

Evaluation of Anti-Melanogenic and Cytotoxic Activities of *Phlomis caucasica* on Human Melanoma SKMEL-3 Cells

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Abstract

Background: Under normal physiological condition, melanosomal melanin acts as a natural UV photoprotective filter. However, after long exposure to UV radiation, melanin has shown to involve in reactive oxygen species (ROS) generation and increases the risk of developing ageing, melasma and melanoma skin cancers. A number of plant polyphenols influenced the melanin biosynthesis and growth of various melanoma cells through their anti-tyrosinase activity as well as antioxidant effect.

Objective: In this study, we evaluated the anti-melanogenic and cytotoxic effects of methanol extract of *Phlomis caucasica* (*Lamiaceae*) on human melanoma SKMEL-3 cells.

Methods: The level of antioxidant and anti-tyrosinase capacity of MeOH extract from *P. caucasica* (MPC) were evaluated in vitro by DPPH radical scavenging and mushroom tyrosinase activity assays, respectively. Moreover, the effects of MPC on the melanin content, cellular tyrosinase activity and cytotoxicity (using MTT assay) on human melanoma SKMEL-3 cells were determined 72 hours after the treatment.

Results: The results showed that MPC weakly inhibited DPPH radicals and mushroom tyrosinase activity with $SC_{50} = 1.037$ mg/mL and $IC_{50} = 1.316$ mg/mL, respectively. The cytotoxicity assay showed that its IC_{50} value was approximately 0.134 mg/mL (0.09791 to 0.1834) on SKMEL-3 cells. The extract had no effect on the melanin content at concentrations of ≤ 0.1 mg/mL. Additionally, no effect on cellular tyrosinase activity was observed at concentrations ranging from 0.001 to 0.25 mg/mL of MPC.

Conclusions: The results obtained suggest that the *P. caucasica* extract can reveal cytotoxic effect on human melanoma SKMEL-3 cells at higher concentrations of 0.1 mg/mL. Therefore, it can be considered as a good candidate for anti-melanoma therapy.

Keywords: Melanin, Tyrosinase, SKMEL-3 Cells, Melanogenesis, Cytotoxicity, *Phlomis caucasica*

1. Background

Melanin, a pigment produced by melanocytes, is responsible for coloring mammalian hair and skin. Melanin formation (melanogenesis) is a very complex process and is regulated by enzymatic and non-enzymatic pathways. It begins with tyrosinase mediated oxidation of L-tyrosine to L-DOPA. This is followed by tightly controlled series of oxidoreduction reactions that involve production of some toxic and mutagenic compounds such as quinones, semiquinones and reactive oxygen species (ROS). Afterwards, tyrosinase over activity increases some toxic and mutagenic intermediates of melanogenesis as well as hyperpigmentation. Moreover, it can affect the melanoma cells' behavior through autophagy effect on tumor cells (1-3). The melanin can protect malignant melanocytes as well as normal melanocytes from numerous physical and chemical damages. On the other hand, over-exposure to UV radiation increases the tyrosinase activity via generation of reactive oxygen species (ROS). ROS can cause exten-

sive cellular damage and decrease enzymatic antioxidant defense. Based on these observations, melanogenesis is involved in several human skin disorders, including pigmentation disorders and melanoma (3-5). Therefore, suppressing melanogenesis through inhibition tyrosinase activity together with increasing antioxidant status can be an effective strategy to achieve both anti-melanogenic and anti-melanoma properties.

Numerous studies have shown that some plant polyphenols influence the melanin biosynthesis and growth of various melanoma cells through their anti-tyrosinase activity as well as antioxidant effect (4, 5). The genus *Phlomis* (*Lamiaceae*) is rich with polyphenols (flavonoids and phenylethanoids/phenylpropanoids), iridoids and essential oils. A number of *Phlomis* species revealed various pharmacological activities such as antioxidant, antimicrobial, immunosuppressive, anti-mutagenic, anti-cancer, anti-diabetic and anti-inflammatory effects (6, 7). Previous studies have described the cytotoxicity

and anti-tumor activity of several *Phlomis* species such as *P. lanceolata* and *P. russeliana* (8, 9). Recently, Salimi et al. confirmed that the cytotoxicity and anti-melanogenesis activity of *P. kurdica* extract on SKMEL-3 cell line (10).

Phlomis caucasica, an endemic species from Iran, has been used in the treatment of throat infections in traditional Iranian medicine (TIM). According to a previous study (11), some flavonoids and phenylethanoid/phenylpropanoid glycosides have been identified from methanol fraction of the aerial parts of *P. caucasica*. Of these compounds, only forsythoside B and acteoside have shown excellent antioxidant activity via DPPH free radicals scavenging assay. On the other hand, two isolated flavonoids, naringenin and kaempferol 3-O-glucoside, have shown very low antioxidant and anti-tyrosinase activity (12). Moreover, some iridoid glycosides were found in this species that have been shown to have opposing effects on melanogenesis, for example geniposide enhanced melanogenesis, while loganin has indicated anti-melanogenic activity (13, 14). Therefore, due to the presence of various compounds in *P. caucasica*, with opposing and complementary effects on melanin production, it can be involved in melanogenesis pathways.

2. Objectives

The present study was designed to investigate the anti-melanogenic and cytotoxic activities of MeOH extract from *P. caucasica* (MPc). We evaluated the level of antioxidant and anti-tyrosinase capacity of MPc by DPPH radical scavenging and mushroom tyrosinase activity assays, respectively. In addition, the effects of MPc on melanin content, cellular tyrosinase activity and cytotoxicity were determined in the human melanoma SKMEL-3 cells.

3. Methods

3.1. Chemicals

Folin-Ciocalteu reagent was obtained from Fluka (Buchs, Switzerland). Tyrosinase from mushroom, butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), kojic acid, quercetin, dimethyl sulfoxide (DMSO), sodium carbonate, potassium dihydrogen phosphate, di-potassium hydrogen phosphate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma, the US. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and trypsin-EDTA were obtained from GibcoBRL, the US. Human melanoma cells (SKMEL-3) were purchased from Pasteur Institute in Iran. All other chemicals and solvents used in experiments were analytical grade and obtained from Merck (Germany).

3.2. Plant Material and Preparation of the Extract

The aerial parts of *Phlomis caucasica* Rech.f. were collected from the Ahar in East Azerbaijan, Iran and were identified by Mr. Yousef Ajani. A voucher specimen (No. 1648 ACECR) was deposited at Herbarium of Medicinal Plant Institute, Academic Center for Education, Culture and Research, Karaj, Iran. The dried powder of *P. caucasica* (100 grams) was extracted with 80% MeOH using percolation apparatus. After filtration, the solvent evaporated in vacuum and concentrated with freeze-dryer. The dried extract (yield 4.63%) was stored at 4°C for experimental studies.

3.3. Determination of Total Phenolic Content

Total phenolic content of the extract was determined according to the Folin-Ciocalteu method (15) with some modifications. Folin-Ciocalteu reagent (100 μ L) was added to the sample and after 5 minutes, the mixture was treated with 0.75 μ L of sodium carbonate 6% (w/v) and was left for 30 minutes. The absorbance was measured at 750 nm using an ELISA reader (Synergy HT, BIO-TEK, USA). The amount of total phenolic content of the sample was expressed as milligrams of gallic acid (GA) equivalents per gram of the dry extract.

3.4. Determination of Total Flavonoid Content

Total flavonoids content of the extract was estimated as the aluminum chloride colorimetric method by 96-well plate (16). Twenty μ L of the extract was mixed with water (80 μ L) followed by the addition of NaNO₂ (15%, 6 μ L). After shaking, 6 μ L of 10% aluminum chloride (AlCl₃), 80 μ L of NaOH (4%) and 80 μ L distilled water were added. After 15 minutes, the absorbance of mixtures was measured at 510 nm with the ELISA reader (Synergy HT, BIO-TEK, USA). Total flavonoid contents were calculated as quercetin (Q) equivalents per gram of the extract.

3.5. Determination of DPPH Radicals Scavenging Effect

The technique using 96-well microplate was applied to determine the DPPH free radicals scavenging activity of the extract (17). Methanolic DPPH solution (150 μ M) was prepared and the solution was kept in the dark at 4°C. Different concentrations (160 μ L) of the extract (0.001 - 10 mg/mL) were mixed with 40 μ L of DPPH solution. The plate was shaken and then incubated at 25°C for 30 minutes in a dark place. The UV absorbance was recorded at 517 nm using an ELISA reader. Inhibition percentage 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. BHT was used as a positive control. A dose response curve was plotted to determine the SC₅₀ value. SC₅₀

is defined as the concentration of sample adequate to obtain 50% of a maximum scavenging capacity.

3.6. Determination of Mushroom Tyrosinase Inhibitory Activity

Tyrosinase inhibitory activity was carried out according to the procedure as explained by Sarkhail et al. (14, 16). The extract was dissolved in dimethyl sulfoxide (DMSO) to make the test concentrations (0.001-10 mg/mL). A mixture of 80 μ L phosphate buffer (50 mM), 20 μ L of mushroom tyrosinase solution (125 units/mL in 50 mM phosphate buffer, pH 6.8) and 40 μ L of sample solution was pre-incubated at room temperature for 10 minutes. Finally, a reaction was performed by adding 40 μ L L-tyrosine (2 mM) for 15 minutes at room temperature. The amount of dopachrome in the reaction was quantified at 475 nm using an 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 a positive control. The concentration at which half the original tyrosinase activity is inhibited (IC_{50}) was determined for each sample.

3.7. Cell Culture and Treatment

Human malignant melanoma cells (SKMEL-3) were obtained from cell bank of Pasteur Institute, 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, various doses of the extract (0.001 to 0.5 mg/mL) were added.

3.8. Cytotoxicity Assay

Cytotoxicity was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) reduction method (18). After drug treatments, cells were incubated with MTT solution (5 mg/mL) for 4 hours 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 if the DMSO had any effect on the concentration used.

3.9. Melanin Content Assay

Melanin content was measured as described previously by Chan et al. (4) with slight modifications. The human melanoma cells were seeded with 3×10^5 cells/well in 3 mL of medium in 6-well culture plates and were incubated overnight to allow cells to adhere. The cells were exposed to various concentrations of the extracts

for 72 hours. At the end of the treatment, the cells were washed with PBS and lysed with 800 μ L of 1 N NaOH (Merck, Germany) containing 10% DMSO for 1 hours at 75°C. The melanin content was determined using a microplate reader at 405 nm absorbance.

3.10. Cellular Tyrosinase Activity Assay

For cellular tyrosinase activity assays (4), SKMEL-3 cell (2×10^5 cells/well in 3 mL of media in 6 well plates) were incubated overnight in a humidified CO₂ incubator at 37°C and 5% CO₂ to allow cells to adhere. The cells were treated with increasing concentration of test extract for 72 hours. Finally, the cells were washed with PBS (pH 6.8) and lysed with mammalian protein extraction (M-PER) reagent. The lysates were then clarified by centrifugation at 13,000 rpm for 15 minutes at 4°C. The protein concentration was evaluated by the Bradford using bovine serum albumin as the standard. The reaction mixture containing 40 μ g protein (adjusted to 100 μ L with 0.1 M PBS, pH 6.8) and 100 μ L of 5 mM L-Dopa was added to each well of a 96-well plate. After incubation at 37°C for 1 hour, the absorbance was determined at 475 nm with a microplate reader. Tyrosinase activity in the protein was calculated by the following formula: tyrosinase activity (%) = $[OD_{475nm}$ of sample/ OD_{475nm} of control] $\times 100$.

3.11. Statistical Analyses

The results were reported as mean \pm SEM and all experiments were performed in triplicate. Data subjected to independent t tests and the group means were compared using the ANOVA followed by Tukey post hoc test. $P < 0.05$ was considered as statistically significant. All analysis performed using Graph Pad Prism 6.

4. Results

4.1. Total Phenolic and Flavonoid Contents of MPC

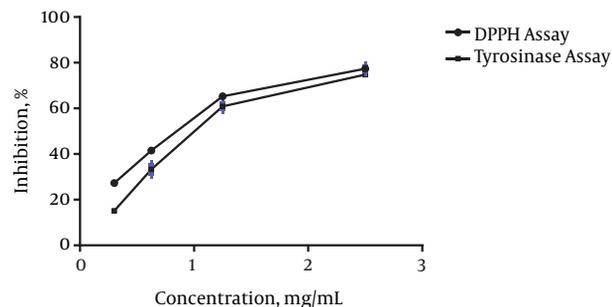
The content of total phenols of *P. caucasica* methanol extract was measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent (standard curve equation: $y = 0.287x + 0.375$, $R^2 = 0.999$). This value was 75.84 ± 0.33 mg GA/g of dry extract. The flavonoid content of the methanol extract was measured in terms of quercetin equivalent (the standard curve equation: $y = 0.124x + 0.129$, $R^2 = 0.998$) and was 28.11 ± 0.86 mg Q/g extract.

4.2. DPPH Radical Scavenging and Mushroom Tyrosinase Activity of MPC

P. caucasica methanol extract exhibited scavenging activity against the DPPH radicals in a concentration-dependent manner with SC_{50} value of 1.037 mg/mL (Figure

1). BHT was used as a positive control in this test and its SC_{50} value (0.71 mg/mL) was lower than the extract. As shown in Figure 1, the inhibitory effect of *P. caucasica* MeOH extract on mushroom tyrosinase activity was increased with increasing sample concentrations. The IC_{50} of the extract was 1.316 mg/mL while the IC_{50} value of kojic acid (0.05 mg/mL) was far lower than that of the extract.

Figure 1. DPPH Radical Scavenging and Anti-Tyrosinase Activity of MeOH Extract from *P. caucasica* (MPc)



Data are expressed as mean \pm SEM (n = 3).

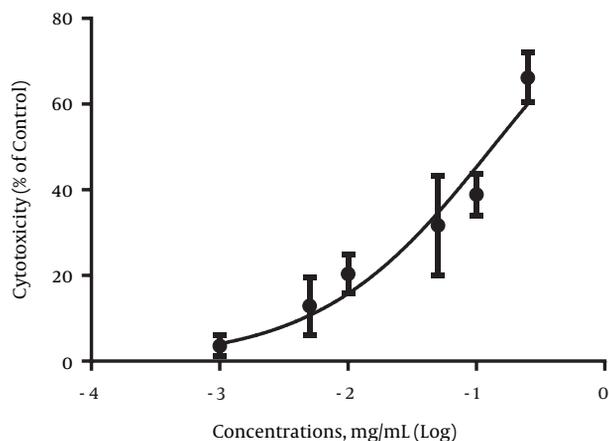
4.3. Cytotoxicity, Melanin Content and Tyrosinase Activity of MPc on SKMEL-3 Cells

In this study, we used human melanoma cell (SKMEL-3) as the melanotic cell model in assaying the inhibitory effect of MPc on melanogenesis and cell proliferation. The cell viability in the presence of MPc was estimated by MTT assay. The data obtained indicated that the cytotoxicity significantly increased in a concentration-dependent manner compared to the control ($P < 0.05$). The IC_{50} was established as 0.134 mg/mL (95% confidence limit, 0.09791 ± 0.1834) against SKMEL-3 cells, 72 hours after treatment (Figure 2). As shown in Figure 3, the extract had no significant reduction effect on melanin content of SKMEL-3 cells in the range of 0.001 to 0.1 mg/mL when compared to control ($P < 0.05$). Though the melanin content significantly increased near 150% when the cells were cultured with 0.25 mg/mL of MPc. As shown in Figure 4, cellular tyrosinase activity was neither significantly decreased nor increased in cultured SKMEL-3 cells in the range of 0.001 - 0.25 mg/mL of MPc.

5. Discussions

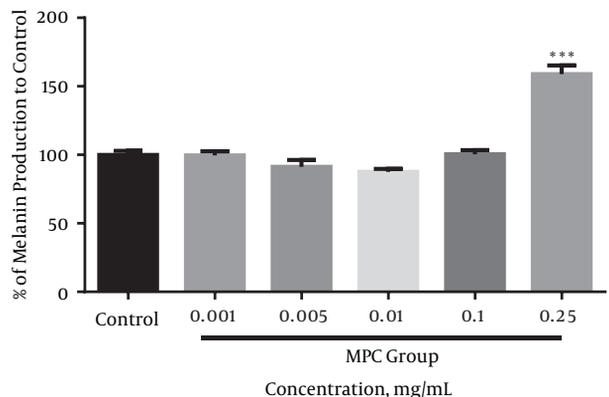
Under normal physiological conditions, melanosomal melanin acts as a natural UV photoprotective filter for melanocytes and keratinocytes. However, after long exposure to UV radiation, melanin has shown to involve in reactive oxygen species (ROS) generation and increases the risk

Figure 2. Cytotoxic Effects of Various Concentrations of MeOH Extract from *P. caucasica* (MPc) on SKMEL-3 Cell Were Determined After 72 Hours of Exposure to MTT Colorimetric Assay



Results were expressed as percentages relative to the control, and are presented as mean \pm SEM of at least three independent experiments.

Figure 3. The Effects of Various Concentrations of MeOH Extract From *P. caucasica* (MPc) on Melanin Content in Human Melanoma SKMEL-3 Cells

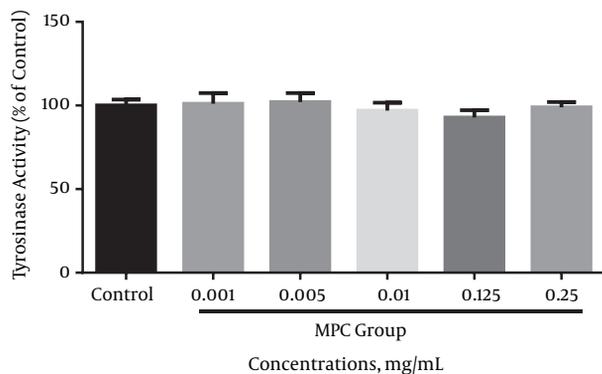


Data are presented as mean \pm SEM (n = 3) and expressed as % of control. $P < 0.05$ was considered as statistically significant.

of developing ageing, melasma and melanoma skin cancers (5, 19).

In the present study, we aimed to evaluate the effect(s) of *P. caucasica* MeOH extract on the cytotoxicity and melanogenesis in SKMEL-3 cells. A number of plant polyphenols influenced the melanin biosynthesis and growth of various melanoma cells through their anti-tyrosinase activity as well as antioxidant effects (4, 5). Generally, B16-F10 murine melanoma cell line is an available model for studying the effects of extracts/compounds on melanin production. Although this cell line is similar in many melanogenic pathways with normal human

Figure 4. The Effects of Various Concentrations of MeOH Extract From *P. caucasica* (MPc) on Cellular Tyrosinase Activity in Human Melanoma SKMEL-3 Cells



Data are presented as mean \pm SEM (n = 3) and expressed as % of control. $P < 0.05$ was considered as statistically significant.

melanocyte, it is different from human melanoma cell lines in several pathways (20). Accordingly, we preferred to use a human melanoma cell line for investigation of anti-melanoma and anti melanogenesis activities of MPc. Many published studies confirmed that melanin can increase the resistant of melanotic melanomas to chemo-, radio- and phototherapy. Therefore, anti-tyrosinase and antioxidant compounds have been able to prevent cellular mutations and tumor cells growth and increase therapeutic effectiveness of anti-melanoma drug through melanogenesis inhibition (3, 4).

P. caucasica is rich with different glycoside compounds including flavonoids, phenylethanoids/phenylpropanoids, and iridoids. The earlier study showed that the (11) polyphenolic compounds from MeOH fraction of *P. caucasica* had a wide range of free radical scavenging activities in DPPH assay. For example, two phenylpropanoid glycosides, acteoside and forsythoside B, exhibited significant antioxidant activities with SC_{50} values between 4 to 5 $\mu\text{g}/\text{mL}$ that were comparable to the Trolox® ($SC_{50} = 2.6 \mu\text{g}/\text{mL}$), while some isolated flavonoids from this fraction such as naringenin, kaempferol 3-O-glucoside, chrysoeriol 7-O-rutinoside, and chrysoeriol 7-O-glucoside have shown very weak antioxidant properties (518.6, 160.5, 98.6 and 94.7 $\mu\text{g}/\text{mL}$, respectively). Naringenin, as a well-known flavanone of Citrus fruits, enhanced tyrosinase activity and melanin synthesis by increasing the expression of melanogenic enzymes (21). Recently, the mechanisms underlying the activities of naringenin on melanogenesis have been investigated by Huang et al. (22). They explained that naringenin had no cytotoxic effects on B16-F10 melanoma cells at concentrations ranging from 3 to 50 μM . This

compound can act as a potential tyrosinase activator and melanogenesis stimulator in B16-F10 cells by promoting the expression of tyrosinase, microphthalmia-associated transcription factor (MITF) and induce melanogenesis through the Wnt- β -catenin-signalling pathway.

In addition, iridoid glycosides, a main chemical group which are found in the genus *Phlomis* plants, have been shown to possess various pharmacological properties (6, 22-25). For example, geniposide, an iridoid glycoside was found in *P. younghusbandii* (26) enhanced melanogenesis by activating extracellular signal-regulated kinase 1/2 kinase, a downstream kinase of c-kit signaling and stimulation of stem cell factor/c-kit signaling in norepinephrine-exposed normal human epidermal melanocyte (13). On the other hand, another iridoid glycoside, named shanzhi-side methyl ester, has been isolated from some species of *Phlomis* such as, *P. younghusbandii* (27) and *P. rigida* (28), and has shown an anti-melanogenic activity near $53.7 \pm 0.41\%$ reduction of melanin content at 100 μM in the α -MSH-stimulated B16 melanoma cells with almost no toxicity to the cells (14). Loganin and sweroside were found in some *Phlomis* species and indicated anti-melanogenic activity in 0.1 mM, but they were not good radical scavengers (29).

In this study, the experimental findings showed that the DPPH radical scavenging and mushroom tyrosinase activity of MPc were significantly lower than positive controls. The weak radical scavenging capacity and anti-tyrosinase activity of MPc may be due to its low polyphenolics content and/or the presence of some compounds such as naringenin with minor radical scavenging and tyrosinase stimulation effects. In addition, MPc is neither an enzymatic inhibitor nor an activator for cellular tyrosinase at concentrations $\leq 0.25 \text{ mg}/\text{mL}$ on SKMEL-3 cells. The results have shown that MPc had no inhibitory effect on melanin formation in the range of 0.001 - 0.1 mg/mL ; however, it significantly increased ($P < 0.05$) melanin content at 0.25 mg/mL on melanoma cells with no effect on tyrosinase activity. As the extract revealed cytotoxicity on SKMEL-3 cells at higher concentrations of 0.1 mg/mL ($IC_{50} = 0.134 \text{ mg}/\text{mL}$), suggesting that, along with its melano-cytotoxic effect, MPc can increase melanogenesis in the remaining viable cells. These findings showed that MPc possibly has melanogenesis and cytotoxic activity in melanoma skin cancer.

5.1. Conclusions

The results indicated that the MeOH extract of *P. caucasica* contains a number of compounds with cytotoxic effect on SKMEL-3 cells. Also, some of these compounds can be involved in melanogenesis. In conclusion, to gain the therapeutic agents from MPc, further studies are needed

to investigate its safety and the molecular mechanisms underlying the cytotoxic and melanogenic effects on human melanoma cells.

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Footnotes

Authors' Contribution: Parisa Sarkhail designed, analyzed and wrote this article, Mona Salimi, Pantea Sarkheil, Soodabeh Saeidnia and Fatemeh Heidarneshad collected and analyzed the data. All authors read and approved the final manuscript.

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Conflict of Interest: The authors declare that there are no conflicts of interest.

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