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Research Article
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Effects of Chinese Herbal Extracts on Tyrosinase Activity and Melanogenesis

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Abstract

Tyrosinase is a key enzyme for the biosynthesis pathway of melanin pigment, which is the most important determinant of the colour of skin and hair colour. Hair turns grey due to a decrease in the production of melanin in the hair bulb, which gives the hair its colour. In previous study, Chinese herbal have been found to increase melanin production and prevent the graying of hair. We demonstrated that 2, 3, 5, 4′-Tetrahydroxystilbene 2-O-β-D-glucoside (THSG), gallic acid, physcion, and emodin are the major compounds of *Fallopia multiflora* in high performance liquid chromatography (HPLC) analysis. In our preliminary screening using mushroom tyrosinase, 2, 3, 5, 4′-Tetrahydroxystilbene 2-O-β-D-glucoside, gallic acid, and physcion were found to show significant increased activity for tyrosinase. We also investigated that Chinese herbal extracts (*Fallopia multiflora, Ligustrum lucidum ait, Angelica sinensis, and other Chinese herbal*) to show significant increased activity for mushroom tyrosinase and melanogenesis in mouse melanocytes. Therefore, Chinese herbal extracts can be a potential agent for hair blackening to be used in cosmetic products.

Keywords: Chinese herbal extracts; *Fallopia multiflora; Melanogenesis;* High performance liquid chromatography; 2,3,5,4′-Tetrahydroxystilbene 2-O-β-D-glucoside (THSG)

Abbreviations

L-DOPA: L-3,4-Dihydroxyphenylalanine; DMSO: L-Tyrosine, Dimethyl Sulfoxide; THSG: 2,3,5,4′-Tetrahydroxystilbene 2-O-β-D-Glucoside; α-MSH: α-Melanocyte-Stimulating Hormone; PBS: Phosphate Buffered Saline; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal Bovine Serum; *FM: Fallopia multiflora*

Introduction

Melanin is polyphenol-like biopolymer with a complex structure and colours varying from yellow to black, [1] which was formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of tyrosinase. [2] Tyrosinase (monophenol monooxygenase EC 1.14.18.1) is a copper-containing enzyme, which is widely found in plants and animals. [3] It is known to be a key enzyme for melanin biosynthesis in plants, microorganisms and mammalian cells. Many tyrosinase inhibitors have been tested as cosmetics and pharmaceuticals for reducing melanin in epidermal layers. [4-8] Pigmentation is highly heritable, being regulated by genetic, environmental, and endocrine factors that modulate the amount, type, and distribution of melanin in the skin, hair, and eyes. [9-12] Various studies have indicated that the number of functioning DOPA-positive melanocytes in non-exposed human skin decreases with age by 8-20 % of the surviving population each decade. However, in UV-irradiated skin there are approximately twice as many melanocytes as in unexposed areas, but there is still a comparable decrease in melanocytes with age. It is surprising that, unlike hair colour, there is no loss of skin pigmentation with age [13]. The colour of mammalian skin and hair is determined by a number of factors, the most important of which is the degree and distribution of melanin pigmentation. Hair turns grey due to a decrease in the production of melanin in the hair bulb, which gives the hair its colour. In previous study, Chinese herbal have been found to increase melanin production and prevent the greying of hair. In our preliminary screening using mushroom tyrosinase, the extract of *Fallopia multiflora* was found to show significant increased activity for tyrosine hydroxylation. *Fallopia multiflora* (FM), also known as *Polygonum multiflorum*, is a traditional Chinese herb used mainly for anti-aging, kidney tonic, hair blackening, etc. 2,3,5,4′-Tetrahydroxystilbene 2-O-β-D-glucoside (THSG) is the active component on FM, which responsible for health properties include (1) Liver protection; (2) Inhibition of protein kinase C and suppressing tumour growth; (3) Modulation of immune function. In our preliminary screening using mushroom tyrosinase, the extract of FM was found to show significant increased activity for tyrosine hydroxylation. The active component of FM including THSG, gallic acid, physcion and emodin, which were characterized as the principal stimulators from FM extract. [14-18] In previous study, Chinese herbal have been found to increase melanin production and prevent the greying of hair. To clarify the mechanism of Chinese herbal, we studied its increased action on the melanin synthetic enzyme and its effects on the melanin production in cultured mouse melanocytes.

Materials and Methods

Chemicals and reagents

Mushroom tyrosinase (EC 1.14.18.1), L-tyrosine, dimethyl sulfoxide (DMSO), trypsin/EDTA, 2,3,5,4′-Tetrahydroxystilbene 2-O-β-D-glucoside (THSG) ,α-melanocyte-stimulating hormone (α-MSH), Phosphate buffered saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA.). Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-BRL (New York, NY, USA). Other chemicals were purchased from Aldrich.

Samples preparation

Two grams of Chinese herbal mixture (Fallopia multiflora, Ligustrum lucidum ait, Angelica sinensis, and other Chinese herbal) powder were accurately weighed and extracted with 40ml different solution (50% methanol, 75% methanol, 100% methanol, 60% ethanol, and 95% ethanol) by refluxing for 1 h. After filtering, the filtrate was

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transferred into a flask and was then evaporated to dryness. Chinese herbal mixture (*Fallopia multiflora, Ligustrum lucidum ait, Angelica sinensis, and other Chinese herbal*) were prepared by EHerber Biotech Enterprise Inc, Taiwan.

Analysis of chemical fingerprint

Sample preparation: accurately weighed about 2g of *Fallopia multiflora* powder into an around-bottom flask and added 40ml NH_3 (aq)/ CH_3OH (volume ratio1:19). It was refluxed for 1 hr in water bath, cooled and filtered. Small amount of methanol was used to rinse the powder. The methanol was combined with the pre-filtrate and evaporated to dry. 8-9ml methanol was added to dissolve the residue (ultrasonic used if needed) and then filled with methanol to 10 ml.HPLC method: the samples in each extract was quantified using a high performance liquid chromatography (HPLC) with a C18 Inertsil ODS-3V column (4.6 mm × 250 mm,5 um) and Column temperature 40°C The flow rate was 1.0 ml/min, the injection volume was 10 µl, and the eluent was monitored at 203 nm. The mobile phase consisted of water (A) and acetonitrile (B) with a flow rate of 1.0 mL/min. The gradient elution was programmed as follows: 0 min:, 95 % A and 5 % B; 50 min: 10 % A and 90 % B; 60 min: 10 % A and 90 % B.

Enzyme assay

Mushroom tyrosinase was purchased from Sigma. The enzyme activity was monitored by dopachrome formation at 475 nm for an appropriate period (not exceeding 10 min, unless otherwise noted). The assay was performed as previously described [19]. First, L-tyrosine aqueous solution was mixed with phosphate buffer (pH 6.8) and incubated at 25°C for 10 min. Then, the solution and mushroom tyrosinase were added in this order to the mixture. This assay solution (1ml: contains 0.8 mM L-tyrosine, 0.9M phosphate sodium buffer at pH 6.8 and 40.0 units/ml mushroom tyrosinase) was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm.

Cell culture

Mouse melanocyte (B16) was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 3 d culture, the cells were harvested after trypsinization, counted by tryptan blue inclusion under microscopy, [20] and assayed for melanin content and cellular tyrosinase activity.

Cell viability

The cells were harvested after trypsinization. Counted by tryptan blue inclusion under microscopy and assayed for cell viability.

Melanin assay

The melanin content was determined by the published protocol with minor modifications. [21] The cells were washed with PBS and lysed with 1 ml of 1N NaOH. The relative melanin content was determined by optical density at 490 nm and the melanin content was calculated as µg/106 cells.

Statistics

All the experiments were repeated at least three times. Data were expressed as means ± SD and analyzed using one-way analysis of variance (ANOVA) followed by a Tukey's test to determine any significant differences. A value of $P < 0.05$ was considered statistically significant.

Results

Samples preparation

Different solvents will yield different extracts and extract compositions. In this study, we investigated the effect of different extracts (50% methanol, 75% methanol, 100% methanol, 60% ethanol, and 95% ethanol) on the tyrosinase activity. In Table 1, the results showed that different extracts affect tyrosinase activity. The 60% and 95% ethanol extract of FM have stronger increases tyrosinase activity than 50% methanol, 75% methanol, and 100% methanol. Therefore, samples preparation were extracted with 95% ethanol by refluxing for 1 h.

Effect of *Fallopia multiflora* and active components on melanin synthesis and cellular tyrosinase activity

To give the most chemical information and best separation in the chromatograms, the HPLC analysis was investigated in this study. Figure 1 showed that FM content five major components using 95% ethanol extraction. Five major components were gallic acid (peak 1), catechin (peak 2), THSG (peak 3), emodin (peak 4), and physcion (peak 5).These results showed that THSG (peak 3), and emodin (peak 4) were the major components of FM (Figure 1,2 for structures). In our preliminary screening using mushroom tyrosinase, the 60% and 95% ethanol extract of FM were found to show significant increased activity for tyrosinase (Table 2). To evaluate the increased effect of FM on tyrosinase, we compared the five active components on FM including THSG, gallic acid, catechin, physcion, and emodin to investigate their effects on mushroom tyrosinase activity. The results showed that THSG and gallic acid, physcion, and emodin were increased effects to 286.2%, 140.5%, 123.9%, and 111.3% respectively on mushroom tyrosinase.

Effect of Chinese herbal on melanin synthesis and tyrosinase activity

In Figure 3A, the Chinese herbal extracts (samples were prepared by EHerber Biotech Enterprise Inc, Taiwan) to show significant increased activity for tyrosinase ($p<0.05$). We also compared with the tyrosinase stimulators 8-methoxypsoralen (8MOP) served as positive controls. Photographs in Figure 3B show the colour of pellets of B16 cells treated with different Chinese herbal extracts and α-MSH. The Chinese herbal and α-MSH treated cells were markedly increase pigmented compared with control. Treatment with Chinese herbal extracts and α-MSH for 3 d evidently increased the cellular content of melanin (Figure 3C). When B16 cells were treated with Chinese herbal extracts, melanin production was greatly increased 18 %~53 % of that in control ($p<0.05$). The data showed that no significant effect on cell viability (as shown in Figure 3D). The results indicated that Chinese

	Concentration	Tyrosinase activity (% of control)
Fallopia multiflora (52% methanol)	200 µg/ml	112.7±0.9
Fallopia multiflora (75% methanol)	200 µg/ml	117.6±2.1
Fallopia multiflora (100% methanol)	200 µg/ml	116.3±1.2
Fallopia multiflora (60% ethanol)	200 µg/ml	133.7±0.8*
Fallopia multiflora (95% ethanol)	200 µg/ml	144.9±3.9*
Control		100.0±2.9

Table 1: Different extracts of *Fallopia multiflora* on Mushroom Tyrosinase. Significant differences were determined by Student's *t*-test; *$p>0.05$ compared to control.

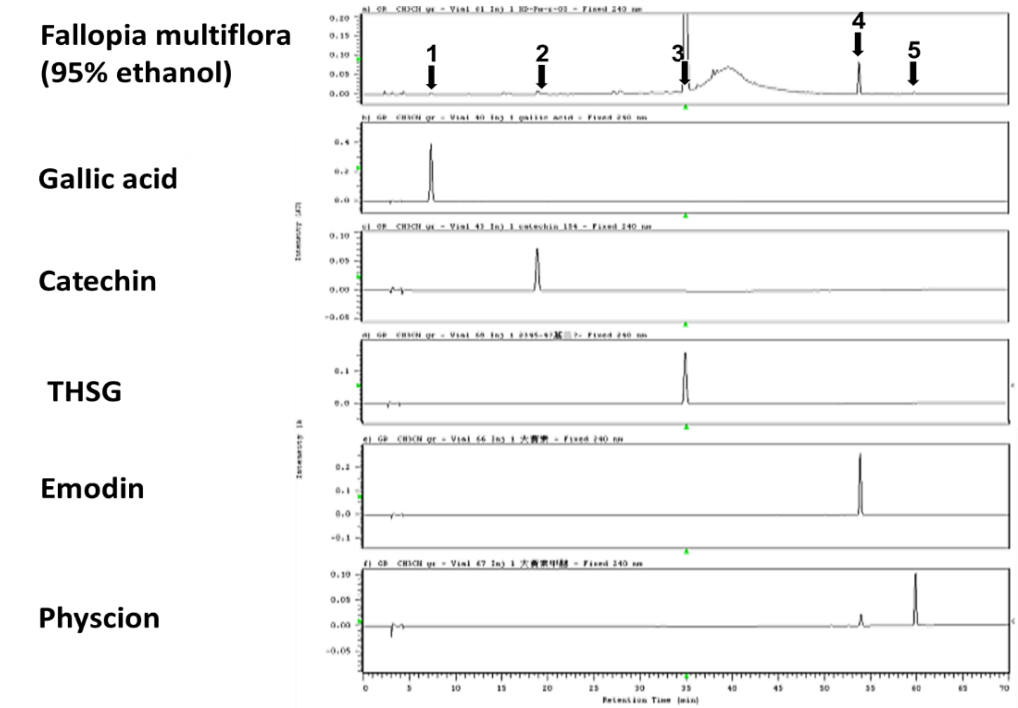

Figure 1: High performance liquid chromatography (HPLC) analysis of *Fallopia multiflora*.

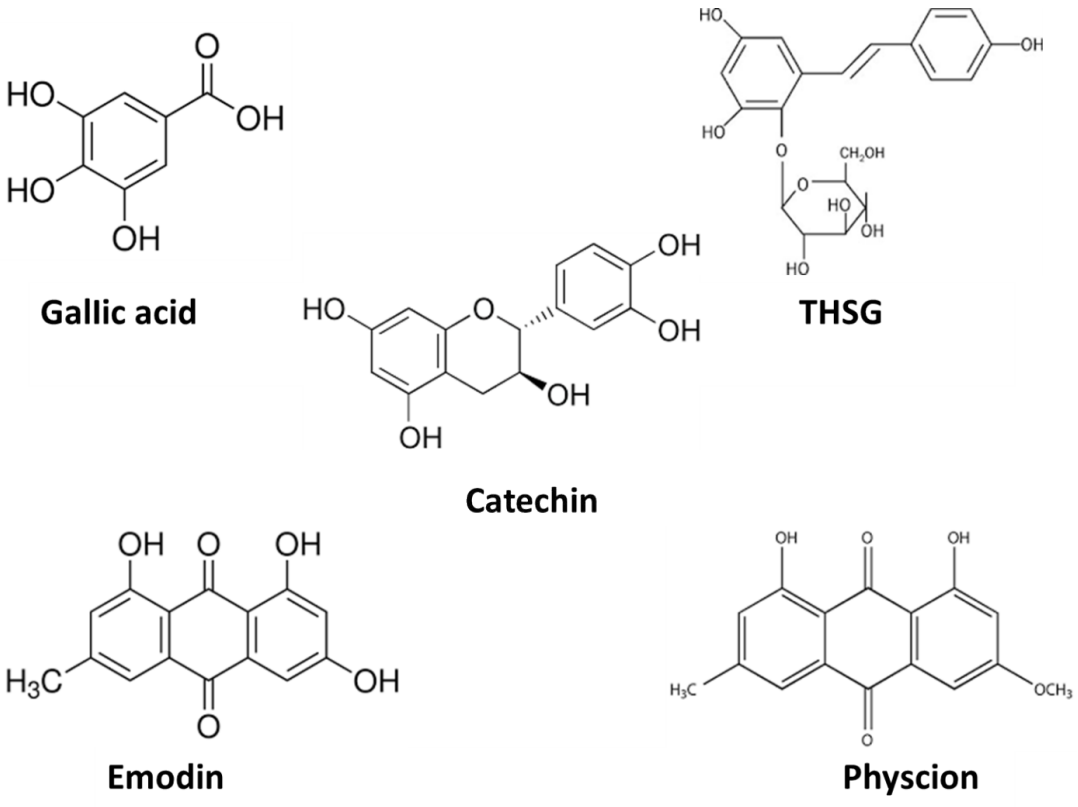

Figure 2: Chemical Structure of THSG, gallic acid, catechin, physcion, and emodin.

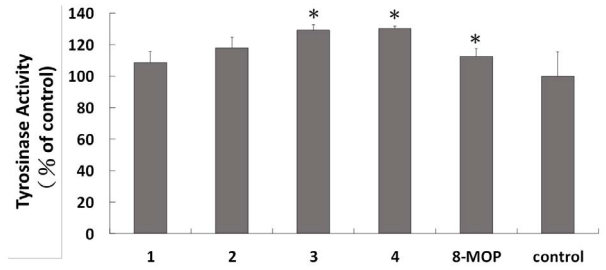

Figure 3A: Effects of Chinese herbal extracts on Mushroom Tyrosinase. 8-MOP: Tyrosinase stimulators 8-methoxypsoralen served as positive controls. 1: *Fallopia multiflora*; 2: *Fallopia multiflora, Ligustrum lucidum ait*; 3: *Fallopia multiflora, Ligustrum lucidum ait, Angelica sinensis;* 4: *Ligustrum lucidum ait, Angelica sinensis,* and other Chinese herbal. Bars represent means ±S.E. of at least three independent experiments. Significant differences were determined by Student's *t*-test; *p>0.05 compared to control

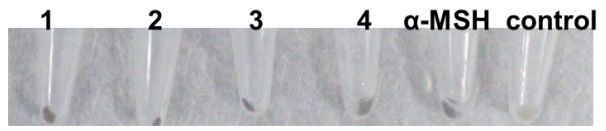

Figure 3B: Effects of Chinese herbal extracts on Melanin Synthesis, and Cell Growth of B16 Cells. Tyrosinase stimulatorsα-melanocyte-stimulating hormone (α-MSH) served as positive controls. (A) Macroscopic view of cell pellets.

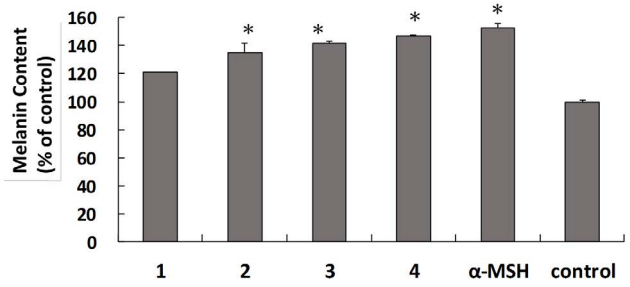

Figure 3C: Quantification of melanin synthesis.

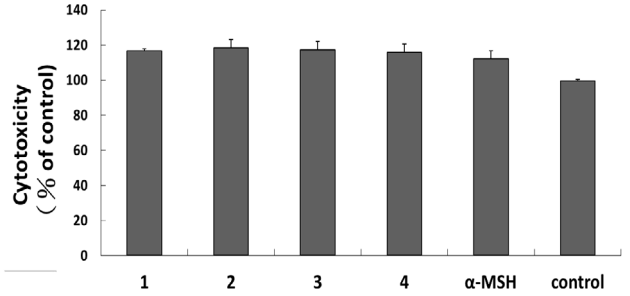

Figure 3D: Results of a cell cytotoxicity assay. 1: *Fallopia multiflora*; 2: *Fallopia multiflora, Ligustrum lucidum ait*; 3: *Fallopia multiflora, Ligustrum lucidum ait, Angelica sinensis;* 4: *Ligustrum lucidum ait, Angelica sinensis,* and other Chinese herbal. Bars represent means ±S.E. of at least three independent experiments. Significant differences were determined by Student's *t*-test; *p>0.05 compared to control.

	Concentration	Tyrosinase activity (% of control)
THSG	150μm	286±6.3
Gallic acid	150μm	140.5±2.1*
Catechin	150μm	101.7±7.1
Physcion	150μm	123.9±7.3*
Emodin	150μm	113.8±9.7
control		100.0±2.9

Table 2: Effects of THSG, gallic acid, catechin, physcion, and emodin on Mushroom Tyrosinase. Significant differences were determined by Student's *t*-test; * p>0.05 compared to control.

herbal extracts showed the increased pigmenting ability and had no influence on cells viability.

Discussion

Many investigations have focused on the specific mechanisms involved in melanogenesis in order to develop new therapeutic agents for hair greying and skin pigmentation abnormalities. Tyrosinase is known to be a key enzyme for melanin biosynthesis in plants, microorganisms and mammalian cells. However, hair greying is an obvious sign of human aging, yet little is known about its causes. It can be caused by age-related loss of melanocytes in the bulge of the hair follicle, or loss of melanization signals [22]. In previous study, Chinese herbal have been found to increase melanin production and prevent the greying of hair. Therefore, the aim of our study is to screen Chinese herbal, which can increase melanin production and prevent the greying of hair. In our preliminary screening using mushroom tyrosinase, the extract of FM was found to show significant increased activity for tyrosine hydroxylation. We demonstrated that THSG, gallic acid, physcion, and emodin are the major compounds of *Fallopia multiflora* in high performance liquid chromatography (HPLC) analysis. THSG, gallic acid, and physcion have been reported to increased activity for mushroom tyrosinase effects. We also investigated that Chinese herbal extracts (FM, *Ligustrum lucidum ait, Angelica sinensis*, and other Chinese herbal) to show significant increased activity for mushroom tyrosinase and melanogenesis in mouse melanocytes. After treatment with Chinese herbal extracts, tyrosinase activity was increased to 8.1 %~34.7 % of that in control. The results indicated that Chinese herbal extracts showed the increased pigmenting ability and had no influence on cells viability. When B16 cells were treated with Chinese herbal, melanin production was greatly increased 18 %~53 % of that in control. In the present study, we demonstrated that Chinese herbal extracts showed the increased pigmenting ability and had no influence on cells viability. In conclusion, these results of low cytotoxicity, high increasing of melanin synthesis and lack of effect on cytotoxicity suggest that Chinese herbal extracts can be a potential agent for hair blackening to be used in cosmetic products.

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