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In vitro anti-melanogenic effect of *Perilla frutescens* leaf extracts

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ABSTRACT

Introduction: *Perilla frutescens* (L.) Britt leaves contain various phenolic compounds, especially flavonoids, which potentially inhibit tyrosinase, the key enzyme catalyzing the melanin synthesis pathway. This work aimed to investigate the anti-melanogenic effect of *Perilla frutescens* extracts through the inhibition of non-cellular as well as cellular tyrosinase activities.

Methods: The total extract from *Perilla frutescens* leaves was obtained by percolation method with 50% ethanol followed by liquid–liquid partition with ethyl acetate (EA) and chloroform (CF) to obtain the EA, CF and EA/CF extracts. The mushroom tyrosinase inhibitory effect of the obtained extracts was screened by dopachrome formation reactions from its substrate 3,4-dihydroxy-L-phenylalanine. The extracts with potential activity were further evaluated for the anti-melanogenic effects in the B16F10 melanoma cell line.

Results: EA and EA/CF extracts significantly inhibited mushroom tyrosinase activity with the IC_{50s} of 0.14 and 0.07 mg/mL, respectively, in the same range of that from kojic acid (0.12 mg/mL). Consistently, in B16F10 cells, these extracts inhibited tyrosinase enzyme; their IC_{50} values were 2 times lower than that of kojic acid. Moreover, both EA and EA/CF extracts remarkably reduced melanin levels in a concentration-dependent manner.

Conclusion: The EA and EA/CF extracts from *Perilla frutescens* leaves were able to inhibit melanogenesis by reducing tyrosinase activity.

Implication for health policy/practice/research/medical education:
The results of this study showed the promising anti-melanogenic effect of various extracts from *Perilla frutescens* leaves providing the scientific evidence for the topical application of Perilla for skin whitening or pigmentation prevention.
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Introduction

Melasma is a common disorder of hyperpigmentation that affects millions of people worldwide. Melasma is characterized by the overproduction and chronic deposition of melanin in the epidermis, which causes uneven brown spots on sun-exposed skin, especially on the face, which affects the aesthetics and causes distress in these patients (1, 2). The treatment of melasma is high cost and usually less effective than expected. A number of options for topical treatment of melasma are available containing kojic acid, hydroquinone, azelaic acid, retinoids, topical steroids, as well as chemical peels, lasers, and intense pulsed light therapy (3). These modalities of treatment modify various stages of melanogenesis; one of the most common approaches involves the inhibition of tyrosinase, a type 3 membrane-bound protein and a key enzyme in the rate-limiting step of melanogenesis process (4,5). Tyrosinase catalyzes the hydrolysis of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and the oxidation of the latter to dopaquinone. Dopaquinone is then polymerized to form melanin (6). However, the long-term use of cosmetic products containing high concentrations of these lightening agents may lead to many undesirable effects, such as allergic reactions, erythema, and post-inflammatory hyperpigmentation (4,7). Thus, effective and safe preparations are always being sought to incorporate in the melasma therapy.

Perilla (*Perilla frutescens* (L.) Britt is an annual herbaceous plant belonging to the family Lamiaceae (5). This herb is widespread in East Asia countries, such as

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Japan, China, South Korea, India, and Vietnam (6, 8). Perilla leaves vary from green to purple with a minty odor. They are widely used for garnishing, culinary, and natural medicine (9). Many polyphenols, such as catechin, rosmarinic acid, caffeic acid, and ferulic acid, were found in Perilla herbal plant. Rosmarinic acid is the main phenolic compound in leaves with an elevated concentration during the reproductive stage of the plant (10,11). Perilla leaves also contain flavonols, such as apigenin and luteolin (10). Many previous studies demonstrated that polyphenols and flavonoids were potent inhibitors of the tyrosinase enzyme (12-14). Apigenin also expressed the inhibitory effect against mushroom tyrosinase, and luteolin was found to reversibly and non-competitively suppress tyrosinase by molecular docking and chromatography techniques (15).

In this study, total ethanolic extract from dried Perilla leaves was prepared by the percolation method followed by fractionation with solvents having different polarities. These extracts were preliminarily screened for its potent anti-tyrosinase effect through the mushroom tyrosinase inhibitory assay. The most effective extracts were further investigated for their anti-melanogenesis by the inhibition of intracellular tyrosinase from murine B16F10 melanoma cells and the melanin content assay. Kojic acid, a well-known tyrosinase inhibitor and commonly incorporated in skin-whitening preparations, was used as a positive reference (16,17).

Materials and Methods

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were purchased from Invitrogen Corporation (USA). Mushroom tyrosinase from *Agaricus bisporus*, 3,4-dihydroxy-L-phenylalanine (L-DOPA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), kojic acid, bovine serum albumin (BSA) and synthetic melanin were obtained from Sigma–Aldrich (USA). Na_2HPO_4 and NaH_2PO_4 were from Guangdong (China), and the B16F10 murine melanoma cells (KCLB 80008) were from the Korean Cell Line Bank (Seoul, Korea).

Preparation of Perilla extracts

Perilla frutescens (L.) Britt plants were collected in Hanoi, Vietnam in June 2019. The medicinal plants were previously authenticated by Prof. Thi Dep Tran, department of botany, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City, Viet Nam. A voucher specimen was stored at this department for future reference (TT1216).

Perilla leaves were harvested, washed, and dried in the shade until the humidity was under 15%. The dried leaves were further ground into a dry powder with the size of 3-mm cubes. Perilla leaf powder (1900 g) was exhaustively extracted by percolation with 50% ethanol until the extract no longer reacted with 5% $FeCl_3$ in ethanol. After filtration, the obtained filtrate was condensed by vacuum evaporation at 55°C to obtain the total Perilla (TP) extract with an extraction yield of 13.25%. The quantitative determination by a reverse phase HPLC method showed the estimated concentration of rosmarinic acid and luteolin in the TP extract about 1.178 ± 0.011 and 0.105 ± 0.001% (w/w), respectively (18). The TP extract (140 g) was then dispensed in distilled water (14 L) and divided into 2 equal parts. The first one was liquid-liquid partitioned with ethyl acetate (EA extract, extraction yield of 3.48%). The second one was partitioned with chloroform (CF extract, extraction yield of 2.8%); the aqueous phase was then shaken with ethyl acetate (EA/CF extract, extraction yield of 0.77%). The organic solvents were evaporated under vacuum and the condensed extracts were stored at 2–8°C.

Mushroom tyrosinase assay and IC_{50} determination

The Perilla extracts were prepared in various solvents at the maximum soluble concentrations in reaction mixture: 3.4 mg/mL TP extract in 50 mM sodium phosphate (NaP) buffer pH 6.8, 0.75 mg/mL EA extract in EtOH, 0.5 mg/mL CF and 1 mg/mL EA/CF extracts in DMSO. Mushroom tyrosinase and L-DOPA were dissolved in 50 mM NaP buffer pH 6.5 at 100 U/mL and 10 mM, respectively. In the 96-well plate, the reaction mixture (120 µL) contained 80 µL of NaP buffer, 10 µL of Perilla extracts, and 30 µL of tyrosinase solution. The mixture was incubated for 5 minutes at 25°C. The reaction was initiated by adding 80 µL of L-DOPA solution. The absorbance (Abs) of the reaction mixture was monitored at 490 nm every 10 seconds for 10 minutes in a microplate reader (ELISA iMark™ Microplate Absorbance Reader, Bio-Rad, USA) to determine the reaction velocity v (Abs/min) (19). The percentage of tyrosinase inhibition was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = \left(1 - \frac{v_{CE} - v_C}{v_{BE} - v_B}\right) \times 100,$$

where CE is the test sample, C is the test sample without the enzyme, BE without the extract, and B without both the enzyme and the extract. Each measurement was performed in triplicate.

The most effective Perilla extracts from the tyrosinase inhibitory assay were further examined to determine the IC_{50} values (the concentration causing 50% inhibition of enzyme activity). Kojic acid was used as a reference inhibitor. A linear regression equation describing the relationship between the logarithm of the extract's concentration (a= logC) and the percentage of tyrosinase inhibition (y) of the Perilla extracts was established as y = ax + b, from which the IC_{50} value was inferred as follows:

$$IC_{50} = 10^{\frac{50-b}{a}}$$

Cell culture

Murine B16F10 skin melanoma cells were grown in DMEM supplemented with 10% FBS and 1% streptomycin in T25 flasks. The cells were incubated in humidified 5% CO_2 atmosphere at 37°C and sub-cultured every 4–5 days after reaching 90% confluence.

Cell proliferation assay (MTT assay)

MTT, a yellow tetrazolium salt, can only be reduced in the mitochondria of viable cells to create a crystal blue compound, formazan. Thus, the formation rate of formazan is proportional to the number of viable cells and can be quantified spectrophotometrically (20). The suspended cells were seeded into each well (3×10^3 cells/well) and incubated for total adherence in 24 hours. The culture medium was removed, the cells were then incubated with the Perilla extracts for 24 hours. The MTT dye (5 mg/mL in NaP buffer pH 6.8) was added and incubated for 3 h. After that, the supernatant was carefully removed and the insoluble formazan product was dissolved by 100 µL DMSO. The absorbance of this mixture (Abs_{sample}) was measured by a microplate reader (ELISA Therma, Biotek, USA) at 550 nm. Each treatment was repeated three times. The maximal absorbance was determined by incubating cells with culture media (no exposure to Perilla extracts) and was considered as 100% viability ($Abs_{control}$). Cell viability percentage was calculated as follows:

$$\text{Cell viability (\%)} = Abs_{sample}/Abs_{control} \times 100$$

Cellular tyrosinase inhibitory assay

Cellular tyrosinase assay was carried out according to the previously described method (11). Briefly, a predetermined number of B16F10 melanoma cells (3×10^3 cells/well) were seeded into a 96-well plate in 100 µL of the culture medium and left overnight for adherence in humidified 5% CO_2 atmosphere at 37°C. Perilla extracts or kojic acid at different concentrations were added to each well. After 24-hour exposure to the treatments, the cells were washed with 100 µL of NaP buffer pH 6.8 and lysed with 100 µL of NaP buffer pH 6.8 containing 1% of 5 mM EDTA prepared in Triton X-100. The cell lysates were centrifuged at 13000 rpm (Himac CT15E, Hitachi, USA) in 15 minutes at 4°C to collect the cell free extract (CFE). The protein concentrations of the CFE were quantified by the Bradford method using BSA as protein standard (21). For the cellular tyrosinase assay, a volume of CFE corresponding to 40 µg protein and 100 µL of 5 mM L-DOPA were pipetted into each well and incubated for 1 hour at 37°C. The absorbance of the mixture (Abs_{sample}) was measured at 475 nm using a microplate reader (ELISA Therma, Biotek, USA). Each sample was carried out in 3 wells and repeated twice. The maximal absorbance was determined by incubating cells with free media (no exposure to Perilla extracts nor kojic acid) and was considered as 100% activity ($Abs_{control}$). Tyrosinase activity was calculated as follows:

$$\text{Tyrosinase activity (\%)} = Abs_{sample}/Abs_{control} \times 100$$

Melanin content assay

Melanin levels were carried out as previously described by Hosoi et al (22). A predetermined number of B16F10 melanoma cells (3×10^3 cells/well) was seeded into a 96-well plate and incubated using similar conditions as in the cellular tyrosinase assay experiment. Perilla extracts or kojic acid at different concentrations were added to each well. After 24-hour exposure, the cells were washed with 100 µL of NaP buffer pH 6.8 and lysed in 200 µL of 1 N NaOH in buffer containing 10% (*v/v*) DMSO by heating at 80°C for 1 hour. The absorbance of the lysate was determined at 405 nm by a microplate reader (ELISA Therma, Biotek, USA). The maximal melanin content was obtained in untreated cells and was set at 100% content (Control). The melanin content of samples (Perilla extracts or kojic acid) was expressed as relative (%) to the control (22).

Statistical analysis

The results were presented as the mean ± SD of 3–4 independent experiments. Comparisons among groups were determined by the ANOVA test and between two groups by the Student's *t* test with $P < 0.05$.

Results

Effects of *Perilla frutescens* (L.) Britt on mushroom tyrosinase activity and IC_{50} determination

The mushroom tyrosinase inhibitory activity of the extracts is presented in Table 1. The inhibitory effects of EA and EA/CF extracts were higher than that of the TP and the CF extracts at the highest concentration that could be prepared. Hence, these two extracts were chosen for further determination of IC_{50} values with kojic acid as a positive control (Table 2). The results showed that the IC_{50} value of the EA extract was approximately equal to that of kojic acid while, interestingly, the EA/CF extract even had a lower IC_{50} value than that of kojic acid. This indicated that Perilla extracts could potentially contain strong tyrosinase inhibitors.

Table 1. Mushroom tyrosinase inhibitory effects of Perilla extracts

Perilla extracts	Mushroom tyrosinase inhibition (%)
TP extract	46.58 ± 14.52
CF extract	37.42 ± 12.93
EA extract	68.44 ± 0.57
EA/CF extract	68.74 ± 6.48

TP, total Perilla; EA, ethyl acetate; CF, chloroform.
Data are expressed as mean ± SEM.

Table 2. IC_{50} of potent Perilla extracts and kojic acid

Sample	Concentration range(mg/mL)	Regression equation	IC_{50} (mg/mL)
EA extract	0.006-0.75	y = 28.213x + 73.749 (R^2 = 0.996)	0.14
EA/CF extract	0.0002-1.00	y = 16.579x + 68.679 (R^2 = 0.998)	0.07
Kojic acid	0.01-4.26	y = 33.537x + 80.685 (R^2 = 0.980)	0.12

EA, ethyl acetate; CF, chloroform.

Table 3. Effect of Perilla extracts and kojic acid on viability (%) of B16F10 cells

Concentration (µg/mL)	240	120	60	30	15	7.5
EA extract	29.71 ± 1.75	31.85 ± 1.22	32.83 ± 1.92	33.59 ± 2.26	34.05 ± 2.62	33.98 ± 2.86
EA/CF extract	31.20 ± 2.19	32.50 ± 3.75	34.97 ± 2.14	34.79 ± 2.78	34.41 ± 2.91	35.28 ± 3.33

EA, ethyl acetate; CF, chloroform.
Data are expressed as mean ± SEM.

Cytotoxicity of Perilla extracts on the B16F10 cells

Table 3 shows the proportion of the B16F10 viable cells under the treatment of Perilla extracts and kojic acid. Both EA and EA/CF extracts produced high cytotoxicity in B16F10 melanoma cells. Only 33.98% and 35.28% of the cells treated by the lowest concentration tested (7.5 µg/mL) of EA and EA/CF extracts, respectively, were still alive. However, when the concentration of the extracts increased, the cell viability did not decrease proportionally. In fact, the percentage of viability at concentrations ranging from 7.5 µg/mL to 240 µg/mL of Perilla extracts was not statistically different.

Effects of Perilla extracts on cellular tyrosinase activity in the B16F10 cells

The regression equation of the correlation between logarithm concentration of Perilla extracts and the percentage of cellular tyrosinase inhibition and their IC_{50} value are presented in Table 4.

Results showed that the cellular tyrosinase inhibitory effect of Perilla extracts and kojic acid was concentration-dependent. The B16F10 cells exposed to higher concentrations of these treatments had lower tyrosinase activity. The IC_{50} of kojic acid was about 2.6 times and 2 times higher than that of EA and EA/CF extracts, respectively. This proved that both investigated Perilla extracts exhibited a relatively strong tyrosinase inhibitory activity on the B16F10 melanoma cell line.

Table 4. Regression equation and IC_{50} of the Perilla extracts and kojic acid in cellular tyrosinase inhibitory assay

Samples	Regression equation	IC_{50} (µg.mL^{-1})
EA extracts	y = 16.788x + 15.286 (R^2 = 0.9322)	116.90
EA/CF extracts	y = 16.001x + 15.153 (R^2 = 0.956)	150.59
Kojic acid	y = 17.67x + 6.2688 (R^2 = 0.9919)	298.46

EA, ethyl acetate; CF, chloroform.

Effects of Perilla extracts on the melanin content

Both EA and EA/CF extracts of *Perilla frutescens* (L.) Britt and kojic acid remarkably reduced the melanin level in a concentration-dependent manner (Figure 1).

The melanin content of B16F10 melanoma cells under EA/CF extract treatment gradually decreased by 30.78% at the concentration of 15 µg/mL to 44.21% at 240 µg/mL compared to the control, and the reduction proportions at each concentration were not statistically different between the two extracts. Particularly, at the concentration of 240 µg/mL, the melanin level of EA/CF extract group was statistically lower than that of the kojic acid group (P<0.05).

Discussion

Tyrosinase inhibitors are common agents in skin-whitening cosmetics and drugs to treat hyperpigmentation (23). Our study aimed at investigating the anti-melanogenetic effect of Perilla extracts by using the mushroom tyrosinase assay and the cellular-derived tyrosinase from the murine B16F10 melanoma cells. The mushroom tyrosinase system is frequently used to screen for anti-melanogenesis agents (24). The cell line B16F10, a mouse melanoma cell that produces tyrosinase, was applied as it could be easily handled and cultured (25).

In the MTT test, the number of viable cells under the treatment of EA and EA/CF fractions was remarkably lower than that of the negative control group. Thus, these extracts were quite cytotoxic to the B16F10 melanoma cells. However, the viable cell rates among various increasing concentrations were not statistically different. This may also result from the high number of cells seeded into wells or extended incubation period.

The results from the screening of mushroom tyrosinase inhibitory assay showed that EA and EA/CF extracts were the most promising agents. In detail, compared to the known inhibitor kojic acid, EA extract presented similar inhibitory potency while EA/CF extract was even

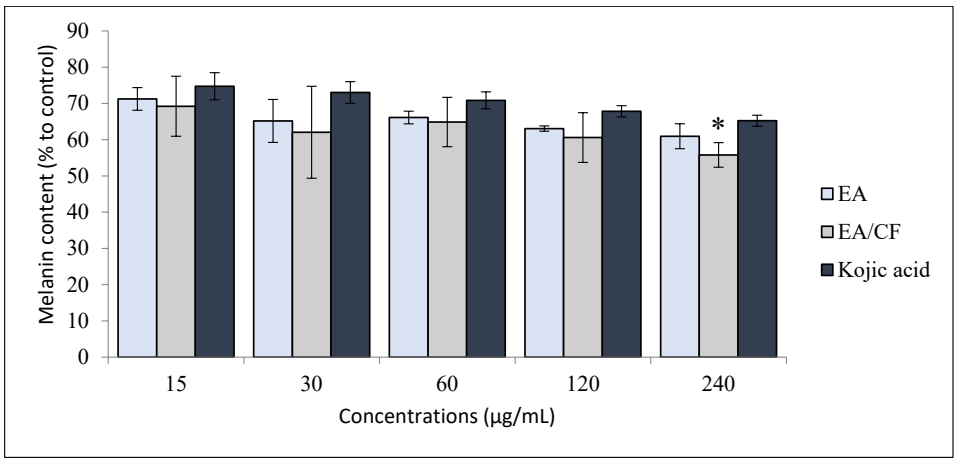

Figure 1. Melanin content in B16F10 melanoma cells after exposure to Perilla extracts and kojic acid. * $P < 0.05$ compared to kojic acid. (EA: Ethyl Acetate; CF: Chloroform).

more active. However, the intracellular assay indicated that the tyrosinase inhibitory activity of these extracts is much stronger than that of kojic acid. This may result from the heterogeneity between the mushroom tyrosinase and the human tyrosinase. The mushroom tyrosinase presents freely in the cytoplasm in tetrameric form, while the human tyrosinase is a membrane-bound monomer. Furthermore, the sequence similarity between them is only 23% (26,27). Many studies have indicated that the inference would be more credible when using tyrosinase produced from viable cells rather than mushrooms to screen for melanogenesis inhibitory effect (21,25,28).

Corresponding to the decrease in tyrosinase activity, the level of melanin in B16F10 melanoma cells also remarkably decreased compared to the negative control group and was almost equivalent to the kojic acid group. This is probably because the EA and EA/CF leaf extracts concentrate a range of polar substances such as polyphenols, particularly flavonoid compounds, which have been proved to possess anti-tyrosinase effect as well as remarkable antioxidant effect (29,30). The antioxidants were also well-known to depress the hyperpigmentation and melanin synthesis (31,32). Perilla extracts contain both luteolin and rosmarinic acid possessing the o-diphenolic structure, which is mandatory for the oxidation/reduction step of melanin synthesis process (33). Thanks to the o-diphenolic structure, these compounds exhibit the competitive inactivation of tyrosinase enzyme through the inhibition of diphenolase activity (33,34). Luteolin and rosmarinic acid were also named as suicide inactivators with two proposed inhibitory mechanisms along with the shape-changing of tertiary and quaternary structure of tyrosinase (35,36).

Conclusion

The promising results from this study suggested the anti-melanogenesis effects of Perilla extracts, especially EA and EA/CF extracts. Further work should be performed to investigate phytochemical components in these extracts and subcellular mechanisms involved in tyrosinase inhibitory activity.

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Authors' contributions

TTAN and THD performed experiments and drafted the manuscript. TTHP contributed to analyze the data and edit the manuscript. NTH conceived the idea, the protocol for the experiments, and completed the manuscript. All authors critically reviewed and approved the final manuscript for publication.

Conflict of Interests

Authors declare no conflict of interests.

Ethical considerations

This study was approved by the Scientific Committee of faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh city, Viet Nam (Number 4380/QĐ-ĐHYD). Text plagiarism, data fabrication and redundant publication have been carefully observed by the authors.

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