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EFFECTS OF METHANOL EXTRACT OF *LINDERA MYRHHA* ON THE MELANIN SYNTHESIS OF B16F10 MELANOMA CELLS

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ABSTRACT

To evaluate the potential of herbal species in treatment of hyperpigmentation, this study focuses on the effect of methanol extract of Lindera myrhha on the melanin synthesis in B16F10 melanoma cells. Lindera myrhha showed low cytotoxicity at even high concentration. The methanol extract of this plant could inhibit the melanin synthesis in the dose dependent manner. At the concentration of 200 µg/ml, it could inhibit 42.58% of melanin formation in the B16F10 melanoma cells. The methanol extract of Lindera myrhha slightly inhibited in vitro mushroom tyrosinase activity (5.45%) at a concentration of 200 µg/ml and had a significant inhibitory effect on cellular tyrosinase activivity (36.35%). This plant also showed high free radical scavenging activity with IC_{50} = 41 µg/ml. This is the first report on activity of this kind in Lindera myrhha. In conclusion, this plant could be used as an effective whitening ingredient in cosmetic products.

Keywords: inhibition, melanin, *Lindera myrhha*, tyrosinase.

1. Introduction

Skin color or pigmentation is a result of melanin content, which synthesizes in melanosomes of the melanocytes (Sulaimon and Kitchell, 2003). The amount, type and distribution of melanin, determines the phenotypic spectra of the skin color. In humans, melanin is found in skin, hair, the pigmented epithelium underlying the retina, the inner ear, and pigment bearing neurons of certain deep brain nuclei. Melanin provides many benefits for human beings. One of the most recognized benefits involves ultraviolet rays of the sun. Melanin provides a natural protection against harmful effects of these rays. Melanin also plays an important role in absorbing heat from the sun. Also, being important for sharpness of vision, melanin serves to minimize the number of light beams that enter the eye. The other functions of melanin include antibiotic, cation chelator, and free radical scavenger of the superoxide (Alhaidari et al., 1999). The copper-containing enzyme tyrosinase (a tyrosine hydroxylase; EC 1.14.18.1) oxidizes the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and DOPA to DOPA quinone, which are the first two rate-limiting steps in the melanin synthesis pathway. At least two additional melanosomal enzymes are involved in the eumelanogenesis pathway that produces brown and black

pigments, including TRP-1 (DHICA oxidase), and TRP-2 (DOPAchrome tautomerase). Depending on the incorporation of a sulfur-containing reactant (e.g. cysteine or glutathione) into the products, the melanogenesis pathway diverges, producing eumelanins or pheomelanins (amber and red pigments) or both (Figure 1) (Nguyen et al., 2008). After synthesized, melanosome is tranfered to the adjunction keratinotyes (Karolien et al., 2006).

Melanin plays an important role in preventing ultraviolet (UV) light-induced skin damage. However, increased levels of epidermal melanin synthesis can darken the skin. Various dermatologic disorders result in the accumulation of excessive levels of epidermal pigmentation. They include melasma, lentigines, and postinflammatory hyperpigmentation. Because of the visible nature of dermatologic diseases, they have a considerable psychological effect on affected patients. Disfiguring facial lesions can significantly affect a person's overall emotional well-being and can contribute to decreases in social functioning, productivity at work or school, and self-esteem (Cayce et al., 2004). Therefore, a number of whitening compounds have been screened for their effectiveness in reducing melanogenesis. Many of the traditionally used skin lightening products such as hydroquinone, corticosteroids and mercury-containing products are still used in many countries, in spite of serious health concerns, including irreversible cutaneous damage, ochronosis, accumulating of mercury in the body (Barr et al., 1973; Findlay and De Beer, 1980; Phillips et al., 1986). Other skin whitening agents like Arbutin, Kojic acid and Vitamin C is low efficacy and unstable (Petit and Pierard, 2003). These adverse effects have led to the search for safer, plant-based skin lightening products.

Lindera myrrha belongs to Lauraceace family. This plant has been found in many provinces of Vietnam such as Thanh Hoa, Nghe Tinh, and Ha Son Binh. It has been used as the traditional drugs in Vietnam, China, and Korea. This traditional drug is reputed for its prevention of cold accumulation, distending pain in the chest and abdomen, cold of insufficiency type in the lower abdomen, frequent urination, and other syndromes. The chemical composition of this drug has not been well-studied. Some researchers showed that the main active components of this plant are alkaloids such as aporhine, arosine and arosinine (Phan et al., 1994). The related specie, *Lindera aggregate* contains some notable compounds such as norisodoldine, linderalactone, lindeneol, boldine, reticuline (Sun et al., 2006). Previous studies showed that this plant exhibited the anti-inflammation, anti-obesity, anti-tumors and anti-virus activities (Cao et al., 2015; Joshi and Mathela, 2012). However, the skin depigmenting activities have not been investigated yet. In this study, the effects of *Lindera myrhha* on the melanin synthesis of B16F10 melanoma cells was investigated.

2. Materials and methods

2.1. Materials

Mushroom tyrosinase (Sigma Aldrich), L-DOPA (3,4-dihydroxy-L-phenylalanine), Arbutin, DMSO (dimethyl sulfoxide) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate) were

purchased from Sigma Chemical Co. (St. Louis, USA). DMEM media, fetal bovine serum, trypsin-EDTA, phosphate buffered saline (PBS), Penicillin/streptomycin were purchased from Invitrogen Corp. (CA, USA). Methanol, Hexane, Chloroform (99.5% purity) was purchased from Samchun Chemical Co. (South Korea).

2.2. Extraction and fractionation method

The 100 g of dry radix *Lindera myrrha* powder was extracted in 400 ml methanol at 40^{o}C, 4 hours for 3 times. The resulting mixtures was filtrated and evaporated in a rotary evaporator at 40^{o}C under reduced pressure to obtain 60g methanol extract.

2.3. Cell culture

B16F10 murine melanoma cells were purchased from ATCC (American Type Culture Collection). B16F10 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% (v/v) fetal bovine serum, 100 units/ml of penicillin and 100 units/ml of streptomycin at 37^{o}C in a humidified, CO_2-controlled (5%) incubator. The cells were sub-cultured every three days until a maximal passage number of 30 were achieved.

2.4. Melanin assay

Cells were seeded into a 6-well plate (Falcon, USA) at a density of 6 x 10^4 cells per well. After 24 hours of cultivation, the medium was replaced with fresh medium containing various concentrations of methanol extract of radix *Lindera myrrha*. After 2 days incubation, the adherent cells were washed with phosphate buffered saline (PBS) and detached from the plate by Trypsin-EDTA. The cells were collected in a test tube and washed twice with PBS. Melanin of cells were extracted by mixture of NaOH 1N 10% DMSO at 80^{o}C for 1 hour. The melanin content was determined at 475 nm using an ELISA (enzyme-linked immunosorbent assay) microplate reader.

2.5. Cell Toxicity Assay

MTT assay was used to determine cell toxicity. After treatment and incubation of the samples for 48 hours, the medium was removed and 100 µl of 0.5 mg/mL MTT solution was added to each well and incubated at 37^{o}C for 4 hours. The MTT solution was then removed and 200 µl Dimethyl Sulfoxide (DMSO) was added to each well. The formazan formation was measured by absorbance at 570 nm in ELISA reader.

2.6. Mushroom Tyrosinase assay

Reaction mixtures consisting of 100 µl of sample, 22 UI 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 10 min of incubation, absorbance was measured 470 nm in ELISA reader. Each sample was measured in triplicate. Kojic acid was used as positive control**.**

2.7. Cellular tyrosinase assay

B16F10 cells were seeded in 6-well plates at a density of 6 x 10^4 cells per well and cultured for 24 hours. After being treated with samples for 48 hours, the cells were washed with PBS and lysed with lysis buffer (0.1 M phosphate buffer pH 6.8 containing 1% Triton

X-100). The cells were then disrupted by sonication for 1 hour at 4ºC, and lysates were clarified by centrifugation at 13.000 rpm for 20 min. After quantifying the protein content using a protein assay kit (Bio-Rad, USA), 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 0.1 M PBS (pH 6.8) was assayed on a 96-well plate at 37ºC. After 30 min incubation, the absorbance was measured at 475 nm using an ELISA reader.

2.8. Free radical scavenging activity assay

The radical scavenging activity of a sample was determined by the DPPH method. A sample was dissolved in a DMSO with different concentrations. Reaction mixtures consisting of 100 µl of sample and 100 µl of DPPH solution were assayed on a 96-well plate. The absorbance at 517 nm of the solution was measured after 30 minutes. The radical scavenging activity was calculated using the following equation: % scavenging activity = [*A* control - *A*sample]/ *A* control*100. Each sample was measured in triplicate. The 50% inhibitory concentration (IC_{50}; concentration of sample required to scavenge 50% of DPPH radicals) values were determined by the method of probit-graphic interpolation for eight concentration levels.

3. Results and discussion

3.1. Effect of radix Lindera myrrha on cell viability in B16F10 melanoma cells

To examine whether radix *Lindera myrrha* has cytotoxic effects, the researchers treated B16F10 melanoma cells with the *Lindera myrrha* methanol extract at various concentrations ranging from 0 to 200 µg/ml, and cell viability was determined by using MTT assay. The results indicated that radix *Lindera myrrha* did not show any effect on cell viability even at high concentration (200 µg/ml) (Figure 1). It should conclude that this plant is safe and could be used in cosmetic purpose.

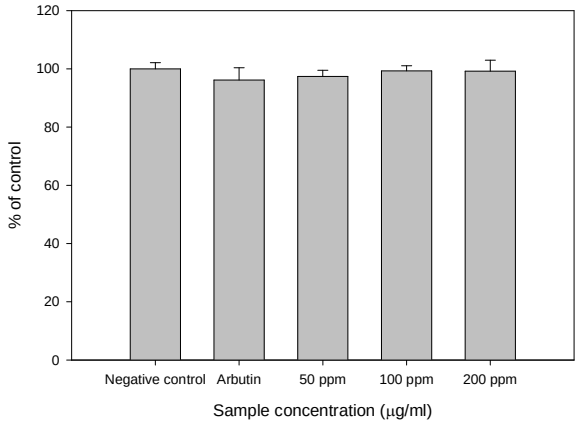

Figure 1. *Effect of Methanol extract of Lindera myrrha in cell viability of B16F10 melanoma cells*

3.2. Effect of Lindera myrrha on melanin synthesis in B16F10 melanoma cells

To investigate the effect of radix *Lindera myrrha* on melanin synthesis, B16F10 murine melanoma cells were exposed to this plant extract from 50 μg/ml to 200 μg/ml for 48 hours, and then melanin contents were measured. As shown in Fig. 2, the melanin synthesis was significantly inhibited in a dose-dependent manner. At a concentration of 50 μg/ml, this sample can inhibit 19.16% of melanin synthesis, as same as the melanin inhibitory effect of Arbutin at the concentration of 200 μg/ml). Several medicinal plants have been screened for melanin inhibition. These include *Morus alba, Pharbitis nil, Spatholobus suberectus* and *Glycyrrhiza uralensis* which can inhibit 16.3 ± 4.8%, 25.7 ± 1.9%, 11.1 ± 5.3%, and 12.1 ± 0.1% at the concentration 100 μg/ml, respectively (Petit and Pierard, 2003). Thus, *Lindera myrrha* is a potent melanin inhibitor.

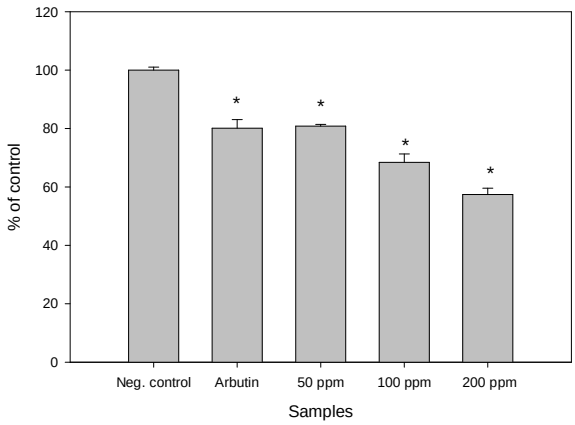

Figure 2. *Effect of Methanol extract of Lindera myrrha in cell viability of B16F10 melanoma cells, * p<0.05: statistically significant vs. value of control group*

3.3. Effect of radix Lindera myrrha on tyrosinase activities

Two kinds of tyrosinase activities, cell-based intracellular tyrosinase extracted from the B16F10 cells and direct mushroom tyrosinase, were used in this study. The methanol extract of radix *Lindera myrrha* could slightly directly inhibited of mushroom tyrosianse. At the concentration of 200 μg/ml, it could inhibit 5.45% of mushroom tyrosinase. For intracellular tyrosinase activity, the methanol extract of radix *Lindera myrrha* could significantly inhibit the cellular tyrosinase in the dose dependent manner. At the concentration of 200 μg/ml, this extract could inhibit 36.35% of cellular mushroom activity (Table 1). Compared to Arbutin, the methanol extract of *Lindera myrrha* exhibited higher inhibitory effect on tyrosinase activities. Compared with the inhibition of mushroom tyrosinase, this plant showed the higher inhibitory effect on cellular tyrosinase activity. It means that in addition to direct inhibit the enzyme activity, the methanol extract may affect on tyrosinase at transcriptional or expression level.

Table 1. *Effect of methanol extract of radix Lindera myrrha on Tyrosinase activiies*

Concentration (µg/ml)	Tyrosinase activity (% of control)	
	Cellular tyrosinase	Mushroom tyrosinase
50	83. 15 ± 3.15	100 ± 2.46
100	74.96 ± 2.23	98.12 ± 2.37
200	63.45 ± 1.67	94.45 ± 1.42
Arbutin	80.08 ± 2.61	97.31 ± 3.45

3.4. Effect of radix Lindera myrrha on free radical scavenging activy

To examine whether *Lindera myrrha* has an antioxidant activity, DPPH assay was used. As shown in Figure 3, *Lindera myrrha* has a good antioxidant activity with IC_{50}= 41 µg/ml.

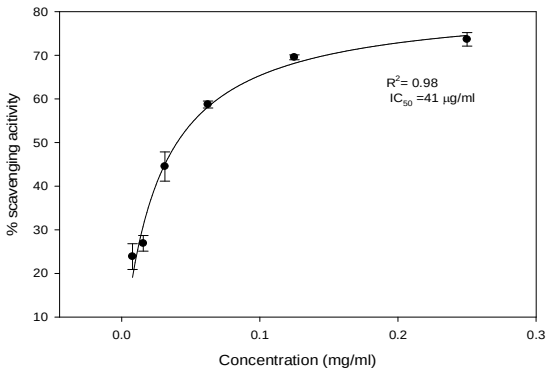

Figure 3. *Effect of Methanol extract of Lindera myrrha on free radical scavenging activity*

Lindera is a typical and widely distributed plant genus, has good economic value and effective therapeutic properties for treating various diseases. *L. aggregata, L. obtusiloba, L. glauca, and L. umbellata* have been used as traditional medicines for treating urinary tract and cardiovascular diseases, chronic gastritis and rheumatoid arthritis, stroke, and pain. Some of components from other *Lindera* species were investigated the antioxidant activity; the essential oil of *L. pulcherrima* leaves shown highest free radical scavenging activity compared with other major compounds, with IC_{50} of 87 µg/ml (Joshi and Mathela, 2012). A chalcone (flavokawain B) from *L. oxyphylla* and lignans from *L. umbellate* exhibited good DPPH scavenging activity, with IC50 value of of 21.5–26.3 µg/ml (Kuroda et al., 2011). This report is the first one on the bioactivities of *Lindera myrrha* and its potential for hyperpigmentation treatment.

4. Conclusions

Several melanogenesis inhibitors have been reported and are being used as cosmetic additives for the treatment of hyperpigmented skin disorders. However, many of them are of limited effectiveness, cause reactions or side effects after long-term use and are difficult to formulate. In this study, the researchers found that the methanol extract of *Lindera myrrha* could inhibit the melanin synthesis in the dose dependent manner. Moreover, it did not show any cell cytotoxicity effect. The methanol extract of this plant has both effect on inhibition of tyrosinase activity and free radical scavenging activity. In conclusion, *Lindera myrrha* could be a promising plant that could be used as skin-whitening agent for treating hyperpigmentation. The isolation and identification of active compounds in this plant is still in process.

❖ ***Conflict of Interest:*** *Authors have no conflict of interest to declare.*

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ẢNH HƯỞNG CỦA CAO CHIẾT METHANOL LOÀI Ô DƯỢC (LINDERA MYRHHA) LÊN QUÁ TRÌNH TỔNG HỢP MELANIN TRÊN DÒNG TẾ BÀO MELANOMA B16F10

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TÓM TẮT

Để đánh giá tiềm năng của các loài dược liệu trong việc điều trị các triệu chứng tăng sắc tố da, nghiên cứu này tiến hành khảo sát sự ảnh hưởng của cao chiết methanol từ loài Ô dược (Lindera myrhha) lên quá trình tổng hợp hắc tố melanin trên dòng tế bào melanoma B16F10. Ô dược được chứng minh là có độc tính tế bào thấp ngay cả ở các nồng độ cao. Cao chiết methanol của loài này có hoạt tính ức chế sự tổng hợp melanin tăng dần theo nồng độ. Ở nồng độ 200 µg/ml, cao methanol có thể ức chế quá trình tổng hợp melanin của tế bào B16F10 đến 42,58%. Cao methanol ở nồng độ 200 µg/ml cũng được chứng minh có thể ức chế in vitro hoạt tính của mushroom tyrosinase (5,45%) và có hoạt tính ức chế mạnh đối với hoạt tính tyrosinase nội bào (36,35%). Ngoài ra, loài ô dược còn có hoạt tính ức chế gốc tự do khá mạnh với IC_{50} = 41 µg/ml. Đây là công bố đầu tiên về các hoạt tính của loài Ô dược (Lindera myrhha). Những kết quả của nghiên cứu này góp phần chứng minh tiềm năng của loài này trong việc ứng dụng như một thành phần làm trắng da trong mĩ phẩm.

Từ khóa: hoạt tính ức chế, melanin, *Lindera myrhha*, tyrosinase.