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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 IC_{50}=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 activivity (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. lychnophora* 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. lychnophora* (Sterculiaceae) is distributed in Vietnam, Thailand, Malysia, Indonesia as well as the southeastern 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

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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% CO_2 at 37 °C, 72 h. The C57BL/6 Melan-a cells were cultured in RPMI 1640 medium containing 200 nM TPA (tetradecanoyl phorbol acetate) with 10% CO_2 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 were extracted by mixture of NaOH 1 N 10% DMSO at 80 °C for 1 h and determined at 405 nm using an ELISA microplate reader. The percentages of total melanin content were calculated with respect to the OD value of the control group. Arbutin (200 μg/ml) was used as a positive standard agent.

For C57BL/6 melan-a cells, the cells were seeded at a density of 1×10^5 cells per well. Cells were treated for 72 h with samples taken every day. The melanin content was measured as mentioned above [22].

5. Assay for Cell Viability

Cell viability was determined by MTT assay, which was based on the cleavage of a 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) molecule by mitochondrial dehydrogenases in viable cells. Cells were seeded into a 96-well plate (Falcon, USA) at a density of 2.5×10^3 cells per well. After 24 h incubation, the cultured medium was removed and new medium containing test substances was added to each well in quintuplicate. After 48 h incubation, cell viability was assayed by MTT solution. After 4 h incubation, the supernatants were removed and produced crystal (formazan), which was solubilized in 100 μl of 100% DMSO. The optical densities were determined at 540 nm using an ELISA microplate reader. The percentages of viable cells in each well were calculated with respect to the OD value of living cells of the control group [22].

6. Assay of Cellular and *In-vitro* Mushroom Tyrosinase

Cells were seeded in 6-well plate at a density as mentioned above and cultured for 24 h. After being treated with samples for 48 h, the cells were washed with ice-cold PBS and lysed with lysis buffer (0.1 M phosphate buffer pH 6.8 containing 1% Triton X-100). The cells were disrupted by sonicating for 1 h at 4 °C. 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 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].

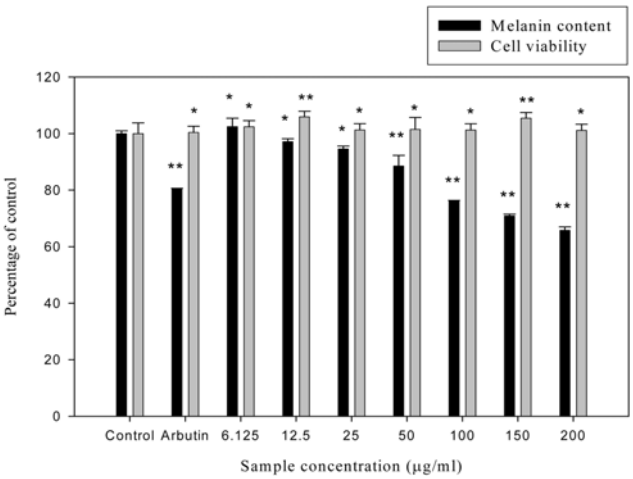

Fig. 1. Effects of methanol extract of *S. lychnophera* Hance on melanin synthesis and cell viability of B16F10 murine melanoma cells. B16F10 cells were treated with *S. lychnophera* methanol extract for 2 days and the cells were then harvested. Melanin content and cell viability were measured as described in Materials and Methods. Data were expressed as the change of the melanin content level and cell viability relative to the untreated control. Each determination was made in triplicate and data shown are means±S.D. *P<0.05, **P< 0.01: statistically significant vs. value of control group.

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).

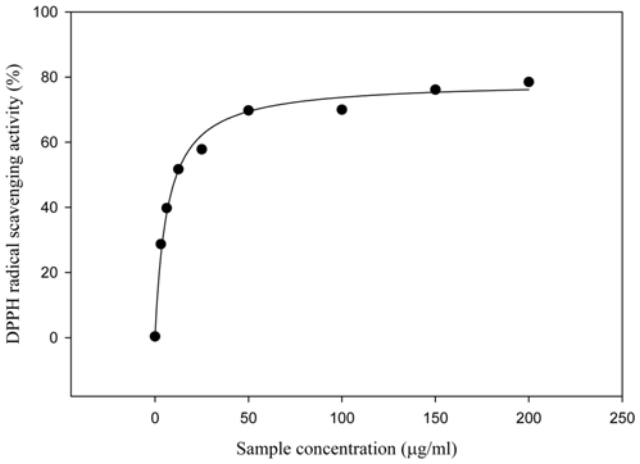

Fig. 3. DPPH radical scavenging activity of methanol extracts from *S. lychnophera* Hance.

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).

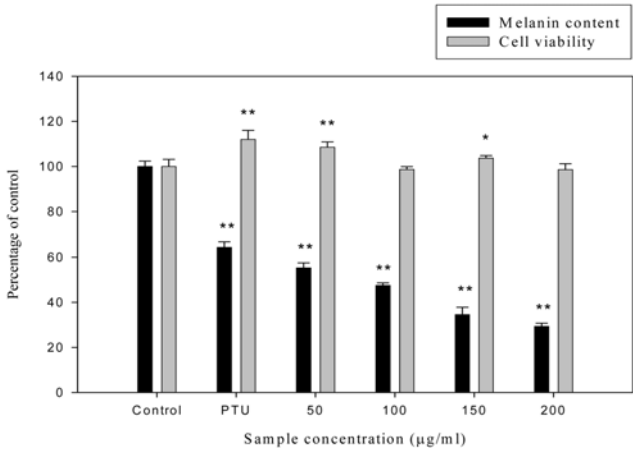

Fig. 2. Effects of methanol extract of *S. lychnophera* Hance on melanin synthesis and cell viability of melan-a cells. Melan-a cells were treated with *S. lychnophera* methanol extract for 3 days and the cells were then harvested. Melanin content and cell viability were measured as described in Materials and Methods. Data were expressed as the change of the melanin content level and cell viability relative to the untreated control. Each determination was made in triplicate and data shown are means±S.D. *P<0.05, **P<0.01: statistically significant vs. value of control group.

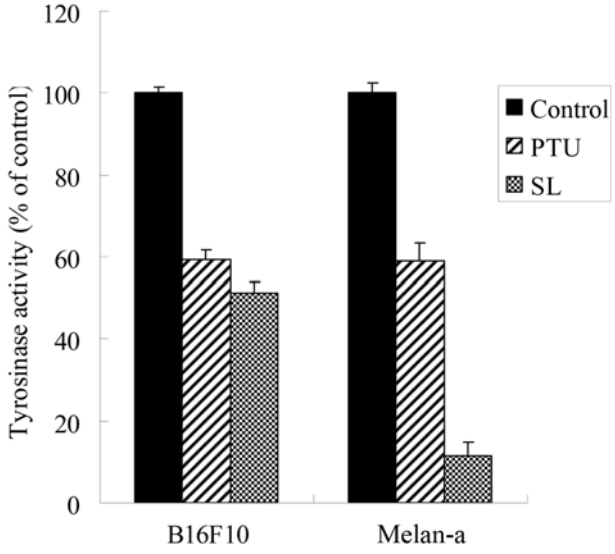

Fig. 4. Effect of *S. lychnophera* Hance on tyrosinase activity in B16F10 and melan-a cells. B16F10 cells and melan-a cells were treated with *S. lychnophera*, harvested, and tyrosinase extracted from the treated cells. The cellular tyrosinase was then incubated with 40 μl of 5 mM L-DOPA at 37 °C for 20 min. Each bar represents the means±SD of three separate experiments.

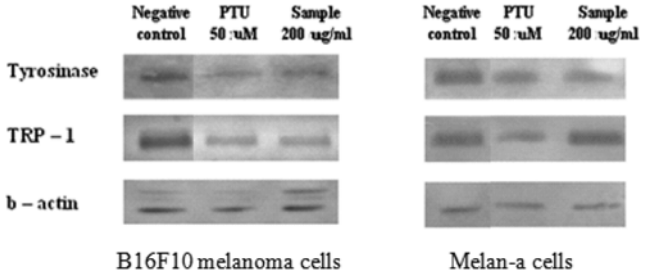

Fig. 5. The effect of methanol extract of *S. lychnophera* Hance on the expression levels of melanogenic enzyme in B16F10 murine melanoma cells and C57BL/6 Melan-a mice wild type cells. B16F10 melanoma cells and melan-a cells were treated with *S. lychnophera* at concentration 200 μg/ml for two days and then harvested. Total protein was extracted and subjected to Western blotting as described in Materials and Methods.

To assess the effect of the SL extract on the cellular tyrosinase activity, B16F10 murine melanoma cells and C57BL/6 melan-a mice wild type cells were treated with the crude extract at a concentration 200 μg/ml. Then cellular tyrosinases were extracted and incubated with 40 μl of 5 mM L-DOPA at 37 °C, 30 min. The dopachrome content, created products were measured at absorbance 475 nm by using ELISA reader. The inhibitory effect on cell - based tyrosinase, the key enzyme of melanogenesis, is shown in Fig. 4. As compared to non-treated cells, at a concentration of 200 μg/ml, methanol extract-treated cells significantly inhibited intracellular tyrosinase activity with 48.65% at B16F10 treated cells and 88.56% at C57BL/6 melan-a cells. It should be noted that the reduced melanin contents were mainly attributed to the suppression of tyrosinase expression, rather than the direct inhibition of tyrosinase activity.

4. Inhibitory Effect of the Methanol Extract on Melanogenic Enzymes Expression

Three melanocyte-specific enzymes, tyrosinase, tyrosinase related protein 1 (Tyrp1), and tyrosinase-related protein 2 (Dct), are involved in catalytic processes that convert tyrosine to melanin. To characterize the amount of those enzymes during the decreased pigmentation by SL, their expression levels were examined by Western blot analysis. As shown in Fig. 5, cells treated with 200 μg/ml of the SL methanol extract had significantly decreased tyrosinase and Tyrp1 protein levels.

CONCLUSION

We examined the effects of SL on melanogenesis in B16F10 murine melanocytes and melan-a cells. SL decreased tyrosinase activity and the melanin content in both cell lines in a dose-dependent manner. This herb also possessed a high free scavenging activity. In addition, it was found that SL decreased tyrosinase and TRP-1 protein expression. Consequently, it was proposed that the depigmenting effect of SL might be due to the combinatory effect of inhibition of tyrosinase expression or enhancement of tyrosinase degradation with a high free radical scavenging activity. These results suggest that SL may be used as skin depigmenting agent.

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