Original Article

Anti-melanogenesis and anti-tyrosinase properties of *Pistacia atlantica* subsp. *mutica* extracts on B16F10 murine melanoma cells

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Abstract

Pistacia atlantica (P. atlantica) subsp. *mutica* has been used in traditional medicine and is famous for its medicinal properties. The aim of this study was to evaluate the effect of methanol (MeOH), *n*-hexane, dichloromethane (CH_2Cl_2), *n*-butanol (BuOH), ethyl acetate (EtOAc), water extracts and essential oil of *P. atlantica* subsp. *mutica* on melanin synthesis and oxidative stress in B16F10 melanoma cell line. The B16F10 cells viability after treatment with increasing concentrations of different extracts of the plant (0.2-200 µg/mL) was measured using resazurin. Essential oil composition was identified by gas-chromatography-mass spectrometry (GC-MS) analysis and inhibitory effect on synthesis of melanin, mushroom tyrosinase activity, cellular tyrosinase, and oxidative stress were evaluated by the colorimetric and fluorometric methods. The data showed extracts at concentrations 0.2-200 µg/mL, did not show significant toxicity on melanoma cells but concentrations of 200 µg/mL of essential oil had cytotoxic effect. *Pistacia atlantica* subsp. *mutica* could inhibit the mushroom tyrosinase activity. Also the amount of melanin in B16F10 cells declined. In addition, the ability of *P. atlantica* subsp. *mutica* extracts in decreasing the amount of reactive oxygen species in melanoma cells revealed remarkable antioxidant activity. In addition, all concentrations of essential oil had no significant effect in this study. The melanogenesis inhibitory and antioxidant effects of *P. atlantica* subsp. *mutica* on B16F10 cells may suggest the potential whitening activity of the plant for using in dermatological skin care products and for prevention of skin aging in cosmetic industry.

Keywords: Anti-tyrosinase; Melanogenesis; *P. atlantica* subsp. *mutica*.

INTRODUCTION

Melanin is a skin pigment which is synthesized in melanosomes and is transferred to keratinocytes throughout the physiological process called melanogenesis. Melanin plays an important role in the protection against UV damage and determines the color of skin, hair and eyes. Excess production of melanin is attributed in melanoma and abnormal pigmentation of the skin (1-3). The key enzyme in the melanin biosynthesis is tyrosinase which catalyzes two separate reactions, the oxidation of 3,4-dihydroxy-phenylalanine (DOPA) to dopaquinone and hydroxylation of L-tyrosine to the DOPA (4). Tyrosinase or polyphenol oxidase is a copper containing mixed-function enzyme found in microorganisms, animals, and plants (5). Tyrosinase is the most influencing factor for the browning of fruits and vegetables. Overproduction and accumulation of melanin may result in a large number of skin disorders including melasma, freckles, solar melanosis, and age spots (6). Therefore, tyrosinase inhibitors have attracted much interest in food and cosmetic industries.

In many living organisms, oxidation is essential for the production of energy to fuel biological processes.

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However, hydrogen peroxide (H_2O_2) and other -reactive oxygen species (ROS)- cause cell death and tissue damage in many physiologic and pathologic phenomena including ROS increment following UV irradiation during melanogenesis process. Also it is well known that UV-induced production of ROS is involved in the pathogenesis of several skin conditions including aging, wrinkles, photosensitivity, and malignancy (7). Thus, finding natural sources of antioxidants with anti-tyrosinase activity helps to modify skin damages related to excess melanogenesis (4).

The genus *Pistacia* is a member of the Anacardiaceae family comprising about 9 species and is mostly distributed in Mediterranean region, Europe, and some parts of Asia (8,9). *Pistacia atlantica (P. atlantica)* Desf. has 3 subspecies namely: *P. atlantica* subsp. *kurdica* (Zohary) Rech. f., *P. atlantica* subsp. *mutica* (Fischer & C. A. Meyer) Rech. f. and *P. atlantica* subsp. *cabulica* (Stocks) Rech. f. The three mentioned subspecies of *P. atlantica, P. khinjuk* Stocks, and *P. vera* L. are grown in Iran (10). The fruits of *P. atlantica* subsp. *mutica* namely Baneh, is round to oval with 0.5-0.7 cm in diameter. The antioxidant and anticancer activity of the hull of the *P. atlantica* has been related to high total phenolic content of the plant. *P. atlantica* has traditionally been used for relieving upper abdominal discomfort and pain, dyspepsia, and peptic ulcer (11-13). However, there are no studies on melanogenesis inhibitory activity of *P. atlantica* subsp. *mutica.* So, in this project we choose B16F10 melanoma cells for studying the antioxidant and anti-melanogenic properties of *P. atlantica* subsp. *mutica* extracts. The aim of this study was to investigate the inhibitory effect of methanol (MeOH), *n*-hexane, dichloromethane (CH_2Cl_2), *n*-butanol (BuOH), ethyl acetate (EtOAc), water (H_2O) extracts and essential oil of *P. atlantica* subsp. *mutica* fruit on melanogenesis and to evaluate the potential antioxidant capacity of the plant on B16F10 melanoma cells.

MATERIALS AND METHODS

Chemicals

Mushroom tyrosinase from the *Agaricus bisporus*, kojic acid, resazurin, L-3,4- dihydroxyphenylalanine (L-DOPA), dichloro- dihydro-fluorescein diacetate (DCFH-DA), egtazic acid (EGTA), dimethyl sulfoxide (DMSO), phenylmethylsulfonyl fluoride, β- glycerophosphate, β-mercaptoethanol, phosphate buffered saline (PBS), sodium orthovanadate, tris-buffered saline tween 20 (TBST) purchased from Sigma (USA). Fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-EDTA were obtained from GibcoBRL (Grand Island, USA). Melanoma cell line (B16F10, Cat. No C540) was purchased from the Pasteur Institute of Iran (Tehran, I.R. Iran). All other chemicals and solvents were from Merck (Germany).

Preparation of extracts

P. atlantica subsp. *mutica* was collected in May 2014 from Bardaskan Mountains, Khorasan Razavi province, northeast of Iran and identified by Mrs. M. Souzani. A voucher specimen (No:13069) was deposited in the herbarium of School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, I.R. Iran. Unripe fruit of *P. atlantica* subsp. *mutica* (200 g) were grounded by a blender (Toos chekan Co, I.R. Iran) and then percolated with MeOH at room temperature for 24 h according to the previously reported protocol (14). After extraction, the solvent was evaporated using rotary evaporator and then freeze-dried. Methanol extract (94 g) was further fractionated by solvent-solvent partition to give five different fractions including MeOH, *n*-hexane, CH_2Cl_2, BuOH, EtOAc, and H_2O.

Isolation of the essential oil

The unripe fruit of *P. atlantica* subsp. *mutica* (150 g) was subjected to hydro- distillation using a Clevenger-type apparatus for 3 h. The colorless oil was obtained with a yield of 0.8% (v/w). The obtained essential oil was dried over anhydrous sodium sulphate and stored at 4 °C in dark until further testing.

Gas-chromatography and gas-chromato- graphy-mass spectrometry

The gas-chromatography (GC) analysis was performed using a Varian CP-3800 equipped with a FID detector, interfaced with a fused- silica column (CP-Sil 8CB, 50 m × 0.25 mm,

film thickness 0.12 μm) under following condition: oven temperature 50-250 °C with the rate of 3 °C/min; injector temperature 260 °C, split ratio 1:5, with carrier gas, N_2 flow rate 2 mL/min; detector temperature 280 °C.

Gas-chromatography-mass (GC-MS) analyses were performed using an Agilent 5975 apparatus equipped with a HP-5 MS column (30 m × 0.25 mm i.d., 0.25 μm film thicknesses) interfaced with a quadruple mass detector and a computer equipped with Wiley 7n.L library. Oven temperature 50-250 °C with the rate of 3 °C/min, injector temperature 250 °C, injection volume: 0.1 μL, split injection with split ratio of 1:50, with the carrier gas (helium) flow rate 1 mL/min, ion source: 70 eV, ionization current: 150 μA, and scan range: 35-465. Identification of the components of the essential oil was based on retention gas chromatography obtained with reference to *n*-alkanes series (C6-C20) on HP-5MS column, comparison of their mass spectra and fragmentation patterns reported in literature, and computer matching with the Wiley 7n.L library (15). Quantification of the relative amount of the individual components of unripe fruit was performed according to the area percentage method without consideration of calibration factor.

Cell culture

Melanoma cell line, B16F10, maintained at 37 °C in a humidified atmosphere (90%) containing 5% CO_2. Cells were cultured in RPMI-1640 (Bioidea, Iran) with 10% (v/v) FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The stock solution of the *P. atlantica* subsp. *mutica* was prepared at 50 μg/mL in DMSO and kept at -40 °C. Kojic acid (2 and 4 mM) was used as positive control in all experiments. Cells were cultured in 96-well plates in a density of 10^5 cells/mL. The activity of the extract in each experiment was calculated using the following equation:

$$\text{Percent of activity} = \frac{Absorbance\ of\ the\ sample}{Absorbance\ of\ the\ control} \times 100$$

Cell viability assay

Resazurin is a cell health indicator uses the reducing power of live cell and converts to resorufin. Resazurin is a blue, non-toxic, non-fluorescent and cell permeable compound that converts to red in color and highly fluorescent resorufin in live cells (16). About 10^4 B16F10 melanoma cells were seeded in each well of 96-microwell plate and treated with various concentrations of extracts and essential oil of *P. atlantica* subsp. *mutica* (0.2-200 μg/mL). After 4 h incubation, the absorbance of resazurin and resorufin was measured at 570 nm and 600 nm using H4 Hybrid Multi-Mode microplate reader (BioTek, Winooski, USA). Each experiment was done in triplicate. The half maximal inhibitory concentration (IC_{50}) was calculated using GraphPad software from the concentration-effect curve: log concentration *vs* response.

Mushroom tyrosinase activity assay

The activity of mushroom tyrosinase in oxidation of L-DOPA was measured spectrophotometrically as described previously (17), with some modifications. Briefly, 160 μL of 5 mM L-DOPA (in 100 mM sodium phosphate buffer pH 6.8) and 20 μL of the same buffer with and without *P. atlantica* subsp. *mutica* extracts and essential oil (10-1000 μg/mL) were mixed with 20 μL of mushroom tyrosinase (200 units/mL) and then incubated at 37 °C for 30 min. The absorbance was measured at 475 nm with Synergy H4 Hybrid Multi-Mode microplate reader (BioTek, Winooski, USA).

Determination of melanin content in melanoma cells

Melanoma cells, B16F10, were seeded at a density of 10^5 cells per well in 96-well culture plates and incubated for 24 h. They were then incubated with different concentrations (0.2-200 μg/mL) of *P. atlantica* subsp. *mutica* extracts and essential oil for 24 h. The melanin content was measured as described previously (18). After treatment, the cells were collected using trypsin. They washed with PBS. The cell pellets were solubilized in 50 μL solution of sodium hydroxide (2 M) for 60 min at 60 °C. The melanin content was measured by measuring the absorbance at 405 nm with Synergy H4 Hybrid Multi-Mode microplate reader (BioTek, Winooski, USA).

Cellular tyrosinase activity assay

The oxidation of DOPA to DOPA chrome was analyzed by spectrophotometry as indicator of tyrosinase activity (18). 10^6 cells of B16F10 melanoma were plated in each well of 96-well plate overnight. After treating of cell with different concentrations (0.2-200 μg/mL) of *P. atlantica* subsp. *mutica* extracts and essential oil for 24 h, The cells were detached using trypsin; washed with PBS, and lysed with 50 μL sodium phosphate buffer (pH 6.8, 100 mM) containing 1% triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 10,000 rpm for 20 min at 4 °C. 100 μL of each lysate (each containing 100 mg protein) was mixed with 30 μL of 5 mM DOPA in 96 well plate and incubated at 37 °C for 2 h, the absorbance was measured at 475 nm with Synergy H4 Hybrid Multi-Mode microplate reader (BioTek, Winooski, USA).

Determination of cellular reactive oxygen species level

Reactive oxygen species level was measured as described previously with minor modifications (19). Melanoma cells, B16F10, (2×10^4) were seeded in 96-well plates overnight and then were treated with different concentrations (0.2-200 μg/mL) of *P. atlantica* subsp. *mutica* extracts and essential oil for 24 h. Then cells incubated with 50 μL H_2O_2 (24 mM) at 37 °C for 30 min. Then 50 μL of DCFH-DA were added to the cells and the fluorescence intensity of DCF was measured at 504 nm emission and 524 nm excitation using a Synergy H4 microplate reader (BioTek, USA).

Western blotting analysis

Melanoma cells, B16F10, were cultured in 75 cm^3 flasks with and without methanol extract (0.5-100 μg/mL) for 24 h. The cells were then lysed in a buffer (50 mM tris-HCl, pH 7.4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 10 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 0.1% deoxycholic acid sodium salt). Equal amount of proteins (50 μg) were loaded on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 2 h in 10% skim milk in TBST (20 mM tris-HCl pH 7.4, 100 mM NaCl, and 0.1% tween 20) buffer at room temperature. After washing with TBST buffer, the membrane was then incubated overnight with a primary antibody: 1:300 (rabbit anti-tyrosinase antibody (Santa Cruz Biotechnology, CA)). After rinsing 3 times with TBST buffer, the membranes were then incubated for 2 h with anti-rabbit IgG (1:2000) as secondary antibody (cell signaling). Rinsing 3 times with TBST buffer was repeated and the protein bands were detected using the enhanced chemiluminescent (ECL) prime western blotting detection system (BioRaD, USA) (20). Anti-β-actin antibody was used as the loading control.

Statistical analysis

The relative results of the experiments were presented as the mean ± SD of the three independent measurements. Analysis of variance and IC_{50} calculation were performed with GraphPad Prism 6.0 using one-way ANOVA test and the means were compared by Dunnett tests. $P < 0.05$ stands for statistically significant difference between extract-treated cells and control. Two-way ANOVA test and bonferroni comparison post-test was used to compare the effect of different extracts in each assay.

RESULTS

Essential oil composition

Gas-chromatography has been used for quantification and Gc-Mass for identification of the compounds. The results of both GC and GC-MS analysis are presented in the quantitative and qualitative identification table of the compounds. In the case of *P. atlantica* subsp. *mutica*, 68 constituents were identified in the essential oil and accounted for 99.8% of the total oil composition (Table 1). The grouped compounds of the essential oil was determined as monoterpene hydrocarbons 86.5%, oxygenated monoterpenes 3.7%, sesquiterpene hydrocarbons 8.7%, oxygenated sesquiterpens 0.1%, and the miscellaneous 0.8%. The major components of the essential oil were β-E-ocimene 29.7%, myrcene 17.1%, β-Z-ocimene 17.0%, α-pinene 10.2%, and E-caryophyllene 7.1%.

Table 1. Chemical compositions of volatile oil of oleoresins of *Pistacia atlantica* subsp. *mutica* obtained by hydro-distillation.

NO	Compound	RI[1]	Percentage
1	Tricyclene	924	0.2
2	**α-Pinene**	**938**	**10.2**
3	Camphene	954	0.9
4	Verbenene	965	*t*[2]
5	β-Pinene	978	0.3
6	**Myrcene**	**988**	**17.1**
7	δ-2-Carene	1002	*t*
8	α-Phellandrene	1003	0.5
9	δ-3-Carene	1010	0.1
10	α-Terpinene	1015	0.2
11	ρ-Cymene	1026	0.2
12	Limonene	1028	7.9
13	**β-Z-Ocimene**	**1037**	**17.0**
14	**β-E-Ocimene**	**1048**	**29.7**
15	γ-Terpinene	1058	0.2
16	Terpinolene	1086	1.5
17	Perillene	1089	0.1
18	α-Fenchol	1115	0.1
19	α-Campholenal	1127	*t*
20	Allo-ocimene	1130	0.2
21	Neo-allo-ocimene	1142	0.2
22	*Cis*-beta-terpineol	1144	0.1
23	Neo-3-thujanol	1147	0.1
24	Pinocarvone	1163	*t*
25	Borneol	1168	0.2
26	Alpha-phellandren-8-ol	1169	0.1
27	Terpinen-4-ol	1175	0.3
28	α-Terpineol	1186	1.9
29	Methyl salicylate	1187	*t*
30	Myrtenal	1193	*t*
31	Myrtenol	1194	*t*
32	Methyl chavicol	1197	*t*
33	Unknown	1199	0.1
34	Verbenone	1203	*t*
35	*Trans*-carveol	1217	*t*
36	Carvone	1242	*t*
37	Carvotanacetone	1245	*t*
38	Piperitone	1253	*t*
39	Vitispirane	1274	*t*
40	Bornyl acetate	1286	0.4
41	3Z-Hexenyl tiglate	1319	0.1
42	Hexyl tiglate	1332	*t*
43	Linalool propanoate	1338	*t*
44	Eugenol	1356	*t*
45	α-Copaene	1377	*t*
46	3Z-Hexenyl-3Z-hexanoate	1383	*t*

Table 1. (continued**)**

NO	Compound	RI[1]	Percentage
47	α-Gurjunene	1406	0.1
48	**E-Caryophyllene**	**1416**	**7.1**
49	*Cis*-thujopsene	1430	0.1
50	Unknown	1448	0.1
51	α-Humulene	1453	0.9
52	*Trans*-β-farnesene	1457	*t*
53	γ-Muurolene	1479	0.1
54	Germacrene-D	1483	0.1
55	Gamma-amorphene	1493	0.1
56	Viridiflorene	1495	0.1
57	Cuparene	1502	0.1
58	δ-Amorphene	1513	0.1
59	δ-Cadinene	1521	0.1
60	γ-Cuparenene	1531	0.1
61	α-Cadinene	1534	*t*
62	α-Calacorene	1547	*t*
63	Palustrol	1566	0.1
64	(Z)-3-Hexenyl benzoate	1571	0.4
65	n-Hexyl benzoate	1577	0.2
66	2E-Hexenyl benzoate	1586	*t*
67	Hexadecane	1600	*t*
68	Heptadecane	1699	*t*
	Major grouped compounds		
	Monoterpene hydrocarbons		86.5
	Oxygenated monoterpenes		3.7
	Sespuiterpene hydrocarbons		8.7
	Oxygented sesquiterpenes		0.1
	Miscellaneous compounds		0.8
	Total identified		99.8

[1] RI, The Kovats retention indices relative to C8-C20 n-alkanes were determined on CP-Sil 8CB capillary column; [2] t, trace > 0.05%.

Effect of extracts on cell survival

The viability of cells was monitored with resazurin. Melanoma cells, B16F10, were seeded in 96-well plate. After 24 h cells treated with different concentrations (0.2-200 μg/mL) of *P. atlantica* subsp. *mutica* extracts, results showed that the extracts had no significant cytotoxic effect on B16F10 cells at the concentrations used in this study (Fig. 1). Doxorubicin as the positive control significantly induced cell death ($P < 0.05$).

Effect of extracts on mushroom tyrosinase activity

The effect of *P. atlantica* on inhibition of the mushroom tyrosinase in oxidation of L-DOPA was assessed. The results indicated that mushroom tyrosinase activity was inhibited by all different extracts, but the concentration of 1000 μg/mL of *n*-hexane extract had no significant effect in this test. Kojic acid (2 and 4 mM) was used as positive control ($P < 0.05$) (Fig. 2).

Effect of extracts and essential oil on the synthesis of melanin

To study the effect of different extracts and essential oil of *P. atlantica* subsp. *mutica* on melanin synthesis, the melanin content of extract-treated B16F10 melanoma cells was assessed. Kojic acid (2 and 4 mM) was utilized as a positive control.

The results showed that MeOH, CH_2Cl_2, and EtAc extracts (0.2-200 µg/mL), *n*-hexane (2-200 µg/mL), and H_2O extract (20 and 200 µg/mL) had inhibitory effect on melanin synthesis ($P < 0.05$) (Fig. 3). However, all of the concentrations of essential oil and BuOH extract had no significant inhibitory effect on melanin synthesis.

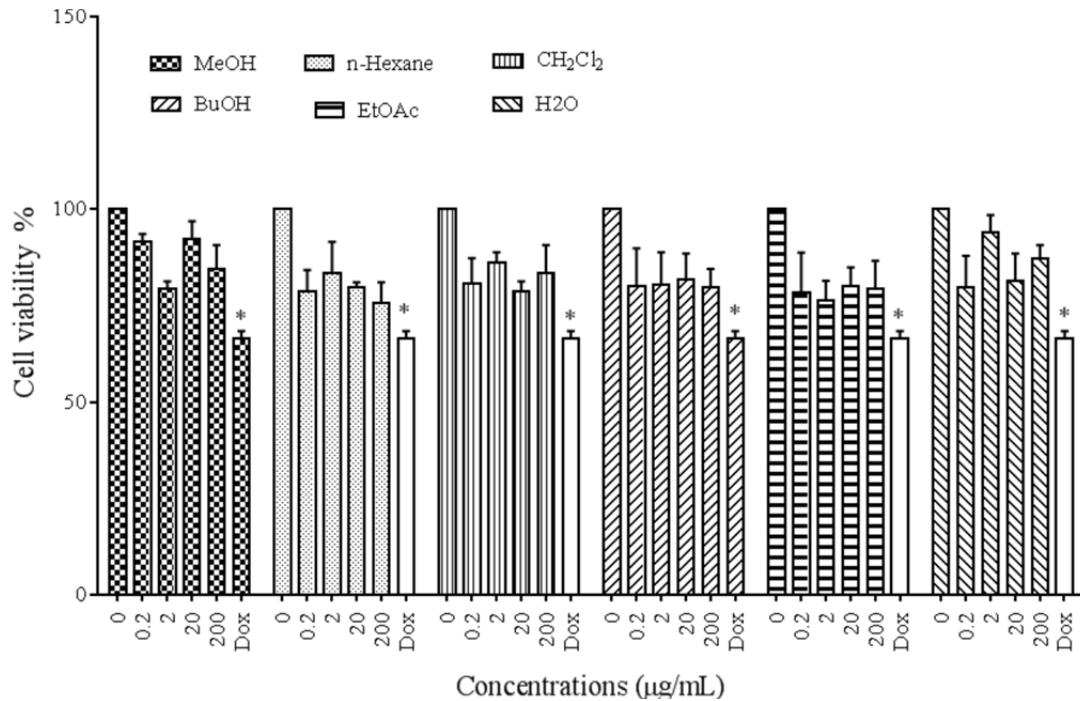The results of essential oil are not shown in the figures.

Fig. 1. Cytotoxic effects of *Pistacia atlantica* subsp. *mutica* extracts on melanoma cell viability. *$P < 0.05$, as compared to control.
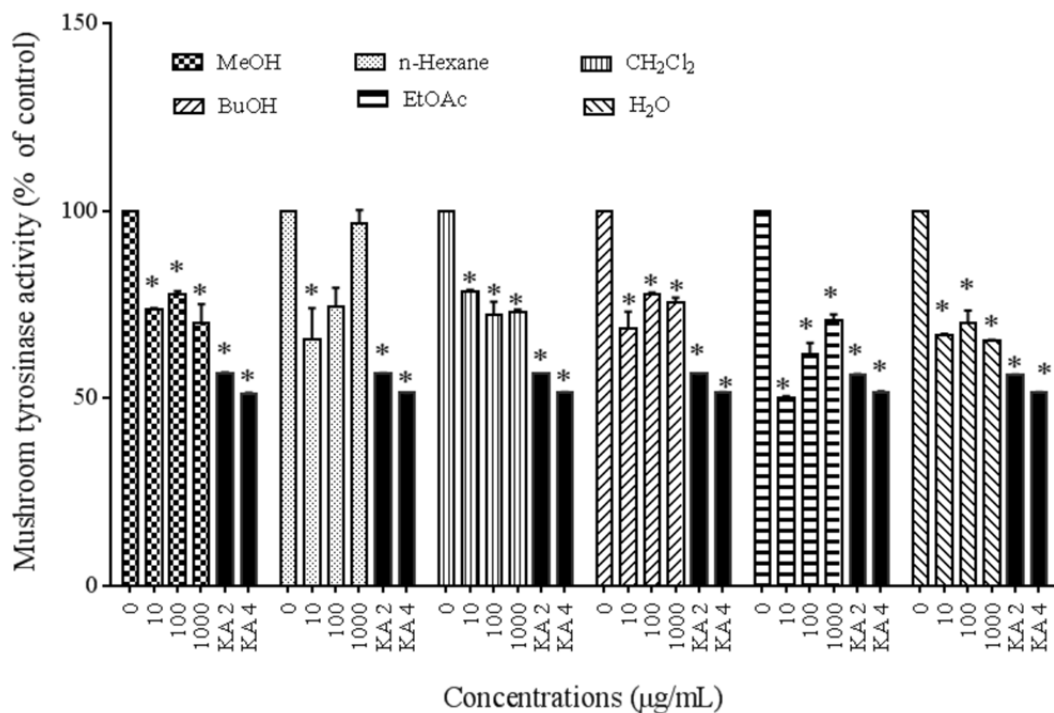
Fig. 2. Effect of *Pistacia atlantica* subsp. *mutica* extracts on mushroom tyrosinase activity. *$P < 0.05$, as compared to control.
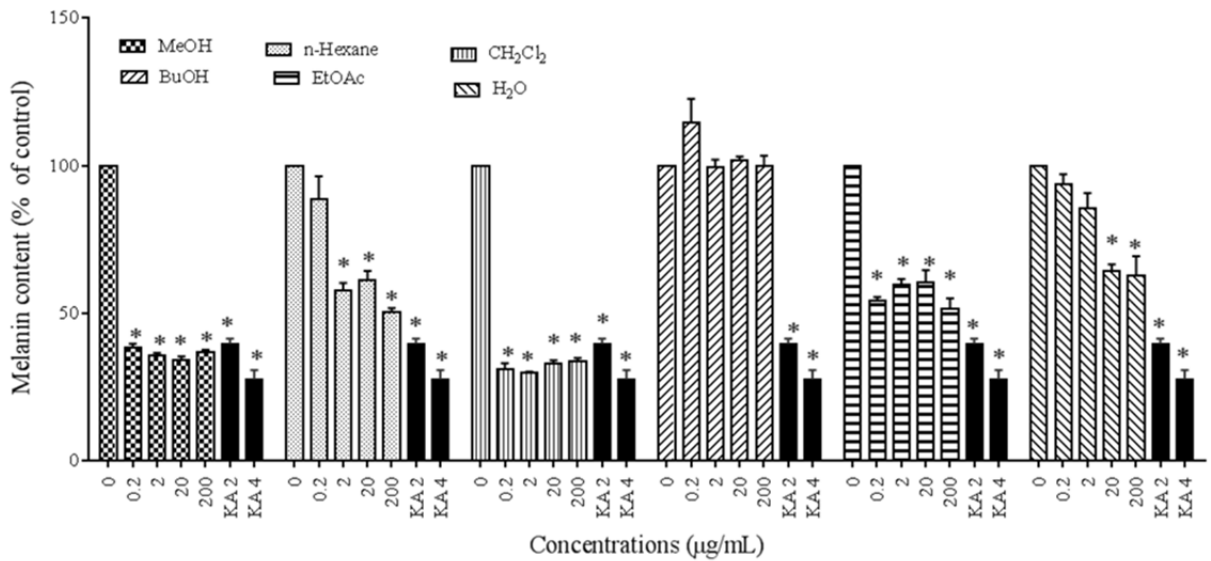
Fig. 3. Effect of *Pistacia atlantica* subsp. *mutica* extracts on melanin content. *$*P < 0.05$* as compared to control.
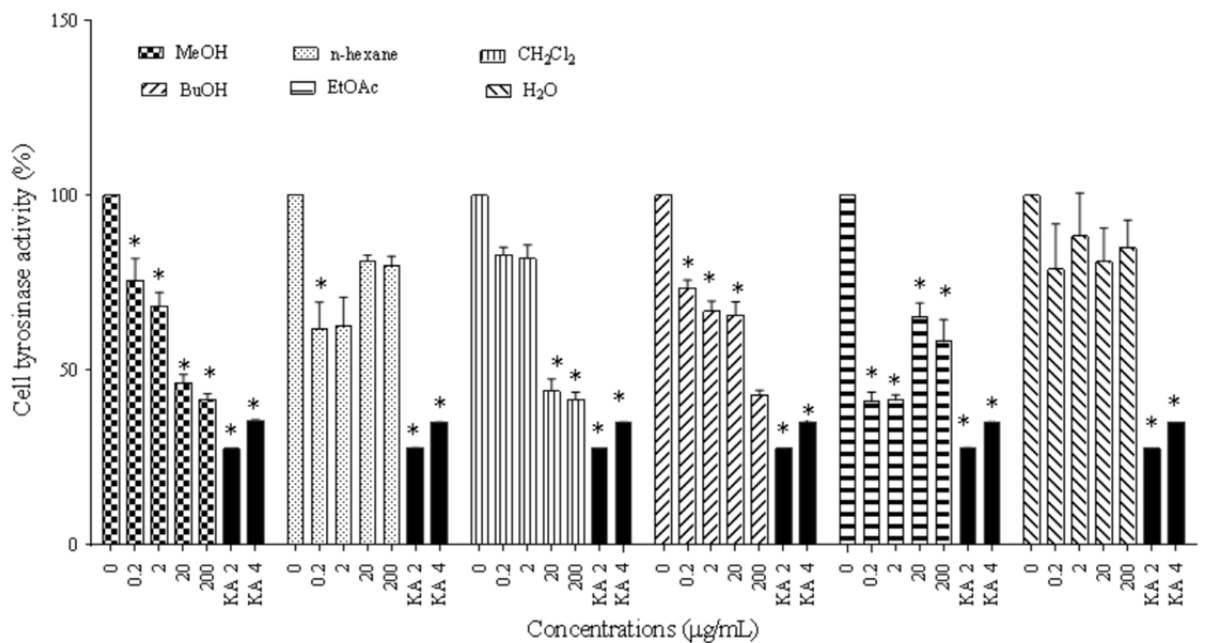

Fig. 4. Effect of *Pistacia atlantica* subsp. *mutica* extracts on cellular tyrosinase activity. *$*P < 0.05$* as compared to control.

Effect of extracts on cellular tyrosinase activity

To evaluate the mechanism of the inhibitory effect of *P. atlantica* subsp. *mutica* extract on melanogenesis in particular, we evaluated the intracellular tyrosinase activity in B16F10 melanoma cells. The results indicated that MeOH, EtOAc, and BuOH extracts (0.2-200 μg/mL), *n*-hexane (0.2 μg/mL), and CH_2Cl_2 (20 and 200 μg/mL) extracts of *P. atlantica* subsp. *mutica* could significantly inhibit cellular tyrosinase activity ($P < 0.05$) (Fig. 4) but water extract had no inhibitory effect on cellular tyrosinase activity.

Effect of extracts on cellular reactive oxygen species level

The intracellular ROS level as indicative of antioxidant capacity of *P. atlantica* subsp. *mutica* was measured in cells treated with 24 mM H_2O_2 alone or with different extracts in B16F10 melanoma cells. The results indicated that all extracts concentrations of *P. atlantica* subsp. *mutica* except for the 0.2 μg/mL of CH_2Cl_2 extract could significantly suppress the oxidative stress induced by H_2O_2 ($P < 0.05$) (Fig. 5).

Effect of extracts on cellular tyrosinase protein level

The intracellular effect of *P. atlantica* subsp. *mutica* on melanogenic related proteins such as tyrosinase as indicator of melanogenesis was evaluated by western blot.

As shown in Fig. 6, tyrosinase protein levels were significantly decreased by *P. atlantica* subsp. *mutica* extract treatment at the concentration of 0.5 and 10 μg/mL ($P < 0.05$). β-Actin was used as internal control.

Effects of essential oil on different parameters

Essential oil at 0.2-200 μg/mL revealed no significant effect on all above-mentioned parameters, except at concentration 200 μg/mL, which exhibited cytotoxic effect on melanoma cells. Hence, the results of essential oil are not included in the result section as no inhibitory effects were observed.
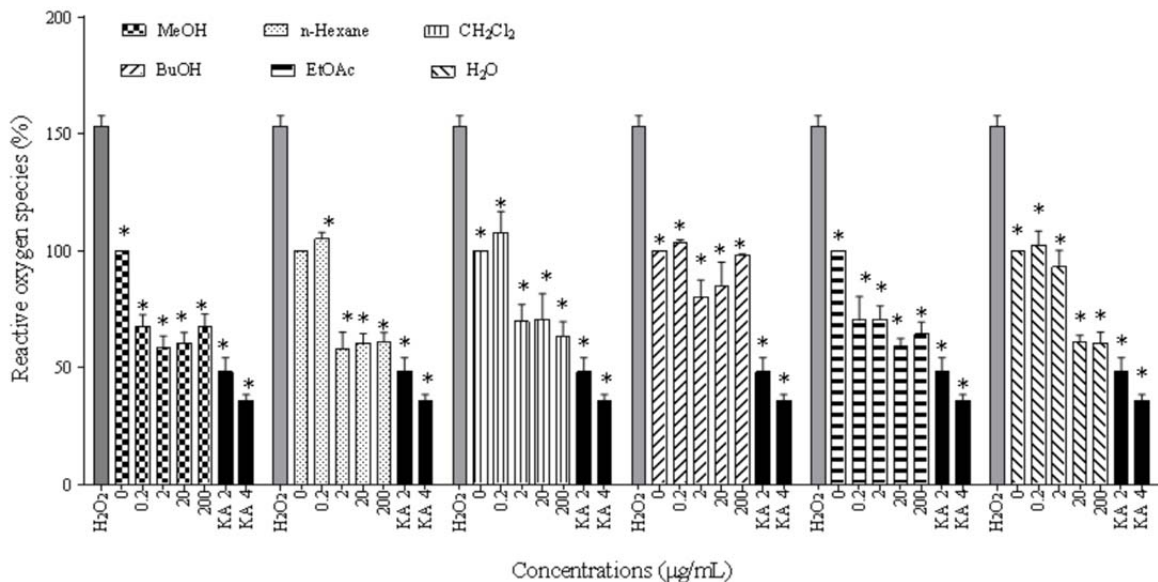

Fig. 5. Antioxidant effect of *Pistacia atlantica* subsp. *mutica* extracts on cellular reactive oxygen species (ROS) levels. *$P < 0.05$ as compared to control.
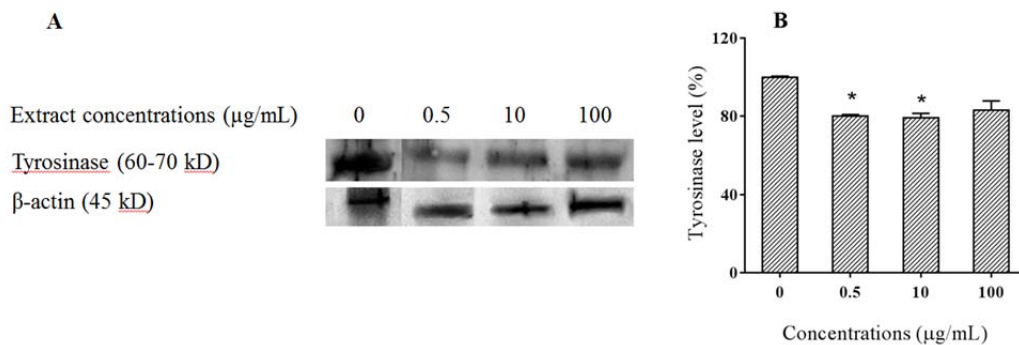

Fig. 6. Effect of *Pistacia atlantica* subsp. *mutica* on cellular tyrosinase protein level. A, Western blotting analysis of tyrosinase and β-actin proteins expression in B16F10 cells; B, the level of tyrosinase in cells normalized to the related β-actin band in comparision with control, *$P < 0.05$.

DISCUSSION

Natural based cosmetic products are widely advertised by commercial agencies for their safety and multifunctional activity. Finding new anti-melanogenic agents with antioxidant activity from natural sources is one of the interests in formulation of the products used for hyperpigmentation disorders. In medicinal and cosmetic products used as skin whitening agents, inhibition of the melanin formation, scavenging free radicals and inhibition of the tyrosinase activity that important for treating related skin disorders (21).

In this study the antioxidant activity of different extracts of *P. atlantica* subsp. *mutica* was evaluated and their effects on the tyrosinase activity and melanin synthesis were analyzed. The MeOH and EtOAc extracts of *P. atlantica* subsp. *mutica* showed significant antioxidant activity at 2, 20 µg/mL and 20, 200 µg/mL which may be attributed to the polyphenols and flavonoids widely found in the plant. The results showed all of the extracts especially MeOH, EtOAc, and H2O extracts could significantly inhibit the mushroom tyrosinase activity. Also, The MeOH, EtOAc, and BuOH extracts were able to decrease the cellular tyrosianse activity which was in accordance with reduction in the melanin production in cells. In this study, all of concentration of essential oil had no significant inhibitory effect against cellular tyrosianse, melanin synthesis, and antioxidant activity. Due to the more activity of the MeOH extract in different experiment in comparison with other extracts we have chosen the mentioned extract for western blot analysis. To sum up, the anti-melanogenic effect of this extract was confirmed in all assays in B16F10 cells, being comparable to the positive control kojic acid (Table 2).

The MeOH, CH2Cl2, and EtOAc extracts have decreased cellular tyrosinase activity, melanin content, and ROS. Semipolar nature fractions may extract phytochemicals which are responsible for antimelanogenic activity such as polyphenols and flavonoids. The n-hexane fraction and essential oil were less active indicating that plant phytochemicals with nonpolar and volatile nature have not significant effect on the melanogenesis process (22,23).

Polyphenols and flavonoids act as inhibitors of ROS production and could be responsible for the anti-melanogenic properties of plant extracts (24-27). The major compounds in the extract of *P. atlantica* subsp. *mutica* are quercetin, luteolin, isoquercetin, rutin, luteolin 7-lactate, and phenolic compounds such as p-coumaric acid, caffeic acid and gallic acid which are responsible for antioxidant activity (28). Rutin has been reported to inhibit the activity of tyrosinase and is a potent anti-pigment agent due to the presence of hydroxyl groups (29). Interestingly quercetin, luteolin, and isoquercetin also have many hydroxyl groups in their structure making them suitable for interaction with tyrosinase resulting in the anti-tyrosinase activity (21,30). Recently it has been shown that effect of flavonoids like luteolin and quercetin are mainly mediated via the modulation of transcriptional factor of melanogenesis associated transcription factor (MITF) and/or the melanogenesis enzymes tyrosinase, DCT or tyrosinase-related protein 1 (TYRP-1) (31). P-coumaric acid and caffeic acid inhibited the mushroom tyrosinase activity and were 10- and 3-fold more potent than kojic acid (32).

Table 2. The half maximal inhibitory concentration (IC$_{50}$) values (µg/mL) for different extracts of *Pistacia atlantica* subsp. *mutica.*

Factors	Extracts					
	n-Hexane	EtOAC[1]	*n*-BuOH[2]	CH2Cl2[3]	MeOH[4]	H2O
Mushroom tyrosinase	85.45	73.73	82.21	75.99	60.78	79.70
Melanin	71.01	57.59	95.23	64.98	54.71	84.61
Cellular tyrosinase	66.95	53.51	59.64	62.51	50.61	90.01
ROS[5]	50.04	36.01	78.24	42.42	34.41	56.29

[1] EtOAC, ethyl acetate; [2] BuOH, *n*-butanol; [3] CH2Cl2, dichloromethane; [4] MeOH, methanol; [5] ROS, reactive oxygen species.

Gallic acid displays tyrosinase inhibitory activity and reduces dopaquinone back to L-DOPA through a redox cycling, similar to ascorbic acid (33). Similarly, in the present study *P. atlantica* subsp. *mutica* decreased the level of tyrosinase protein in cells which is strongly correlated with the presence of quercetin, luteolin, isoquercetin, rutin, and other polyphenols in the plant.

There are many reports about plants to have the anti-melanogenic and antioxidant activity that decrease oxidative stress, which may have a high potential for the treatment of skin disorder. For example, the different extracts of aerial parts of *P. atlantica* reduced oxidative stress, which may be attributed to the polyphenols, flavonoids, and anthocyanin widely found in the plant (34). In another study, the MeOH extract of *P. vera* decreased the melanin secretion which was attributed to some antioxidant compounds in this plant. This study indicated the kojic acid, as a positive control, showed an IC$_{50}$ value of 0.05 mg/mL and higher concentrations of *P. vera* can be used as an effective agent for skin disorders treatment such as melanoma cancer (35). Gourine, *et al.* showed the aerial parts of *P. atlantica* have the powerful antioxidant properties (36). Also MeOH and CH$_2$Cl$_2$ fractions of *Nepeta satureioides* indicated potential effects against melanin production in B16F10 melanoma cells and may contribute in cosmetic formulations of skin care products (37). The derivatives of kojic acid had inhibitory activity on tyrosinase. It seems that the presence of free hydroxyl group and methyl substitute in this compound confirmed inhibitory activity. In this study kojic acid (positive control) indicated an IC$_{50}$ value of 0.28 mM (38). The phenylpropanoid glycosides and flavonoids isolated from *Teucrium polium* L. var. *gnaphalodes* showed antioxidant and tyrosinase inhibitory activities. The authors exhibited jaranol showed the highest tyrosinase inhibitory activity (IC$_{50}$ 0.041 mM) and poliumoside was the best antioxidant among the tested compounds. Kojic acid showed an IC$_{50}$ value of 0.02 mM (39).

Decrease in the protein level of tyrosinase by *P. atlantica* subsp. *mutica* and antioxidant properties and reduced ROS were confirmed

the use of this agent as anti-melanogenesis agent. This study for the first time has determined the inhibitory effect of *P. atlantica* subsp. *mutica* on melanogenesis in B16F10 melanoma cells. In order to prevent accumulation and overproduction of melanin in skin, inhibition of tyrosinase has an important role. Hence, respect to the reported anti-oxidant effect of *P. atlantica* subsp. *mutica,* flavonoid and phenolic compounds are important components of the plant responsible for anti-melanogenesis and antityrosinase activities of *P. atlantica* subsp. *mutica.* Both antioxidant and anti-melanogenic activity of *P. atlantica* subsp. *mutica* may offer suitable agent for skin whitening formulations and could be included in cosmetic product for skin care formulations.

CONCLUSION

In conclusion, this study for the first time showed that MeOH and EtOAc extracts of *P. atlantica* subsp. *mutica* has strong tyrosinase inhibitory activity which is in accordance with melanin reduction. Moreover, *P. atlantica* subsp. *mutica* also demonstrated high scavenging activity and antioxidant properties, which could be attributed mainly to its high levels of flavonoids and total polyphenols. The results suggested that the ability of *P. atlantica* subsp. *mutica* to reduce melanin production may correlate to depletion of cellular ROS and its inhibitory action on the signaling pathway regulating tyrosinase activity. Since the semipolar extract had significant anti-tyrosinase and anti-melanogenic effects, therefore, we conclude the compounds responsible for these effects are accumulated in the extract, not in essential oils.

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