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Anti-melanogenic activity of *Viola odorata* different extracts on B16F10 murine melanoma cells

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ABSTRACT

Objective(s): In previous studies, antioxidant activity of *Viola odorata* L. has been demonstrated. In this study, we have investigated the anti-melanogenic effect of extract and fractions of the plant in B16F10 cell line.

Materials and Methods: Impact of different increasing concentrations of extract and fractions of *V. odorata* was evaluated on cell viability, cellular tyrosinase, melanin content and mushroom tyrosinase as well as ROS production in B16F10 murine melanoma cell line.

Results: *Viola odorata* had no cytotoxicity on B16F10 cells compared to control group. Kojic acid as positive control had significant decreasing effects on cellular and mushroom tyrosinase activity, melanin content and ROS production (P<0.001, for all cases). *V. odorata* (1-20 µg/ml) decreased all measured parameters including cellular tyrosinase and melanin content as well as ROS production and among all extract and fractions ethyl acetate fraction had the best effect (P<0.05).

Conclusion: *Viola odorata* had promising anti-melanogenic activity through inhibition of cellular tyrosinase activity and ROS production as well as melanin content. More basic and clinical studies need to aver its impact.

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Introduction

Skin is the vastest organ covering about 16% of total body weight of the adult (1). Furthermore, the skin is a multi-functional barrier protecting internal organs from physical and chemical injuries, pathogens, dehydration and ultraviolet (UV) irradiation as well as controlling body temperature, excretion of wastes, production of vitamin D and playing important role in psychosocial relations (2, 3). Skin tone is an individual characteristic that is most variable in range of pale to nigra in different racial origin and dependent to polymeric and non-protein compound named melanin, which found in basal epidermis layer (4).

Melanin is the main pigment in primates, synthesized in melanosomes in melanogenesis process and transferred to the other part of epidermis. Melanin cares against harmful impact of oxidative stress and DNA damage, and plays scavenging role in both internal and external sources of free radical (3, 5). Melanogenesis is started with tyrosinase (EC: 1.14.15.1), which oxidize the tyrosine to dopaquinone. In the presence of glutathione or cysteine (thiols moiety), dopaquinone in consecutive stages is converted to red pigment called pheomelanin, while in the absence of glutathione or cysteine, black pigment named eumelanin is made (6). Tyrosinase is the key and rate-limiting enzyme in melanogenesis process and has a binuclear copper active site which catalyzes the hydroxylation of phenols to catechols and consecutively oxidized catechols to quinones (6, 7). Despite the protective role of melanin, excessive accumulations of melanin cause hyper-pigmentation areas in the skin which named spot (8).

Viola odorata L. is a grassy and perennial plant (Figure 1) that belongs to Violaceae (9). In previous studies, many pharmacological properties of *V. odorata* has been demonstrated including: anti-bacterial (10), anti-inflammatory (11), anti-cancer (12, 13), anti-pyretic (14), hypnotic and sedative (9, 15), blood pressure lowering (14), diuretic and laxative (16), as well as anti-oxidant activity (17).

Pigmentation disorders is the third cause of skin consultation mainly in patients in range of 15-30 and 40-54 years old (18). So, in the present study we extracted the aerial parts of *V. odorata* with methanol. The extract then fractionated with ascending polarity solvents including *n*-hexane,

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Figure 1. The picture shows the leaves and flowers of *V. odorata*. (Ref: http://americanvioletsociety.org/Violet_Keys/Viola_odorata_002_L.htm; photographed by Susan Polan)

dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (BuOH), and finally water (H_2O) to investigate the anti-melanogenic activity of the plant on B16F10 murine melanoma cell line.

Materials and Methods

Preparation of plant extracts

Viola odorata was collected from Mashhad, Razavi Khorasan Province, Northeast of Iran in the flowering stage in month of July 2013. The plant was identified by Mrs. Suzani and voucher sample has been deposited at the herbarium of School of Pharmacy, Mashhad University of Medical Sciences, Iran (No. 12855).

The leaves were separated, dried in shadow and powdered. Then, 327 g of leaves powder were macerated with 95% methanol for 24 hr at controlled room temperature (23-27 °C). The mixture was percolated as described previously (19). The extract was concentrated via a rotary evaporator and subsequently freeze dried. This yielded about 15.3% (50 g) extract.

Thereafter, the methanol extract was re-dissolved in 95% of methanol, transferred to a decantation funnel and then fractionated with following solvents: *n*-hexane, CH_2Cl_2, EtOAc, BuOH and finally H_2O as schemed in Figure 2 (20).

Dried extract/fractions were dissolved in final concentration of DMSO lesser than 1% (v/v) plus completed RPMI-1640 medium. To evaluate the DMSO at this concentration had no significant effect on experiments; the same concentration was examined in separated group in each tests.

Cell culture

B16F10 murine melanocyte cell line was obtained from Pasture Institute, Tehran, Iran. B16F10 cells were cultured in completed RPMI-1640 medium (10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate and HEPES buffer) at 37 °C in 5% CO_2. Cells

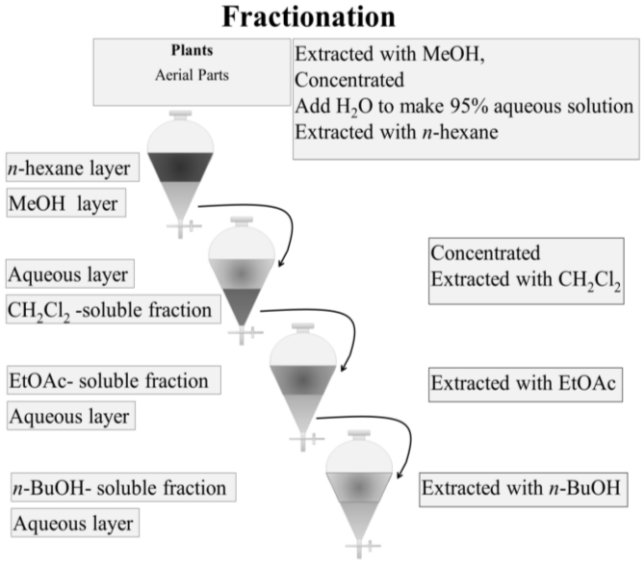

Figure 2. Extraction and fractionation of *Viola odorata* aerial parts

were passaged every 3 days until achieved confluent monolayers melanocytes, and then harvested using 0.05% trypsin and 0.54 mM ethylenediaminetetraacetic acid (EDTA). Kojic acid (2 mM) and medium containing DMSO were used as negative and positive control, respectively in all experiments.

Cell viability assay

Cell viability was examined using alamarBlue® dye. Upon entering this dye to viable cells, blue and non-florescent resazurin is converted to purple and detectable florescent dye (21).

About 10^4 B16F10 cells/100 µl were cultured in flat-96 well plate overnight. Next, a range of 1 to 32 µg/ml of *V. odorata* extract and fractions were added to B16F10 cells. After 48 hr, 15 µl of alamarBlue® solution (0.1 mg/ml) was added to each well and incubated at 37 °C for 3 hr. The absorbance was read using micro-plate reader (awareness Inc., US) at 570 and 600 nm. The assay was performed in triplicate and repeated four times for each sample. The cell viability was expressed as percent of live cells.

Analysis of mushroom tyrosinase function

The activity of mushroom tyrosinase as the enzyme which is responsible for converting levodopa (L-dopa) to dopamine was examined using spectrophotometric method as previously described (22, 23). Briefly, 160 µl of L-Dopa at the concentration of 5 mM with extract and fractions of *V. odorata* (1-64 µg/ml) were added to flat-96 well plate and incubated with 20 µl of mushroom tyrosinase (200 unit/ml) at 37 °C for 30 min. The absorbance was measured at 475 nm.

Measurement of cellular tyrosinase activity

Cellular tyrosinase activity was performed using spectrophotometric method with measurement of

oxidation of Dopa to Dopachrome (22, 23). In brief, B16F10 melanoma cells were cultured at density of 10^6 cells/ well in flat-6 well plate. Cells were mixed with or without different concentrations (1, 5, 10 and 20 µg/ml) of extract of *V. odorata* as well as kojic acid (2 mM) and incubated for 24 hr. The supernatant was removed and rinsed cells with phosphate buffer saline (PBS) twice. Next, cells were detached, transferred to 1.5 ml micro-tube and centrifuged at 1100 rpm for 7 min. 100 µl of lysis buffer contains 100 mM Na_3PO_4 (pH=6.8) was mixed with cells for 30 min on ice. After centrifugation at 10000 rpm at 4 °C for 20 min, 100 µl of 5 mM L-dopa was incubated with 100 µl of supernatants (each contain 100 µg the protein) at 37 °C for 2 hr. Cellular tyrosinase activity was measured by measuring the absorbance of each wells compared to control group at 475 nm.

Assessment of melanin content

B16F10 cells were cultured at density of 10^5 cells per well of flat-six well plate overnight. *Viola odorata* extract and fractions (1, 5 and 10 µg/ml) were added to well, and incubated for 24 hr. Then, the supernatants were removed and wells rinsed with PBS twice. Cells were detached and centrifuged at 1100 rpm for 7 min. 100 µl of 2 M NaOH was added to cells, incubated at 100 °C for 30 min and optical density measured using micro-plate reader (Awareness Inc., US) at 405 nm (22).

Evaluation of in vitro reactive oxygen species (ROS) level

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay is a fluorimetric probe for detection of oxidative stress (24). Reactive oxygen species level was measured as described previously with minor modifications (25). Twenty thousands of B16F10 melanoma cells were cultured in a flat-96 well plate and incubated overnight. Different concentrations (1, 5 and 10 µg/ml) of *V. odorata* extract and fractions were incubated with cells for 24 hr. Next, H_2O_2 at concentration of 24 mM was added to each well and incubated at 37 °C for 30 min. Then, 2.5 µl of DCFH-DA was added to the wells, and incubated for 30 min. The intensities were measured using ELISA reader (Awareness Inc., US) at excitation wavelength of 504 nm and emission wavelength of 524 nm.

Statistical analysis

The results were expressed as means±SEM. Group-data in comparison to un-treated group were performed using one way analysis of variance (ANOVA) with Dunnett's *post hoc* test by GraphPad Prism ® 6 (GraphPad Software, San Diego, CA) software. Significance was considered at $P<0.05$.

Results

The effect of extract and fractions of V. odorata on cell viability

All different concentrations of the extract and fractions of *V. odorata* led to a minimal decrease in cell viability which was not significant (Figure 3).

The effect of extract and fractions of V. odorata on cellular tyrosinase activity

All tested concentrations of both methanol extract and CH_2Cl_2 fraction, and three higher concentrations of BuOH fraction of *V. odorata* significantly decreased the cellular tyrosinase activity ($P<0.05$, Figure 4). Furthermore, three lower concentrations of both H_2O and EtOAc fractions, and two lower concentrations of *n*-hexane fraction of *V. odorata* led to a significant decrement ($P<0.05$ to 0.001, Figure 4).

The effect of extract and fractions of V. odorata on melanin content

Three concentrations of methanol extract, BuOH and EtOAc fractions and two lower concentrations of both H_2O and CH_2Cl_2 fractions could significantly decrease the melanin content in cells in comparison to negative control group ($P<0.01$, Figure 5).

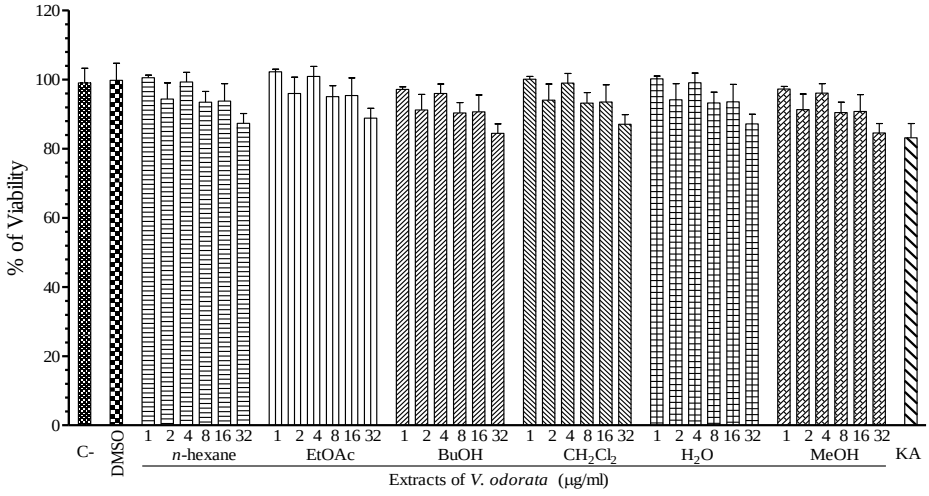

Figure 3. The effect of extract and fractions of *Viola odorata* on cell viability. Data were presented as mean±SEM

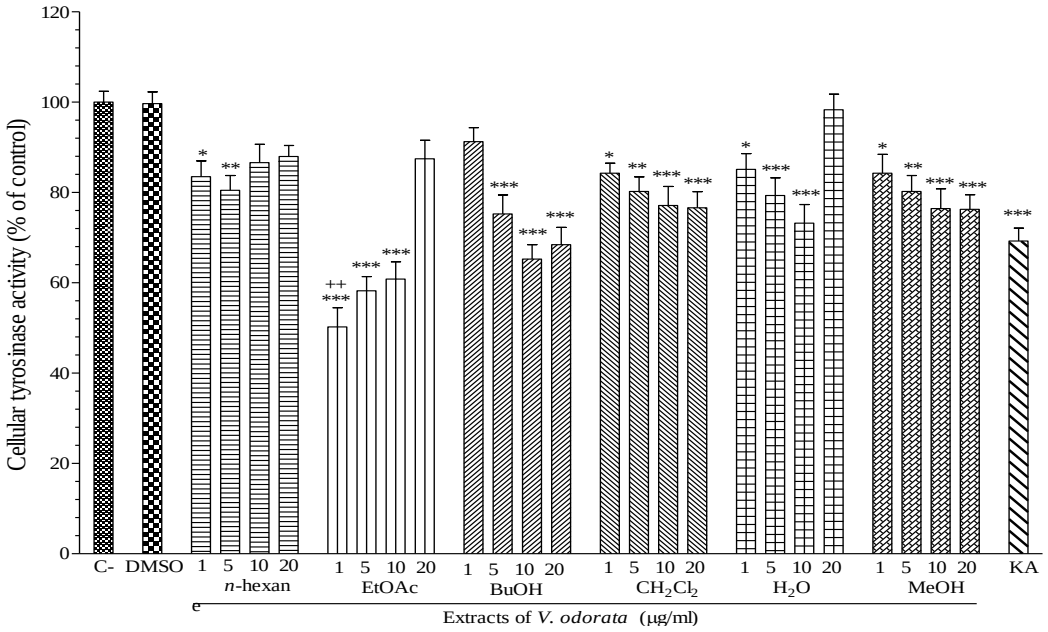

Figure 4. The effect of extract and fractions of *Viola odorata* on cellular tyrosinase activity. Data are presented as mean±SEM. **P*<0.05, ***P*<0.01 and ****P*<0.001 compared to negative control. ++*P*<0.01: compared to KA

The effect of methanol extract and different fractions of V. odorata on mushroom tyrosinase activity

All tested concentrations of extract and fractions had non-significant lowering effect on mushroom tyrosinase activity (Figure 6).

The effect of methanol extract and different fractions of V. odorata on ROS production

Concentrations of 1-10 µg/ml of methanol extract, and BuOH and EtOAc fractions significantly decreased ROS production, while the concentrations of 1 and 5 µg/ml of both H_2O and CH_2Cl_2 fractions of *V. odorata* had significant decreased ROS in comparison to control untreated group (*P*<0.01 to 0.001, Figure 7).

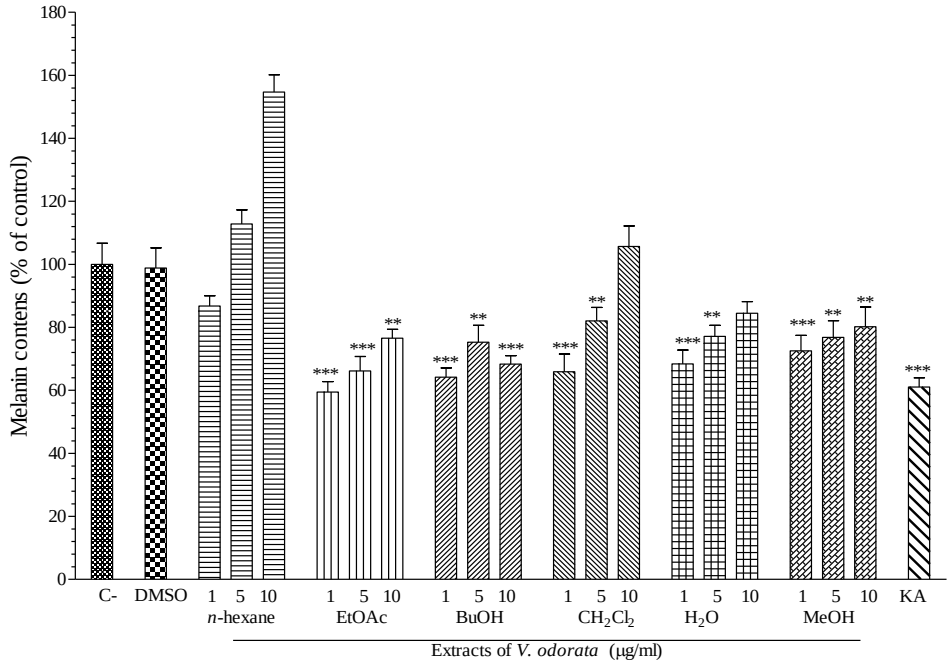

Figure 5. The effect of extract and fractions of *Viola odorata* on melanin content. Data were presented as mean±SEM. ***P*<0.01 and ****P*<0.001 compared to negative control

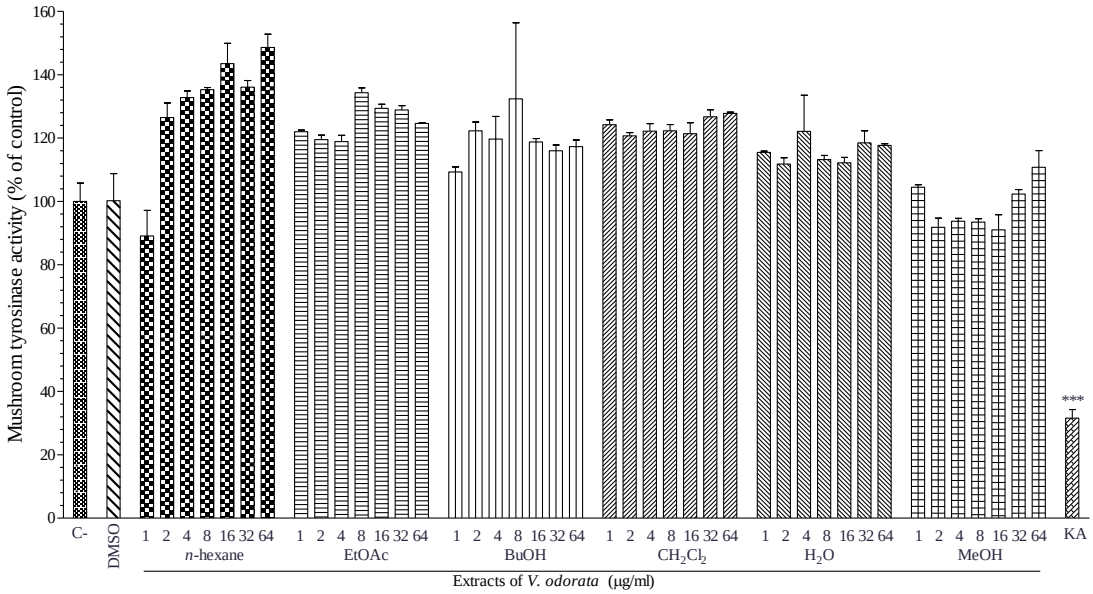

Figure 6. The effect of extract and fractions of *Viola odorata* on mushroom tyrosinase activity. Data were presented as mean±SEM. **P*<0.05, ***P*<0.01 and ****P*<0.001 compared to negative control

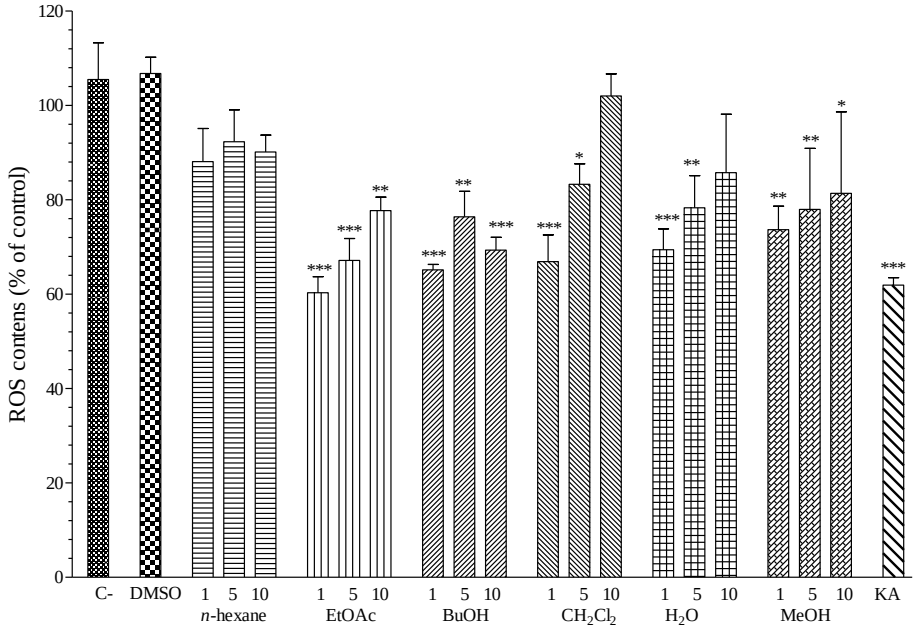

Figure 7. The effect of extract and fractions of *Viola odorata* on *in vitro* reactive oxygen species (ROS) production. Data were presented as mean±SEM. **P*<0.05, ***P*<0.01 and ****P*<0.001 compared to negative control

Discussion

According to available information, this study is the first original research on antimelanogeic activity of *V. odorata.* In this study the effect of different extracts and fractions of *V. odorata* including methanol extract and *n*-hexane, CH_2Cl_2, BuOH, EtOAc and H_2O fractions on proliferation, cellular and mushroom tyrosinase activity, melanin content and ROS production were examined using B16F10 murine melanoma cell line.

Methanol extract and fractions of *V. odorata* showed no significant effect on cell viability as mentioned in Figure 3. Although *V. odorata* at concentrations 1-32 µg/ml had minimal effect on viability of cells but it was not significant. Based on our results, concentrations equal and less than 32

µg/ml were chosen for more evaluations.

Hyperpigmentation disorders are considered as inflammatory problems and have different causes such as excessive activity of melanocytes and increased melanin aggregation that leads to dark areas called spot. In addition, many factors can lead to spots including pregnancy, oral contraceptive pills, aging, excessive use of anti-acne drugs, cosmetics, perfumes and some chemical materials, etc.(6).

Melanin is composed and secreted by melanocytes during a regular and complex process in human skin (26). Sunlight exposure and especialy UV radiation stimulates melanocytes to synthetize melanin to protect skin (7). Skin pigmentation is determined by different physiologic processes of melanocytes creation, expression of the melanosomes residuals, melanosomes transferring to keratinocytes and melanin distribution in the skin (27-29). Successful treatment usually obtained by combining two or more mechanisms through synergism effect (30). Furthermore, it has been demonstrated that tyrosinase has an important role in melanin formation and transferring to adjacent cells (6).

Kojic acid (5-hydroxy-2-hydroxy methyl-4-pyron) is a natural compound that is obtained from *Aspergillus niger*, *Acetobacter* spp. and *Penicillium* spp., and is used at concentrations of 1 to 4% as skin whitening agent (31). In this study, kojic acid, as positive control, was used at concentration of 2 mM and it significantly decreased cellular and mushroom tyrosinase activity, melanin content and ROS production. It has been shown that kojic acid inhibits tyrosinase through chelating Cu^{2+} in the active site of the enzyme (32), antioxidant and free radical scavenging activity (7). In contrast of good efficacy, limited application of kojic acid is due to toxicity and the side effect of the substance in inducing allergic dermatitis (33).

Herbal products are so popular among people due to naturalness and the belief to be non-toxic substitutes, but studies have been shown the toxicity of some plant. In melanocytes, decrease in the skin pigments production and the toxic impact limited the use of plant for hyperpigmentation problem. Hydroquinone is one of the compounds which irritates skin, induces toxicity for melanocytes and causes contact dermatitis and skin depigmentation (34). Also, it has carcinogenic effects which have been reported on rodents (35). Hydroquinone derivatives, ether monobenzyl hydroquinone and mono methylether hydroquinone, could also inhibit tyrosinase and have toxicity similar to hydroquinone (36, 37).

Rate limiting and first step of melanin synthesis is oxidation of tyrosine to dopa-quinone through acting tyrosinase (38). Tyrosinase catalyze and oxides the monophenols and diphenols to quinines (6). Considering the results, methanol extract of *V. odorata* at concentrations 1-20 µg/ml could significantly decrease tyrosinase activity in a concentration dependent manner. Among all extract and fractions of *V. odorata,* EtOAc extract that contains medium polarity components such as flavonoids was more effective than other extracts.

In the next step, the melanin content was measured to confirm the cellular tyrosinase results. All extract and fractions except the *n*-hexane extract, could significantly decrease melanin content. However, EtOAc fraction had stronger effects than other extract and fractions which supports the results obtained in cellular tyrosinase test.

Herbal antioxidants such as glutathione, phytic acid and ubi-quinone (39, 40) could decrease the intracellular oxidative stress and play a protective role against disorders associated with oxidative stress (7). Herbal antioxidants could decrease the oxidation of tyrosin to dopaquionone and melanin synthesis in the melanogenesis pathway (41). Aging process will be accelerate in all body parts including skin as a result of the gradual accumulation of ROS which causes skin damages in the skin exposed to sunlight. Melanin has an important role in absorbing free radicals which is produce in cytoplasm of the cells irritated by UV irradiation (42). Antioxidants have important role in decreasing free radicals and the melanin content. Considering the results, extract and fractions of *V. odorata* could reduce ROS induced by hydrogen peroxide and the EtOAc extract was more effective than other extract and fractions. Furthermore, it has been shown that concentrations between 25-800 µg/ml of *V. odorata* have antioxidant activity in radical scavenging by DPPH, Fe^{2+} chelating, nitric oxide and hydrogen peroxide chelating tests (17).

In addition, according to investigations and literature survey we have done, there is no similar study on the genus Viola which emphasizes more investigations on other species.

Conclusion

In summary, it can be suggested that different extracts and fractions of *V. odorata* could decrease total melanin content, cellular tyrosinase activity and oxygen free radicals in B16F10 cells and could have promising therapeutic effects on skin inflammatory diseases such as hyperpigmentation. In addition, our evaluation presented that the ethyl-acetate fraction had best anti-melogenic activity compared to other fractions or extract. However, we suggest that more basic and clinical studies will be done on ethyl-acetate fraction to establish this effect.

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Conflict of interest

Authors declare that there is no conflict of interest.

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