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중의 약재로부터 피부 미백제의 탐색

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The Study on the Whitening Effects of Traditional Chinese Medicines

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요 약: 본 연구에서는 미백 화장품 원료를 개발하기 위해 중국에서 전통적으로 사용되어 오던 47종의 천연약재 또는 복합처방 중에서 멜라닌 생성 저해 효과를 지닌 원료를 찾고자 하였다. 그 중 버섯 티로시나제 활성 실험에서는 저해 효과를 보이지 않았으나, B16-F10 멜라닌 생성세포(B16-F10 melanoma cell)를 이용하여 류기노와 단삼을 선별하였으며 단삼에서 단삼소(丹蔘素, α,3,4-trihydroxybenzenepropanoic acid sodium salt)를 분리하여 B16-F10 멜라노마 세포를 이용하여 멜라닌 생성 억제에 관한 실험을 실시하였다. 류기노와 단삼소는 농도에 따라 멜라닌 생성을 억제하였으며, 류기노는 300 μg/mL의 농도에서 약 60%, 단삼소는 100 μg/mL의 농도에서는 약 50 %의 멜라닌 생성 저해 효과를 보였다. 따라서 연구 결과로써 얻어진 2종의 원료는 새로운 천연 미백 소재로 적용할 수 있을 것으로 기대된다.

Abstract: In order to search for new whitening cosmetic ingredients from Chinese herbal extracts including Chinese herbs and complex prescriptions from TCM (Traditional Chinese Medicine), we screened about 47 TCM extracts collected from China. We tested their inhibitory effects on melanogenesis by using *in vitro* tyrosinase inhibition assay and B16 melanoma cells. We selected *Siphonostegia chinensis* and *Salvianic miltiorrhiza* Bunge. We isolated *Danshensu* (α,3,4-trihydroxybenzenepropanoic acid sodium salt) from *Salvianic miltiorrhiza* Bunge extract and tested its inhibitory effect on melanin formation in B16-F10 melanoma cells. *Danshensu* suppressed melanin synthesis up to about 50 % at a concentration of 100 μg/mL. *Siphonostegia chinensis* suppressed melanin synthesis up to about 60 % at a concentration of 300 μg/mL. The results showed that these extracts could be used as new natural active ingredients for whitening cosmetics.

Keywords: *melanin, TCM, B16-F10 melanoma, whitening, Siphonostegia chinensis, sodium danshensu*

1. Introduction

Recently, people of all ages and both sexes wish to have bright skin tone as one of the requirements for the beauty. They want to have fair skin complexion and put lots of effort to get bright skin tone. Therefore, many cosmetics with natural materials have been developed for the use of depigmentation.

Melanin is a skin pigment. It is a substance that determines the skin color. Dark-skinned people have more melanin than light-skinned people. Melanin is produced by cells called melanocytes. It provides some

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protection against skin damage from the sun, and the melanocytes increase melanin formation as a defense mechanism against ultraviolet light of the sun. However, the abnormal pigmentation such as freckles and chloasma causes a serious skin problem[1,2].

Melanogenesis stimulated by UV irradiation occurs in plant and mammalian cells by an enzymatic oxidation process starting from L-tyrosine. Recent reports indicated that α-melanocyte stimulating hormone (α-MSH)[3], a typical activator of the cyclic AMP system, stimulates tyrosinase activity in human melanocytes and accompanies the increased expression of tyrosinase[4-6]. It is likely that the cyclic AMP system is an essential pathway for accentuation of the melanogenesis process. However, recent reports suggest that the stimulating effect on the melanin synthesis is closely involved with the activation of a protein kinase C (PKC)[7]. Tyrosinase catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-DOPA) and subsequently of L-DOPA to dopaquinone, and oxidative polymerization of several dopaquinone derivatives eventually produce melanin[3]. On the other hand, it has been reported that superoxide dismutase (SOD) is one of the key factors that reduce melanin production caused by UV irradiation[8]. Aside from avoiding UV exposure, inhibition of melanogenesis may be the least invasive-to-the-skin method for maintaining skin whiteness, and in fact such agents are used increasingly in cosmetic products[9,10].

By blocking various points of the melanogenetic pathway, depigmentation agents in the skin could inhibit melanin biosynthesis and could also be used to treat local hyperpigmentation or spots caused by a local increase in melanin synthesis or uneven distribution. Various ingredients for skin-whitening cosmetics are developing to reduce melanogenesis. Up to now, natural materials have been used for the inhibitory agents of melanin formation, and active components such as kojic acid, arbutin, vitamin C, and hydroquinone have been used for whitening agents.

In this study, we investigated the inhibitory effect of melanin biosynthesis with natural plant extracts, which are well known folk remedies for whitening in China by using melanin content assay on B16-F10 melanoma cells.

2. Materials and Methods

2.1. Preparation of Plant Extract

In order to search for new whitening cosmetic ingredients from Chinese herbal extracts including Chinese herbs and complex prescriptions from TCM, we screened about 47 TCM extracts collected from China. TCM were authenticated by a Professor, the director of Institute of Chinese Matira Medicine, Shanghai University of TCM. Collected plants were dried in the shade at room temperature and stored in a dark, cold room until needed. Dry plants were extracted twice with 70 % (v/v) ethanol (10 times as much as the weight of the dried plants) for 24 h at room temperature. The extracts of plants were filtered through Whatman paper (No. 5) and then evaporated at 60 ℃. After the evaporation, the viscous residue was lyophilized in order to yield the product.

2.2. Preparation of Sodium Danshensu

Dry roots of *Salvianic miltiorrhiza* Bunge. (3,000 g) were cut into thin slices and extracted with boiling water (15 L × 2) for 60 min. After filtration, the liquid extract was concentrated to a small volume under vacuum at 60 ℃. Appropriate volumes of ethanol (EtOH) were then added to the concentrated water extracts to achieve final 80 % ethanol concentrations, then the solution was allowed to stand for an hour at 4 ℃, and filtered. The filter liquor was evaporated under reduced pressure to yield a residue, which was suspended in H_2O. The suspension was then treated with EtOAc, after removal of the solvent, to get a residue. The ethyl acetate-soluble fraction was subjected to silica gel column chromatography (CC) using a $CHCl_3$-Me_2CO-HCOOH gradient system to give sixty fractions (frs. 1 ~ 60). Fraction 53 was further purified by Sephadex LH-20 chromatography eluting with a mixture of H_2O-MeOH (1 : 1) to afford salvianic acid A (danshensu). Salvianic acid A (danshensu) was dissolved in ethanol and adjusted pH with $NaHCO_3$ to stand for overnight

at room temperature, and to get salvianic acid A sodium salt as needle type solid.

2.3. *In vitro* Mushroom Tyrosinase Activity Inhibition Assay

Inhibition of mushroom tyrosinase activities of arbutin, *siphonostegia chinesis* and sodium danshensu are generally assayed with a spectrophotometer. The tyrosinase activity inhibition assay was carried out as previously described by Vanni *et al.*[11]. The reaction mixture consisted of 1 mL of L-tyrosine solution (0.3 mg/mL) and 0.9 mL of sample solution at each concentration. After pre-incubation in a water bath for 20 min at 37 ℃, 0.1 mL of 1,250 U/mushroom tyrosinase was added to the reaction mixture and incubated for another 10 min at 37 ℃. The optical density at 480 nm was measured using a spectrophotometer. The percentage of inhibition was calculated according to the following formula:

$$\text{Tyrosinase activity inhibition (\%)} = [1-(OD_s-OD_b)/OD_c]\times 100$$

Where, OD_s is the absorbance at 480 nm in the presence of the sample, OD_b is the absorbance of the blank at 480 nm, and OD_c is the absorbance of the control at 480 nm.

The results were reported in terms of IC_{50} (IC_{50}: concentration needed to reduce 50 % of tyrosinase activity). Arbutin, a representative tyrosinase inhibitor, was used as a control.

2.4. Cell Culture and Treated Samples

B16-F10 melanoma cell line (ATCC, CRL-6475) was used in this study. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), and they were supplemented with 10 % fetal bovine serum (FBS, Sigma, USA) and 1 % anti-biotics (Gibco, USA) at 37 ℃ in a humidified incubator with 5 % CO_2. 1×10^5 cells of B16-F10 melanoma were seeded on 6-well plates and cultured for 24 h. Then, media were replaced with fresh media, and samples were treated to triplicate cultures.

2.5. Measurement of Melanin Content and Cell Viability in B16-F10 Melanoma Cells

After 3 days of culture, the cells were disrupted with 1 N sodium hydroxide and homogenized by a sonicator. For the analysis, 200 μL of each crude cell extract was transferred into a 96-well plate. The relative melanin content was measured at 470 nm on an ELISA reader [4].

Cell viability was evaluated by MTT assay. The culture medium was removed from the 6-well plate and treated with 250 μg/ml MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphnyltetrazolium bromide] solution in PBS per well, and it was incubated at 37 ℃ for additional 4 h. Then, the medium containing MTT was discarded, and MTT formazan product was extracted with 1 ml DMSO (dimethyl sulfoxide). The amount of formazan in the culture medium was determined by the absorbance measured at 570 nm by an ELISA reader[12].

The inhibition percentage of melanin formation per cell was calculated as:

$$\text{Inhibition (\%)} = (1-A/B) \times 100$$

where, A is the percentage of melanin formation, and B is the percentage of cell viability.

2.6. Statistical Analysis

For all experiments, data from the three cell populations of each sample were combined and expressed as means ± standard deviation. A p-value of < 0.05 was considered significant.

3. Results and Discussion

3.1. Screening of TCM on Melanin Formation of B16-F10 Melanoma Cells

In order to find TCMs' melanin formation inhibition capacity in B16-F10 melanoma cells, melanin contents were measured by a spectrophotometer. The results are shown in Table 1 and Table 2. Out of 47 samples tested, Table 1 contains the results on melanin formation inhibition activity for 31 Chinese herbal

Table 1. Inhibitory Effects of Chinese Herbal Extracts from TCM on Melanin Formation in B16-F10 Melanoma Cells. The Assay was Carried out at a Concentration of 100 μg/mL, and Arbutin was Used as a Positive Control

Sample	Cell viability (% control)	Inhibition (%)
Trichosanthes kirilowii aximowicz (seed)	48.72	-41.24
Drynaria fortunei (rhizome)	63.90	-27.97
Selaginella puluinata	69.04	-33.35
Salvia miltiorrhiza Bunge	83.39	40.65
Verbena officinalis L.	81.82	-11.88
Ampelopsis radix	91.37	19.71
Tribulus terrestris (fruit)	77.67	-34.82
Pharbitis nil (seed)	77.60	-17.53
Brassica campestris ssp. napus var. pekinensis (seed)	89.27	-6.27
Imperata cylindrica var. koenigii (rhizome)	96.16	-5.43
Atractylodes japonica	100.24	6.74
Hedyotis diffusa	72.50	-6.97
Acanthopanax senticosus (root)	88.71	-70.66
Plantago asiatica (seed)	90.92	-60.58
Magnolia officinalis	68.55	50.90
Cyperus rotundus (rhizome)	82.43	-19.06
Rosa laevigata	92.94	19.42
Siphonostegia chinensis	97.71	39.12
Eclipta prostrata	73.32	-11.86
Portulaca grandiflora	89.44	-78.54
Crocus sativus L.	85.55	-10.35
Magnolia denudata	61.71	-48.69
Chrysanthemum indicum	96.46	4.62
Polygala tenuifolia (root)	47.69	98.33
Ligularia Speciosa (rhizome)	82.86	-12.73
Sanguisorba hakusanensis (rhizome)	73.15	31.23
Melia toosendan (fruit)	65.18	20.91
Cyathula officialis (root)	89.14	-30.46
Astragalus membranaceus var. mongholicus	77.24	-60.07
Cynanchi Radix	23.62	88.78
Myristica fragrans (seed)	42.61	65.23
Arbutin (250 μM)	98.69	56.32

extracts. Meanwhile, Table 2 shows the results on melanin formation inhibition activity for 15 complex prescriptions. This experiment was performed with the concentration of 100 μg/mL. As a result, we observed 13 samples which inhibit melanin formation to above 15 percentages. Among those samples, 6 samples - Modanhyup, Mokgiyeooksan, *Cynanchi Radix*, *Polygala tenuifolia* (root), *Myristica fragrans* (seed) and Cheonsimryeon - have lower cell viability of less than 50 percentages, and therefore excluded as candidates. And another 5 samples were also removed from the candidate list: *Ampelopsis radix*, *Magnolia officinails*, *Rosa laevigata*, *Sanguisorba hakusanensis* (rhizome) and *Melia toosendan* (fruit). The reason is that even

Table 2. Inhibitory Effects of Complex Prescriptions from TCM on Melanin Formation in B16-F10 Melanoma Cells. The Assay was Carried out at a Concentration of 100 μg/mL, and Arbutin was Used as a Positive Control

Sample	Cell viability (% control)	Inhibition (%)
Myungdangsam	88.96	-2.72
Kimkukkungryeo palbaksan	58.07	-29.89
Myeonbakyeookbang	90.70	2.11
Semyeonyoeokgo	72.00	-4.06
Jayunsumyeonbang	60.18	-6.85
Yangchunbakseolgo	88.27	-6.76
Oknyodohwaboon	76.38	-18.28
Eumgeohyohumbang	43.92	-154.83
Umseochumda	84.05	12.89
Oryungsan	66.46	-17.67
Modanhyup	19.86	94.65
Mokgiyeooksan	2.69	93.96
Ceonsimryeon	15.38	75.29
Gibakgihwanghwan	75.59	-2.19
Igongsangami	82.66	-17.5
Arbutin (250 μM)	98.69	56.32

with cell death, the sample reacted with MTT reagents to produce a color reaction. Therefore, the value of cell viability was high, and it was shown as if it had melanin formation inhibition activity.

3.2. Isolation and Identification of *Sodium Danshensu* from *Salvia Miltiorrhiza Bunge*

Salvianic acid A sodium salt (sodium danshensu) was obtained as white needles, with mp 256.7 ~ 257.8 ℃. The molecular formula of sodium danshensu was determined as $C_9H_9NaO_5$ by ESIMS at *m/z* 221.1 for $[M+H]^+$ (Figure 1). The ^1H NMR spectrum showed six proton signals, of which three aromatic proton signals formed an ABX spin system, [δ_H 6.73 (1H, d, *J*=2.0 Hz, H-2), 6.65 (1H, d, *J*=7.8 Hz, H-5), 6.59 (1H, dd, *J*=7.8, 2.0 Hz, H-6)] and other three protons formed a oxygen-bearing ethyl unit [δ_H 2.96 (1H, dd, *J*=13.7, 3.3 Hz, H-7), 2.63 (1H, dd, *J*=13.7, 8.2 Hz, H-7), 4.04 (1H, dd, *J*=8.2, 3.3 Hz, H-8)]. The ^{13}C NMR spectrum showed nine carbon signals, including one carbonyl signal at δ_C 180.9, and aromatic carbon signals at δ_C 131.9, 117.7, 144.5, 145.8, 115.9, 121.9 as well as aliphatic carbon signals at δ_C 41.9 and 74.9. (Table 3).

Table 3. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) Data for Sodium Danshensu

No	^1H	^{13}C
1		131.9
2	6.73 (1H, d, J=2.0 Hz)	117.7
3		144.5
4		145.8
5	6.65 (1H, d, J=7.8 Hz)	115.9
6	6.59 (1H, dd, J=7.8, 2.0 Hz)	121.9
7	2.96 (1H, dd, J=13.7, 3.3 Hz) 2.63 (1H, dd, J=13.7, 8.2 Hz)	41.9
8	4.04 (1H, dd, J=8.2, 3.3 Hz)	74.9
9		180.9

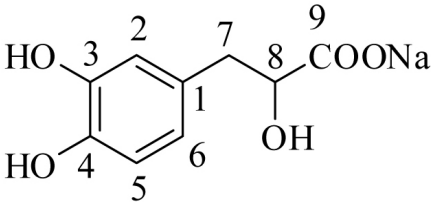


Figure 1. Structural formula of *sodium danshensu*.

3.3. Evaluation on *in vitro* Mushroom Tyrosinase Inhibition Activity

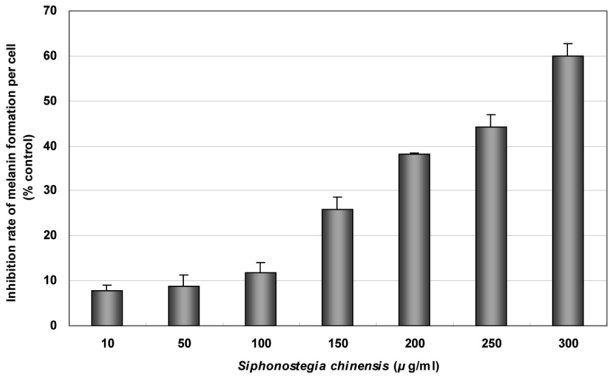
Experiment on tyrosinase activity inhibition was done to *Siphonostegia chinensis* and sodium danshensu that did not have inhibitory effect against mushroom tyrosinase activity. The capacity of each samples are shown in Table 4. *Siphonostegia chinensis* showed 8.60 % of mushroom tyrosinase activity inhibitory effect at 400 μg/mL, but this is not able to be evaluated as a good effect. Other samples did not show mushroom tyrosinase activity inhibition effect at all.

3.4. Effects of *Siphonostegia chinensis* and Sodium Danshensu on Melanin Formation of B16-F10 Melanoma Cells

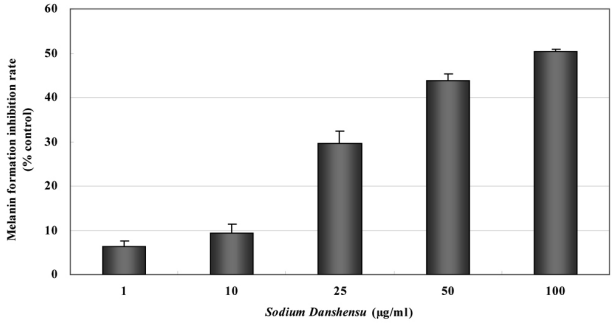
Siphonostegia chinensis and sodium danshensu extracts showed inhibitory effect of melanin formation. So we measured melanin formation inhibition effect

Table 4. Comparison of *in vitro* Mushroom Tyrosinase Inhibition Activity

Sample	Concentration (μg/mL)	Inhibition (%)
Arbutin	100.00	61.87 ± 0.33
Siphonostegia chinensis	13.33	-10.56 ± 0.56
	400.00	8.60 ± 2.04
Sodium Danshensu	13.33	-4.10 ± 1.25
	400.00	-19.95 ± 4.26



(A)



(B)

Figure 2. Inhibitory effects of *Siphonostegia chinensis* (A) and sodium danshensu (B) on Melanin Formation in B16-F10 Melanoma cells.

according to various concentrations, which indicated its inhibitory effects with dose dependence. The results are shown in Figure 2. *Siphonostegia chinensis* increases the inhibition of melanin formation up to 60 % of the control value at 300 μg/mL (Figure 2A). And sodium danshensu showed that it has melanin formation inhibition activity dose-dependently. It has melanin formation inhibition capacity up to 50.51 % at concentration of 100 μg/mL (Figure 2B). These results suggest that *Siphonostegia chinensis* and sodium danshensu effectively inhibit melanin synthesis in B16-F10 melanoma cells.

4. Conclusions

47 TCM extracts from China were screened for melanin formation inhibitory effects by using *in vitro* mushroom tyrosinase assay and B16-F10 melanoma cell. The extracts of *Siphonostegia chinensis* and *Salvia miltiorrhiza* Bunge were shown to inhibit of melanin formation in B16-F10 melanoma cells.

Salvia miltiorrhiza Bunge is a famous herb of TCM with the effects of promoting blood circulation, relieving blood stasis, clearing heat from the blood, resolving swelling and tranquilizing the mind[13]. It has been officially listed in the *Chinese Pharmacopoeia*[14]. The main biologically active ingredients in root of *Salvia miltiorrhiza* Bunge include abietane-type diterpenes such as dihydrotanshione I (Dih), tanshinone I (Tan I), cryptotanshinone (Cry) and tanshinone IIA (Tan IIA) and water-soluble compounds such as sodium Danshensu[15,16]. We isolated sodium danshensu which is the main biological active ingredients in the root of *Salvia miltiorrhiza* Bunge and investigated its melanin formation inhibition activity. The results indicate that *Siphonostegia chinensis* extract and sodium danshensu may be useful ingredients in skin-whitening cosmetics. Further investigation of *Siphonostegia chinensis* and complex prescriptions is in progress along these lines.

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