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Antioxidant activities and phenolic constituents of *Cephalotaxus oliveri* Mast. aerial parts

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Abstract: The antioxidant activity and the responsible chemical constituents in *Cephalotaxus oliveri* Mast. aerial parts were analyzed in the present study. The DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid)), reducing power and total phenolic content assays indicated that the methanol extract of the aerial parts was the most potent radical-scavenger and reducing agent and had the highest content of phenolics among the tested extracts. The high positive linear correlations implied that the four assays had a similar capacity to predict the antioxidant potential of the aerial parts and that the present phenolic compounds contribute significantly to the DPPH and ABTS radical scavenging activities and to the reducing power of the extracts. The plant also displayed considerable superoxide anion radical scavenging activity. LC–MS/MS and GC–MS analyses resulted in the identification of 22 compounds in the methanol extract, of which 15 were phenolic compounds. The total amount of the phenolic compounds in the methanol extract determined by the HPLC method was more than 5.62 mg g^{-1} dry weight. The considerable antioxidant potential and a high content of phenolic antioxidants suggest that *C. oliveri* aerial parts are a potential source of natural antioxidants.

Keywords: radical scavenging activity; reducing power; total phenolic content; LC–MS/MS; HPLC; GC–MS.

INTRODUCTION

Cephalotaxus is the single genus of the coniferous Cephalotaxaceae.[1] *Cephalotaxus* plants have been used in traditional Chinese medicine for the treatment of human malignant tumors, rheumatism, dyspepsia, abdominal distension and the like.[2] Among the *Cephalotaxus* species, *Cephalotaxus oliveri* Mast. is endemic to the subtropical forests of China, which have a scattered distribution.[1] Hitherto, a total of eight compounds, including three cephalotaxine-type alka-

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loids, three bisflavones and two homoerythrina alkaloids, have been demonstrated to be present in *C. oliveri*.[3–5] The plant is rich in two anticancer cephalotaxine-type alkaloids, *i.e.*, harringtonine and homoharringtonine.[3] Harringtonine and homoharringtonine are clinical drugs in use in China for the treatment of acute non-lymphocytic leukemia and chronic myelogenous leukemia.[3,5] Moreover, homoharringtonine is in clinic trials in the USA for the treatment of chronic myelogenous leukemia and other cancers.[6]

ROS (reactive oxygen species) include both oxygen radicals, such as superoxide, hydroxyl, peroxyl and hydroperoxyl radicals, and some non-radical oxidizing agents, such as hydrogen peroxide, hypochlorous acid and ozone.[7] At normal physiological levels, ROS function as "redox messengers" in intracellular signaling and regulation, whereas excessive ROS damage cellular macromolecules, such as lipids, proteins, and nucleic acids, through inducing oxidative modification and promote cell death.[7] As results, they cause inflammation or lesions on various organs and are associated with diseases such as atherosclerosis, arthritis and cancers.[8] Together with endogenous defense systems, regular supplements of exogenous antioxidants limit or prevent the dangerous effects caused by excessive ROS.[9] Due to the severe side effects of synthetic antioxidants, such as butylated hydroxyanisole (BHA), natural antioxidants from medicinal plants, vegetables, and fruits have received increasing attention and are considered as better alternatives to synthetic antioxidants.

The aerial parts (*i.e.*, branches with leaves) of *C. oliveri* are normally discarded as waste during pruning. To maximize the exploitation of the limited plant resources, it is necessary to evaluate the bioactive potential and analyze the chemical profile of the waste aerial parts. To the best of our knowledge, no data on the antioxidant activities and responsible components of *C. oliveri* aerial parts are hitherto available. Thus, in this study, the antioxidant activities of *C. oliveri* aerial parts were evaluated. The chemical constituents responsible for the activities were also analyzed.

EXPERIMENTAL

Plant materials and chemicals

The aerial parts of *C. oliveri* were collected from the South China Botanical Garden, Guangzhou, China, on January 25, 2008, and identified by Mr. Qiang Wei, a senior engineer from the Garden.

BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid)), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), Folin–Ciocalteu reagent, gallic acid, trichloroacetic acid, ferric chloride, potassium persulfate, potassium ferricyanide, NADH (β-nicotinamide adenine dinucleotide), NBT (nitroblue tetrazolium), PMS (phenazin methosulfate), protocatechuic acid, gentisic acid, catechin, vanillic acid, epicatechin, caffeic acid, syringic acid, epicatechin gallate, *p*-coumaric acid, taxifolin, apigenin, and emodin were pur-

chased from Sigma–Aldrich (St. Louis, MO). All other reagents were of analytical or HPLC grade.

Sample preparation

The collected plant materials were washed, air-dried at room temperature (*ca*. 23 °C), then ground to a powder and passed through a 20-mesh sieve. The sieved powder was extracted exhaustively with water, methanol, acetone, ethyl acetate, chloroform and hexane (3:50, w/v), respectively, at 220 rpm in a shaker at room temperature and filtered through Whatman No. 1 filter paper (Advantec, Tokyo). All obtained extracts were lyophilized and kept in the dark at –20 °C until use.

The method described by Neo *et al.*[10] with some modification was used to treat the plant material for the chemical constituent analysis by the LC–MS/MS and HPLC methods. In brief, 20 ml of supernatant after centrifugation following the methanol extraction was mixed well with 5 ml water and then evaporated at 50 °C under vacuum to a smaller volume to remove the methanol. The residual aqueous phase was centrifuged to remove the insoluble components. The supernatant was extracted three times with hexane to remove the possibly present free fatty acids and other lipid contaminants. After the removal of hexane at 50 °C under vacuum, the free phenolics (soluble free phenolics, SFP) in the aqueous phase were extracted exhaustively with diethyl ether–ethyl acetate (1:1, v/v) with a solvent to aqueous phase ratio of 1:1 (v/v). The diethyl ether–ethyl acetate extracts were pooled and treated with anhydrous sodium sulfate to remove moisture, filtered, evaporated to dryness under vacuum and finally re-dissolved in 2.25 ml methanol. The precipitate pellet was added into 12 ml water and 5 ml 10 M NaOH and then shaken at 220 rpm overnight. The hydrolyzed solution was adjusted to pH 2. The released phenolics (esterified phenolics, EFP) were extracted exhaustively with diethyl ether–ethyl acetate (1:1, v/v). The diethyl ether–ethyl acetate extracts were combined, evaporated to dryness, and dissolved into 0.75 ml of methanol. The treatment procedure of the methanol extract for chemical analyses by LC–MS/MS and HPLC is schematically presented in Fig. 1.

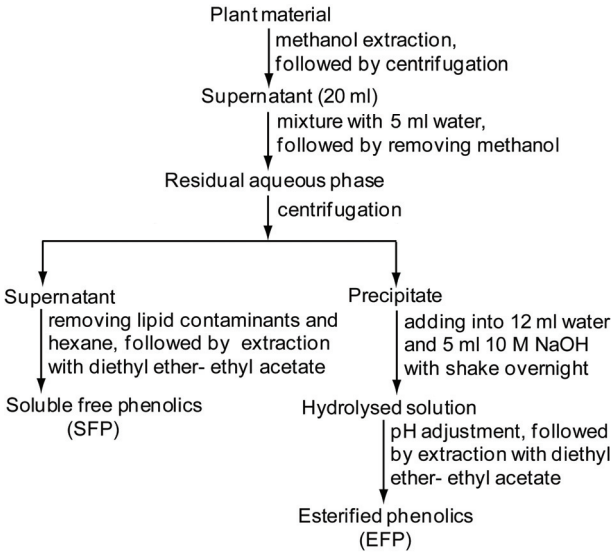

Fig. 1. Treatment scheme of the methanol extract of *C. oliveri* aerial parts for chemical component analyses by LC–MS/MS and HPLC.

DPPH radical scavenging activity

The DPPH radical scavenging effect of the *C. oliveri* extracts was assessed according to the method described by Zeng *et al*.[11] with slight modification. In brief, 0.1 ml of the extract solutions or the positive controls BHA and BHT were mixed with 2 ml freshly prepared DPPH solution in ethanol and then incubated for 30 min at room temperature. The DPPH radical scavenging activities were assayed spectrophotometrically in a microplate reader (PowerWave XS, BioTek Instruments, Inc.) at 517 nm. The DPPH radical scavenging activity is reported in μmol of Trolox equivalents per gram extract (μmol TE g^{-1} dry matter) and as the IC_{50} value (defined as the concentration in mg ml^{-1} that caused a 50 % inhibition of the DPPH radical).

ABTS radical scavenging activity

The ABTS radical scavenging activity was assayed using the method described by Zeng *et al*.[11] with some modifications. Briefly, ABTS radicals were generated in a mixture of 7 mM ABTS and 2.45 mM potassium persulfate in water at room temperature in the dark during 12––16 h. An aliquot of 0.10 ml extract solution or the reference compounds BHA and BHT was introduced into 2.6 ml of diluted ABTS radical solution with absorbance of 0.8 at 734 nm and mixed thoroughly. The reactive mixture was allowed to stand at room temperature for 6 min and absorbance was recorded immediately afterwards at 734 nm in a microplate reader (PowerWave XS). ABTS radical scavenging activity of the extracts is expressed in term of TEAC (Trolox equivalent antioxidant capacity, μmol TE g^{-1} dry extract). The scavenging activity is also reported as the IC_{50} value (defined as the concentration in μg ml^{-1} that caused a 50 % inhibition of the ABTS radical).

Reducing power

The reducing power of the extracts was determined by the method of Zeng *et al*.[11]. Extract solution or the positive controls BHA and BHT (80 μl), sodium phosphate buffer (200 μl, 0.2 M, pH 6.6) and potassium ferricyanide (200 μl, 10 mg ml^{-1}) were mixed and incubated at 50 °C for 20 min. The mixture was then placed into a refrigerator at 4 °C to rapidly lower its temperature. Then, trichloroacetic acid (200 μl, 100 mg ml^{-1}) was added and incubated for 5 min to stop the reaction. Afterwards, the resultant mixture (680 μl) was mixed with 680 μl distilled water and 68 μl ferric chloride (10 mg ml^{-1}). Finally, the absorbance of the resulting mixture was measured in a microplate reader (PowerWave XS). A higher absorbance indicates a stronger reducing power.

Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity of the extracts of *C. oliveri* was measured by the method of Zhang *et al*.[12] with a slight modification. Briefly, 3 ml of 16 mM Tris–HCl buffer (pH 8.0), containing 1 ml of NBT (150 μM) solution, 1 ml of NADH (468 μM) solution and 1 ml of extract solution or the positive control gallic acid at 0.10 mg ml^{-1} were mixed at room temperature and the mixture was measured as the blank. Then, 1 ml of PMS solution (60 μM) was added. After incubation at room temperature for 5 min, the absorbance of the resulting mixture was measured at 560 nm. Superoxide radical scavenging activity was calculated as scavenging activity (%) = (absorbance of control – absorbance of sample)/(absorbance of control – absorbance of blank)×100.

Total phenolic content

Total phenolic content of *C. oliveri* extracts was estimated by the Folin–Ciocalteu colorimetric assay according to the method described by Zeng *et al*.[11] Briefly, 1.0 ml of extract

solution was mixed with 5 ml of 0.20 M Folin–Ciocalteu reagent. After incubation at room temperature for 6 min, 4 ml of sodium carbonate (75 g l^{-1}) was added. Absorbance of the resulting blue-colored solution was measured in a microplate reader (PowerWave XS) at 760 nm after incubation at room temperature for 2 h with intermittent shaking. The amount of total phenolic content was calculated as gallic acid equivalents (GAE, mg g^{-1} dry extract).

Phenolic constituents in methanol extract analyzed by LC–MS/MS

LC–MS/MS under the negative ion multiple reaction monitoring (MRM) mode was used for the phenolic analysis of the methanol extract using an Agilent 1200 series LC (Agilent, Santa Clara, USA) instrument fitted with a binary pump, an on-line degasser and an Applied Biosystem API 2000 mass spectrometer (Life Technologies Corporation, California, USA) equipped with an ESI (electrospray ionization) interface. The whole LC–MS/MS system was controlled by Analyst software (Life Technologies Corporation, California, USA). The HPLC separation was performed on a reverse-phase Zorbax C18 column (250 mm×i.d. 4.6 mm, 5 μm, Agilent, Santa Clara, CA).

The employed mobile phases A and B were acidified water (0.1 % acetic acid, v/v) and methanol, respectively. For the analysis of the SFP fraction, the gradient elution was programmed as follows: from 5 to 35 % B in 10 min; from 35 to 45 % B in 25 min; from 45 to 60 % B in 10 min; from 60 to 70 % B in 10 min and from 70 to 100 % B in 5 min and kept at 100 % B for 10 min. To analyze the EFP fraction, the gradient elution was programmed as follows: from 5 to 10 % B in 2 min and kept at 10 % B for 3 min; from 10 to 40 % B in 10 min; from 40 to 50 % B in 5 min; from 50 to 90 % B in 25 min and from 90 to 100 % B in 10 min and kept at 100 % B for 10 min. The flow rate was 1 ml min^{-1} and the injection volume was 10 μL. A T-type phase separator was used to split the effluent from the HPLC column into the mass spectrometer. The conditions for the MS analysis were the same as those used by Zeng *et al.*[11]. The collision energy for each compound was optimized. Nitrogen was used as the nebulizing and collision gas in the MS analysis.

The phenolic compounds in the methanol extract of *C. oliveri* aerial parts were characterized and identified by MS/MS by comparison with the MS data and the LC retention times of authentic standards.

Phenolic content determination by HPLC analysis

The content of phenolic compounds in the methanol extract was determined by the HPLC method using a Waters HPLC system with a 600 pump, a 600 controller, a 2487 dual--wavelength absorbance detector, an in-line degasser and Empower software (Waters Corporation, Massachusetts, USA). The LC conditions for the separation of the component on a reverse-phase Zorbax C18 column (250 mm×i.d. 4.6 mm, 5 μm, Agilent, Santa Clara, CA) were the same as for the LC part in the LC–MS/MS analysis. The quantification was performed using the external standard method with the wavelength set at 280 nm. The amount of phenolic compounds is expressed as μg g^{-1} dry extract.

GC–MS determination of the chemical constituents in the methanol extract

The chemical constituents in the methanol extract of *C. oliveri* aerial parts were also analyzed by the GC–MS method, which was realized using an Agilent 7890A GC system (Agilent, Palo Alto) together with an Agilent 5975C mass spectrometer operating in the EI mode and equipped with an HP-5MS capillary column (30 m×0.25 mm×0.25 μm).

The electron energy was set at 70 eV with a mass range at *m*/*z* 25–800. The GC injector temperature and the MS source temperature were set at 280 and 230 °C, respectively. Helium was used as the carrier gas at a flow rate of 1 ml min^{-1}. The oven temperature was pro-

grammed from 60 to 120 °C at a rate of 3 °C min^{-1}, held isothermal for 5 min, raised to 155 °C at 5 °C min^{-1}, held isothermal for 10 min; raised to 170 °C at 3 °C min^{-1}, held for 5 min; raised to 200 °C at 3 °C min^{-1}, held for 10 min; raised to 250 °C at 5 °C min^{-1}, held for 10 min; to 270 °C at 10 °C min^{-1} and then held for 15 min. An aliquot of 1.0 µl methanol extract dissolved in acetone was injected automatically in the splitless mode.

The components were identified based on a comparison of their gas chromatographic retention indices and mass spectra to those from NIST05 MS library and from the literature. Retention indices were calculated for each separate component against *n*-alkane standards (C8–C40, Sigma–Aldrich, Switzerland) on the HP-5MS column.

Statistical analysis

All data are presented as means ± *SD*. Differences at $p < 0.05$ were considered statistically significant. All the statistical analyses were performed with Origin 6.0 (Microcal Software, Inc., Northampton) or Excel 2003 (Microsoft Corporation, Redmond, WA).

RESULTS

DPPH radical scavenging activity

The DPPH radical scavenging effect of *C. oliveri* extracts compared with BHA and BHT is presented in Fig. 2. Water extract (WE) and methanol extract (ME) exhibited a similarly strong ability to quench the DPPH radical ($p > 0.05$) with IC_{50} values of 0.96 and 0.82 mg ml^{-1}, respectively. These values are comparable to the IC_{50} value of BHT (0.55 mg ml^{-1}). In contrast, the hexane extract (HE) showed the weakest scavenging effect ($p < 0.01$), with only about 10 % activity at 10 mg ml^{-1}. The DPPH radical scavenging activity expressed as Trolox equivalents of the extracts were 316.1±1.0, 331.7±4.8, 250.0±2.6, 60.3±1.8, 45.3±2.1 and 12.8±1.8 µmol TE g^{-1} for WE, ME, acetone extract (AE), ethyl acetate extract (EE), chloroform extract (CE) and HE, respectively. The TEAC values of WE and ME were more than 24 times that of HE. The results showed the order of DPPH radical scavenging activities of the extracts as ME > WE > AE > EE > CE > HE ($p < 0.01$).

ABTS radical scavenging activity

The ABTS radical scavenging activity of the extracts and BHT and BHA are shown in Fig. 3. For CE and HE, when the assay concentrations were > 2.5 mg ml^{-1}, the absorbance of the extract solutions *per se* at 734 nm apparently affected the results. They had only very weak ABTS radical scavenging activity with inhibition of only 27.3±0.2 and 10.4±0.6 %, respectively, at 2.5 mg ml^{-1}. The respective IC_{50} values of WE, ME, AE and EE were 0.76, 0.49, 0.82 and 3.36 mg ml^{-1}, while the respective values of BHA and BHT were 0.054 and 0.080 mg ml^{-1}. These result suggested that the extracts had only weak or moderate ABTS radical scavenging activity. In addition, the TEAC values of the extracts ranged from 22.5±1.7 to 621.6±1.7 µmol TE g^{-1} dry weight. ME had the strongest ABTS scavenging activity with a TEAC value 27 times higher than that of HE. The results

indicated that the order of ABTS radical scavenging activity of the extracts was ME > WE > AE > EE > CE > HE ($p < 0.01$).

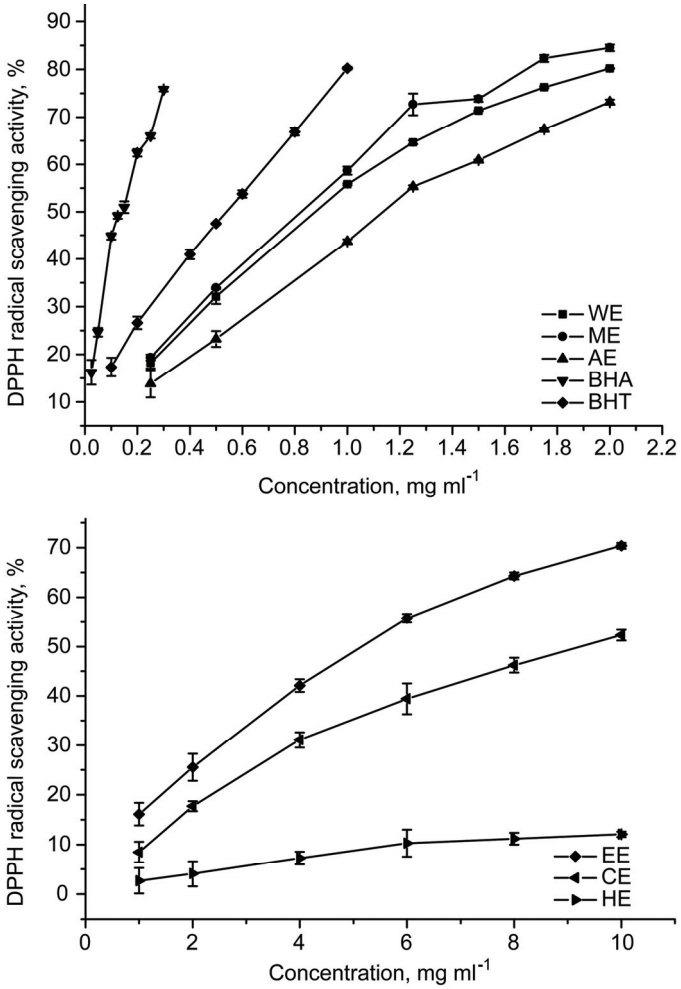

Fig. 2. DPPH radical scavenging activities of different extracts of *C. oliveri* aerial parts and positive controls. WE, water extract; ME, methanol extract; AE, acetone extract; EE, ethyl acetate extract; CE, chloroform extract; HE, hexane extract. The values are the mean of three determinations ± *SD* (standard deviation).

Reducing power

The reducing powers of the extracts of *C. oliveri* aerial parts are indicated in Fig. 4. Two-way ANOVA analysis suggested that the order of reducing power of the extracts ME > WE > AE > EE > CE > HE, which is consistent with the results in the DPPH and ABTS assays. At the concentrations of 0.8 mg ml^{-1} for

ME and 1.5 mg ml^{-1} for WE, their reducing power was equal to that of BHT. However, CE and HE had very weak reducing power.

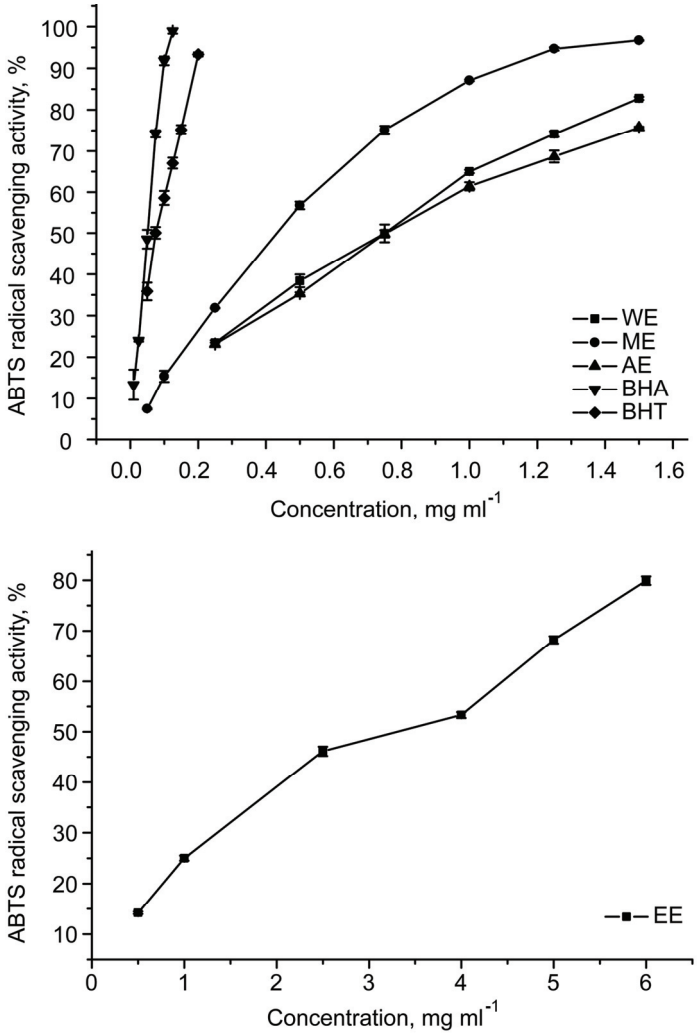

Fig. 3. ABTS radical scavenging activities of different extracts of *Cephalotaxus oliveri* aerial parts and positive controls. WE, water extract; ME, methanol extract; AE, acetone extract; EE, ethyl acetate extract. The values are the mean of three determinations ± *SD* (standard deviation).

Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activities of the extracts at 0.1 mg ml^{-1} are shown in Fig. 5. Two-sample *t*-tests suggested that the descending order of the scavenging activity of the extracts is EE (49.5±1.2 %) > CE (44.9±1.3 %) >

HE (41.6±0.6 %) > ME (33.2±0.6 %) ≥ AE (32.6±0.8 %) > WE (29.4±1.0 %), while the activity of gallic acid was 57.5±0.3 %. This means that EE had the strongest superoxide anion scavenging activity, which was comparable to that of gallic acid.

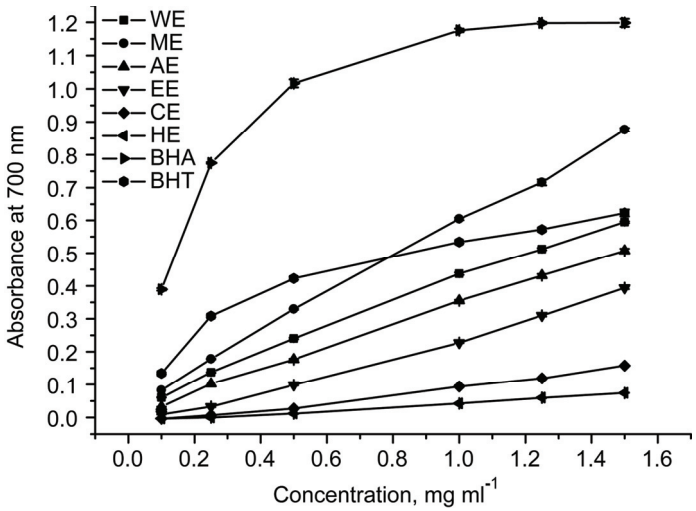

Fig. 4. Reducing power of different extracts of *C. oliveri* aerial parts and positive controls. WE, water extract; ME, methanol extract; AE, acetone extract; EE, ethyl acetate extract; CE, chloroform extract; HE, hexane extract. The values are the mean of three determinations ± *SD* (standard deviation).

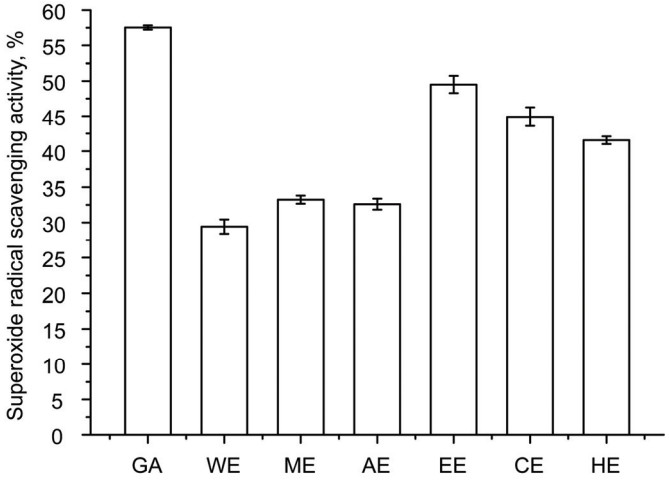

Fig. 5. Superoxide radical scavenging activities of different extracts of *C. oliveri* aerial parts and positive control at 0.1 mg ml^{-1}. GA, gallic acid; WE, water extract; ME, methanol extract; AE, acetone extract; EE, ethyl acetate extract; CE, chloroform extract; HE, hexane extract. The values are the mean of three determinations ± *SD* (standard deviation).

Total phenolics content

The total phenolic content of the six extracts are presented in Table I, which varied extensively ($p < 0.01$) from 7.2±0.2 to 96.1±1.8 mg GAE g^{-1} dry weight. ME had the highest total phenolic content at 96.1±1.8 mg GAE g^{-1} dry weight, followed by WE, AE, EE, CE and HE in descending order. The total phenolic content of ME was about 13 and four times larger than those of HE and CE, respectively.

TABLE I. Total phenolics content (GAE, mg g^{-1}) in the extracts of *C. oliveri* aerial parts (means of three replicates ± *SD* (standard deviation) as mg g^{-1} dried extract)

WE[a]	ME[b]	AE[c]	EE[d]	CE[e]	HE[f]
78.23±1.48	96.07±1.85	68.98±2.66	47.96±0.44	23.65±0.43	7.21±0.23

[a]Water extract; [c]methanol extract; [c]acetone extract; [d]ethyl acetate extract; [e]chloroform extract; [f]hexane extract

Phenolic components in the methanol extract determined by LC–MS/MS and HPLC

In total, 12 phenolic compounds were identified in the methanol extract of *C. oliveri* aerial parts by LC–MS/MS analysis and their MS data and content are given in Table II. All the 12 compounds were detected in the SFP fraction but only three in the EFP fraction. Apigenin, *p*-coumaric acid, and emodin were present in both the SFP and EFP fractions. The content of most of the individual

TABLE II. Phenolic compounds in the methanol extract of *C. oliveri* aerial parts identified by ESI-MS/MS and their content determined by HPLC

No.	Compound	M *m/z* $[M–H]^-$	MS/MS *m/z*	Retention time min[a]		Content[b], μg g^{-1}		
				SFP	EFP	SFP	EFP	Total[c]
1	Protocatechuic acid	153	109, 108, 91	10.01	–[d]	54.0±0.2	–	54.01
2	Gentisic acid	153	109	10.98	–	602.1±6.8	–	602
3	Catechin	289	221, 245, 203	11.62	–	256.0±3.6	–	256
4	Vanillic acid	167	108, 123, 152	14.19	–	184.6±2.2	–	185
5	Epicatechin	289	221, 245, 109	14.27	–	2205.8±9.9	–	2206
6	Caffeic acid	179	135	14.40	–	87.8±1.7	–	87.8
7	Syringic acid	197	182, 167, 123	14.83	–	Trace	–	Trace
8	Epicatechin gallate	441	169	16.43	–	ND[e]	–	ND[e]
9	*p*-Coumaric acid	163	119	18.54	20.52	129.9±5.3	110.9±3.5	240
10	Taxifolin	303	125, 285	19.07	–	2349±3.3	–	235
11	Apigenin	269	117, 149, 151	49.48	32.33	817.5±5.3	677.4±3.5	1495
12	Emodin	269	225, 241	63.18	45.73	14.9±0.6	247.0±2.1	262
Total[f]								5620

[a]Retention time in LC–MS/MS analysis; [b]means of three replicates ± *SD* as μg g^{-1} dry methanol extract; [c]sum of content of phenolic compounds in both SFP and EFP fractions; [d]absent in the fraction; [e]Content not determined; [f]sum of content of all identified phenolic compounds

phenolic compounds was larger than 100 μg g^{-1} dry methanol extract, especially epicatechin and apigenin, the contents of which were larger than 1.0 mg g^{-1} dried methanol extract. The total amount of the phenolic compounds identified was more than 56.2 mg g^{-1} dried methanol extract.

Chemical components detected by GC–MS analysis

Based on the comparison of the retention indices and mass spectra to those from NIST05 MS library and the literature, ten compounds as shown in Table III were identified in the methanol extract of *C. oliveri* aerial parts by GC–MS analysis. The identified compounds are characterized by the presence of three phenolic compounds, *i.e.*, ferruginol, 2,5-bis(1,1-dimethylethyl)phenol and α-tocopherol. In addition, an important phytosterol, *i.e.*, β-sitosterol, was also detected in the methanol extract.

TABLE III. Chemical components in the methanol extract of *C. oliveri* aerial parts analyzed by GC–MS

No.	*Rt*[a] / min	Compound	*RI*[b]
1	13.305	1-(4-Methylphenyl)ethanone	1188
2	28.580	2,4-Bis(1,1-dimethylethyl)phenol	1516
3	28.931	Dihydroactinidiolide	1526
4	43.855	6,10,14-Trimethyl-2-pentadecanone	1846
5	48.507	Hexadecanoic acid, methyl ester	1922
6	51.432	Hexadecanoic acid	1969
7	69.720	Ferruginol	2330
8	70.482	4,8,12,16-Tetramethylheptadecan-4-olide	2342
9	95.559	α-Tocopherol	3119
10	100.709	β-Sitosterol	3291

[a]Retention time of elution from HP-5MS column; [b]Retention index on an HP-5MS column in reference to C8–C40 *n*-alkanes

DISCUSSION

Antioxidant potential

Foods or extracts of medicinal plants are mixtures with multi-functions. It is easily understood that multiple reaction characteristics and mechanisms, and different phase localizations are usually involved, hence any single method will be not accurate and comprehensive enough to reflect the antioxidant profile of a mixed or complex system.[13] Therefore, more than two different methods are typically used. In the present study, DPPH, ABTS, superoxide anion scavenging, reducing power and the Folin–Ciocalteu colorimetric assays were used to test the antioxidant potential of extracts from *C. oliveri* aerial parts. They are chemical methods associated with electron donation or hydrogen donation and can be spectrophotometrically assessed.[14]

DPPH and ABTS radicals are two artificial free radicals that are widely used to test the antioxidant potential of plant extracts or food materials. The antioxidant effect is determined by measuring the reduction of the radicals in the test systems. The most widely and commonly used method recently to evaluate the DPPH and ABTS radicals scavenging activities is to monitor the decrease of UV absorption at 517 and 734 nm, respectively, with a UV spectrophotometer.[14] The results obtained in this study implied that the DPPH and ABTS scavenging activities of all the extracts were dose-dependent, as is the case with BHA and BHT. Generally, except water, the solvents with higher polarity showed better extractability of the antioxidants scavenging DPPH and ABTS radicals from *C. oliveri* aerial parts. For each solvent extract, the TEAC value in the ABTS test was overtly higher than that in the DPPH test. One of the reasons for this may be the color interference by some components, such as anthocyanins (λ_{max} 475–485 nm), with DPPH chromogen,[15] which resulted in lower measured TEAC values.

The reducing power was measured by direct electron donation to reduce the Fe^{3+}/ferricyanide complex to ferrous products, while electron-donating activity is an important mechanism of phenolic antioxidant action. Ferrous ions can be monitored spectrophotometrically at 700 nm. Increased absorbance at 700 nm indicates increased reducing power. The reducing power of all the extracts of *C. oliveri* aerial parts was dose-dependent. The reducing powers of WE and ME were strong and comparable to that of BHT.

Superoxide anion radical contributes to tissue damage and various diseases. In this study, superoxide anion radical was generated by the non-enzymatic PMS–NADH–NBT system. EE had the strongest superoxide anion radical scavenging activity with *ca*. 50 % inhibition at a concentration of 0.1 mg ml^{-1}.

Phenolic compounds are ubiquitous secondary metabolites in plants. It has been well demonstrated that the antioxidant activity of plant materials is well correlated with the content of phenolic compounds they produce. In the present study, except HE, all the five other extracts had a high total phenolic content (GAE > 20 mg g^{-1} dry extract).[16] The results implied that *C. oliveri* aerial parts contain a high level of phenolic compounds and that ME had the highest antioxidant potential, whereas HE had the lowest potential. This result suggested that high polarity organic solvents extract the phenolic compounds better from *C. oliveri* aerial parts than those of lower polarity.

Correlations of the assays determining the antioxidant potential of the extracts

Highly positive linear correlations between the total phenolic content and DPPH radical scavenging activity (R = 0.9466), ABTS radical scavenging capacity (R = 0.9914) and reducing power (R = 0.9870) suggest that the phenolic compounds are significantly responsible for the antioxidant ability, the scavenging of DPPH and ABTS radicals and the reducing power of the extracts, which is

consistent with previous results showing the antioxidant activity of *C. sinensis*.[17] Highly positive linear correlations were also present between the reducing power assay and the DPPH (R = 0.9609) and ABTS assays (R = 0.9977), which implies that the constituents present in the extracts scavenging DPPH and ABTS radicals are also able to reduce ferric ions and that the reducing ability seems contributive to the free radical-scavenging capacity of the extracts. In addition, the highly positive linear relationship between DPPH and ABTS radicals scavenging activity (R = 0.9883) shows that the two methods have similar predictive capacities for the antioxidant activity of *C. oliveri* extracts. However, such relationships did not exist between superoxide radical scavenging activity and total phenolic content and other antioxidant activities of the extracts. Such different behaviors may be due to differences in the main active components and mechanisms of the action of scavenging different radicals and reaction with the test probes.[18]

Chemical components

For the first time, the chemical profile of *C. oliveri* using the LC–MS/MS and GC–MS methods is reported. Phenolic compounds are the characteristic components in the chemical profile. The LC–MS/MS analysis resulted in the identification of 12 phenolic compounds in the methanol extract of *C. oliveri* aerial parts, including four hydroxybenzoic acids (*i.e.*, protocatechuic acid, gentisic acid, vanillic acid and syringic acid), two hydroxycinnamic acids (*i.e.*, caffeic acid and *p*-coumaric acid), three flavan-3-ols (*i.e.*, catechin, epicatechin and epicatechin gallate), two flavanones (*i.e.*, taxifolin and apigenin) and one phenolic anthraquinone (*i.e.*, emodin). Except *p*-coumaric acid, apigenin and emodin, all the other identified phenolic compounds were detected only in the SFP fraction, which means that they were present in the soluble free form in the methanol extract. Apigenin and *p*-coumaric acid were present in both the soluble free form and bound form, while emodin had a much higher content in the EFP fraction than in the SFP fraction, which means that most of emodin in the methanol extract of *C. oliveri* was present in the bound form. The GC–MS analysis suggested the presence of ten organic compounds in the methanol extract of *C. oliveri* aerial parts, of which three were phenolic compounds. As a result, a total of 15 phenolic compounds were identified in the methanol extract by both the LC–MS/MS and GC–MS methods.

Due to the reactivity of phenol moiety (hydroxyl substituent on the aromatic ring), most phenolic compounds can behave as antioxidants. The presence of at least 15 phenolic compounds implies *C. oliveri* should have antioxidant potential. Phenolic acids are ubiquitous in plant food (*e.g.*, fruits, vegetables and coffee)[19] and are the main phenols consumed by humans. The phenolic acids identified in this research are all prominent naturally occurring ones and their role as dietary antioxidants has received increasing attention.[20] They possess various potent an-

tioxidant activities.[20,21] The other phenolic compounds also have potent antioxidant activities. For example, of the tocopherols and tocotrienols in the vitamin E group found in food, only α-tocopherol meets human vitamin E requirements. α-Tocopherol is the most powerful soluble lipid natural antioxidant known.[22] It scavenges peroxyl radicals extremely rapidly, which maintains the integrity of long-chain polyunsaturated fatty acids in the membranes of cells and thus maintains their bioactivity.[22,23] Moreover, it possibly plays a role in the prevention of cancer and atherosclerosis.[22] In addition, β-sitosterol is one of the most abundant phytosterols, which has demonstrated, to some extent, DPPH and hydrogen peroxide scavenging activity and strong nitric oxide radical-scavenging activity. It also inhibits the autoxidation of methyl linoleate and polyunsaturated fatty acid oxygenation.[24] The presence of the components with antioxidant potency confirms and contributes to the antioxidant activities of the *C. oliveri* extracts.

CONCLUSIONS

Some natural antioxidants have remarkable pharmaceutical and therapeutic potentials. They are in the central area in the development of functional foods and can be used as food additives to prevent the lipid oxidation of foods. The considerable antioxidant activities and the presence of important phenolic antioxidants imply that the aerial parts of *C. oliveri*, usually cut off as waste, could be a source of natural antioxidants.

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ИЗВОД

АНТИОКСИДАТИВНА АКТИВНОСТ И САДРЖАЈ ФЕНОЛА У НАДЗЕМНИМ ДЕЛОВИМА БИЉКЕ *Cephalotaxus oliveri* MAST.

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У овој студији је анализирана антиоксидативна активност и хемијски састав надземних делова биљке *Cephalotaxus oliveri* Mast. Тестови са DPPH (2,2-дифенил-1-пикрилхидразил) и ABTS (2,2'-азинобис(3-етил-бензотиазолин-6-сулфонска киселина)), као и тестови за одређивање редукујућег потенцијала и садржаја фенола су показали да метанолни екстракт надземних делова биљке има највећи потенцијал за хватање радикала и редукцију, због велике концентрације фенола. Велика позитивна корелација између четири теста указује на сличне могућности тестова у процени антиоксидативног потенцијала. Биљка је, такође, показала да има значајан капацитет хватања супероксид анјонског радикала. Методама LC–MS/MS и GC–MS идентификована су 22 једињења у метанолном екстракту, од којих су 15 фенолна једињења. Укупни садржај фенола у метанолном екстракту је одређиван методом HPLC и чинио је више од 56,2 mg g^{-1} суве масе. Значајан антиоксидативни потенцијал и висок садржај

фенолних антиоксиданаса указују да би надземни делови *C. oliveri* могли бити коришћени као извор природних антиоксиданаса.

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