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Short Communication
Furocoumarin from Radix *Angelica dahurica* and Synthetic Analogue as Potential Agent for Treatment of Vitiligo

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Abstract

Background and Objective: Imperatorin, isolated from medicinal plant Radix *Angelica dahurica*, has the same furocoumarin nucleus with psoralen and 8-Methoxypsoralen. The purpose of this study was to investigate the melanocyte activity of imperatorin and its synthetic derivative. **Methodology:** The effects of imperatorin and its derivative on the activity of tyrosinase and the content of melanin were tested on A375 cells. The possibility of healing vitiligo in Guinea pigs was assessed by using Lillie iron staining, DOPA staining and ELISA assay. **Results:** Psoralen, 8-Methoxypsoralen, imperatorin and its derivative all increased cellular tyrosinase activity and the contents of melanin in a dose-dependent manner. These four compounds significantly increased the number of melanocyte mm^{-2} compared with the model control group ($p<0.01$). The activity of tyrosinase in plasma and cholinesterase in serum were increased contrast to the model control ($p<0.01$). **Conclusion:** Imperatorin and its derivative can induce the production of melanocytes in vitiligo animal model, which could be helpful in treating vitiligo and clarifying structure-activity relationship.

Key words: Psoralen derivatives, imperatorin, melanin, tyrosinase, vitiligo

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Vitiligo is a common depigmenting disorder characterized by the loss of functioning epidermal melanocytes. It occurs worldwide, with an incidence rate of between 0.1 and 2%[1]. Vitiligo is caused in many ways, such as genes, immune system and melanocytes[2]. In most cases, skin depigmentation of vitiligo patients was resulted from melanocyte loss and obstruction of the melanin synthesis[3]. Melanin synthesis is regulated by enzymatic cascade, such as tyrosinase (TYR), tyrosinase-related protein 1 and protein 2. TYR is viewed as the rate-limiting enzyme of melanogenesis and it can catalyze the hydroxylation of tyrosine into 3,4-Dihydroxyphenylalanine (DOPA) and oxidize DOPA into dopaquinone[4].

Traditional Chinese Medicine and their extracts are widely used to treat vitiligo. In 1930s, 8-Methoxypsoralen and 5-Methoxypsoralen were isolated from the *Psoralea corylifolia* L[5]. These compounds with chemical structure of the furan ring fused with coumarin (benzo-α-pyrone) showed strong photosensitivity and were applied to the treatment of psoriasis and vitiligo in clinical. Continuous researches proved that linear furocoumarins, such as psoralen, imperatorin, have photosensitive activity[6].

Experimental results from our laboratory displayed that imperatorin isolated from *Angelica dahurica* Benth. Et Hook. f. could increase TYR activity *in vitro*. Further, a new imperatorin derivative was synthesized for further research, whose solubility in water was much better than imperatorin's. This study will be focused on the effect of imperatorin and its derivative on the melanocyte activity. Four furocoumarin compounds with different side chain at C-8 position (Fig. 1a) were submitted to the activity assay of melanogenesis in A375 human melanoma cells and hydroquinone-induced vitiligo model animals.

MATERIALS AND METHODS

Chemicals and animal: Psoralen (FCM-I) and 8-Methoxypsoralen (FCM-II) was purchased from Shanghai Baoman biological technology Co., Ltd. (HPLC purity:≥90%). 9-[2-(Dimethylamino) ethoxy] -7H-furo[3,2-g]-chromen-7-one (FCM-IV) were synthesized in laboratory[7] (HPLC purity:≥ 98%).

Guinea pigs (250±20 g) were bought from the Laboratory Animal Center of Xi'an Jiaotong University and housed at 25±1°C and a relative humidity of 50%.

Preparation of imperatorin: The powders of *Angelica dahurica* (5.0 kg) were refluxed with 40 L of 95% ethanol for 2 h. The filtrate was concentrated to obtain a crude extract (0.7 kg). Crude extract was extracted with 1.4 L of ethyl acetate to obtain an ethylacetate-soluble fraction (365 g). This fraction was separated by silica gel column chromatography with petroleum ether-ethyl acetate from 100:0-100:7 to obtain a crude product of imperatorin (1.37 g) whose purity was above

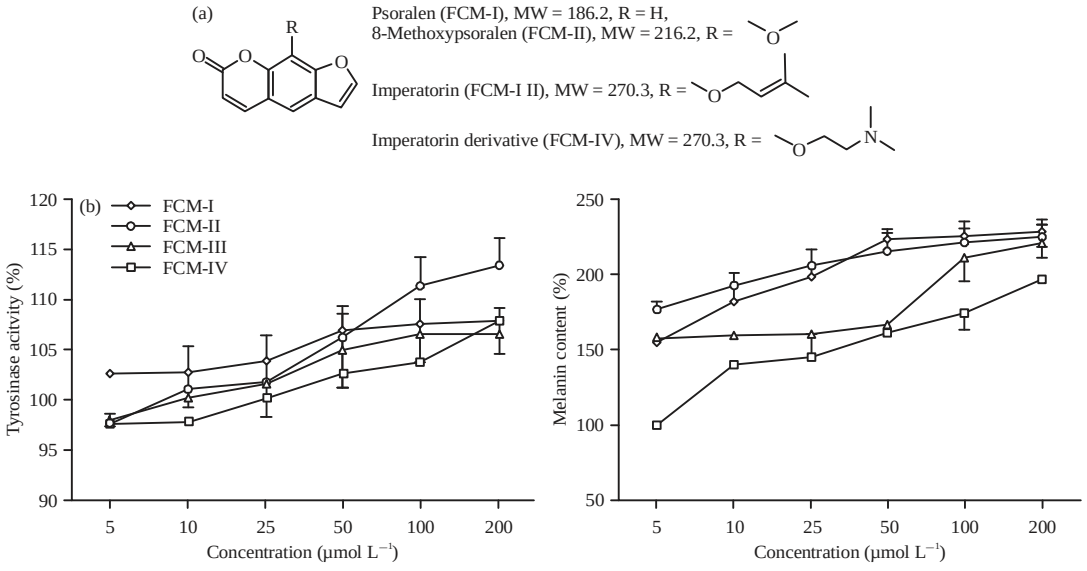

Fig. 1(a-b): (a) Chemical structures and the effects on activity of tyrosinase and (b) Content of melanin A375 cells of FCM-I, FCM-II, FCM-III and FCM-IV. (Data are presented as Mean±SD)

80%. After being recrystallized, FCM-III (1.10 g) was obtained, which the content of imperatorin was above 92%.

Tyrosinase activity and contents of melanin in cells: A375 cells were plated at a density of 1×10^5 cells in 96-well plates and incubated for 24 h. FCM-I, FCM-II, FCM-III and FCM-IV were introduced separately to cells with different concentrations (5, 10, 25, 50, 100, 200 μmol L^{-1}). Cells cultured without compounds were taken as the control. After 48 h incubation, the cells were washed with phosphate buffer solution free of Ca^{2+} and Mg^{2+}. Cells were lysed and added L-DOPA solution (10 g L^{-1}). Following 2 h incubation, the optical densities (OD) was measured at 490 nm.

$$\text{Tyrosinase activity (\%)} = \frac{\text{OD of compounds}}{\text{OD of control}}\times100$$

A375 cells were plated at a density of 2×10^5 cells in 24-well plates and incubated for determination the contents of melanin. The subsequent operation was same as the tyrosinase activity determination.

Experimental groups *in vivo*: Animals were divided into seven groups, each group with 5 males and 5 females. About 0.5 g hydroquinone was dissolved in 100 mL distilled water and 0.3 g FCM-I, FCM-II, FCM-III and FCM-IV were dissolved in 100 mL 75% ethanol-water solution. The initial 10 days, group B-G were treated with 0.5% hydroquinone. Then group B were treated with hydroquinone solution, used as model controls, the other animals were administrated with hydroquinone solution and 75% ethanol (group C) and FCM-I (group D), FCM-II (group E), FCM-III (group F), FCM-IV solution (group G). Group A was treated with distilled water alone as negative control. All the solutions (0.5 mL) were completely massaged on the 4×4 cm shaved skin twice a day for 40 days.

Epidermal depigmentation: To observe objectively the pigment change, the photos were taken on every 10 days. Guinea pigs were anesthetized with 10% chloral hydrate. The skin were excised from the control and treated areas and divided into three pieces for Lillie iron staining, conventional histopathological study and DOPA staining.

The fresh skin was embedded in paraffin, sectioned 10 μm samples. The sections were hydrated in distilled water and placed in ferrous sulfate solution for 1 h. They were stained by the potassium ferricyanide-acetic aciod solution and washed in glacial acetic acid. Van Gieson dye was used as comparison staining. The sections were dehydrated with alcohol, cleared in dimethyl benzene and mounted in neutral balsam. The sections were also stained with Haematoxylin-Eosin (H&E) according to standard procedures.

The frozen skin was cut into 10 μm sections with freezing microtome (Leica CM1900, Germany). The sections were fixed in 10% formalin-phosphate buffer solution for 20 min, washed and immersed in the DOPA-PBS at 37°C about 4 h. Then the specimens were washed and restrained with the nuclear fast red. The specimens were dehydrated in graded ethanol batches, cleared in dimethyl benzene and mounted in neutral balsam.

The extent of depigmentation was counted by using an image-pro plus soft to estimate the integral optical density (IOD) under the microscope (400X).

ELISA test: Blood samples were collected from the abdominal aorta. The enzyme activities of CHE, MAO (Guinea pig CHE Elisa Kits and MAO Elisa Kits Huamei Biotech Co., Ltd. China) and TRY (Rat TYR Elisa Kits, Elabsicience Biotechnology Co., Ltd. China) were detected quantitatively by ELISA method.

Statistical analysis: Dual compare means between the groups were made using the Student's t-test for data analyze.

RESULTS

Tyrosinase activity and contents of melanin: With the concentration of four compounds increasing, FCM-I, FCM-II, FCM-III and FCM-IV can increase the TYR activity and contents of melanin in A375 cells (Fig. 1b). The results is consistent with some studies that have indicated FCM-II promotion TYR activity, melanin synthesis and melanocyte proliferation[8].

Animal experiment: The skin of the Guinea pigs appeared some small white patches (Fig. 2b-g), though the Guinea pigs had a transient irritant effect, produced redness and desquamation on treated area with hydroquinone, on the 30th days, distant white patches or white hairs were found on the exposed skin of guinea pigs in the model control group and the vehicle control group (Fig. 2b and c). With continuous administration of the four compounds, white patches or white hairs decreased. On the 50th days, not only the white patches and white hairs died away, but also redness and desquamation disappeared in group D-G. The skin treated with distilled water alone, as negative control, was smooth and didn't appear redness, desquamation and depigmentation (Fig. 2a). Those results suggested that the redness, desquamation and depigmentation were probably due to the stimulus of hydroquinone. The Guinea pigs which received hydroquinone showed no depigmentation.

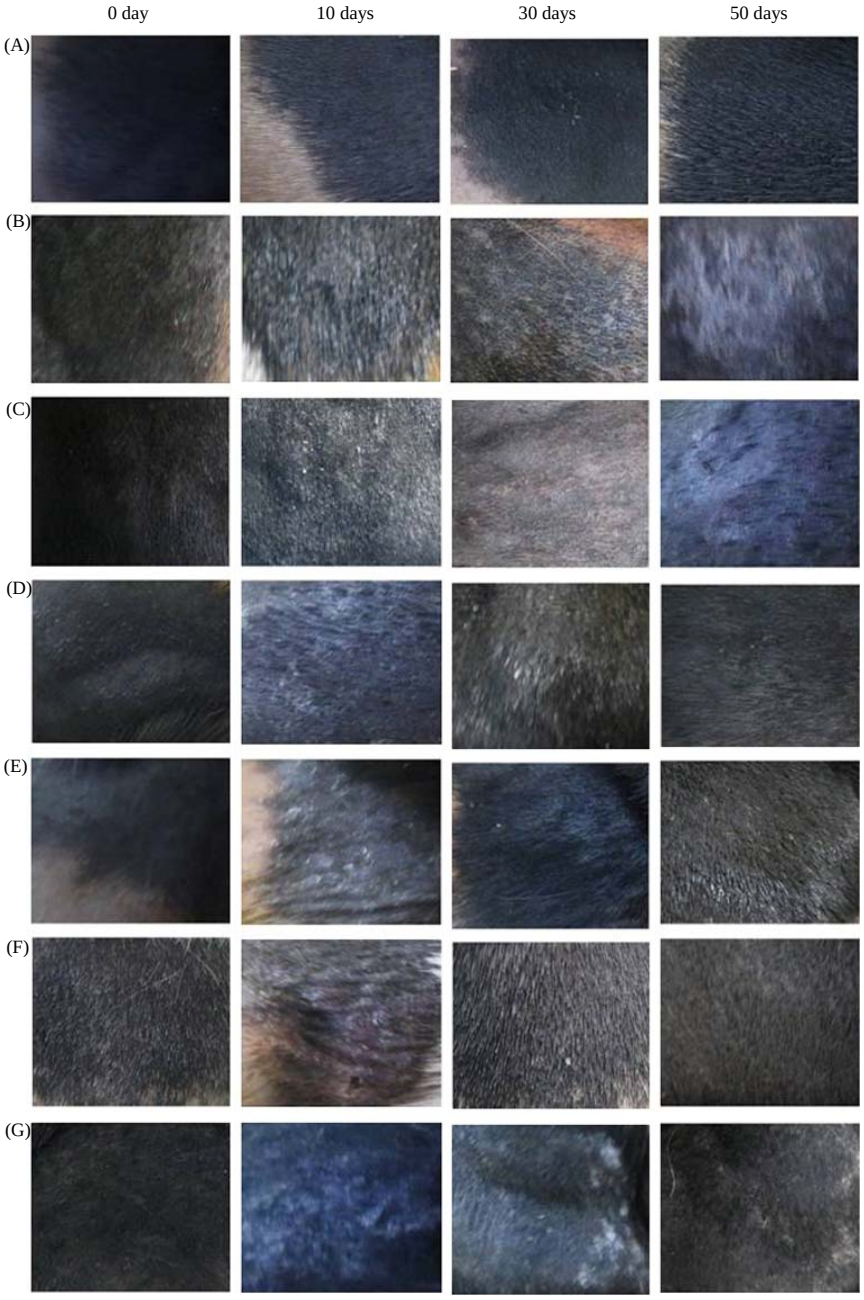

Fig. 2: Pigmentation changes of the epidermis tissue treated with hydroquinone and test compounds in the animal experiment
(A) Distilled water, (B) 0.5% hydroquinone, (C) 75% ethanol, (D) FCM-I, (E) FCM-II, (F) FCM-III, (G) FCM-IV

However, the visible pigmentation was observed in group D-G compared with model control group or vehicle control group.

The microscopic results of skin sections showed that there was no obvious change in microstructure of the skin in all experimental groups (Fig. 3 H&E staining A-G). The pigmentation responses were clearly characterized by the DOPA and the Lillie iron staining, which the number of melanocyte was corresponded with changes in the number of Lillie iron$^+$ and DOPA$^+$ cells mm^{-2} in the skin[9]. The specimens in group D E, F and G showed significantly more melanocyte number mm^{-2} than those in Group B or Group C (Fig. 3, DOPA and Lillie iron staining). Melanocyte loss appeared in model control group and vehicle control group, which the melanocyte number had a significant reduction ($p<0.01$) compare with the negative control group (Fig. 3, DOPA

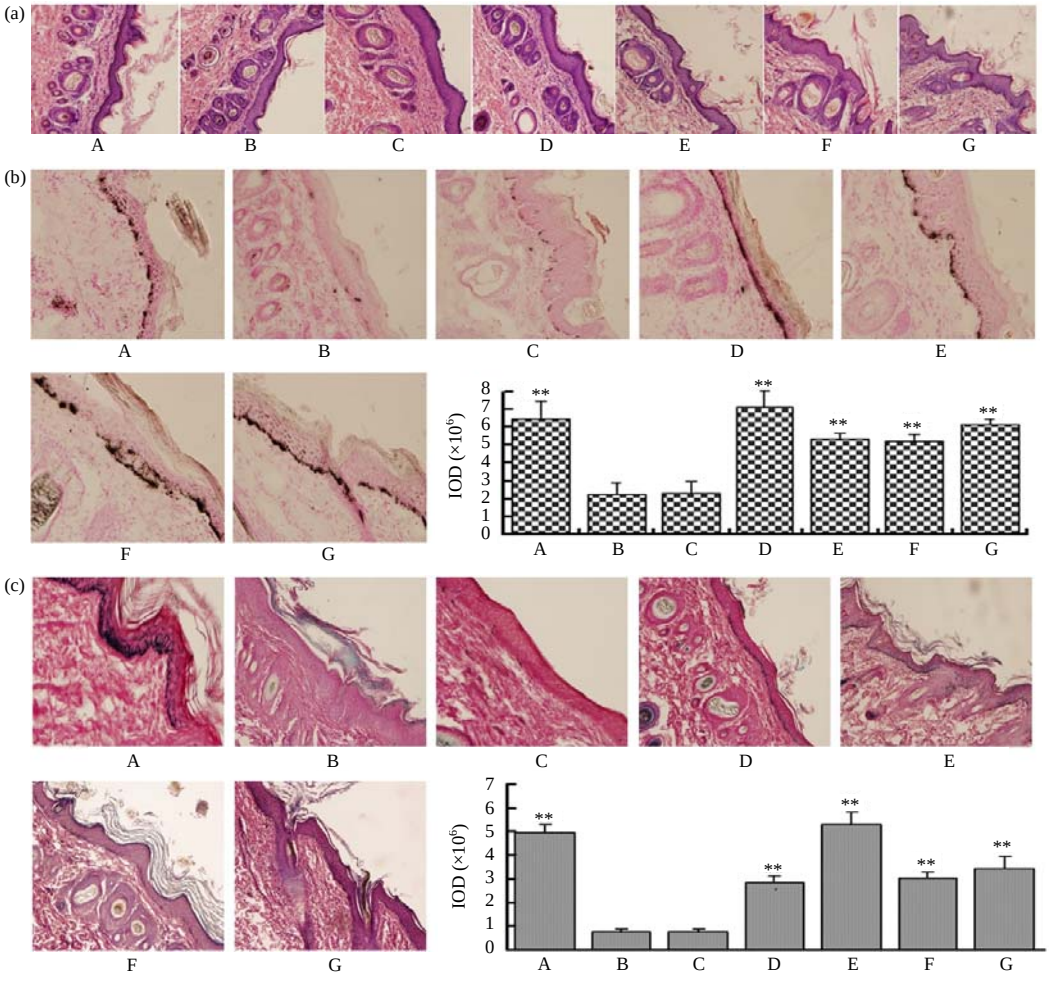

Fig. 3(a-c): Histopathology of epidermis tissue by (a) H&E staining, (b) DOPA staining and (c) Lillie iron staining
(A) Distilled water, (B) 0.5% hydroquinone, (C) 75% ethanol, (D) FCM-I, (E) FCM-II, (F) FCM-III, (G) FCM-IV. Data are presented as Mean±SD, **: p<0.01 compared with B

Table 1: Effect of FCM-I, II, III and IV on the activities of CHE, MAO and TYR

Groups	TYR (pg mL^{-1})	CHE (U mL^{-1})	MAO (U mL^{-1})
A	21.26±2.16	5.21±0.76	4.42±0.98
B	18.03±2.08^{b}	1.71±0.26^{b}	9.75±1.24^{b}
C	17.17±2.71^{b}	1.74±0.31^{b}	9.40±1.18^{b}
D	21.43±4.14^{c}	4.85±0.67^{d}	4.07±0.91^{d}
E	21.77±2.29^{d}	4.74±0.55^{d}	3.23±0.32^{d}
F	21.54±4.51^{c}	4.67±0.68^{d}	3.03±0.45^{d}
G	23.20±2.70^{d}	5.12±0.65^{d}	4.38±0.54^{d}

Data are presented as Mean±SD (n = 10), a: p<0.05 compared with A, b: p<0.01 compared with A, c: p<0.05 compared with B, d: p<0.01 compared with B, CHE: Cholinesterase, MAO: Monoamine oxidase, TYR: Tyrosinase

staining A and Lillie iron staining A). Continuous treatment with the 4 compounds significantly increased the melanocyte number compared with model control group (p<0.01). The pigmentation may be inferred from increasing melanocyte number or melanin production.

ELISA test: Compared with group A, TYR activity reduced signally in group B and C (p<0.01) (Table 1). The treatment with FCM-I, FCM-II, FCM-III or FCM-IV significantly increased the TYR activity compared with group B (p<0.01 or p<0.05). Compared with group A, CHE contents in group B or C significantly reduced (p<0.01) and those in the Group D-G had no different (p>0.05). However, the CHE contents in the group D-G significantly increased (p<0.01) than those in group B or C. As presented in the Table 1, the serum in model group and vehicle group expressed significant high level of MAO activities compared to group A and group D-G (p<0.01).

DISCUSSION

Vitiligo is a common skin disorder characterized by depigmentation of the skin due to melanocytes loss[10]. In this

study, the induced depigmentation by the hydroquinone in the Guinea pigs indicated melanocytes loss in the lesional skin, which is similar to lesions of human vitiligo. It was reported that hydroquinone react with cellular macromolecules, such as proteins and DNA and cause melanocyte death[11]. Though the Guinea pigs appeared side effects during experiment, such as redness and desquamation, the discomfort was transient and tolerable. The further study results indicated that the animals with vitiligo expressed high level of MAO activities in serum and low level of TYR and CHE activities in plasma, which was similar to the characteristics of patients with vitiligo. Melanocytes were studied by the DOPA staining method, which is a specific method for identifying melanocytes in the skin. Tyrosinase is a melanogenesis regulating enzyme and converts tyrosine into DOPA through oxidation. The animal specimen treated with FCM-I, FCM-II, FCM-III and FCM-IV showed a higher number of melanocytes evidenced through dark brown DOPA-stained cytoplasm.

Radix *Angelica dahurica* has the function of eliminating wind and dampness. In recent years, it is widely used in treating psoriasis and vitiligo. Imperatorin was isolated from *Angelica dahurica* and a new compound was synthesized as its derivative. The two compounds have the same parent nucleus with psoralen[12] and 8-Methoxypsoralen that have been applied to vitiligo treatment for many years. In addition, they have a side chain at C-8 position, which is similar structure to 8-Methoxypsoralen.

CONCLUSION

The present study has shown that imperatorin and its derivative, as psoralen and 8-Methoxypsoralen, increased cellular tyrosinase activity and melanin content and induced the production of melanocytes of vitiligo animal model. These results will help to develop the new candidate drug for treatment vitiligo.

SIGNIFICANCE STATEMENTS

Vitiligo occurs worldwide with having a significant impact on the patient's life quality. This study discovered that the furocoumarin nucleus is the key chemical structure to increase melanocyte production. The more effective compounds are expected to optimize a side chain at C-8 position of parent nucleus. The further studies are suggested to discover the mutual influence between chemical structure and melanocyte production.

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