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Original article

Influence of Four *Phlomis* Species on Melanogenesis in Human Malignant Melanoma (SKMEL-3) Cells

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Abstract

Background and objectives: Phytochemical studies have shown that the *Phlomis* species are rich in polyphenolics and iridoid glycosides and many of them have shown potential value in different biological and pharmacological activities. In this study, we evaluated the effect of *Phlomis persica*, *P. brugieri*, *P. olivieri* and *P. anisodontea* extracts on melanin production in human malignant melanoma (SKMEL-3) cells. **Methods:** The anti-tyrosinase activity of the extracts was investigated using mushroom tyrosinase assay**.** Cytotoxicity potentials were examined through MTT assay on SKMEL-3 cell line and then the level of melanin formation in SKMEL-3 cells was determined. **Results:** The anti-tyrosinase activity of the *Phlomis* extracts was not remarkable (about 0.1 mg/mL). All extracts significantly increased the melanin content in SKMEL-3 cells at 0.25 mg/mL and among them *P. anisodontea* extract seemed to be more efficient in stimulating melanin production. **Conclusion:** Based on our results, we suggest these total extracts particularly *P. anisodontea* extract contain the potent skin browning agents that can be used in pharmaceutical and cosmetic products.

Keywords: cytotoxicity; melanogenesis; *Phlomis* species; SKMEL-3 cells; tyrosinase

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Introduction

Melanin is the main pigment responsible for the numerous colorations found in human, animals, plants, fungi and in a wide variety of microorganisms. Mammalian melanins are divided in two large groups, eumelanin (the dark brown to black pigments) and pheomelanin (reddish or yellowish pigments) that varying amounts of them determine the skin, eye and hair color [1]. Melanins possess a broad range of biological activities, such as anti-microbial [2], anti-tumor, radical scavenger, anti-inflammatory [3] and immune-modulating properties [4]. Despite the fact that melanin acts as an ultraviolet absorbant and plays important roles to protect skin against UV radiation, it can promote oxidative DNA damage and induction of melanin-dependent melanoma [5]. The most common melanogenesis disorders are related to the pigment-producing, which may be reduced, absent or increased. The level of melanin formation depends on tyrosinase activity that plays a key role in the process of melanogenesis. Therefore, tyrosinase inhibitors and stimulators can be used, respectively for treatment of skin hyperpigmentation (freckles, age spots and melanoma) and hypopigmentation problems (white hair, vitiligo and albinism) or may be candidates for cosmetic products (skin-whitening or –tanning agent) [6-8].

For centuries, herbal extracts/compounds have been used for better management of overall skin conditions and diseases. Up to now, numerous

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plant extracts have been identified as a melanogenesis inhibitor such as *Glyceriza glabra*, *Morus alba* and *Marrubium* specie **,** while some plants such as *Moricandia arvensi*, *Daphne gnidium*, *Pyrostegia venusta* are well-known as melanogenesis stimulators [8,9].

Genus of *Phlomis* (Lamiaceae) is represented by about 100 species and distributed extensively in Asian, European and North African regions. Accumulating evidence demonstrated different pharmacological effects of *Phlomis* genus, most of them were due to the presence of phenolic, flavonoid as well as iridoid glycosides [10,11]. According to previous studies, some polyphenolics found in *Phlomis* genus exert apoptosis induction and inhibition of cell proliferation through their anti-melanogenic properties. In contrast to their anti-melanogenic activity, melanogenesis-promoting properties were also reported for some kinds of flavonoids including kaempferol, rhamnetin, apigenin, naringenin and luteolin [12-15].

Our previous results demonstrated the controversial effects of *Phlomis kurdica* and *P. caucasica* on cell viability and melanogenesis process in melanoma cells. Indeed, we found that *P. caucasica* significantly increased melanin content on melanoma cells, while *P. kurdica* showed inhibitory effect on melanogenesis process at similar concentrations [16,17]. These results motivated us to investigate the efficiency of four *Phlomis* species including, *Phlomis persica*, *P. brugieri*, *P. olivieri* and *P. anisodontea* on melanin production in SKMEL-3 cell line for the first time.

Material and Methods

Chemicals and reagents

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma, USA. Human malignant melanoma cells (SKMEL-3) were obtained from Pasteur Institute of Iran. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and trypsin-EDTA were obtained from GibcoBRL, USA. All other solvents and chemicals were prepared from Merck co. (Germany).

Preparation of crude extract and fractions

The aerial parts of *Phlomis persica*, *P. brugieri*, *P. olivieri* and *P. anisodontea* were collected from the west and north-west of Iran (Kordestan, Azarbayjan) in July 2011. A voucher specimen (No: 1610, 1581, 1631, 1580) was deposited at the Herbarium of Medicinal Plant Institute, Academic Center for Education, Culture and Research, ACECR Karaj, Iran. The dried powder of plants (100 g) was extracted through maceration with 80% aqueous MeOH using percolation apparatus three times during one week at room temperature. The solvent extractions were filtered and then evaporated in vacuum and concentrated by using freeze-dryer. The dried extracts were kept at 4 °C before experimental studies. All measurements were carried out in triplicates.

Mushroom tyrosinase inhibitory activity assay

Mushroom tyrosinase activity was carried out according to the procedure explained by Salimi et al.) [18]. Each plant extract was dissolved in MeOH to make the test concentrations (0.1-10 mg/mL). A mixture of 20 µL of mushroom tyrosinase solution (125 units/mL in 50 mM phosphate buffer, pH 6.8), 80 µL phosphate buffer (50 mM), and 40 µL of sample solution was incubated at room temperature. The reaction was completed after 10 min by adding 40 µL L-tyrosine (2 mM). Kojic acid was used as the positive control. The amount of produced dopachrome in the reaction was determined after incubation (15 min) at 475 nm using the ELISA reader. The percentage of inhibition of tyrosinase was calculated as follows:

tyrosinase inhibition (%) = $[(A-B)/A] \times 100$

where A = absorbance at 475 nm without test sample and B = absorbance at 475 nm with test sample. Kojic acid was used as the positive control. The concentration at which half the tyrosinase activity was inhibited (IC_{50}) was determined for each sample.

Cytotoxicity assay (MTT assay)

The human malignant melanoma (SKMEL-3) cell line was obtained from the cell bank of Pasteur Institute of Iran (NCBI No. C458). The cells were seeded in DMEM containing 10% fetal bovine serum, 100 mg/mL streptomycin, 100 U/mL penicillin G and incubated for 72 h at 37 °C and 95% humidity, under 5% carbon dioxide. Cell viability was determined using the MTT reduction method [19], Briefly, a density of 5×10^3 cells/well were plated in 96-well plates and after 24 h of incubation, the concentrations of 0.5 mg/mL of each extract was added. The cells were

incubated with MTT solution (5 mg/mL) at 37 °C for 72 h to produce formazan crystals. Finally the medium was discharged and the crystals were dissolved with DMSO and the absorbance was measured at 545 nm using ELISA reader. IC_{50} was calculated as the concentration of samples which inhibited 50% of cell viability.

Melanin content assay

Melanin content assay was conducted as previously described by Salimi et al. [16] The SKMEL-3 cells were seeded in 96-well plates at 3×10^5 cells/ well and incubated overnight to allow cells to adhere. The cells were exposed to the different concentrations (0.001, 0.005, 0.01, 0.1, 0.25 mg/mL) of the extracts for 72 h. Following treatment, the cells were washed with PBS and then dissolved by 800 µL of 1 N NaOH containing 10% DMSO for 1 h at 75 °C. The melanin content was determined using a microplate reader at 405 nm absorbance.

Statistical analyses

All results were presented as mean ± SEM. The group means were compared using the ANOVA test followed by Tukey post hoc test. $P<0.05$ was considered as statistically significant. All analysis performed using Graph Pad Prism 6.

Results and Discussion

In the present study, the mushroom tyrosinase inhibitory activity of four endemic *Phlomis* species, including *P. persica*, *P. brugieri*, *P. olivieri* and *P. anisodontea* in free cell culture was evaluated. Among samples, *P. brugieri* methanol extract showed the highest inhibition tyrosinase activity on mushroom tyrosinase with IC_{50} value 0.98 mg/mL and *P. anisodontea* extract displayed the lowest inhibition activity with IC_{50} value 1.41 mg/mL (table 1). The results were similar to our previous reports regarding *Phlomis* species [16,17]; however, the obtained findings were less effective than kojic acid (IC_{50}= 0.049) and other plants extracts [20].

The quantity of melanin content can be affected by the viable cell number; thus, the cytotoxicity of the samples was determined on SKMEL-3 cells. The ability of the treated cells to decrease MTT to a purple formazan product was measured at the highest concentration (0.5 mg/mL) that had been carried out in in our previous studies [16,17]. The results revealed that all samples at concentration of 0.5 mg/mL showed cytotoxicity around 50% toward the cells (table 1).

Table 1. Effect of total extract of *Phlomis persica*, *P. olivieri*, *P. brugieri* and *P. anisodontea* on mushroom tyrosinase activity, cytotoxicity and melanin content in human melanoma SKMEL-3 cells

***Phlomis* species**	**Mushroom tyrosinase inhbition (IC_{50} mg/mL)**	**Cytotoxicity (%) at 0.5 mg/mL**	**Melanin content (%) at 0.25 mg/mL**
P. anisodontea	1.41	47.17 ± 8.26	137.60 ± 3.81
P. persica	1.28	44.79 ± 5.93	139.50 ± 4.58
P. olivieri	1.31	52.00 ± 4.66	128.70 ± 2.74
P. brugieri	0.99	46.89 ± 8.36	131.40 ± 7.93

Values expressed as means ± SD (n = 3)

Thereafter, melanin production potency of the samples in this cell line was evaluated at non-toxic concentrations (≤0.25 mg/mL). The results showed that the extract of *P. persica*, *P. brugieri* and *P. olivieri* significantly ($p< 0.05$) stimulated melanogenesis ability in SKMEL-3 cells at higher concentrations than that of *P. anisodontea*. As shown in figure 1, the melanin production in the *P. anisodontea* extract treated cells at concentrations of 0.1 and 0.25 mg/mL increased to 123.2±4.64 and 137.6±3.81 (% of control), respectively, thus this species seemed to be more efficient in stimulating melanin production than other samples. Previous reports have shown that theses samples demonstrated cytotoxic activity against MDBK normal cell line and some human cancer cell lines (MCF-7 and A-549) at concentrations higher than 0.5 mg/mL. In the present research, we expected these plants to have cytotoxic effects on SKMEL-3 cells at concentration higher than 0.5 mg/mL [21]. In addition, we found that the total extract of *P. caucasica* [16] similar to *P. anisodontea*, significantly increased ($p<0.05$) the melanin content at concentration of 0.25 mg/mL on SKMEL-3 cells but in comparison with *P. anisodontea*, it revealed high cytotoxic activity.

Many studies have showed that several polyphenolic compounds were found in *Phlomis* species which could enhance melanogenesis, while some of them act as melanogenesis inhibitors [12-15]. For example, kaempferol, rhamnetin, apigenin and luteolin, indicated melanogenesis-promoting actions; in contrast, acetoside acted as a melanogenesis inhibitor [12,13].

According to the results, the difference of melanogensis activity of *Phlomis* species can be correlated to the type and amount of phenolics and flavonoid content and other compounds.

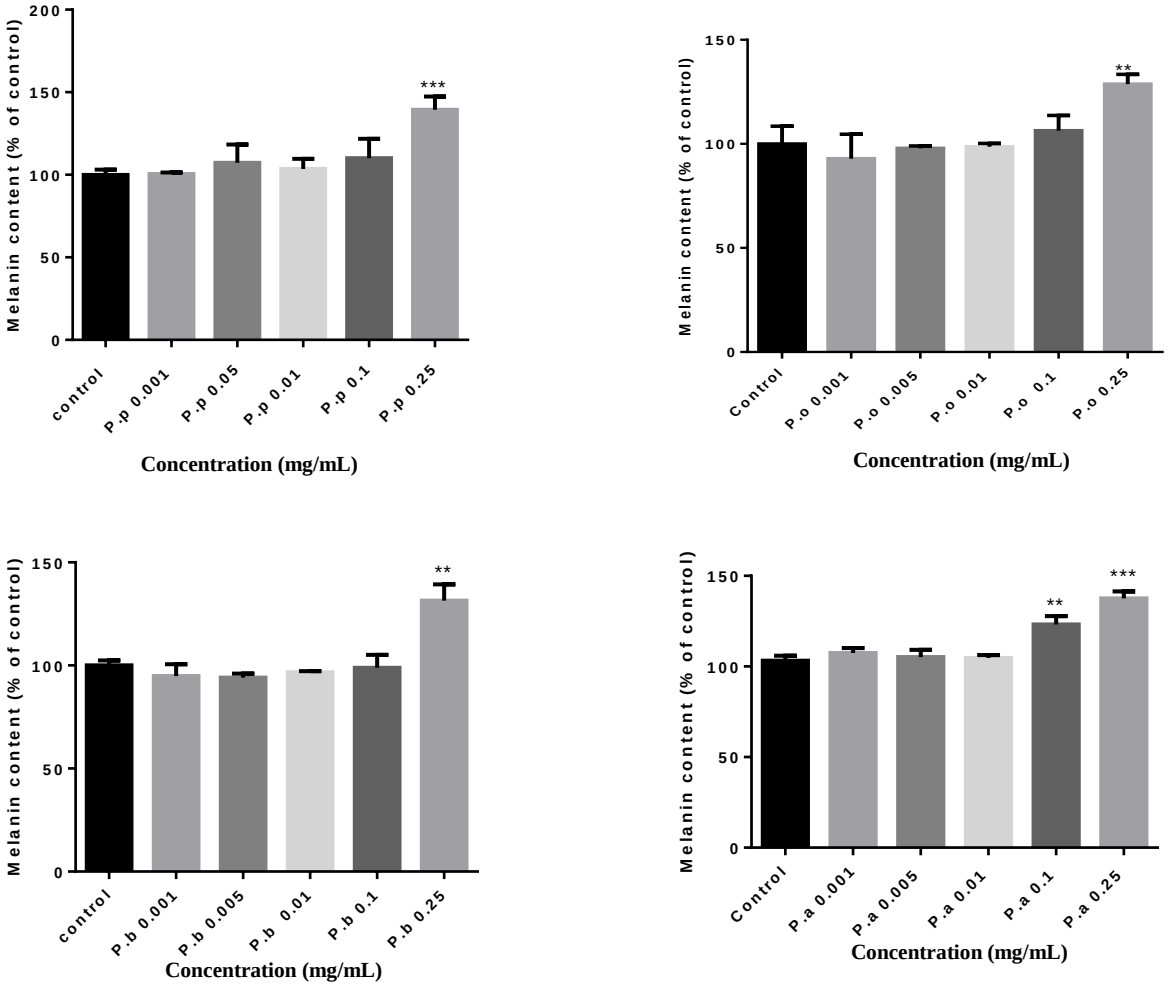

Figure 1. The effects of total extract of *Phlomis persica* (P.p), *P. olivieri* (P.o), *P. brugieri* (P.b) and *P. anisodontea* (P.a) at concentrations between 0.001 and 0.25 mg/mL on melanin production in human melanoma SKMEL-3 cells. Values have been expressed as mean ± SEM (n = 3); * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to the control

In conclusion, the results of this study displayed that the extracts of *Phlomis persica*, *P. brugieri*, *P. olivieri* and *P. anisodontea* increased melanogenesis at non-toxic concentrations. Moreover, our findings suggested that the total extract of these plants particularly *P. anisodontea* may contain the potent skin browning compound (s) that can be used in pharmaceutical and cosmetic products.

Acknowledgments

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Author contributions

Parisa Sarkhail, Mona Salimi and Soodabeh Saeidnia designed and supervised all experiments; Marjan Nikan was involved in the plants extraction and anti-tyrosinase study; Parisa Sarkhail and Mona Salimi performed melanogenesis study and analyzed the data and wrote the manuscript; Mahdieh Kurpaz-mahmoodabadi contributed in the plants collection and edited the manuscript. All authors have read and approved the final manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

SKMEL-3: human malignant melanoma skin cancer cell line; MDBK: Madin-Darby bovine kidney epithelial cells; MCF-7: human breast cancer cell line; A-549: adenocarcinomic human alveolar basal epithelial cells; MTT: 3-(4, 5-

dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; EDTA: ethylenediaminetetraacetic acid; DMSO: dimethyl sulfoxide