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Antioxidant, Anti-wrinkle, Whitening, and UV-protective Effects of *Polygonum tinctorium* Flower

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Abstract : This study was carried out ot investigate the antioxidant, anti-wrinkle, whitening, and UV-protective effects of polygonum tinctorium flower extracts (PTFE). The antioxidantive effects were determined by measuring2,2-diphenyl-1-[picrylhydrazyl (DPPH) and ABTS free radical- scavenging activities. In addition, the elastase, tyrosinase, and melanogenic inhibitory potential of PTFE were estimated. The protective effect of PTFE against UV-induced cytotoxicity in HaCaT keratinocytes was also measured. Tesults showed that DPPH and ABTS free radical-scavenging activities of PTFE increased in a dose-dependent manner, withjalf maximal inhibitory concentration (IC_{50}) values of 40.70 and 31.59μg/mL, respectively. The capacity of PTFE to inhibit elastase and tyrosinase, key enzymes know to be involved in skin wrinkling and melanogenesis, was also investigated. PTFE showedmoderate anti-tyrosinase (IC_{50}: 444.15 μg/mL) and anti-elastase activities. Furthermore, PTFE reduced α –melanocyte stimulating hormone-induced melanin production in B16/F10 murine melanoma cells, indicating that it has anti-melanogenic effects. Finally, we investigated the cellular protective effects of PTFE for potential use in promoting human skin health. PTFE efficiently protected HaCaT keratinocytes against UV-induced cellular toxicity. These results suggest that PTFE possesses several biological activities that confer protection against skin aging and melanogenesis. Further investigations will focus on cell-based *in vitro* assays and identification of the major active components mediating itsanti-aging and anti melanogenesis effects.

Introduction

Aging is a biological process that induces the progressive loss of structural integrity and physiological changes inthe skin[1]. It occurs through two main pathways: the intrinsic (genetically determined) and extrinsic (environmentally mediated) pathways[2]. Wrinkle formation is a striking feature of intrinsic and extrinsic skin aging, which are both associated with over-accumulation of free radicals and a decrease of collagen, elastin, melanin, and hyaluronic acid in human epidermis[3]. Over-accumulation of free radicals are also associated with the decrease of elastin and melanin through activation of skin disease-related enzymes, such as elastase and tyrosinase, which further contributes to skin aging[4]. The skin possesses elaborate defensive mechanisms that interact with free radicals to obviate their deleterious effects[5]. These include non-enzymatic and enzymatic substances that function as potent antioxidant or oxidant-degrading systems[6]. Methods based on a single

relatively stable reagent, such as DPPH• and ABTS•+, have become most popular for the screening of antioxidants, owing to their simple set-up and ease of control[7].

Tyrosinase is known to be a key enzyme involved inmelanin biosynthesis, where it catalyzes the first two stages of melanogenesis[8]. Melanin is largely responsible for the hair and skin color in mammals. It also serves as a photoprotective agent against the harmful effects of UV radiation. Hence, tyrosinase inhibitors may be clinically helpful in dealing with hyperpigmentation, such as stains and freckles[9].

Elastase is a member of the chymotrypsin family of proteases involved in the degradation of the extracellular matrix that includes elastin, leading to skin aging[10]. Chronic UV exposure results in elastase secretion and activation, leading to the loss of elastin, which further contributes to wrinkles and sagging skin[11]. Therefore, elastase inhibitors could also be clinically useful for protection against skin diseases[12]. Medicinal plants have been used as functional ingredients in the cosmetic industry, as elastase and tyrosinase inhibitors have become increasingly important to prevent anti-wrinkle and hyperpigmentation through the inhibition of enzymatic activities[13].

Polygonum tinctorium (Indigo plant), an annual herb, is a species of flowering plant in the buckwheat family that shows antipyretic, analgesic, and antiphlogistic properties[14]. This plant has been a source for the indigo blue textile dye since ancient times in East Asia, including Korea[15]. In some local areasin Korea, the indigo plant has been utilized as a natural remedy for wound healing and skin problems[16]. These findings led us to consider the potential use of the *P. tinctorium*flowersas herbal medicines orcosmetic supplements. In this study, we evaluated the antioxidant, anti-wrinkle, whitening, and UV-protective properties of *P. tinctorium* flower extracts (PTFE)by using *in vitro* assays and identified the potential application of this plant as an active ingredient for cosmetic products.

Experimental

Plant materials and extract preparation

P. tinctorium flowers were collected from Ilchul Land Park on Jeju Island in October 2015. Voucher specimens were deposited at the Jeju National University and the identity of the vouchers and fresh plants were verified by a specialist,Ms. Choon Il Kang. Approximately 35 g of air-dried and powdered *P. tinctorium* flowers were subjected to extraction with 70% ethanol for 24 h with stirring. The ethanol extract was filtered using sterilized Whatman filter paper No.1 to obtain a particle-free extract. The ethanol extract was concentrated by evaporation at < 50℃ using a rotary evaporator and vacuum oven to obtain dry powder. The extract was stored until use.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay

The DPPH radical-scavenging capacity of PTFE was determined according to the method described by Kim et al. [17]. The free radical-scavenging activity of was measured by assessing the decrease in absorbance of a solution of DPPH, 180 μL of DPPH solution (0.2 mM in ethanol) was mixed with various concentrations (3.9, 7.8, 15.6, 31.3, 62.5 and 125 μg /mL) of PTFE and incubated in the dark at 25°C for 10 min. Thereafter, the absorbance was measured at 517 nm. Absorbance was also estimated for the negative control consisting of only ethanol. The DPPH radical-scavenging activity was calculated using the following equation:

DPPH scavenging activity = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$

ABTS radical-scavenging assay

The ABTS radical-scavenging assay of PTFE was performed as described previously, with slight modification[18]. The ABTS radical cation was generated by the reaction between 7 mM ABTS and2.45 mM potassium persulfate stored in the dark for16 h. Prior to use, the radical cation was diluted with ethanol to obtain an initial absorbance of approximately 0.78 (±0.02) at 700 nm. Various concentrations (3.9, 7.8, 15.6, 31.3, 62.5 and 125 μg /mL) of PTFEwas then mixed with 180 μL of ABTS solution. The test mixture was incubated in the dark at 25°C for 10 min, and the absorbance of the sample (Abs_{sample}) and ethanol-onlycontrol ($Abs_{control}$) was measured. The percentage of inhibition was calculated based on the following formula:

ABTS radical-scavenging activity (%) = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$

Tyrosinase inhibition assay

The mushroom tyrosinase inhibition activity was performed by measuring DOPA chrome formation upon oxidation of tyrosine by mushroom tyrosinase, as described previously[19]. In brief, PTFEwas dissolved in 70% ethanol and seven different concentrations (15.6, 31.3, 62.5, 125, 250, 500, and 1000 μg/mL) were prepared. Thereafter, 70 μL of PTFEwas combined with 30 μL of mushroom tyrosinase (500 units/mLin 20 mM phosphate buffer) in triplicate. After pre-incubation of the test mixture (100 μL) at 37°C for 5 min, 110 μL of substrate (2 mM L-tyrosine) was added to each well. The test mixture (200 μL) was incubated for 10 min at 37°C, and tyrosinase activity was determined by measuring the absorbance at 475 nm. A similar mixture without PTFE and a solution of hydroquinone-*O*-β-glucopyranoside (arbutin) were used as the vehicle and positive control, respectively. Each treatment was replicated three times, and the percentage of tyrosinase inhibition was calculated as follows:

inhibition (%) = $[1 - (Abs_{sample} - Abs_{blank})/Abs_{control}] \times 100$

Porcine pancreatic elastase (PPE) inhibition assay

ThePPE inhibitory activity was assayed by monitoring the release of ρ-nitroanilide from the substrate *N*-Succ-$(Ala)_3$-*p*-nitroanilide (SANA) as described previously, but with minor modifications[20]. The inhibitory activity was determined by measuring the intensity of the color produced upon cleavage of SANA by PPE. In brief, PPE (0.1 mg/Ml, 10 μL) and SANA (6.5 mM, 5 μL) were added to Tris-hydrochloride (HCl) buffer (0.2 M, 165 μL) containing PTFE to a final volume of 200 μL. The test mixture was incubated for 15 min at 25°C, and absorbance changes due to the formation of ρ-nitroaniline was measured at 405 nm. Each treatment was replicated three times, and the percentage of PPE inhibition was calculated as follows:

inhibition (%) = $[1 - (Abs_{sample} - Abs_{blank})/Abs_{control}] \times 100$

Cell culture

The B16F10 murine melanoma cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were maintained as sub-confluent culturein Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a 95% air/5% CO_2 humidified atmosphere.

Cell viability assay

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. B16F10 cells were cultured in 24-well plate for 18 h, followed by treatment with various concentrations (25, 50, and 100 μg/mL) of PTFEfor 48 h. MTT was added to the cells and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance was measured at 540 nm. Cell viability was expressed as a percentage relative to the control group.

Measurement of melanin content

The amount of melanin in B16F10 cells was measured according to a previously published method with slight modifications. The cells were treated with PTFE and α-melanocyte stimulating hormone for 48 h at 37°C. Thereafter, the medium was removed and the cells were washed with cold phosphate-buffered saline (PBS) and pelleted using centrifugation. The cell pellet was dissolved in 1 M NaOH for 1 h at 80°C. Spectrophotometric analysis of melanin content was performed at an absorbance of 405 nm. Each experiment was performed in triplicate.

Measurement of MTT by UVB irradiation

The human keratinocyte cell line, HaCaT, was maintained at 37℃ in a humidified atmosphere of 95% air and 5% CO_2 in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/ml streptomycin. HaCaT cells (2×10^5 cells/mL) were seeded in 96-well culture dish, and maintained in a tissue culture incubator. The cells were irradiated usingUV irradiation by using a UV lamp

(Bio-Link BLX-312, Vilber Lourmat GmbH, France). HaCaT cells (2.0×10^5 cells/mL) were seeded in 96-well plate for 24 h. Subsequently, the medium was replaced with PBS and cells were simultaneously treated with various concentrations of PTFE and irradiated with 25 mJ/cm^2 UVB. After irradiation, the medium was replaced with fresh medium containing PTFE and cells were further cultured for 24 h. MTT was added to the cells for 4 h and formazan crystals were dissolved in 200 µL DMSO. viability was expressed as a percentage relative to the UV-irradiated group without PTFE treatment.

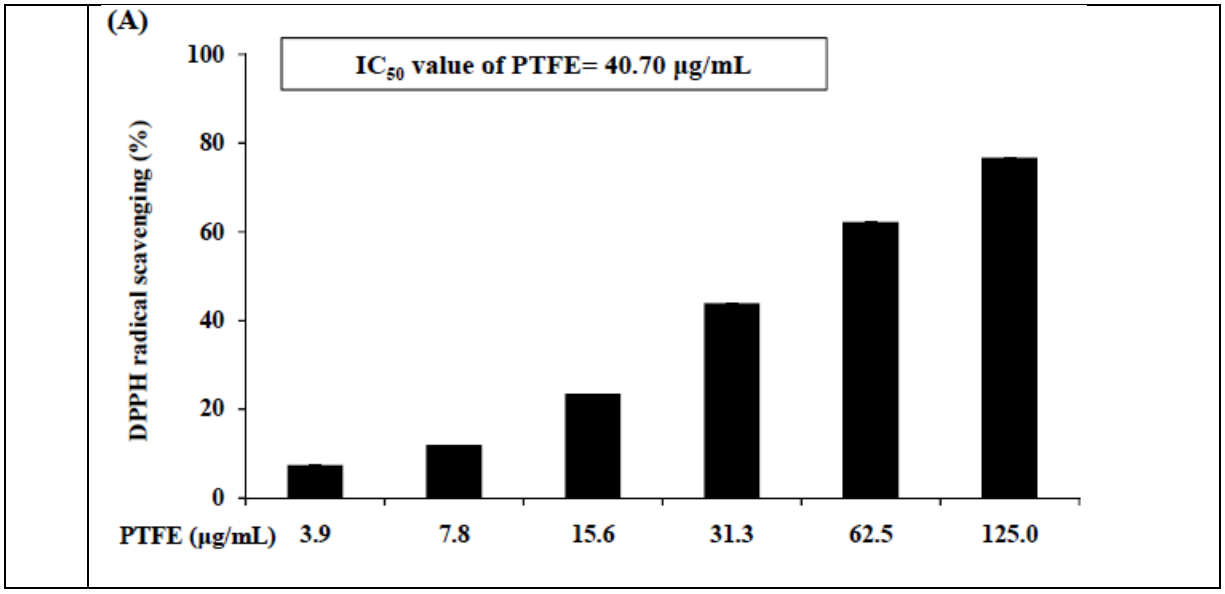
Data analysis

All data were expressed as mean ± standard deviation (SD) of at least triplicate experiments. Student's *t*-tests and one-way analysis of variance (ANOVA) were used for statistical analyses, and $P < 0.05$ was considered significant.

Results and discussion

Antioxidant and elastase inhibitory activity

In this study, the antioxidant activity of PTFE was evaluated using DPPH and $ABTS^+$ radical-scavenging assays. As shown in Figure 1, the DPPH radical-scavenging activity of PTFE was presented as the IC_{50} value (µg/mL), defined as the concentration of the test solution that efficiently scavenged half of the DPPH free radicals. Results showed that PTFE showed significant radical-scavenging activity ($P < 0.05$) with an IC_{50} valueof 40.70 µg/mL. DPPH is a relatively stable free radical and the assay determines the ability of natural products to reduce DPPH• to the corresponding hydrogen by converting the unpaired electrons to form pairs. This conversion is driven by the antioxidant[21].The free radical-scavenging activity of ABTS has also been widely used to evaluate the antioxidant activity of natural products obtained from bioresources[22]. In the ABTS scavenging activity model,PTFE (3.9-125 µg/mL) significantly scavenged $ABTS^+$ in a concentration-dependent manner (Fig 1B). Elastase is known to degrade elastin, a protein closely related to the elasticity and restoration of skin, and this degradation process induces wrinkles and a lack of elasticity[23].The elastase inhibitory potential of PTFE is shown in Figure 1C. The inhibition rate of PTFE at concentrations of15.6 and 125µg/mL were20.9% and 24.0%, respectively, indicating that PTFE may have a moderate inhibitory effect.



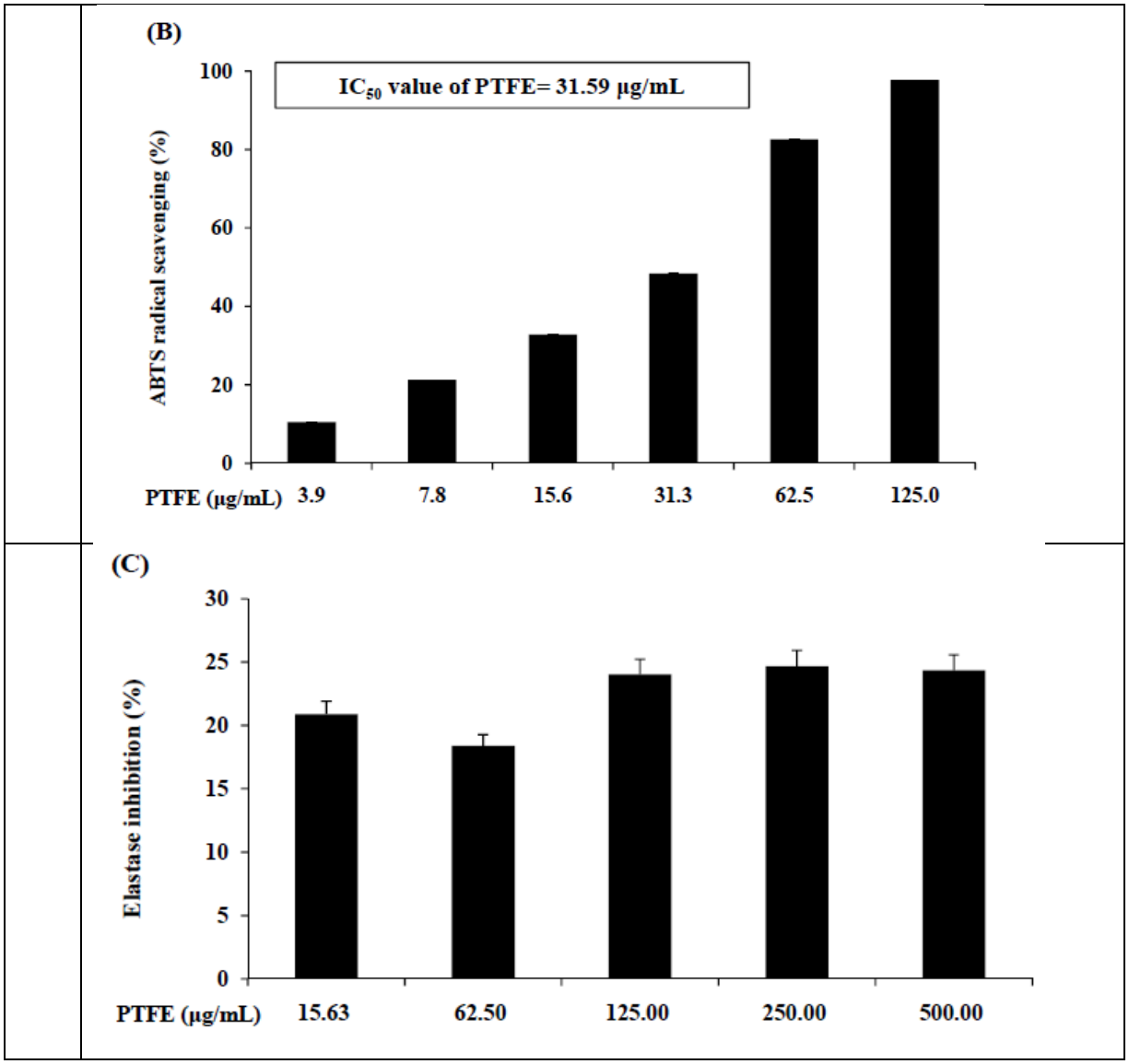


Fig.1. Antioxidant and elastase inhibitory activities of *Persicaria tinctoria* flower extracts (PTFE). The antioxidant capacity of PTFE was measured using the (A) DPPH and (B) ABTS scavenging assays. (C) Inhibition of porcine pancreatic elastase by PTFE.

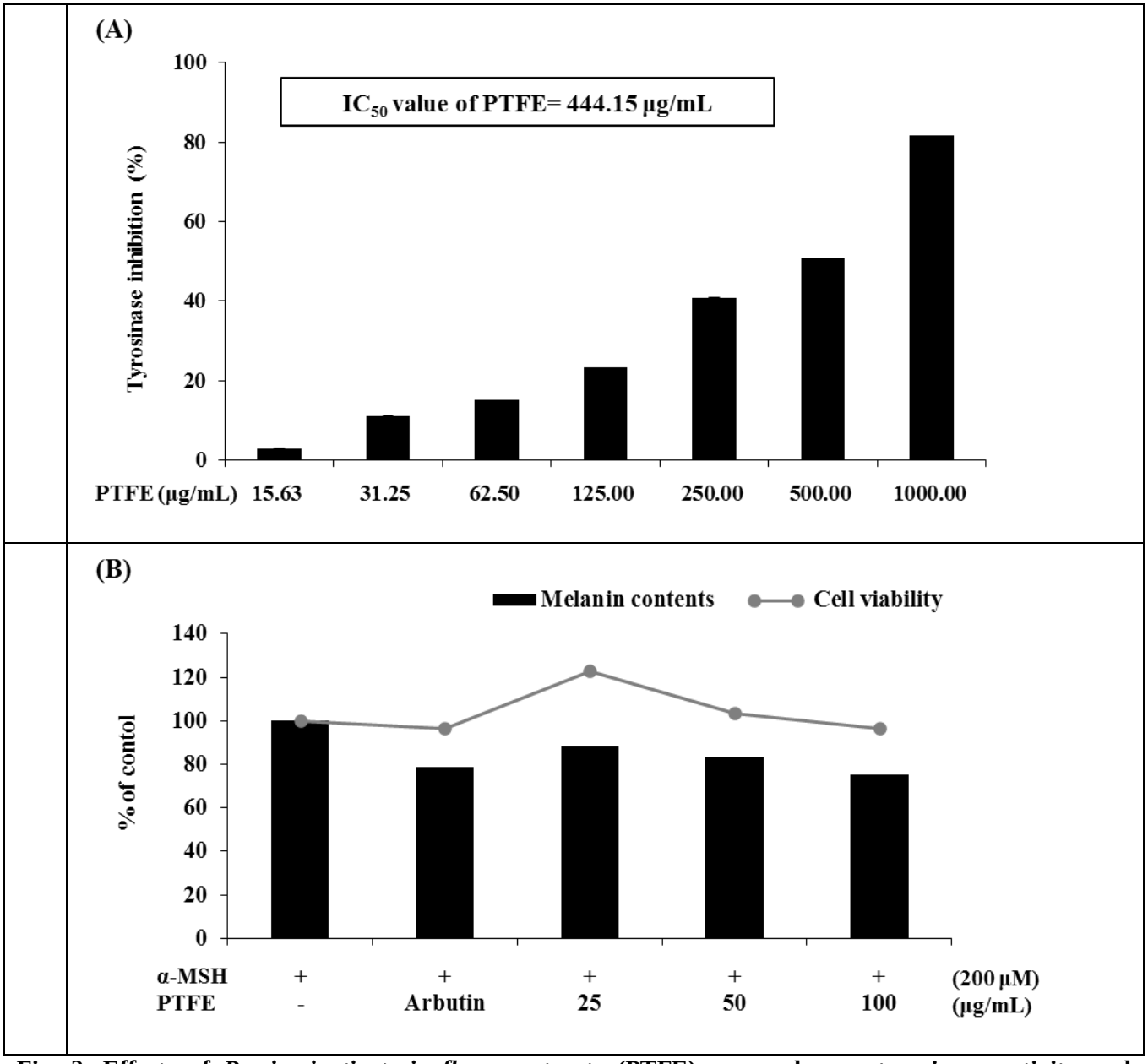


Fig. 2. Effects of *Persicaria tinctoria flower* extracts (PTFE) on mushroom tyrosinase activity and melanogenesis of B16F10 cells. (A) The potential effects of PTFE on melanin production were determined based on its effects on mushroom tyrosinase activity. (B)B16F10 cells (2.0×10^4/mL) were pre-incubated for 18 h. The melanin content in these cells was assayed after further incubation with α-melanocyte stimulating hormone (200 nM) and PTFE for 72 h at 37°C in a 5% CO_2 atmosphere.

Mushroom tyrosinase and melanogenic inhibition capacity

The capability of PTFE to inhibit tyrosinase activity can be translated to its potential use as a skin-whitening agent. When tyrosinase enzymatic activity is inhibited, melanin production is reduced, resulting in fairer skin[24]. The tyrosinase inhibitory effect of PTFE was determined using mushroom tyrosinase is,as shown in Figure 2A. The assay was conducted at various concentrations of PTFE ranging from 15.6 to 1000 µg/mL. The tyrosinase inhibitor, arbutin, was used as the positive control. PTFE showed 23.4% and 50.8% inhibition at concentrations of 125 and 500 µg/mL, respectively. Its IC_{50} value was 444.2 µg/mL. To investigate the effect of PTFE on melanin synthesis, the cytotoxicity of PTFE on B16F10 murine melanoma cell line was measured usingMTTassay. Results indicated that the number of viable, activated murine melanoma cellswas not altered by PTFE, indicating that the inhibition of melanin production by PTFE was not due to cellular toxicity (Fig 2B). To further determine whether PTFE regulates melanin synthesis, melanin content was measured after treatmentwith PTFE. The cells were pretreated with PTFE at doses ranging from 25 to 100 µg/mL. PTFE treatment significantly reduced melanin production in a dose-dependent manner when compared to the control (Fig. 2B).

Effects of PTFE on UVB-induced HaCaT cell death

The protective effect of PTFE against UVB-induced HaCaT cell death was determined usingMTT assay. As shown in Figure 3, UVB treatment at 25 mJ/cm^2 reduced HaCaT cell viability to approximately 50% of control viability. Increasing the dose of UVB further reduced cell viability (data not shown).PTFE, at concentrations rangingfrom25-100 µg/mL,restored the viability of UVB-treated HaCaT cells, with the highest viability closely approaching 120%, i.e., the viability of non-UVB-treated control cells (Fig 2).

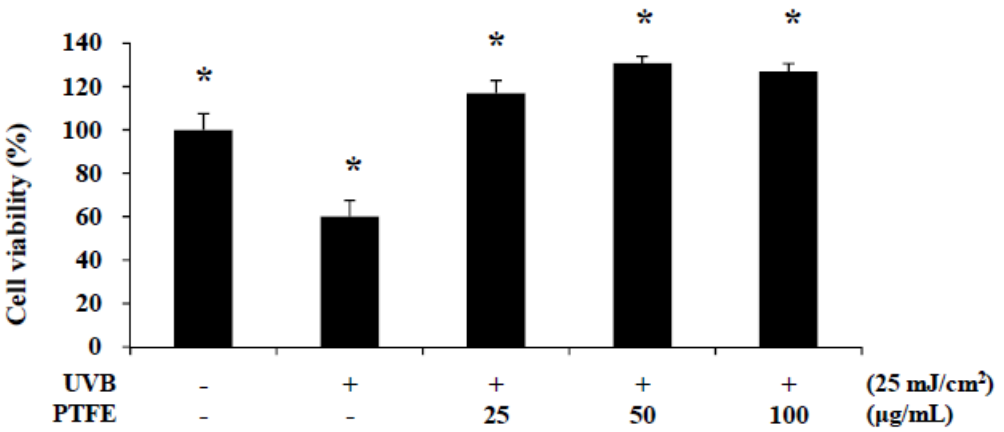


Fig. 3: Effects of the *Persicaria tinctoria* flower extracts (PTFE) on the cell viability of UVB-induced keratinocytes. The effect of PTFE on cell viability in UVB induces HaCaT cells. The cells were treated with or without PTFE, then irradiated with UVB (25 mJ/cm^2). After 24 h, the cell viability was measured by MTT assay.

Conclusion

This is the first study to investigate the antioxidant, anti-elastase, anti-tyrosinase, hypopigmenting, and UV-protective activity of PTFE. The free radical-scavenging and elastase inhibitory activities of PTFE suggest that they can restore skin elasticity, thereby slowing down the onset of wrinkles. PTFE also inhibited melanin production in melanoma cells and decreased mushroom tyrosinase activity, which shows its potential to prevent skin pigmentation. In addition, PTFE showed excellent protective effect against UVB‐induced cytotoxicity in human keratinocytes. In conclusion, PTFE may be practically applicable as an active ingredient in cosmetics for the prevention of skin aging. However, further work is needed to identify the active compound(s) and its mechanism of action.

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