Original Article

Determination of Anti-melanogenic Activity of *Phlomis kurdica* in Human Melanoma SKMEL-3 Cells

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Abstract

The present study was designed to investigate the anti-melanogenic and cytotoxic activities of methanol extract of *Phlomis kurdica*. The antioxidant and anti-tyrosinase activity of MeOH extract from *P. kurdica* (MPk) were examined by DPPH radical scavenging and mushroom tyrosinase activity assays (*in vitro*), respectively. Furthermore, the effect of MPk on the melanin content, cellular tyrosinase activity and cytotoxicity was studied on human melanoma SKMEL-3 cells (*in vivo*). The results showed that the MPk inhibited DPPH radicals and mushroom tyrosinase activity in a dose dependent-manner, but these effects were weaker than positive controls. The extract revealed cytotoxic effect in SKMEL-3 cells at high concentrations (> 0.2 mg/mL). Moreover, at concentration of 0.25 mg/mL, it reduced melanin content and cellular tyrosinase activity about 7% and 28% of control, respectively. These findings suggest that the MPk can be considered as a cytotoxic extract in melanoma skin cancers and exhibited inhibitory effect on melanogenesis process.

Keywords: antioxidant, cytotoxicity, melanin, Phlomis kurdica, SKMEL-3 cells, tyrosinase.

1. Introduction

Melanin, a pigment produced by cells called melanocytes, is responsible for coloring mammalian hair and skin. The tyrosinase, a multifunctional copper-containing enzyme oxidase, is the key enzyme involved in the biosynthesis of melanin through enzymatic reactions [1]. Tyrosinase overactivity leads to

hyperpigmentation that causes some dermatological disorders, such as freckles, age spots or melasma and melanoma cancers [2]. Melanoma, a type of skin cancer develops from melanocytes (pigment cells), occurs almost on parts of the body that have been overexposed to ultraviolet (UV) radiation. Some synthetic depigmenting agents in drug formulations have exhibited poor skin penetrations and side effects including allergic contact dermatitis, cytotoxicity, and mutagenicity [3]. Additionally, the most melanomas are resistant to conventional chemotherapy and there is no effective treatment for metastatic malignant melanomas [4]. For these reasons, finding new potential anti-melanogenic agents are definitely required.

The genus *Phlomis* (*Lamiaceae*), comprising nearly 100 species is widely distributed in Asia and Europe. About 17 species are found in Iran, including *P. kurdica* which grows wildly in the west of Iran [5]. Some *Phlomis* species have been used in folk medicine for treatment of different conditions such as stimulants, tonics, diuretics and for the treatment of ulcers and haemorrhids. Several biological activities such as antioxidant, antimicrobial, anti-inflammatory, immunosuppressive, anti-mutagenic, anti-nociceptive, antifibriel, anti-allergic, and anti-diabetic effects have been reported from *Phlomis* plants. The genus *Phlomis* have been found to be rich in essential oils, phenolics, flavonoids, phenylpropanoids, and iridoid glycosides [5, 6]. Bader *et al*. [7] reported the presence of 14 phenolic compounds including flavonoids, phenylpropanoids, and phenolic acid glycosides in the aerial parts of *P. kurdica*. Some of these compounds such as luteolin 7-O-β-d-glucopyranoside, jaceoside, and luteolin 7-O-sophoroside have shown a capable inhibitory activity on human lactate dehydrogenase that is known as an essential enzyme for survival and progression of cancer cells [7]. Previous pharmacological studies have reported the presence of many phenolic compounds showing multiple biological effects such as antioxidant, anti-tyrosinase, melanogenic or anti-melanogenic activity as well as cytotoxicity [8-10].

Considering these data, we investigated the effects of MeOH extract from *Phlomis kurdica* (MPk) on the scavenging of DPPH radicals and inhibition of mushroom tyrosinase activity. Secondly, the depigmenting activity and cytotoxicity of MPk were evaluated on human melanoma cell line (SKMEL-3 cells) to find a new natural source for treatment of various hyperpigmentation disorders.

2. Materials and Methods

2.1. Chemicals and Reagents

Mushroom tyrosinase, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), kojic acid, quercetin, Dimethyl sulfoxide (DMSO), potassium dihydrogen phosphate, di-potassium hydrogen phosphate and the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma, USA. Dulbecco's

modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and trypsin-EDTA were obtained from GibcoBRL, USA.

2.2. Plant Material and Extract Preparation

The aerial parts of *Phlomis kurdica* Rech.f. were collected from the west of Iran (Sanandaj to Marivan) and identified by Mr. Yousef Ajani. A voucher specimen (No. 1582 ACECR) was deposited at Herbarium of Medicinal Plant Institute, Academic Center for Education, Culture and Research, Karaj, Iran. The dried powder of *P. kurdica* (100 grams) was extracted with 80% MeOH using Percolation apparatus for 7 days at room temperature. After filtration, the solvent was evaporated in vacuum and concentrated with freeze dryer. The dried extract (yield 4.63%) was stored at 4°C for experimental studies. All measurements were performed in triplicate.

2.3. Determination of DPPH Scavenging Effect

The stable radical DPPH was used to measure the free radical scavenging activity by the method of Blois [11] with some modifications. Methanol DPPH solution (150 μM) was prepared and kept in the dark at 4°C. 160 μL of different extract concentrations (0.1-10 mg/mL) were mixed with 40 μL of DPPH solution. After shaking the plate, the mixture was incubated at 25 °C for 30 min in a dark place and then the UV absorbance was recorded at 517 nm using the ELISA reader (Synergy HT, BIO-TEK, USA). All tests were run in triplicate. Percentage inhibition was calculated using the following formula: DPPH radical scavenging activity (%) = $[(A-B)/A] \times 100$ where A = absorbance at 517 nm without test sample and B = absorbance at 517 nm with test sample. Quercetin was used as the positive control. A curve was plotted to determine the SC_{50} values. SC_{50} is defined as the concentration of sample sufficient to obtain 50% of a maximum scavenging capacity.

2.4. Determination of Mushroom Tyrosinase Inhibitory Activity

Tyrosinase inhibitory activity was carried out according to the procedure as explained by Sarkhail *et al*. [12]. The extract was dissolved in dimethyl sulfoxide (DMSO) to make the test concentrations (0.1-10 mg/mL). A mixture of 80 μL of phosphate buffer (50 mM), 20 μL of mushroom tyrosinase solution (125 units/mL in 50 mM phosphate buffer, pH 6.8) and 40 μL of sample solution was pre-incubated at room temperature for 10 min in triplicate. Then, a reaction was performed by adding 40 μL L-tyrosine (2 mM) for 15 min at room temperature. The amount of dopachrome in the reaction was measured 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 of the original tyrosinase activity is inhibited (IC_{50}) was determined for each sample.

2.5. Cell Culture and MTT Assay

Human malignant melanoma cells (SKMEL-3) were obtained from cell bank of Pasteur Institute of Iran (NCBI). Cells were cultured in DMEM containing 10% fetal bovine serum, 100 U/mL penicillin G, 100 mg/mL streptomycin and maintained at 37°C, 95% humidity and 5% carbon dioxide. For anti-proliferative studies, SKMEL-3 cells were plated at a density of 5×10^3 cells/well in 96-well plates. After 24 h of incubation, extracts (0.001 to 0.5 mg/mL) were added. Cytotoxicity was measured using the 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) reduction method [13]. After drug treatments, cells were incubated with MTT solution (5 mg/mL) for 4 h at 37°C. The formazan crystals were dissolved in DMSO and the color violet was measured at 545 nm using microplate reader. Solvent control (DMSO) was included to check that the DMSO had no effect at the concentration used.

2.6. Melanin Content Assay

Melanin content was measured as described previously by Chan *et al*. [14] with slight modifications. The human melanoma cells were seeded at 3×10^5 cells/ well in 6-well culture plates and incubated overnight to allow cells to adhere. The cells were exposed to concentrations (0.001, 0.005, 0.01, 0.1, 0.25 mg/mL) of the extracts for 72 h. At the end of the treatment, the cells were washed with PBS and lysed with 800 μL of 1 N NaOH (Merck, Germany) containing 10% DMSO for 1h at 75 °C. The melanin content was determined using a microplate reader at 405 nm absorbance.

2.7. Cellular Tyrosinase Activity Assay

For cellular tyrosinase activity assay [14], SKMEL-3 cell (2×10^5 cells/well) were seeded overnight in 6 well plates in a humidified CO_2 incubator at 37°C and 5% CO_2 to allow cells to adhere. Then, cells were treated with increasing concentrations of extract (0.001-0.25 mg/ml) for 72 h. Afterwards, the cells were washed with PBS (pH 6.8) and lysed with mammalian protein extraction (M-PER) reagent. The lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4°C. The protein concentration was determined by Bradford using bovine serum albumin as the standard. The reaction mixture consisting of 40 μg protein (adjusted to 100 μl with 0.1 M PBS, pH 6.8) and 100 μl of 5 mM L-Dopa was added to each well of 96-well plate. After incubation at 37°C for 1 h, the absorbance was measured at 475 nm using a microplate reader. Tyrosinase activity was calculated by the following formula: tyrosinase activity (%) = [OD_{475nm} of sample/OD_{475nm} of control] × 100.

2.8. Statistical Analyses

The results were reported as mean ± SEM. Data subjected to independent t tests and the group means were compared using ANOVA

followed by Tukey post hoc test. $P < 0.05$ was considered as statistically significant. All analysis performed using Graph Pad Prism 6.

3. Results and Discussion

Free radicals can induce biological damage and aggravate the progression of cells to a pathological condition such as inflammation, aging, carcinogenesis, neurodegenerative diseases, diabetes and AIDS. Several studies confirmed that inactivation of antioxidant enzymes, and stimulation of melanogenesis in skin produced through oxidative stress. Under normal physiological condition, melanosomal melanin acts as a natural UV photoprotective filter. But after long UV exposure, melanin can be involved in ROS generation. Thus, melanogenesis inhibition through increasing antioxidant activity as well as decreasing tyrosinase activity can improve the treatment of

Table 1. Antioxidant and anti-tyrosinase activities of *P. kurdica* extract.

Sample extract	DPPH assay (SC_{50} mg/mL)[b]	Mushroom tyrosinase assay (IC_{50} mg/mL)[c]
MPk[a]	5.25	1.56
Quercetin	0.06	-
Kojic acid	-	0.49

[a] MPk: MeOH extract of *Phlomis kurdica*; [b] SC_{50} is the mean 50% free radical scavenging activity, [c] IC_{50} is the mean 50% tyrosinase inhibition.
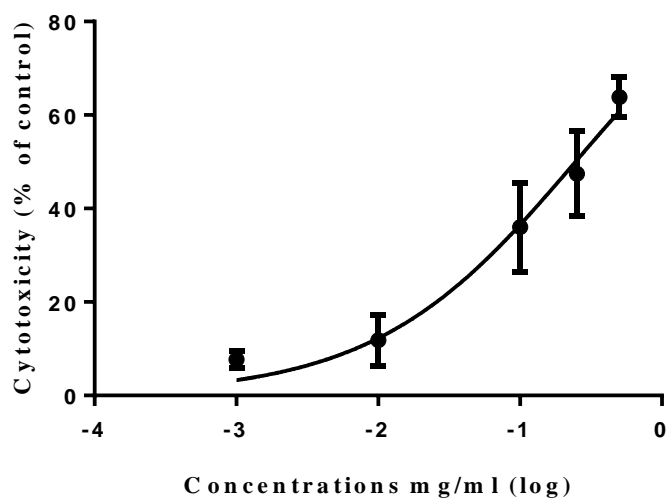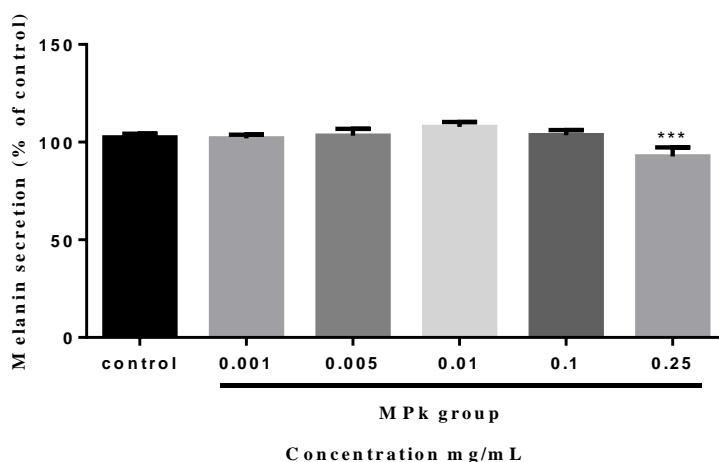

Figure 1. Cytotoxic effects of various concentrations of MeOH extract from *P. kurdica* (MPk) in SKMEL-3 cell were determined by MTT assay after 72 hours incubation. Results were expressed as percentages relative to the control, and are presented as mean ± S.E.M of at least three independent experiments.

hyperpigmentation disorders [10, 15].

In this study, the antioxidant activity of MeOH extract from *P. kurdica* (MPk) was examined by DPPH radical scavenging assay. In addition, evaluation of the anti-tyrosinase activity of MPk was determined by mushroom tyrosinase inhibition assay and compared with kojic acid, as a well-known tyrosinase inhibitor. The results showed that the MPk inhibited DPPH radicals (SC_{50} = 5.25 mg/mL) and the activity of mushroom tyrosinase (IC_{50} = 1.56 mg/mL) in a concentration-dependent manner; however, these effects were weaker than positive controls, quercetin and kojic acid, respectively (Table 1). The lower antioxidant activity of MPk may be due to prooxidants and the presence of some compounds with no or minor radical scavenging effect [16].

In many studies, B16-F10 murine melanoma cell line has been used as a model to evaluate the effects of extracts/compounds on melanogenesis process. Although B16-F10 cell is similar to normal human melanocyte in many melanogenic pathways, it is different from human melanoma cell lines in various cellular behaviors [17]. In this study, we evaluated the cytotoxic and anti-melanogenic activity of MPk in human melanoma SKMEL-3 cells, as a malignant melanotic melanoma with low melanin content in cell culture, not tumor cell [5]. Previous studies indicated that the maximum cellular tyrosinase inhibitory and cytotoxic effects of kojic acid were found after 72 hours incubation time [18]. Therefore, we considered possible cytotoxic effects of MPk on their survival and proliferation status of SKMEL-3 cells after 72 h.
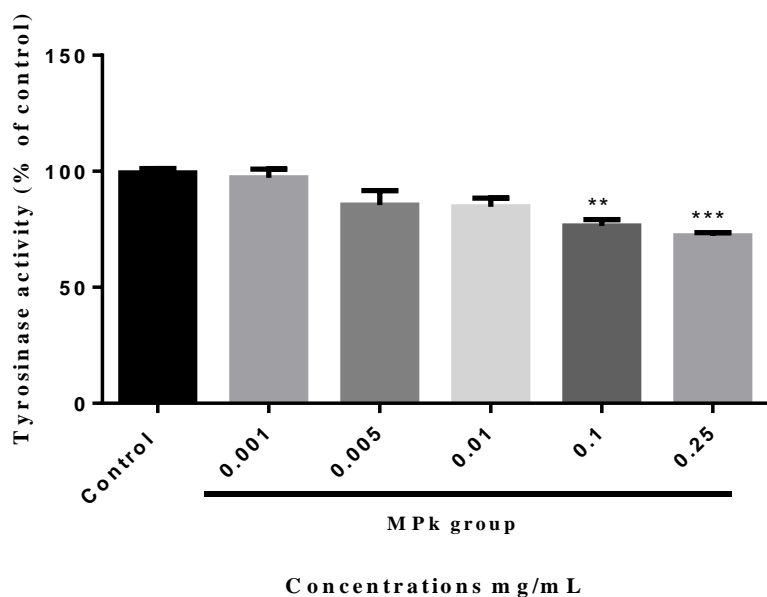

Figure 2. Effect of various concentrations of MeOH extracts from *P. kurdica* (MPk) on melanin production in SKMEL-3 cells after 72 hours incubation. Results were expressed as percentages relative to the control, and are presented as mean ± S.E.M of at least three independent experiments. *** *P*<0.001 compared with the control.

The results of MTT assay revealed that MPk had significant cytotoxic activity (~63%) at 0.5 mg/mL after 72 hours of incubation (Figure 1) with IC_{50} value of 0.23 mg/mL (95% confidence limit, 0.1786 - 0.3389 mg/mL).

As shown in Figure 2, no reduction of melanin content was observed after 72h treatment at lower concentration of extract (0.1 mg/mL). However, it decreased melanin production up to 7% at concentration of 0.25 mg/mL. Furthermore, the treated SKMEL-3 cell with various concentrations of MPk showed (Figure 3) a significant ($P<0.05$) decrease in tyrosinase activity at two concentrations, 0.1 and

0.25 mg/mL (76.39 and 72.22 % of control, respectively). Our results are in agreement with previous studies suggesting the presence of some active compounds in MPk involve in reduction of melanin content. It has been reported that phenylpropanoid acteoside inhibited tyrosinase activity and melanin synthesis in both *in vitro* and *in vivo* assays. It reduced melanogenesis in cultured B16-F10 cells through activating ERK signaling, which degrades MITF, tyrosinase and TRP-1 production [19]. Although the tyrosinase inhibitory effect of the extract can be one of the main mechanisms in reduction of melanin

Figure 3. Effect of various concentrations MeOH extract from *P. kurdica* (MPk) on cellular tyrosinsae activity on SKMELl-3 cell. Results were expressed as percentages relative to the control, and are presented as mean ± S.E.M of at least three independent experiments. ** $P<0.01$ and *** $P<0.001$ compared with the control.

content in melanoma cells, the level of melanin can be certainly influenced by the cytotoxicity of the extract on the cells. Based on the recent study, phenolic compounds can play an important role in cytotoxic activity of MPk on SKMEL-3 cells. For example, some isolated phenolics from *P. kurdica* displayed the inhibitory activity on human lactate dehydrogenase. This enzyme is known as an essential enzyme for survival and progression of cancer cells [8]. The cytotoxicity of MPk in SKMEL-3 cells can be related to its inhibitory activity on human lactate dehydrogenase. However, the different mechanisms may be involved in anti-cancer effect of MPk.

Skin cancer, including melanoma and non-melanoma forms, is a growing health problem in all countries and usually remains resistant to conventional chemotherapy. Thus, it is important to find novel effective chemo-preventive agents that can inhibit and control hyperpigmentation skin disorders, especially skin cancers. According to the results described above, MPk can be considered as a cytotoxic extract in melanoma skin cancers and may exhibit inhibitory effect on melanogenesis process. However, this hypothesis needs to be confirmed in further studies.

Acknowledgments

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