, and as a remedy against, pharyngitis. It has also been used for the treatment of tussis and constipation since ancient times in China [18]. The original plant, *S. lynchnophera* (Sterculiaceae) is distributed in Vietnam, Thailand, Malysia, Indonesia as well as the southeast-ern part of China. Though it has been reported that polysaccharides, fatty acids and alkaloids were isolated from Pandahai, the direct effect of SL on melanogenesis has not been reported yet [19-21].

In this study, we showed that SL inhibited melanin synthesis in B16F10 melanoma and melan-a cells. The free radical scavenging activity, tyrosinase activity and the expression of melanogenic enzymes such as tyrosinase, tyrosinase related protein-1 were also investigated.

MATERIALS AND METHODS

1. Materials and Reagents

B16F10 murine melanoma cells were obtained from the American Type Culture Collection (ATCC), the C57BL/6 Melan-a mice wild type cells were obtained from Amore-pacific. Mushroom tyrosinase, 3, 4-dihydroxy-L-phenylalanine (L-DOPA), Phenylthiourea (PTU), arbutin, Dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 2, 2-diphenyl-2-picrylhydrazyl hydrate (DPPH) and tetradecanoyl phorbol acetate (TPA) were purchased from Sigma Chemical Co. (St. Louis, USA), DMEM media, RPMI 1640 media, fetal bovine serum (FBS), Phosphate buffered saline (PBS), 10X Trypsin EDTA, 10X Penicillin/Streptomycin were purchased from Gibco Laboratory (Invitrogen Corp. CA, USA), PVDF membrane (Invitrolon, Carlsbad, USA) was purchase from Sigma. Goat polyclonal tyrosinase antibody, Goat polyclonal Trp-1, Goat polyclonal Trp-2 antibody, and anti-goat HRP-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc.

2. Plant Extraction

The dried samples were extracted three times with five volumes of a methanol 99.5% at 40 °C for 24 h. The resulting mixtures were...
filtrated and concentrated to dryness at 40°C under vacuum evaporator to produce the methanol extract.

3. Cell Culture

B16F10 cells were cultured in DMEM medium at a humidified atmosphere with 5% CO2 at 37 °C, 72 h. The C57BL/6 Melan-a cells were cultured in RPMI 1640 medium containing 200 nM TPA (tetradeanol phorbol acetate) with 10% CO2 at 37 °C for five days. Both DMEM and RPMI medium were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100,000 IU/L Penicillin and 100 mg/L Streptomycin. Cells were passaged by brief treatment with Trypsin/EDTA and resuspended in fresh medium.

4. Measurement of Melanin in Cultured B16F10 Murine Melanoma and C57BL/6 Melan-a Mice Wild Type Cells

For B16F10 melanoma cells, the cells were seeded into a 6-well plate (Falcon, USA) at a density of 6×10^4 cells per well. After 24 h of cultivation, the medium was replaced with fresh medium containing various concentrations of samples in triplicate. After 48 h incubation, the adherent cells were washed with phosphate buffered saline (PBS) and detached from the plate by trypsinization. The cells were collected in test tubes and washed twice with 1 ml PBS. The total melanin of cells was extracted by mixture of NaOH (0.1 M phosphate buffer pH 6.8 containing 1% Triton X-100). The cell lysate was centrifuged at 13,000 rpm, 4°C for 20 min and protein concentration of the supernatants was determined by BCA protein assay kit (Pierce). Then the cell lysates were adjusted to the same amount of protein with a lysis buffer. Reaction mixtures consisting of 40 μg of protein, 40 μl of 5 mM L-DOPA and 0.1M PBS (pH 6.8) were assayed on a 96-well plate at 37 °C for 30 min. Absorbance was measured at 475 nm with an ELISA reader. Phenylthiourea (PTU) was used as a standard agent.

For in vitro mushroom tyrosinase assay, reaction mixtures consisting of 100 μl of sample, 125 U mushroom tyrosinase, 40 μl of 5 mM L-DOPA, and 0.1 M PBS (pH 6.8) were assayed on a 96-well plate at 37 °C. After 20 min, absorbance was measured as described above. Each sample was measured in triplicate. Kojic acid was used as positive control [21].

7. Assay of Free Radical Scavenging Activity (DPPH Assay)

The effect of samples on DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate) was estimated on a 96-well plate. The mixture of 100 μl sample and 100 μl of 0.3 mM DPPH in ethanol was allowed to stand for 30 min at 37 °C in the dark. The absorbance at 517 nm was measured. Vitamin C was used as a standard agent. The ability to scavenge the DPPH radical was calculated as follows: scavenging activity (%)=[1−(absorbance of samples at 517 nm/absorbance of control at 517 nm)]×100 [21].

8. Western Blotting Analysis

The treated cells were harvested and washed with ice-cold PBS 2 times and lysed in a cold lysis buffer. An aliquot of lysate was used to determine the protein concentration by the BCA method. Seventy mg per ml of proteins per lane were separated by 10% SDS-polyacrylamide gel electrophoresis. The resolved proteins were transferred to a PVDF membrane (Invitrogen, Carlsbad, USA) at 250 mA for 2 h. The membrane was blocked shaking with 5% skim milk for 2 h and washed with 1×TBS. The membrane was then incubated 10 h with tyrosinase C19 goat polyclonal IgG antibody, TRP-1, TRP-2, and β-actin. The tyrosinase, TRP-1, TRP-2 and β-actin were then further incubated with rabbit polyclonal anti-goat IgG-HRP antibody. Bound antibodies were detected by using an Amersham ECL system. The expression of β-actin was used as a normalizing control [1].
9. Statistical Analysis
Data were expressed as mean values±SD. The statistical significant differences from the control were analyzed by the Student’s t-test.

RESULTS AND DISCUSSION

1. The Effects of Methanol Extract of SL on Melanogenesis in B16F10 Melanoma and Melan-a Cells
To investigate the effect of *S. lychnophera* (SL) on melanin synthesis, B16F10 murine melanoma cells were exposed to this plant extract from 12.5 µg/ml to 200 µg/ml for 48 h, and then melanin contents were measured. As shown in Fig. 1, the melanin synthesis was effectively inhibited in a dose-dependent manner. At a concentration of 200 µg/ml, this sample can inhibit 32.9% of melanin synthesis, compared with arbutin, which inhibits 20% of melanin content at the same concentration. The depigmenting effect of SL on melan-a immortalized melanocyte cell line was also employed. When melan-a cells were treated with a various concentrations of SL extract, inhibition of melanin synthesis was displayed in dose-dependent manner (Fig. 2). PTU (50 µM) was used as a positive control. This compound is known to inhibit tyrosinase and induces its degradation near the Golgi complex in melan-a cells [23]. To examine whether SL has cytotoxic effects, we treated B16F10 and melan-a cells with this herb at various concentrations, and cell viability was determined by using MTT assay. The results indicated that SL did not show any effect on cell viability even at high concentration (200 µg/ml) (Fig. 1 and 2).

2. The Effects of Methanol Extract of SL on Anti-oxidant Activity
It is well-known that reactive oxygen species (ROS) play significant roles in the regulation of melanocyte proliferation and melanogenesis. ROS scavengers such as antioxidants could down-regulate hyperpigmentation [24]. To examine whether SL has antioxidant activity, DPPH assay was used. As shown in Fig. 3, SL showed a significant free radical scavenging activity with IC$_{50}$=11.02 µg/ml. Vitamin C was used as a positive control. The IC$_{50}$ of vitamin was 5.06 µg/ml in this experiment (data not shown).

3. Effect of the Methanol Extract on In-vitro Mushroom and Cellular Tyrosinase Activity
The direct effects of SL on tyrosinase activity were investigated by using mushroom tyrosinase. L-DOPA oxidation activities of mushroom tyrosinase were slightly directly affected by methanol extract of SL (23.4% at a concentration of 200 µg/ml). Kojic acid (0.7 mM) was used as a positive control and it completely inhibited mushroom tyrosinase activity (data not shown).
radiation with a high free radical scavenging activity. These results suggest that SL may be used as skin depigmenting agent.

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