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Original Article

Inhibitory effects of phenylethanoid glycosides on melanin synthesis in cultured human epidermal melanocytes

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Abstract: Objective: To investigate the inhibitory effects of phenylethanoid glycosides (PhGs) derived from cistanche on melanogenesis in cultured human epidermal melanocytes, and explore the structure-activity relationship through comparison of the inhibitory effect of ten kinds of PhGs. Methods: L-dopa assay was performed to examine the effect of PhGs on tyrosinase activity. Alkaline lysis method was used to detect the content of melanin in cultured human epidermal melanocytes after treated with ten kinds of PhGs. Results: PhGs reduced tyrosinase activity in a dose-depend manner. The content of melanin was reduced by PhGs. Compared with the positive control, significant elevation was noticed in the inhibitory effect of poliumoside, 2'-acetylacteoside, acteoside, oleuropein and calceolarioside B ($P < 0.01$), respectively. On the contrary, the inhibitory effects of forsythoside A/B was relatively weaker compared with positive control group ($P < 0.01$). Conclusions: PhGs exhibit significantly inhibitory effect on melanogenesis in human epidermal melanocytes. The suppression ability is related to the number of phenolic hydroxyl and aglycone, steric hindrance, substituents on the middle glucopyranose, and the location of phenolic hydroxyl.

Keywords: Phenylethanoid glycosides, melanogenesis, structure-activity relationship, acteoside, tyrosinase

Introduction

Excessive synthesis of melanin regulated by tyrosinase is the primary cause of pigment disorder which causes great threats to the public health [1]. Nowadays, extensive studies have been conducted with a favor to reduce the melanin production for the treatment of the pigment disorder [2-5].

Herba Cistanches, a Chinese herbal medicine, has been commonly used in China mainland with the goals of nourishing kidney and replenishing essence and blood [6]. According to the chemical analysis, phenylethanoid glycoside (PhG) is one of the main constituents in cistanche, and more than 40 kinds of PhGs have been isolated until now. To date, an increasing number of studies reveal the potential efficiency of cistanche in enhancing learning and memory, preventing bone loss, and the potential toxicity in reproduction system [7, 8]. In this study, we aim to investigate the efficiency of cistanche in the management of pigment disorder.

In this study, we purified the total PhGs from cistanche, and determined their effects on tyrosinase activity in primary cultured human skin melanoma cells, based on which to investigate the effects of PhG on pigmentation. This study will provide theoretical support for the development of the novel anti-pigmentation drugs with the PhGs.

Materials and methods

Preparation and determination of PhGs

Preparation of PhGs was performed as previously described [9]. The air-dried cistanches material was powered and extracted by percolation with 80% EtOH. The percolate was evaporated under pressure, followed by filtration of the residual fluid. The filtrate was concentrated and chromatographed on a SP-825 macropo-

Table 1. Gradient of the mobile phase

Time	Phosphoric acid solution (%)	Acetonitrile (%)
0 min	85	15
20 min	80	20
30 min	70	30

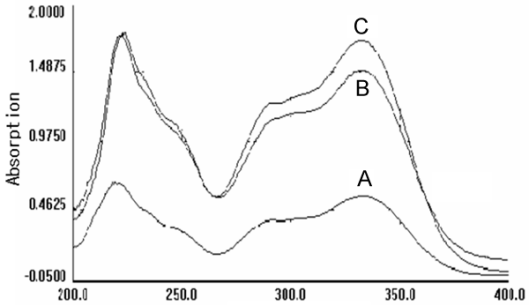

Figure 1. UV spectra of the echinacoside (A), acteoside (B) and PhGs (C).

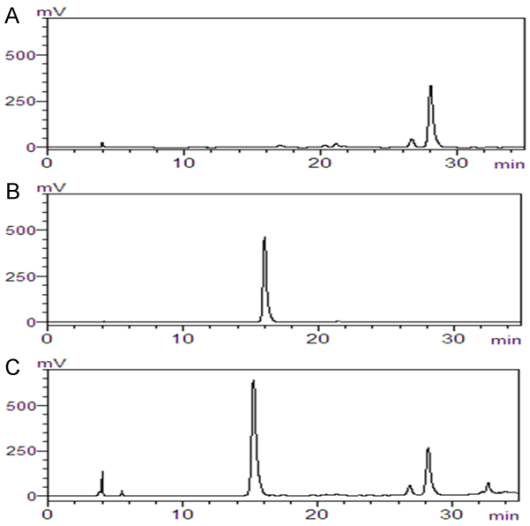

Figure 2. HPLC chromatography of acteoside (A) echinacoside (B) and standard sample (C).

rous resin column using 0, 30%, 50%, 70% and 90% EtOH in water as eluants. The 30%-50% EtOH eluants were concentrated and dried under pressure to obtain PhG rich fraction. UV spectrophotometry was performed, and the content of the PhG was 87.6%. Echinacoside served as control.

Determination of echinacoside and verbascose by HPLC

The content of echinacoside and verbascose was determined using HPLC [10]. A Hypersil-ODS-2 column (4.6 mm×250 mm, 5 μm) was used and maintained at room temperature. The mobile phases were methyl cyanides and water containing 0.4% phosphoric acid (v/v) with a gradient elution (**Table 1**) at a flow rate of 0.75 ml/min. The wavelength was set to 333 nm. The content for echinacoside and verbascose was 35.2% and 18.7%, respectively.

Isolation and culture of melanocytes

Melanocytes were isolated and cultured from foreskin obtained from infants undergoing surgery. The foreskin was washed using phosphate buffered saline (PBS) supplemented with 100 μ/ml penicillin, 100 μg/mL streptomycin thrice completely. Subcutaneous fat and connective tissue were removed in the aseptic condition, epidermis and dermis were isolated with 0.25% cleavage enzyme at 37°C. The epidermal tissue suspension was filtered through a 100 mesh filter to remove the undigested cells, followed by centrifugation. The cells were washed with 254 medium and were inoculated into cell culture flasks. The cells were harvested after a confluence of 80%. The identification of cultured melanocytes was performed using the DOPA staining according to the previous study.

Effects of PhGs on tyrosinase activity

Human melanoma cells (5×10^4/mL) were inoculated on 96-well plate, and cultured for 24 hours followed by adding different concentrations of PhGs (200 μL/well). Subsequently, the culture medium was discarded after cultured for 48 h, followed by washing with PBS twice. Afterwards, 1% TrionX-100 (50 μL/well) was added, and was stored at -80°C for 30 min. Then, 10 μL 0.25% DOPA solution was added after incubation at 37°C. After incubation at 37°C for 2 h, the absorbance was measured using ELISA at a wavelength of 490 nm. The inhibition rate of tyrosinase was calculated according to the formula (1-experimental group A490/control group A490) ×100%. The experiment was performed at least in triplicate.

Cell proliferation determination

Human melanocytes (10^5/mL) were placed in 96-well plates and incubated for 24 h at 37°C in 5% CO_2. After that, the cells were divided into: blank control; arbutin group, in which the cells were treated with various concentrations of arbutin (50-1000 μg/mL), served as positive

Table 2. The inhibitory effect of drugs on tyrosinase activity

Group	Concentration (μg/mL)	A_{490}	Inhibition rate
Blank	-	0.1372±0.0294	-
PhGs group	100	0.0843±0.0148	27.95%
	200	0.0744±0.0702	38.56%
	400	0.0638±0.0602	45.78%
Arbutin group	100	0.1067±0.0067	22.23%
	200	0.0862±0.0035	37.11%
	400	0.0780±0.0342	43.15%

Table 3. IC_{50} of drugs

Drug	IC_{50} (μg/mL)
Arbutin	1282.79
Echinacoside	907.06
Poliumoside	659.48
Forsythiaside A	891.57
Forsythiaside B	943.54
Oleuropein	793.95
Acteoside	1169.21
Calceolarioside B	807.6
Salidroside	772.99
Isoacteoside	964.35
2'-acetyl verbascoside	885.3
PhGs	716.51

control; and PhGs group, which was subject to different concentrations of PhGs (50-1000 μg/mL). After incubation for 24 h, MTT (5 mg/mL) was added to each well. Supernatant was discarded after incubation for 4 h, followed by the addition of 150 μL DMSO. The absorbance was measured at 490 nm. The drug concentration at 50% inhibition rate (IC_{50}) was calculated according to the formula (1-experimental group A490/control group A490) ×100%.

Cell viability determination

Human melanocytes were seeded in 96-well plates at a density of 10^5/mL and incubated for 24 h at 37°C in 5% CO_2. To determine the potential effects of PhGs on the melanocytes, the cells were treated with 200 μL 254 culture fluid (blank control), 400 μg/mL arbutin (positive control), and 400 μg/mL PhGs respectively. After incubation for 24 h, MTT (5 mg/ml) was added, followed by the addition of 150 μL DMSO after removal of supernatant. The absorbance was measured at 490 nm.

Effects of PhGs on melanin content

The content of melanin was determined according to the method proposed by Hosoi et al. [11]. 6 with slight modifications. The cells (2×10^5/mL) grown on a 6-well dish were treated with 400 μg/mL PhGs for 48 h. After treating with 0.25% trypsin, the cells were harvested by centrifugation at 1,000 rpm for 5 min. The supernatant was discarded, and the pellet was washed twice with PBS. The melanin and precipitate were solubilized by incubating with 100 μL of NaOH (1 M) at 37°C for 8 h. The absorbance of the solutions was measured at 470 mm. The inhibition rate of PhG on the melanin content was calculated according to the formula (1-experimental group A470/blank group A470) ×100%.

Statistical analysis

Measurement data were presented as mean ± standard deviation. Student's t test was used for the inter-group comparison. All data were analyzed by SPSS 13.0. $P < 0.05$ was considered to be statistically significant.

Results

UV spectra of PhGs

The UV spectra of test solution, echinacoside standards and verbascoside standards solution were scanned, and the results showed that UV spectrums of them were consistent (**Figure 1**), the maximum absorption wavelength was 333 nm. Thus, the echinacoside or verbascoside could be used as standards. The total PhGs were determined using UV. In this study, the contents of total PhGs were determined using the echinacoside as the standard, and the content of the total PhGs was 87.6% (**Figure 2**).

Effects of PhGs on tyrosinase activity

PhGs reduced tyrosinase activity in a dose-dependent manner (**Table 2**). Compared with the blank group, the tyrosinase activity was significantly reduced in arbutin group and PhG group ($P < 0.01$). Meanwhile, compared with the arbutin group, significant increase was noticed in the inhibitory effects of PhGs on tyrosinase activity ($P < 0.01$).

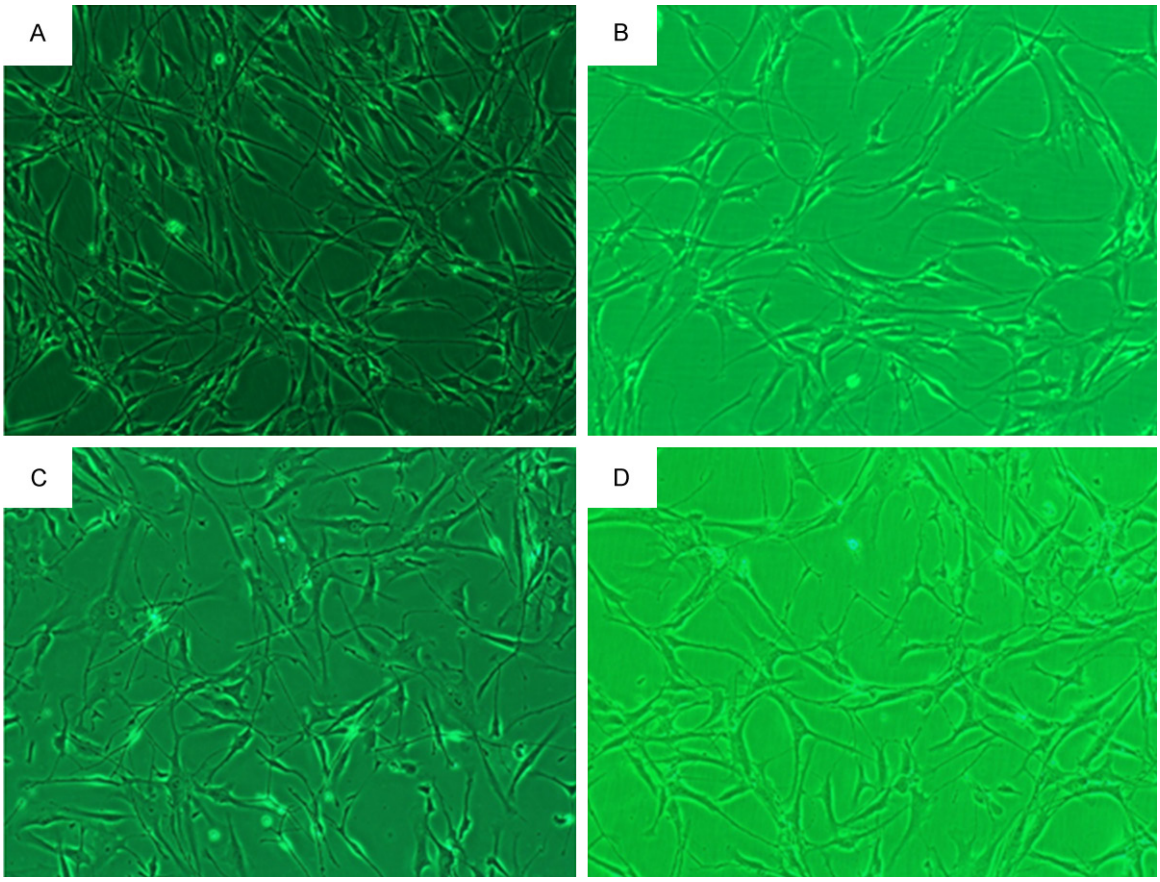

Figure 3. The morphology of melanocytes in the blank control (A), verbascose group (B), PhGs group (C) and arbutin group (D). The images were observed under a magnification of 100×.

Cell proliferation determination

To exclude the possibility that the inhibitory effects of PhGs on melanogenesis might be caused by the inhibition of cell growth, cell proliferation was determined using the MTT method. The results showed that IC_{50} of 10 kinds of PhGs (echinacoside, poliumoside, foliumoside A, foliumoside B, oleuropein, acteoside, calceolarioside B, salidroside, ialidroside, and 2'-acetyl verbascoside) was in the range of 650-1200 μg/mL, which indicated that the cytotoxicity of PhGs on melanacytes was low (**Table 3**).

Cell viability determination

A concentration of 400 μg/mL was used to test the inhibitive effects on the melanacytes according to the half lethal dose. The results indicated such concentration showed a lower inhibition rate on cell growth. As shown in **Figure 3**, shrinkage was observed in few cells, together with cell expansion and delayed proliferation. Taken together, we confirmed that PhGs had an inhibitory effect on melanogenesis at noncytotoxic concentrations (400 μg/mL).

Effects of PhGs on melanin content

To determine the efficiency of PhGs on the production of melanin, we determined the melanin production in cells exposed to the major 10 PhGs above mentioned. Results showed that the content of melanin was reduced by PhGs especially in poliumoside, followed by 2'-acetyl verbascoside, verbascoside, oleuropein, calceolarioside B, salidroside, echinacoside, arbutin, isoacteoside, forsythoside A, and forsythoside B, respectively (**Table 4**). Compared with the positive control, significant elevation was noticed in the inhibitory effect of poliumoside, 2'-acetylacteoside, acteoside, oleuropein and calceolarioside B ($P < 0.01$), respectively. On the contrary, the inhibitory effects of forsythoside A/B were relatively weaker compared with positive control group ($P < 0.01$, **Table 4**).

Table 4. The inhibitory effect of drugs on synthesis of melanin

Drug	Melanin content	Inhibition rate
Blank	0.1651±0.0023	-
Positive control	0.1112±0.0019$^{\#}$	32.65%
PhGs	0.1132±0.0028$^{\#}$	31.43%
Echinacoside	0.1110±0.0043$^{\#}$	32.77%
Poliumoside	0.0955±0.0025$^{\#,**}$	42.16%
Forsythiaside A	0.1177±0.0050$^{\#,*}$	28.71%
Forsythiaside B	0.1197±0.0021$^{\#,*}$	27.50%
Oleuropein	0.1017±0.0014$^{\#,**}$	38.40%
Verbascoside	0.1008±0.0080$^{\#,**}$	38.95%
Calceolarioside B	0.1047±0.0016$^{\#,**}$	36.58%
Salidroside	0.1076±0.0012$^{\#}$	34.83%
Isoacteoside	0.1120±0.0012$^{\#}$	32.16%
2'-acetyl verbascoside	0.0992±0.0024$^{\#,**}$	39.92%

$^{\#}P < 0.01$, compared with the blank control group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the positive control group.

Discussion

The inhibition of tyrosinase activity could reduce the melanogenesis. The role in pigmentation of drug was reflected by the melanin content in melanocytes [12]. Therefore, in this study, the inhibitory effects of PhGs on melanogenesis were investigated, the relationship of structure-activity was explored. Our study provides theoretical basis for the development of PhGs drugs for pigment disorder.

As previously described, PhGs could significantly inhibit the mushroom tyrosinase activity [13]. In this study, the total PhGs were separated from cistanche, and the content of PhGs was up to 87%, the content of echinacoside and verbascoside was more than 53%, which was significantly improved compared with the previous extraction. Therefore, the effect of PhGs on the melanogenesis could be reflected more accurately.

The tyrosinase activity could be activated by UV, and tyrosine was catalyzed to produce dopa, which is the rate-limiting reaction in melanogenesis [14]. Although melanin has the function of shielding UV, the accumulation of melanin is a serious skin disease, which reduces the social function and causes great threats to the public health [15]. In this study, the primary cultured human melanoma cells were used, which could reflect the normal physiological condition of skin objectively. It is an ideal experimental model for evaluating the effects of PhGs on melanogenesis and exploring the possible mechanism.

The tyrosinase inhibitor-arbutin with high efficiency and low toxicity was developed in the last century [16]. The arbutin as the active component in anti-pigmentation has been used in chemical and pharmaceutical fields widely. In this study, the arbutin was used as a positive control. The results showed that PhGs could inhibit tyrosinase activity and reduce the content of melanin, indicating that PhGs had inhibitory effects on melanogenesis. Compared with the positive control, significant elevation was noticed in the inhibitory effects of poliumoside, 2'-acetylacteoside, acteoside, oleuropein and calceolarioside B respectively. On the contrary, the inhibitory effects of forsythoside A/B were relatively weaker compared with positive control group. This study suggested that PhGs had strong activity in anti-pigmentation. It provides theoretical support for the treatment of pigment disorder.

Normal melanogenesis plays important role in protecting skin, resisting UV and preventing overheating [17]. To exclude the possibility that the inhibitory effects of PhGs on melanogenesis might be caused by the inhibition of cell growth, cell proliferation was determined using the MTT method. The results showed that IC_{50} of 10 kinds of PhGs was in the range of 650-1200 μg/mL, which indicated that the cytotoxicity of PhGs on melanacytes was low. A concentration of 400 μg/mL was used to test the inhibitive effects on the melanacytes according to the half lethal dose. The results indicated such concentration showed a lower inhibition rate on cell growth.

The inhibitory effects of PhGs on melanogenesis were related to the 2-hydroxy-benzeneethanol, 2-hydroxy-benzene propylene group and glycolsyl group [13, 18]. Containing the same structure of 2-hydroxy-benzeneethanol and 2-hydroxy-benzene propylene group, the inhibitory effects of single glucoside-calceolariaoside B were stronger compared with diglucoside-forsythoside A and three glycosidase-echinacoside, which indicated the inhibitory effect of PhGs would be smaller when the steric hindrance and the number of glycoside were larger. As the single glycosides containing no 2-hydroxy-benzene propylene group, the activity of salidroside and oleuropein was related to the

number of phenol hydroxyl group in benzene ethanol, the location of phenol hydroxyl group in benzene ethanol, and the substituent group of glycosidic. When the number of phenol hydroxyl group especially ortho two phenol hydroxyl was more in PhGs, the inhibitory effect on melanogenesis was stronger. The inhibitory effect of salidroside was weaker than calceolariaoside B, which may be related to the structure of α, β-conjugated unsaturated ester in 2-hydroxy-benzene propylene increased the planar conjugate property of benzene ring. Containing the same number and location of saccharide groups and phenolic hydroxyl groups, the content of melanin was reduced by PhGs especially in poliumoside, followed by 2'-acetyl verbascoside, verbascoside, oleuropein, calceolarioside B, salidroside, echinacoside, arbutin, isoacteoside, forsythoside A, and forsythoside B, respectively. Therefore, we deduced that the inhibitory effects of PhGs on melanogenesis were related to the substituent groups on the central glucose.

In deed, there are limitations in this study. It is hard to identify which component of PhG is responsible for the inhibitory effects as there are so many components in PhGs. We could only confirm that PhGs showed inhibitory effects on melanin synthesis in cultured human epidermal melanocytes. In future, we will focus on identifying which component is effective for the inhibitory effects.

In summary, PhGs is a compound composed of phenylethyl group, benzene acryloyl group and glycosyl group. The structure is related to the biological effect of PhGs. In the further, studies with more kinds of PhGs are needed to clarify the correlation between structure and activity.

Disclosure of conflict of interest

None.

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