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Research Article

Antioxidant Property and Inhibition of Tyrosinase and Melanin Synthesis of the Korean Fir (*Abies koreana* Wilson) Needle Extracts

Ji Hye Kim, Min Young Kim *

Toxicology Laboratory, Department of Biotechnology (Biomaterials), College of Applied Life Science, Jeju National University, Jeju, 63243, Republic of Korea

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**Corresponding author:*
E-mail: jeffmkim@jejunu.ac.kr

ABSTRACT

Korean fir (*Abies koreana* Wilson) is traditionally used in folk medicine for its antibacterial, memory-enhancing, and anti-inflammatory properties. In this study, we evaluated the antioxidant and skin-whitening effects of the methanol and ethanol extracts of Korean fir needles. The extracts were tested for their antioxidant capacity using various assays, including radical scavenging (1,1-diphenyl-2-picrylhydrazyl, O_2^-, H_2O_2 and $NO^·$), SOD-like, ferrous ion chelating, and reducing power assays. The total phenolic and flavonoid contents were determined by the Folin-Ciocalteu method. The non-toxic doses of the extracts were determined by MTT assay using human malignant melanoma SK mel-100 cells, and the tyrosinase activity and melanin contents were measured using an enzyme-substrate assay. The results showed that the antioxidant activity of the Korean fir needle extracts increased in a dose-dependent manner, as confirmed by their radical scavenging activities in the 2,2-diphenyl-1-1-picrylhydrazyl and 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) assays. The Korean fir needle extract significantly reduced tyrosinase activity and melanin content in a dose-dependent manner ($p < 0.01$), suggesting its potential use as a skin-whitening agent. The methanol extracts of the Korean fir needles exhibited significantly higher phenolic (8306 mg gallic acid equivalents/100 g) content, with higher superoxide (IC_{50} = 4.22 mg/mL) and nitric oxide (IC_{50} = 1.50 mg/mL) radical scavenging activities and inhibition of tyrosinase and melanin synthesis than those of ethanol extracts ($p < 0.05$). Overall, our results demonstrate the potential of Korean fir (*Abies koreana* Wilson) needles as a source of tyrosinase inhibitors and antioxidants for inhibiting melanin biosynthesis, which could have applications in the cosmetic and pharmaceutical industries.

Keywords: Antioxidant, Extraction solvents, Korean fir (Abies koreana), Melanin and tyrosinase inhibition

Introduction

Melanin production is responsible for skin color and plays a crucial role in protecting the skin from UV damage [1-3]. However, overproduction and accumulation of melanin can result in various dermatological disorders. Thus, melanogenic inhibitors have become essential components in medications and cosmetics aimed at preventing hyperpigmentation [1-3].

Tyrosinase, the key enzyme that catalyzes melanin synthesis in melanocyte cells [1-6], can be inhibited to prevent melanin biosynthesis by inhibiting melanocyte metabolism and proliferation [1, 4-6]. Tyrosinase inhibitors are critical in skin-whitening research. Several inhibitors have been identified from both natural and synthetic sources [1, 4-6], but their use has been limited due to side effects such as coloration, odor, and cytotoxicity. Recent studies are focused on developing skin-whitening agents from safe, natural products [3-6].

Reactive oxygen species (ROS) are generated during several intracellular pathways, leading to oxidative stress [7-10]. ROS are considered to be a major contributor to age-related symptoms and

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the pathogenesis of many diseases [7-10]. Oxidative stress can cause skin wrinkling and is associated with various skin diseases in humans [7-10]. It has also been suggested to play a role in the pathogenesis of human skin cancers [7-10], as well as being involved in the pathogenesis of several allergic and inflammatory skin diseases [7-10]. By altering gene and protein function, ROS can disrupt intracellular and extracellular homeostasis, thereby impairing skin function [7-10].

Korean fir (*Abies koreana* Wilson) is a popular ornamental conifer planted in cold-climate gardens and preferred as Christmas trees in North American and European countries [11-13]. This species is endemic to Korea, where it is currently distributed in the southern part of the Korean peninsula and on Jeju Island, Korea [11-13]. Previous studies have shown that Korean fir (*Abies koreana* Wilson) is used in the traditional medicine to treat various disorders including colds, stomachache, indigestion and pulmonary diseases [11-13]. In addition, the needles of this plant are used commercially in the medical industries to treat atopic dermatitis and asthma [11-13]. Moreover, the supercritical carbon dioxide extract from the needles of Korean fir (*Abies koreana* Wilson) exhibited memory enhancing effect in mice and antimicrobial activity [11-13]. However, the utilization of Korean fir as tyrosinase inhibitor and antioxidant substances have not been explored.

In this study, we examined the inhibitory effects of extracts from Korean fir (*Abies koreana* Wilson) on melanogenesis, as well as their antioxidant properties. These results offer potential applications in the development of tyrosinase inhibitors or antioxidants to impede melanin biosynthesis and auto-oxidation, particularly in functional food or cosmetic products. Furthermore, the outcomes of this research will improve our understanding of the impact of various commonly used extraction solvents on the efficiency of phenolic compound extraction, and identify the most suitable solvent for effectively recovering phenolic compounds and boosting antioxidant capacity from Korean fir extracts.

Material and Methods

Preparation of Korean fir extracts

The needle like leaves of Korean fir (*Abies koreana* Wilson) were collected in September 2019 from Camellia Hill, which is located in Andeokmyeon, Seogwipo-si, Jeju-do Province, South Korea. Voucher specimens (AKW-0812) have been deposited in our laboratory for future reference. To prepare the solvent extracts of Korean fir, samples were freeze-dried. The powder (100 g) was extracted with ethanol (70%) and methanol(100%) by constant shaking for 72 h, and then purified with a Sep-Pak C18 cartridge and a 0.45 μm membrane filter (Waters Corporation). The extracted liquid was concentrated using a rotary vacuum evaporator (Buchi Rotavapor R-200, New Castle, DE, US), and the yield of solvent extracts was determined. The residues were taken to the laboratory for further analysis.

Total phenol and flavonoid contents

The total phenol and flavonoid content were quantified according to the protocol previously described [14]. To measure the total polyphenol contents, 30 μL of the extracts, 30 μL of 95% ethanol, 150 μL of distilled water, and 15 μL of Folin-Ciocalteu reagent were added to 96 well microplates. The reaction was allowed to proceed for 5 min at room temperature, followed by an additional 1 h reaction with 15 μL of Folin-Ciocalteu reagent. The optical density was measured at 725 nm using a microplate reader (Dynex Technologies, Inc., Chantilly, VA, US). A gallic acid standard curve was generated from the concentration range of 0-400 μg/mL. Total phenol values were expressed as gallic acid equivalents (mg/100 g of dry mass).

The total flavonoid content of the Korean fir needle extracts was measured using the aluminum chloride colormetric assay. An aliquot (15 μL) of each extract was mixed with 4.5 μL of 5% sodium nitrite, 60 μL of distilled water, and 4.5 μL of 10% aluminum chloride and left at room temperature for 5 min. Two milliliters of 1 M sodium hydroxide were added to the mixture and then filled up to 150 μL with distilled water. The absorbance of reaction mixture was measured at 510 nm. A rutin standard curve was used to calculate the flavonoid content.

Radical scavenging activity

The free radical scavenging activity of plant extracts was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) following the method described by Moon and Kim [14]. Briefly, 100 μL of the plant extracts were mixed with 100 μL of 0.4 mM DPPH solution in a glass tube and allowed to

react for 10 min in the dark at room temperature. The absorbance was measured at 517 nm.

The superoxide anion scavenging activity of Korean fir needle extracts was measured using a method previously described [14]. Superoxide radicals were generated in a mixture containing 50 mM sodium carbonate buffer (pH 10.5), 3 mM xanthine, 3 mM ethylene diamine tetra acetic acid (EDTA), 0.5 mM nitroblue tetrazolium (NBT), and 0.15% bovine serum albumin. The solution of Korean fir needle extracts was added to the mixture, and the reaction was initiated by the addition of xanthine oxidase (XO) (0.25 units/mL). The reaction mixture was incubated at room temperature for 25 min, and the absorbance was measured at 560 nm using a microplate reader.

The ability of Korean fir needle extracts to scavenge hydrogen peroxide was determined according to a previously described method [14]. Briefly, a solution of hydrogen peroxide (20 µL, 10 mM) and 80 µL of extract were mixed with 100 µL of 100 mM phosphate buffer (pH 5.0). After incubation at 37 °C for 5 min, a freshly prepared solution of 1.25 mM ABTS (30 µL) and 1 U/mL peroxidase (30 µL) were added to the reaction mixture. The absorbance of the hydrogen peroxide at 405 nm was determined spectrophotometrically 10 min later at 37 °C against a phosphate buffer blank without hydrogen peroxide.

The nitric oxide radical inhibition was measured by the Griess reduction method [14]. Sodium nitroprusside in phosphate-buffered saline (10 mM, pH 7.0) was added to the extracts, and the reaction mixtures (100 µL) were incubated at 25 °C for 3 h. Then, an equal volume of Griess reagent was added and allowed to stand for 5 min. The absorbance of these solutions was measured at 540 nm against the corresponding blank.

The results for the scavenging activity of the radicals, obtained from triplicate analyses, were expressed as IC_{50} values (mg/mL), which is the dose required to cause 50% inhibition. All samples were analyzed in triplicate, and L-ascorbic acid was used as a positive control.

Superoxide dismutase (SOD)-like activity

The SOD-like scavenging activity of Korean fir needle extracts was determined using the method described by Kim *et al.* [15]. Briefly, 200 µL of Korean fir needle extracts with different concentrations (0.125-2 mg/mL) were mixed with 20 µL of 7.2 mM pyrogallol solution and 260 µL of Tris-HCl buffer (50 mM Tris, 10 mM EDTA, pH 8.5). The mixture was incubated at room temperature for 10 min, and the reaction was terminated by adding 10 µL of 1N HCl to the mixture. The autooxidation of pyrogallol was monitored at 420 nm using a microplate reader.

Ferrous ion chelating activity

The ferrous ion chelating activity was determined as previously described [14]. Freshly prepared $FeCl_2$ (2 mM) was mixed with 250 µL of Korean fir needle extracts. Then, 10 µL of 5 mM ferrozine was added to the mixture, and absorbance readings were taken after exactly 10 min at 25 ºC.

Reducing power assay

The Fe^{3+} reducing power of Korean fir needle extracts was carried out as described previously [14]. Different concentrations of the extract (200 µL, 0.125-2 mg/mL) were mixed with 200 µL of 200 mM phosphate buffer (pH 6.6) and K3Fe(CN)6 (200 µL, 1%). After incubation for 20 min at 50 °C, 200 µL of 10% trichloroacetic acid solution was added to the mixture and then centrifuged at 800 × g for 10 min. One hundred µL of the upper layer of solution was mixed with deionized water (100 µL) and FeCl3 solution (20 µL, 0.1%), and the absorbance was measured at 700 nm.

Tyrosinase inhibition assay

The tyrosinase inhibitory activity of Korean fir needle extracts was carried out using a previously described method [16]. Briefly, mushroom tyrosinase (40 µL, 110 unit/mL) was mixed with 100 µL of phosphate buffer (0.175 M, pH 6.8) and 40 µL of 10 mM tyrosine and 20 µL of Korean fir needle extracts with different concentrations (0.125, 0.25, 0.5, 1, and 2 mg/mL). The mixture was then incubated for 15 min with shaking at room temperature. Following incubation, the absorbance of the mixture was determined at 475 nm using a Spectra MR microplate reader. The concentration of the extract that caused a half maximal inhibition of the tyrosinase activity (IC_{50}) was obtained from a semilog plot of Korean fir needle extract concentrations against the percentage of enzyme inhibition. Kojic acid was used as a positive control for the assay.

Cell culture

The human malignant melanoma SK mel-100 cell line was kindly gifted from Dr. G. N. Wogan (Massachusetts Institute of Technology, MA, USA). The SK mel-100 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cells were cultured in an incubator under 37 ºC and 5% CO2. All experiments were performed in triplicate and repeated thrice to ensure reproducibility.

Cell viability assay

To determine the cytotoxicity of Korean fir needles on SK mel-100 cells, we performed 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays using a cell proliferation kit I (Roche, Indianapolis, IN, USA) [17]. Briefly, we seeded SK mel-100 cells in 96-well plates and replaced the culture medium with a solution containing DMEM (10% FBS) with different concentrations of Korean fir needle extracts (0.05-0.4 mg/mL). The cells were allowed to incubate for 48 h before adding 10 µL of MTT (5 mg/ml) into each well. After incubating the cells at 37 ºC in the dark for 4 h, we solubilized the tetrazolium crystals by adding 10% SDS (100 µL). We incubated the plate overnight at 37 ºC and read the plate using a microplate reader at 550 nm. We calculated the relative percentage of cell survival by dividing the absorbance of treated cells by that of the control in each experiment.

Melanin content

SK mel-100 cells were seeded at 1×10^3 cells/well in 3 mL of medium in 6-well culture plates and incubated overnight to allow cells to adhere. The cells were exposed to various concentrations (0.05-0.2 mg/mL) of the Korean fir needle extracts for 48 h in the presence or absence of 0.1 µM of α-melanocyte-stimulating hormone (α-MSH; Sigma-Aldrich, St. Louis, MO, USA). At the end of the treatment, the cells were washed with PBS (pH 7.2) and lysed with 300 µL of 1 M NaOH containing 10% DMSO for 1 h at 80 °C. The amount of melanin was measured at 405 nm using a microplate reader.

Statistical analysis

All data are presented as mean ± standard deviation of triplicate values. Significant differences between the groups were performed by using SPSS program (SPSS Inc. Chicago, IL, USA) using two-tailed Student's *t*-test. A dose-response curve was plotted to determine IC_{50} values. Correlations among data obtained were analyzed using Pearson's correlation coefficient. A *p*-value less than 0.05 and 0.01 was considered statistically significant.

Results and Discussion

Determination of total phenol and flavonoid

The impact of solvents on the recovery of total phenol content is depicted in Figure 1. Generally, extraction solvents significantly affected the recovery of total phenol content, with ethanol solvent (8305 mg GAE/100 g) exhibiting a higher recovery yield compared to the methanol solvent (7877 mg GAE/100 g) used in this study ($p < 0.05$). This suggests that methanol is a relatively more efficient extraction solvent for polyphenolic compounds from Korean fir. Flavonoids are the most abundant secondary metabolites of phenolic compounds in plants, with more than 4,000 individual compounds identified [18-19]. Due to the various health benefits associated with flavonoids, it is necessary to determine the impact of solvents on the extraction yields of flavonoids to achieve maximum extraction [18-19]. However, in the current study, there was no significant difference between the total flavonoid contents obtained in the methanol-based (6779 mg RE/100 g) and ethanol-based (6585 mg RE/100 g) extractions.

Antioxidant capacities of the Korean fir extracts

Free radicals are closely associated with oxidative damage, and antioxidants act as reducing agents to limit this damage by donating electrons to free radicals and passivating them [20-22]. Eventually, these antioxidants can neutralize the free radicals before they cause damage [20-22]. Many secondary metabolites synthesized by plants act as antioxidants [23]. Therefore, the current study aimed to investigate the free-radical scavenging ability of Korean fir in vitro. As shown in Table 1, methanol extracts of Korean fir needles exhibited significantly higher DPPH, superoxide, and nitric oxide scavenging activities than ethanol extracts ($p < 0.05$).

DPPH is a dark-colored crystalline powder composed of stable free-radical molecules [24]. It is a common antioxidant assay and a well-known

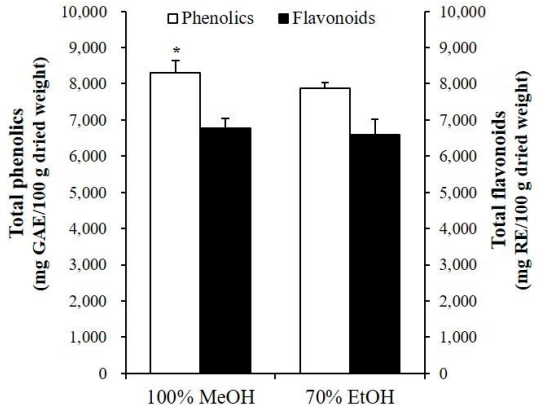

Figure 1. Total phenolic and flavonoid contents of the Korean fir (*Abies koreana*) extracts. Values are the mean of three replications (n=3). $^{*}p < 0.05$ compared to EtOH extract by Student's *t*-test.

radical [24]. Many plant extracts have been reported to scavenge DPPH radicals *in vitro* [25]. Superoxide is generated during cellular respiration and is less toxic, but it can be converted into highly reactive hydroxyl radicals in the presence of iron [26].

Additionally, superoxide anions produced as a result of incomplete oxygen metabolism damage biomolecules directly or indirectly by forming hydrogen peroxide, hydroxyl radical, peroxynitrite, or singlet oxygen [27-28]. Therefore, it is necessary to remove or neutralize superoxide radicals to protect cells from their deleterious effects [27-28]. Nitric oxide is an important cellular signaling molecule involved in many physiological and pathological processes. It is a powerful vasodilator with a short half-life of a few seconds in the blood [29-31]. The nitric oxide radical is toxic after reacting with oxygen or superoxide anion radicals [29-31]. Both methanol and ethanol extracts of Korean fir reduced the generation of nitric oxide in a concentration-dependent manner.

The IC_{50} concentrations (concentrations at which 50% of DPPH, superoxide, and nitric oxide are scavenged) were higher in methanol extract (1.12, 4.22, and 1.50 mg/mL, respectively) than in ethanol extract (1.22, 5.67, and 1.67 mg/mL, respectively). Methanol extracts had a higher total phenolic content (Figure 1), which increased the DPPH, superoxide, and nitric oxide scavenging activities. This may be due to the fact that methanol treatment can eliminate compounds of different natures that interfere with the antioxidant activities of phenolic compounds. The DPPH, superoxide, and nitric oxide scavenging activities of Korean fir may be due to the presence of flavonoids and other polyphenols in the extracts, as indicated in the current study (Figure 1). However, methanol and ethanol extracts showed similar scavenging capacities for hydrogen peroxide radicals, SOD-like, ferrous ion chelating, and reducing power activities (Table 1).

Correlation between antioxidant components and antioxidant activity

Phenolic compounds in plants have been reported to effectively scavenge free radicals [32]. However, there is no information available on the contribution of individual phenolics to the overall antioxidant capacity of Korean fir. To further investigate the contribution of the predominant individual phenolic compounds to the antioxidant capacity of phenolic extracts of Korean fir, a correlation analysis was conducted (Table 2). Significant positive correlations ($p < 0.01$) were observed between the TPC and the antioxidant activities in both ethanol and methanol extracts (r^2 = 0.900-1.000, $p < 0.05$), implying that the antioxidant capacity of Korean fir primarily originates from its

Table 1. Antioxidant activity of the Korean fir (*Abies koreana*) extracts

Solvents	Radical scavenging (IC_{50}, mg/mL)				Superoxide dismutase-like (IC_{50}, mg/mL)	Ferrous ion chelating (IC_{50}, mg/mL)	Reducing power (EC_{50}, mg/mL)
	DPPH	Superoxide	Hydrogen peroxide	Nitric oxide			
100% MeOH	1.12 ± 0.004	4.22 ± 0.088*	0.13 ± 0.002	1.50 ± 0.098*	1.29 ± 0.030	4.48 ± 0.665	1.99 ± 0.080
70% EtOH	1.22 ± 0.064	5.67 ± 0.010	0.13 ± 0.002	1.67 ± 0.064	1.29 ± 0.040	4.49 ± 0.636	1.87 ± 0.110

Note: MeOH=methanol; EtOH=ethanol. IC_{50} and EC_{50} mean the effective concentration at which the antioxidant activity was 50% and at which the absorbance was 0.5, respectively, which was obtained by interpolation from linear regression analysis. Each value is expressed as mean ± standard deviation (n = 3). $^{*}p < 0.05$ compared to EtOH extract by Student's *t*-test.

Table 2. Coefficients of correlation between total phenolics and antioxidant activities of the Korean fir (*Abies koreana*) extracts

	Solvents	Radical scavenging						
		DPPH	Hydrogen peroxide	Nitric oxide	Superoxide	Superoxide dismutase-like	Ferrous ion chelating	Reducing power
Total phenolics	100% MeOH	1.000*	1.000*	0.997*	0.902*	1.000*	0.900*	0.926*
	70% EtOH	0.964*	0.939*	0.943*	0.973*	0.983*	0.963*	0.737

Note: All values are absolute value of correlation coefficients; $^{*}p < 0.05$ is considered statistically significant.

phenolic substances. This positive relationship between total phenolic content and antioxidant activity has been reported previously [33]. However, no significant correlation was found between the total phenolic content and the reducing power activity in the ethanol extract ($r^2 = 0.737$).

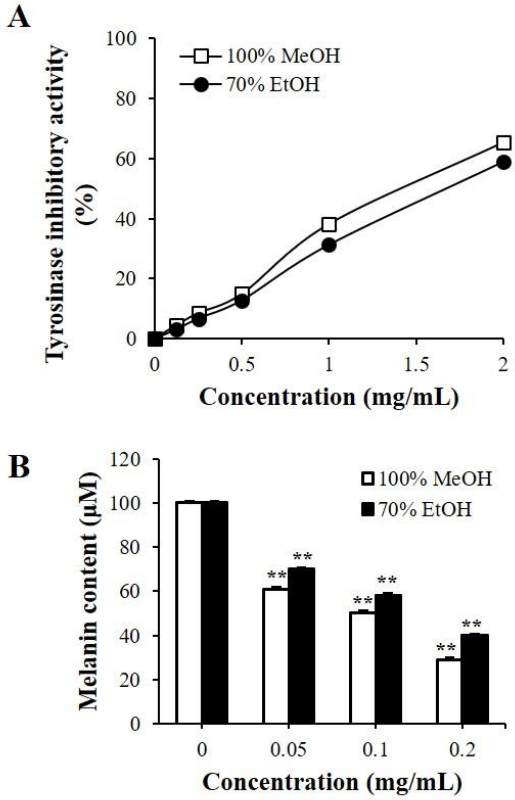

Figure 2. Effect of the Korean fir (*Abies koreana*) extracts on tyrosinase inhibitory activity (A); and melanin secretion (B) on human melanoma SK mel-100 cells. $^{**}p < 0.01$ compared to DMSO control by Student's *t*-test.

Effects of the Korean fir extracts on intracellular tyrosinase activity

Modulating the biosynthetic pathway of melanogenesis is a primary target in the development of skin-whitening agents [4-6], and one well-known biomolecule in this process is tyrosinase [4-6]. As a rate-limiting enzyme in melanin formation, tyrosinase catalyzes the first step in the oxidation of L-DOPA [4-6], and many skin-whitening agents have been screened for their ability to inhibit its activity [4-6]. In this study, methanol and ethanol extracts of Korean fir exhibited potent inhibitory effects on the DOPA oxidase activity of mushroom tyrosinase, with inhibitory activity increasing with extract concentration (Figure 2A). At a test concentration of 2 mg/mL, both methanol and ethanol extracts from Korean fir needles exhibited tyrosinase inhibitory activity of 65.4% and 59.0%, respectively (Figure 2A). These findings suggest that Korean fir needle extracts have potential as tyrosinase inhibitors, which could be useful in the development of anti-browning and skin-whitening agents.

Effect of the Korean fir needle extracts on SK mel-100 cells cell viability

The MTT assay was used to evaluate the effect of Korean fir needle extracts on SK mel-100 cell viability. The cells were treated with various extract concentrations (0.05-0.4 mg/mL) for 72 h, and the MTT assay was performed to assess cell viability, with results expressed as a percentage of control. The Korean fir needle extract did not show any cytotoxic effect on SK mel-100 cell proliferation (data not shown). Therefore, further experiments were conducted with extract concentrations ranging from 0.05 to 0.2 mg/mL.

Effects of the Korean fir extracts on melanin production

The effects of Korean fir extracts on melanin production were also evaluated. Changes in melanin content in SK-mel 100 cells treated with Korean fir extracts were assessed for depigmentation activity. The ethanol fraction significantly attenuated melanin content in a dose-dependent manner (Figure 2B) ($p < 0.01$). Moreover, inhibition of

melanin synthesis was related to the level of tyrosinase inhibition (Figure 2A). The methanol extract had an even stronger inhibitory effect on melanin production than the ethanol extract. In the cosmetics industry, melanin content in human skin can have a negative impact on skin whitening effects [4-6]. Therefore, there is a need to identify new compounds that can reduce melanin content in human skin, and the use of inhibitors is paramount in the cosmetics industry [4-6]. This study aimed to identify new components to reduce melanin synthesis in cells, and some promising possibilities were found.

Conclusion

This study demonstrated that Korean fir needle extracts possess potent antioxidant capacity and inhibit melanin synthesis, indicating their potential as promising candidates for the prevention and treatment of hyperpigmentation, as well as in skin whitening agents. Future investigations will involve the evaluation of the antioxidant and anti-tyrosinase activities of each isolated compound, as well as their skin whitening activity in both cellular and in *vivo* systems.

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