

Research Article

HPLC Analysis and Antioxidant Evaluation of Acteoside-Rich *Osmanthus fragrans* Extracts

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Received 16 April 2020; Revised 7 August 2020; Accepted 13 August 2020; Published 2 September 2020

Academic Editor: Seyed Mohammad Taghi Gharibzadeh

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A method for the separation and quantification of acteoside by reverse-phase high-performance liquid chromatography (HPLC) was developed and validated. Hot water and ethanolic extracts of *Osmanthus fragrans* leaves and flowers were analyzed for acteoside content. Excellent linearity was obtained, with an r^2 higher than 0.999. The precision, specificity, and accuracy of our method were excellent, suggesting that it can be conveniently used for the quantification of acteoside in the crude extract of *O. fragrans*. The hot water and ethanol extracts were analyzed, and their biological activities were tested. The extraction yields, marker (acteoside) contents, and antioxidant activities of the leaf and flower extracts were analyzed. The antioxidant activity was confirmed by measuring the 2,2-diphenyl-2-picrylhydrazyl radical scavenging activity, reducing power, and total phenolic content. The acteoside content tended to be higher in the 100% ethanol extract of *O. fragrans* compared to those with the other extraction conditions tested. Overall, almost all extracts prepared with ethanolic solvents tended to produce better antioxidant activity than those prepared with hot water. These results suggest that the ethanolic extract of *O. fragrans* could serve as a potential antioxidant and anti-inflammatory pharmaceutical source, and our validated method would be useful for the quality control of *O. fragrans* extracts.

1. Introduction

Osmanthus fragrans (Oleaceae) is a well-known ornamental plant in Asia [1]. It is widely cultivated as an ornamental plant for its fragrant flowers in Korea, Taiwan, Japan, China, and elsewhere. *O. fragrans* has been used for treating weakened vision, asthma, coughs, toothaches, stomachaches, diarrhea, and hepatitis. However, modern evidence for the biomedical use of *O. fragrans* is limited [2]. Recently, various biological activities of *O. fragrans*, such as antipotent [3], antihypoxic [4], anti-inflammatory [5],

antiallergic [2], and neuroprotective [6] activities, were reported. Zhou et al. reported that the extract of *O. fragrans* flowers showed antihypoxic activity, and 80% acetone and 95% ethanol (EtOH) extracts contained about 3–7% acteoside (Figure 1) [4]. Hung et al. reported that the 75% EtOH extract of *O. fragrans* flowers had an antiallergic effect in a mouse model at an oral dose of 1 g/kg/14 day [2]. According to their results, the extract of *O. fragrans* flowers could be administered at a dose of about 4.8 g/day to a 60 kg human. However, pharmacologically active substances supporting their results have not been reported; only total

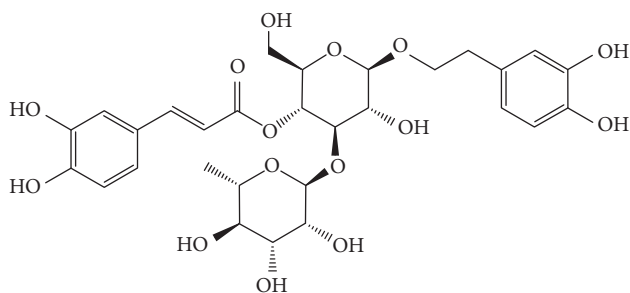


FIGURE 1: Chemical structure of acteoside (verbascoside).

phenolic compounds and total flavonoid concentrations have been reported (367 mg/g phenolics and 45 mg/g flavonoids) [7].

Few studies have reported the possibility of medicinal applications of *O. fragrans*. Recently, Lu et al. reported a study showing that extracts of *O. fragrans* flowers had very low toxicity, suggesting that *O. fragrans* flowers could be developed as a natural drug [1]. They used 80% EtOH for the extraction and partially purified the extract with adsorptive macroporous resin to prepare an extract containing 52% acteoside. Extracts of *O. fragrans* flowers showed no toxicity for 90 days at a dose of 2 g/kg/day. This is equivalent to 90 days of administration of 1 g/kg as acteoside. Based on these results, the acteoside dose calculated for humans is 4.86 g/60 kg [1]. The pharmacological activity of *O. fragrans* has been reported in several studies. However, there is a lack of research on the origin of the source materials and their association with biologically active compounds. Recently, researchers have reported the biological activities and identified several components of the flower extracts of *O. fragrans*. Hung et al. identified five compounds, including tyrosyl acetate, (+)-phillygenin, (8E)-ligustroside, rutin, and acteoside from the dichloromethane extract of *O. fragrans* flowers [7]. Among them, phillygenin, rutin, and acteoside showed strong antioxidant activity. Liu et al. identified 16 compounds from *O. fragrans* var. *aurantiacus* flowers; among them, ligustroside and (+)-pinoselinol were identified as showing the best inhibition of nitric oxide production in lipopolysaccharide-induced RAW 264.7 cells [8].

We reviewed the results of previous studies extensively and confirmed that acteoside is a major component of *O. fragrans* and that *O. fragrans* could be developed as a safe medicinal source. However, the flower parts of *O. fragrans* are hard to industrialize owing to low yields. In a preliminary study, we found that the main component of *O. fragrans* was acteoside and that it was present in large amounts in the flowers and leaves. In the present study, we established a quality control method by high-performance liquid chromatography (HPLC), which could separate and quantify acteoside in the extracts of *O. fragrans* leaves and flowers. To obtain an acteoside-rich extract, leaf and flower extracts were prepared from *O. fragrans* and *O. fragrans* var. *aurantiacus*, which are distinctive plants grown in Korea. Subsequently, the extraction yields, acteoside contents, and antioxidant activities of the leaf and flower

extracts were examined. Antioxidant activity was confirmed by measuring the 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, and total phenolic content.

2. Materials and Methods

2.1. Plant Materials. Leaves and flowers of *O. fragrans* and *O. fragrans* var. *aurantiacus* were supplied by the Jeollanamdo Forest Resource Research Institute (Naju, Korea). A voucher specimen (MNUCSS-OF-01~04) was deposited at the Mokpo National University (Muan, Korea). Air-dried and powdered *O. fragrans* leaves and flowers (20 g) were subjected to extraction twice with 20, 40, 60, 80, and 100% EtOH (100 mL) at room temperature for 48 h or subjected to extraction with hot water (100°C) for 4 h. The extraction was repeated twice. The resultant solution was evaporated, dried, and stored at -20°C for further experiments.

2.2. Identification of Acteoside in *O. fragrans* Extracts. All analyses were performed on a Waters Alliance 2695 HPLC system with a photodiode array detector. The analytical column used was an Agilent Zorbax extended C18 (5 μm, 150 mm × 5 mm), with the mobile phase consisting of a mixture of solvent A (acetonitrile) and B (water containing 0.2% phosphoric acid) and employing a gradient elution (from 10/90 to 100/0, v/v) at a flow rate of 1 mL/min (Table 1). The column temperature was maintained at 25°C, and the detection wavelength was set at 270 nm for acteoside [(2R, 3R, 4R, 5R, 6R)-6-[2-(3,4-dihydroxyphenyl)ethoxy]-5-hydroxy-2-(hydroxy-methyl)-4-[(2S, 3R, 4R, 5R, 6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxyoxan-3-yl] (E)-3-(3,4-dihydroxy-phenyl) prop-2-enoate.

The acteoside standard was purchased from ChemFaces Biochemical Co., Ltd. (Wuhan, China) and had a purity of >98% (Figure 1).

2.3. Method Validation. For the validation of the analytical method used for the quantification of acteoside in the extract of *O. fragrans*, specificity, linearity, sensitivity, accuracy, precision, and recovery were determined as previously described [9].

2.4. DPPH Free Radical Assay. Antioxidant activity was determined using a DPPH radical-scavenging assay. In brief, 1 mL of sample solution was mixed with DPPH sample solution (0.4 mM). The mixture contents were allowed to react at room temperature for 10 min. The absorbance of the mixture at 517 nm was then measured using a microplate reader (Perkin Elmer, Waltham, MA, USA) [10].

2.5. Ferric-Reducing Antioxidant Power (FRAP) Test. The reducing power of the sample was determined using a modified FRAP test [10]. In brief, the sample was mixed with sodium phosphate buffer (0.2 M) and potassium ferricyanide (1%, w/v) followed by incubation at 50°C for 20 min. Subsequently, trichloroacetic acid solution (10%, w/v) was added

TABLE 1: Analytical conditions of high-performance liquid chromatography for the analysis of acteoside.

Time (min)	A	B
0	10	90
7	10	90
18	100	0
19	10	90
30	10	90

A: acetonitrile; B: 0.2% phosphoric acid.

to the reaction mixture followed by centrifugation at 12000 ×g for 10 min. The supernatant was mixed with distilled water and iron (III) chloride solution (0.1%). The absorbance of the resulting solution was measured at 700 nm. The reducing power of the samples is expressed as ascorbic acid equivalents.

2.6. Determination of Total Phenolic Content. Total phenolic content was determined using the Folin–Ciocalteu assay. The sample was mixed with sodium carbonate solution (2%, w/v) and Folin–Ciocalteu’s phenol reagent (10%, v/v) for 10 min, and its absorbance was measured at 750 nm. The data are expressed as milligrams of gallic acid equivalents per gram of sample [10].

2.7. Statistical Analysis. The *t*-test was used for comparing two unpaired means, and a *p* value <0.05 was considered statistically significant. Data are expressed as mean ± standard deviation.

3. Results and Discussion

3.1. Extraction Yields of *O. fragrans* Extracts. To date, there have been no reports on yields of extracts prepared from *O. fragrans* leaves (OFL), *O. fragrans* flowers (OFF), *O. fragrans* var aurantiacus leaves (OFVL), and *O. fragrans* var aurantiacus flowers (OFVF). As shown in Table 2, the hot water and 20–100% EtOH extraction yields ranged from 6.12% to 42.22%. The best overall yield was obtained by the hot water extraction of *O. fragrans* flowers. This study is the first report of the extraction yields determined according to the extraction conditions for two species of *O. fragrans*. Our data could provide optimal extraction information when *O. fragrans* is developed as a pharmaceutical material in the future.

3.2. Method Validation

3.2.1. Limit of Detection (LOD), Limit of Quantification (LOQ), and Linearity. The LOQ of an individual analytical procedure is the lowest amount of analyte in a sample that can be determined with suitable precision and accuracy. The LOD and LOQ for acteoside were found to be 0.26 and 0.78 µg/mL, respectively. The LOD and LOQ values derived from this experiment were less than 1 µg/mL and were sufficient for the analysis of *O. fragrans*. The calibration curve was linear over an acteoside concentration range of

TABLE 2: Extraction yields of the *Osmanthus fragrans* leaves (OFL), *O. fragrans* flowers (OFF), *O. fragrans* var. aurantiacus leaves (OFVL), and *O. fragrans* var. aurantiacus flowers (OFVF) (*n* = 1).

	OFL	OFF	OFVL	OFVF
Hot water	11.93	42.22	17.67	25.71
20% EtOH	8.41	11.00	14.89	15.53
40% EtOH	16.53	16.66	14.67	18.59
60% EtOH	15.68	17.44	14.44	17.76
80% EtOH	15.67	17.13	12.41	15.88
100% EtOH	9.53	6.12	6.99	11.23

6.25–100 µg/mL. The calibration curves exhibited good linear regression ($r^2 = 0.999$) (Table 3).

3.2.2. Precision, Accuracy, and Repeatability. The intraday and interday precision and accuracy data are shown in Table 4. The developed method was found to be precise, with the obtained RSD values being below 2.0%, and the accuracy was in the range of 98.33–103.42%. The recovery data are shown in Table 5. These results indicate that the developed method was reproducible with good precision and accuracy.

3.3. Acteoside Contents in *O. fragrans* Extracts. The acteoside contents in the *O. fragrans* extracts were determined by HPLC analysis. As shown in Figure 2, acteoside was identified as the main compound in the extract of *O. fragrans*. We will identify other bioactive substances in this plant in the future. In the present study, we used acteoside as the main target, and tried to develop an acteoside-rich extract as a drug.

The acteoside contents resulting from various extraction conditions are shown in Figure 3. The acteoside content of the *O. fragrans* extracts tended to be significantly higher in ethanolic extracts than in hot water extracts. All three types of extracts, except OFL, showed more than 10% acteoside content in the 100% EtOH extract. In particular, the content of the 100% ethanol extract of OFVF tended to be the highest (13.86%). OFF also showed a high content, of 11.91%, indicating that the maximum acteoside content can be obtained from flowers. The acteoside contents of the 100% and 80% EtOH extract of OFVL were 10.81% and 11.14%, respectively. In particular, if the leaves are superior to flowers in terms of acteoside content, further research on leaf extracts should be conducted. We found no existing reports on biologically active phytochemicals of *O. fragrans* leaves.

3.4. Antioxidant Properties of *O. fragrans* Extracts. The antioxidant activities of various extracts of *O. fragrans* were determined by measuring the DPPH-scavenging activity, reducing power, and total phenolic content. As shown in Figure 4, the extraction conditions produce higher DPPH-scavenging activity in the 40/60/80% EtOH for OFL and 60% EtOH for OFF than in the hot water.

The reducing power was tested for 100 µg/mL of each extract and was calculated as ascorbic acid equivalents (µg/mL eq. ascorbic acid). As shown in Figure 5, the extraction condition produce higher reducing power in

TABLE 3: Linearity and sensitivity of the present method.

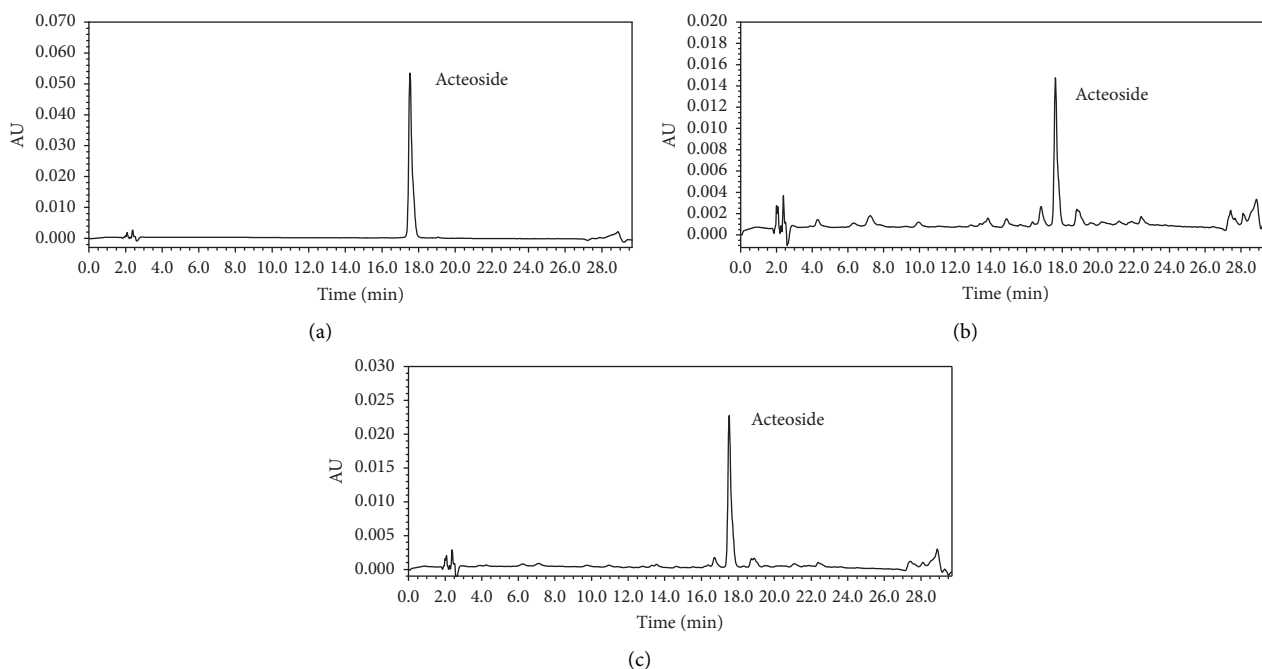
Analyte	Retention time (min)	r^2	Linear range ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)	LOD ($\mu\text{g/mL}$)
Acteoside	12.28	0.999	6.25–100	1.79	0.53

TABLE 4: Intraday and interday precision and accuracy values for the present method ($n = 3$).

Analyte	Conc ($\mu\text{g/mL}$)	Intraday		Inter-day	
		RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
Acteoside	12.5	0.30	98.33	1.12	98.10
	25	0.32	102.59	0.27	102.75
	50	0.20	103.42	0.52	103.27

TABLE 5: Recovery of the present method ($n = 6$).

Analyte	Added ($\mu\text{g/mL}$)	Recovery (%) (mean \pm SD)	RSD (%)
Acteoside	12.5	99.91 \pm 0.81	0.76
	25	100.74 \pm 1.30	1.25
	50	103.56 \pm 0.41	0.39

FIGURE 2: Typical high-performance liquid chromatography chromatograms of (a) acteoside standard ($100 \mu\text{g/mL}$), (b) *Osmanthus fragrans* leaf (0.2 mg/mL), and (c) *Osmanthus fragrans* var. *aurantiacus* (0.2 mg/mL).

the 40/60/80/100% EtOH for OFL, 60/80/100% EtOH for OFF, 60/80% EtOH for OFVL, and 60/80% EtOH for OFVF than in the hot water. In particular, the 60 and 80% EtOH extracts of OFVL showed the best reducing power among all extracts ($176.83 \pm 4.53 \mu\text{g/mL}$ eq. ascorbic acid).

The total phenolic compounds, determined using the Folin–Ciocalteu method, were expressed as gallic acid equivalents, with reference to a standard curve ($r^2 = 0.999$). The total phenolic contents of the extracts are shown in Figure 6. The extraction conditions produce higher total phenolic content in the 40/60/80/100% EtOH for OFL, 60/80/100% EtOH for OFF,

60/80/100% EtOH for OFVL, and 60/80/100% EtOH for OFVF. Particularly, the 80% EtOH extract of OFVL and the 100% EtOH extract of OFL showed the highest total phenolic content. However, in terms of productivity, the extraction yield of the 100% EtOH extract of OFL (9.53%) was lower than that of the 80% EtOH extract of OFVL (12.41%). Thus, the 80% extract of OFVL showed the best phenolic content.

In summary, the flower and leaf extracts of *O. fragrans* and *O. fragrans* var. *aurantiacus* were prepared, and their extraction yields, marker compound (acteoside) content, and antioxidant activities were investigated. Overall, almost all extracts

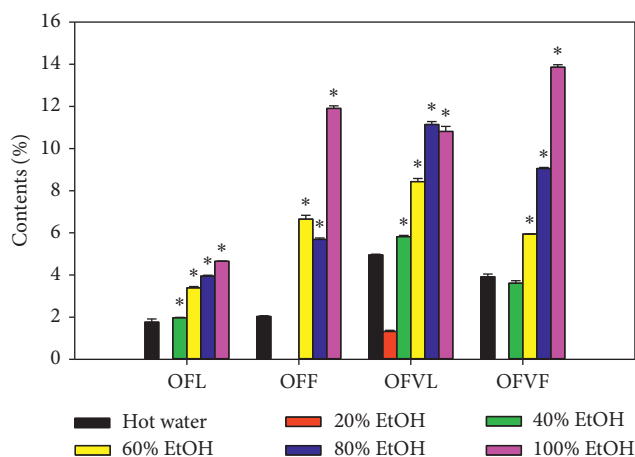


FIGURE 3: Acteoside contents in *Osmanthus fragrans* leaves (OFL), *O. fragrans* flowers (OFF), *O. fragrans* var. *aurantiacus* leaves (OFVL), and *O. fragrans* var. *aurantiacus* flowers (OFVF) ($n=3$; *significantly higher than the “hot water” extract).

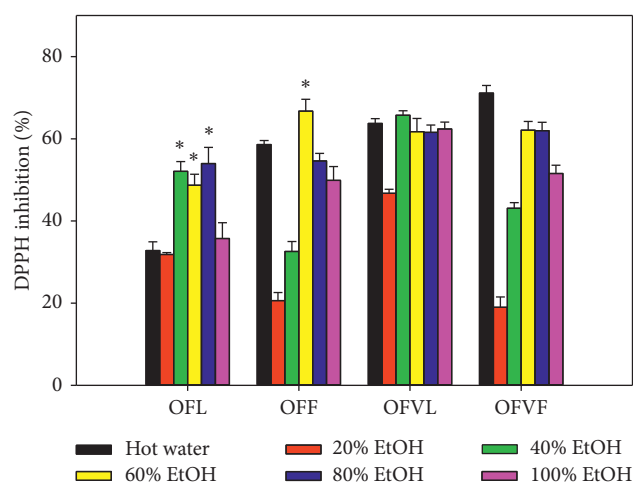


FIGURE 4: DPPH radical scavenging activity of various extracts of *Osmanthus fragrans* ($n=3$; *significantly higher than the “hot water” extract).

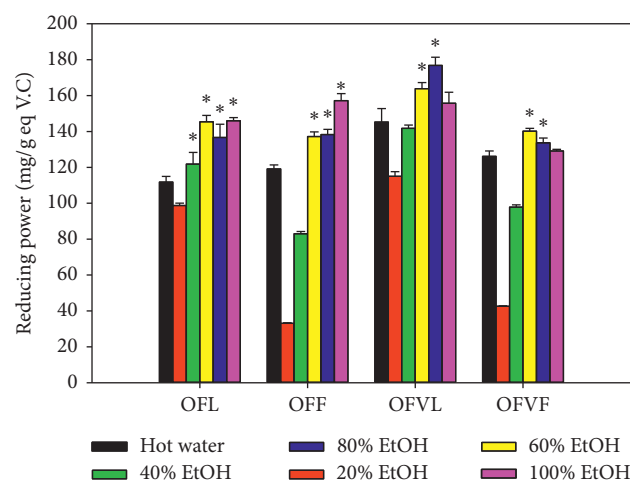


FIGURE 5: Reducing power of various extracts of *Osmanthus fragrans* ($n=3$; *significantly higher than the “hot water” extract).

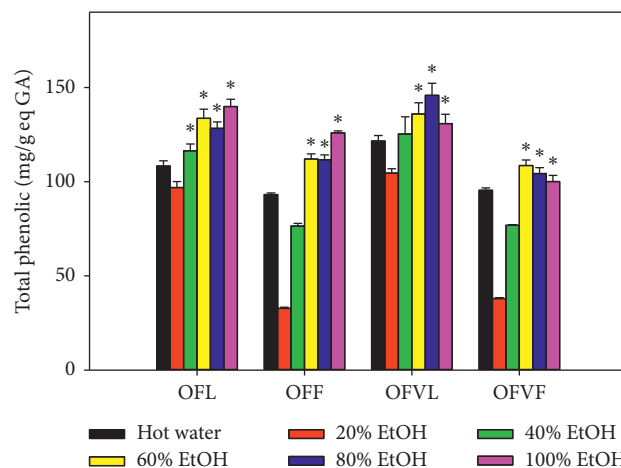


FIGURE 6: Total phenolics of various extracts of *Osmanthus fragrans* ($n=3$; *significantly higher than the “hot water” extract).

prepared using EtOH-based extraction solvents tended to produce better antioxidant activity than those prepared using hot water. These results suggest that the ethanolic extract of *O. fragrans* could serve as a potential antioxidant and anti-inflammatory pharmaceutical source, and our validated method would be useful for the quality control of *O. fragrans* extracts.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Sung-Ho Lee and Seong-Wook Seo contributed equally to this work.

Acknowledgments

This study was carried out with the support of the “R&D Program for Forest Science Technology (Project no. 2019141A00-2021-AB02)” provided by the Korea Forest Service (Korea Forestry Promotion Institute).

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