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Original Article

Melanogenesis Inhibitory Effects of Ethanol Extract of *Perilla frutescens*'s Leaves on B16 Melanoma Cells

Thuan Van Tran [a, b, d], Quynh-Nhu Nguyen-Ho[a, d], Truong-Van Nguyen-Thien[c, d], Quang Ton-That[c,d], Thuoc Linh Tran [a,b,d], Hieu Tran-Van[*a, b, d]

[a]*Department of Molecular and Environmental Biotechnology, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, Vietnam,* [b]*Laboratory of Biosensors, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, Vietnam,* [c]*Department of Organic Chemistry, Faculty of Chemistry, University of Science, Ho Chi Minh City, Vietnam,* [d]*Vietnam National University, Ho Chi Minh City, Vietnam.*

Abstract

Perilla frutescens (L.) Britt. is a Vietnamese herb containing many polyphenol compounds potential on antioxidants and tyrosinase inhibitors for cosmetic applications. Natural compounds and products have got more attention as they have less undesirable side effects and satisfy the diverse needs of consumers that include anti-melanogenesis, antioxidation, etc. This study evaluated the antioxidant capacity, inhibition of melanin synthesis in B16 melanoma cells of perilla leaves ethanol extract (PEE). PEE showed the high antioxidant properties *in vitro* by 2, 2′-diphenyl-1-picrylhydrazyl radical assay (DPPH) and Ferric reducing antioxidant power (FRAP). In addition, PEE has inhibitory activities for mushroom tyrosinase (TYR). The results showed that concentrations of 100 - 300 µg/mL of PEE were not affected on B16 cell viability. In B16 cells, melanin content and cellular tyrosinase activity were significantly decreased in PEE-treated cells in a dose-dependent manner. These results suggested that *Perilla frutescens (L.)* Britt. had a great potential as natural sources which could be used as agents in skincare products.

Keywords: antioxidant, cosmetic. melanogenesis, melanin, Perilla frutescens, tyrosinase,

1. Introduction

Perilla frutescens (L.) Britt (Perilla frutescens var crispa) is an annual herb and belongs to the family Lamiaceae. Perilla is a native and popular herb in East Asian countries such as Japan, China, Vietnam, etc. In Vietnam, Perilla (Vietnamese name: tía tô) is not only a spiced vegetable rich in nutritional value, but also from it has long been used as a folk remedy to treat poisoning, colic, vomiting, cough, *etc.* [1, 2].

Nowadays, 271 phytochemical compounds are discovered and isolated from perilla that

Corresponding Author: Hieu Tran-Van, *Department of Molecular and Environmental Biotechnology, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, Vietnam, Laboratory of Biosensors, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, Vietnam, Vietnam National University, Ho Chi Minh City, Vietnam.*

Tel: +8428.62884499

E-Mail: tvhieu@hcmus.edu.vn

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included phenolic acids, flavonoids, anthocyanins, and so on [3]. In traditional medicine, the present of biochemical compounds in perilla leads to its beneficial biological activity for human health such as antioxidant activity, antibacterial and antifungal activity, anti-tumor effect, and anti-allergic effect [3, 4]. Recently, perilla has been interested in anti-aging and whitening effects due to its high content in phenolic acids that have potential applications in cosmetic [5, 6].

Melanin is a broad term for a group of natural compounds found in most organisms. In the human skin, melanin is synthesized in melanosomes of melanocytes, and it is transferred to keratinocytes, which is called melanogenesis. Melanocytes produce melanin through the action of tyrosinase (TYR), the protein related to tyrosinase 1 (TRP-1), and the protein related to tyrosinase 2 (TRP-2). Notably, TYR plays the most important role in the path of melanin biosynthesis [7, 8]. When there are activating effects from UV, these enzymes enhance melanin synthesis that leads to the increment of melanin. Although having high content in phenolic acids, leaves of perilla have been not yet examined the inhibitory ability of melanogenesis.

In this present study, ethanol extract of perilla's leaves (PEE) was prepared to investigate the inhibitory effect of the extract on melanin production and TYR activity in B16 murine cells. In addition, antioxidant activity and mushroom TYR inhibitory activity of PEE was also evaluated.

2. Material and Methods

2.1. Chemical and Reagent

Acid ascorbic, arbutin, acid kojic, dimethyl sulfoxide (DMSO), bovine Serum Albumin (BSA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-isobutyl-1-methylxanthin (IBMX), phenylmethylsulfonyl fluoride (PMSF), L-3,4 dihydroxyphenylalanine (L-DOPA), synthetic melanin, and Triton X-100 were purchased from Sigma (St. Louis, MO). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), trypsin/EDTA were from Biobasic (Canada). All reagents were at analytical grade.

2.2. Plant Material and Extraction Procedure

The leaves of *Perilla frutescens var. crispa* were collected in February 2019 in Ho Chi Minh city, Vietnam and were authenticated by botanist Dr. Viet Hoang, Department of Ecology and Evolutionary Biology, Faculty of Biology and Biotechnology, VNUHCM-University of Science, Ho Chi Minh City. A voucher specimen (PFC 2019) has been deposited at the Department of Organic Chemistry, Faculty of Chemistry, VNUHCM-University of Science. The dried leaves of *Perilla frutescens var. crispa* were ground into powder. The material (1.0 kg) was

then extracted under reflux with ethanol to obtain ethanolic extract (200.0 g).

2.3. Antioxidant Activities In Vitro

DPPH Radical Scavenging Activity was used to determine antioxidant activity of PPE [9]. 20 µL of different concentrations were pipetted into 980 µL DPPH solution (1.98 mg/mL in methanol). After 30 min of incubation, the result of reactions was measured at wavelength 517 nm by using a spectrophotometer. Positive control was ascorbic acid and negative control was solution without PPE. Antioxidant activity was demonstrated via the reduction of DPPH absorbance.

2.4. Reducing Antioxidant Power (FRAP) Method

FRAP solutions were prepared by mixing 10 mM TPTZ, 20 mM $FeCl_3.6H_2O$, 300 mM acetate buffer (pH 3.6) in the volume ratio of 1:1:10 at 37 ^oC. The reaction mixture contained 900 µL FRAP reagent and 100 µL different concentration of sample. After 30 min, ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex was reduced to ferrous (Fe^{2+} -TPTZ) with intense blue color and maximum absorption at 593 nm. The absorbance of different concentrations of $FeSO_4$ at 593 nm was use plot in the calibration curve [10].

2.5. Mushroom Tyrosinase Inhibitory assay

Tyrosinase inhibition assays were performed with L-DOPA as substrate [11]. 90 µL mixture contained concentrations of samples and phosphate buffer (pH 6.8, 50 mM), which were added in 96-well microplate with 30 µL tyrosinase (250 U/mL). After 15 min, 80 µL L-DOPA (10 mM) was mixed and the reaction mixture was incubated for 10 min at room temperature. A mount of Dopachrome was determined by measurement of absorbance at 492 nm. Kojic acid was used as a positive control. Percentage inhibition of enzyme activity was calculated according to the following equation:

$$\text{Inhibition (\%)} = 100\times(A_0 - A_1)/A_0$$

where A_0, and A_1 are absorbance values of control, and samples, respectively.

2.6. Cell Culture

B16 murine cells (ATCC) were cultured in complete growth media that included DMEM high- glucose (Sigma), 10% FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin. Cells were maintained in an incubator with 5% CO_2, 37℃. In three days, cells would be sub-cultured to maintain cell growth. Cells were not used over 20 passages.

2.7. Cell Viability

MTT assay was used to test B16 cell viability with PEE [12, 13]. Cells were seeded in 96-well microplate with $1x10^4$ cells/100 µL/well. Overnight, fresh media with different concentrations (0 to 800 µg/mL) of samples that were added to each well. After incubating plates for 48h, MTT (0.5 mg/mL) were incubated in 3h and formazan crystals were solubilized in DMSO. The darker solution absorbed in 550 nm that were detected by the microplate reader (Microplate Reader Thermo Multiskan Ascent 96).

2.8. Melanin Content Assay

The melanin content was measured as described previously with some modifications. B16 cells were seeded in a 6-well plate ($2x10^5$/well). After 24h, the media were replaced by fresh media with IBMX (100 nM), and different sample concentrations. At the end of cell culture, cells were washed by PBS and lysed in 20 mM sodium phosphate (pH 6.8), and 1% Triton X-100 [14]. After centrifugation at 13,000 rpm in 20 min, the melanin pellets were solubilized in 150 µL NaOH 1N (containing 10% DMSO) by ultrasound for 45 min, and at 80℃. The melanin solutions were measured at 450 nm by the microplate reader (Microplate Reader Thermo Multiskan Ascent 96). The percentage of melanin content was calculated using the following equation:

Melanin contents (%) = $100*OD_1/OD_0$. OD_1 is OD of control; OD_0 is OD of sample.

2.9. Intracellular Tyrosinase Activity

Intracellular tyrosinase activity was evaluated as described in a previous study with some modifications. Cells were maintained in a 6-well plate with 100 nM IBMX and 100-300 µg/mL of sample [15]. Cells were harvested by cold PBS and lysis solution that contain 20mM sodium phosphate, 1% Triton X-100, and phenylmethylsulfonyl fluoride (PMSF). After freezing and thawing, the lysates were centrifuged at 13,000 rpm for 20 min. Then, 70 µL of supernatants were incubated at 37℃ in 10 min. L-DOPA (10 mM) was added to microplate in 10 min and absorbance was measured at 492 nm using the microplate reader (Microplate Reader Thermo Multiskan Ascent 96). The protein content in the supernatants was measured by Bradford assay, with BSA as the protein standard.

2.10. Statistical Analysis

An analysis of variance (ANOVA) and Student's t-test was used to determine the significance of different means. P values less than 0.05 were statistically significant.

3. Results and Discussion

3.1. Antioxidant Activities In vitro

Nowadays, various methods for determining antioxidant activity *in vitro* are widely used due to their efficiency and convenience. These methods are intended to analyze antioxidant capacity of extracts because compositions and contents of antioxidant agents in extracts have different antioxidant mechanisms. In this study, we used two method to evaluate antioxidant capacity of PEE including scavenging radical activity of DPPH, and ferric-ion-reducing capacity.

The present data showed PEE had a medium ability to neutralize DPPH free radicals, and the IC_{50} value of 41.41 µg/mL. Vitamin C was used as a positive control and its IC_{50} was 1.27 µg/mL (Table 1).

Principle of ferric reducing antioxidant power assay is ability of antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ. One-gram dry weight of extract (DE) could reduce 13.72 mol Fe^{3+} to Fe^{2+} (Table 1).

Reports from the analysis of Central Laboratory for Analysis showed that PEE contained phenolic, flavonoid, anthocyanin at a concentration of 11.58 mg/g dry weight (DW),

Table 1. Antioxidant capacity and mushroom tyrosinase inhibitory activity of PEE.

	DPPH IC_{50} (μg/ml)	**FRAP (mol Fe^{2+}/1g DE)**	**Inhibition of tyrosinase IC_{50} (μg/ml)**
PPE	41.41	13.72	1259.00
Positive Control	1.27 (Vitamin C)		31.06 (Kojic acid)

8.46 mg/g DW, and 3.53 mg/g DW, respectively. Phenolic, flavonoid, and anthocyanin are polyphenol compounds that have been shown to have high antioxidant capacity [١٧ ,١٦]. There have been many previous studies on the polyphenols of perilla that had a high resistance to oxidation via *in vitro* methods [18, 3] which was in line with the antioxidant activity of PEE in this study because the extract contained phenolic, flavonoid, and anthocyanin cyclic compounds.

In a study of Lee *et al*. (2017) showed that Korean perilla's extracts had antioxidant resistance ranging from 63% to 86% at a concentration of 100 μg/mL, which was consistent with our results (100 μg/mL of PEE scavenged 86.96% radical activity of DPPH). Different from our results, the research of Yueliang Zhao *et al*., (2019) showed an antioxidant activity of IC_{50} as 12.15 μg/mL [19] Methanol extract of perrila at a concentration of 1.5-25 μg/mL had scavenging abilities on DPPH radicals was in the range of 6.7-63.1% in study of Lin *et al.* [20]. Antioxidant ability of those extracts was higher than that of our PEE because of its high contents of phenolic, and flavonoid.

3.2. Mushroom TYR Inhibitory Activity

Inhibitory tyrosinase ability is a valuable feature of ingredient in cosmetic products. Oxidant L-DOPA activity of mushroom tyrosinase was reduced by PEE. The following equation presented the formula for percentage inhibiting tyrosinase activities (y) of PEE and its concentrations (x): $y = 16.377\ln(x) - 66.911$. IC_{50} value of PEE was 1259 μg/mL that was high in comparison to IC_{50} of Kojic acid (IC_{50} =31.06 μg/mL) (Table 1). In a study of Jung-Hee Hwang *et al*. showed that the extract of *Perilla frutescens var. Acuta*'s leaves inhibited 52.35 ± 1.18% tyrosinase activity at 666.67 μg/mL [6]. These results implied that perilla leaf extract had the ability to inhibit tyrosinase, however, the inhibitory ability was poor because the amount of polyphenol compounds accounts for a small proportion of the extract. On the other side, extracts of different parts of perilla had different inhibitory tyrosinase ability. In Mungmai's study, methanol extract of *perilla frutescens var crispa* seeds inhibited 79% tyrosinase activity at 80 μg/mL [21].

3.3. Cell Viability

Before further evaluation, PEE was determined its effect on viability of B16 melanoma cells by MTT assay. A range of concentration of 0 to 800 μg/mL was treated on B16 cell for 48h at 37°C. As shown in Fig. 1, PEE had no cytotoxic effect on B16 at concentration of 100 to 300 μg/mL. However, concentrations of 400 to 800 μg/mL significantly reduced B16 cell viability. Positive control was DMSO 10% that almost destroyed cell viability (15.88 ± 1.79%).

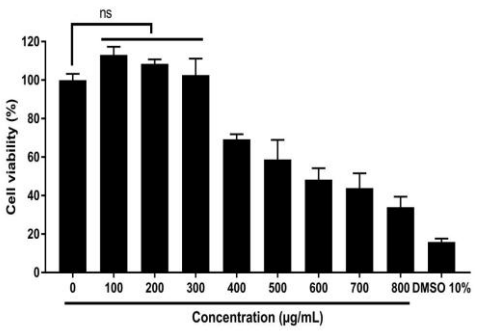

Figure 1. Effect of perilla ethanol extracts on cell viability of B16 cells.

In a previous study, methanol extract of above-ground portion of *Perilla frutescens* induced melanoma cell apoptosis that resulted in decline of B16 viability at the concentration of 100 - 400 µg/mL [22]. In line with the published result, ethanol extract of perilla's leaves (PEE) had just toxicity on B16 cells at the concentration of 400 - 800 µg/mL, thus PEE doses ranging from 100 to 300 µg/mL were used to further investigation.

3.4. Effect of PEE on Melanin Content

Melanin content in B16 was measured to demonstrate inhibitory melanogenic effect of PEE. The IBMX agent was used to stimulate intracellular cAMP level that leads to enhance melanin synthesis. Fig. 2.A showed that melanin content of cell IBMX-treated dramatically increased. PEE treatment in B16 strongly prevented melanin production on B16 cells. In comparison between inhibitory potencies of PEE and Arbutin, 300 µg/mL PEE exhibited more effective anti-melanogenic activity than 2 mM Arbutin, a positive control. In other studies, extracts of seeds and aerial parts of perilla also had a great ability to decrease melanin production [21, 6]. These studies implied the herbal plant showed a huge potential as a whitening agent in cosmetic products.

3.5. Effect of PEE on Intracellular Tyrosinase Activity

Tyrosinase is a crucial element in melanogenesis, therefore effect of PEE on intracellular tyrosinase activity assay was also investigated. PEE effectively inhibited tyrosinase activity compared to IBMX-only-treated B16 cells at concentration of 200 to 300 µg/mL (Figure 2B). Two mM Arbutin and 200 µg/mL PEE had comparable inhibition of tyrosinase activity. These results proved that PEE significantly reduced melanin synthesis by

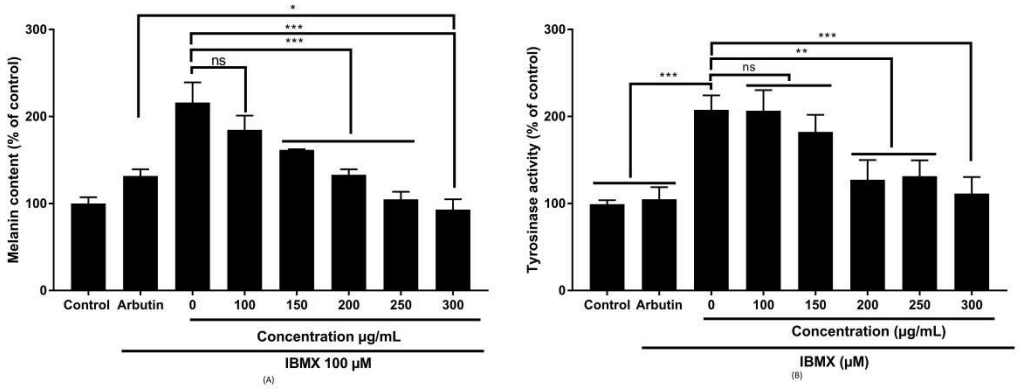

Figure 2. Effect of perilla ethanol extracts on Melanin production (A) and Intracellular tyrosinase activity (B) in B16 Cells.

directly inhibiting cellular tyrosinase activity in IBMX-stimulated B16 melanoma cells.

IBMX is a phosphodiesterase inhibitor, which increases intracellular cAMP, thereby activating protein kinase A (PKA). PKA is an enzyme capable of regulating the transcription factor Microphthalmia-associated transcription factor (MITF), which plays a key role in melanogenesis. MITF is directly related to the expression of important enzymes such as tyrosinase, TRP-1, and TRP-2 [23, 7]. The increase in expression of these factors leads to an increase in melanin production in B16 cells. Two mM Arbutin and 300 μg/mL PEE significantly reduced intracellular melanin and intracellular tyrosinase activity. Arbutin is powerful inhibitor of tyrosinase, thereby reducing melanin synthesis on B16 cells. However, PEE has not been shown to effectively inhibit mushroom tyrosinase activity. Therefore, PEE was likely to decrease the expression of factors in the melanin synthesis pathway or indirect regulation of tyrosinase activity, although the mechanism was not yet clear.

4. Conclusion

Ethanol extract of *Perilla frutescens*'s leaves as a natural product that has multi-function: free radical scavenging activity, ferric-ion-reducing capacity, and effective anti-melanogenesis activity. This study proved that *Perilla frutescens* have a great whitening capability *in vitro*. In further studies, *Perilla frutescens'*s leaves should be evaluated in melanogenesis regulation on cell *(in vitro)*, and inhibition of melanin production on animal model *(in vivo).*

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