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Anti-Melanogenesis Activity of Supercritical Carbon Dioxide Extract from *Perilla frutescens* Seeds

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Abstract

Perilla frutescens seed (PFS) oil is reported to inhibit skin photoaging; however, its effect on melanogenesis has not yet been investigated. Herein, we tested the anti-melanogenesis activity of an oil-based extract from PFS with supercritical carbon dioxide ($scCO_2$). In a cell culture system, B16 mouse melanoma cells were treated with the PFS $scCO_2$ extract and other samples. The PFS $scCO_2$ extract decreased melanin production by approximately 90% in B16 mouse melanoma cells without cytotoxicity at 100 μg/mL. This effect was greater than that of the well-known melanogenesis inhibitor, kojic acid. Although a hexane-extracted PFS oil and a squeezed PFS oil also decreased melanin production in the B16 cells, the inhibitory effect of the PFS $scCO_2$ extract was higher than both of these. Chemical analysis of the PFS $scCO_2$ extract and squeezed PFS oil showed that almost 90% of the components of both oils were α-linolenic acid, linoleic acid, and oleic acid. Furthermore, the ratio of those three fatty acids across both samples was almost the same. When the three fatty acids were mixed in the same ratio as in the PFS $scCO_2$ extract, the IC_{50} of the mixture for melanin production in B16 melanoma cells was identical to that of the PFS $scCO_2$ extract. However, the IC_{50} of the squeezed PFS oil was approximately 6.6 times higher than that of the mixture. Although those fatty acids are the main inhibitory ingredients against melanin production in all of the extracts, some factor(s) in the squeezed PFS reduce their affinity with the cells. These results indicated that the PFS $scCO_2$ extract could be a superior melanogenesis inhibitor. Although its main ingredients are probably the same as those of the squeezed PFS oil, it is necessary to extract with $scCO_2$ for stronger anti-melanogenesis activity.

Keywords

Perilla frutescens, Melanogenesis, Supercritical Carbon Dioxide, B16 Mouse Melanoma Cells

1. Introduction

Melanogenesis is involved in the production of melanin by melanocytes and the subsequent distribution of melanin to the basal cells of the epidermis [1]. Melanin is vital in the development of skin color [2] and protection of skin from ultraviolet light [3]; however, excessive melanin production leads to adverse effects, such as blotches and freckles on the skin, and therefore, many melanogenesis suppressors have been previously investigated. Examples of these suppressors include arbutin [4] [5], kojic acid [6] [7], 4-n-butylresorcinol [8], derivatives of ascorbic acid [9] [10] [11], tranexamic acid [12] [13] [14], and crude extracts of plants, such as aloe (*Aloe ferox*) [15], shrubby sophora (*Sophora flavescens*) [16], and rice bran [17].

Perilla frutescens (L.) Britton var. *frutescens* is a traditional herbal grass native to East Asia [18]. Extracts from *P. frutescens* leaves have shown anti-inflammatory activity [19] [20] [21] and *P. frutescens* seeds (PFSs), which are generally used as a raw material of edible oil, contain many fatty acids including linolenic acid, linoleic acid, and oleic acid [18] [22] [23]. PFS oil has been reported to inhibit skin photoaging [22], and medicinal and nutritional benefits are associated with the consumption of the oil [24] [25] [26]. Yet, the effects of PFS oil on melanogenesis have not been investigated, even though the three aforementioned fatty acids are known to show anti-melanogenesis activity [27] [28].

The use of supercritical carbon dioxide ($scCO_2$) fluid as an extractant for plant oils has gained attention. The $scCO_2$ dissolves in lipids [29] and because the critical temperature and pressure of carbon dioxide (CO_2) are relatively low [30], extraction via $scCO_2$ does not require large-scale equipment. We have previously reported that a $scCO_2$ extract of Kuji amber inhibits melanin production in B16 mice melanoma cell lines [31]. The anti-melanogenic effect of the $scCO_2$ extract is greater than that of an ethanol extract obtained from the same amber, indicating that $scCO_2$ is more efficient than ethanol in extracting anti-melanogenic compounds from Kuji amber [31]. This suggests that $scCO_2$ may also represent a valuable extractant for other natural products, such as PFS.

Herein, we examined the anti-melanogenesis effects of the PFS $scCO_2$ extract and compared these effects to those of kojic acid, a well-known melanogenesis inhibitor, and PFS oils. We further evaluated the content ratio of some fatty acids in the PFS $scCO_2$ extract and squeezed PFS oil, as well as examined if these fatty acids are the main active ingredients contributing to the anti-melanogenesis activity.

2. Materials and Methods

2.1. Preparation of Test Samples

2.1.1. Reagents and Materials

Kojic acid was purchased from Tokyo Kasei Co., Ltd. (Tokyo, Japan), whilst *α*-Linolenic acid and linoleic acid were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Oleic acid was purchased from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan), and PFS oil, produced via the squeezing method (squeezed PFS oil), and PFS powder were purchased from PAL LLC (Miyagi, Japan).

In addition, cold-pressed PFS oil produced from Chinese PFS was purchased from Tencho Foods Industries Co., Ltd. (Aichi, Japan).

2.1.2. PFS $scCO_2$ Extract

The PFS powder acquired from PAL LLC was used for $scCO_2$ and for hexane extraction. This powder, which contains only approximately 15% of the total oil, is essentially the residue after the PFS is squeezed to yield oil. Extraction using $scCO_2$ was carried out as previously described [31], but with some minor modifications: 1.0 g of PFS powder was packed into a 10 mL extraction container before CO_2 was passed in at 40˚C under 20 MPa at a flow rate of 3.0 mL/min. The $scCO_2$ containing PFS oil was then discharged into a glass bottle and evaporated at room temperature (22˚C - 27˚C) and normal pressure (approximately 0.1 MPa). This extraction was performed using a PU-2086 plus CO_2 pump, CO-2060 plus oven, and BP-2080 plus back pressure regulator (JASCO Corporation, Tokyo, Japan).

2.1.3. Hexane-Extracted PFS Oil

Hexane-extracted PFS oil was produced similarly to the ethanol extracts as previously described [31], with some minor modifications. In this case, 30 g of PFS powder, which was the residue of the squeezed PFS oil described above, was soaked and then stirred in 90 mL of *n*-hexane. After overnight treatment, the solution was centrifuged at 2600 g for 10 min. Next, the supernatant was filtered using filter paper and a 0.22 μm nylon membrane before the filtrate was evaporated using a rotary evaporator. Finally, the remaining oil was collected.

2.2. Cell Culture

B16 mouse melanoma cells (JCRB0202) were purchased from JCRB Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan). Cell culture was performed under 5% CO_2 at 37˚C in Eagle's minimum essential medium (Fujifilm Wako Pure Chemical Corporation), which contained 10% (v/v) fetal bovine serum (Biological Industries, Cromwell, CT, USA) and penicillin-streptomycin (100 U/mL and 100 μg/mL; Fujifilm Wako Pure Chemical Corporation). The density of cell seeding was 8×10^3 cells/well in a 96-well plate for cytotoxicity analysis, and 2.4×10^5 cells/well in 6-well plates for the determination of melanin content. After overnight culturing, 100 μM of 3-iso-

butyl-1-methylxanthine (IBMX) (Fujifilm Wako Pure Chemical Corporation) was added to all the cells, except for the unstimulated group. Additionally, the cells stimulated by IBMX were treated with the test samples in triplicate. The cells were then incubated for 72 h.

2.3. Cytotoxicity

Cytotoxicity of the test samples was evaluated as previously described [31]. Cells in the 96-well plate were incubated for 2 h at 37˚C with 0.5 mg/mL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Dojindo Laboratories, Kumamoto, Japan) dissolved in the medium. After washing the cells with phosphate-buffered saline (PBS) (Fujifilm Wako Pure Chemical Corporation), the MTT formazan inside the cells was dissolved using dimethyl sulfoxide (Fujifilm Wako Pure Chemical Corporation). The absorbance of the solutions was determined at 545 nm using a microplate reader (CHROMATE MODEL 4300; Awareness Technology Inc., Palm City, FL, USA), and absorbance at 630 nm was deducted as background. The cell viability of each group was recorded as a percentage of the viability of the control group.

2.4. Melanin Production

Melanin content in the cells was measured as described previously [31], with some minor modifications. Initially, B16 melanoma cells in 6-well plates were rinsed with PBS and then lysed in 1 M NaOH at room temperature. Absorbance at 450 nm was determined, and the absorbance at 630 nm was deducted as the background. Authentic synthetic melanin (Fujifilm Wako Pure Chemical Corporation) was then used to generate a standard curve.

Simultaneously, protein concentrations of the cell solutions were determined using the Takara BCA Protein Assay Kit (Takara Bio Inc., Shiga, Japan).

The melanin/protein concentration in the unstimulated group was subtracted from that in each well. Finally, the melanin/protein concentration for each group was recorded as a percentage of those in the control group.

2.5. Microscopic Photography

The cells were photographed with an inverted microscope (AE2000; SHIMADZU, Kyoto, Japan), while removing phase contrast to clarify the black color of melanin.

2.6. Chemical Analysis of Fatty Acids

Analyses of *α*-linolenic acid, linoleic acid, and oleic acid, all of which are reported to be rich in PFS oil [22], were carried out with gas chromatography (GC). Percentages of the three fatty acids in the PFS $scCO_2$ extract and the squeezed PFS oil were determined as follows: Samples (40 mg) were mixed with heptadecanoate as the internal standard and saponified with 1.5 mL of 0.5 M sodium hydroxide methanol solution, before heating at 100˚C for 9 min. Then,

they were methyl esterified with 2 mL of boron trifluoride methanol complex methanol solution and heated at 100˚C for 7 min. Subsequently, 3 mL of *n*-hexane and 5 mL of saturated salt solution were added, followed by the collection of their hexane phases, and analysis with GC.

The analysis conditions for GC were as follows: Machine, 7890B (Agilent Technologies, Inc., Santa Clara, CA, USA); detector, FID; column, DB-23 (Agilent Technologies, Inc.) (0.25 mm i.d. × 30 m, 0.25 μm film thickness); temperature of injection port, 250˚C; temperature of detector, 250˚C; temperature of column, 50˚C (1 min) to 170˚C (10˚C/min) to 210˚C (1.2˚C/min); sample introduction system, split (1:20); flow rate of hydrogen, 35 mL/min; flow rate of air, 300 mL/min; flow rate of nitrogen (make up gas), 20 mL/min; pressure of helium (carrier gas), 115 kPa; injection volume, 1 μL.

2.7. IC_{50} of Melanin Production

α-Linolenic acid, linoleic acid, and oleic acid were mixed in the same ratio (63.0:13.6:10.8) as the PFS $scCO_2$ extract. Then, various concentrations of this mixture, the PFS $scCO_2$ extract, and squeezed PFS oil were added to B16 melanoma cells in 6-well plates and their effects on melanin production were measured as described above. The IC_{50} of melanin production for each sample was calculated from the logarithmic curve with the equation below, as described previously [31].

$$IC_{50} = 10^{\log(A/B)*(50-C)/(D-C)+\log(B)}$$

A: The concentration; inhibitory ratio is just higher than 50%.
B: The concentration; inhibitory ratio is just lower than 50%.
C: The inhibitory ratio of *B*.
D: The inhibitory ratio of *A*.

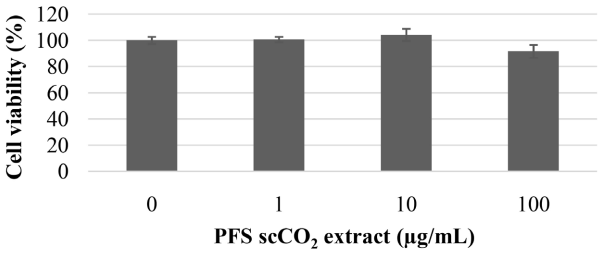
2.8. Statistical Analysis

Homoscedasticity of sample variances was examined via the Hartley test. Differences in data were analyzed via one-way analysis of variance, and further assessment was performed using Dunnett's test or Tukey's test. Data were indicated as the mean ± standard deviation. Values of $p < 0.05$ were deemed significant.

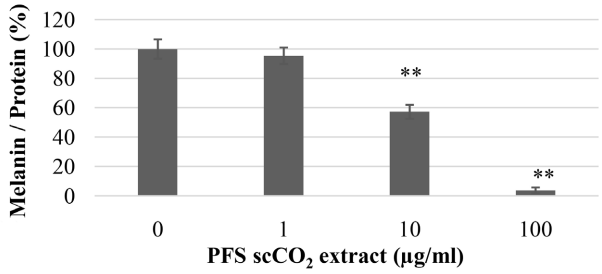
3. Results

3.1. Inhibition of Melanin Production by the PFS $scCO_2$ Extract

The PFS $scCO_2$ extract did not show cytotoxicity at 100 μg/mL (**Figure 1(a)**). However, the extract dose-dependently reduced melanin production in the cells. At 100 μg/mL, the reduction in melanin production was estimated to be over 90% than that in the control (**Figure 1(b)**). Based on microscopic photography, many cells with black pigment were observed in the control group because of the stimulation of melanin production by IBMX [31] [32] [33] (**Figure 2(a)**).



(a)



(b)

Figure 1. Anti-melanogenic effect of the PFS $scCO_2$ extract on B16 mice melanoma cells. Cell viability (a) and melanin production (b) in B16 mouse melanoma cells treated with various concentrations of the PFS $scCO_2$ extract were measured. **: $p < 0.01$ (compared to 0 μg/mL), Dunnett's test (n = 3). PFS, *Perilla frutescens* seed; $scCO_2$, supercritical CO_2.

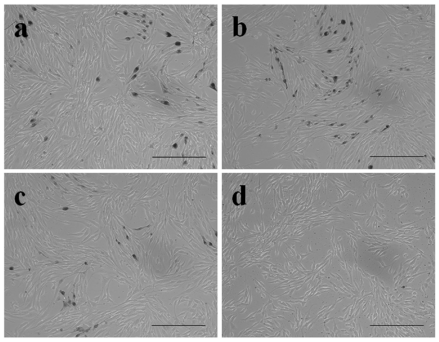


Figure 2. Microscopic photography of B16 mouse melanoma cells treated with various concentrations of the PFS $scCO_2$ extract. Cells not treated with the PFS $scCO_2$ extract (a) and cells treated with 1 μg/mL (b), 10 μg/mL (c), and 100 μg/mL (d) of the extract were photographed without phase contrast. Bar = 250 μm. PFS, *Perilla frutescens* seed; $scCO_2$, supercritical CO_2.

Although the number of black cells was not affected by PFS $scCO_2$ extract at 1 μg/mL (**Figure 2(b)**), those cells dose-dependently decreased at 10 and 100 μg/mL (**Figure 2(c)** and **Figure 2(d)**).

3.2. Comparison of the Inhibitory Effect of the PFS $scCO_2$ Extract and Kojic Acid on Melanin Production

To determine the strength of the inhibitory effect of the PFS $scCO_2$ extract on melanin production in B16 melanoma cells, the effect was compared to that of kojic acid, a renowned melanogenesis suppressor [34]. Kojic acid showed in-

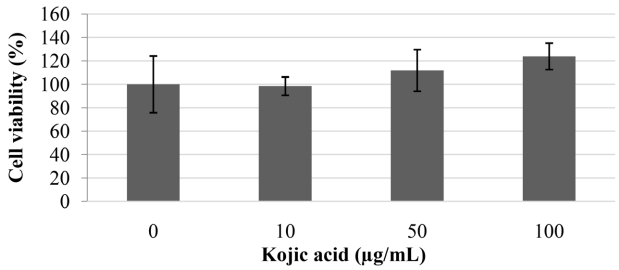
creases in the viability of the cells in some instances, although these were not deemed significant (Figure 3(a)). At a concentration of 100 μg/mL, the PFS $scCO_2$ extract and kojic acid decreased melanin production in the cells compared to the control group by 96.8% and 65.0 %, respectively ($p < 0.01$) (Figure 3(b)); the inhibitory effect of the former was considerably higher than that of the latter ($p < 0.05$).

3.3. Comparison of the Inhibitory Effect of the PFS $scCO_2$ Extract, the Hexane-Extracted PFS Oil and the Squeezed PFS Oil on Melanin Production

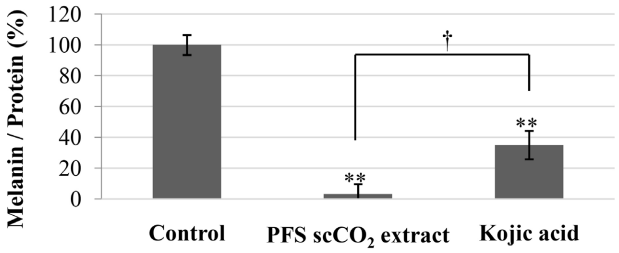
Neither the hexane-extracted PFS oil nor the squeezed PFS oil showed cytotoxicity against B16 cells at 100 μg/mL similar to the PFS $scCO_2$ extract (Figure 4(a)). At this concentration, the hexane-extracted PFS oil and the squeezed PFS oil considerably decreased melanin production in the cells by 72.2% and 64.2%, respectively, compared to the control group ($p < 0.01$). However, their inhibitory effects were significantly lower than those of the PFS $scCO_2$ extract ($p < 0.01$) (Figure 4(b)).

3.4. Chemical Analysis of Fatty Acids

The total content of α-linolenic acid, linoleic acid, and oleic acid was 87.4% in the PFS $scCO_2$ extract, compared to 88.7% in the squeezed PFS oil. The ratio of these three fatty acids among both samples was the same (Table 1).



(a)



(b)

Figure 3. Comparison of inhibitory effect of the PFS $scCO_2$ extract and kojic acid on melanin production. (a) Cell viability of B16 mouse melanoma cells treated with various concentrations of kojic acid. (b) Melanin production in cells treated with 100 μg/mL of the PFS $scCO_2$ extract and kojic acid. **: $p < 0.01$ (compared to the control), †: $p < 0.05$ (compared between two groups), Tukey's test (n = 3). PFS, *Perilla frutescens* seed; $scCO_2$, supercritical CO_2.

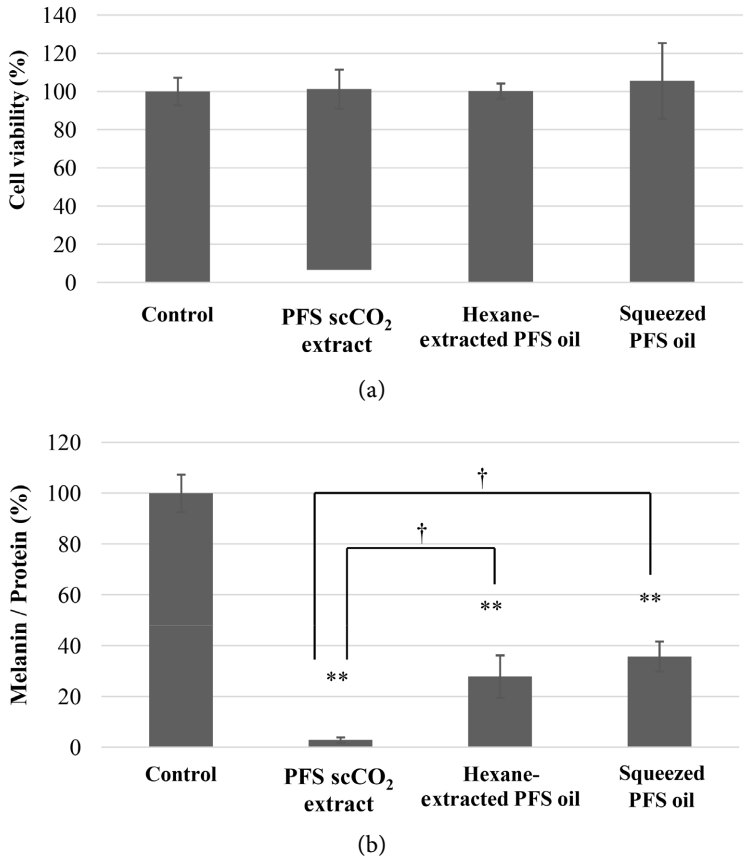


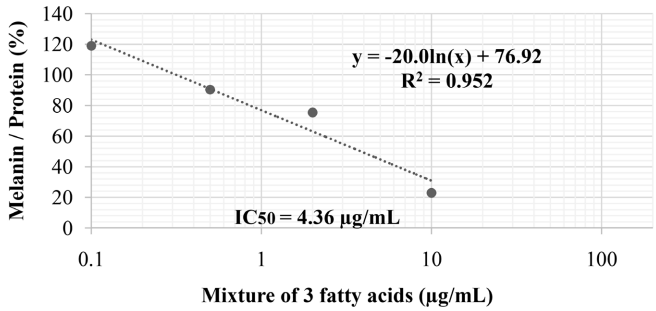
Figure 4. Comparison of the inhibitory effect of the PFS $scCO_2$ extract, the hexane-extracted PFS oil and the squeezed PFS oil on melanin production. Cell viability (a) and melanin production (b) in cells treated with 100 μg/mL of the PFS $scCO_2$ extract, the hexane-extracted PFS oil and the squeezed PFS oil. **: $p < 0.01$ (compared to the control), †: $p < 0.05$ (compared between two groups), Tukey's test (n = 3). PFS, *Perilla frutescens* seed; $scCO_2$, supercritical CO_2.

Table 1. Concentration of *α*-linolenic acid, linoleic acid, and oleic acid in the PFS $scCO_2$ extract and the squeezed PFS oil.

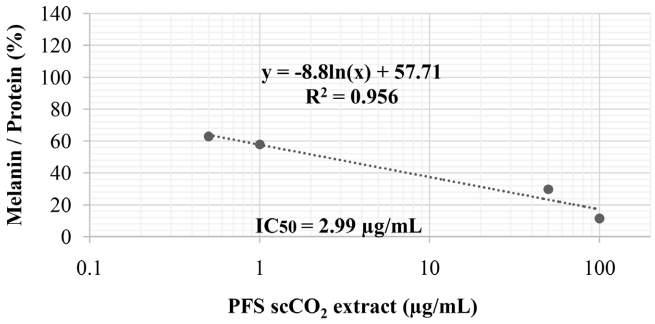
Fatty Acid	Concentration	
	PFS $scCO_2$ extract	Squeezed PFS oil
α-Linolenic acid	63.0%	63.2%
Linoleic acid	13.6%	12.5%
Oleic acid	10.8%	13.0%

3.5. IC_{50} of Melanin Production

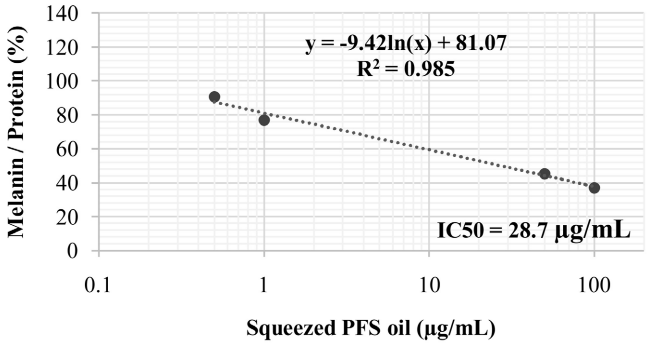
The IC_{50} of the mixture of *α*-linolenic acid, linoleic acid, and oleic acid to melanin production in B16 melanoma cells was 4.36 μg/mL (**Figure 5(a)**), similar to that of the PFS $scCO_2$ extract (2.99 μg/mL, **Figure 5(b)**). However, the IC_{50} of the squeezed PFS oil was 28.7 μg/mL (**Figure 5(c)**), approximately 6.6 times higher than that of the mixture.



(a)



(b)



(c)

Figure 5. Dose dependency of anti-melanogenic effect on B16 mouse melanoma cells. Melanin production in cells treated with various concentrations of the mixture of the three fatty acids (a), the PFS $scCO_2$ extract (b) and the squeezed PFS oil (c). PFS, *Perilla frutescens* seed; $scCO_2$, supercritical CO_2.

4. Discussion

PFS oil has been reported to inhibit skin photoaging [22], and the consumption of the oil is also associated with other medicinal and nutritional benefits, for example, the prevention of some diseases, such as cardiovascular disorders, cancer, inflammatory, rheumatoid arthritis, and the promotion of antioxidant activity [24] [25] [26]. However, the effect of PFS oil on melanogenesis has not yet been examined.

B16 mice melanoma cell is a major cell line for cancer research [34]. This cell line has also dedicated for the field of melanogenesis research [31] [32] [33]. Herein, we examined the effect of PFS oil on melanogenesis with it.

The results of this investigation confirm that PFS oils, specifically the PFS $scCO_2$ extract, significantly reduced the amount of melanin produced by B16 cells. Furthermore, the PFS $scCO_2$ extract reduced melanin production of cells by more than 90%, without cytotoxicity, at a concentration of 100 μg/mL (**Figure 1** and **Figure 2**).

The PFS $scCO_2$ extract showed an inhibitory effect higher than that of kojic acid (**Figure 3(b)**), a well-known anti-melanogenesis compound [35]. Therefore, the PFS $scCO_2$ extract could represent a new effective melanogenesis inhibitor and a valuable whitening agent.

The inhibitory effect of the PFS $scCO_2$ extract was considerably higher than that of other PFS oils obtained from the same PFS powder via different methods (the hexane-extracted PFS oil and the squeezed PFS oil) without cytotoxicity (**Figure 4**). Similar results were obtained when the effect of the PFS $scCO_2$ extract was compared to that of a cold-pressed PFS oil produced from Chinese PFS (**Figure S1**). This suggests that the anti-melanogenesis activity of the PFS $scCO_2$ extract is higher than that of PFS oils obtained via other methods, regardless of where the PFS is produced.

The total content and ratio of *α*-linolenic acid, linoleic acid, and oleic acid in the PFS $scCO_2$ extract and the squeezed PFS oil was almost the same as that previously reported in *Perilla frutescens* oils [16] [19]. Because the IC_{50} of the mixture of the three fatty acids to the production of melanin in B16 melanoma cells was not different from that of the PFS $scCO_2$ extract (**Figure 5(a)** and **Figure 5(b)**), it was suggested that those fatty acids are the main inhibitory ingredients of the extract. However, the IC_{50} of the squeezed PFS oil (**Figure 5(c)**) was an estimated 6.6 times higher than that of the mixture, indicating that some factor(s) in the squeezed PFS oil reduce(s) the affinity of the fatty acids to the cells.

5. Conclusion

From the results we have shown here, the PFS $scCO_2$ extract could be one of the most effective melanogenesis inhibitors. Herein, for the first time, we confirmed that some PFS oils significantly reduced the melanin production of B16 cells, particularly the PFS $scCO_2$ extract. Interestingly, the use of $scCO_2$ to extract from PFS was necessary for stronger anti-melanogenesis activity, despite the main inhibitory ingredients likely being the same as other PFS oils.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplement

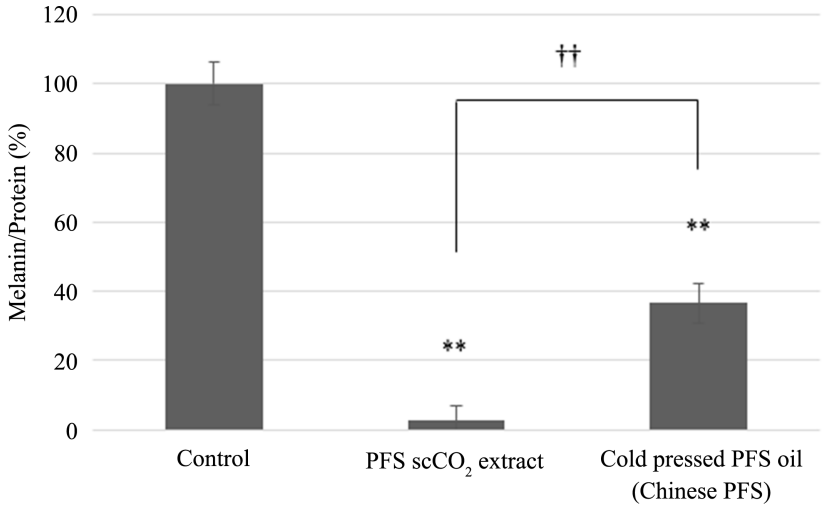

Figure S1. Melanin production in B16 mice melanoma cells treated with 100 μg/mL of the PFS $scCO_2$ extract and the cold-pressed PFS oil (Chinese PFS). **: $p < 0.01$ (compared to the control), ††: $p < 0.01$ (compared between two groups), Tukey's test (n = 3). PFS, *Perilla frutescens* seed; $scCO_2$, supercritical CO_2.