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Polyphenol Profiles and Antioxidant Properties of Ethanol Extracts from *Osmanthus Fragrans* (Thunb.) Lour. Flowers

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Key words: ethanol extracts of *Osmanthus fragrans* flowers, antioxidant, polyphenol profiles, cultured endothelial cells

This study evaluated the antioxidant activity of ethanol extracts of *Osmanthus fragrans* (Thunb.) Lour. flowers (EOF) and identified phenolic compounds in EOF using liquid chromatography-mass spectrometry. Nine compounds, 3-*O*-caffeoylquinic acid, caffeic acid 4-*O*-glucoside, salidroside, 5-*O*-coumaroylquinic acid, 4-*O*-coumaroylquinic acid, acteoside, ligustroside, fucosterol and arjunolic acid were identified. To our knowledge, caffeic acid 4-*O*-glucoside, 5-*O*-coumaroylquinic acid and 4-*O*-coumaroylquinic acid have not been detected in EOF. *In vitro* antioxidant activity analysis demonstrated that EOF possessed strong DPPH and ABTS radicals scavenging activity with EC_{50} values at 0.26±0.06 mg/mL and 0.36±0.01 mg/mL, respectively, and reducing power with $Ab_{0.5}$ value at 13.04±0.16 μg/mL. The value of oxygen radical absorbance capacity (ORAC) was 333.23±13.39 μmol Trolox/g. Antioxidant activity assay in human umbilical vein endothelial cells (HUVEC) showed that the activity of superoxide dismutase (SOD) was significantly improved and the reactive oxygen species (ROS) was removed effectively from cells when treated with EOF of 300~3000 μg/L.

Abbreviations: TEAC: Trolox equivalent antioxidant capacity; **ORAC:** oxygen radical absorbance capacity; **EOF**: ethanol extracts of *Osmanthus fragrans* flowers; **OF**: *Osmanthus fragranslour* (Thunb.) Lour; **HUVEC**: human umbilical vein endothelial cells; **SOD**: superoxide dismutase; **ROS**: reactive oxygen species; **ABTS:** 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic Acid Ammonium Salt); **DPPH:** 1-diphenyl-2-picryhydrazyl; **LDH:** lactic dehydrogenase; **BCA**: bicinchoninic acid; **TNF-**α: tumor necrosis factor-α; **TPC:** total phenolic content; **FRAP**: ferric reducing antioxidant power.

INTRODUCTION

Osmanthus fragranslour (Thunb.) Lour. (OF), native to Asia and widely cultivated in China, Taiwan, Japan, Korea and other places in the world, belongs to the Oleaceae family [Omura *et al.,* 2000]. OF flowers are valued for their delicate fruity-floral apricot aroma. They are especially valued as an additive for some cake, wine, tea and other beverages to increase or improve their taste in this country. OF has also been in use as a medicinal plant for thousands of years in China, and possesses a broad spectrum of biological effects, including anticancer, anti-inflammatory and antioxidant activities [Huang *et al.,* 2015; Lee *et al.,* 2011a]. The biological properties of OF have been considered due to phenolic compounds in various parts of the plant [Wang *et al.,* 2009].

Previous studies described several functional compounds of OF, including verbascoside [Hung *et al.,* 2012], salidroside [Ouyang *et al.,* 2015], ligustroside [Liu *et al.,* 2015], (+)-phillygenin, phillyrin, (-)-phillygenin [Lee *et al.,* 2011b], and pomolic acid [Yoo *et al.,* 2013]. Ethanol extracts of OF flower (EOF) were shown to be characterized by a high content of total phenolics (TPC) and a strong antioxidant activity [Ouyang *et al.*, 2015; Wu *et al.*, 2009; Lee *et al.*, 2007; Huang *et al.*, 2015]. The phenolic extracts of OF exhibited evident augments in ferric reducing antioxidant power assay (FRAP) and radical scavenging ability *in vitro* [Wu *et al*., 2009; Lee *et al*., 2007; Zeng *et al*., 2014]. The extracts also inhibited lipid per-oxidation initiated by ferrous chloride in rat brain, liver, heart and kidney mitochondria [Lee *et al*., 2007], and the growth of *P. gingivalis*, especially in iron-limited culture medium, and markedly upregulated the expression of the cytoprotective enzyme and nucleoprotein Nrf2 [Huang *et al*., 2015].

The present study examined phenolic profiles and antioxidant properties of EOF. Liquid chromatography diode array detection and time of flight mass spectrometer (LC-DAD--TOF-ESI-MS2) were employed to analyze phenolic profiles. The antioxidant properties of phenolic compounds from OF flowers and their effects on the removal of ROS in cell--free and cytokines-stimulated endothelial cells (EC) were further assessed.

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MATERIALS AND METHODS

Chemicals

HPD100 macro porous resin was purchased from Cangzhou Bon Adsorber Technology Ltd. (Hebei province, China). RPMI-1640 medium, fetal bovine serum (FBS), and TNF-α were purchased from Gibco/Invitrogen (Shanghai, China). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), ROS, BCA, and SOD assay kit and Radio Immunoprecipitation Assay (RIPA) lysis buffer were bought from Beyotine Technology Inc. (Nanjing, China). ABTS (2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic Acid Ammonium Salt), DPPH (1,1-diphenyl-2-picrylhydrazyl), Trolox (6-hydroxy--2,5,7,8-tetramethylchroman carboxylic acid), Folin-Ciocalteu phenol reagent, and gallic acid were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Ethanol (analytical grade) and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetic acid (HPLC grade) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Water used to prepare solutions or mobile phase was purified using Milli-Q-Integral System (Merk Millipore, MA, USA). Seed cells of HUVEC were purchased from the CELL RESEARCH Biological Technology Ltd. (Shanghai, China).

Plant materials and extraction

Dried *Osmanthus fragrans* var. thunbergii flowers was obtained from 'Fangzhi Lin' ecological garden (Nanjing, China). The dried material (100 g) was extracted with ethanol-water (1000 mL, 70:30, v/v) using an Ultrasonic sonicator (KQ5200E, Kunshan, China) at 40 kHz and 100 W for 2 h at room temperature. The extract was filtered through a 0.45 μm nylon Acrodisc 25 filter (Gelman, Ann Arbor, MI, USA).

The filtrates were concentrated with a rotary evaporator (RE-52A, Shanghai, China) and frozen to dry with a vacuum frozen dryer machine (EYELA FDU-1200, Tokyo, Japan). The dried extracts (about 10 g) were dissolved with ultra-pure water and adsorbed by HPD100 macroporous resin placed in the plexiglass column (inside diameter 15 mm, outside diameter 20 mm, height 350 mm), then purged with ultra-pure water to remove proteins and sugars and eluted with ethanol-water (1000 mL, 50:50, v/v) again. The elute (20 mL) was used to measure TPC, phenolic compositions, and antioxidant properties. The rest was concentrated with a rotary evaporator and frozen to dried again. The powder was stored at −20°C until used.

Mass spectrometry

LC-DAD-TOF-ESI-MS^2 consists of a liquid chromatograph (LC) with a diode array detector (DAD) coupled to time of flight mass spectrometer (TOF) [Agilent 1100 LC/MS (SL), USA]. A 250 mm×4.6 mm, 5 μm, Symmetry C18 column (Waters Corp., Milford, MA, USA) and sentry guard column were used at a flow rate of 0.6 mL/min. The column oven temperature was set at 35°C. The mobile phase consisted of a combination of A (water) and B (methanol), both containing 1% acetic acid. The following gradient elution scheme was expressed by B (v/v): 0–5 min, 5%-20%; 5–15 min, 20%–25%; 15–25 min, 25%-35%; 25–30 min, 35%-38%; 30–40 min, 38%-42%; 40–50 min, 42%-48%; 50–55 min, 48%-50%; 55–65 min, 50%-60%; 65–75 min, 60%-75%; 75–80 min, 75%-80%; 80–85 min, 80%-83%; 85–90 min, 83%-85%; 90–95 min, 85%–20% and 95–100 min, 20%–5%. The re-equilibration time was 10 min. The injection volume was 10 μL. The DAD was set at 280 nm for flight time monitoring of the peak intensity and full spectra (190–650 nm) were continuously recorded for plant component identification. Mass spectra were simultaneously acquired using electro spray ionization in the negative ion modes at fragmentation voltages (3.5 KV) over the range of m/z 100–1000 and MS^2 data were collected in the total ion counting model.

Antioxidant activity *in vitro*

Total phenolics content (TPC)

TPC of EOF was determined with using Folin--Ciocalteu'a phenol reagent [Spanos & Wrolstad, 1990]. Extract solution (1 mL) was mixed with 1 mL of 2 N Folin-Ciocalteu reagent for 6 min. After the addition of 3 mL of 7.5% Na_2CO_3 solution, the volume was made up to 10 mL with ethanol-water (50:50, v/v) solvents, followed by an incubation for 2 h at room temperature. Absorbance of the mixture was measured at 765 nm using UV-5500/Ultraviolet visible spectrophotometer (Shanghai, China). TPC was calculated from the calibration curve of gallic acid ($y = 0.031x - 0.007$, $r = 0.9995$) and expressed as milligram of gallic acid equivalent per gram of OF flowers (dry weight).

ABTS radical scavenging activity

The ABTS radical-scavenging activity was measured as performed by Re *et al.* [1999] with modifications. All measurements were performed as follows: 100 μL of EOF in various concentrations (0.002–1.0 mg/mL) were mixed with 3 mL of ABTS radical cation solution. Then the mixture was vibrated for 20 s, followed by standing for 6 min. The absorbance was measured at 734 nm using the same volume of ethanol-water (50:50, v/v) solvents as the blank sample. The ABTS radical cation solution was the control sample. Trolox was used as an antioxidant standard. Trolox equivalent antioxidant capacity (TEAC) was expressed as millimole Trolox equivalents per gram OF flowers (dry weight) (μmol Trolox /g).

$$\text{ABTS radical scavenging activity (\%)} = \frac{1 - (A_{\text{sample}} - A_{\text{blank}})}{A_{\text{Control}}} \times 100$$

The concentration of extract providing 50% of radical scavenging (EC_{50}) was calculated from the plot of ABTS radical scavenging activity against sample concentration by non--linear regression analysis.

DPPH radical scavenging activity

This DPPH assay was investigated by the modified method of Hatano *et al.* [1989]. In brief, 2 mL EOF of various concentrations (0.002–1.0 mg/mL) and 1 mmol/L DPPH solution (1.0 mL) were well mixed and left to stand in the dark at room temperature for 30 min. The absorbance was measured at 517 nm and the sample in ethanol was used as the blank, while DPPH radical in ethanol solution was used as a control.

Then, the DPPH radical scavenging activity was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{1-(A_{sample}-A_{blank})}{A_{Control}} \times 100$$

The concentration of extract providing 50% of radical scavenging (EC_{50}) was calculated from the plot of DPPH radical scavenging activity against sample concentration by non-linear regression analysis.

Reducing power

The reducing power of EOF was determined according to the method of Oyaizu *et al*. [1986] with minor modifications. One mL of EOF of various concentrations (0.002–1.0 mg/mL) was mixed with 2.5 mL of phosphate-buffered saline (PBS) (2.0 mol/L, pH 6.6) and 2.5 mL of potassium ferricyanide (1.0%). The mixture was incubated at 50°C for 20 min. Afterwards, 1.0 mL of trichloroacetic acid (10%) had been added to the mixture, the mixture was centrifuged at 2,500×g for 10 min. Then, 2.5 mL of the supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. Without sample addition was the control sample. The absorbance of the final mixture was measured at 700 nm using the UV-5500 spectrophotometer (Shanghai, China). The reducing power was calculated using the following formula:

$$\text{Reducing power} = A_{sample} - A_{Control}$$

The extract concentration providing 0.5 of absorbance ($Ab_{0.5}$) was calculated from the graph of absorbance at 700 nm against extract concentration by linear regression analysis.

ORAC assay

The ORAC assay was carried out as described by Mertz *et al.* [2009] with minor modifications. TriStar LB 941 microplate reader (Berthold Technologies, Germany) was used with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The final assay solution contained 100 μL of 40 μmol/L fluorescein working solution, 50 μL of phosphate buffer (blank), Trolox standard or EOF. The mixture was pre-incubated at 37°C for 10 min before 50 μL of the prepared AAPH (60 mmol/L) was added. The fluorescence of the mixture solution was recorded every minute for a total of 50 min. The ORAC values were expressed as micromoles of Trolox equivalents per gram of OF flowers (dry weight) (μmol Trolox/g).

Antioxidant activity of EOF in HUVEC

Endothelial cell culture and treatment

The 5th to 6th passage cells were used for all experiments at 80%~90% confluence. The cells were quiescenced in a reduced serum medium for 4 h prior to experiment. In a separate set of experiments, the cells were treated with EOF of 30 μg/L (test 1), 300 μg/L (test 2) and 3000 μg/L (test 3), for 18 h, followed by TNF-α (10 μg/L) stimulation for 6 h. Only RPMI-1640 was used as control 1. RPMI-1640 supplemented with TNF-α (10 μg/L) was control 2.

Determination of ROS contents in endothelial cells

After growth medium was removed, the cells were washed using PBS. Twenty-five μmol of DCFH-DA were dissolved in the treatment medium without FBS, and incubated at 37°C and 5% CO_2 for 20 min. The cells were washed with PBS three times. After all these procedures, fluorescence intensity (λ_{ex}=485 nm, λ_{em}=538 nm) was monitored using a microplate reader and detected by Ultra View VOX (PerkinElmer Company, USA) at the same time. Cells were observed at 10% of the laser intensity and photographed. The content of ROS was expressed by fluorescence intensity.

Activity of SOD in cells

First, cells were centrifuged at 2,000×g for 10 min, growth medium was discarded, washed by PBS, and then centrifuged at 2,000×g for 10 min again, supernatants were removed. Finally, 80 μL of the RIPA lysis buffer were added to cells and the mixture was centrifuged at 10,000×g for 10 min, supernatants were collected for the measurement of the activity of SOD, which was determined using BCA and SOD assay kit according to the instructions.

Statistical analysis

All data were presented in mean value ± standard deviation (SD). The data were analyzed by one-way ANOVA using the SAS 8.2 statistical software. Differences were considered significant at $p<0.05$. The fitted curve and value of EC_{50} was obtained through the Globe model (non-linear regression model) from GraphPad prism 5.0 software [Khan *et al.,* 2012; Kis *et al.,* 2009]. The equation was as follows:

$$Y = \text{Bottom} + \frac{\text{Top}-\text{Bottom}}{1+10^{(\text{LogEC }50-X)\times \text{HillSlope}}}$$

where: Top and Bottom were plateaus in the units of the Y axis. EC_{50} was the concentration of agonist that gives a response half way between Bottom and Top. Hill slope describes the steepness of the family of curves. A HillSlope of 1.0 is standard, and you should consider constraining the Hill Slope to a constant value of 1.0. A Hill slope greater than 1.0 is steeper, and a Hill slope less than 1.0 is shallower.

RESULTS AND DISCUSSION

Polyphenol profiles of EOF analysis

A chromatogram of EOF was shown in Figure 1 and the corresponding chromatographic peaks of the nine compounds were labeled as 1–9. The retention times (TR), molecular ions ($[M-H]^-$) and major fragment ions of the peaks were listed in Table 1. Proposed cleavage positions of compound salidroside, acteoside, ligustroside, fucosterol, and arjunolic acid were displayed in Figure 2.

The peak 1, eluted at 3.7 min, had a NI quasi-molecule ion at m/z of 352.8 (Table 1). The unknown compound lost inner glucose residue (162 amu) [Ma *et al.,* 2000] and yielded the Y_0^* ion at m/z 191.2. Other important fragment-ion peaks

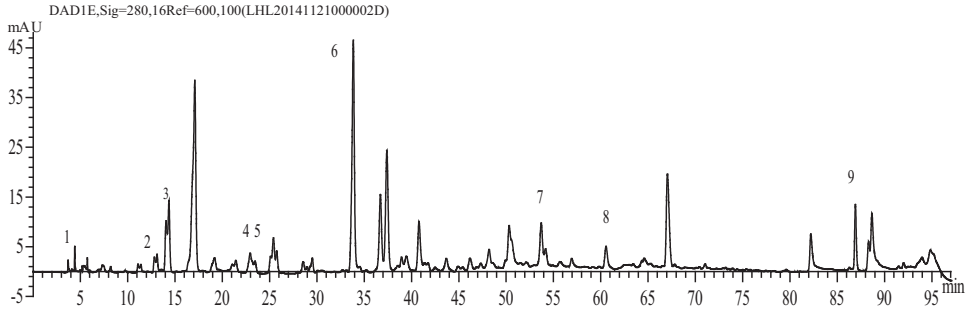

FIGURE 1. Chromatogram of ethanol extract of *Osmanthus fragrans (Thunb.) Lour.* flowers at 280 nm.

at m/z 179.1, m/z 173.1 and m/z 135.2 were consistent with that of 3-*O*-caffeoylquinic acid [Lin & Harnly, 2010]. 3-*O*--Caffeoylquinic acid was isolated and identified from the ethyl acetate fraction of the alcohol extract of the OF [Yin *et al.,* 2013].

The peak 2 was eluted at 12.8 min. It showed that the peak of the highest mass was m/z 341.0 $[M-H]^-$. The loss of a glucose moiety (162 amu) from it produced Y_0* ion at m/z 178.7, which further produced a fragment ion at m/z 160.8 by H_2O group (18 amu) dissociation. These diagnostic fragments were the same with that of caffeic acid 4-*O*-glucoside [Lin & Harnly, 2010].

The peak 3 was eluted at 14.0 min. It showed that the highest m/z value was 599.1 in the negative mode. The molecular complexes ($[2M-H]^-$) can be generated when adducts were with acid molecules [Barnes *et al.,* 1994; de Rijke *et al.,* 2003; Tian *et al.,* 2002]. The molecule ion was detected at m/z 298.9 ($[(M-H)/2]^-$). Then the molecular ion formed m/z 178.8 through a loss of a 120 amu (Figure 2). These fragments were conformed to that of salidroside and identified in the extract from pulps of EOF [Ouyang *et al.,* 2015].

The peaks 4 and 5 were eluted at 22.9 min and 23.2 min, respectively. The molecular ion $[M-H]^-$ of the two peaks (m/z value) is 337.0. The Y_0* ion at m/z 190.8 was formed as a result of a *p*-Co (146.2 amu) loss from the quasi-molecular ion of the peak 4. A *p*-Co and H_2O were lost from the quasi-molecular ion of peak 5 and yielded the Y_0* ion at m/z 172.8. These fragments matched that of 5-*O*-coumaroylquinic acid and 4-*O*--coumaroylquinic acid, respectively [Clifford *et al.,* 2003].

The peak 6 was eluted at 33.8 min, with a molecule ion at m/z 623.4. The Y_0^* fragment at m/z 461.2, a 162 amu difference between this ion and the precursor $[M-H]^-$ ion corresponds to the loss of a glucose residue (Figure 2). These fragments were consistent with that of acteoside [Lin & Harnly, 2010], which was isolated from the $CHCl_3$ sub-extract of OF [Hung *et al.,* 2012].

The peak 7 had a retention time of 53.7 min, with molecular ions at m/z 523.1 and yielded the Y_0* fragment at m/z 361.0 after loss of the inner glucose residue (162 amu). Cleavage at the glycosidic *O*-linkages with a concomitant H-rearrangement leads to the elimination of monosaccharide residues (Figure 2). And other diagnostic fragment-ion at m/z 291.0 and 259.0 were confirmed to ligustroside. The chemical compound has been identified from OF [Hung *et al.,* 2012; Liu *et al.,* 2015].

The peak 8, eluted at 60.6 min, had a molecular ion at m/z 411.1. Y_0* ion at m/z 248.9 was obtained by the second--order mass spectrum. The cleavage position was shown in Figure 2. And other important fragments such as m/z 493.0, 216.8 and 575.0, were the same with that of fucosterol. Fucosterol was isolated and identified from the ethyl acetate fraction of OF [Lee *et al.,* 2011a].

The peak 9, eluted at 86.9 min, had a NI molecular ion at m/z 487.4. The loss of a water moiety (18 amu) from

TABLE 1. Peaks assignments of the ethanol extract of *Osmanthus fragrans (Thunb.) Lour.* (EOF) flowers.

Peaks. no.	TR (min)	$[M-H]^-$ (*m/z*)	Y_0^* (*m/z*)	Diagnostic fragments (*m/z*)	Identification
1	3.7	352.8	191.2$[M-H-161.6]^-$	179.1(a)/173.1(b)/135.2(c)	3-*O*-Caffeoylquinic acid
2	12.8	341.0	178.7$[M-H-162.3]^-$	160.8(a)/134.9(b)	Caffeic acid 4-glucoside
3	14.1	599.1	298.9$[(M-H)/2]^-$	178.8(a)/118.8(b)	Salidroside
4	22.9	337.0	190.8$[M-H-P\ Co]^-$	162.8(a)/172.8(b)	5-*O*-Coumaroylquinic acid
5	23.2	337.0	172.8$[M-H-P\ Co-H_2O]^-$	190.8(a)/162.8(b)	4-*O*-Coumaroylquinic acid
6	33.8	623.4	461.2$[M-H-162.2]^-$	315.0(a)/179.1(b)/161.0(c)	Acteoside
7	53.7	523.1	361.0$[M-H-162.1]^-$	291.0(a)/259.0(b)	Ligustroside
8	60.6	411.1	248.9$[M-H-162.2]^-$	493.0(a)/216.8(b)/575.0(c)	Fucosterol
9	86.9	487.4	469.3$[M-H-H_2O]^-$	425.7(a)/443.7(b)/371.5(c)	Arjunolic acid

Note: a, b, c and d indicate the size of the ion abundance, respectively. a>b>c>d.

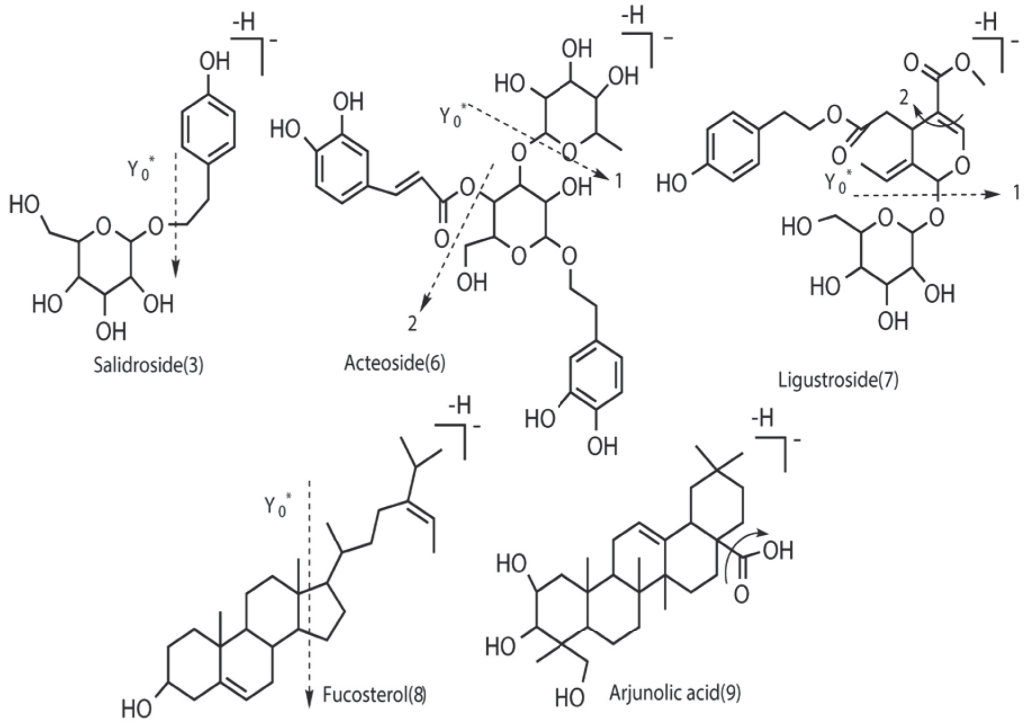

FIGURE 2. Proposed fragmentation positions of EOF compounds.

the molecular ion produced Y_0* ion at m/z 469.3, which further produced a fragment ion at m/z 425.7 by the CO_2 group (44 amu) dissociation (Figure 2). These diagnostic fragments were the same with that of arjunolic acid. Arjunolic acid was isolated and identified from the ethyl acetate fraction of alcohol extract of the OF [Yin *et al.,* 2013].

The 9 phenolic compounds were identified from OF extracts using ethanol-water (70:30, v/v) as solvents. 3-*O*--Caffeoylquinic acid, salidroside (peak 3), acteoside (peak 6), ligustroside (peak 7), fucosterol (peak 8), and arjunolic acid (peak 9) have been reported from anhydrous alcohol extracts of OF. Three compounds: caffeic acid 4-*O*-glucoside (peak 2), 5-*O*-coumaroylquinic acid (peak 4), and 4-*O*-coumaroylquinic acid (peak 5) have not been reported to be associated with EOF. So further studies on polyphenol profiles of ethanol--water extracts from OF are indicated.

Total phenolics content of OF flowers

TPC of OF flowers was 18.06 mg/g (dry weight) (Table 2). Li *et al.* [2014] obtained a similar result – 16.0 mg/g. In turn, Lee *et al.* [2007] and Zeng *et al*. [2014] reported higher contents of total phenolics in OF flowers – 193 μg/mg d.w. and 47.45 mg/g, respectively. The value determined in the present study shows that the content of total phenolic compounds in OF flowers is higher than that estimated for commonly consumed legumes and cereals [Weidner *et al*., 1999; Orak *et al*., 2016] and comparable to TPC noted for many edible flowers and fruits of herbs [Zeng *et al*., 2014; Li *et al*., 2014; Arfan *et al*., 2007].

Antioxidant properties of EOF *in vitro*

The reducing power assay is an electron transfer method based on the reduction of the Fe^{3+}/ferricyanide complex to its ferrous form in the presence of antioxidants [Oyaizu *et al*., 1986]. Reducing power against sample concentration was fitted according to the linear regression. With rising concentration, the reducing power of Trolox and EOF were also ascending. The reducing power of EOF was slightly higher than that of Trolox under the same concentration, and close to 3.0 at the concentration of 1 mg/mL (Figure 3). The $Ab_{0.5}$ of EOF and Trolox were 0.13 and 0.15 mg/mL, respectively (Table 2). Lee *et al*. [2007] obtained a lower value for the ethanolic extract of *Osmanthus fragrans* flowers – 7.74 μg/mL – using FRAP assay, in which the ferric tripyridyl triazine (Fe(III)-TPTZ) complex is reduced to the ferrous form. Whereas, as in our study, the difference between values for Trolox and EOF was slight.

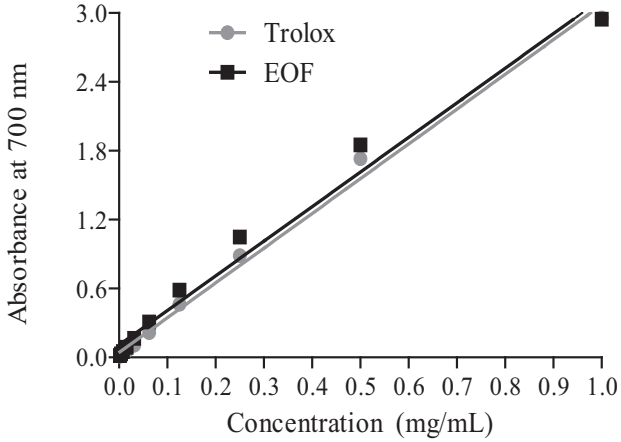

FIGURE 3. The effects of concentration of EOF and Trolox on the reducing power.

Each value is mean ± standard derivation (SD) of three replicate analyses.

TABLE 2. Antioxidant properties of EOF.

	DPPH EC_{50} (mg/mL)	ABTS EC_{50} (mg/mL)	Reducing Power Ab_{50} (mg/mL)	ORAC (μmol Trolox/g)	TEAC (μmol Trolox/g)	TPC (mg/g)
EOF	0.26 ± 0.06^{a}	0.36 ± 0.01^{a}	0.13 ± 0.02^{b}	333.23 ± 18.39	285.20 ± 2.15	18.06 ± 0.24
Trolox	0.01 ± 0.00^{b}	0.25 ± 0.01^{b}	0.15 ± 0.01^{a}	-	-	-

Note: The ABTS, DPPH and Reducing power results are expressed per mL of extract and ORAC, TEAC and TPC – per g of *Osmanthus Fragrans* (Thunb.) Lour. flowers (dry weight). Values are mean ± SD (n = 3). [a–b] Values in each group are significantly different ($p< 0.05$).

The EC_{50} of DPPH and ABTS assays of EOF was higher than the result obtained for Trolox (Table 2). The DPPH EC_{50} of the ethanolic and acetonic extracts of OF flowers was determined by Lee *et al*. [2007] and Wu *et al*. [2009], respectively. The reported values (9.99 μg/mL and 16.90 μg/mL) were lower than that in the present study. The reason partly could be the use of various extraction solvents and differences in results expression. In turn, Wu *et al*. [2009] estimated EC_{50} of OF acetonic extract using ABTS assay and expressed it as mg Trolox equivalent/g of extract (516.3 mg Trolox/g). EC_{50} of Trolox and EOF from Table 2 calculated in this way provided a comparable value.

The ORAC assay is a next method used to evaluate the antioxidant capacity of food ingredients and products. Oxygen radical absorbance capacity of OF flowers was shown in Table 2. Literature provides no data for ORAC of OF, but the obtained value – 333.23 μmol Trolox/g flowers (dry weight) – was comparable to results noted for some edible flowers used as herbs or food ingredients [Xiong *et al*., 2014].

Antioxidant activities of EOF in HUVEC

Removal of ROS

The fluorescence intensity of control 2 was significantly higher than that of control 1 ($p<0.001$), which showed that TNF-α-induced cells produced more ROS than normal cells (Figure 4). This result was consistent with the previous studies [Hwa *et al.,* 2011; Kim *et al.,* 2005]. The fluorescent intensity was not significantly different from that of control 2 when 30 μg/L of EOF was used, which suggested that the removal effect of ROS was not distinct. The fluorescence intensities were significantly lower than that of control 2 ($p<0.01$), which indicated that ROS was removed effectively at the concentrations of 300 μg/L and 3000 μg/L of EOF (Figure 4). ROS can trigger cells apoptosis, endothelial dysfunction, sclerosis, cancer, aging and inflammation *etc*. [Lenz *et al*., 2008; Calhoun *et al.,* 2008]. Thus, EOF is potentially beneficial to individuals with those diseases.

SOD activity in EC

The activity of SOD in control 2 was significantly lower than that in control 1 ($P<0.001$), indicating that the activity of SOD in cytosol was inhibited in TNF-α-treated cells. When the concentration of EOF was 30 μg/L, the activity of SOD was comparable to that of control 2, which indicated that there was no effect on the activity of SOD in the cells stimulated with TNF-α. The activity of SOD in HUVEC treated with 300 μg/L or 3000 μg/L of EOF was significantly higher than that of control 2 ($P<0.01$), which suggests that the activity of SOD was enhanced in cells exposed to high concentrations of EOF (Figure 5). The results were in accordance with Huang *et al* [2015] showing that the ethanol extract

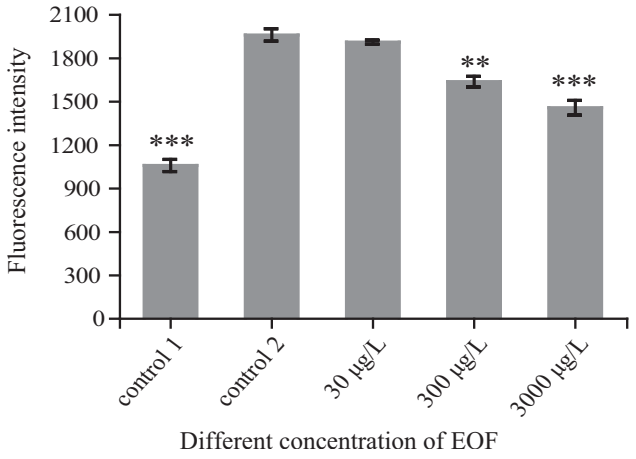

FIGURE 4. The effect of concentration of EOF on the content of ROS.

HUVEC were treated with EOF of 30 μg/L, 300 μg/L and 3000 μg/L for 18 h, followed by TNF-α (10 μg/L) stimulation for 6 h. Only treated with RPMI-1640 was control 1. RPMI-1640 and TNF-α (10 μg/L) was control 2. Each value is mean ± SD of three replicate analyses. *, **, ***: $p < 0.05$, $p < 0.01$ or $p < 0.001$ versus TNF-α alone.

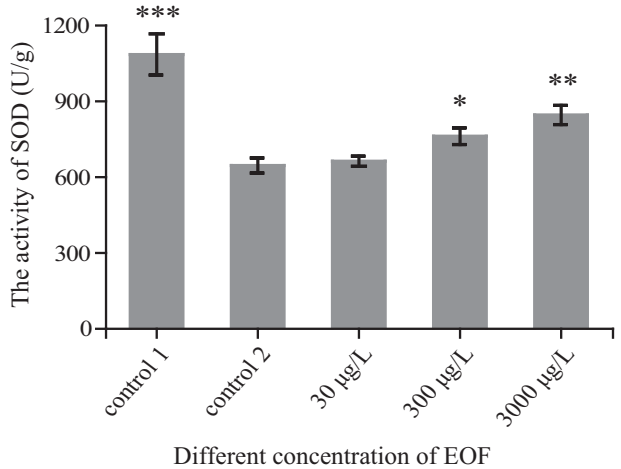

FIGURE 5. The effect of different concentration of EOF on the activity of SOD.

HUVEC were treated with EOF of 30 μg/L, 300 μg/L and 3000 μg/L for 18 h, followed by TNF-α (10 μg/L) stimulation for 6 h. Only treated with RPMI-1640 was control 1. RPMI-1640 and TNF-α (10 μg/L) was control 2. Each value is mean ± standard derivation (SD) of three replicate analyses. *, **, ***: $p < 0.05$, $p < 0.01$ or $p < 0.001$ versus TNF-α alone.

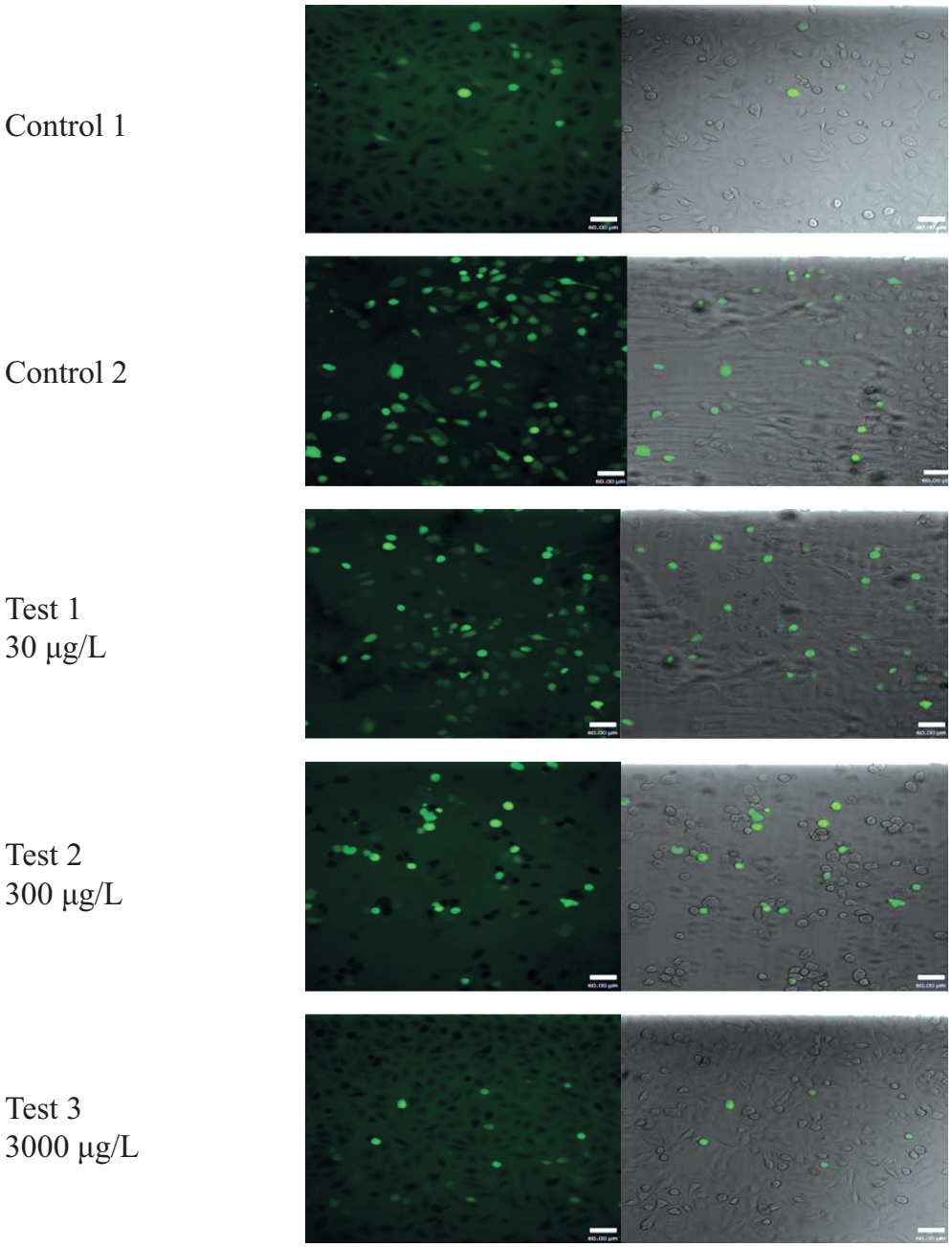

FIGURE 6. The effect of different concentration of EOF on cell viability in HUVEC.

HUVEC were treated with EOF of 30 μg/L (Test1), 300 μg/L (Test 2) and 3000 μg/L (Test 3) for 18 h, followed by TNF-α (10 μg/L) stimulation for 6 h. Each photo on the left side was illustrated in GFP detected by Ultra View VOX., and the ones on the right side were with DIC. Vehicle (RPMI-1640) only was control 1. Vehicle + TNF-α (10 μg/L) was control 2.

of OF powder increased the expression of SOD in the LPS--induced inflammatory responses.

ROS content and cellular morphology in HUVEC

The effect of different concentrations of EOF on the ROS contents and growth status of cells were determined morphologically using Ultra View VOX. Each photo on the left side was illustrated in GFP, the ones on the right side were with DIC, from which the growth state of the cells could be clearly seen. The shapes of cells in normal growth state appeared spherical. The cells with a higher ROS contents were demonstrated in green fluorescence with the excitation of moderate laser, and showed irregular or spindle shapes, which suggests the viability of the cells was affected (Figure 6).

The number of cells with irregular or spindle shape in control 2 was more than that in control 1, which indicated that TNF-α had affected the viability of cells. The number of cells on low viability was less in Test 2 and Test 3 than that in control 2, which indicated that EOF had a protective effect on TNF-α--treated cells. The growth state of cells in Test 1 and control 2 were almost the same, which showed that the EOF at low concentration may not affect the growth state of the cells. The fluorescence intensity of the cells detected under DIC model was consistent with the results shown in Figure 4.

CONCLUSION

In conclusion, 3-*O*-caffeoylquinic acid, caffeic acid 4-*O*-glucoside, salidroside, 5-*O*-coumaroylquinic acid, 4-*O*-coumaroylquinic acid, acteoside, ligustroside, fucosterol, and arjunolic acid were characterized in ethanol-water (70:30, v/v) extracts of EOF. EOF showed strong DPPH and ABTS radicals scavenging activity and reducing power. The content of ROS was significantly inhibited and the activity of SOD was increased by EOF in cytokine-stimulated EC.

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CONFLICT OF INTERESTS

Authors declare no conflict of interests.

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