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Identification of Antioxidants in Essential Oil of Radix Angelicae Sinensis Using HPLC Coupled with DAD-MS and ABTS-Based Assay

SU-YING LI, YUE YU, AND SHAO-PING LI*

Institute of Chinese Medical Sciences, University of Macau, Taipa, Macao SAR, China

Radix Angelicae sinensis (named *danggui* in Chinese), a commonly used traditional Chinese medicine, has multiple pharmacological activities. The essential oil of danggui is usually considered to be its main active fraction. However, to date, studies on the antioxidant potentials of danggui have focused on water-soluble compounds. In this paper, the antioxidant activity of the commercial essential oil of danggui was investigated by DPPH radical scavenging assay, ABTS radical scavenging assay, and β-carotene bleaching test. Antioxidant constituents in the essential oil were identified using HPLC coupled with DAD-MS and ABTS-based assay. The results showed that the essential oil of danggui has concentration-dependent antioxidant activity, which can be attributed to its component (coniferyl ferulate). This is the first report on the antioxidant activity of essential oil from danggui; its antioxidant potential was lower than those of positive controls, ascorbic acid and BHA.

KEYWORDS: Radix Angelicae sinensis; antioxidant; coniferyl ferulate; 2,2-diphenyl-1-picrylhydrazyl (DPPH); 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) diammonium salt (ABTS); β-carotene bleaching test

INTRODUCTION

Oxygen free radicals or reactive oxygen species (ROS) are well recognized to play a dual role in biological systems, because they can be either harmful or beneficial to living systems (*1*). Actually, beneficial effects of ROS involve physiological roles in cellular responses to noxia. However, the high concentration of ROS can also induce the damage of cell structures, including lipids and membranes, proteins, and nucleic acids (*2*), which lead to a number of diseases (*3*, *4*), such as cancer, atherosclerosis and cardiovascular diseases, inflammatory lung diseases, immune dysfunctions, and neurodegenerative disorders. In the search for novel antioxidants, the current focus is toward those of plant origin (*5*−*9*).

Radix Angelicae sinensis (named *danggui* in Chinese), commonly used in traditional Chinese medicines, has multiple pharmacological activities (*10*, *11*), including antitumor, antioxidation, antiradiation, cardiovascular disease prevention, and estrogenic activities. The essential oil of Radix Angelicae sinensis (*AS* oil) is usually considered to be the main fraction responsible for its pharmacological activities (*12*, *13*). However, to date, studies on antioxidant potentials of danggui have focused on water-soluble compounds (*14*−*17*).

In this study, the antioxidant activity of *AS* oil was investigated, and its antioxidant constituent was identified using HPLC coupled with DAD-MS and ABTS-based assay. Finally, the antioxidant activity of the identified component was confirmed on the basis of three assays, including DPPH radical scavenging assay, ABTS radical scavenging assay, and β-carotene bleaching test.

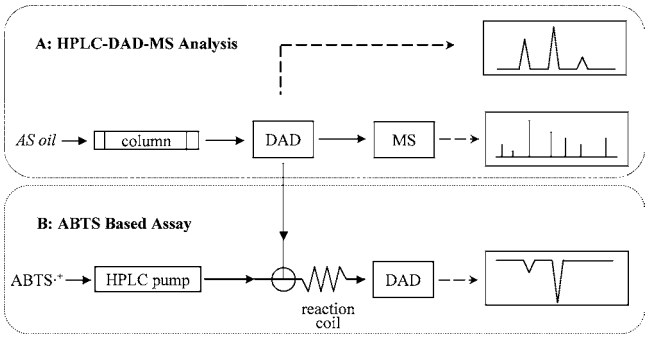

Figure 1. Diagrammatic scheme of HPLC coupled with DAD-MS and ABTS-based assay for rapid screening and identification of antioxidants in essential oil from Radix Angelicae sinensis.

MATERIALS AND METHODS

Materials. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Tween 20, L-ascorbic acid (VC), and butylated hydroxyanisole (BHA) were purchased from Sigma (St. Louis, MO). 2,2′-Azinobis(3-ethylbenzthiazolinesulfonic acid) diammonium salt (ABTS), β-carotene crystalline, and linoleic acid were purchased from International Laboratory (San Bruno, CA). Potassium persulfate was from Fluka (Seelze, Germany), and disposable cuvettes for spectrometry were from VWR International (Milano, Italy). HPLC grade acetic acid, acetonitrile, and ethanol were purchased from Merck (Darmstadt, Germany). Analytical grade chlo-

* Author to whom correspondence should be addressed (telephone +853-397 4692; fax +853-2884 1358; e-mail lishaoping@hotmail.com).

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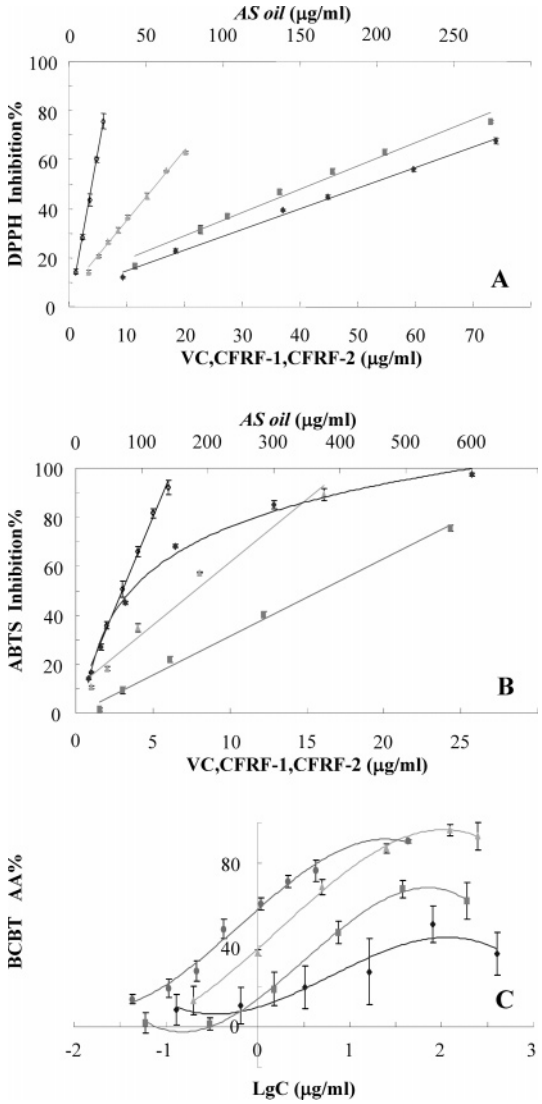

Figure 2. Antioxidation of essential oil from Radix Angelicae sinensis (*AS* oil, ◆), CFRF-1 (■), CFRF-2 (▲), VC (◇), and BHA (●) evaluated by (**A**) DPPH, (**B**) ABTS, and (**C**) βCBT antioxidant activity assays.

roform was purchased from UNI-CHEM (Belgrade, Serbia and Montenegro). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Billerica, MA).

AS oil, extracted using supercritical fluid extraction (SFE), was purchased from Guangzhou Honsen Sunshine BioScience and Technology Co. Ltd. Coniferyl ferulate and its rich fractions 1 (CFRF-1) and 2 (CFRF-2) were prepared from *AS* oil using chromatographic methods in our laboratory with purities (determined by HPLC) of >98, 67, and 92%, respectively. Coniferyl ferulate (purity > 98%), the structure of which was confirmed by its UV, MS, ^{1}H NMR, and ^{13}C NMR data compared with the literature (*18*, *19*), was used as the reference for the quantitation of coniferyl ferulate in different fractions.

Methods. *DPPH Radical Scavenging Activity Assay.* The DPPH radical scavenging activity assay was performed as in refs *20*−*22* with minor modification. In brief, a series concentration of sample in ethanol was prepared, and then 0.5 mL of the sample solution mixed with 0.5 mL of 0.15 mM DPPH radical solution freshly prepared in methanol. Ethanol and L-ascorbic acid were used as negative and positive control, respectively. After incubation for 15 min at room temperature in the dark, the absorbance of reactant in a 1-cm path length disposable plastic cuvette was measured at 515 nm on UV−vis spectrometer DU-640 (Beckman Coulter). The decreasing absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity. The measurements of DPPH radical scavenging activity were carried out

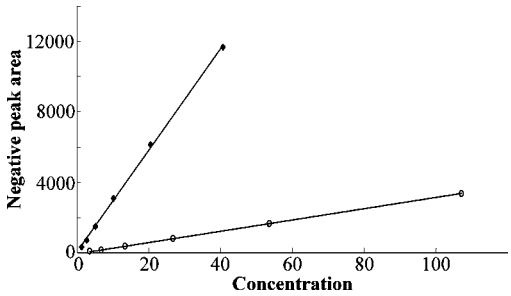

Figure 3. Linear correlations of negative peak area with the concentration of essential oil from Radix Angelicae sinensis (*AS* oil, ◆, mg/mL) and L-ascorbic acid (◇, μg/mL).

triplicate. This activity is given as a percentage of DPPH radical scavenging, which is calculated with the equation

$$\% \text{ inhibition} = [(\text{negative control absorbance} - \text{sample absorbance})/\text{negative control absorbance}] \times 100$$

ABTS Radical Scavenging Activity Assay. An improved ABTS decolorization assay was used, which has been applied for both lipophilic and hydrophilic compounds (*23*, *24*). ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS solution (7 mM in water) with 2.5 mM potassium persulfate (final concentration) for 12 h at 4 °C in the dark (stock solution). Then the $ABTS^{\bullet+}$ stock solution was diluted with ethanol to an absorbance of approximately 1.0 at 750 nm, which could exist stably for at least 2 days. The reaction was initiated by the addition of 10 μL of test sample into 990 μL of the diluted $ABTS^{\bullet+}$ solution to produce 10−80% $ABTS^{\bullet+}$ scavenging of the blank absorbance. Decrease of absorbance was measured after 5 min of incubation at room temperature in the dark. The radical scavenging activity of the tested samples, expressed as $ABTS^{\bullet+}$ scavenging percentage, was calculated according to the same formula as used in the DPPH radical scavenging activity assay. The measurements were carried out in triplicate. Ethanol and L-ascorbic acid were used as negative and positive control, respectively.

β-Carotene Bleaching Test (βCBT). The βCBT was performed according to the literature (*25*−*27*) with minor modification. In brief, 0.5 mL of β-carotene in chloroform (1.0 mg mL^{-1}) was mixed with 20 μL of linoleic acid and 0.2 mL of Tween 20 in a glass conical flask. The mixture was then evaporated to remove chloroform at 40 °C using a rotary evaporator (Büchi, Flawil, Switzerland). The residue was added to 50 mL of oxygenated Milli-Q water (oxygenated for 10 min at the oxygen pressure of 50 kPa) and mixed well to prepare an emulsion. Five milliliter aliquots of the emulsion were pipetted into different test tubes containing 0.2 mL of the sample ethanol solution. The tubes were placed at 50 °C in a water bath with gentle shaking, and the absorbance at 460 nm was taken at zero time ($t = 0$). A mixture prepared as above without β-carotene served as blank. Negative (ethanol) and positive (BHA) controls were also performed simultaneously. Measurement of absorbance was continued at an interval of 15 min until the absorbance of negative control decreased to about 0.1. The antioxidant activity (AA) of the samples was evaluated in terms of bleaching of the β-carotene using the formula

$$\text{AA\%} = 100 \times [1 - (A_0 - A_t)/(A^\circ_0 - A^\circ_t)]$$

where A_0 and A°_0 are the absorbance values measured at zero time of the incubation for test sample and negative control and A_t and A°_t are the absorbance last measured in the test sample and negative control, respectively.

HPLC Coupled with DAD-MS and ABTS-Based Assay. HPLC coupled with DAD-MS and ABTS-based assay was performed using the method developed by Koleva et al. (*28*) with modification. **Figure 1** is the schematic profile of HPLC-DAD-MS analysis (line A) and continuous flow ABTS assay (line B). HPLC-DAD-MS analysis was performed on an Agilent series 1100 (Agilent Technologies) liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler, and a diode array detection (DAD) system and an ion-

Table 1. Antioxidant Capacities of the Essential Oil from Radix Angelicae Sinensis (*AS* Oil) and Coniferyl Ferulate (CF) Rich Fractions 1 (CFRF-1) and 2 (CFRF-2) Determined by Three Assays

	positive control[a]	*AS* oil	CFRF-1	CFRF-2
relative amount of CF (%)		9	67	92
DPPH assay				
test range (μg/mL)	1.19–5.95	34.69–555.00	5.70–91.24	3.38–33.75
linear range (μg/mL)	1.19–5.95	34.69–277.50	11.40–72.99	3.38–20.25
regression equation	$Y = 12.989X - 1.926$	$Y = 0.224X + 6.383$	$Y = 0.951X + 9.720$	$Y = 2.869X + 6.363$
R^2	0.994	0.994	0.980	0.995
IC_{50} (μg/mL)	4.0	194.7	42.4	15.2
95% confidence interval of IC_{50} (μg/mL)	3.9–4.1	191.1–198.5	41.0–43.6	15.0–15.4
ABTS assay				
test range (μg/mL)	1.00–6.00	18.75–600.00	0.97–97.35	0.64–64.42
linear range (μg/mL)	1.00–6.00	18.75–150.00	1.52–24.34	1.01–16.11
regression equation	$Y = 15.139X + 4.193$	$Y = 0.400X + 10.473$	$Y = 3.164X - 0.151$	$Y = 5.138X + 10.048$
R^2	0.989	0.973	0.992	0.976
IC_{50} (μg/mL)	3.0	98.8	15.9	7.8
95% confidence interval of IC_{50} (μg/mL)	2.9–3.1	92.2–104.1	15.3–16.3	7.3–8.2
β-carotene bleaching test				
test range (μg/mL)	0.04–42.69	0.13–404.23	0.06–187.21	0.04–247.77
linear range (μg/mL)	0.21–2.13		0.30–37.44	0.20–24.78
regression equation	$Y = 41.992X + 58.726$		$Y = 32.325X + 16.452$	$Y = 36.585X + 38.612$
R^2	0.929		0.977	0.978
IC_{50} (μg/mL)	0.6		11.0	2.0
95% confidence interval of IC_{50} (μg/mL)	0.5–0.7		8.7–12.9	1.7–2.5

[a] L-Ascorbic acid is used as positive control for the DPPH and ABTS assays, whereas BHA is used for β-carotene bleaching test.

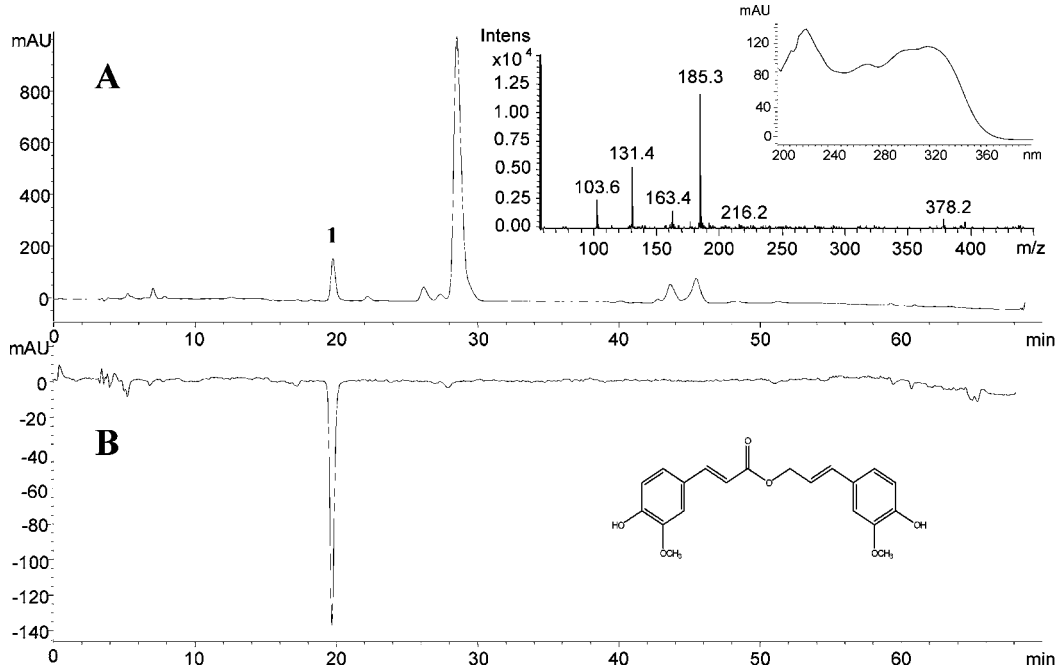

Figure 4. Chromatograms of essential oil from Radix Angelicae sinensis (*AS* oil) analyzed by HPLC coupled with (**A**) DAD-MS and (**B**) ABTS-based assay. (Insets) UV spectra, MS, and structure of coniferyl ferulate (**1**).

trap mass spectrometer with electrospray ionization interface, controlled by Agilent LC/MSD Trap software. A Zorbax ODS C18 column (250 × 4.6 mm i.d., 5 μm) with a Zorbax ODS C18 guard column (12.5 × 4.6 mm i.d., 5 μm) was used. The separation was achieved by a step gradient elution of 1% aqueous acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL/min. The column temperature was 25 °C. The elution conditions applied were as follows: 0−10 min, isocratic 30% B; 10−11 min, linear gradient 30−45% B; 11−20 min, isocratic 45% B; 20−35 min, linear gradient 45−52% B; 35−36 min, linear gradient 52−62% B; 36−50 min, isocratic 62% B; 50−60 min, linear gradient 62−100% B. The injection volume was 10 μL, and the detection wavelength was set at 270 nm. Mass spectrometry was carried out in the positive scan mode from *m*/*z* 50−1400 u. ESI-MS conditions were as follows: drying gas, N_2, 10 L/min; temperature, 350 °C; pressure of nebulizer, 40 psi; and capillary voltage, 4.0 kV.

The eluent from DAD of line A was split into two streams (flow ratio 19:1) using an adjustable flow splitter (Alltech, Deerfield, IL). The minor stream was introduced into MS. The major one went to line B, which was used for the ABTS assay, through 100 cm of 0.13 mm i.d. PEEK tubing. Line B, controlled by Agilent ChemStation software, consisted of an Agilent HPLC pump connected with a reaction coil (500 cm × 0.25 mm i.d. PEEK tubing) through a T-connector and an Agilent DAD detector. The continuous flow rate of the diluted $ABTS^{\bullet+}$ solution, prepared as mentioned under ABTS Radical Scavenging Activity Assay, was set to 0.5 mL/min, and any bleaching of the initial color was detected as a negative peak at 750 nm. L-Ascorbic

acid was used as positive control, for which HPLC-DAD-MS analysis was performed with isocratic elution of 1% aqueous acetic acid and acetonitrile (9:1) with detection at 244 nm. The other conditions were the same as described above.

Statistical Analysis. The data are presented as mean ± SD. The IC_{50} value and 95% confidence interval of each sample were calculated on the basis of linear regression of the antioxidant capacities to the concentrations, which was performed by SPSS 11.5 for Windows (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Antioxidant Activities of *AS* Oil. The antioxidant activities of *AS* oil were evaluated using three methods including two based on the free radical scavenging capacity, that is, DPPH radical scavenging assay, ABTS radical scavenging assay, and β-carotene bleaching test. The results show that *AS* oil has concentration-dependent antioxidant activity, whereas its potency is much poorer than that of L-ascorbic acid or BHA (**Table 1**; **Figure 2**). There have been many reports for the antioxidation of danggui, but the antioxidant components are focused on its aqueous soluble compounds (*14*−*17*). This is the first report on the antioxidant potential of *AS* oil, which is helpful to elucidate the pharmacological activities of danggui.

In βCBT, the mechanism of bleaching of β-carotene is a free radical mediated phenomenon resulting from the hydroperoxides of linoleic acid. β-Carotene undergoes rapid discoloration in the absence of an antioxidant. **Figure 2C** shows that *AS* oil has considerable antioxidant activity by retaining β-carotene in the medium, which suggests that *AS* oil can inhibit lipid peroxidation reactions by scavenging free radicals. In this study, the samples were tested at low concentration because of their strong hydrophobic property. Therefore, the IC_{50} of *AS* oil could not be calculated (**Table 1**).

Identification of Antioxidant in *AS* Oil Using HPLC Coupled with DAD-MS and ABTS-Based Assay. A conventional procedure for the screening and identification of antioxidants in herbal extracts is separating and purifying chemical compounds with the guidance of a bioassay, which is tedious and time-consuming work. Recently, a technique was developed to measure the radical scavenging activity of individual compounds on-line when they elute from an HPLC column (*29*, *30*). This technique makes it possible to directly identify active constituents in complex matrices. Thus, in the present study, HPLC coupled with DAD-MS and ABTS-based assay was used for the identification of antioxidant constituent(s) in *AS* oil, because the on-line DPPH system has slow reaction kinetics (*28*).

For validation of the on-line HPLC method, the calibration curve of negative peaks at different concentrations of the reference compound, L-ascorbic acid, with the same injection volume was obtained (**Figure 3**). The result shows that HPLC coupled with DAD-MS and ABTS-based assay is available for screening and identification of antioxidants. Therefore, *AS* oil was applied onto the system. As shown in **Figure 4**, compound **1** has significant free radical scavenging activity. This antioxidant compound was identified as coniferyl ferulate (**1**) on the basis of its UV spectra, MS data (**Figure 4**), and previous results (*31*, *32*), which was confirmed by comparing its retention time, UV, and MS with those of pure coniferyl ferulate.

To confirm the antioxidation of *AS* oil mainly attributed to the effect of coniferyl ferulate, two coniferyl ferulate rich fractions, CFRF-1 and CFRF-2 (67 and 92%, respectively), were prepared and their antioxidant activities evaluated using the same three methods mentioned above. The results show that the antioxidant capacity of *AS* oil is in strong accordance with the relative amount of coniferyl ferulate in the oil (**Figure 2**; **Table 1**), which suggest that coniferyl ferulate is the major antioxidant constituent.

In conclusion, coniferyl ferulate is elucidated as the main antioxidant component in *AS* oil, for which antioxidant activity is first reported. By comparison with the conventional procedure mentioned above, HPLC coupled with DAD-MS and ABTS-based assay is a powerful tool for the rapid screening and identification of antioxidants in complex matrices such as herbal extracts.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) diammonium salt; DPPH, 2,2-diphenyl-1-picrylhydrazyl; SFE, supercritical fluid extraction; *AS*, Radix Angelicae sinensis; CFRF, coniferyl ferulate rich fraction; βCBT, β-carotene bleaching test; BHA, butylated hydroxyanisole; AA, antioxidant activity; IC_{50}, concentration (μg/mL) providing 50% inhibition.

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