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Original Article

Inhibitory effects of different fractions of *Nepeta satureioides* on melanin synthesis through reducing oxidative stress

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Abstract

Nepeta satureioides Boiss. has been used in traditional medicine of eastern countries and is famous for its medicinal properties. The aim of this study was to evaluate the effect of methanol (MeOH), *n*-hexane and dichloromethane (CH_2Cl_2) fractions of the extract on melanin synthesis and oxidative stress in B16F10 melanoma cell line. The B16F10 cell line viability after treatment with increasing concentrations of different fractions of the plant (5-60 µg/mL) was measured using MTT assay. The inhibitory effect on synthesis of melanin, mushroom tyrosinase activity, cellular tyrosinase and oxidative stress were determined by the colorimetric and fluorometric methods. The data showed that at concentrations below 60 µg/mL, fractions did not show significant toxicity on melanoma cells. The amount of melanin synthesis by MeOH and CH_2Cl_2 fractions and mushroom tyrosinase activity by the MeOH fraction declined in B16F10 cells. In addition to the capacity of MeOH, *n*-hexane and CH_2Cl_2 fractions in decreasing the amount of reactive oxygen species (ROS) in melanoma cells, all fractions revealed remarkable antioxidant activity. The melanogenesis inhibitory and antioxidant effects of *N. satureioides* on B16F10 cells may suggest this plant as a new pharmaceutical agent in reducing skin pigment and skin aging in cosmetic industry.

Keywords: Melanogenesis; Tyrosinase; Melanoma; Nepeta satureioides Boiss; Lamiaceae

INTRODUCTION

Natural products with melanin synthesis inhibitory activity have received much attention because they are associated with hyperpigmentation disorders in humans such as lentigo, nevus, ephelis, post-inflammatory state and melasma mask of pregnancy. Increased number of melanocytes or activity of melanogenic enzymes causes hyperpigmentation (1). Melanin is a group of natural pigment produced by the oxidation of the amino acid tyrosine. Although melanin is involved in many processes such as thermoregulation, cation chelation and antibiotic activity but photoprotection and absorbing of ultra violet radiation (UVR) are known as the main function of melanin. Therefore, melanin is responsible for protection of skin cells from UVR damage.

Tyrosinase is one of the main enzymes in the regulation of the first two steps of melanin synthesis pathway and catalyzes the oxidation of L-tyrosine to 3,4-dihydroxyphenyl-L-alanine (L-DOPA) which is then oxidized to dopaquinone and finally produces melanin (2).

Plant extracts with the tyrosinase inhibitory activity reduced melanogenesis and used in pharmaceutical and cosmetic industries (2). It has been reported that hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) are produced in the melanogenesis process by UV irradiation.

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ROS is involved in the regulation of melanin synthesis and UV-induced melanogenesis could be repressed with inhibition of ROS generation (3). Also it is well known that exposure to UV radiation increases the ROS production that is involved in the pathogenesis of several skin conditions including aging, wrinkles, photo-sensitivity and malignancy (4). The B16F10 murine melanoma cell line is considered as a good model for studying human melanoma (5). *Nepeta* (Lamiaceae) contains about 250 perennial species found mostly in Europe and Asia as well as North Africa (6). There are 79 species of this genus found in Iran of which 38 species are endemic to the country (7). *Nepeta satureioides* Boiss. is an annual herb, which is grown in Central Asia, Afghanistan, Pakistan and Iran (8).

The genus *Nepeta* from Lamiaceae, consist of 79 herbaceous perennial and rarely annual species growing in Iran. Thirty eight of these species are endemic to the country. *N. satureioides* Boiss. is one of the aromatic plants. This medicinal plant is traditionally used for common cold, sinusitis and as a carminiative. Infusion provided from the aerial parts of *N. satureioidesis* are used in folk medicine as a carminative and for cure of colds and bronchitis (9-11). However, there are no scientific reports on melanogenesis inhibitory activity of *N. satureioides*. Thus, the aim of this study was to investigate the inhibitory effect of methanol (MeOH), *n*-hexane and dichloromethane (CH_2Cl_2) fractions of *N. satureioides* on melanogenesis and to evaluate the potential antioxidant characteristics of the plant on B16F10 melanoma cells.

MATERIALS AND METHODS

Preparation of extracts

N. satureioides was collected in June 2013 from Gonabad altitude of Razavi Khorasan province located in the northeast of Iran. A voucher specimen of the plant with ID 11259 was deposited in the herbarium of School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. Aerial parts of *N. satureioides* (100 g) were grounded by a blender (Toos shekan Co, Iran) and then perculated with MeOH at room temperature for 24 h according to previously reported protocol (12). After extraction, the solvent was evaporated using a rotary evaporator and then freeze dried. The freeze dried extract (10 g) was then subjected to subsequent fractionation with solvent of increasing polarity including *n*-hexane, CH_2Cl_2, ethylacetate, *n*-butanol and water.

Toxicity assessment of the extracts on melanoma cell line

B16F10 melanoma cell line (Cat. No C540) was purchased from the Pasteur Institute of Iran (Tehran, Iran) and 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) fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. In order to evaluate the cytotoxic effect of different fractions of *N. satureioides* on cells, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cytotoxic assay was performed (13). To monitor cell viabilities, cells (2×10^4 cells per well) seeded in a 96-well plate overnight. The cells then exposed to different concentrations of fractions (5 - 60 µg/mL) for 24 h.

Determination of melanin content of melanoma cells

B16F10 cells were cultured in a 12-well plate overnight and then were treated with different concentrations (5-40 µg/mL) of MeOH, *n*-hexane and CH_2Cl_2 fractions of *N. satureioides*. After 24 h, the cells were detached by trypsin and then were washed with PBS. The cell pellets were solubilized in 100 µL solution of sodium hydroxide (2 M) for 30 min at 100 °C. Melanin content was compared with that of the control by their absorptions at 405 nm (14).

Determination of mushroom tyrosinase activity in melanoma cells

Tyrosinase activity was performed according to the method of Hyun, *et al*. with some modifications (15). Briefly in a 96-well microplate, 10 µL of each sample with different concentrations of the fractions

(5-40 µg/mL) and 10 µL of mushroom tyrosinase in the phosphate buffer solution (pH = 6.8) were added. The plate incubated for 30 min at 37 °C and the amount of dopachrome produced in the mixture was measured by a spectrophotometer at 490 nm. The percentage of inhibition was calculated by the following equation:

Inhibition of tyrosinase activity (%) = A× 100/ (B-A)

where, A is the mean absorption values measured for the different fraction concentrations and B is the average absorption values in the control group.

Determination of cellular tyrosinase activity in melanoma cells

The tyrosinase activity in B16F10 cells was determined by measuring the rate of oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) (16). In summary, 2×10^4 B16F10 cells were plated in a 24-well plate. The cells were then incubated with MeOH, *n*-hexane and CH_2Cl_2 fractions of the plant at 5-40 µg/mL for 24 h.

Then cells were washed with PBS and were lysed in PBS containing 1% Triton X-100 and mixed with 2 mL of L-DOPA (2 mg/mL) for 20 min. After incubation for 2 h at 37°C then the absorbance was read at 475 nm using a micro-plate reader (BioTek, USA).

Determination of cellular ROS levels

B16F10 melanoma cells (10^4 × 2) were seeded in 96-well plates overnight and then were treated with different concentrations of MeOH, *n*-hexane and CH_2Cl_2 of plants at 5-40 µg/mL for 24 h. Then cells incubated with 50 µL H_2O_2 (24 mM) at 37 °C for 30 min. Then 50 µL of dichloro-dihydro-fluorescein diacetate (DCFH-DA) were added to the cells and the fluorescence intensity of dichloro-fluorescein (DCF) was measured at 528 nm emission and 485 nm excitation using a Synergy H4 microplate reader (BioTek, USA).

Statistical analysis

All experiments were repeated in triplicate and the relative results were presented as the mean ± SD of the three independent measurements. Analysis of variance was performed using one-way ANOVA test with GraphPad Prism 5.0 and the means were compared by Dunnett tests. $P < 0.05$ stands for statistically significant difference between extract-treated cells and control.

RESULTS

Effect of different fractions of N. satureioides on cell survival

In this study, MTT assay was performed in order to monitor cell viability. B16F10 cell lines were seeded in a 96-well plate. After 24 h cells were treated with different concentrations of MeOH, *n*-hexane and CH_2Cl_2 fractions of *N. satureioides*. As illustrated in Fig. 1, results showed that treatment of the cells with concentration of 60 µg/mL of MeOH, *n*-hexane and CH_2Cl_2 fractions significantly reduced their cell viability ($P < 0.05$) and induced the cell death ($P \leq 0.05$).

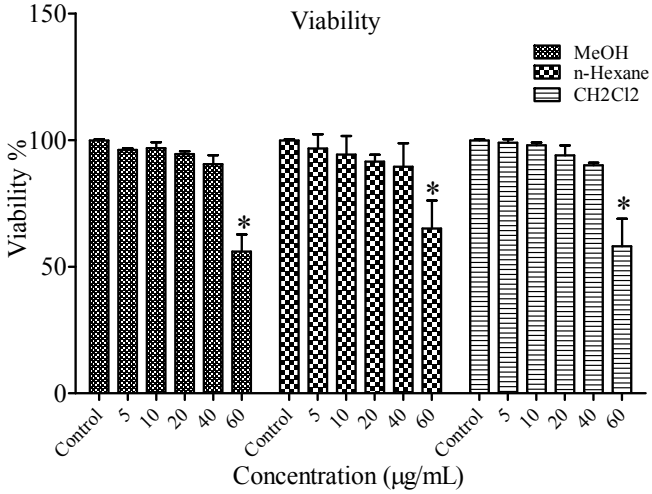

Fig. 1. Effect of different concentrations of MeOH, n-hexane and CH_2Cl_2 fractions of *N. satureioides* on cell viability *($P < 0.05$).

Effect of different fractions of N. satureioides on the synthesis of melanin

To determine the antimelanogenic activity, the inhibitory effect of different fractions of MeOH, *n*-hexane and CH_2Cl_2 of *N. satureioides* were assessed on melanin content of B16F10 cells. Kojic acid (2 mM) was utilized as a positive standard. The results showed that the concentration of 15, 20 and 40 µg/mL of MeOH and 20 and 40 µg/mL of CH_2Cl_2 fractions had inhibitory effect on melanin synthesis (Fig. 2).

Effect of different fractions of N. satureioides on mushroom tyrosinase activity

The tyrosinase enzyme inhibition experiment using L-DOPA as substrate and mushroom tyrosinase as enzyme source was performed to assess the inhibitory effect of *N. satureioides* on mushroom tyrosinase activity. The results indicated that mushroom tyrosinase activity was inhibited only by the concentration of 20 µg/mL of MeOH fraction. Kojic acid (2 mM) was used as positive control (Fig. 3).

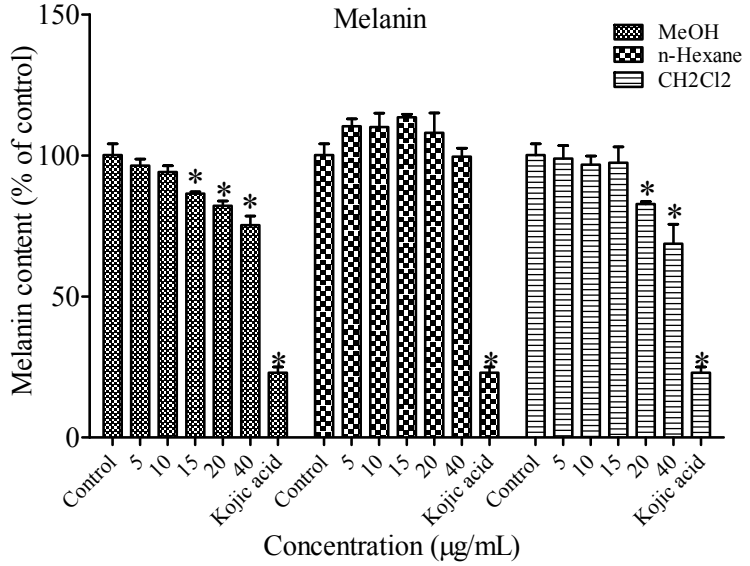

Fig. 2. Effect of different extracts of *N. satureioides* on melanin content in B16F10 murine melanoma cells $*P < 0.05$ as compared to control.

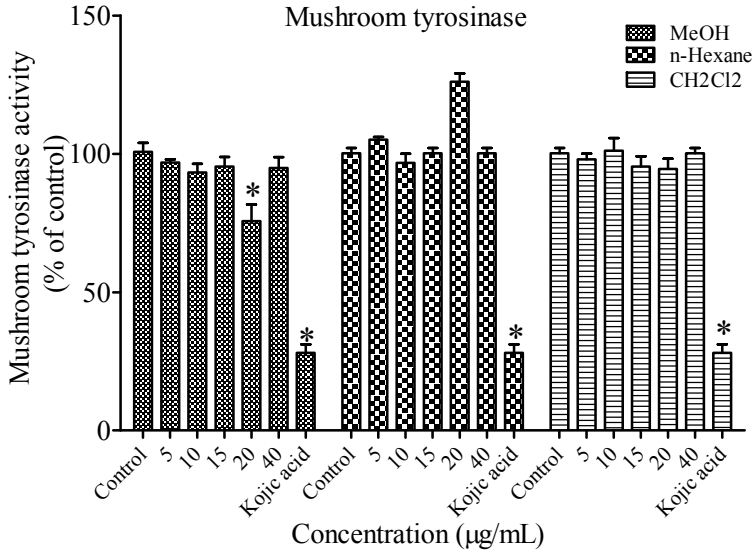

Fig. 3. Effect of different extracts of *N. satureioides* on mushroom tyrosinase activity in B16F10 murine cells. $*P < 0.05$ as compared to control.

Effect of different fractions of N. satureioides on cellular tyrosinase activity

To evaluate the mechanism of the inhibitory effect of *N. satureioides* extract on melanogenesis in particular, we determined intracellular tyrosinase activity in B16F10 melanoma cells. The results indicated that all fractions of *N. satureioides* at 20 µg/mL and *n*-hexane and CH_2Cl_2 fractions at 40 µg/mL could significantly inhibit cellular tyrosinase activity (Fig. 4).

Effect of N. satureioides on cellular ROS cellular level

The intracellular ROS levels as an indicative of antioxidant capacity of *N. satureioides* was measured in cells treated with 24 mM H_2O_2 alone or with MeOH, *n*-hexane and CH_2Cl_2 fractions in B16F10 melanoma cells.

As shown in Fig. 5 cells pretreated with plant extracts could significantly suppress the oxidative stress induced by H_2O_2 (Fig. 5).

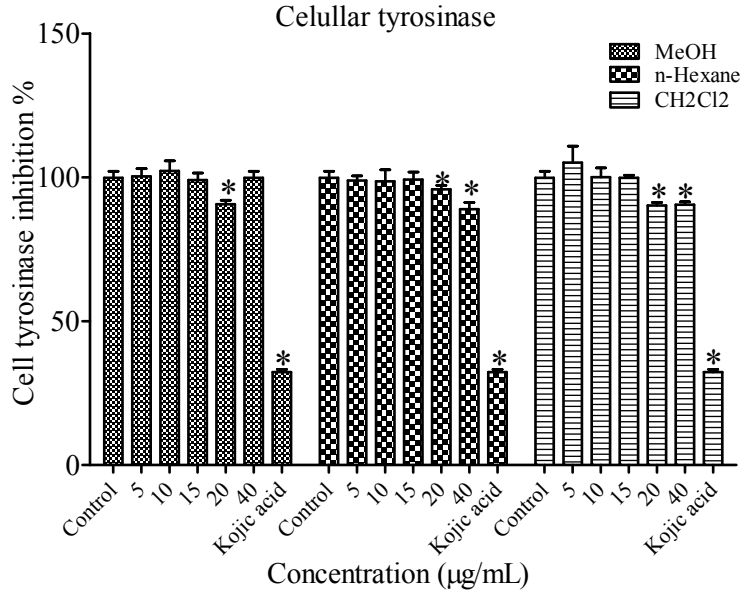

Fig. 4. Effect of different extracts of *N. satureioides* on cellular tyrosinase activity in B16F10 murine melanoma cells. *P < 0.05 compared to control.

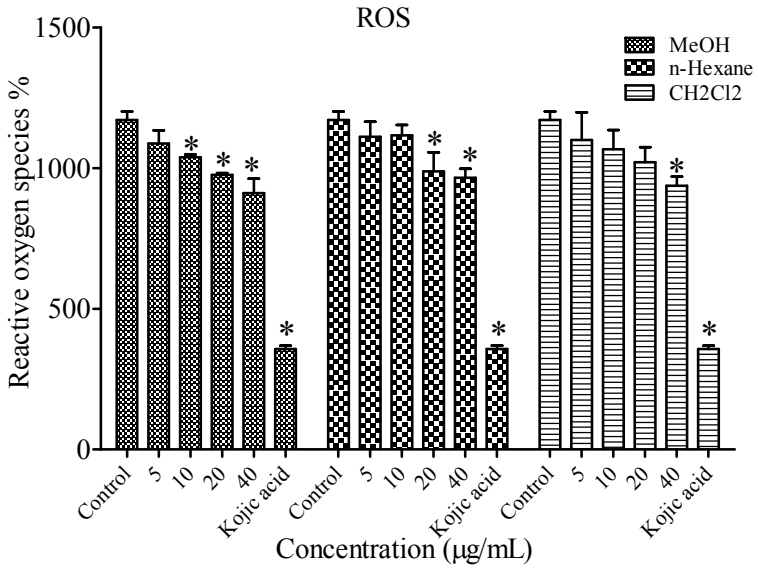

Fig. 5. Antioxidant effects of different extracts of *N. satureioides* on cellular reactive oxygen species (ROS) levels in B16F10 murine melanoma cells. *P < 0.05 compared to control.

DISCUSSION

Chemicals and natural base preparations have been widely used for the prevention of skin hyper pigmentation disorders. Inhibition of the melanin formation, inhibition of the tyrosinase activity, scavenging of free radicals and preventing the translocation of melanosomes from melanocytes in to epidermal cells are pathways mainly affected by agents used in the treatment of hyperpigmentation (17).

In the current study, to identify the appropriate concentration of the plant fractions which do not affect the viability of B16F10 melanoma cells, the MTT assay was performed with *N. satureioides* different fractions. The MTT assay is a common colorimetric assay to determine the dead cells from living cells. Different concentrations (5-60 µg/mL) of MeOH, n-hexane and CH_2Cl_2 fractions for 24 h showed, at concentration equal or higher than 60 µg/mL all fractions were significantly cytotoxic. Consequently, we have chosen concentration below 60 µg/mL to determine the inhibitory effect of *N. satureioides* on melanogenesis. The results indicated that MeOH fraction could significantly inhibit the mushroom tyrosinase activity at 20 µg/mL. In order to distinguish if inhibition in the mushroom tyrosinase activity is resulted from the inhibition of endogenous tyrosinase activity, the inhibition in intracellular tyrosinase activity by *N. satureioides* was measured. Although *n*-hexane and CH_2Cl_2 fractions did not show significant effect on mushroom tyrosinase activity but all fractions were able to reduce the cellular tyrosianse activity which was in accordance with the decrease in melanin production in the cells for MeOH and CH_2Cl_2 fractions. In order to evaluate the antioxidant properties of this plant extract, intracellular ROS levels was measured. DCFH-DA was used as indicator for ROS measurement. DCFH-DA is hydrolyzed by the endogenous esterases to dichloro-dihydro-fluorescein (DCFH) and then DCFH reacts with ROS such as H_2O_2 to produce DCF. The fluorescent intensity of DCF as a product of oxidation of DCFH by intracellular radicals is compared in samples (18). Our results indicated all fractions of *N. satureioides* were able to reduce the free radical levels in a dose dependent manner.

Main component of *Nepeta* species are nepetalactones, iridoids and their glucosides, diterpenes, triterpenes and flavonoids which are responsible for biological activities such as antibacterial, antifungal and antiviral (9).

This is the first attempt to evaluate the inhibitory effect of *N. satureioides* on melanogenesis in B16F10 melanoma cells. The results of the present study confirmed the inhibitory effect of *N. satureioides* on melanogenesis process may facilitate through antioxidant capacity of the plant. In accordance to our results some other studies have been reported the combination effect of antioxidant and antimelanogenic for chestnut flower extract (19), and *Magnolia grandiflora* L. (20) as shown for *N. satureioides*.

The MeOH and CH_2Cl_2 fractions have decreased cellular tyrosinase activity, melanin content and ROS. Semipolar nature of MeOH and CH_2Cl_2 may extract phytochemicals which are responsible for antimelanogenic activity. Although the *n*-hexane fraction could decrease ROS and cell tyrosinase activity but it could not show any inhibitory effect on melanin content which may indicate that plant phytochemicals with nonpolar nature have not significant effect on the melanogenesis process (21,22).

There are many reports about plants to have the inhibitory effect on melanin synthesis and tyrosinase which may have a high potential for the treatment of skin disorder. For example Phetdee, *et al.* showed tamarind seed coat extract can reduce melanin production by 20-32% (23).

In another research ethanol leaf extracts (500 µg/mL) of *Aloe ferox* Mill, *A. aculeata* Pole-Evans, *A. pretoriensis* Pole-Evans, and *A. sessiliflora* Pole-Evans exhibited 60%, 31%, 17%, and 13% inhibition of tyrosinase activity respectively. Also bark extract of *Harpephyllum caffrum* Bernh. (500 µg/mL) showed good inhibitory effect on tyrosinase and melanin production so it has the potential to be used as the source of chemical ingredients for antpigmentation therapy (24).

The potential inhibitory effect of *Magnolia grandiflora* L. flower extract, *Paeonia suffruticosa* Andrews, chestnut flower extract and leaf extracts from wild bitter melon on melanogenesis also have been reported (20,25). Some compounds isolated from methanolic extract of the flower buds of *Cananga odorata* showed the inhibitory effect on melanogenesis which may be effective in skin diseases (26).

Based on our results *N. satureioides* with dual actions of antioxidant and antimelanogenic activities may contribute in skin whitening and could be included in cosmetic formulations of skin care products.

CONCLUSION

In this study, MeOH and CH_2Cl_2 fractions of *N. satureioides* showed potential effects against melanin production in B16F10 melanoma cells. This is the first report about the effect of *N. satureioides* on melanin production. It is found that *N. satureioides* also expressed antioxidant activities. The results suggested that the ability of *N. satureioides* to decrease melanin production may be attributed to its inhibitory action upon the signaling pathway regulating tyrosinase activity or depletion of cellular ROS.

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