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Original Article

# Anti-Melanogenic Activity of Different Extracts from Aerial Parts of *Nepeta glomerulosa* on Murine Melanoma B16F10 Cells

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## Abstract

*Nepeta glomerulosa* Boiss. is a medicinal plant used in traditional medicine. The aim of this study was to evaluate the anti-melanogenesis inhibitory activity of Methanol (MeOH), *n*-hexane, acetate (EtAc), dichloromethane ($CH_2Cl_2$), *n*-butanol (BuOH), and $H_2O$ extracts isolated from *N. glomerulosa* in B16 melanoma cell line. The B16F10 cell line viability after treatment with increasing concentrations of different extracts of the plant (50-200 µg/ml) was measured by using MTT. The inhibitory effect on synthesis of melanin, mushroom tyrosinase activity, cellular tyrosinase, and effect on oxidative stress was determined by the colorimetric and fluorometric method. The data showed that at concentrations <200 µg/ml, all extracts did not show significant toxicity on melanoma cells. The amount of melanin synthesis by MeOH and $CH_2Cl_2$ extracts and mushroom tyrosinase activity by the MeOH extract declined in B16F10 cells. In addition to the capacity of MeOH, *n*-hexane and $CH_2Cl_2$ extracts in decreasing the amount of reactive oxygen species (ROS) in melanoma cells all extracts revealed the remarkable antioxidant activity. Our results showed the melanogenesis inhibitory and antioxidant effects of *N. glomerulosa* on B16F10 cells may recommend a novel agent in diminishing skin pigmentation and skin aging in cosmetic industry.

*Keywords:* Melanogenesis, Melanoma, *Nepeta glomerulosa*, Tyrosinase

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## 1. Introduction

Melanin, which is a mixture of heterogeneous biopolymers, is responsible for the color of human skin, eyes, and the hair. It is synthesized through melanosomes, the membrane-bound granules in the melanocytes and it then moved to keratinocytes. Varieties of environmental, genetic and hormonal factors regulate the melanogenesis process [1-4]. It is found that ROS involves in the regulation of melanin synthesis. Exposure to ultraviolet (UV) radiation increases the hydrogen peroxide ($H_2O_2$) and other reactive oxygen species (ROS) within the melanogenesis process [5].

Tyrosinase, which is one of the main enzymes in the regulation of melanin synthesis, hydrolysis tyrosine to L-DOPA (3, 4-dihydroxyphenyl-L-alanine), then oxidizes L-DOPA to DOPA quinone and finally produces melanin [6]. In addition, microphthalmia-associated transcription factor (MITF) and other factors such as tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2) contribute in production of melanin [7, 8]. Natural products such as kojic acid [9], ascorbic acid derivatives [10], retinoic acid [11], azealic acid [12] and arbutin [13] which can target tyrosinase, are commercialized and used for treatment of some of the hyperpigmentary skin conditions such as melasma, lentigo, freckling, and other dermatological disorders [14].

Although catechins [15], hydroquinone [16], aloesin [17] and 4, 4′-dihydroxybiphenyl [18] are known as chemical with tyrosinase-inhibitory properties, the effectiveness of these products is limited because of their adverse effects, modest skin penetrations, and poor formulation stabilities [19-21]. So, plant extracts with inhibitory effect on melanin synthesis could be suitable choice due to their relatively lower side effects.

The genus *Nepeta* belongs to the family of Lamiaceae with approximately 250 species that most of them are widely distributed in Eurasia [22, 23]. It was found that 79 species of this genus are growing in Iran [24] from which 38 species are endemic [25]. Different biologic activities such as antinociceptive (*N. italica*) [26], antiviral (*N. cataria*) [27], and antimicrobial (*N. crispa*) have been reported for *Nepeta* species [28]. *Nepeta glomerulosa* is a medicinal plant found especially in Razavi Khorasan and Isfahan provinces. The aerial parts of *N. glomerulosa* have been used empirically in

folk medicine as diuretic, antiseptic, antispasmodic, antitussive, anti-asthmatic, and febrifuge agents [29].

In the present study, the anti-melanogenesis inhibitory activity of methanol (MeOH), *n*-hexane, ethyl acetate (EtAc), dichloromethane ($CH_2Cl_2$), *n*-butanol (BuOH), and $H_2O$ extracts isolated from *N. glomerulosa* in B16 melanoma cells were investigated.

## 2. Materials and Methods

### *2.1. Preparation of Extracts*

*N. glomerulosa* was collected in June 2013 from Gonabad to Ferdows altitude Razavi Khorasan province, Northeast of Iran. A voucher specimen (No:13062) has been deposited in the Herbarium of School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran. The dried powder of aerial parts of *N. glomerulosa* (100 g) were grounded by a blender and then extracted with MeOH at room temperature for 24 h. After extraction, the solvent was evaporated using rotary evaporator and then freeze dried. The extract (10 g) was then subjected to subsequent extractation with solvent of increasing polarity including *n*-hexane, $CH_2Cl_2$, EtAc, *n*-BuOH, and $H_2O$.

### *2.2. Cell culture*

B16F10 (melanoma cell line) was purchased from the Pasteur Institute (Tehran, Iran) and maintained under standard culture conditions (37°C in a humidified atmosphere (90%) containing 5% CO2). The Cells were seeded and propagated 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 extracts on melanoma cells, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxic assay was performed [30]. To monitor cell viabilities, the cells ($2\times10^4$ cells per well) were seeded in 96-well plate overnight. The cells were then exposed to different concentrations of different extracts for 48 h (0-200 µg/ml).

### *2.3. Mushroom Tyrosinase Activity Assay*

Tyrosinase activity was performed according to the method of Hyun et al. (2008) with some modifications [31]. Briefly in a 96-well microplate 100 µl of each sample with different extracts of *N. glomerulosa* (100 µg/ml) and 10 µl of mushroom tyrosinase in a phosphate buffer solution (pH=6.8) were added together. The plate was incubated for 30 min at 37 °C and the amount of dopachrome produced in this mixture was measured by spectrophotometer at 490 nm. The percentage of inhibition was calculated by the following equation: inhibition of tyrosinase activity (%) = A× 100/B, where B is average absorption values in the control group, and A is the mean

absorption values measured for concentration of the different factions.

### *2.4. Determination of Melanin Content in Melanoma Cells*

B16F10 cells were seeded in plate 12 wells overnight and then were treated with different extracts of *N. glomerulosa* (100 µg/ml). After 24 h, the cell pellets were resuspended in 100 µl solution of sodium hydroxide (2 M) for 30 min at 100°C until it was completely dissolved. Melanin content in the solution was determined and compared with control by comparison of the absorptions at 405 nm using a micro-plate reader (BioTek, USA) [32].

### *2.5. 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) [33]. In summary, $2\times10^4$ B16F10 cells were plated in 24-well plate. The cells were then incubated with different extracts of *N. glomerulosa* (100 µg/ml) for 48 h. Then the treated and untreated 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, the absorbance at 475 nm was read using a micro-plate reader (BioTek, USA).

### *2.6. Determining The Level of Cellular ROS*

B16F10 melanoma cells ($10^4\times2$) were seeded in 96 well plates overnight and then were treated with different extracts of *N. glomerulosa* (100 µg/ml) for 48 h. Then, the cells incubated with 50 µl $H_2O_2$ (24 mM) at 37°C for 30 min. Then, 50 µl of DCFH-DA were mixed to the cells and the fluorescence intensity of DCF was measured at 528 nm emission and 485 nm excitation using a Synergy H4 microplate reader (BioTek, USA).

### *2.7. Western Blotting*

B16F10 Cells were harvested and treated with different extract of *N. glomerulosa* extract (100 µg/ml). After incubation for 24 h, the cells were washed twice with cold PBS buffer and centrifuged at 1500 rpm then cell pellet lysed in lysis buffer, containing Tris 50 mM pH 7.4, 2 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 10 mM β-glycerol-phosphate, 0.2% W/V sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and complete protease inhibitor cocktail (Sigma, P8340), was then centrifuged at 10,000 rpm for 30 min at 4°C. Utilizing bovine serum albumin as standard protein concentration was quantified by Bradford method. Briefly, equal amounts of protein extracts (50 µg) from each extract were loaded on 12% sodium dodecyl sulfate-polyacrylamide

(SDS) gel and after electrophoresis transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked overnight in 5% skim milk in PBST buffer (PBS with 0. 05% Tween-20) and after a brief wash with PBST buffer, incubated overnight with (4°C) anti-tyrosinase (H-109) [sc-15341] and anti-MITF (microphthalmia-associated transcription factor) (H-50) [sc-25386] antibodies. Later, the primary antibody was removed. Subsequent washing the membranesin PBST buffer, the PVDF incubated with goat anti-mouse antibody (1:2000) labeled with horseradish peroxidase was performed at room temperature for 2 h.

Protein bands were visualized and analyzed by an enhanced chemiluminescence (Pierce ECL Western blotting substrate) and Alliance gel doc (Alliance 4.7 Gel doc, UK). The relative amounts of MITF and tyrosinase compared to total b-actin protein were normalized and analyzed with, UV TEC software (UK).

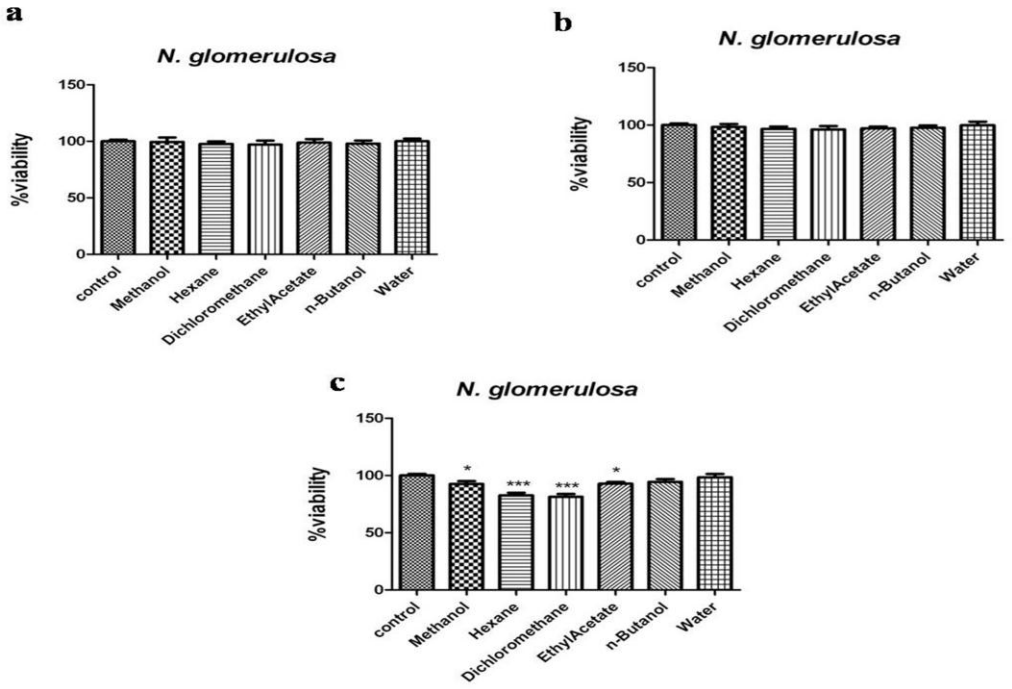


**Figure 1**. a) Effect of different extracts of *N. glomerulosa* extract (50 µg/ml) b) (100 µg/ml) c) (200 µg/ml) on cell viability. After incubation of B16F10 melanoma cells with concentration of 50-200 µg/ml of extracts of *N. glomerulosa* in a 96-well plate for 48h, cell viability was determined by MTT assay. Only 200 µg/ml of some extracts significantly reduced their cell viability. Data are expressed as mean±SD for triplicate samples. *($P < 0.05$): Statistically significant difference between extract-treated cells and control.

*2.8. Statistical Analysis*

All experiments were repeated in triplicate and the relative results are presented as the mean ± standard error of the mean (SEM) 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 values less than 0.05 were considered as significant.

## 3. Results and Discussion

*3.1. Effect of Different Extracts of N. glomerulosa on Cell Survival*

Results showed that treatment of the cells with concentration of 200 µg/ml of MeOH, *n*-hexane, EtAc, and $CH_2Cl_2$ extracts significantly reduced their cell viability ($p < 0.05$) and induced the cell death ($p \leq 0.05$) (Figure 1).

*3.2. Effect of Different Extracts of N. glomerulosa on the Synthesis of Melanin*

To determine the antimelanogenic activity of different extracts of MeOH, *n*-hexane, EtAc, $CH_2Cl_2$, *n*-BuOH and $H_2O$extracts of *N. glomerulosa*, the inhibitory effect on melanin content in B16F10 cells was assessed. Kojic acid was utilized as a positive standard. The results showed the concentration of 100 µg/ml of MeOH, EtAc, and *n*-butanol extracts have inhibitory effect on melanin synthesis (Fig. 2).

*3.3. Effect of Different Extracts of N. glomerulosa 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. glomerulosa* on mushroom tyrosinase activity. The results indicated that mushroom tyrosinase activity was inhibited only by the concentration of 100 µg/ml of EtAc extract and kojic acid used as positive control (Figure 3).

*3.4. Effect of Different Extracts of N. glomerulosa on Cellular Tyrosinase Activity in Melanoma Cells*

To evaluate the mechanism of the inhibitory effect of *N. glomerulosa* extract on melanogenesis in particular, we performed intracellular tyrosinase activity in B16F10 melanoma cells. The results indicated that MeOH extract of *N. glomerulosa* at 100 µg/ml could significantly inhibit cellular tyrosinase activity (Figure 4).

*3.5. Effect of N. Glomerulosa on Cellular ROS Level*

The intracellular ROS levels as indicative of antioxidant capacity of *N.glomerulosa*was measured in cell treated with 24 mM $H_2O_2$ alone or with different extracts of *N. glomerulosa* in B16F10 melanoma cells. As

shown in Figure 5, only MeOH and n-butanol extracts could significantly suppress the oxidative stress induced by $H_2O_2$ (Figure 5).

### *3.6. Effect of N. Glomerulosa on Expression of Melanogenesis-Related Proteins*

To test effect of *N. glomerulosa* extract on regulation of the expression of melanogenesis-related proteins, MITF and

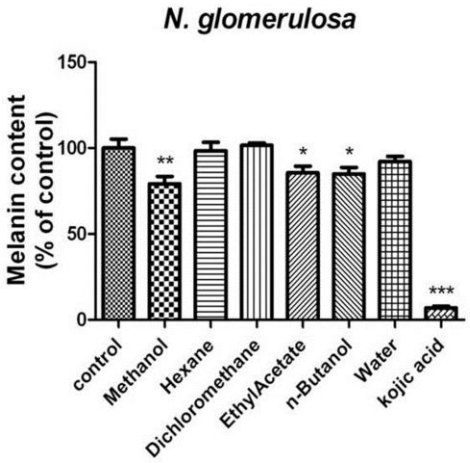


**Figure 2**. Effect of *N. glomerulosa* on melanin content in B16F10 murine melanoma cells. The results showed the concentration of 100 µg/ml of MeOH, EtAc, and *n*-butanol extracts have inhibitory effect on melanin synthesis. *($P<0.05$): Statistically significant difference between all extracts -treated cells and control.

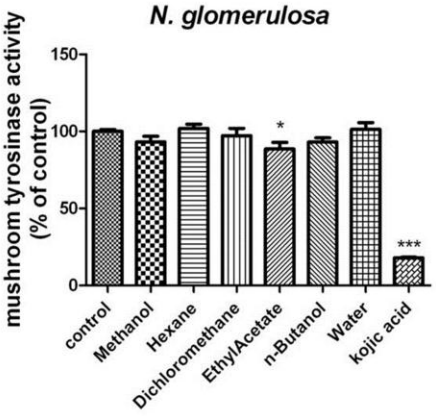


**Figure 3**. Effect of *N. glomerulosa* extracts on mushroom tyrosinase in B16F10 murine cells. The results indicated that mushroom tyrosinase activity was inhibited only by the concentration of 100 µg/ml of EtAc extract. *($P < 0.05$): Statistically significant difference between all extracts -treated cells and control.

tyrosinase level were assayed by western blot (Figure 6). It was found that MeOH extract of *N. glomerulosa* could reduce the level of tyrosinase but the protein contents of MITF were not noticeably changed after treatment.

*3.7. Discussion*

Nowadays, research about plants compounds have taken more attention due to their effects on human health and economy and are substituting with chemical drugs because of great biological activities and low costs of their natural synthesis [34].

In melanogenesis process, about more than 100 gens are involved in encoding important structural, enzymatic, and regulatory proteins [35]. Tyrosinase is one the most important enzyme in the regulation of melanogenesis [36]. Moreover, contributions of ROS inmelanogenes have been demonstrated in many studies [37, 38]. This study was planned to evaluate the anti-melanogenic effect of different extracts obtained from aerial parts of *N. glomerulosa* on B16F10 cells. In the first step, in order to select the suitable concentration of the plant extract which does not influence the cell viability and growth of B16 melanoma cells, the MTT assay was performed. The MTT assay is a common colorimetric test to determine dead cells from living cells [39].

As shown in figure 1 only the concentration of 200 µg/ml of *N. glomerulosa* had cytotoxic effect on B16F10 and reduced the cell viability compared to

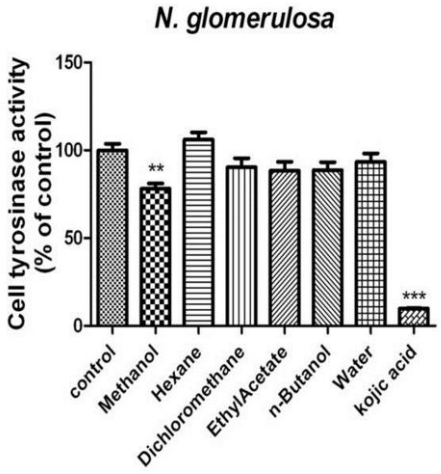


**Figure 4**. Effect of *N. glomerulosa* extracts on cellular tyrosinase in B16F10 murine melanoma cells. The results indicated that MeOH extracts of *N. glomerulosa* at 100 µg/ml could significantly inhibit cellular tyrosinase activity *($P < 0.05$): Statistically significant difference between all extracts -treated cells and control.

control. Therefore, according to MTT assay, we have selected only the concentration of 100 µg/ml of each extract of *N. glomerulosa* to determine the inhibitory effect on melanogenesis. Tyrosinase is a key enzyme in the melanin synthesis pathway which converts L-tyrosine to L-dopa and then oxidized it to dopachrome. Mushroom tyrosinase is extensively utilized as a target enzyme in monitoring potential inhibitors of melanogenesis [32]. The results indicated EtAc extract could significantly inhibit the mushroom tyrosinase activity. To clarify the true inhibitory effect of the *N. glomerulosa* on melanogenesis, B16F10 melanin content and intracellular tyrosinase activity were examined at the same concentration.

The results shown in figure 2 indicated that the MeOH extract of *N. glomerulosa* presents a greater inhibitory effect on melanin production than other extracts. These data supported that *N. glomerulosa* blocks melanogenesis and also MeOH, EtAc, and n-butanol extracts decreased the melanin content in B16F10 melanoma cells.

Western blot analyzing in this study indicated that MeOH extracts of *N. glomerulosa* decreased the protein expression levels of tyrosinase. MITF play the role of transcritption factor of tyrosinase. In our research, *N. glomerulosa* extract did not affect the MITF expression.

To confirm the antioxidant capacity of *N. glomerulosa* in a cellular environment, intracellular ROS levels were evaluated. DCFH-DA diffuses via the cell membrane and was hydrolyzed by means of esterase to DCFH, which is able to react with free

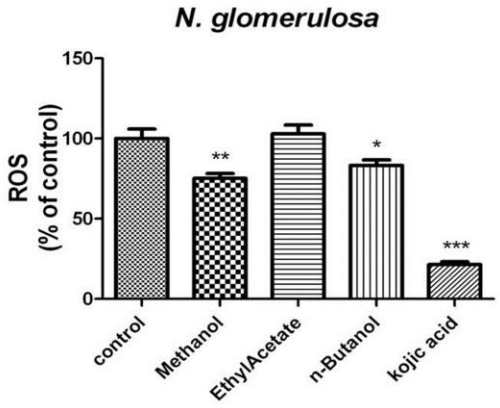


**Figure 5**. Antioxidant effects of *N. glomerulosa* extracts and cellular reactive oxygen species (ROS) level in B16F10 murine melanoma cells. MeOH and n-butanol extracts could significantly suppress the oxidative stress induced by $H_2O_2$ Results were expressed as percentages relative to control, and are presented as mean±SD of triplicate samples. *($P < 0.05$): Statistically significant difference between all extracts -treated cells and control.

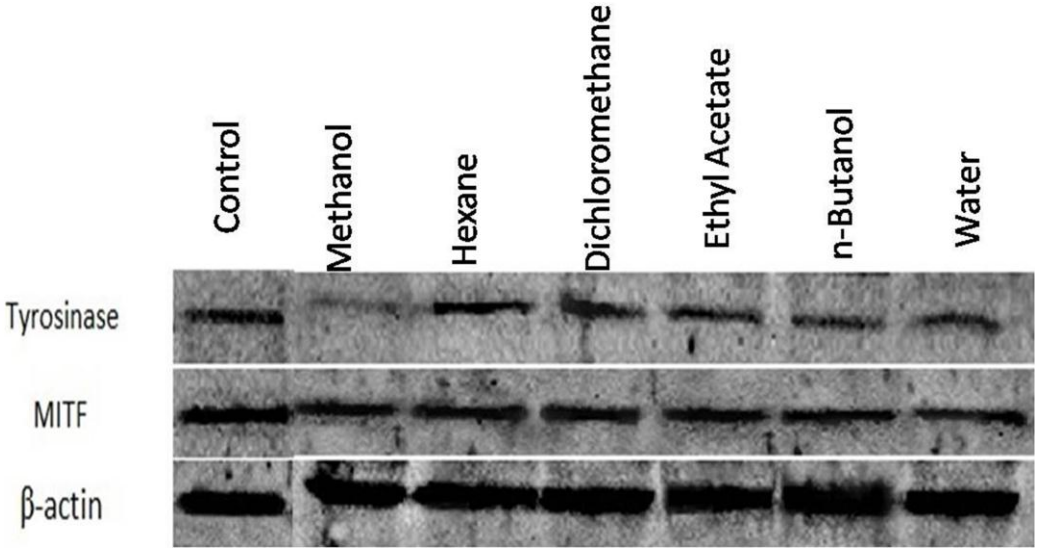


**Figure 6**. Effect of *N. glomerulosa* on expression of melanogenesis-related proteins. Only MeOH extract of *N. glomerulosa* could reduce the level of tyrosinase but the protein contents of MITF were not noticeably changed after treatment.

radical products specially $H_2O_2$ leading to generate the nonfluorescent compound namely DCF. Fast increase in DCF level exhibits the oxidation of DCFH by intracellular radicals [40]. The results revealed that MeOH and *n*-butanol extracts could deplete intracellular ROS in B16F10 melanoma cells. The skin is exposed to UV and environmental oxidizing pollutants. UV irradiation is a potent inducer of ROS production and the main type of ROS produced on the skin surface is $^1O_2$, which is oxidized to unsaturated acyl residues in the sebum layer and produced lipid peroxides [41]. In order to frustrate and response to the oxidative damage, skin is equipped with several ROS elimination systems to protect cells. Chronic exposure to solar UV radiation causes several skin disorders [42]. So, there is a demand for detection and production of new and efficient photo-protective agents with skin whitening properties for prevention of skin disorders.

Up to now, there is no report about the effect of *N. glomerulosa* extract on melanin production. This is the first report to verify the inhibitory effect of *N. glomerulosa* extract on melanogenesis in B16F10 melanoma cells.

## 4. Conclusion

This study is the first report about the effect of *N. glomerulosa* on the process of melanin production. In the present study, it is found that *N. glomerulosa* can inhibit the melanin synthesis also possess antioxidant

activities. As a valuable antioxidant plant *N. glomerulosa* is suggested for future mechanistic and clinical investigations. It is also worthwhile to perform bio-guided fractionation on *N. glomerulosa* to elucidate compounds which are responsible for tyrsinase inhibitory activity.

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