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DPPH–HPLC–DAD analysis combined HSCCC for screening and identification of radical scavengers in *Cynomorium Songaricum* Rupr.

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A combinative method using 2,2-diphenyl-1-picrylhydrazyl (DPPH)–high performance liquid chromatography (HPLC)–diode array detector (DAD) and high-speed counter-current chromatography (HSCCC) has been developed to screen and separate radical scavengers from water extract of *Cynomorium songaricum* Rupr. Under the target-guidance of DPPH–HPLC–DAD experiment, 3 compounds were found to possess potential antioxidant activities. In order to identify the chemical structures of those compounds, the HSCCC method with a two-phase solvent system composed of ethyl acetate–water (1:1; v/v) was developed to isolate and purify the active compounds. Three compounds, 14.3 ± 0.7 mg of (+)-catechin (1), 15.2 ± 0.6 mg of (-)-epicatechin (2) and 11.3 ± 0.4 mg oleuropein (3) were obtained from 600 mg of the crude sample with purities of 97.8%, 98.3% and 95.6%, respectively, as determined by HPLC. Their structures were elucidated by electrospray ionization-mass spectrometry (ESI-MS), ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR. Among them, oleuropein was obtained from *C. songaricum* for the first time. The peak order of the three compounds in HSCCC chromatogram was completely different with that in HPLC chromatogram. Even both HSCCC and HPLC method that employed in this paper were under reversed phase mode. The elution order of HSCCC was determined by the hydrophobicity of the compounds, while the elution order of HPLC may be affected by some other interactions between the stationary phase and the compounds such as hydrogen bond and steric effect. In addition, antioxidant activities of the three compounds were evaluated by the methods of DPPH radical scavenging assay. All of them showed high radical scavenging activities with the EC_{50} values being 16.97 ± 0.02, 11.15 ± 0.09 and 41.32 ± 0.20 μg mL^{-1} for (+)-catechin, (-)-epicatechin and oleuropein, respectively.

1 Introduction

Cynomorium songaricum Rupr. is an obligate root parasitic plant that mainly grows in Northwestern China. The stem of *C. songaricum* is a popular tonic in traditional Chinese medicine. This herb is also frequently added to teas and wines. It is also consumed by the local people as vegetable or food. *C. songaricum* has been widely used for the treatment of digestive disorders, dyspepsia, "kidney" deficiency, lumbago and diarrhea[1]. This herb has other potential activities such as anti-hypoxic and antianoxic actions[2]. Gang Zhao et al. discovered that *C. songaricum* extracts can functionally modulate transporters of γ-aminobutyric acid and monoamine[3]. Dae Young Yoo et al. found that *C. songaricum* extracts can enhances novel object recognition, cell proliferation and neuroblast differentiation in the mice via improving hippocampal environment[4]. An ursolic acid-enriched extract of *C. songaricum* was reported having the ability to protect against carbon tetrachloride hepatotoxicity and gentamicin nephrotoxicity in rats[5]. Those bioactivities may be attributed partly to the antioxidants contained therein.

The human body has a complex system of natural enzymatic and non-enzymatic antioxidant defenses which counteract the harmful effects of free radicals and other oxidants[6]. Free radicals not only cause premature aging and wrinkles, but also are the primary causes of diseases including cancer[7], cardiovascular disease[8] and other chronic diseases[9]. Recently, increasing efforts have been made in the search for natural radical scavengers from *C. songaricum*. Yi Lu et al. discovered the good protective effect of the ethyl acetate extracts of *C. songaricum* on staurosporine-induced apoptotic cell death in SK-N-SH neuroblastoma cells[10]. The extracts also showed a good scavenging activity without inhibition of xanthine oxidase. Flavan-3-ol oligomers from the stems of *C. songaricum* were found to show potent antioxidative activity[11]. Flavan-3-ol derivatives, including 3 cysteine conjugates and 3 acetylcysteine conjugates, were prepared using *C. songaricum* and edible reagents. All of the compounds showed strong radical-scavenging activity[12]. Of all the study mentioned above, the solvent for extracting contained a portion of organic solvent, such

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as ethyl acetate, acetone and so on. It is well known that traditional Chinese medicine was mostly decocted by water for taking, so it is important to study the antioxidants in water extract of *C. songaricum.*

In a previous study, the antioxidant peak in the juice or residue extract were characterised by HPLC coupled with a diode array detector (DAD) through spiking with free radicals. To identify their chemical structures, the antioxidant peaks were further investigated using HPLC–ESI–MS–MS[13]. While the instruments of HPLC–ESI–MS–MS are expensive so as to difficultly popularize. High-speed counter-current chromatography (HSCCC) is a type of liquid chromatography that works without a solid stationary phase, and it allows large-scale purification [14]. It has many advantages such as low risk of sample denaturation, total sample recovery, large load capacity, and low cost[15,16], which has been widely used in preparative separation of active compounds from traditional Chinese herbs and other natural products[17]. In this paper, a method of DPPH–HPLC–DAD analysis combined HSCCC for screening and identification of radical scavengers in *C. songaricum* was established. The major radical scavengers were screened using HPLC coupled with DAD and then separated by HSCCC.

2 Experimental

2.1 Apparatus

Chromatographic analysis was performed on an Agilent 1260 Series LC system with a G1311C quaternary pump, a G1328C manual injector, a G1315D DAD detector and Agilent Chemstation software (Agilent, USA). Separations were carried out on a ZORBAX SB-Aq analytical column (250 mm × 4.6 mm i.d., 5 μm, Agilent, USA).

The HSCCC instrument employed in the present study was a TBE-300B high-speed counter-current chromatograph (Tauto Biotechnique, Shanghai, China) with three multilayer coil separation columns connected in series (i.d. of the tubing = 1.5 mm, total volume = 295 mL) and a 20 mL sample loop. The revolution radius was 5 cm, and the β values (β = r/R, where r is the rotation radius or the distance from the coil to the holder shaft, and R is the revolution radius or the distances between the holder axis and central axis of the centrifuge) of the multilayer coil varied from 0.5 at internal terminal to 0.8 at the external terminal. The revolution speed of the apparatus could be regulated with a speed controller in the range between 0 and 1000 rpm. The HSCCC system used in the present study was equipped with a DC-0506 constant-temperature circulating implement, a TBP5002 constant flow pump (Tauto Biotechnique, Shanghai, China) and a Sanotac UV detector (Sanotac Scientific Instruments Co., Ltd, Shanghai, China). The data were collected with an EasyChrom-1000 chromatography workstation (Hanbon Science & Technology Co., Ltd, Jiangsu, China).

Absorbance measurements were recorded on a T6 new century spectrophotometer (Puxi Tongyong instruments Co., Ltd, Beijing, China) during the whole experiment.

2.2 Chemicals and reagents

The stem of *C. songaricum* was purchased from Alashan Left Banner (Inner Mongolia, China). A voucher specimen was identified by Professor huanyang Qi at Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences. Ethanol of industrial grade was procured from sugar factory of Huangyang town (Gansu, China). Acetonitrile of HPLC grade were obtained from Jiangsu Hanbang science and technology Co., Ltd (Jiangsu, China). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich Co., Ltd (St. Louis, USA). Ethyl acetate, *n*-butanol, *n*-hexane and methanol of analytical grade were from Tianjin Fuyu Fine Chemicals Co., Ltd (Tianjin, China). Ultrapure water was obtained with a Spring-R10 water purification system (Research Scientific Instrument Co., Ltd, Xiamen, China).

2.3 Preparation of the crude sample

The stems of *C. songaricum* (1 kg) were crushed and extracted three times with 10 L of water for 3 h per extraction. The extracts were filtered, combined and concentrated under reduced pressure to fluidextract. The fluidextract was separated with macroporous adsorption resins. Dynamic adsorption/desorption experiments were performed in a glass column (2.25 cm × 22.7 cm) packed with GS-6 resin with a bed volume (BV) of 30 mL. The adsorption process was performed by loading the fluidextract onto the glass column above. The adsorbate-laden column was firstly washed with distilled water and then desorbed with ethanol–water (70:30, v/v) solution. The eluent was concentrated under reduced pressure and dried under vacuum to yield 60 g residue.

2.4 DPPH–HPLC–DAD analysis for the radical scavengers.

Stock solutions of the crude sample was prepared in methanol with a concentration of 5.0 mg mL^{-1} and kept in fridge at 4 °C for use. The blank sample was prepared by adding 0.3 mL of the stock solutions to 2.4 mL of methanol, while the spiking sample was prepared by adding 0.3 mL of the stock solutions to 2.4 mL of DPPH solution (3.0 mg mL^{-1}). Both the blank sample and the spiking sample were shaken and allowed to stand for 30 min in the dark at room temperature. Chromatographic analysis of the blank sample and the spiking sample was carried out on an Agilent 1260 liquid chromatograph system. The mobile phase consisted of water (A) and acetonitrile (B) with the elution profile as follows: 0–7 min, 5–12% B (linear gradient, v/v); 7–17 min, 12% B (equilibration, v/v); 17–35 min, 12–30% B (linear gradient, v/v); 35–40 min, 30% B (equilibration, v/v). The flow rate was 1.0 mL min^{-1}. The injection volume was 20 μL. The detection wavelength was 210 nm.

2.5 Isolation of radical scavengers by HSCCC

2.5.1 Measurement of partition coefficient

The two-phase solvent system of HSCCC was mainly selected according to the partition coefficients (K) of target components. Measurement of K value was as follows. 10 mg of crude sample was added to a test tube, to which 2 mL of each phase of two-phase solvent system were added. The test tube was shaken vigorously.

After the equilibration was established, both the upper phase and lower phase were analyzed by HPLC. The *K* value was defined as the peak area of component in the upper phase (A_U) divided by that of in the lower phase (A_L) ($K = A_U/A_L$).

2.5.2 Preparation of two-phase solvent system and sample solution

A two-phase solvent system comprising ethyl acetate–water at a volume ratio of 1:1 was used for the HSCCC separation. The selected solvent system was thoroughly equilibrated in a separation funnel at room temperature. Then, the upper phase and the lower phase were separated and degassed by ultrasonication for 20 min before use.

The sample solution for the HSCCC separation was prepared by dissolving 600 mg of the crude sample in 20 mL of the lower phase. The solution was filtrated to remove any solid impurities. Then, it was injected into the HSCCC.

2.5.3 HSCCC operation

In HSCCC procedure, the multi-layer coil column was first completely filled with the upper phase of the solvent system as stationary phase. Then, the apparatus was rotated at 900 rpm. Meanwhile, the lower phase was pumped into the coil column at a flow rate of 3.0 mL min^{-1}. After reaching hydrodynamic equilibrium, as indicated by the mobile phase front emerging at the tail outlet, the prepared sample solution was injected into the column through the inject valve. The temperature of the apparatus was set at 25 °C and the effluent from the column was monitored at 280 nm. After the separation, the retention of stationary phase (S_f) is expressed as a percentage of a volume of the stationary phase (*V*s) relative to the total column capacity (*V*c), that is $S_f = [Vs/Vc] \times 100\%$.

2.6 Compound identification by NMR spectroscopy and ESI-MS

The ^1H NMR and ^{13}C NMR spectral of the isolated compounds were recorded on a Varian Inova-400 FT-NMR spectrometer (USA) in methanol-d_4 using TMS as an internal reference. The chemical shifts (δ) are reported in ppm and the coupling constants (*J*) are reported in Hertz (Hz). The mass spectrometer employed was a micrOTOF-Q II quadrupole time-of-flight mass spectrometer (Bruker Daltonics Inc., USA). The structures of compounds were elucidated by comparison of their NMR spectral with the literature values and were confirmed by ESI-MS data.

2.7 DPPH radical scavenging activity

The radical scavenging activity was performed by the DPPH assay[18] to evaluate the antioxidant activity of the purified (+)-catechin, (-)-epicatechin and oleuropein from the stem of *C. songaricum.* DPPH radical in methanol (40 μg mL^{-1}) was prepared and this solution (5.0 mL) was added to antioxidant sample solutions in methanol (5.0 mL) at different concentrations. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm. Rutin was used as the positive control. All

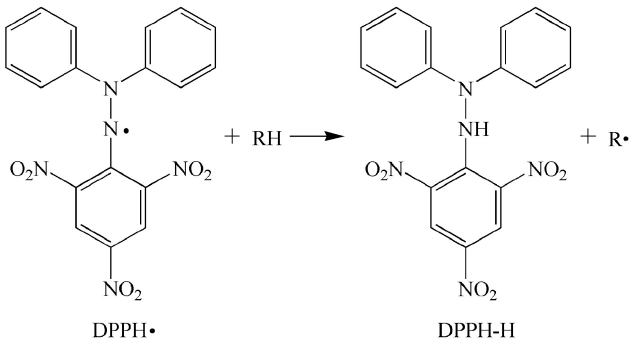

Fig. 1 Reaction of the radical scavenger with DPPH•

determinations were performed in triplicate. The DPPH radical scavenging activity was calculated using the following formula:

DPPH radical scavenging activity (%) = $(A_0\text{-}A_1)/A_0 \times 100\%$

where A_0 is the absorbance of the control (DPPH solution without test sample) and A_1 is the absorbance in the presence of the test sample.

3 Results and discussion

3.1 Screening radical scavengers with DPPH–HPLC–DAD

Several methods have been developed to evaluate the antioxidant activities of pure compounds and plant extracts. Among free radical scavenging methods, DPPH method is rapid, simple and inexpensive in comparison to other test models[6]. The DPPH reagent is a stable organic nitrogen radical which has a deep purple colour with maximum absorption at 517 nm[19]. When a solution of DPPH is mixed with a substrate (RH) that can donate a hydrogen atom, DPPH will turn into the reduced form with the loss of the violet colour. Meanwhile, the compound-derived free radicals have different spectral characteristics or they may further react with DPPH• and thus have different retention characteristics. On this basis, after the reaction happened, the peak areas of the radical scavengers would significantly decrease in the HPLC chromatogram[20]. The general reaction of DPPH and radical scavenger is shown in Fig. 1.

The blank sample and the spiking sample were analyzed using HPLC–DAD method. The chromatograms are shown in Fig. 2. It was observed that many peaks disappeared or significantly diminished after spiking with DPPH. Among those peaks, peaks A, B and C had large areas in the chromatogram of the blank sample. Therefore, those compounds were the major potential antioxidants in the water extract of *C. songaricum*.

3.2 HSCCC separation

Appropriate solvent system plays an important role in separation by HSCCC. To achieve a successful separation using HSCCC, the suitable solvent system should provide an ideal range of partition coefficient (*K*, 0.5–2) for the target compounds and a proper separation factor ($\alpha \geq 1.5$) between adjacent peaks. Six different solvent systems

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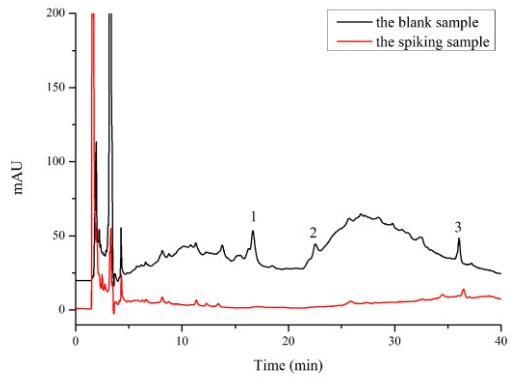

Fig. 2 HPLC–DAD chromatograms of the blank sample and the spiking sample. Column: ZORBAX SB-Aq (250 × 4.6 mm i.d., 5 μm); mobile phase, (A) water and (B) acetonitrile in gradient mode as follows: 0-7 min, 5-12% B;7-17 min, 12% B; 17-35 min, 12-30% B; 35-40 min, 30% B; detection wavelength: 210 nm; flow rate: 1.0 mL min^{-1}.

Table 1 The *K* values of the three compounds in different solvent systems

No	Solvent system (volume ratio)	K_1	K_2	K_3
1	*n*-butanol–ethyl acetate–water (2:3:5)	0.86	1.46	2.46
2	*n*-butanol–ethyl acetate–water (1:4:5)	0.72	1.40	1.50
3	ethyl acetate–water (1:1)	0.50	0.74	1.17
4	*n*-hexane–ethyl acetate–methanol–water (1:19:1:19)	0.36	0.56	0.67
5	*n*-hexane–ethyl acetate–methanol–water (1:9:1:9)	0.21	0.29	0.35
6	*n*-hexane–ethyl acetate–methanol–water (1:5:1:5)	0.20	0.24	0.31

were examined to obtain an appropriate range of *K* value for the compound 1-3. It could be seen from Table 1 that *K* value of ethyl acetate–water (1:1) system was more acceptable than that of others. Therefore was selected for next HSCCC separation.

In HSCCC process, the high flow rate of the mobile phase will shorten the separation time, while the low flow rate phase may improve the resolution. The flow rates of 2.0 and 3.0 mL min^{-1} were tested in this study, respectively. When the flow rate was 2.0 mL min^{-1}, the retention of the stationary phase was 62.7% and the separation time was 300 min. When the flow rate was 3.0 mL min^{-1}, the retention of the stationary phase was 54.2% and the separation time was 200 min. However, the resolution of the compounds under the condition of the high flow rate did not decreased obviously compared to the one of the low rate. Therefore, 3.0 mL min^{-1} was selected as the optimal flow rate. The separation of HSCCC under the optimized conditions was shown in Fig. 3. Based on the HPLC analysis, three compounds were obtained in one-step separation and yielded 11.3 ± 0.4 mg oleuropein (peak A in Fig. 3), 15.2 ± 0.6 mg epicatechin (peak B in Fig. 3) and 14.3 ± 0.7 mg catechin (peak C in Fig. 3). The purities were 95.6%, 98.3% and 97.8%, respectively (Fig. 4). The molecular structures are shown in Fig. 5. Among them, oleuropein was obtained from *C. songaricum* for the first time.

The traditional silica gel column chromatography plays an important role in separating compounds from natural products. However, there are still some drawbacks of the method such as long experiment process and irreversible adsorption. Some less stable compounds will degrade or denature during the separation process. So they can hardly be obtained by silica gel column chromatography. In comparison, HSCCC is a more rapid separation technology. Besides, there is no irreversible adsorption in HSCCC because both the stationary phase and mobile phase are liquid. Therefore, HSCCC can be a complementary to silica gel column chromatography in discovering compounds in natural products.

The separation modes of HPLC and HSCCC method employed in this paper were both reversed phase mode. It would be reasonable

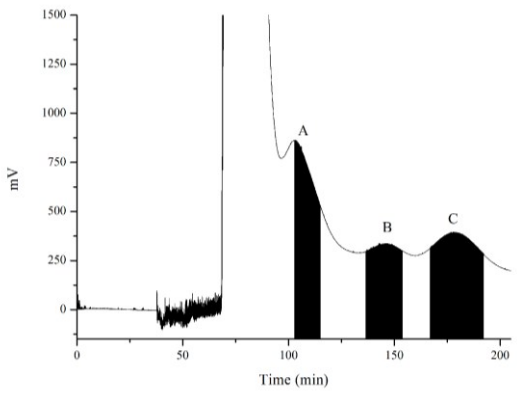

Fig. 3 HSCCC chromatogram of the extract of *C. songaricum.* The shadow shows how the fractions were collected. Solvent system: ethyl acetate–water (1:1, v/v); sample size: 600 mg; stationary phase: upper phase; mobile phase: lower phase; revolution speed: 900 rpm; detection wavelength: 280 nm; flow rate: 3.0 mL min^{-1}; separation temperature: 25 °C, retention of stationary phase: 54.2%.

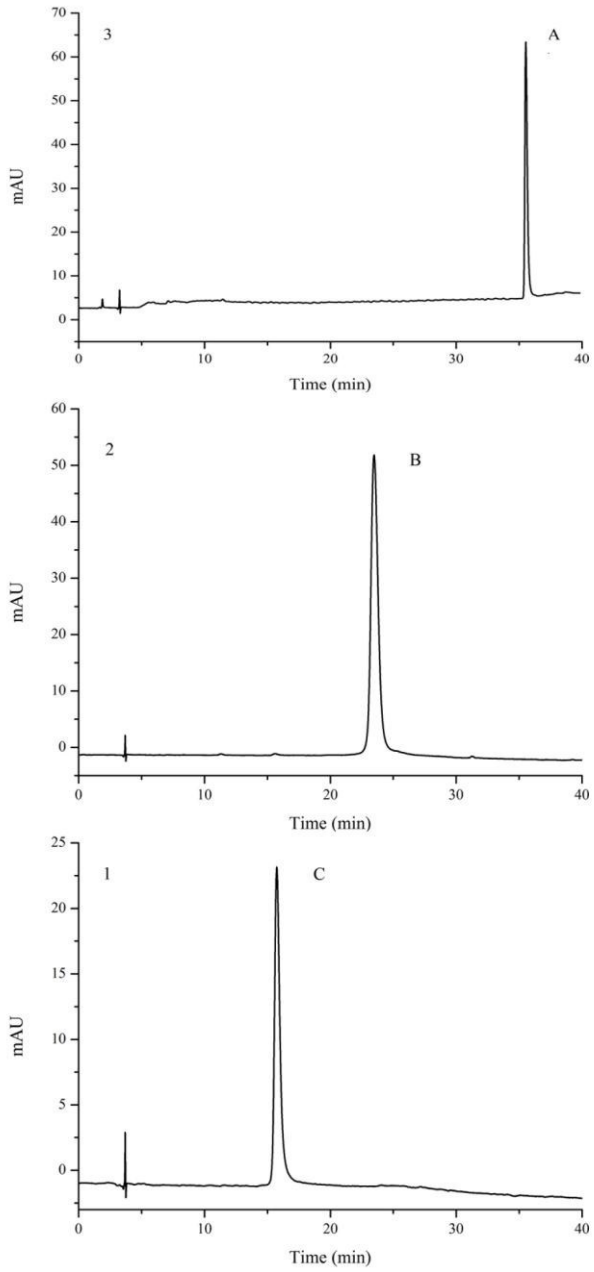

Fig. 4 HPLC chromatogram of HSCCC peak A (compound 3), peak B (compound 2) and peak C (compound 1). Column: ZORBAXSB-Aq (250 mm × 4.6 mm i.d., 5μm); mobile phase, (A) water and (B) acetonitrile in gradient mode as follows: 0-7 min, 5-12%B; 7-17min, 12%B; 17-35min, 12-30%B; 35-40min, 30%B; detection wavelength: 280 nm; flow rate: 1.0 mL min^{-1}.

that the peak orders of HPLC chromatogram and HSCCC chromatogram were the same, while the truth was just the opposite. The peak order of the three compounds was 1, 2 and 3 in HPLC chromatogram in Fig 1. However, it is interesting that the

Table 2 The 1-octanol/water partition coefficient (log $P_{o/w}$) values of the compounds

	catechin (1)	epicatechin (2)	oleuropein (3)
log $P_o/_w$	0.31[22]	0.10[22]	-0.97[23]

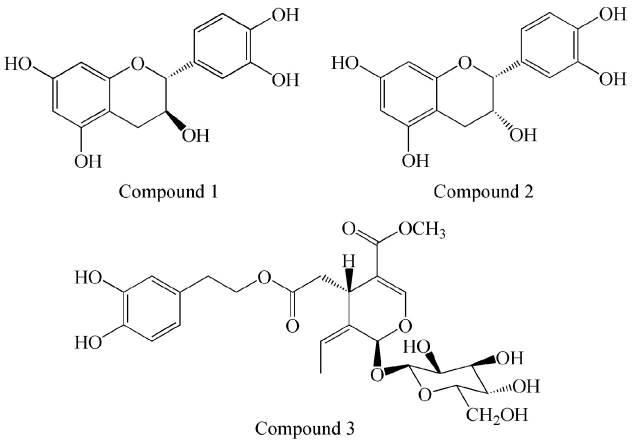

Fig. 5 Chemical structures of compounds 1–3 isolated from *C. songaricum*. 1, Catechin; 2, Epicatechin; 3, Oleuropein.

peak order was completely reversed in HSCCC chromatogram. The peak order of the three compounds was 3, 2 and 1 in HSCCC chromatogram in Fig. 3. Generally, in a reversed phase chromatographic mode, the compound with stronger hydrophobicity would be eluted later than the compound with weaker hydrophobicity. The partition coefficient for 1-octanol/water (log $P_o/_w$) is generally used as a measure of hydrophobicity[21]. The log $P_o/_w$ values of the three compounds which separated by HSCCC in this paper was listed in Table 2. It can be seen that the log $P_o/_w$ value is decreased in sequence from compound 1 to compound 3, which means the peak order is in accordance with the hydrophobicity order of the three compounds in HSCCC separation, while the peak order was contrary to the hydrophobicity order of the three compounds in HPLC separation. It can be concluded that the elution order of HSCCC separation of the three compounds was determined by their hydrophobicity, while the elution order of HPLC separation of the three compounds was not merely determined by the hydrophobicity. There are some other interactions between the stationary phase and the compounds in HPLC separation which may affect the elution order. The oleuropein molecule (compound 3) contains many –OH, –O– and C=O. These functional groups may form hydrogenbond with the –OH which was not endcapped in the stationary phase of HPLC column. That might extend the retention time of oleuropein. Catechin (compound 1) and epicatechin (compound 2) are isomers. The different retention of catechin and epicatechin was probably due to the different steric effect when they interact with the stationary phase of HPLC column. The fact that mentioned above confirmed that HSCCC and HPLC have different selectivity in the separation of some compounds. HSCCC is orthogonal to conventional chromatographic methods[24].

3.3 Structure identification

The structure identification of the isolated compounds was carried out by ESI-MS, ^{1}H NMR and ^{13}C NMR spectroscopy as well as comparison with the published literatures. Data of each compound is as follows:

Catechin (1) white powder, ESI-MS m/z: 313 $[M+Na]^+$; ^{1}H-NMR (400 MHz, methanol-d_4): δ 6.83 (1H, d, *J* = 1.5 Hz, H-2′), 6.72 (1H, dd, *J* = 1.5, 8.0 Hz, H-6′), 6.70 (1H, d, *J* = 8.0 Hz, H-5′), 5.92 (1H, d, *J* = 2.0 Hz, H-8), 5.85 (1H, d, *J* = 2.0 Hz, H-6), 4.89 (1H, d, *J* = 5.0 Hz, H-2), 4.55 (1H, m, H-3), 3.96 (1H, m, 3-OH), 2.82 (1H, dd, *J* = 5.4, 16.0 Hz, H-4α), 2.47 (1H, dd, *J* = 8.1, 16.0 Hz, H-4β). ^{13}C-NMR (100 MHz, methanol-d_4): δ 158.6 (C-9), 158.4 (C-7), 157.7 (C-5), 147.0 (C-3′, 4′), 133.0 (C-1′), 120.8 (C-6′), 116.9 (C-5′),116.0 (C-2′), 101.6 (C-10), 97.1 (C-6), 96.3(C-8), 83.6 (C-2), 69.6(C-3), 29.3 (C-4). Those data were in agreement with earlier published data for catechin [25].

Epicatechin (2) white powder, ESI-MS m/z: 313 $[M+Na]^+$; ^{1}H-NMR (400 MHz, methanol-d_4): δ 6.97 (1H, d, *J* = 1.5 Hz, H-2′), 6.78 (1H, d, *J* = 3.5 Hz, H-6′), 6.74 (1H, d, *J* = 1.5 Hz, H-5′), 5.93 (1H, d, *J* = 2.2 Hz, H-8), 5.91 (1H, d, *J* = 2.2 Hz, H-6), 4.86 (1H, s, H-2), 4.81 (1H, s, H-3), 4.17 (1H, s, 3-OH), 2.83 (1H, dd, *J* = 4.1, 16.4 Hz, H-4α), 2.70 (1H, dd, *J* = 4.1, 16.4 Hz, H-4β). ^{13}C-NMR (100 MHz, methanol-d_4): δ 158.8 (C-9), 158.5 (C-7), 158.2 (C-5), 146.8 (C-3′), 146.6 (C-4′), 133.1 (C-1′), 120.2 (C-6′), 116.7 (C-5′), 116.1 (C-2′), 100.9 (C-10), 97.2 (C-6), 96.7(C-8), 80.7 (C-2), 68.3(C-3), 30.1 (C-4). Those data were in agreement with earlier published data for epicatechin[25].

Oleuropein (3): a yellow gum, ESI-MS m/z: 563 $[M+Na]^+$; ^{1}H-NMR (400 MHz, methanol-d_4): δ 5.90 (s, 1H, H-1), 7.50 (s, 1H, H-3), 3.87 (m, 1H, H-5), 2.39 (m, 1H, H-6), 6.04 (m, 1H, H-8), 1.64 (d, 3H, *J* = 6.0 Hz, H-10), 3.70 (s, 3H, OMe), 4.06 (m, 2H, H-1′), 2.67 (m, 2H, H-2′), 6.66 (s, 1H, H-4′), 6.68 (d, *J* = 8.0 Hz, 1H, H-7′), 6.53 (d, *J* = 8.0 Hz, 1H, H-8′), 4.79 (d, *J* = 8.0 Hz, 1H, glc-1"); ^{13}C-NMR (100 MHz, methanol-d_4): δ 96.0 (C-1), 155.9 (C-3), 110.1 (C-4), 32.6 (C-5), 42.0 (C-6), 174.0 (C-7), 125.7 (C-8), 131.2 (C-9), 14.3 (C-10), 169.5 (C-11), 52.7 (OMe), 67.7 (C-1′), 36.1 (C-2′), 131.5 (C-3′), 117.8 (C-4′), 147.0 (C-5′), 145.7 (C-6′), 117.2 (C-7′), 122.1 (C-8′), 101.7 (glc-1"), 75.5 (glc-2"), 78.7 (glc-3"), 69.9 (glc-4"), 79.1 (glc-5"), 63.5 (glc-6"). Those data were in agreement with earlier published data for oleuropein[20].

3.4 DPPH scavenging activity

The antioxidant activities of target-isolated compounds from *C. songaricum* were measured spectrophotometrically by DPPH radical scavenging activity assay in comparison with rutin as standard antioxidant. EC_{50} (50% radical scavenging activity concentration of the antioxidant) was used to evaluated the scavenging activity and a lower EC_{50} value indicates a higher scavenging activity. The results showed that (+)-catechin ,(-)-epicatechin and oleuropein had potent free radical scavenging capacities with IC_{50} values of 16.97 ± 0.02, 11.15 ± 0.09 and 41.32 ± 0.20 μg mL^{-1}, respectively. The IC_{50} value for rutin was 21.49 ± 0.15 μg mL^{-1}. In the present study, (-)-epicatechin presented the highest hydrogen donating capability to act as antioxidant.

Conclusions

In this article, DPPH–HPLC–DAD method followed by HSCCC experiments was successively developed for the fast screening and purification of potent antioxidants from water extract of *C. songaricum* systematically. Three major constituents including (+)-catechin, (-)-epicatechin and oleuropein with potential antioxidant activity were screened and isolated using this method. Among them, oleuropein was obtained from *C. songaricum* for the first time. In addition, we found that the elution order of the three compounds in HSCCC separation was determined by their hydrophobicity, while the elution of HPLC may be affected by some other interactions between the stationary phase of the column and the compounds such as hydrogen bond and steric effect. Results of the present study indicated that the combinative method using DPPH–HPLC–DAD and HSCCC was a speedy, efficient and reproductive technique to systematically isolate antioxidant compounds from complex natural products.

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References

1 G.-D. Liu, G.-L. Chen, W. Li and C.-X. Li, *Biochemical Systematics and Ecology*, 2013, **48**, 34-41.
2 G. Shi, W. Jiang, L. Cai and G. Sui, *International journal of biological macromolecules*, 2011, **48**, 788-792.
3 G. Zhao, J. Wang, G. W. Qin and L. H. Guo, *Neurochemical research*, 2010, **35**, 666-676.
4 D. Y. Yoo, J. H. Choi, W. Kim, H.Y. Jung, S. M. Nam, J. W. Kim, Y. S. Yoon, K.-Y. Yoo, M.-H. Won and I. K. Hwang, *BMC Complementary and Alternative Medicine*, 2014, **14**, doi:10.1186/1472-6882-14-5.
5 J. Chen, H. S. Wong, H. Y. Leung, P. K. Leong, W. M. Chan, N. Chen and K. M. Ko, *Journal of Functional Foods*, 2014, **7**, 330-341.
6 M. N. Alam, N. J. Bristi and M. Rafiquzzaman, *Saudi pharmaceutical journal : SPJ : the official publication of the Saudi Pharmaceutical Society*, 2013, **21**, 143-152.
7 V. L. Kinnula and J. D. Crapo, *Free radical biology & medicine*, 2004, **36**, 718-744.
8 U. Singh and I. Jialal, *Pathophysiology : the official journal of the International Society for Pathophysiology / ISP*, 2006, **13**, 129-142.
9 M. Valko, D. Leibfritz, J. Moncol, M. T. Cronin, M. Mazur and J. Telser, *The international journal of biochemistry & cell biology*, 2007, **39**, 44-84.
10 Y. Lu, Q. Wang, M. F. Melzig and K. Jenett-Siems, *Phytotherapy research : PTR*, 2009, **23**, 257-261.
11 C.-M. Ma, N. Sato, X.-Y. Li, N. Nakamura and M. Hattori, *Food chemistry*, 2010, **118**, 116-119.
12 H. C. Meng and C. M. Ma, *Food chemistry*, 2013, **141**, 2691-2696.
13 G. Shui and L. P. Leong, *Journal of Chromatography A*, 2004, **1022**, 67-75.
14 J. Zhang, X. Huang, X. Sun, D. Pei and D. Di, *New Journal of Chemistry*, 2014, **38**, 3796.
15 Y. Q. Zhang, J. G. Luo, C. Han, J. F. Xu and L. Y. Kong, *Journal of pharmaceutical and biomedical analysis*, 2015, **102**, 276-281.
16 X.-Y. Huang and D.-L. Di, *TrAC Trends in Analytical Chemistry*, 2015, **67**, 128-133.

17 Y. Liu, G. Wang, X. Huang, Y. Liu and D. Di, *New Journal of Chemistry*, 2014, **38**, 1150.
18 A. Tapia, J. Rodriguez, C. Theoduloz, S. Lopez, G. E. Feresin and G. Schmeda-Hirschmann, *Journal of ethnopharmacology*, 2004, **95**, 155-161.
19 D. Biswal, N. R. Pramanik, S. Chakrabarti, N. Chakraborty, K. Acharya, S. S. Mandal, S. Ghosh, M. G. B. Drew, T. K. Mondal and S. Biswas, *New J. Chem.*, 2015, **39**, 2778-2794.
20 X. Wang, C. Li, Y. Liu, H. Li and D. Di, *Biomedical chromatography : BMC*, 2011, **25**, 373-380.
21 A. Sarkar and G. E. Kellogg, *Current Topics in Medicinal Chemistry*, 2010, **10**, 67-83.
22 A. Yanagida, H. Murao, M. Ohnishi-Kameyama, Y. Yamakawa, A. Shoji, M. Tagashira, T. Kanda, H. Shindo and Y. Shibusawa, *Journal of chromatography. A*, 2007, **1143**, 153-161.
23 N. Nenadis, L.-F. Wang, M. Z. Tsimidou and H.-Y. Zhang, *Journal of Agricultural an Food Chemistry*, 2005, **53**, 295-299.
24 M. Englert, L. Brown and W. Vetter, *Analytical chemistry*, 2015, **87**, 10172-10177.
25 Y. Sun, H. Lin, Z. Liu, S. Yang and J. Wang, *Separation and Purification Technology*, 2014, **134**, 171-177.

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