Food Sci. Biotechnol. 26(1): 213-220 (2017)
DOI 10.1007/s10068-017-0029-y

Food Science
and
Biotechnology

Anti-oxidative and anti-inflammatory activities of devil's club (*Oplopanax horridus*) leaves

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Received June 28, 2016
Revised November 8, 2016
Accepted November 14, 2016
Published online February 28, 2017

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pISSN 1226-7708
eISSN 2092-6456

Abstract This study aimed to investigate the anti-oxidative properties of the ethanolic extracts of the devil's club (*Oplopanax horridus*) leaves, stems, and roots. Furthermore, the anti-inflammatory activity of the leaf extract was analyzed. The leaf extract had higher total phenolic and flavonoid contents and anti-oxidative activity (radical scavenging, reducing power, and inhibition of lipid oxidation) than the root and stem extracts. The leaf extract also had anti-inflammatory effects. It significantly reduced lipopolysaccharide (LPS)-induced nitric oxide (NO; 71.0% at 50 μg/mL), tumor necrosis factor (TNF)-α (87.6% at 100 μg/mL), and interleukin (IL)-6 (36.2% at 100 μg/mL) production in murine RAW 264.7 macrophages. Furthermore, LPS-induced inducible nitric oxide synthase (iNOS) expression was decreased by the leaf extract (IC_{50}=24.4 μg/mL). The ultra performance liquid chromatography-diode array detector (UPLC-DAD) analysis showed that the leaf extract contained gallic acid, protocatechuic acid, chlorogenic acid, and maltol. These findings suggest that the leaf extract could be utilized as a functional food material because of its anti-oxidative and anti-inflammatory activities.

Keywords: *Oplopanax horridus*, anti-oxidative activity, anti-inflammatory activity, phenolic compounds, UPLC-DAD

Introduction

Crude extracts of medicinal plants have been widely used as sources of traditional medicines to treat a variety of human disorders. Recently, bioactive natural products have become increasingly important (1). *Oplopanax horridus* (Sm.) Miq., commonly known as devil's club, belongs to the Araliaceae family, the same family as Asian ginseng, American ginseng (*Panax quinquefolius* L.), and eleuthero (*Acanthopanax senticosus*). *Oplopanax horridus* (OH) is occasionally referred to as "Alaskan ginseng" or "Pacific ginseng", although they are in different genera (2). Accumulative evidences have reported the beneficial effects of OH against rheumatoid arthritis, autoimmune diseases, eczema, type-II diabetes, and infections (3). Moreover, OH has been commercially available in North America as a devil's club extract with respiratory stimulant and expectorant effects (3).

Previous studies have shown that OH extract and its constituents possess hypoglycemic, anti-bacterial, and anti-fungal properties (4-6). In recent pharmacological studies, OH extract was found to have a potential anti-cancer capability on a variety of human tumor cell lines (7,8). Although the roots, stems, and leaves are the major sources of pharmaceutical products from natural plants, the studies of OH have been mainly focused on the root bark (9). OH has been utilized by the natives of the Pacific Northwest (10). The root bark is the most commonly used plant part of OH, and the supplements contain root bark as the main ingredient. As most previous studies have focused on the roots of OH, the biological activity of the leaf extract has not been elucidated yet. In addition, many studies on OH phytochemical isolation have been conducted on the root bark extract (9). To the best of our knowledge, no information about OH leaf extract is available in the literature, and the effect of the OH leaf extract has not been evaluated. The present study was performed to examine the anti-oxidative effect of extracts derived from the leaves, stems, and roots of OH, and the results from the three different components of OH were compared. In addition, the anti-inflammatory effects were estimated, and the OH leaf extract was analyzed as a source of natural products.

Materials and Methods

Plant material and preparation of extracts OH was purchased from Pacific Botanicals Co. Ltd. (Chicago, IL, USA). It was separated into the roots, stems, and leaves and was washed thoroughly with distilled water. The plants were cut into small pieces, dried, and pulverized to fine powder using a mechanical grinder. Five grams of milled and dried OH leaves, stems, and roots were extracted with 100 mL of 70% ethanol for 1 h at room temperature with stirring, and the extracted solution was filtered through Whatman No. 4 filter paper (GE Healthcare Life Sciences Co., Buckinghamshire, UK) and evaporated. The extracts were freeze-dried to obtain powder and were stored at −80°C until use. For the extracts from each part of the plant, an aliquot was freeze-dried under vacuum, and the yield was determined.

Determination of the total phenolic and flavonoid contents The total phenolic content was determined using Folin-Ciocalteu reagent (11). A 200 μL aliquot of each extract (0.1 mg/mL) was dissolved in ethanol and mixed with 0.5 mL of Folin–Ciocalteu reagent (previously diluted 1:50 v/v with water) and 1.5 mL of a 20% sodium carbonate solution. The total volume of the solution was adjusted to 5 mL with distilled water. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured in triplicate. The concentration of total phenolic compounds in each extract was expressed as gallic acid equivalents (GAE) in milligrams per gram extract by referencing the gallic acid standard calibration curve.

The total flavonoid content was determined using a previously published method (12), wherein 0.1 mL of 2% aluminum trichloride ($AlCl_3$) in ethanol was mixed with the same volume of each extract (0.1 mg/mL). The absorbance was measured at 430 nm after 10 min. The total flavonoid content was determined using a standard curve with quercetin as the standard and was expressed as mg quercetin equivalent (QE) per gram extract. Measurements were performed in triplicate.

Determination of anti-oxidative activity

***DPPH free radical scavenging activity*:** The free radical scavenging capacity of the extracts was determined using a DPPH (2,2-diphenyl-1-picrylhydrazyl) assay according to a previously published method (13), with some modifications. In this assay, 0.2 mL of extract solution was added to 0.8 mL of 0.4 mM DPPH solution, and the mixture was shaken and left for 20 min in the dark at room temperature. The absorbance was then measured at 517 nm. The decrease in absorption was related to the radical scavenging activity (percent inhibition) of the samples. The percent inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \left[\frac{A_c - A_s}{A_c}\right] \times 100 \quad (1)$$

where A_c is the absorbance of a standard that was prepared using the same conditions but without samples and A_s is the absorbance of the samples (1–100 mg/mL). Vitamin C (1 mg/mL) was used as a positive control.

***ABTS radical scavenging activity assay*:** The anti-oxidant activity was measured using a modified ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assay (14). First, 1.0 mM AAPH (2,2'-azobis-(2-amidinopropane)-dihydrochloride) and 2.5 mM of ABTS were dissolved in 100 mM potassium phosphate-buffered solution (PBS; pH 7.4). The $ABTS^{\bullet-}$ radical solution was heated at 70°C for 30 min and then cooled to room temperature. After filtration using a 0.45 μm syringe filter, the $ABTS^{\bullet-}$ solution was diluted to obtain an absorbance of 0.65±0.02 at 734 nm. A 0.98 mL aliquot of the $ABTS^{\bullet-}$ solution was mixed with 0.02 mL of sample solution, and the absorbance at 734 nm was measured after 20 min. PBS was used as the blank, and the control comprised 0.98 mL of $ABTS^{\bullet-}$ radical solution mixed with 0.02 mL of water. The anti-oxidant capacity was evaluated by measuring the free radical scavenging activity of the extracts. The sample stock solution and vitamin C were diluted in distilled water, and a fresh radical solution was prepared before the experiment. The percent free radical scavenging activity of the extracts (1–100 mg/mL) and vitamin C (1 mg/mL), which was used as a positive control, was calculated as follows, and calibration curves were obtained:

$$\% \text{ free radical scavenging activity} = \left(100 - \frac{A_s}{A_c}\right) \times 100 \quad (2)$$

where A_c is the absorbance of the control and A_s is the absorbance of the sample.

***Ferric reducing anti-oxidant power (FRAP) assay*:** Determination of the total anti-oxidant activity in the extract was performed using a modified FRAP assay (15). The stock solution included a 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 mL $C_2H_4O_2$) at pH 3.6, a 10 mM TPTZ (2,4,6-tripyridyl-striazine) solution in 40 mM HCl, and a 20 mM $FeCl_3 \cdot 6H_2O$ solution in distilled water. The acetate buffer (25 mL) and TPTZ (2.5 mL) were mixed with $FeCl_3 \cdot 6H_2O$ (2.5 mL). The temperature of the solution was increased to 37°C before use; 150 mL of extracts (10–100 μg/mL) were allowed to react with the FRAP solution (2.85 mL) for 30 min in the dark, and the absorbance was measured at 593 nm. Vitamin C (1 μg/mL) was used as a positive control.

***Superoxide dismutase (SOD)-like activity*:** The SOD-like activity of the extracts was determined using an SOD assay kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). Twenty microliters of sample solution (10–100 μg/mL) was added to 200 μL of the kit working solution. The mixture was incubated at 37°C for 20 min after gentle shaking, and 20 μL of the kit enzyme working solution was added. The absorbance of the mixtures was measured spectrophotometrically at 450 nm (Infinite M200; Tecan Trading AG, Männedorf, Switzerland). The SOD-like activity was calculated according to the following equation:

$$\% \text{ SOD-like activity} = \left[(A_{blank1} - A_{blank3}) - \frac{(A_{sample} - A_{blank2})}{(A_{blank1} - A_{blank3})} \right] \times 100 \quad (3)$$

where blank 1 was a mixture of the working solution and enzyme working, blank 2 contained the sample solution with the working solution and dilution buffer, and ddH_2O was added to the sample solution in blank 3. Trolox (10 μg/mL) was used as a positive control.

***Thiobarbituric acid (TBA) assay*:** A TBA assay was used to assess lipid peroxidation using a previously published method (16) with a few modifications. The testing samples were added to an aqueous solution containing 200 μL of Tris buffer (pH 7.4), 300 μL of 1 M KCl, 400 μL of 1% SDS, 10 μL of cod liver oil, 40 μL of 1.0 μM $FeCl_2$, and 20 μL of 0.5 μM H_2O_2. The solution was incubated for 18 h at 37°C with shaking. The reaction was stopped by the addition of trichloroacetic acid (35% w/v, 1.0 mL) followed by TBA (0.75%, w/v, 2.0 mL), and the mixture was heated at 95°C for 40 min. After centrifugation at 6,350x*g* for 5 min to remove the precipitated protein, the color intensity of the thiobarbituric acid reactive substances (TBARS) in the supernatant was determined by measuring the absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = [(A\ B)/A] \times 100\%$$

where A and B are the absorbance of the negative control and extract solution (1 mg/mL), respectively. Vitamin C (1 mg/mL) was used as a positive control.

Determination of anti-inflammatory activity

***Cell culture*:** A murine macrophage cell line, RAW 264.7, was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/L streptomycin, and 100 IU/mL penicillin at 37°C in a 5% CO_2 atmosphere (HERAcell 150; Thermo Electron Corp., Waltham, MA, USA).

***MTT assay for cell viability*:** RAW 264.7 cells were seeded at a density of 2×10^5 cells per well into 96-well plates. After 24 h of growth, the cells were pretreated with the extracts for 2 h at 37°C and then treated with lipopolysaccharide (LPS; 1 μg/mL) for an additional 12 h. After treatment, the cells were assayed for viability using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. The optical density of the cells was measured using a microplate reader (Infinite M200; Tecan Trading AG) at 570 nm.

***Nitrite assay*:** RAW 264.7 cells were pretreated with the indicated extract concentrations for 2 h and then treated with LPS (1 μg/mL) for an additional 22 h. The nitrite concentrations in the suspended media were determined using a Griess reagent. The Griess reagent and an equal volume of cell supernatant were mixed, and the nitrite concentrations were measured at 540 nm against a standard sodium nitrite curve.

***Measurement of tumor necrosis factor (TNF)-α and interleukin (IL)-6 levels*:** RAW 264.7 cells were plated at a density of 2×10^5 cells per well in a 24-well plate. The cells were pretreated with the indicated extract concentrations for 2 h and then induced with 1 μg/mL LPS for an additional 22 h. The concentrations of TNF-α and IL-6 were determined using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

***Western blotting*:** Equal amounts of proteins were separated using 15% sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The blots were probed with primary and secondary antibodies, and the immuno-reactive bands were developed with enhanced chemiluminescence (Bio-Rad Laboratories, Hercules, CA, USA). All the primary and secondary antibodies, which included inducible nitric oxide synthase (iNOS) and β-actin, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

***Identification of an active compound by ultra performance liquid chromatography-diode array detector (UPLC-DAD)*:** UPLC-DAD analyses were performed using a Waters Acquity ultra performance liquid chromatographic system (Waters, Milford, MA, USA). Chromatographic separations were performed on an Acquity UPLC HSS T3 column (100×2.1 mm, 1.8 μm, Waters) at 40°C with a mobile phase flow rate of 0.4 mL/min. The mobile phase comprised (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. A linear gradient elution program was applied as follows: 0 min, 5% B; 3–5 min, 7% B; 7–9 min, 10% B; 11–13 min, 12% B; 15–17 min, 15% B; 18 min, 18% B; 21–24 min, 20% B; 30 min, 45% B; 32–34 min, 100% B; 36–42 min, 5% B. The injection volume was 10 μL, and the identification of each compound was based on a combination of the retention time and spectral matching. Chlorogenic acid, gallic acid, maltol, and protocatechnic acid were procured from Sigma-Aldrich.

Statistical analysis All the experimental values obtained are expressed as the mean±standard deviation (SD). Differences among groups were examined for statistical significance using one-way analysis of variance (ANOVA) followed by Duncan's test. The results were considered statistically significant when the *p* value was less than 0.05 or 0.01. Furthermore, the correlations were analyzed and statistical significance was identified at the level of $p<0.05$.

Results and Discussion

Total phenolic and flavonoid content (TPC and TFC) The TPC in the ethanolic extracts of the leaves, stems, and roots of OH are presented in Table 1. Among the three extracts, the lowest level of TPC was found in the stem extract, whereas the leaf extract contained a relatively high TPC. Phenolic compounds are found in many types of plants and are known to have some biological effects, such as anti-

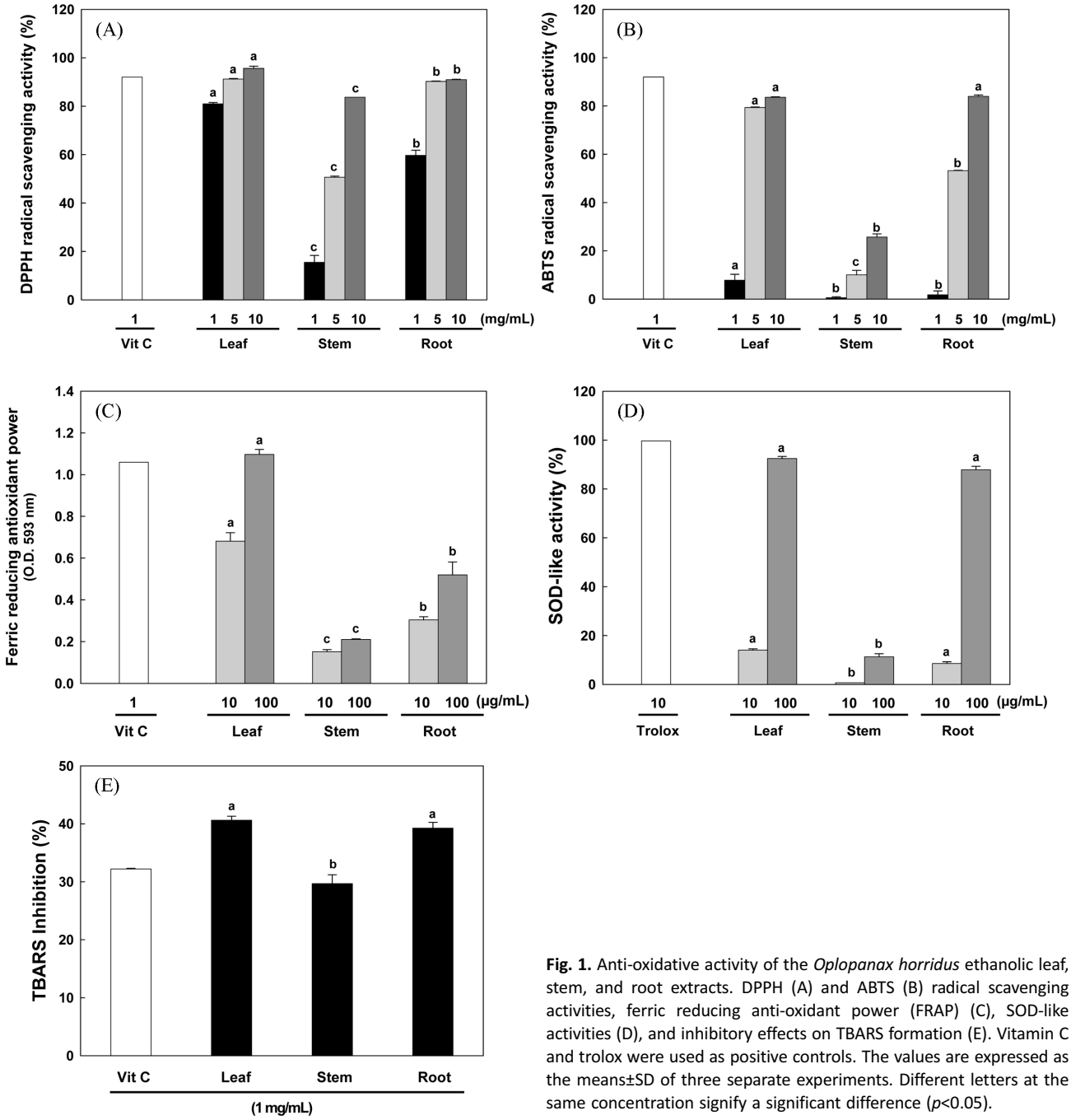


Fig. 1. Anti-oxidative activity of the *Oplopanax horridus* ethanolic leaf, stem, and root extracts. DPPH (A) and ABTS (B) radical scavenging activities, ferric reducing anti-oxidant power (FRAP) (C), SOD-like activities (D), and inhibitory effects on TBARS formation (E). Vitamin C and trolox were used as positive controls. The values are expressed as the means±SD of three separate experiments. Different letters at the same concentration signify a significant difference (p<0.05).

oxidant activity. The crude extracts of phenolic-rich plant materials are gaining the food industry's attention because they delay the oxidative deterioration of lipids and increase the quality and nutritional value of foods (17). The TFC in the leaf extract was greater than that in the root extract, followed by the content in the stem extract (Table 1). Flavonoids are a class of secondary plant metabolites that possess anti-oxidant and chelating properties (17). By comparing the different plant parts, it was found that the leaf extract had higher TPC and TFC than the extracts from the roots or stems, whereas the yield of the extract from the roots was the greatest, followed by stems and leaves, as shown in Table 1.

Table 1. Total phenolic and flavonoid contents of *Oplopanax horridus* ethanolic leaf, stem, and root extracts

	Leaf	Stem	Root
Total phenolics (mg GAE/g of dry material)	54.3±0.3[a1)]	16.3±0.2[c]	35.4±0.0[b]
Total flavonoids (mg QE/g of dry material)	5.2±0.6[a]	1.3±0.1[c]	2.0±0.1[b]
Yield (% w/w)	10.0±0.0[a]	12.5±2.0[a]	13.5±2.0[a]

[1)]The data are expressed as the mean±SD of three separate experiments. Different letters in the same row indicate significant differences (p<0.05).

Table 2. Correlation coefficients among the anti-oxidant parameters, total phenolic content, and total flavonoid content of the extracts from *Oplopanax horridus*

	TPC[1)]	TFC[2)]	DPPH[3)]	ABTS[4)]	FRAP[5)]	SOD[6)]	TBA[7)]
TPC		0.8775	0.9620	0.9739	0.9417	0.7949	0.8446
TFC			0.7235	0.7534	0.9871	0.4580	0.5226
DPPH				0.9988	0.8186	0.9269	0.9569
ABTS					0.8440	0.9082	0.9421
FRAP						0.5714	0.6346
SOD							0.9958

[1)]TPC, total phenolic content.
[2)]TFC, total flavonoid content.
[3)]DPPH, radical-scavenging activity.
[4)]ABTS, ABTS radical-scavenging activity.
[5)]FRAP, ferric reducing anti-oxidant power.
[6)]SOD, superoxide dismutase-like activity.
[7)]TBA, thiobarbituric acid assay.

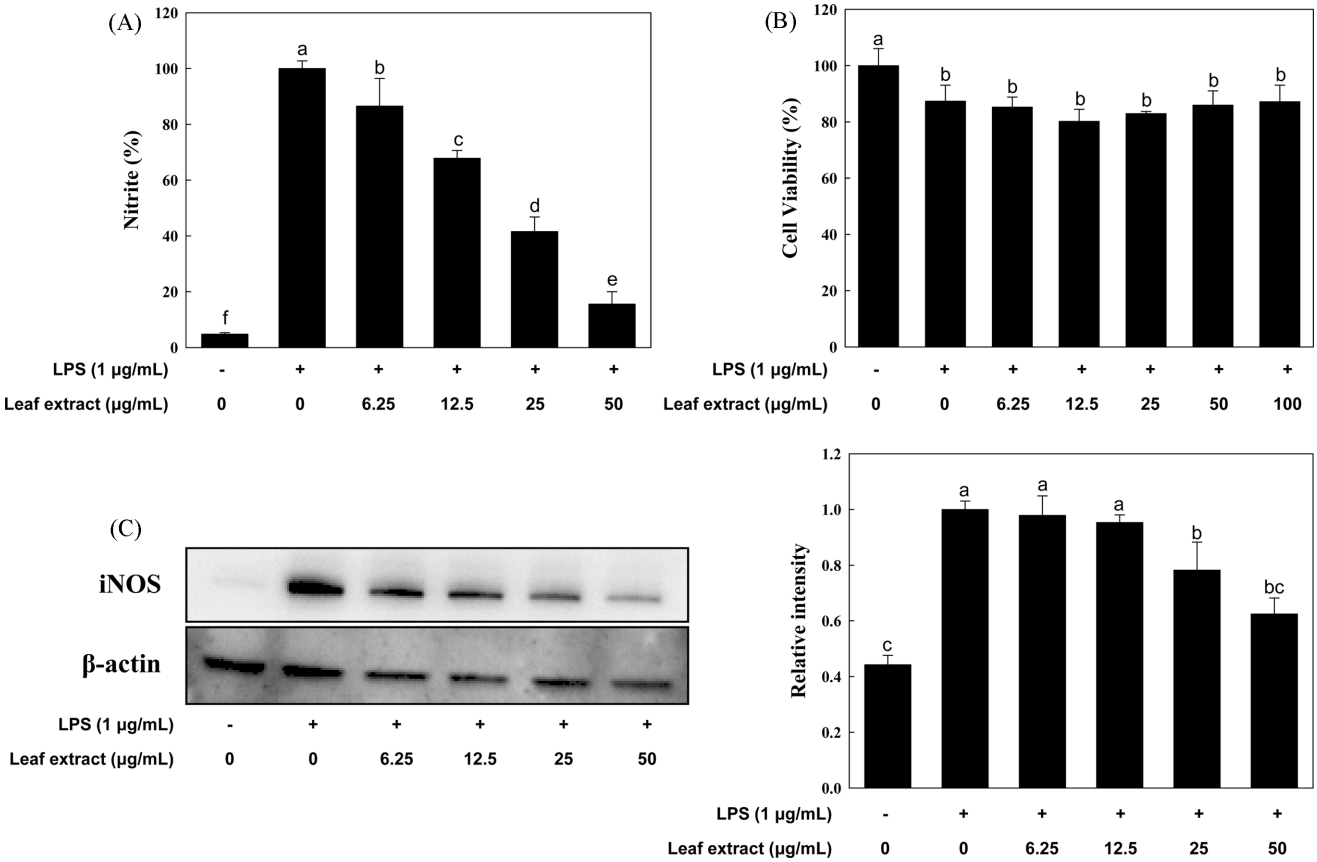


Fig. 2. Inhibitory effects of the *Oplopanax horridus* ethanolic leaf extract on LPS-induced NO production (A), cell viability (B), and iNOS protein expression (C) in RAW 264.7 cells. The values are expressed as the means±SD of three separate experiments. Values with different letters are significantly different from the LPS-untreated cells (p<0.05).

Anti-oxidative activity There are many methods to determine the anti-oxidant activity of plant extracts, with each having its own limitations (18). Because the anti-oxidant ability of a plant extract is determined by a mixture of various anti-oxidants with different modes of actions, it is necessary to use multiple methods to determine the exact *in vitro* anti-oxidant ability of a plant extract (19). For the comprehensive and accurate evaluation of the anti-oxidant activity of the three different extracts of OH, four *in vitro* assays were conducted: radical scavenging (DPPH and ABTS), FRAP activity, SOD-like activity, and inhibition of TBARS formation.

***The radical scavenging activities (DPPH and ABTS assays)*:** The DPPH and ABTS methods for screening anti-oxidant activity allow convenient measurement of the free radical removing effect with high sensitivity. The absorbance decreases when the DPPH free

radicals are removed by anti-oxidants by giving an electron or hydrogen to form a stable DPPH molecule (20). Figure 1A and 1B show the percent inhibition of the DPPH and ABTS radicals caused by different OH extracts. At a dose of 1 mg/mL, the percent inhibition of the DPPH radical by the leaf extract was 81.05%, whereas the percent inhibition was 15.53% and 56.65% for the stem and root extracts, respectively. The DPPH radical removing activity of the leaf extract was significantly better than those of the root and stem extracts at all concentrations tested (p<0.05) (Fig. 1A).

The ABTS assay is frequently used to estimate the anti-oxidative effect of foods (21). The anti-oxidative capacities of the extracts and vitamin C were measured against the $ABTS^{+}$ radical. As shown in Fig. 1B, the percent inhibition value of the ABTS radical by the leaf extract was 93.31% at 5 mg/mL; the percent inhibition was 27.24% and 69.82% for the stem and root extracts at the same concentration, respectively. In the DPPH and ABTS assays, the leaf extract had a higher anti-oxidant activity than the stem and root extracts. In this study, the results revealed that the OH leaf extract has good ability to remove free radicals and can act as a primary anti-oxidant.

***FRAP, SOD-like activity, and inhibition of TBARS formation*:** The extracts from the different parts of OH were tested using the FRAP assay, which is a simple assay that provides fast, reproducible results for anti-oxidant activities (15). Figure 1C shows the differences in reducing power of extracts derived from OH leaves, stems, and roots. The leaf extract had the greatest activity for reducing Fe^{3+} to Fe^{2+}, followed by the root and stem extracts. Previous study reported the reducing activity of the extract is generally associated with the presence of reductones, which show anti-oxidant activity through terminating the free radical chain via hydrogen ion donation (22).

SOD is an important anti-oxidative enzyme that catalyzes the superoxide (O_2) radical dismutation into either hydrogen peroxide or ordinary molecular oxygen. Figure 1D shows the SOD-like activity of the three different parts of OH. The SOD-like activities at 0.10 mg/mL were: leaf (92.45%)>root (87.84%)>stem (11.31%). The leaf and root extracts had greater SOD-like activity than the stem extract (p<0.05). The presence of hydroxyl substituents in aromatic rings is closely associated with the radical scavenging effect by their hydrogen donating capacity (23). TBARS are naturally present in biological specimens. Lipid peroxidation was assessed using the TBARS assay, which is a well-established assay for measuring the autoxidation of foodstuff and assessing lipid peroxidation. This method has been shown to be a sensitive index of lipid peroxidation (24). The TBARS formation inhibition of the leaf and root extracts was significantly greater than the inhibition of the stem extract (Fig. 1E).

The results of this study confirm that the ethanolic extract of leaves had a significantly greater anti-oxidant activity than the stem and root extracts (p<0.05).

***Correlation among the anti-oxidative activity and TPC and TFC*:** The correlation coefficients between the tests for anti-oxidants and TPC and TFC were calculated and are presented in Table 2. TPC had a positive correlation with DPPH (r=0.9620) and ABTS (r=0.9739). The

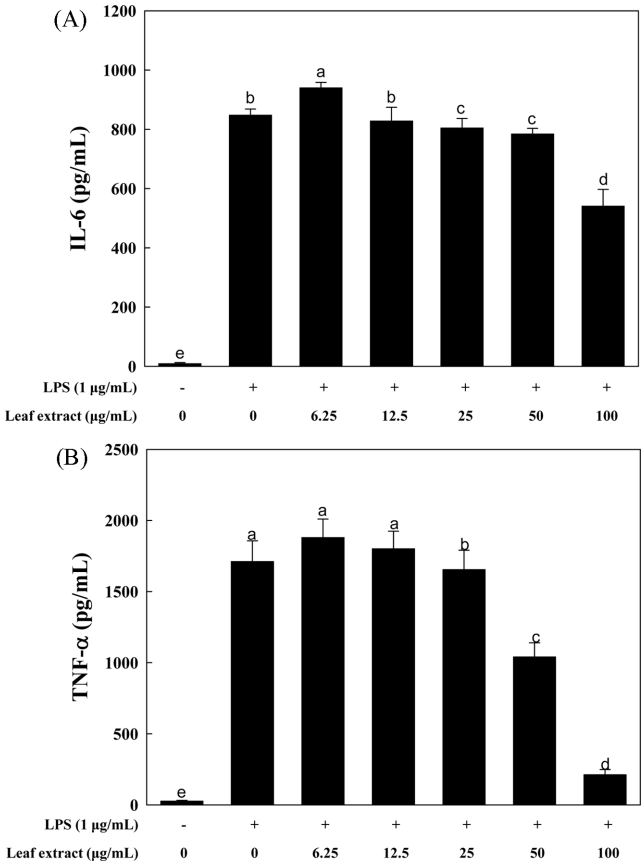
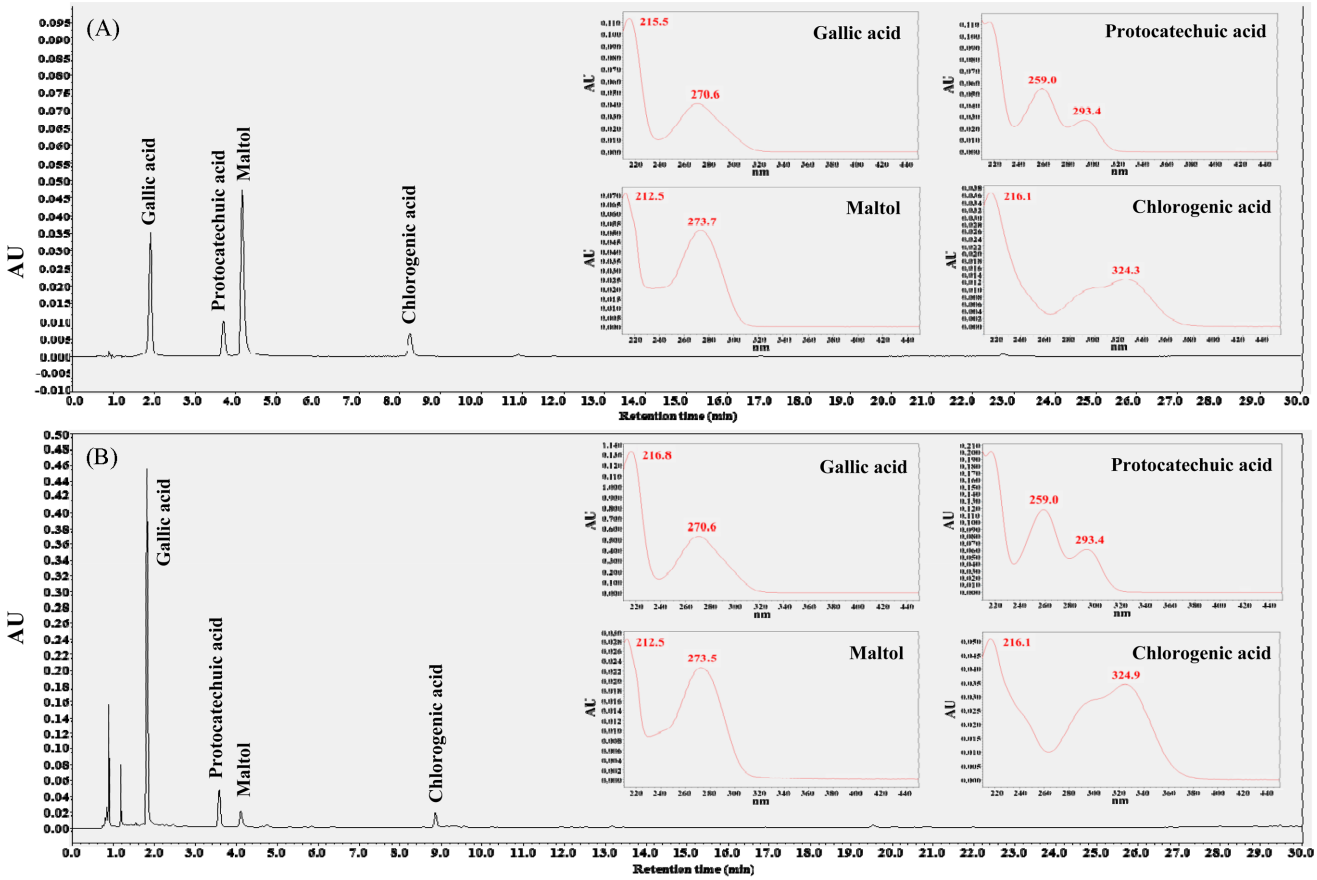


Fig. 3. Inhibitory effects of the *Oplopanax horridus* leaf extracts on LPS-induced IL-6 (A) and TNF-α (B) production in RAW 264.7 cells. The values are expressed as the means±SD of three separate experiments. Values with different letters are significantly different from cells treated with LPS alone (p<0.05).

ABTS and DPPH radical scavenging activities were well correlated (r=0.9988). The DPPH and ABTS assays showed a strong correlation when thirty aqueous plant extracts were evaluated (r=0.906) (25). There was a good correlation between the SOD-like activity and DPPH (r=0.9269) and ABTS radical scavenging activity (r=0.9082). The results of the DPPH and SOD-like activity assay were positively correlated to TPC. The phenolic constituents influence the anti-oxidant activities because of their redox properties (26). There was a robust correlation between the FRAP value and TFC (r=0.9871) and TPC (r=0.9417) of the extracts tested, indicating that the TPC and TFC is a major determinant of the anti-oxidant power of the extracts.

***Anti-inflammatory activity of the OH leaf extract*:** The TPC is closely related to the anti-oxidative and anti-inflammatory activities of plant extracts (27). As the leaf extract had the greatest TPC and anti-oxidant activity, it was used in all subsequent experiments for anti-inflammation.

***Inhibitory effects on LPS-induced NO production and iNOS protein expression*:** The pretreatment of RAW 264.7 macrophages with leaf extract (6.25-50 μg/mL) resulted in a significant concentration-dependent reduction of NO production by LPS treatment (Fig. 2A).



(C) (mg/g)

Gallic acid	Protocatechuic acid	Maltol	Chlorogenic acid
32.97±0.36	7.97±0.28	0.72±0.05	2.70±0.08

Fig. 4. UPLC-DAD chromatograms of a standard mixture (A), *Oplopanax horridus* leaf extract (B), and the phenolic compound content in *Oplopanax horridus* leaf extract (mg/g) (C). The values are expressed as the means±SD of three separate experiments.

MTT assays were performed to confirm the suitable concentration range of the leaf extract for the research experiments. As shown in Fig. 2B, the OH leaf extract was not cytotoxic to the macrophages within the tested doses. These results indicate that OH leaves inhibited LPS-stimulated NO production by 58.4% and 71.0% at 25 and 50 μg/mL, respectively (Fig. 2A).

As shown in Fig. 2C, 1 mg/mL of LPS treatment markedly increased iNOS protein expression in RAW 264.7 macrophages. However, pretreatment with the leaf extract decreased the iNOS expression levels in LPS-stimulated RAW 264.7 macrophages in a concentration-dependent manner (Fig. 2C). NO produced by iNOS is one of the widely known pro-inflammatory mediators that are related to various pathophysiological processes. In previous study, it was reported that iNOS inhibitors could alleviate various inflammatory diseases (28). Thus, inhibition of NO production and iNOS expression is an important therapeutic target for the treatment of diverse diseases caused by inflammation.

***Inhibition of LPS-induced production of TNF-α and IL-6*:** The leaf extract treatment inhibited the secretion of IL-6 and TNF-α in a dose-dependent manner in RAW 264.7 macrophages (Fig. 3). TNF-α, one of important Th1 cytokines, has a wide range of action in inflammation, infection, and immunity (29). IL-6, a major pro-inflammatory cytokine, plays a critical role in the immune system which is secreted from Th2 cells and macrophages (30). NO, IL-6, and TNF-α are well-known pro-inflammatory mediators that trigger the pathogenesis of inflammatory diseases (31). In the present study, the leaf extract of OH decreased LPS-induced NO, IL-6, and TNF-α production, and iNOS protein expression. Therefore, these results suggest that the OH leaf extract has potent anti-inflammatory activity, at least in part through the inhibition of the LPS-induced production of NO, IL-6, and TNF-α.

***Identification of phenolic compounds from the OH leaf extract by UPLC-DAD*:** Four compounds were identified and quantified in OH leaves: gallic acid, protocatechuic acid, maltol, and chlorogenic acid (Fig. 4). Two hydroxybenzoic acids, gallic acid (3,4,5-trihydroxy-

benzoic acid) and protocatechuic acid (3,4-dihydroxybenzoic acid), were detected, and the hydroxycinnamic acid identified in the analysis was chlorogenic acid. The highest content in the OH leaf extract was that of gallic acid, followed by protocatechuic acid, chlorogenic acid, and maltol. Gallic acid is a naturally abundant plant phenol that is found in foods of plant origin. It has attracted considerable interest because of its anti-oxidative activity (32). Protocatechuic acid is a phenolic compound found in many plant foods. Pretreatment with protocatechuic acid had anti-inflammatory effects on the expression of cyclooxygenase (COX)-2, iNOS, and myeloperoxidase, as well as on the levels of nitrite and nitrate in CCl_4-induced rats (33). Chlorogenic acid is one of the most common phenols in the human diet and has been known as a potent anti-oxidative and -carcinogenic agent, both *in vivo* and *in vitro* (34). Maltol, a food additive, is used as a potent flavor enhancer in food products and has excellent anti-oxidative activity (35).

The leaf extract had the highest DPPH and ABTS radical scavenging activities, FRAP, SOD-like activity, and inhibition of TBARS formation among the extracts derived from the OH leaves, stems, and roots. These anti-oxidative activities were closely related to the TPC and TFC. The OH leaf extracts significantly decreased LPS-induced NO and pro-inflammatory cytokine (IL-6 and TNF-α) production. The leaf extract also decreased the protein levels of LPS-induced iNOS. Four compounds were identified and quantified in OH leaves: gallic acid, protocatechuic acid, chlorogenic acid, and maltol. These results suggest that the OH leaf extract can be used as a new functional food material with the potential to improve anti-oxidative and anti-inflammatory activities.

Acknowledgments This research was supported by Main Research Program (E0145201-03) of the Korea Food Research Institute (KFRI) funded by the Ministry of Science, ICT & Future Planning.

Disclosure The authors declare no conflict of interest.

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