Changes in the phenolic compounds profile, antioxidant and anti-melanogenic activity from organs of Petasites japonicas under different extraction methods

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Abstract
The aerial parts of Petasites japonicus, a perennial flowering plant belonging to the Asteraceae family, have been shown to have antioxidant, anti-allergic and anti-inflammatory effects, and its roots have been used as a functional resource for the cosmetic industries. The present study was conducted to investigate the effect of extraction methods including microwave-assisted extraction, autoclave-assisted extraction, and hot water extraction on the polyphenolic composition, antioxidant activity, and anti-melanogenic activity of P. japonicus leaves, stems, and roots. In comparison with other samples, L-HWE (hot water extract of leaves) and L-AAE (autoclave-assisted extract of leaves) exhibited strong DPPH radical scavenging activity (L-HWE, IC50 = 138.7 ± 32 µg/mL; L-AAE, IC50 = 82.3 ± 4 µg/mL), reducing power activity and hydrophilic oxygen radical scavenging activity. Quantitative real-time PCR, western blot, and cellular reactive oxygen species (ROS) analyses indicated that the anti-melanogenic effect of leaf extracts obtained by hot water extraction may result from the inhibition of ROS generation and the downregulation of tyrosinase expression in B16F10 cells. Furthermore, the extraction methods differentially affected the content of polyphenolic compounds and bioavailability of P. japonicus extracts. Taken together, the antioxidant activity and anti-melanogenic effect of leaf hot water extracts suggest that the leaves of P. japonicus could be a beneficial source of natural antioxidants for skincare products.

Keywords: Anti-melanogenic activity, antioxidant activity, Petasites japonicus, polyphenolic compounds.

Resumen
Se ha demostrado que las partes aéreas de Petasites japonicas, una planta con flores perennes perteneciente a la familia Asteraceae, tienen efectos antioxidantes, antialérgicos y antiinflamatorios, y sus raíces se han utilizado como un recurso funcional para las industrias cosméticas. El presente estudio se realizó para investigar el efecto de los métodos de extracción, incluida la extracción asistida por microondas, la extracción asistida por autoclave y la extracción con agua caliente sobre la composición polifenólica, la actividad antioxidante y la actividad antimelanogénica de las hojas, tallos y raíces de P. japonicus. En comparación con otras muestras, L-HWE (extrato de hojas de agua caliente) y L-AAE (extrato de hojas asistido por autoclave) mostraron una fuerte actividad de eliminación de radicales DPPH (L-HWE, IC50 = 138.7 ± 32 µg/mL; L-AAE, IC50 = 82.3 ± 4 µg/mL), reduciendo la actividad energética y la actividad de eliminación de radicales hidrófilos de oxígeno. Los análisis cuantitativos de PCR en tiempo real, transferencia Western y especies reactivas de oxígeno celular (ROS) indicaron que el efecto antimelanogénico de los extractos de hojas obtenidos por extracción con agua caliente puede ser el resultado de la inhibición de la generación de ROS y la regulación negativa de la expresión de tirosinasa en células B16F10. Además, los métodos de extracción afectaron diferencialmente el contenido de compuestos polifenólicos y la biodisponibilidad de los extractos de P. japonicus. En conjunto, la actividad antioxidante y el efecto antimelanogénico de los extractos de agua caliente de las hojas sugieren que las hojas de P. japonicus podrían ser una fuente beneficiosa de antioxidantes naturales para los productos para el cuidado de la piel.

Palabras clave: Actividad antimelanogénica, actividad antioxidante, Petasites japonicus, compuestos polifenólicos.

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1 Introduction

The color of the skin is mainly determined by the amount and distribution of melanin pigments produced by melanocytes, which are pigment-producing cells located at the basal level of the epidermis (Kubo and Matsuda, 1995). Melanogenesis is stimulated by various factors such as ultraviolet (UV) irradiation, cytokines (e.g. interleukin-18, interleukin-33, interferon-γ and prostaglandin E2), growth factors (e.g. angiopoietin-like protein 2), and hormones (e.g. adrenocorticotropic hormone, and α-melanocyte-stimulating hormone) (D’Mello et al., 2016; Satou et al., 2019; Fu et al., 2020), and is identified by the expression of melanocyte-specific genes such as tyrosinase, tyrosinase-related protein 1 (TYRP1), TYRP2, premelanosose protein 17, melanoma antigen recognized by T cells 1, and microphthalmia-associated transcription factor (Słominski et al., 2004; D’Mello et al., 2016). Tyrosinase, a multifunctional copper-containing metalloenzyme with dinuclear copper ions, is the key enzyme in the first two steps of melanin biosynthesis, catalyzing the hydroxylation of L-tyrosine to 3,4 dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone (D’Mello et al., 2016; Pillaiyar et al., 2017). Melanin synthesis appears to be predominantly controlled by the expression and activity of tyrosinase. Therefore, tyrosinase has become the most prominent and successful target of skin pigmentation (Pillaiyar et al., 2017). Although melanin plays a physiological role in protecting the human body, melanin overproduction causes hyperpigmentation, which is the third most common dermatological disorder related to serious esthetic problems owing to its visible nature. (Costin and Hearing, 2007; Yamaguchi et al., 2007; Al-Amin et al., 2016). Therefore, tyrosinase inhibitors have attracted increasing attention as depigmenting agents in hyperpigmentation disorders, and several tyrosinase inhibitors including arbutin, kojic acid, and hydroquinone have been used as skin-whitening agents (Pillaiyar et al., 2017). However, due to the toxicity and/or lack of efficacy of these inhibitors, the screening and identification of more effective and safer tyrosinase inhibitors from natural products have been important in determining alternative prevention and treatment methods (Chen et al., 2015; Zolghadri et al., 2019).

Petasites japonicus (Asteraceae), commonly known as butterbur, is an herbaceous perennial plant mainly distributed and cultivated in East Asian countries including Korea, Japan, and Taiwan (Bang et al., 2005). In ancient oriental medicine, the flower buds were used as apophtegmatic, antitussive, antifebrile, antiasthma, and detoxification agents, and the young leaves were harvested and used as raw materials and raw vegetables (Choi, 2002; Bang et al., 2005). Current pharmaceutical studies have revealed antioxidant, anti-inflammatory, anti-cancer, anti-allergic, and anticoagulant effects of P. japonicus (Choi, 2002; Han et al., 2012; Hwang et al., 2015). In addition, a number of chemical constituents including sesquiterpenes, triterpenes, and various types of phenolic compounds (e.g. flavonoids) have been isolated from this plant (Lee et al., 2019). Since hot water extracts of P. japonicus leaves have been found to exhibit anti-inflammatory activity, hot water extraction (HWE) has been used for the isolation of active polyphenolic compounds including aryltetralin lactone lignans, cimicifugic acid D, fukinolic acid, 3,4-dicaffeoylquinic acid, 3,5 dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, and caffeic acid (Lee et al., 2019), which were identified as the antioxidant, anti-inflammatory and/or anti-melanogenic compounds (Maruyama et al., 2018; Hiemori-Kondo and Nii, 2019; Lee et al., 2019). In addition, petaslignolide A, a furfuran lignan isolated from the leaves of P. japonicas, has been exhibited the neuroprotective effect on the oxidative damage in the brain of mice (Cui et al., 2005; Sok et al., 2009). Furthermore, petasphenol, a polyphenolic compound isolated from the vegetable body of P. japonicus (Mizushima et al., 2002), exhibited antioxidant activity, anti-inflammatory activity, and DNA polymerase inhibitory activity (Mizushima et al., 2007). In root of P. japonicus, 5-O-cafeoylquinic acid, fukinolic acid, 3,5-di-O-cafeoylquinic acid, and 4,5-di-O-cafeoylquinic acid have been found as antioxidant compounds (Kim et al., 2012), indicating that polyphenolic compounds including phenolic acids, flavonoids and lignans are active compounds in P. japonicus. Therefore, we analyzed total phenolic and flavonoid contents from leaves, stems and roots of P. japonicus.

Various methods including maceration, decoction, hot continuous extraction (Soxhlet), microwave-assisted extraction (MAE), ultrasound extraction, and supercritical fluid extraction have been used for the preparation of plant extracts (Pandey and Tripathi, 2014). In addition, autoclave-assisted extraction
(AAE), and MAE have been recognized as green extraction technologies, which are suitable when high extraction efficiency is required in a short time (Suh et al., 2017; Vallejo-Castillo et al., 2019). However, only extraction with organic solvents has been reported for P. japonicus (Kang et al., 2010; Kang et al., 2015; Kim et al., 2015; Choi et al., 2017b). Although these findings demonstrated the potential P. japonicus as a crude drug and dietary health supplement, there are limited studies on the variation in the chemical composition and biological activities of P. japonicus with the use of different extraction methods.

In this study, P. japonicus extracts were prepared from different organs using different extraction methods such as HWE, AAE, and MAE and were subjected to antioxidant and anti-melanogenic activity analyses. In addition, we identified 19 polyphenolic compounds using HPLC analysis.

2 Materials and methods

2.1 Plant material and extraction procedures

The leaves, stems, and roots of P. japonicus were purchased from the natural-herb company (http://natural-herb.co.kr) in South Korea and ground into powder using a blender. The ground material (10 g) was extracted with 500 mL of water (80 °C, 1 h × 3 times, HWE). A multimode microwave extraction system with a microwave frequency of 2.45 GHz and maximum output power 1600 W was used for MAE. The ground material (10 g) was mixed with 500 mL of water, and the suspension was irradiated in the microwave oven at regular intervals (5 min irradiation and 1 min off × 3 times). For AAE, 10 g of ground material with 500 mL of water was autoclaved in an autoclave at 121 °C and 0.15 MPa for 15 min. After filtration, the extracts obtained by different extraction methods were evaporated using a rotary vacuum evaporator (IKA RV8, IKA, Staufen, Germany). The extraction yield was 2.2% for L-HWE (hot-water extraction of leaf), 2.15% for L-MAE (microwave-assisted extraction of leaf), 1.75% for L-AAE (autoclave extraction of leaf), 3.35% for S-HWE (hot-water extraction of stem), 2.65% for S-MAE (microwave-assisted extraction of stem), 3.45% for S-AAE (autoclave extraction of stem), 4.15% for R-HWE (hot-water extraction of root), 3.8% for R-MAE (microwave-assisted extraction of root), and 4.4% for R-AAE (autoclave extraction of root). Then, 10 mg of each extract was re-dissolved in 1 mL of HPLC grade water for further analysis.

2.2 Analysis of total phenolic and flavonoid contents

The total phenolic content (TPC) in each extract was determined by Folin-Ciocalteu assay (Jin et al., 2019). Folin & Ciocalteu’s phenol reagent (2N, 50 µL) was mixed with 100 µL of each extract and incubated for 5 min at room temperature. Then, 0.3 mL of 20% sodium carbonate was added to each mixture. The absorbance of the reaction mixtures was measured at 725 nm using the iMark microplate reader (Bio-Rad, Hercules, CA, USA). The calibration curve was prepared using gallic acid (0-1000 µg/mL, R² = 0.9407, y = 0.014x + 0.0497), and the TPC was expressed in milligram gallic acid equivalents (mg GAE/g extract).

To analyze the total flavonoid content (TFC) of each sample, 100 µL of each extract was mixed with 20 µL of 10% aluminum nitrate (w/v), 20 µL of 1 M potassium acetate, and 860 µL of 80% ethanol as described by Jin et al. (2019). After 40 min of incubation at room temperature, the absorbance was determined at 415 nm. The TFC in each extract was calculated as milligrams of quercetin equivalents (QE) per gram of extract using the equation obtained from the standard quercetin graph (0-1000 µg/mL, R² = 0.9712, y = 0.0126x + 0.0359).

2.3 Analysis of 1,1-diphenyl-2-picrylhydrazil (DPPH) radical scavenging activity

The effects of each extract on DPPH radical were analyzed using the modified method of Jin et al. (2019). In brief, 180 µL of 0.4 mM DPPH in 80% MeOH was plated in 96-well plates, and 1 mg/mL of each sample was added to each well, followed by serial dilution. After 10 min of incubation in the dark, the absorbance values were measured at 520 nm using the iMark microplate reader. The IC₅₀ was calculated from a graph of radical scavenging activity versus extract concentration.

2.4 Analysis of reducing power

To analyze the total reducing power of each extract, different concentrations of the extract (100, 200, and 300 µg/mL) were mixed with 50 µL of 0.2 M sodium phosphate buffer (pH 6.6) and 50 µL of 1% potassium ferricyanide as described by Jin et al. (2019). After
20 min of incubation at 50 °C, the reaction was stopped by adding 250 µL of 10% trichloroacetic acid. The absorbance was measured at 750 nm using a microplate reader.

2.5 Oxygen radical antioxidant capacity (ORAC) assay

In each well of a microplate, 150 µL of 0.08 µM fluorescein was mixed with 25 µL of phosphate buffer (pH 7.0; blank), diluted sample (12.5 µg/mL, 25 µg/mL, and 50 µg/mL), or Trolox (6.25, 12.5, 25, and 50 µM) standard and incubated at 37 °C for 10 min. Immediately following the addition of 25 µL of 2,2’-azobis(isobutyramidine) dihydrochloride (0.12 g/mL), the fluorescence intensity was measured every 1 min for 90 min using the SynergyTM HTX Multi-mode Microplate Reader (BioTek, Winooski, VT, USA) with fluorescence filters (485 nm excitation and 530 nm emission). Data are expressed as µmol Trolox equivalents (TE) as described by Jin et al. (2019).

2.6 Cell viability assay and determination of melanin synthesis inhibitory activity

Cell viability and melanin synthesis inhibitory activity has been determined as described by Jin et al. (2019). Effect of P. japonicus leaf, stem and root extracts on melanin production was analyzed in α-MSH-stimulated B16F10 cells. B16F10 melanoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin in an incubator containing humidified CO2 (5%) at 37 °C. For cell viability analysis, 100 µL of cultured B16F10 cells were plated at a density of 1 × 105 cells/mL in 96-well plates and incubated at 37 °C for 24 h. After the cells were exposed to various concentrations (50 µg/mL, 100 µg/mL, and 200 µg/mL) of each extract with or without 50 nM α-melanocyte-stimulating hormone (α-MSH) for 48 h, the cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested for protein extraction as described by Jin et al. (2019). After protein extraction, equal amounts (10 µg) of proteins were separated by SDS-PAGE, transferred to a PVDF membrane (Millipore, Billerica, MA, USA), and probed with primary antibodies specific to tyrosinase or β-actin, which was used as an internal control for Western blotting. Immunodetection was performed using a chemiluminescence system according to the manufacturer’s instructions.

To investigate the inhibitory effect on melanogenesis, cultured B16F10 cells were treated with each extract and 50 nM α-MSH. After incubation at 37 °C for 48 h, the cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested by centrifugation at 4,000 rpm for 10 min. The pellets were solubilized in 1 N NaOH with 10% DMSO at 65 °C for 1 h. The absorbance of the supernatant was determined at 490 nm. For mock control, cells were treated with the identical volume of DMSO. Data are expressed in terms of melanin content as a percentage compared with the mock control.

2.7 Intracellular reactive oxygen species (ROS) measurement

Cellular ROS levels were determined using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as described by Choi et al. (2017a). B16F10 melanoma cells treated with each extract for 24 h were incubated with 24 mM H2O2 at 37 °C for 30 min to induce oxidative stress. Then, the cells were treated with 20 µM of fresh DCFH-DA and incubated for 30 min at 37 °C in a 5% CO2 incubator. Following washing with PBS, the dichlorofluorescein fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

2.8 Western blot analysis

The expression and protein levels of tyrosinase to determine whether the inhibitory effect of extracts obtained by L-HWE on α-MSH-induced melanin biosynthesis may be attributed to the inhibition of tyrosinase expression. After treatment with different concentrations of the extract in the presence of 50 nM α-MSH for 48 h. B16F10 cells were harvested for protein extraction as described by Jin et al. (2019). After protein extraction, equal amounts (10 µg) of proteins were separated by SDS-PAGE, transferred to a PVDF membrane (Millipore, Billerica, MA, USA), and probed with primary antibodies specific to tyrosinase or β-actin, which was used as an internal control for Western blotting. Immunodetection was performed using a chemiluminescence system according to the manufacturer’s instructions.
2.9 Gene expression analysis by quantitative real-time PCR (qPCR)

Total RNA was extracted using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) and reverse-transcribed into cDNA using the ReverTra Ace® qPCR RT Master Mix with qDNA Remover (TOYOBO, Co., Ltd., Osaka, Japan) according to the manufacturer’s recommendations. qRT-PCR was performed using the SYBR® Green Real-time PCR Master Mix (TOYOBO, Co., Ltd., Osaka, Japan) with the CFX96TM Real-time system (Bio-Rad, Hercules, CA, USA) as described by Jin et al. (2019). The expression levels of each gene were normalized to the level of actin, and specific primer pairs are listed in Table 1.

2.10 HPLC analysis

The polyphenolic composition of each extract was determined using a HPLC system equipped with a UV-vis detector (SPD-10A; Shimadzu, Kyoto, Japan) and Luna 5 μm C18(2) 100 A column (4.6 mm × 250 mm, particle size = 5 μm). The mobile phases consisted of 0.1% trifluoroacetic acid in distilled water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). Linear gradient elution (0 -0.01 min, 90% A; 0.01 -28 min, 60% A; 28 -39 min, 40% A; 39 - 50 min, 10% A; 50 - 55 min, 10% A; 55 - 56 min, 90% A; and 56 - 65 min, 90% A) with a flow rate of 0.7 mL/min was used. All standards were purchased from Sigma-Aldrich. The concentration was calculated by comparing the peak areas of the standards including Rutin (R² = 0.9995, y = 0.5511x - 1.8572), taxifolin (R² = 0.9893, y = 0.4897x - 6.6803), naringin (R² = 0.9843, y = 0.4992x - 16.6003), hesperidin, myricetin (R² = 0.9538, y = 0.6775x - 29.022), quercetin (R² = 0.97012, y = 0.4661x - 18.841), luteolin (R² = 0.952, y = 0.3722x - 7.6698), naringenin (R² = 0.9567, y = 0.2784x + 3.5013), apigenin (R² = 0.9953, y = 0.6976x - 13.779), kaempferol (R² = 0.9852, y = 0.7918x - 24.95), isorhamnetin (R² = 0.9892, y = 0.6953x - 11.211), ramnetin (R² = 0.9981, y = 0.7355x - 7.4283), gallic acid (R² = 0.9885, y = 0.6463x + 2.1345), p-hydroxybenzoic acid (R² = 0.9742, y = 0.5723x + 10.801), chlorogenic acid (R² = 0.9286, y = 0.8628x + 12.256), caffeic acid (R² = 0.9841, y = 0.8796x - 8.5626), syringic acid (R² = 0.9854, y = 0.8647x - 1.0796), p-coumaric acid (R² = 0.9144, y = 0.8515x + 18.684), ferulic acid (R² = 0.9421, y = 1.0452x + 3.3637).

2.11 Statistical analysis

All experimental results are expressed as the mean ± standard error. For statistical analysis, ANOVA was performed using SPSS (IBM, Armonk, NY, USA), and Duncan’s multiple range test was used to determine significant differences (p < 0.05).

3 Results and discussion

3.1 Effects of extraction methods on the phenolic compound contents of P. japonicus leaves, stems, and roots

Phenolic compounds are a large class of plant secondary metabolites consisting of a benzene ring (C6H6) with several hydroxyl groups (Manach et al., 2005). Owing to this structure, it can bind to enzymes and other molecules and exhibit biological activity against antioxidants and various related chronic diseases (Manach et al., 2005; Rein et al., 2013). In addition, flavonoids, a kind of phenolic compound, are secondary metabolites that can protect plants from UV rays, associate with nitrogen-fixing bacteria, and exert other physiological functions. In addition, it has been reported to have a positive effect on health associated with antioxidation of lipids, protection of vitamins and enzymes, antibacterial activity, and cell regeneration (Yao et al., 2004). In general, the growth stage, cultivation environment, and genetic variation of crops greatly influence the phytochemical content in crops (Renaud et al., 2014; Liu et al., 2016; Jin et al., 2019), and a difference in the content has been observed according to the extraction method (Altemimi et al., 2017). The extraction efficiency of phenolic compounds from different organs of P. japonicus was similar for AAE and HWE except MAE. The TPC was higher in the P. japonicus leaf extract (12.19 - 17.24 mg GAE/g), followed by the root and stem extracts (Table 2). In the case of the TFC, the levels of flavonoids were relatively high in the roots (2.136 - 10.74 mg QE/g), especially when HWE was performed (Table 2). The TFC (< 1 mg QE/g) was measured in the leaves and stems of P. japonicus, and the extraction method did not significantly affect the extraction efficiency of flavonoids. In leaves and stems, irrespective of the extraction methods, the TPC was higher than TFC, but in roots those values depended on the extraction methods.
In addition, HPLC analysis was performed to identify the main polyphenolic components of *P. japonicus* extracts. In the leaf extracts, isorhamnetin was identified as the main component, and in the root extract, p-coumaric acid was the main component (Table 2). In particular, rutin, which has various physiological activities such as antioxidant effects, cholesterol-lowering effects, and vascular disease prevention effects (Ganeshpurkar and Saluja, 2017), was around 6 times higher in leaf hot water extracts than in other extracts. In leaf extracts, the extraction efficiency of most polyphenolic compounds, except p-hydroxybenzoic acid, naringin, myricetin, naringenin, and ramnetin, was found dependent on the extraction methods, indicating that the extraction methods affected the composition and content of polyphenolic components in each extract. Thus, antioxidant and whitening activities were investigated to determine the effect of this difference on the physiological activity of *P. japonicus* extracts.

### 3.2 Comparison of antioxidant activity

Antioxidants are molecules that inhibit the oxidation of other molecules. They play a role in protecting organisms from ROS damage; thus, they can prevent and treat various diseases such as cancer, diabetes, and neurological diseases (Surveswaran et al., 2007). Vitamins, flavonoids, carotenoids, and anthocyanins are powerful antioxidants, and changes in the contents of these substances are highly correlated with changes in the antioxidant capacity of plant extracts (Gull et al., 2012). Extracts obtained by MAE of the leaves and roots showed lower DPPH free radical scavenging activity compared with those obtained by other extraction methods. However, in stems, HWE exhibited lowest DPPH free radical scavenging activity, suggesting that effects of extraction methods on DPPH free radical scavenging activity vary with plant organs (Fig. 1a). In addition, extracts obtained by L-HWE, L-AAE, and L-MAE exhibited higher reducing power compared with extracts obtained from other organs (Fig. 1b). Furthermore, ORAC assay results were similar to DPPH free radical scavenging activity (Fig. 1a) and reducing power (Fig. 1b), which showed the high antioxidant activity of all extracts obtained from leaves. In the case of root extracts, a relatively high ORAC activity was observed with R-HWE and R-AAE (Fig. 1c). The antioxidant activity of polyphenolic compounds is mediated by phenolic hydroxyl groups, which can provide hydrogen atoms or electrons to free radicals or form conjugated aromatic systems capable of transporting non-covalent electrons (Dai and Mumper, 2010).

![Fig. 1. Antioxidant activity of *P. japonicus* leaf, stem and root extracts were measured by DPPH free radical scavenging (a), reducing power (b) and ORAC (c) assays. DPPH radical scavenging activity was calculated as IC\textsubscript{50} (50% reduction of DPPH free radical). ORAC values are expressed as µmol of Trolox (TE) equivalents. Bars represent the mean ± S.E. of three independent experiments. Values in the same column with different superscripted letters are significantly different (p < 0.05). Hot-water extraction of leaf (L-HWE), stem (S-HWE) and root (R-HWE). Microwave-assisted extraction of leaf (L-MAE), stem (S-AAE) and root (R-AAE). Autoclave extraction of leaf (L-AAE), stem (S-AAE) and root (R-AAE).](image)

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>Tyrosinase-F</td>
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</tr>
<tr>
<td>Tyrosinase-Rev</td>
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<tr>
<td>β-actin-F</td>
<td>CCCACTCTTAAGAGGAGATG</td>
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<tr>
<td>β-actin-Rev</td>
<td>AGGGAGACAAAGCCTTCAT</td>
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</table>

Table 1. Primer sequences for qPCR analysis.
Table 2. Polyphenolic compounds in *Petasites japonicus* leaf, stem and root extracts.

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<tr>
<th>Compounds</th>
<th>L-HWE</th>
<th>L-MAE</th>
<th>L-AAE</th>
<th>S-HWE</th>
<th>S-MAE</th>
<th>S-AAE</th>
<th>R-HWE</th>
<th>R-MAE</th>
<th>R-AAE</th>
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<tr>
<td>Rutin</td>
<td>16.424</td>
<td>4.23±1.15c</td>
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<td>0.84±0.01b</td>
<td>0.79±0.06a</td>
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<td>0.73±0.01a</td>
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<td>Tacifolin</td>
<td>18.833</td>
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<td>0.42±0.01b</td>
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<td>0.27±0.05a</td>
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<td>n.d</td>
<td>n.d</td>
<td>0.25±0.01a</td>
</tr>
<tr>
<td>Naringenin</td>
<td>58.546</td>
<td>0.26±0.01c</td>
<td>0.24±0.01a</td>
<td>0.29±0.01b</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>0.19±0.01a</td>
<td>0.2±0.00a</td>
</tr>
<tr>
<td>Apigenin</td>
<td>62.579</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>0.23±0.01a</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>67.12</td>
<td>0.34±0.01a</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>0.29±0.01a</td>
</tr>
<tr>
<td>Iorushamnetin</td>
<td>72.443</td>
<td>6.13±0.42c</td>
<td>4.87±0.02b</td>
<td>3.42±0.07a</td>
<td>1.40±0.12a</td>
<td>n.d</td>
<td>2.39±0.02b</td>
<td>1.03±0.02a</td>
<td>4.33±0.45b</td>
</tr>
<tr>
<td>Ramnetin</td>
<td>74.477</td>
<td>0.22±0.01a</td>
<td>0.21±0.04a</td>
<td>0.21±0.01a</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>0.20±0.01a</td>
<td>0.35±0.11a</td>
</tr>
<tr>
<td>Total flavonoid (mg QE/g)</td>
<td>0.90±0.18b</td>
<td>0.54±0.10a</td>
<td>1.28±0.20b</td>
<td>0.81±0.15a</td>
<td>0.50±0.10a</td>
<td>1.04±0.04b</td>
<td>10.74±1.13c</td>
<td>2.13±0.26a</td>
<td>5.54±0.97b</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>22.368</td>
<td>0.17±0.01a</td>
<td>0.52±0.01b</td>
<td>0.97±0.01b</td>
<td>n.d</td>
<td>n.d</td>
<td>0.25±0.01a</td>
<td>0.11±0.01a</td>
<td>n.d</td>
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<tr>
<td>p-hydroxybenzoic acid</td>
<td>33.999</td>
<td>0.47±0.01c</td>
<td>0.23±0.01a</td>
<td>0.44±0.01b</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>35.314</td>
<td>2.43±0.01c</td>
<td>0.30±0.01a</td>
<td>2.15±0.01b</td>
<td>0.21±0.02a</td>
<td>n.d</td>
<td>0.28±0.01b</td>
<td>0.48±0.01a</td>
<td>n.d</td>
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<tr>
<td>Caffeic acid</td>
<td>38.195</td>
<td>1.34±0.01b</td>
<td>0.30±0.04a</td>
<td>2.66±0.01c</td>
<td>0.30±0.01a</td>
<td>n.d</td>
<td>1.07±0.01b</td>
<td>1.72±0.02a</td>
<td>n.d</td>
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<tr>
<td>Syringic acid</td>
<td>39.129</td>
<td>0.04±0.01a</td>
<td>n.d</td>
<td>0.15±0.02b</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>0.24±0.03a</td>
<td>0.44±0.01b</td>
</tr>
<tr>
<td>P-Coumaric acid</td>
<td>42.103</td>
<td>1.02±0.09a</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>17.66±0.18b</td>
<td>9.42±0.02a</td>
<td>20.35±0.10c</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>43.626</td>
<td>3.16±0.01c</td>
<td>1.01±0.03b</td>
<td>0.55±0.01a</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Total phenol (mg GAE/g)</td>
<td>15.83±1.70b</td>
<td>12.19±0.34a</td>
<td>17.24±2.82b</td>
<td>4.12±0.10b</td>
<td>2.31±0.46a</td>
<td>4.51±0.73b</td>
<td>5.93±1.33b</td>
<td>4.17±0.21a</td>
<td>6.74±0.57b</td>
</tr>
</tbody>
</table>

1) µg/g of extract values are the average of triplicate experiments. 2) Retention time. 3) Total flavonoid content analyzed as quercetin equivalent (QE) mg/g of extract; values are the average of triplicate experiments. 4) Total phenolic content analyzed as gallic acid equivalent (GAE) mg/g of extract; values are the average of triplicate experiments. n.d = Not detectable. Note: Hot-water extraction of leaf (L-HWE), stem (S-HWE) and root (R-HWE). Microwave-assisted extraction of leaf (L-MAE), stem (S-MAE) and root (R-MAE). Autoclave extraction of leaf (L-AAE), stem (S-AAE) and root (R-AAE).

Table 3. Correlations between the biological activities and total phenolic and flavonoid contents of *Petasites japonicus* extracts.

<table>
<thead>
<tr>
<th>Correlation</th>
<th>R²</th>
<th>TPC</th>
<th>TFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH free radical scavenging activity</td>
<td>-0.827*</td>
<td>-0.388</td>
<td></td>
</tr>
<tr>
<td>Reducing power</td>
<td>0.899**</td>
<td>0.186</td>
<td></td>
</tr>
<tr>
<td>ORAC</td>
<td>0.951**</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>Anti-melanogenic activity</td>
<td>-0.553</td>
<td>-0.145</td>
<td></td>
</tr>
</tbody>
</table>

Significance at p < 0.05. ** Significance at p < 0.01.

Leaves were the organ that contained higher values of TPC as well as higher values of antioxidant activity than the other organs, whereas stem extracts had a low level of TPC and antioxidant activity (Table 2 and Fig. 1). In addition, leaves contained higher concentration of polyphenolic compounds (10 out of 19 compounds) than other organs (Table 2). Furthermore, Pearson correlation analysis revealed that TPC was strongly correlated with DPPH free radical scavenging activity (R² = -0.827), reducing power (R² = 0.899), and ORAC (R² = 0.951) (Table 3), suggesting that the content of polyphenolic contents was highly correlated with antioxidant activity. A comparison of extracts obtained by HWE and AAE revealed that there was no significant difference in the TPC, and antioxidant activity; however, extracts obtained by MAE showed the lowest TPC and low antioxidant activity, indicating that HWE and AAE are useful for the enrichment of phenolic compounds.

3.3 Anti-melanogenic potential of *P. japonicus* extracts

Most of the tested extracts exhibited an inhibitory effect on α-MSH-induced melanin production in B16F10 cells (Fig. 2a). Among them, extracts obtained by L-HWE (100 µg/mL) inhibited melanin production (30% greater compared with the mock control), whereas extracts obtained by S-AAE, R-MAE, and R-AAE exhibited no effect or a low inhibitory effect on α-MSH-induced melanin synthesis. In stems and roots, extracts obtained by HWE exhibited higher inhibitory effect compared with those obtained by other extraction methods (Fig. 2a).
Fig. 2. Effect of *Petasites japonicus* extracts on melanin content in B16F10 cells. (a) Anti-melanogenic activity of *Petasites japonicus* leaf, stem and root extracts. (b) Dose-dependent anti-melanogenic effects of the hot-water extract obtained from *P. japonicus* leaves (L-HWE) in α-MSH-stimulated B16F10 cells. (c) Effect of L-HWE at different concentrations on cell viability of B16F10 cells. Values are the mean ± S.E. of triplicate experiments. Bars in the same sub-figure with the same lowercase letter are not significantly different (p < 0.05). Hot-water extraction of leaf (L-HWE), stem (S-HWE) and root (R-HWE). Microwave-assisted extraction of leaf (L-MAE), stem (S-MAW) and root (R-MAW). Autoclave extraction of leaf (L-AAE), stem (S-AAE) and root (R-AAE).

In addition, extracts obtained by L-HWE markedly inhibited α-MSH-induced melanin synthesis in a dose-dependent manner (Fig. 2b). No significant reduction in viability was observed for all the tested concentrations of extracts obtained by L-HWE (Fig. 2c), suggesting that the anti-melanogenic activity of extracts obtained by L-HWE was not due to cytotoxicity. Furthermore, extracts obtained by L-HWE reduced tyrosinase accumulation (Fig. 3a) and expression (Fig. 3b) in a dose-dependent manner, indicating that their anti-melanogenic activity could be mediated by the inhibition of tyrosinase expression.

ROS play an important role as signaling molecules that regulate important biological processes such as cell differentiation, proliferation, stress adaptation, and gene expression. However, the excessive accumulation of ROS due to an imbalance between cellular production of ROS and antioxidative mechanisms can cause oxidative damage to cellular components and a variety of neurological diseases (Tan et al., 2018). In particular, UV-induced ROS accumulation is known to be a major factor involved in melanin accumulation, and ROS scavenger and antioxidant compounds have been found to exhibit inhibitory effects on ROS-induced melanin production (Meyskens et al., 2001). Based on these findings, we hypothesized that the anti-melanogenic activity of extracts obtained by L-HWE may be attributed to antioxidant activity. Intracellular ROS levels were increased in B16F10 cells treated with H$_2$O$_2$, and extracts from L-HWE of *P. japonicus* suppressed ROS accumulation induced by oxidative stress in a dose-dependent manner (Figure 4).

Fig. 3. Effect of the hot-water extract obtained from *P. japonicus* leaves (L-HWE) on protein (a) and expression level (b) of tyrosinase in α-MSH-stimulated B16F10 cells. The transcript level of tyrosinase was normalized to the constitutive expression level of β-actin and expressed relative to the values of mock control (b). Values in the same column with different superscripted letters are significantly different (p < 0.05).

Fig. 4. Intracellular reactive oxygen species (ROS) scavenging activity of hot-water extract obtained from *P. japonicus* leaves (L-HWE) in oxidative stress-damaged B16F10 murine melanoma cells. B16F10 cells were treated with various concentrations of L-HWE for 24 h, and 5 µL of 24 mM H$_2$O$_2$ was added for 30 min. The intracellular ROS level was analyzed using the fluorescent probe DCFH-DA. Values in the same column with different superscripted letters are significantly different (p < 0.05).
Taken together, these results indicate that the anti-melanogenic activity of L-HWE is mediated by reduction of ROS accumulation and tyrosinase expression in B16F10 melanoma cells.

## Conclusions

In this study, the variation in bioavailability according to the extraction method was analyzed. For *P. japonicus* leaf extracts, there was no large difference in antioxidant and anti-melanogenic activities according to the extraction method. The methanol extract of *P. hybridus* leaf contained higher level of TPC (122.61 mg GAE/g extract; Arnold et al., 2015) compared with *P. japonicus* leaf extract (Table 2). However, it has been shown that TPC in the methanol extract of *P. japonicus* was 218.32 mg GAE/g extract (Kim et al., 2016). The solubility of phenolic compounds is influenced by the type of solvent (polarity) used, degree of polymerization of phenolics, as well as interaction of phenolics with other substances (proteins, polysaccharides, lipids, terpenes and inorganic compounds) (Naczk and Shahidi, 2004), indicating that the low content of phenolic compounds in the aqueous extract of *P. japonicus* leaf might be due to the presence of impurities including proteins which can reduce the solubility of the phenolic compounds. In the case of *P. japonicus* root extracts, a higher level of flavonoid content (Table 2) and antioxidant activity (Fig. 1) was observed with HWE compared with AAE or MAE. In addition, AAE exhibited the highest extraction efficiency of polyphenolic compounds when the leaves and stems were used (Table 2). The improved extraction efficiency of polyphenolic compounds by AAE may be attributed to an improvement in the solvent absorption rate due to high pressure treatment (Suh et al., 2017). Because of the low investment cost in equipment and operation compared to other technologies, MAE has been widely used for the extraction polyphenolic compounds from plants (Vallejo-Castillo et al., 2019), although MAE exhibited the lowest extraction efficiency of polyphenolic compounds compared with HWE and AAE. It has been shown that extraction efficiency of polyphenolic compounds has been affected by various factors including extraction time, microwave power, and solid:solvent ratio, indicating the significant impact of extraction conditions on the polyphenols and flavonoids extraction (Vallejo-Castillo et al., 2019). Furthermore, a high level of polyphenolic compounds such as rutin, isorhamnetin, chlorogenic acid, and ferulic acid, which are well known pharmaceutically active compounds, was found in extracts obtained by L-HWE, indicating that a change in the chemical composition of the extracts (Table 2) rather than a change in the extraction efficiency according to the extraction method (Table 1) may have a significant effect on the biological activity of the extracts.

In conclusion, the results of the present study indicated the importance of the extraction method. The extraction efficiency of polyphenolic compounds did not affect biological activity, indicating that polyphenolic composition is a major contributor to the biological activity of *P. japonicus* extracts. In addition, we found that the leaf extracts of *P. japonicus* are a useful source of natural antioxidants and anti-melanogenic agents. Further studies will be needed to isolate and characterize active compounds from *P. japonicus* leaves.

## References


