ORIGINAL ARTICLE

Rec. Nat. Prod. 12:4 (2018) 332-339

records of natural products

Antioxidants from *Pedicularis longiflora* var. *tubiformis* (Klotzsch) P. C. Tsoong

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(Received August 23, 2017; Revised November 17, 2017; Accepted November 18, 2017)

Abstract: An efficient method for the screening and isolation of potent antioxidants from a tibetan medicinal plant by employing online HPLC–DPPH assay combined HSCCC has been successfully established. Six major constituents: boschnaloside (**1**), alyssonoside (**2**), leucosceptoside A (**3**), isoverbascoside (**4**), leucosceptoside B (**5**) and verbascoside (**6**) were isolated and purified from the water extract by HSCCC using ethyl acetate-n-butanol-water as two-phase solvent system. The results indicated that the combination of the online HPLC–DPPH assay with HSCCC could be suitable for the screening and separation of antioxidant compounds from extract of this plant or other medicine plants.

Keywords: *Pedicularis longiflora* var. *tubiformis* (Klotzsch) P. C. Tsoong; online HPLC–DPPH; HSCCC; antioxidants. © 2018 ACG Publications. All rights reserved.

1. Introduction

Pedicularis longiflora var. *tubiformis* (Klotzsch) P. C. Tsoong, belongs to a kind of traditional Tibetan medicinal herbs, and is also recorded in Tibetan medicine, mainly distributed in the northwest and southwest of China [1]. According to the records of Qinghai economic plants, it has a good therapeutic effect on hepatitis, cholecystitis, edema, spermatorrhea and other diseases [2]. The genus *Pedicularis* is rich in phenolic compounds, and many phenolic compounds have been isolated from other plants of this genus [3]. Although many plants of this genus in the chemical composition have been studied, few papers were about this plant. Previous chemical and pharmacological studies have preliminarily confirmed that phenylpropanoid glycosides, iridoid glycosides and flavonoids are the main chemical constituents and active substances of this plant [4,5]. These kinds of compounds play an important role in human health owing to their antioxidant activities [6-8]. Therefore, the development of convenient and efficient methods to screen and separate antioxidant active substances with higher activity and safety from this plant is highly desirable.

In this paper, a convenient and efficient of online DPPH-HPLC-DAD analysis combined with HSCCC for screening and separation of antioxidants in *P. longiflora* was successfully developed. Six

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The article was published by ACG Publications
www.acgpubs.org/RNP © July-August EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.35.17.08.142

major compounds, boschnaloside [9], alyssonoside [10], leucosceptoside A [11,12], isoverbascoside [13], leucosceptoside B [14], and verbascoside [15,16], were screened as antioxidants by on-line DPPH-HPLC-DAD analysis and isolated by HSCCC in one step. The purities of the six compounds were 98.1%, 95.7%, 96.6%, 98.3%, 93.2%, and 95.8%, respectively as determined by HPLC. In addition, the DPPH free radical scavenging assay was used to detect the antioxidant activity of the six compounds. All of them showed high radical scavenging activities with the EC50 values being 7.01±0.54, 7.59±0.29, 11.26±0.32, 14.65±0.83, 13.05±0.31, 13.38±0.46 µM, respectively. Moreover, boschnaloside, alyssonoside, leucosceptoside A and leucosceptoside B were first separated from this plant.

2. Materials and Methods

2.1. Reagents and Materials

Analytical grade chemical reagents used for pretreatment and separation were obtained from Tianjin BaiShi chemical industry Co. Ltd (TianJin, China). HPLC grade reagents were purchased from Shandong Yuwang reagent company (Shandong, China). Deionized water was prepared by a Ulupure purification system (Ulupure Corporation, Chengdu, China).
P. longiflora was collected from Hu Zhu, Qinghai province in 2015, and was identified by Professor Qing-bo Gao of Northwest Plateau Institute of Biology, CAS. The complete specimen 200g was stored in the herbarium (Herbarium number: hnwp0330007) of the Northwest Plateau Biological Institute of CAS.

2.2. Apparatus

On-line HPLC–DPPH assay was laboratory assembled including an Agilent 1100 series HPLC with a diode array detector (DAD) and an Agilent 1260 series HPLC with a visual web detector (VWD). Polyetheretherketone (PEEK) tubing (15.0m×0.25mm i.d.) was used as the reaction coils.

The TBE-300A high speed counter current chromatography equipment (Shanghai Tauto Biotech Co., Ltd., Shanghai, China) was used for HSCCC. A model TBP5002 constant-flow pump (Shanghai Tauto Biotech Co., Ltd., Shanghai, China) was used to pump the solvent system. UV absorbance was detected by a model UV-500 (XUYUKJ Instruments, Hangzhou, China) and recorded by N2000 workstation (Zhejiang University Star Information Technology Co., Ltd., Hangzhou, Zhejiang, China). The system temperature was controlled by an HX-1050 constant temperature circulating implement (Shanghai Shunyuhengping Science Instruments Co. Ltd., Shanghai, China).

A Mercury-600BB NMR spectrometer (Varian Co. Ltd., Palo Alto, CA, USA) with tetramethylsilane (TMS) as the internal standard was used to identify the compounds of I-VI.

2.3. Preparation of Crude Sample

The powdered *P. longiflora* (1000 g) were extracted by 70% ethanol for three times (2h each time) under refluxing condition. The extract was concentrated by rotary evaporator under reduced pressure, and 130 g residue was obtained. The residue was first dissolved by deionized water (500 mL) and then extracted six times with petroleum ether (3.0 L). After evaporating the organic solvent, the aqueous fraction was loaded on a macroporous resin column (100 cm × 6.0 cm), which contains 600 g D101 macroporous resin, and eluted stepwise with different proportions of water and ethanol (100:0, 80:20, and 60:40, and 40:60 v/v; 3000 mL for each proportion). The different fractions were concentrated and screened based on the DPPH radical scavenging activity and the 40% aqueous ethanol (water: ethanol=60:40) fraction exhibited considerable antioxidant effect. Therefore, the 40% aqueous ethanol fraction (30 g) may be a good candidate for further isolation and screening of antioxidant components, which prompted us to perform a detailed target-guided chemical investigation.

2.4. Online DPPH Radical-Scavenging Analysis

Antioxidants in 40% aqueous ethanol fraction of *P. longiflora* were screened by Online RP-HPLC-DPPH method. The instrumental composition, connection and detection process was shown in Figure 1. The fraction (stock concentration 20 mg/mL) was analyzed by Agilent 1100 with a Dikma Platisil ODS C_{18} column (4.6×250 mm, id 5μm). The flow rate was 1.0 mL/min, detection wavelength was set at 330 nm and the column temperature was controlled at 25 °C. Methanol - water was used as the mobile phase with a gradient program as follow: 0-45 min, 45-50% methanol. Online DPPH radical-scavenging assays were performed on Agilent 1260 with the flow rate of DPPH reagent (25 μg/ml in methanol) at 0.45 mL/min, and detected at 517 nm by VWD.

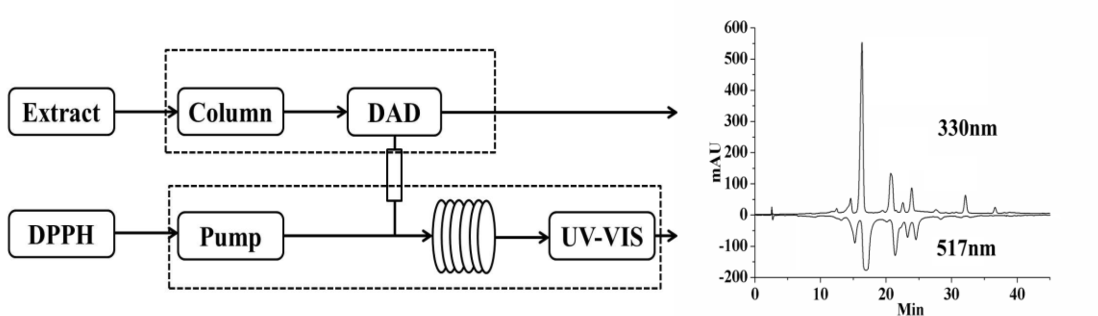

Figure 1. Instrument set-up of on-line system.

2.5. Measurement of Partition Coefficient of Target Compounds

The partition coefficient (K) was expressed as the absorbency of sample in the upper phase divided by that in the lower phase. The K value of crude sample was measured as follow: firstly, 400 mg crude extract was dissolved in a series of pre-equilibrated two-phase-solvent systems. Subsequently, the solution was fully shaken in order to reach the partition equilibrium. At last, the upper and lower phases of equal volumes were evaporated to dryness separately and the residues were diluted into 2 mL methanol, which were analyzed by HPLC.

2.6. Preparation of Two-Phase Solvent System and Sample Solution

Proper solvent system is the key factor for the success of HSCCC separation. The selection of two-phase solvent system plays an important role in the successful separation of high speed countercurrent chromatography. According to the K value of the targeted compounds, ethyl acetate-butanol-water (13:3:9) was chosen as the solvent system in this experiment. The three solvents are mixed in proportion and placed at room temperature. The upper and lower phases are separated and used before use. The 400mg sample was dissolved in the lower phase solution of 5ml as an injection solution.

2.7. HSCCC Separation Procedure

First, the coiled column of HSCCC was filled with the upper phase (stationary phase) of solvent system. The lower phase (mobile phase) of solvent system was pumped through the column at a flow rate of 2.5 mL/min with the apparatus rotated at 950 rpm. 5 mL of sample solution containing 400 mg sample was injected through valve when hydrodynamic equilibrium was reached in the column. The peak fractions were collected manually according to the elution profile monitored with a UV detector at 330 nm. The temperature of the apparatus was set to 30 °C.

2.8. HPLC analysis and identification of HSCCC peak fractions

The six peak fractions isolated by HSCCC were analyzed by HPLC based on the analytic conditions mentioned in section 2.4, and the chromatograms are shown in Fig. 4. Meanwhile, peak fraction identification was carried out by ^{1}H and ^{13}C NMR.

2.9. DPPH radical scavenging activity assays

The assays of offline DPPH radical scavenging activity were performed according to previously reported protocols [17]. Ascorbic acid (VC) was used as positive control. The DPPH stock solution(250μM) which was firstly prepared with ethanol and stored in the dark at room temperature for 12-16 h was diluted with ethanol to an absorbance of 0.700 (±0.020) at 517 nm before use. 20μL of the compounds were mixed with 180μL of DPPH solution. The mixture was reacted for 30min in the dark and then measured at 517nm with a UV/vis spectrophotometer (PerkinElmer Enspire, PE, USA) after The radical scavenging activity was determined by the following equation: % scavenging activity = [Ablank control–Asample]/Ablank control×100%. The EC50 value was obtained through GraphPad Prism 6.02. All determinations were carried out in triplicate.

3. Results and Discussion

3.1. Screening Radical Scavengers Using on-line HPLC–DPPH

The online HPLC-DPPH method provided a rapid screen of antioxidative components in complex mixtures, particularly plant extracts [18]. Combined UV (positive signals) with DPPH (negative signals) radical quenching chromatograms under gradient conditions of the 40% aqueous ethanol fraction of *P. longiflora* are presented in Figure 2. Several eluted phytochemicals in the *P. longiflora* were detected on the UV detector (330 nm). Among them, six main compounds with high content showed obvious antioxidant capacity (negative peak) toward the DPPH radical at the applied concentration.

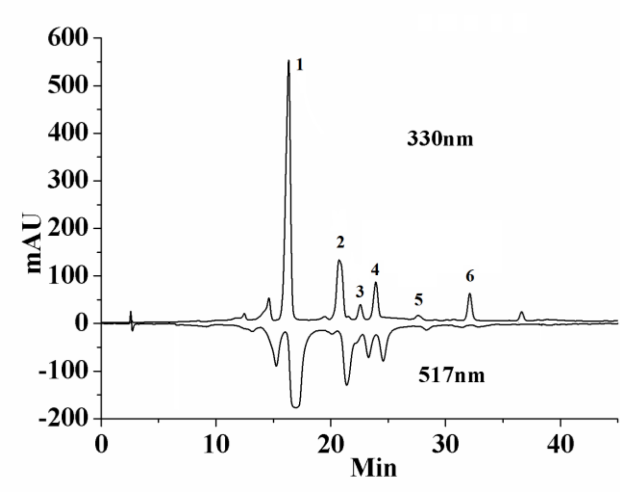

Figure 2. The HPLC chromatograms (330 nm) and the DPPH radical scavenging chromatograms (517 nm) of the crude extract from *P. longiflora*.

3.2. HSCCC Separation

Six compounds including boschnaloside (1, 22 mg), alyssonoside (2, 39 mg), leucosceptoside A (3, 27 mg), Isoverbascoside (4, 38 mg), 27 mg of leucosceptoside B (5, 27 mg) and verbascoside (6, 13 mg), were generated in one step separation within 8 h from 400 mg crude sample by using ethyl acetate-n-butanol-water (13:3:9, v/v/v) as two-phase solvent system at 30°C with flow rate of 2.5 mL/min. The purities of these six compounds were 98.1%, 95.7%, 96.6%, 98.3%, 93.2%, and 95.8%, respectively, analyzed by HPLC

3.3. Structure Elucidation

The chemical structures of the peak fractions separated by HSCCC as show in Fig. 3 were identified by ^{1}H NMR and ^{13}C NMR data as well as comparison with the published reference data. Results for each peak fraction were as follows.

Figure 3. The chemical structures of compounds **1-6**

*Verbascoside (**1**):* yellow needles, ^{1}H NMR (DMSO-d$_6$, 600 MHz) δ = 7.44 (1H, d, J = 15.8 Hz, H-7'), 7.01 (1H, d, J = 1.9 Hz, H-2'), 6.96 (1H, dd, J = 2.0 Hz, J = 8.3 Hz, H-6'), 6.75 (1H, d, J = 7.9 Hz, H-5'), 6.62 (1H, d, J = 2.2 Hz, H-2), 6.61 (1H, d, J = 7.9 Hz, H-5), 6.47 (1H, dd, J = 2.0 Hz, J = 8.1 Hz, H-6), 6.19 (1H, d, J = 15.9 Hz, H-8'), 5.00 (1H, s, Rha H-1), 4.70 (1H, t, J = 9.7 Hz, Glu H-4), 4.34 (1H, d, J = 7.9 Hz, Glu H-1), 3.87-3.08 (9H, m, H-8 or Rha/Glu-H), 2.67 (2H, m, H-7), 0.94 (3H, d, J = 6.2 Hz, Rha H-6); ^{13}C NMR (DMSO-d$_6$, 150 MHz) = 129.1 (C-1), 115.8 (C-2), 143.5 (C-3), 145.0 (C-4), 74.5 (Glu C-5), 119.5 (C-6), 35.1 (C-7), 70.2 (C-8), 125.5 (C-1'), 113.6 (C-2'), 148.5 (C-3'), 145.5 (C-4'), 116.3 (C-5'), 115.5 (C-5'), 121.4 (C-6'), 145.6 (C-7'), 114.7 (C-8'), 165.7 (C-9'), 102.3 (Glu C-1), 74.4 (Glu C-2), 79.1 (Glu C-3), 68.7 (Glu C-4), 60.7 (Glu C-6), 101.3 (Rha C-1), 70.5 (Rha C-2), 70.3 (Rha C-3), 71.6 (Rha C-4), 69.2 (Rha C-5), 18.3 (Rha C-6) [19].

*Alyssonoside (**2**):* yellow needles, ^{1}H NMR (DMSO-d$_6$, 600 MHz) δ = 7.53 (1H, d, J = 15.7 Hz, H-7'), 7.28 (1H, d, J = 1.9 HZ, H-2'), 7.08 (1H, d, J = 1.9 Hz, J = 8.3 Hz, H-6'), 6.77 (1H, d, J = 8.2 Hz, H-5'), 6.62 (1H, d, J = 2.1 Hz, H-2), 6.61 (1H, d, J = 8.0 Hz, H-5), 6.49 (1H, dd, J = 2.6 Hz, J = 8.0 Hz, H-6), 6.41 (1H, d, J = 15.9 Hz, H-8'), 5.01 (1H, s, Rha H-1), 4.77 (1H, d, J = 3.0 Hz, Api H-1), 4.66 (1H, t, J = 9.7 Hz, Glu H-4), 4.37 (1H, d, J = 7.9 Hz, Glu H-1), 3.79 (3H, s, OMe), 3.77-3.07 (15H, m, H-8 or Rha/Glu/Api-H), 2.68 (2H, m, H-7), 0.96 (3H, d, J = 6.2 Hz, Rha H-6); ^{13}C NMR (DMSO-d$_6$,

150 MHz) □ = 129.1 (C-1), 113.9 (C-2), 145.0 (C-3), 143.5 (C-4), 116.3 (C-5), 119.6 (C-6), 35.0 (C-7), 70.5 (C-8), 125.6 (C-1'), 109.3 (C-2'), 147.9 (C-3'), 149.4 (C-4'), 115.5 (C-5'), 123.3 (C-6'), 145.8 (C-7'), 111.0 (C-8'), 165.9 (C-9'), 55.6 (OMe), 102.2 (Glu C-1), 72.8 (Glu C-2), 78.8 (Glu C-3), 68.8 (Glu C-4), 74.4 (Glu C-5), 67.2 (Glu C-6), 109.1 (Api C-1), 75.9 (Api C-2), 78.7 (Api C-3), 73.4 (Api C-4), 63.1 (Api C-5), 101.3 (Rha C-1), 70.2 (Rha C-2), 70.3 (Rha C-3), 71.6 (Rha C-4), 69.4 (Rha C-5), 18.1 (Rha C-6) [20].

*Leucosceptoside A (**3**)*: yellow needles, ^{1}H NMR (DMSO-d_6, 600 MHz) δ = 7.55 (1H, d, J = 15.8 Hz, H-7'), 7.30 (1H, s, H-2'), 7.10 (1H, d, J = 8.3 Hz, H-6'), 6.78 (1H, d, J = 7.9 Hz, H-5'), 6.62 (2H, m, H-2 and H-5), 6.43 (1H, d, J = 8.1 Hz, H-6), 6.05 (1H, d, J = 15.9 Hz, H-8'), 5.03 (1H, s, Rha H-1), 4.72 (1H, m, Glu H-4), 4.35 (1H, d, J = 7.9 Hz, Glu H-1), 3.89-3.10 (9H, m, H-8 or Rha/Glu-H), 3.81 (3H, s, OMe), 2.69 (2H, m, H-7), 0.96 (3H, d, J = 6.2 Hz, Rha H-6); ^{13}C NMR (DMSO-d_6, 150 MHz) □ = 129.1 (C-1), 115.5 (C-2), 143.5 (C-3), 145.0 (C-4), 116.3 (C-5), 119.5 (C-6), 34.9 (C-7), 70.3 (C-8), 125.6 (C-1'), 111.1 (C-2'), 149.4 (C-3'), 147.9 (C-4'), 115.4 (C-5'), 123.1(C-6'), 145.5 (C-7'), 114.1 (C-8'), 165.8 (C-9'), 55.6 (OMe), 102,3 (Glu C-1), 74.5 (Glu C-2), 79.1 (Glu C-3), 68.8 (Glu C-4), 74.6 (Glu C-5), 60.7 (Glu C-6), 101.2 (Rha C-1), 70.5 (Rha C-2), 70.4 (Rha C-3), 71.7 (Rha C-4), 69.1 (Rha C-5), 18.0 (Rha C-6) [19].

*Isoverbascoside (**4**):* , yellow needles, ^{1}H NMR (DMSO-d_6, 600 MHz) δ = 7.45 (1H, d, J = 15.7 Hz), 7.04 (1H, d, J = 2.0 Hz), 6.95 (1H, dd, J = 2.0 Hz, J = 8.4 Hz), 6.74 (1H, d, 8.2 Hz), 6.58 (1H, d, J = 2 Hz), 6.56 (1H, d, J = 8.0 Hz), 6.44 (1H, dd, J = 2.0 Hz, J = 8.0 Hz), 6.28 (1H, d, J = 15.9 Hz), 5.03 (1H, s), 4.35 (1H, d, J = 10.8 Hz, Glu H-6), 4.26 (1H, d, J = 7.6 Hz), 4.18 (1H, m, Glu H-6), 3.88-3.10 (10H, m, Rha/Glu-H), 2.65 (2H, m), 1.07 (3H, d, J = 6.0 Hz); ^{13}C NMR (DMSO-d_6, 150 MHz) = 129.2 (C-1), 115.8 (C-2), 143.5 (C-3), 145.0 (C-4), 116.3 (C-5), 119.5 (C-6), 35.1 (C-7), 70.3 (C-8), 125.5 (C-1'), 113.7 (C-2'), 148.4 ((C-3'), 145.3 (C-4'), 115.5 (C-5'), 121.6 (C-6'), 145.6 (C-7'), 114.8 (C-8'), 165.5 (C-9'), 102.7 (Glu C-1), 73.7 (Glu C-2), 80.8 (Glu C-3), 68.6 (Glu C-4), 74.1 (Glu C-5), 63.5 (Glu C-6), 100.7 (Rha C-1), 70.5 (Rha C-2), 70.4 (Rha C-3), 72.1 (Rha C-4), 68.9 ((Rha C-5), 17.9 (Rha C-6) [19].

*Leucosceptoside B (**5**)*: yellow needles, ^{1}H NMR (DMSO-d_6, 600 MHz) δ = 7.53 (1H, d, J = 15.8 Hz, H-7'), 7.28 (1H, d, J = 1.7 HZ, H-2'), 7.07 (1H, d, J = 1.8 Hz, J = 8.3 Hz, H-6'), 6.79 (1H, d, J = 8.3 Hz, H-5'), 6.77 (1H, d, J = 8.2 Hz, H-5), 6.67 (1H, d, J = 2.2 Hz, H-5), 6.63 (1H, dd, J = 2.1 Hz, J = 8.2 Hz, H-6), 6.40 (1H, d, J = 15.9 Hz, H-8'), 5.01 (1H, s, Rha H-1), 4.77 (1H, d, J = 2.9 Hz, Api H-1), 4.66 (1H, t, J = 9.7 Hz, Glu H-4), 4.37 (1H, d, J = 7.9 Hz, Glu H-1), 3.87-3.08 (15H, m, H-8 or Rha/Glu/Api-H), 3.80 (3H, s, OMe), 3.71 (3H, s, OMe), 2.73 (2H, m, H-7), 0.96 (3H, d, J = 6.2 Hz, Rha H-6); ^{13}C NMR (DMSO-d_6, 150 MHz) = 131.1 (C-1), 116.3 (C-2), 145.9 (C-3), 146.1 (C-4), 112.3 (C-5), 119.4 (C-6), 35.0 (C-7), 70.5 (C-8), 125.5 (C-1'), 111.0 (C-2'), 149.6 (C-3'), 148.0 (C-4'), 115.5 (C-5'), 123.3 (C-6'), 146.3 (C-7'), 113.8 (C-8'), 165.9 (C-9'), 55.7 (OMe), 55.6 (OMe), 102.2 (Glu C-1), 72.8 (Glu C-2), 78.8 (Glu C-3), 68.8 (Glu C-4), 74.4 (Glu C-5), 67.2 (Glu C-6), 109.1 (Api C-1), 75.9 (Api C-2), 78.7 (Api C-3), 73.4 (Api C-4), 63.2 (Api C-5), 101.3 (Rha C-1), 70.2 (Rha C-2), 70.4 (Rha C-3), 71.7 (Rha C-4), 69.4 (Rha C-5), 18.2 (Rha C-6) [19].

*Boschnaloside (**6**)*: yellow needles, ^{1}H NMR (DMSO-d_6,600 MHz) δ = 9.17 (s, 1H), 7.37 (1H, s, H-3), 5.63 (1H, d, J = 3.0 Hz, H-1), 4.69 (1H, d, J = 7.8 Hz, H-1), 3.90 (1H, d, J = 11.4 Hz, Glu H-1), 3.65-3.62 (1H, m, Glu H-2), 3.37-2.92 (5H, m, Glu H-3, H-4, H-5, H-6), 2.29-1.28 (7H, m, H-5, H-6, H-7, H-8, H-9), 1.07 (3H, d, J = 6.0 Hz, H-10); ^{13}C NMR (DMSO-d_6, 150 MHz) δ = 88.0 (C-1), 154.7 (C-3), 117.0 (C-4), 27.6 (C-5), 21.8 (C-6), 24.1 (C-7), 22.8 (C-8), 34.6 (C-9), 7.13 (C-10), 183.6 (C-11), 90.4 (C-1'), 65.2 (C-2'),69.0 (C-3'), 62.2 (C-4'), 68.5 (C-5'), 53.4 (C-6') [21].

3.4. DPPH Scavenging Activity

Target-isolated compounds from *P. longiflora* extract by HSCCC were evaluated in vitro for their antioxidant with VC as the standard antioxidant. Primary assay showed that six compounds exhibited relatively better radical scavenging effect at the concentration of 10.00μM. (Fig. 4) These compounds were further assayed to obtain the EC50 values for antioxidant activities, and a lower EC50 value

indicates a higher antioxidant activity. The results showed that boschnaloside, alyssonoside, leucosceptoside A, isoverbascoside, leucosceptoside B and verbascoside had potent free radical scavenging capacities with EC50 values of 13.38±0.46, 7.59±0.29, 11.26±0.32, 14.65±0.83, 13.05±0.31, 7.01±0.54 µM, respectively. The EC50 value for VC was 12.29±0.68 µM. All of the six target compounds had strong antioxidant activity, and the activities of compounds I, II and III were better than the positive control. This study revealed that the method can precisely screening antioxidant compounds and reduce the experimental task in isolating and purifying non-target phytochemicals.

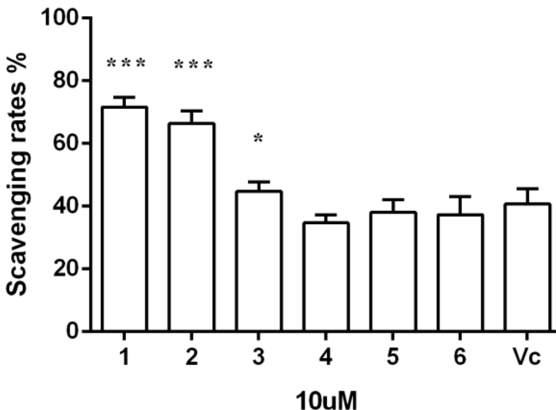

Figure 4. DPPH free radical scavenging rates of six compounds and VC at a concentration of 10.00µM; *p < 0.05 and ***p < 0.001 versus positive control.

As a conclusion, we have successfully developed an efficient method for the screening and purification of potent antioxidants from the water extract of *P. longiflora* by employing online HPLC–DPPH assay combined HSCCC. Six major constituents including boschnaloside, alyssonoside, leucosceptoside A, isoverbascoside, leucosceptoside B and verbascoside with potential antioxidant activity were screened and isolated using this method. Among them, boschnaloside, alyssonoside, leucosceptoside A and leucosceptoside B was obtained from *P. longiflora* for the first time. The present results indicated that the combination of the online HPLC–DPPH experiment with HSCCC was an efficient method to target-guided isolate antioxidant compounds from natural plants.

Acknowledgments

This research was supported by the project of QingHai Science and Technology Department Application Fundamental Research (2015-ZJ-726), West Light Foundation of the Chinese Academy of Sciences 2013, The Open Project of State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University (2017-KF-04) and The Project of Qinghai Science & Technology Department (2016-ZJ-Y01).

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