Anti-Melanogenic Activity and Cytotoxicity of *Pistacia vera Hull* on Human Melanoma SKMEL-3 Cells

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Abstract- *Pistacia vera* seed is a common food and medicinal seed in Iran. It's hull (outer skin) as a significant byproduct of pistachio, is traditionally used as tonic, sedative and antidiarrheal and has been shown to be a rich source of antioxidants. The aim of the present study is to evaluate the anti-melanogenic activity of the pistachio hulls in order to discover a new alternative herbal agent to treat skin hyperpigmentation disorders. In this work, antioxidant and anti-tyrosinase activity of MeOH extract from *Pistacia vera* hull (MPH) were evaluated *in vitro*, respectively, by DPPH radical scavenging and mushroom tyrosinase activity assays. Then the effect of MPH on the melanin content, cellular tyrosinase activity and cytotoxicity (MTT assay) on human melanoma SKMEL-3 cell were determined followed by 72 h incubation. The results indicated that MPH had valuable DPPH radical scavenging effect and weak anti-tyrosinase activity when compared to the well-known antioxidant (BHT) and tyrosinase inhibitor (kojic acid), respectively. MPH, at a high dose (0.5 mg/mL), showed significant cytotoxic activity (~63%) and strong anti-melanogenic effect (~57%) on SKMEL-3 cells. The effect of MPH in the reduction of melanin content may be related to its cytotoxicity. The results obtained suggest that MPH can be used as an effective agent in the treatment of some skin hyperpigmentation disorders such as melanoma.

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Keywords: *Pistacia vera hull*; Antioxidant; Tyrosinase; Melanin; Cytotoxicity

Introduction

Melanin, the main pigment created through melanogenesis, plays an important role in protecting the skin from harmful effects of ultraviolet light (UV) radiation. UV radiation produces reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and the hydroxyl radical that can interfere with melanin synthesis. ROS can induce DNA mutations and excessive melanin accumulation that cause the diverse pigmentation problems skin such as melanoma (1,2). Melanogenesis is regulated by various melanogenic enzymes such as tyrosinase, tyrosinase related proteins (TPR 1 and TPR 2) and microphthalmia-associated transcription factor (MITF). Tyrosinase is known as the main metalloenzyme that catalyzes the initial steps of melanin synthesis: hydroxylation of monophenols (monophenolase activity) and oxidation of diphenols to *O*-quinones (diphenolase activity). Then, *O*-quinone during the process of non-enzymatic reactions converts into melanin. Some toxic and mutagenic compounds such as quinones, semiquinones and reactive oxygen species (ROS) are created during melanogenesis. Thus, tyrosinase over activity not only causes hyperpigmentation, but it can also increase the mutagenic and toxic intermediates of melanogenesis. Moreover, tyrosinase is responsible for melanization in animals and browning in plants and fungi (3,5). As skin hyperpigmentation in human and enzymatic browning in fruits are undesirable, researchers have been interested in finding new potent tyrosinase inhibitors for use in skin whitening and anti-browning of foods. Up to now, many natural and synthetic agents have been shown significant anti-tyrosinase activity and applied for prevention and treatment of hyperpigmentation. However, some of these tyrosinase inhibitors have shown adverse side effects such as erythema, inflammation and other toxicities after prolonged application. For these reasons, herbal products that are capable of displaying antioxidant, anti-tyrosinase and

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anti-melanogenic property in pharmacological studies have always been interested in alternative treatments for skin pigmentation disorders (5,7).

Pistachio (*Pistacia vera* L.), belonging to *Anacardiaceae* family, naturally distributed in central Asia and the Middle East. The English name pistachio is derived from "Pesteh," which is a Persian name. Pistachio nut is intertwined with Iranian culture and get a mention in all Iranian literature, beliefs, traditions, and ceremonials such as Yalda festival and Norooz (New Iranian Year). Iran is the largest producers and exporters the best taste of pistachios (8). Fresh and dry raw or toasted pistachios are commonly applied as food, food additive, and flavoring agent. Moreover, in traditional Iranian medicine (TIM), pistachio fruit kernels are known as effective tonic for heart, brain, liver, and stomach and used for the treatment of coughs (9). Pistachio fruits are rich source of energy with a low glycemic index (10) and an excellent source of heart-healthy fatty-acid profile as well as protein, dietary fiber, potassium, magnesium, vitamin K, and a number of phytochemicals that may contribute to their overall antioxidant activity, including carotenes, vitamin E, and polyphenolic antioxidant compounds (11,12). The pistachio hull (outer skin) which is removed during industrial processing is a significant by-product of pistachio. In Iran, pistachio hulls are used as tonic, sedative, anti-diarrheal and for making jam (12). A few previous studies showed that Iranian pistachio hull is rich in polyphenolic compounds and acts as an antioxidant and antimicrobial agent and to promote wound healing and antimutagenic activity (13-15). The Italian pistachio hull was found to be better antioxidant activity compared to the seed because of its higher phenolic content such as gallic acid, catechin, epicatechin, quercetin, luteolin, naringenin and cyanidin-3-*O*-galactoside (16).

Although in previous studies the total phenolic and flavonoid compounds with antioxidant capacity of *Pistacia vera* hulls have been considered, no information about the anti-melanogenesis and cytotoxic activity of pistachio hulls was reported. Therefore, the present study aims to evaluate the cytotoxic and anti-melanogenic properties of methanol extract of *Pistacia vera* hull (MPH) on human melanoma SKMEL-3 cells in order to discover a new alternative herbal agent to treat skin hyperpigmentation disorders such as melasma and melanoma skin cancers.

Materials and Methods

Chemicals

Tyrosinase from mushroom, butylated hydroxytoluene (BHT), kojic acid, quercetin, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), sodium carbonate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and 3-(4, 5-dimethyl-thiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma, USA. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and trypsin-EDTA were obtained from GibcoBRL, Grand Island, USA. Folin-Ciocalteau reagent, were purchased from Fluka (Buchs, Switzerland). Human malignant melanoma cells (SKMEL-3) were obtained from Pasteur Institute of Iran. All other chemicals and solvents were from Merck (Germany).

Plant material and preparation of the extract

The fruits of pistachio were purchased from the local market in Tehran, Iran and identified by the corresponding author. A voucher specimen (No. 1391-3) was deposited at Pharmaceutical Sciences Research Center (PSRC) laboratory, Tehran University of Medicinal Sciences. The pink and creamy white hulls were separated and then dried at 40° C. Then, 100 grams of dried powder was extracted with 80% aqueous MeOH using percolation apparatus three times at room temperature. After filtration, the combined extracts were evaporated in vacuum and concentrated with freeze-dryer. The dried extract (yield 19.72% w/w) was stored at 4° C for experimental studies. All experiments were performed in triplicate.

Determination of total phenolic content

The total phenolic contents of the extracts were determined according to the Folin-Ciocalteau method (17). To the samples, 100 µL of Folin-Ciocalteu reagent (diluted 10 times) was added and mixed. After 5 min, 0.75 µL of sodium carbonate 6% (w/v) was added to the mixture. The wells allowed standing for 30 min before measuring absorbance 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.

Determination of total flavonoid content

Total flavonoid contents of the extracts estimated as the aluminum chloride colorimetric method by 96-well plate (18). The extract (20 µL) was mixed with water (80 µL) followed by the addition of $NaNO_2$ (15%, 6 µL).

After shaking the mixture for 5 min, 6 µL of $AlCl_3$ (10%), 80 µL NaOH (4%) and (80 µL) distilled water was added. After 15 min, the absorbance of the mixtures was measured at 510 nm with the ELISA reader. Total flavonoid contents were calculated as quercetin (Q) equivalents per gram of the dry extract.

Determination of the scavenging effect on DPPH

The free radical scavenging activity of the extract was evaluated according to Blois method with some modifications (19). DMSO solution (160 µL) of the sample at different concentrations (0.3125 - 2.5 mg/mL) was added to 40 µL of methanol DPPH solution (150 µM) and shaken vigorously. The mixture was incubated at 25° C for 30 min in a dark place, and then the absorbance of the resulting solution was recorded at 517 nm using an ELISA reader (Synergy HT, *BIO-TEK*, USA). The antioxidant activity of MPH was expressed as SC_{50}, which was defined as the concentration of extract required to inhibit the formation of DPPH radicals by 50%. Percentage inhibition was calculated using the following formula:

DPPH radical scavenging activity (%)=[(A−B)/A] ×100 where A=absorbance at 517 nm without test sample and B=absorbance at 517 nm with the test sample. BHT was used as the positive control.

Determination of mushroom tyrosinase inhibitory activity

Tyrosinase inhibitory activity was carried out according to the procedure as described by Momtaz *et al*., (4) with some modifications. The extracts were dissolved in DMSO to make the test concentrations (0.3125-2.5 mg/mL). A mixture of 80 µL 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. Afterward, a reaction was performed by adding 40 µL L-tyrosine (2 mM) as the substrate for 15 min at room temperature. The amount of dopachrome in the reaction was measured at 475 nm using an ELISA reader. The percentage of inhibition of tyrosinase was calculated as follows:

Tyrosinase inhibition (%)=[(A−B)/A] ×100 where A=absorbance at 475 nm without test sample and B=absorbance at 475 nm with the test sample. Kojic acid was used as the positive control. The concentration at which half the original tyrosinase activity is inhibited (IC_{50}) was determined for each sample.

Cell culture and treatment

Human melanoma cells (SKMEL-3) were obtained from the cell bank of Pasteur Institute of Iran (NCBI). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GibcoBRL, Grand Island, USA) containing 10% fetal bovine serum (GibcoBRL), 100 U/mL of penicillin G, 100 mg/mL streptomycin and then maintained at 37° C with 5% carbon dioxide in 95% humidity. The cells were subcultured in a ratio of 1: 3 on every third or fourth day. For proliferation studies, SKMEL-3 cells were plated at a density of 5×10^3 cells/well in 96-well plates. After 24h of incubation, extract samples at concentrations ranging from 0.001 to 0.5 mg/mL were added to the each well and incubated for 72h. Kojic acid was used as a controlled drug at dose of 0.1 mg/mL.

Cytotoxicity assay (MTT assay) for 72 hours

Cytotoxicity was measured using the 3-(4, 5-dimethyl-thiazole-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) reduction method (20). Treated cells were incubated with MTT (0.5 mg/mL in phosphate buffered saline) for 4 h at 37° C. The ability of cells to reduce MTT to a blue formazan product was measured to determine cell survivability. The formazan crystals were dissolved in DMSO, and the color change was measured at 545 nm using a microplate reader. The 50% inhibitory concentration (IC_{50}) value, defined as the amount of extract that inhibits 50% of cell growth, was calculated from concentration-response curves after 72 hours exposure period.

Melanin content assay

Melanin content was quantified as described previously by Salimi *et al*., (21). The human melanoma cells were seeded with 3×10^5 cells/ well in 3 ml of medium in 6-well culture plates and incubated overnight to allow the cells to adhere. The cells were exposed concentration ranged from 0.5 to 0.001 mg/mL of the MPH and kojic acid (0.1 mg/mL) for 72 hours. 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 1 h at 75° C. The melanin content was determined using a microplate reader at 405 nm absorbance.

Statistical analyses

The results were reported as mean±SEM of three replicate determinations and were analyzed by GraphPad Prism 6. The group means were compared using the ANOVA test followed by Tukey *post hoc* test. $P<0.05$ was regarded as statistically significant.

Results

Total phenolic and total flavonoid content

In this study, the total phenolic content of MPH was 395.5±3.95 mg GA/g dry extract powder (the standard curve equation of gallic acid: y=0.287x+0.375, R^2=0.999). In addition, the total flavonoid content of MPH was 98.49±7.58 mg Q/g dry extract powder (the standard curve equation of quercetin: y=0.124x+0.129, R^2=0.998).

DPPH radicals scavenging and anti-tyrosinase activity

The antioxidant and anti-tyrosinase capacity of MPH were evaluated by DPPH radical scavenging and mushroom tyrosinase activity assays, respectively (*in vitro*). The extract revealed dose dependent antioxidant activity with an SC_{50} value of 0.89 mg/mL (Figure 1).

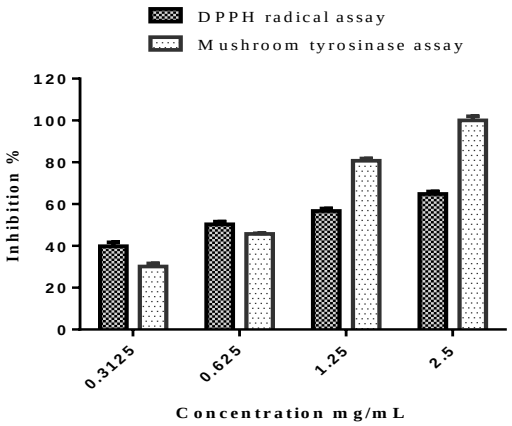

Figure 1. Percent scavenging effect and tyrosinase inhibition of different concentrations of MeOH extract of Pistachio hull (MPH). Values were expressed as mean±SEM (n = 3).

The radical scavenging effects of the extract on DPPH radicals were 39.83±1.85, 50.30±1.31, 56.72±1.16 and 64.93±1.12% of control for 0.3125, 0.625, 1.25 and 2.50 mg/mL. The BHT was used as a positive control in this test exhibited the SC_{50} value of 0.71 mg/mL.

As shown in Figure 1, the tyrosinase inhibitory activity of MPH improved with increasing concentration with an IC_{50} value of 0.72 mg/mL. The inhibitory effects of the extract on mushroom tyrosinase activity were 30.11±1.46, 45.74±0.29, 80.78±1.02 and 100±2.0 % of the control for 0.3125, 0.625, 1.25 and 2.50 mg/mL, respectively. The Kojic acid, as a positive control, showed an IC_{50} value of 0.05 mg/mL.

Cytotoxicity and melanin content

The cytotoxicity and anti-melanogenic property of MPH were evaluated on SKMEL-3 cells, one of a series of malignant human melanoma cell lines. The ability of the cells to reduce MTT to a blue formazan product was measured to determine cell survivability. Cells treated with extract show up to 62.59±1.72 % loss of cell survivability at dose of 0.5 mg/mL. Cytotoxicity of MPH on SKMEL-3 cells was dose dependent manner and IC_{50} of MPH was 0.137 mg/mL; 95% confidence limit, 0.107-0.174 mg/mL. Kojic acid revealed the reduction of cell viability by 50% at dose of 0.1 mg/mL.

As indicated in Figure 2, MPH inhibited the synthesis of melanin in a dose dependent manner (IC_{50}=0.41 mg/mL; 95% confidence limit, 0.338±0.517). At a high concentration of 0.5 mg/mL of MPH, melanin content was significantly (P<0.05) reduced to 43.60±2.41 % in SKMEL-3 cells. MPH and kojic acid showed the similar melanin inhibitory effect at dose of 0.1 mg/mL in this study (about 10%).

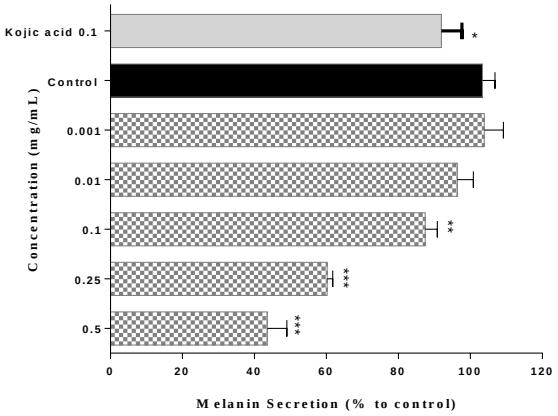

Figure 2. Effect of various concentration of MeOH extract of Pistachio hull (MPH) on melanin secretion on human melanoma SKMEL-3 cells. Denotes *P <0.05, **P <0.01, ***P <0.001 compared to control. All data were presented as mean ± SEM and expressed as % of control.

Moreover, the effective concentration of MPH on melanin reduction was higher than 0.5 mg/mL; while MPH induced cytotoxicity lower this dose on SKMEL-3 cells (Figure 3).

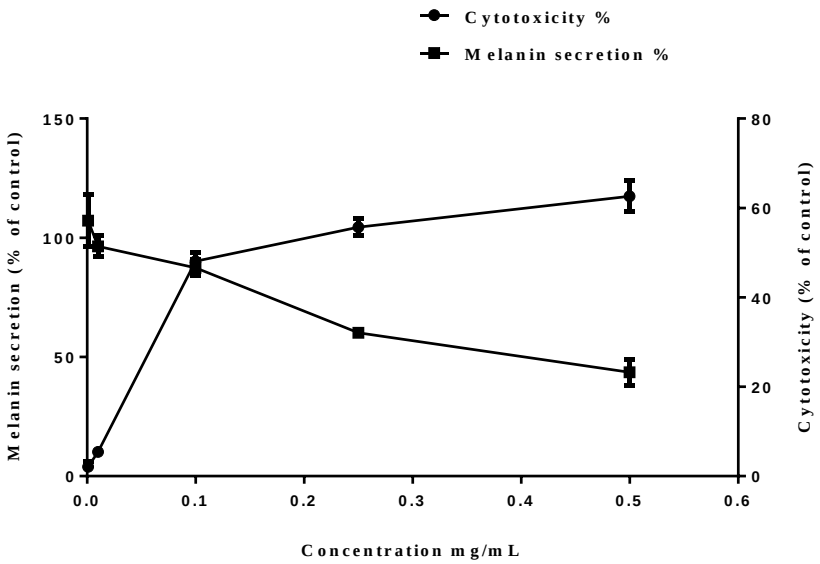

Figure 3. Effect of various concentration of MeOH extract of Pistachio hull (MPH) on melanin secretion and cytotoxicity on human melanoma SKMEL-3 cells. MPH at a high concentration of 0.5 mg/mL had significant (P<0.05) cytotoxic activity (~63%) and strong anti-melanogenic effect (~57%) on human melanoma SKMEL-3 cells after 72 hours.

Discussion

Recent studies have confirmed that many polyphenolic compounds derived from plants had effective antioxidant and anti-tyrosinse activity *in vitro* assay. The level of polyphenols is dependent on the solvent polarity and method of extraction. Methanol solution (80%) is commonly used for extraction of various bioactive compounds such as polyphenols and dissolves a larger portion of polar compounds and nonpolar compounds. Our results showed that MPH is rich in phenolic compounds as reported previously (13,14).

The UV radiation exposure increases ROS generation as well as melanin production. During melanogenesis, some toxic and mutagenic compounds such as quinones, semiquinones, and ROS are produced. Excessive ROS can damage biological processes and increase the risk of developing aging, melasma and melanoma skin cancers. Thus, antioxidant and anti-tyrosinase compounds can reduce the toxic and mutagenic intermediates of melanogenesis as well as melanin (22,23).

Previously, Rajaei *et al.*, (13) confirmed that the extracts of Iranian *P. vera* green hull had a stronger scavenging activity when compared to the BHT in DPPH assay. In another study, Tomaino *et al.*, (16) reported a high scavenging efficiency of fresh *P. vera* hull on DPPH radicals that was related to its high phenolic content, including gallic acid, catechin, epicatechin, quercetin, luteolin, naringenin, and cyanidin-3-O-galactoside, etc. In this work, the antioxidant assay result showed that MPH could efficiently scavenge DPPH radicals and was comparable to BHT. The findings revealed that the antioxidant activities of MPH could be associated with the high contents of phenolic and flavonoid antioxidant such as gallic acid and quercetin.

The results displayed that the inhibitory effect of MPH on mushroom tyrosinase activity was very lower than kojic acid. The little tyrosinase inhibitory effect is probably due to the presence of some MPH polyphenolic compounds such as naringenin that improved tyrosinase activity (24).

Considering the results of the cytotoxicity assay showed that MPH at a high dose of 0.5 mg/mL had significant cytotoxic activity (~63%) and strong anti-melanogenic effect (~57%) on SKMEL-3 after 72 hours (Figure 3). The effect of MPH in reduction of melanin biosynthesis could be explained by cytotoxicity since there was an apparent decrease in the amount of viable cells at higher doses (>0.1 mg/mL). It can be suggested that reduction of the cell viability and decrease in the melanin secretion may be caused by some antioxidant compounds in MPH. On the basis of these data and previous reports, MPH is a good natural source of antioxidant compounds. On the other hand, at higher concentrations of 0.1 mg/mL, it can be used as an

effective agent for skin disorders treatment such as melanoma cancer. However, more research is necessary to explain the mechanism of cytotoxic activity of MPH in melanoma cells.

According to previous studies and our research, *Pistacia vera* hull from Iran is a good natural source of antioxidant compounds. In summary, MPH showed reduction of cell viability at concentrations ranging from 0.1 to 0.5 mg/mL on SKMEL-3 cell line. Thus, it can be suggested that the hulls consist of several potential therapeutic compounds for treatment of some skin hyperpigmentation disorders such as melanoma.

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