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Antioxidant Activities and Melanogenesis Inhibitory Effects of *Terminalia chebula* in B16/F10 Melanoma Cells

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Abstract

To examine the potential of *Terminalia chebula* as a whitening agent, we measured antioxidant activity using DPPH·, $ABTS^{·+}$ assays and ferric-reducing antioxidant power (FRAP) assays, and depigmenting activity using B16F10 melanoma cells. The intracellular reactive oxygen species (ROS) level was monitored by H_2DCFDA fluorescence labeling, and melanin contents in B16F10 melanoma cells by 960 J/m^2 dose of UVA-induced oxidative stress. The radical-scavenging activities of *T. chebula* extract (TCE) were measured in terms of EC_{50} values using DPPH·, $ABTS^{·+}$ assays and FRAP value were 280.0 μg/mL, 42.2 μg/mL and 113.1 μmol $FeSO_4 \cdot 7H_2O$/g, respectively. We found that ROS and melanin concentrations were reduced by TCE treatments of 25 μg/mL under UVA-induced oxidative stress. Tyrosinase activity and melanin contents in α-melanocyte stimulating hormone (MSH)-induced melanoma cells both decreased dose-dependently in the treatment groups. TCE similarly reduced melanogenesis in B16F10 melanoma cells stimulated by α-MSH as compared to arbutin as a positive control. *T. chebula* may prove to be a useful therapeutic agent for hyperpigmentation and an effective component in skin whitening and/or lightening cosmetics.

Key words: *Terminalia chebula*, B16F10 melanoma cell, ultraviolet, skin whitening, melanogenesis

INTRODUCTION

Overexposure to ultraviolet (UV) rays, present in sunlight, will cause the skin to tan. Tanning is a natural mechanism in which the skin produces more melanin to shield and protect itself from the harmful effects of the sun (1). The increase in melanin causes the skin to become darker. The enzyme tyrosinase, one of the key enzymes involved in melanin synthesis, is activated in melanoma cells by the stimulation of free radicals induced from UV irradiation (2) and stress (3). The activation of tyrosinase triggers melanogenesis, a complex series of enzymatic and chemical reactions that result in the production of melanin. However, there are several types of hyperpigmentation that can occur, with common causes being: sun-related (such as freckles), pregnancy-related (hormones stimulate melanin production), and disease-related (such as hyperpituitarism and Addison's disease) (4). Skin without significant dyschromia is an aesthetic goal of people worldwide. Thus, many investigators have isolated depigmenting agents from plants, such as hydroquinone, kojic acid (5), and retinoids (6). Arbutin is a natural phenolic glucoside and is commonly used as a commercial component in functional whitening cosmetics. It is found in various plant species of diverse families such as Ericaceae (*Vaccinium* spp., *Arctostaphylos* spp.), Asteraceae (*Achillea millefolium*), Betulaceae (*Betula alba*), and Rosaceae (*Pyrus communis* L.) (7).

Terminalia chebula (Black Myrobalan or Chebulic Myrobalan), a plant native to southern Asia, found in India and Nepal in the west and spreading eastward to southwestern China (8). *T. chebula* is called the "king of medicines" and is always listed first in *Ayurvedic medica* because of its extraordinary healing powers. *T. chebula* exhibits antioxidant and free radical scavenging activities *in vivo* and *in vitro* (9). Its antimicrobial activity (10) and antitumor activity (11) have also been reported. The depigmentation effect of *T. chebula* was previously reported (12). However, these reports only confirmed that *T. chebula* can inhibit melanin production through tyrosinase inhibition in melanoma cells and hinder tyrosinase activity in mushrooms. The effects of *T. chebula* against UV irradiation have not been reported. One of the biggest causative agents of hyperpigmentation

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is UV radiation. UV radiation has a strong oxidative component, and photo-oxidative stress has been directly linked to the onset of skin melanogenesis. The most damaging and cytotoxic part of solar UV light (100～295 nm) is eliminated in the stratospheric ozone layer; however, the remaining UV radiation that makes it to earth, part of the UVB rays (295～315 nm) and all of the UVA rays (315～400 nm), is responsible for manifold skin diseases. Due to their short wavelength, UVB rays have high energy levels and are therefore biologically very active, causing sunburn. Possessing the longest wavelengths (320～400 nm), 80% of UVA reaches as far as the dermis and the remaining 20% penetrate considerably deeper. UVA can be more dangerous than UVB because it cannot be blocked by glass and its effects are not immediately visible. Even at low-level exposures, UVA light breaks down collagen, which causes wrinkles. Even worse, scientists have found that UVA is the main culprit for many melanomas because it reaches deep into the underlying support structure of the skin (13). The skin whitening effect of *T. chebula* is important not only in terms of tyrosinase inhibition, but also with respect to counteracting oxidative stress caused by UV rays; however, previous research on *T. chebula* has focused on factors that affect tyrosinase in the regulation of melanogenesis. Therefore, the present study aimed to investigate the photo-protective effects of *T. chebula* against UVA radiation-induced damage by examining tyrosinase activity inhibition and melanin accumulation in B16/F10 melanoma cells.

MATERIALS AND METHODS

Materials

B16F10 melanoma cells (CRL 6323) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cell culture reagents such as Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) were purchased from Gibco BRL (NY, USA). Synthetic melanin, arbutin, α-melanocyte stimulating hormone (MSH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO (dimethyl sulfoxide), folin Ciocalteu's phenol reagent, DPPH (2,2-diphenyl-2-picrylhydrazyl hydrate), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS·), and 2′,7′-dichloro-dihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma Chemical Co. (St. Louis, USA). All other reagents were commercially obtained from standard sources.

Preparation of *T. chebula* extract (TCE)

The *T. chebula* plants, cultivated in Taiwan, were purchased at Kyung-Dong traditional market (Seoul, Korea). The medicinal plants were identified and authenticated by Dr. B.W. Kang (College of Life Science and Biotechnology, Korea University). The dried fruits (5 g as dry weight) were ground with a mortar, and then soaked in 150 mL of ethanol followed by refluxing for 3 hr and cooling. The resulting crude extract was filtered and lyophilized to a dry powder. This powder was redissolved in DMSO. The final concentration of DMSO in the media was below 0.1%.

The content of chebulic acid in *T. chebula* ethanolic extract (TCE) was estimated by HPLC and SymmetryPrep™ C18 (7.8×300 mm i.d. 7 μm) (Waters, USA) (14). Chebulic acid was identified by comparison of sample retention times to those of purified single compound under the same analysis conditions. The chebulic acid content of TCE was 8% (Fig. 1).

Antioxidant activities

DPPH scavenging activity was measured using the method described by Quang et al. (15) with some modifications. Briefly, 0.4 mL of 0.2 mM DPPH· ethanolic

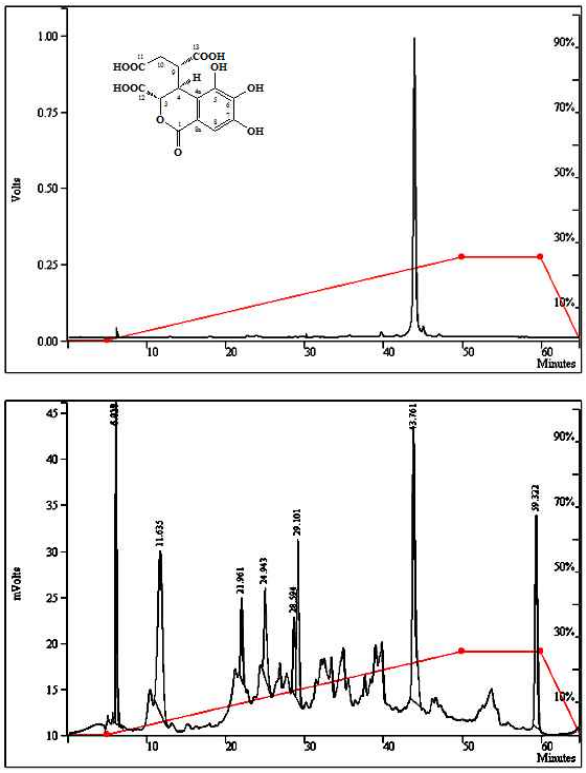

Fig. 1. HPLC chromatogram of chebulic acid (up panel) and TCE (down panel). The column was SymmetryPrep™ C18 (7.8×300 mm). Elution was affected using a linear gradient of the solvent mixtures aqueous 1% (v/v) formic acid (solvent A) and acetonitrile (solvent B). The composition of B was held to 0% for 5 min, increased to 30% in 55 min and held for 10 min, then returned to initial conditions in 5 min at 1 mL/min.

solution was mixed with 0.1 mL of sample in a 96-well plate. The mixture was then vigorously shaken and left to stand for 10 min under subdued light, after which the absorbance was measured at 520 nm. All tests were performed in triplicate. The antioxidant activities of the test samples were expressed as the median effective concentration for radical-scavenging activity (EC_{50}), (i.e., the amount of tested extract required for a 50% decrease in absorbance of DPPH radicals) and expressed in terms of ascorbic acid equivalents.

ABTS radical scavenging activity was determined as previously described by Wang and Xiong (16) and Almajano et al. (17) with slight modifications. The ABTS stock solution was prepared at final concentrations of 7 mM ABTS and 2.45 mM potassium persulfate. The mixture was left in the dark at room temperature for 12～16 hr before use to produce ABTS radical cations ($ABTS \cdot^{+}$). Prior to the assay, the ABTS solution was diluted with 0.2 M sodium phosphate-buffered saline (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. Then, 40 μL of sample at various concentrations were added to 4 mL of the diluted $ABTS \cdot^{+}$ solution. The mixture was shaken vigorously for 30 sec and left in the dark for 6 min. The absorbance of the resultant solution was measured at 734 nm. The results were expressed as the percent of scavenged nitric oxide with respect to the control (without any antioxidant or sample). The scavenging percentage was calculated as the ratio of the absorption of the sample relative to the control without the extract.

The ferric-reducing antioxidant power (FRAP) assay was performed using the method of Lee et al. (9).

Cell cultures and UVA irradiation

B16/F10 melanoma cells were cultured routinely in DMEM medium containing 10% FBS, 50 μg/mL streptomycin, and 50 U/mL penicillin in a humidified 5% CO_2 atmosphere at 37°C. The medium was changed every 2 days.

The cells (1×10^5) were seeded into 24-well plates and allowed to attach to the plates for 12 hr. The culture medium was then replaced with serum-free DMEM containing TCE at specific concentrations. Next, the B16/F10 melanoma cells were exposed to a UVA lamp (Sankyo Danki Spectronics Ltd, Tokyo, Japan) with the emission spectrum centered at 352 nm (960 J/m^2) to induce oxidative stress. Dosimetry was monitored using a Lutron UV-340 light meter (Conrad Electronic, Hirschau, Germany). After UVA irradiation, complete DMEM medium with the appropriate compound was immediately added to the wells for continuous cultivation for 1 hr or 2 days. Another 24-well plate was treated under the same conditions and was kept in the dark to serve as a control. After 1 hr, the treated cells were examined for cell viability using with MTT assay, along with intracellular reactive oxygen species level (18). Finally, melanin content was measured in the cells that were continuously incubated for 2 days after UV treatment, using the method described below.

Cell cytotoxicity

Cell cytotoxicity was quantified using a colorimetric MTT assay to measure mitochondrial activity in the viable cells by the method of Mosmann (19) with minor modifications. At the end of the treatment time, all wells were aspirated and refilled with MTT solution (1 mg/mL) and then incubated for 3 hr at 37°C. The formazan crystals that formed in the actively metabolizing cells were extracted with 10% sodium dodecylsulfate (SDS) (20). The absorbance was measured at 540 nm using a microplate spectrophotometer (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA) and the results were expressed as a percentage of the untreated control (% of control).

Assessment of intracellular ROS level

Intracellular ROS levels were measured by staining the cells with DCFH-DA, which oxidizes to highly fluorescent dichlorofluorescein (DCF) by ROS, using the methods of Wang and Joseph (21) with minor modifications. As described above, the B16F10 melanoma cell cultures were replaced with serum-free DMEM containing compounds at the designated concentrations for 4 hr. The cells were then washed once with PBS and incubated in 20 μM DCFH-DA in phenol-red-free Hanks balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. After 30 min, the cells were exposed to UVA-irradiation at 1.7 W/m^2 for 564 sec (960 J/m^2). One hour later the cells were trypsinized and centrifuged at $60 \times g$ for 5 min. The pellet was washed, re-suspended in phosphate buffered saline (PBS) containing 1% FBS, and the fluorescence intensity of DCF was measured at an excitation of 495 nm and an emission of 527 nm by a microplate fluorometer (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA, USA).

Determination of tyrosinase inhibition activity

Tyrosinase activity was determined based on a modified version of a previously described method (22). The B16F10 melanoma cells were cultured at 2×10^4 cells/well in 60 mm culture dishes and then incubated for 24 hr. The cells were treated with various concentrations of TCE or 25 μM arbutin as a positive control, and then 100 μM α-melanocyte stimulating hormone (α-MSH) was added. The cells were incubated for 3 days and then harvested by trypsinization, washed twice with ice-cold

PBS, and collected by centrifugation. The harvested cells were lysed in 1% Triton X-100 and 0.1 mM phenylmethanesulphonylfluoride (PMSF) in PBS. After lysis for 30 min to release tyrosinase from the melanosome membrane, the cellular extracts were clarified by centrifugation at 10,000×*g* for 30 min at 4°C. The reaction mixture consisted of 0.05% L-DOPA solution and cell-extracted protein (30 μg) in 0.2 mL of PBS. The protein content in the supernatant was analyzed using a Bio-Rad protein assay with BSA standards (23). Dopachrome formation at 37°C was monitored every 10 min for 1 hr by measuring the absorbance at a wavelength of 405 nm using a microplate spectrophotometer.

Determination of melanin contents

The melanin contents of the samples were measured using a modified version of a previously described method (24). The cell density and treatment for the determination of melanin content were the same as the above-mentioned procedure for the determination of tyrosinase inhibition activity. After 3 days, the collected medium and trypsinized cells were centrifugated at 1000×*g* for 10 min. Each precipitate was combined and washed twice with PBS and then dried. The dried cell pellets were dissolved in 250 μL of 1 N NaOH containing 10% DMSO at 80°C for 1 hr and then centrifuged for 30 min at 21200×*g*. The optical densities of the supernatants were measured at 405 nm and compared with standard curves of synthetic melanin. The melanin levels were expressed against cell pellet protein concentrations using a Bio-Rad protein assay with BSA standards.

Statistical analysis

The results are expressed as the mean±standard deviations (SD). All statistical analyses were performed using the SigmaStat 3.5 program (Jandel Scientific, San Rafael, CA). The differences among groups were evaluated by one-way analysis of variance (ANOVA) and Duncan's multiple range tests. A level of $p<0.05$ was used as the criterion for statistical significance.

RESULTS AND DISCUSSION

Antioxidant Activities of TCE

The antioxidant activities of the TCE were measured using three *in vitro* assays, namely ABTS·$^+$, DPPH·, and FRAP, and the results are given in Table 1. The TCE's EC_{50} values for radical-scavenging activity against DPPH· and ABTS·$^+$ were 280.0±0.6 μg/mL and 42.2 ±1.0 μg/mL, respectively, and its FRAP value was 113.1 ±0.7 μmol $FeSO_4$ g/L. The DPPH· scavenging effect of TCE was 5.7-fold lower than that of ascorbic acid (54.1±0.2 μg/mL), but its ABTS and FRAP values were higher than ascorbic acid (68.7±0.5 μg/mL and 101.0 ±1.9 μmol $FeSO_4$ g/L).

Table 1. Antioxidant activities of *T. chebula* extract (TCE)[1] and L-ascorbic acid as a positive control

	TCE (μg/mL)	L-Ascorbic acid (μg/mL)
DPPH scavenging activity (EC_{50})[2]	280.0±0.6	54.1±0.2
ABTS scavenging activity (EC_{50})[2]	42.2±1.0	68.7±0.5
TRAP (μmol$FeSO_4$/g)	113.1±0.7	101±1.9

Values are means±SD (n=5).
[1]Dry weight basis of the original sample of plant parts.
[2]Amount of sample necessary to decrease the initial radical concentration by 50%.

Both DPPH· and ABTS·$^+$ radical-scavenging assays have been widely used to measure the antioxidant capacities of natural extracts based on their ability to reduce radical cations. The DPPH· assay is applied widely to measure the antioxidant activities of polyphenolics and colorants. This assay determines a decrease in sample absorbance at 520 nm; therefore, colored compounds such as anthocyanins and carotenoids present in the test sample may have spectra that overlap with DPPH· at 520 nm (25). For this reason, Awika et al. (18) suggested that the ABTS·$^+$ approach is better than the DPPH· radical-scavenging assay for evaluating the antioxidant activities of phenolic phytochemicals. In contrast, the FRAP assay measures the antioxidant potentials of "antioxidants" to reduce the Fe^{3+}/2,4,6-tripyridyl-s-triazine (TPTZ) complex present in a stoichiometric excess to the blue colored Fe^{2+} form (9). *T. chebula* is yellowish and has many active phenolic constituents such an ellagic acid, 2,4-chebulyl-β-D-glucopyranose, chebulinic acid, casuarinin, chelanin, and 1,6-di-O-galloyl-β-D-glucose (9). The antioxidant activity of chebulic acid TCE was 2.5 times lower than that of EGCG (epigallocatechin gallate), but their activities were found to be similar in the FRAP assay (14). Thus, we presumed that the antioxidant power of TCE resulted from part of the chebulic acid.

Effects of TCE against UVA irradiation by intracellular ROS level

UVA penetrates into deeper layers of the skin and activates the melanin pigment. This then affects connective tissue and blood vessels, and as a result, the skin gradually loses its elasticity and starts to wrinkle. Therefore, large doses of UVA cause premature aging. The mechanisms of this UVA damage are not fully understood, but a popular hypothesis assumes that UVA increases oxidative stress in cells (4). Tyrosinase is regarded as an important system for eliminating oxidative stress in the skin. This enzyme is able to use superoxide to produce melanin, and increased melanin production associated

with enhanced tyrosinase activity was found in melanocytes during exposure to UV (26).

To study the potential protective capacity of TCE toward UVA-induced intracellular ROS, we exposed B16F10 melanoma cells to UVA (960 J/m^2; 1.7 W/m^2, 564 sec) with or without TCE. As shown in Fig. 2A and 2C, there were no cytotoxic effects with the sample alone at concentrations less than 50 μg/mL, but at the dose of 100 μg/mL, cell viability was 90%. However, cell viability was greatly reduced by 50 μM arbutin in the presence of UVA exposure. Hu et al. (27) reported that hydroquinone and arbutin increased cytotoxicity under UVA irradiation. As shown in Fig. 2, the 960 J/m^2 dose of UVA irradiation did not cause cytotoxicity, but it did cause a 2.6-fold increase in endogenous ROS compared to non-exposed melanoma cells. The addition of TCE (10～100 μg/mL) under these conditions resulted in a dose-dependent reduction of UVA-induced intracellular ROS formation, showing reductions of approximately 14%, 33%, 41%, and 44% with treatments of 10, 25, 50, and 100 μg/mL of TCE, respectively. The extract was shown to be more photo-stable against UVA-mediated oxidative toxicity as compared with 50 μM arbutin. In addition, UVA-induced melanin production was decreased in the B16/F10 melanoma cells at TCE concentrations less than 25 μg/mL. However, the melanin levels showed an obvious increase at TCE concentrations over 50 μg/mL, compared to cells treated with UVA alone (Fig. 3). We assume that this may be due to the fact that the extract used in this study was a crude extract, consisting of various compounds, and thus, the higher doses reduced its effectiveness or increased the concentration of adverse compounds.

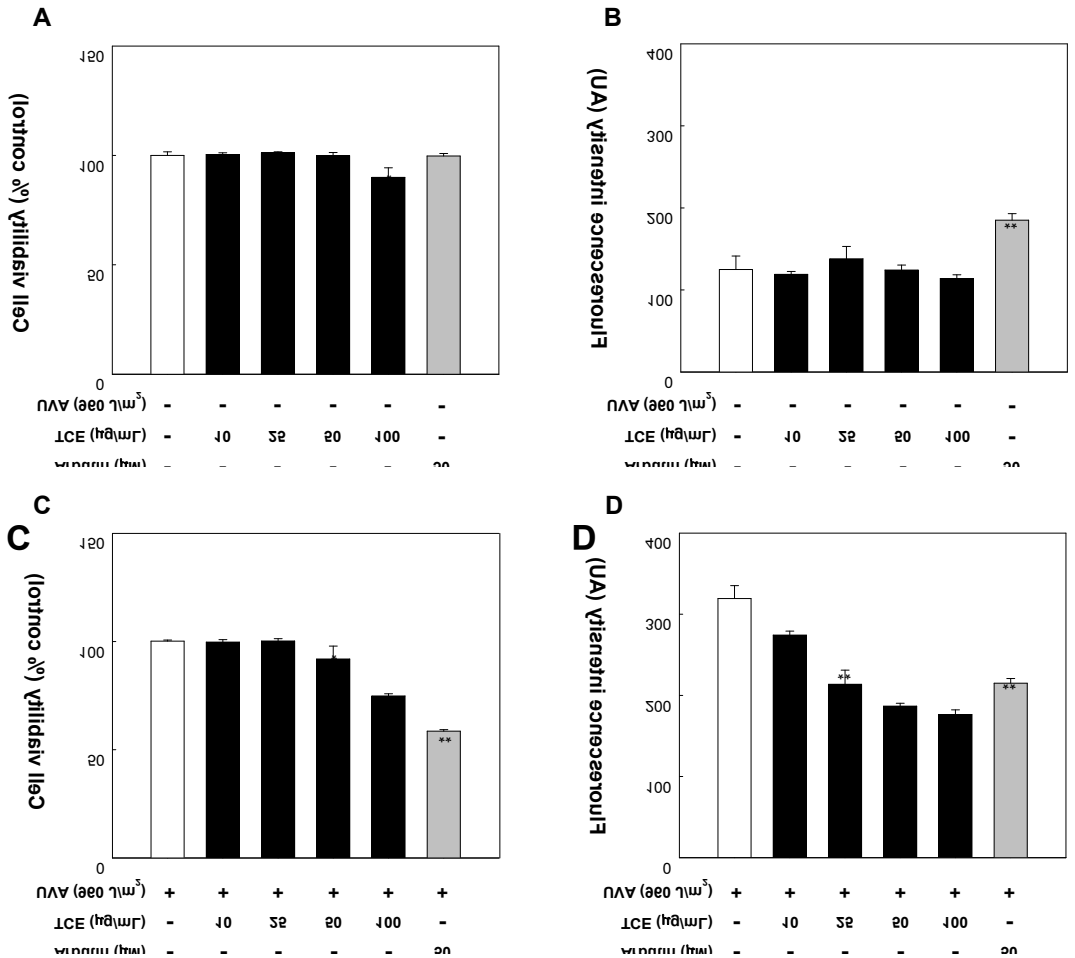

Fig. 2. Cytotoxicity and intracellular reactive oxygen species levels in B16/F10 melanoma cells exposed to *T. chebula* extract without/with UVA irradiation. Cytotoxicity (A and C) and intracellular ROS levels (B and D) of melanoma cells following treatment with concentrations ranging from 10 to 100 μg/mL of extract in the presence or absence of 960 J/m^2 UVA irradiation were measured by MTT assay and 2′,7′-dichlorodihydrofluorescein staining, as described in the Materials and Methods. The results of cytotoxicity by MTT assay were normalized by taking 100% as viability of the untreated control (UVA- and *T. chebula*-). Results are the mean±SD from 3 separate experiments. Significant differences were tested between the treatments and the untreated cells of samples, $^{*}p<0.05$; $^{**}p<0.01$.

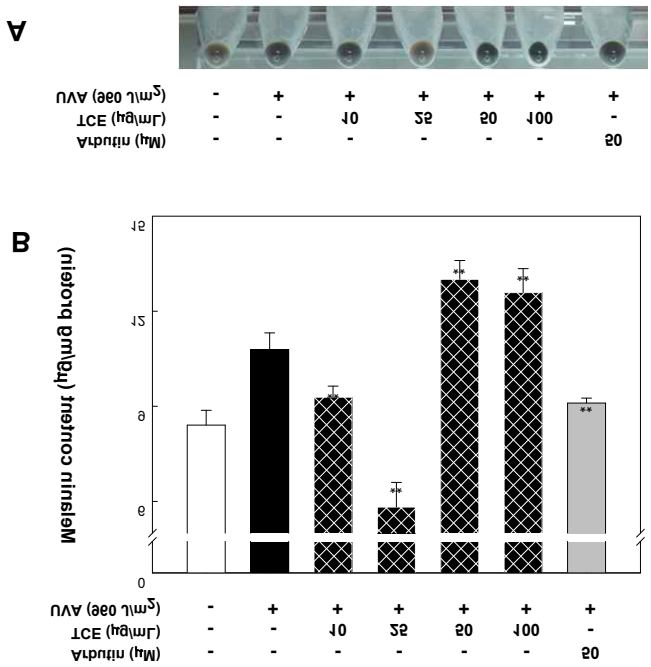

Fig. 3. Melanin content in B16/F10 melanoma cells exposed to *T. chebula* extract with UVA irradiation. After UVA irradiation, cells were continuously incubated for 2 days followed by measurements of melanin content. The melanin contents were measured as described in the Materials and Methods. Panel A is photograph of pellets of B16/F10 melanoma cells. Panel B is melanin content in B16/F10 melanoma cells. Results are the mean±SD from 3 separate experiments. Significant differences were tested between the treatments and the untreated cells (UVA- and *T. chebula*-), $^{**}p<0.01$.

Cytotoxicity of TCE

To further study melanogenesis, the cells were incubated with various concentrations of TCE (5～200 μg/mL) for 3 days to investigate cytotoxicity, and then cell proliferation was examined using the MTT assay. As shown in Fig. 4, there were no cytotoxic effects at TCE concentrations less than 10 μg/mL, but at doses of 25, 50, 100, and 200 μg/mL, cell viabilities were 93.6, 80.9, 69.2, and 63.5%, respectively. As shown in the box within Fig. 4, arbutin also presented a cytotoxic effect of 63.5% at 100 μM (27.2 μg/mL). The safety and effect of arbutin as a positive control have been reported by some researchers. Arunga et al. (28) reported that the EC_{50} value of arbutin in melanin biosynthesis was 111 μM, but this concentration resulted in cell toxicity. We also observed cytotoxicity at the 100 μM arbutin concentration. Therefore, we used 50 μM arbutin as a positive control. From the results in Fig. 3, there were no cytotoxic effects at TCE concentrations less than 10 μg/mL. Thus, we used TCE at concentrations less than 10 μg/mL to determine melanin production, since destroying the melanoma cells would cause confusion between whitening activity and cytotoxicity.

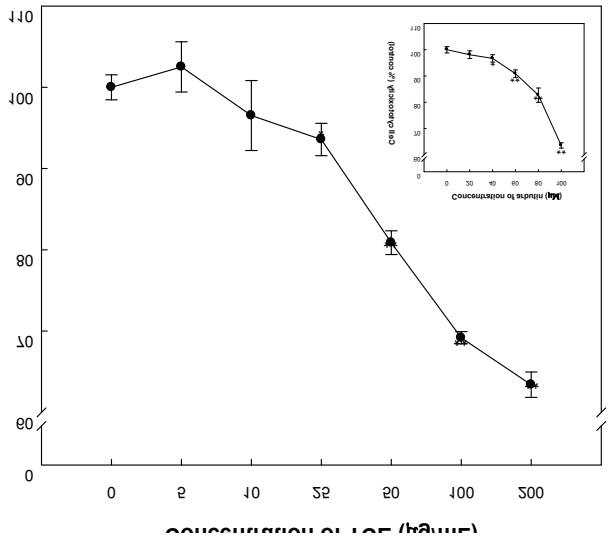

Fig. 4. Cytotixicity of *T. chebula* extract (TCE) and arbutin as a positive control using the MTT assay. The B16F10 melanoma cells were treated with various concentrations of TCE or arbutin for 72 hr. The data were normalized by taking 100% as viability of the untreated control. Results are the mean± SD from 3 separate experiments. Significant differences were tested between the treatments and the control, $^{*}p<0.05$; $^{**}p<0.01$.

Effects of TCE on tyrosinase activity and melanin production in B16F10 melanoma cells stimulated by α-MSH

To examine the effects of TCE on melanogenesis in B16F10 melanoma cells stimulated by α-MSH, the cells were cultured in the presence of 1～10 μg/mL of TCE for 3 days. As shown in Fig. 5, the color of the conditioned medium or the collected cell pellet from the cells treated with TCE became visibly lighter in a dose-dependent manner. The microphotographs show that the cells treated with TCE had decreased melanin spots as compared to the melanoma cells stimulated by only α-MSH. The quantitative determinations of melanin content in these cells are shown in Fig. 6. Melanin content decreased dose-dependently, showing approximately 31.0, 23.6, and 21.9 μg/mg protein upon treatment with 1, 5, and 10 μg/mg of TCE, respectively. At the 10 μg/mL concentration, the TCE had greater inhibition of melanin synthesis (65.6%) compared to 50 μM arbutin (25.2 μg/mg protein, 50.7%), but the difference was not significant. In the α-MSH-stimulated B16F10 melanoma cells, TCE also inhibited tyrosinase activity in a dose-dependent manner (Fig. 6). At the concentration of 10 μg/mL, TCE inhibited α-MSH stimulated tyrosinase activity by 67.3%. The tyrosinase activity of the cells treated with 10 μg/mL of TCE was similar to that of the non-stimulated melanoma cells, at 56.4%, while the treatment of 50 μM arbutin presented an activity of 73.4%. We confirmed that the

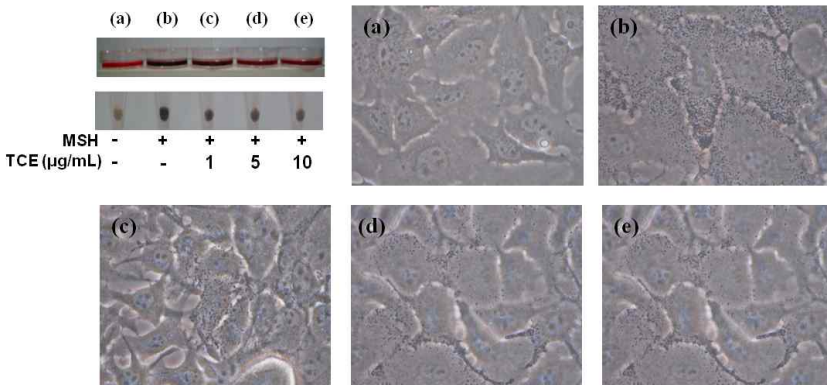

Fig. 5. Inhibitory effects on melanogenesis in B16/F10 melanoma cells exposed to *T. chebula* extract (TCE) with α-MSH. The cells were treated with various concentrations of TCE and 100 μM α-MSH was added followed by incubation for 3 days.

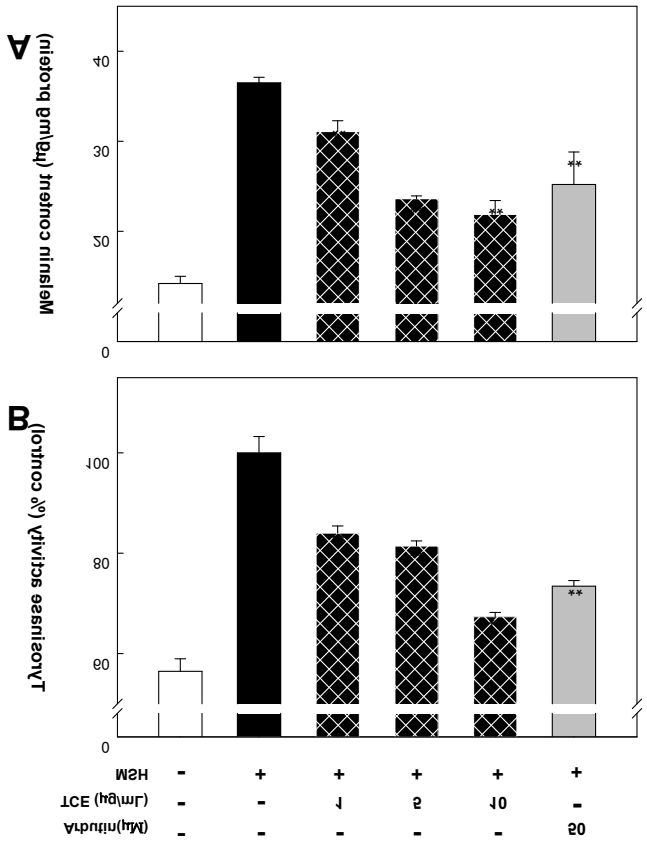

Fig. 6. Inhibitory effects on melanin biosysthesis (A) and tyrosinase activities (B) in B16/F10 melanoma cells exposed to *T. chebula* extract (TCE) with α-MSH. Results are the mean ±SD from 3 separate experiments. Significant differences were tested between the treatments and the control, $^{**}p<0.01$.

10 μg/mL concentration of TCE similarly reduce melanogenesis in B16F10 melanoma cells stimulated by α-MSH as 50 μM (13.6 μg/mL) arbutin, which is commonly used as a commercial cosmetic component in functional whitening cosmetics.

Skin whitening products have become increasingly popular in the past few years. Effective skin whitening technology is a global skin care need for many consumers. Several cosmetic and pharmaceutical companies have tried to find inhibitors for melanogenesis. The regulation of cellular pigmentation can be controlled at the different stages of melanogenesis. In particular, tyrosinase inhibitors and antioxidants can be used to inhibit cellular pigmentation, since the melanin producing process involves enzymatic and nonenzymatic oxidation reactions. Currently available compounds such as arbutin, hydroquinone, kojic acid, licorice extract, and vitamin C are used in cosmetics to lighten skin complexion and in pharmaceutical formulations to ameliorate hyperpigmented abnormalities (6).

T. chebula is a traditional Indian herb with many reported medicinal benefits. Several researchers have confirmed the healing properties of this herb. In previous research, we reported its antioxidant activities and protective effect against oxidative stress in liver by oral administration (9). We also isolated and identified chebulic acid from *T. chebula*, and identified its antioxidant activity (14). To our knowledge, the radical scavenging activity of *T. chebula* fruit against UV-induced ROS is being reported for the first time. Its extract has been used as a dietary supplement, and also has wide applicability in cosmetics for facial scrubs, masks, body wraps, hair wraps, and hair rinses, such as shampoos and conditioners. However, *T. chebula* has not yet been used for skin whitening. Based on our current study, we suggest that *T. chebula* may have beneficial properties as a skin whitening agent, however, it presented toxic and adverse effects at high concentrations (Fig. 3 and Fig. 4). At this point, we presume those results were due to low purity of the extracts.

CONCLUSION

In this study, we confirmed that *T. chebula*: (i) has antioxidant capabilities comparable to that of ascorbic acid; (ii) displays radical scavenging ability against UV-induced intracellular ROS and can reduce melanin accumulation induced by UV irradiation; and (iii) can reduce melanogenesis through tyrosinase inhibition in B16F10 melanoma cells stimulated by α-MSH. As evidenced by

the above, *T. chebula* acted not only as a tyrosinase inhibitor, but also provided antioxidant activity. However, the active compound and its mechanism and safety remain unclear. The isolation and the structural elucidation of active constituents in *T. chebula* extracts will provide useful leads in the development of skin-whitening agents. Therefore, our future efforts are focused on isolating and identifying the principle active compounds found in TCE, as well as its inhibitory mechanisms on melanin production. When these points are clarified, we hope to show that *T. chebula* will prove to be a useful therapeutic agent for reducing hyperpigmentation and that *T. chebula* will be an effective component in whitening and/or lightening cosmetics.

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