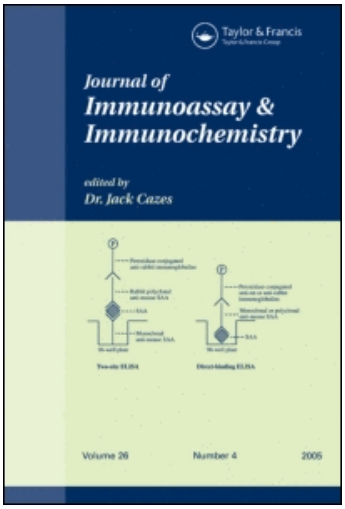

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Shu Yun Shi[ab]; Yu Ping Zhang[a]; Hong Hao Zhou[b]; Ke Long Huang[a]; Xin Yu Jiang[a]
[a] School of Chemistry and Chemical Engineering, Central South University, Changsha, P. R. China [b] Pharmacogenetics Institute of Central South University, Changsha, P. R. China

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SCREENING AND IDENTIFICATION OF RADICAL SCAVENGERS FROM *NEO-TARAXACUM SIPHONANTHUM* BY ONLINE RAPID SCREENING METHOD AND NUCLEAR MAGNETIC RESONANCE EXPERIMENTS

Shu Yun Shi,[1,2] Yu Ping Zhang,[1] Hong Hao Zhou,[2] Ke Long Huang,[1] and Xin Yu Jiang[1]

[1]*School of Chemistry and Chemical Engineering, Central South University, Changsha, P. R. China*
[2]*Pharmacogenetics Institute of Central South University, Changsha, P. R. China*

□ *An online rapid screening method, the high-performance liquid chromatography (HPLC)–diode array detector (DAD)–radical scavenging detection (RSD)–electrospray ionization (ESI)–mass spectroscopy (MS)/MS system, was developed for the screening and identification of radical scavengers from Neo-Taraxacum siphonanthum, a new species found in China in 1989. For further characterization, the target compounds were isolated by silica column chromatography, preparative high-performance liquid chromatography (HPLC), HSCCC, and Sephadex LH-20 column chromatography and elucidated on the basis of ultraviolet (UV), ESI-MS/MS, and nuclear magnetic resonance (NMR) spectroscopy, as well as the chemical analysis. Eighteen antioxidative polyphenols (5 caffeic acid derivatives and 13 flavonoid derivatives) were characterized from Neo-T. siphonanthum. The distribution of all compounds was discussed in a chemosystematic context, which suggested that the genera Neo-Taraxacum and Taraxacum might relate chemosystematically.*

Keywords antioxidant activity, chemotaxonomy, HPLC-DAD-RSD-ESI-MS/MS, *Neo-Taraxacum siphonanthum*, NMR, phenolic metabolites

INTRODUCTION

Neo-Taraxacum siphonanthum (Asteraceae) was a new species found in Inner Mongolia, China, in 1989, and was initially ascribed to the genus *Taraxacum* because of the related morphological characters of leaves.[1] However, the particular bisexual ligulate flowers distinguished this species from those in the *Taraxacum* genus; then *Neo-T. siphonanthum* was subjected to a new genus, *Neo-Taraxacum*.[2] This species has now commonly been

Address correspondence to Shu Yun Shi, School of Chemistry and Chemical Engineering, Central South University, Changsha 410083, P. R. China. E-mail: shuyshi@gmail.com

used by Chinese local people for both dietary and medicinal purposes. However, to the best of our knowledge, there have seldom been published reports on the chemical constituents and pharmacological properties of this species.[3] Our previous research showed that the methanolic extract exhibited a high DPPH radical scavenging activity,[3] and therefore it might be a good candidate for further development as antioxidant remedies.[4] Moreover, antioxidants have received a great amount of attention as being primary preventive ingredients against various diseases such as cancer, inflammation and cardiovascular diseases.[5–8] Therefore, further comprehensive chemical and pharmacological research of *Neo-T. siphonanthum* is warranted.

The standard procedure of searching for active metabolites was activity-guided fractionation followed with biological screening. However, bioassay-guided fractionation of crude plant extracts was time-consuming, labor-intensive, and expensive, which also led to loss of activity during the isolation and purification process due to dilution effects or decomposition.[9] Moreover, owing to the existence of hundreds of bioactive compounds with different types of structures in the natural products, the fast separation and identification of these were almost impossible. Several attempts have been made to accelerate the isolation, identification, and bioactivity evaluation processes. Recent publications demonstrated the efficiency of the online coupling of high-performance liquid chromatography (HPLC) separation and activity determination of radical scavenging compounds (HPLC-RSD).[10–14] Such techniques apply for a rapid and selective detection of radical scavenging compounds from complex extract. Thus, it is no longer necessary to purify every constituent for offline assays, leading to very significant reductions of costs and time to obtain results. And the integration of biological and chemical screening into bioactive compound discovery programs reduces the chance of missing novel and unidentified compounds, preventing replication during separation. A combination of HPLC-RSD method with online mass spectroscopy (MS)/MS for the analysis of radical-scavenging compounds would permit the rapid determination of antioxidant activity and provide the structural identification of the antioxidant compounds involved, and this technique was fast, sensitive, and required only minor sample preparation.[15] On the other hand, because of the lack of standard substances, the complete elucidation of complex plant secondary metabolites frequently requires the aid of more effective auxiliary techniques, such as nuclear magnetic resonance (NMR).

Therefore, the aim of the present study was to screen the radical scavengers in *Neo-T. siphonanthum* by the hyphenated method HPLC–diode array detector (DAD)–RSD–electrospray ionization (ESI)–MS/MS and subsequently characterize their chemical structures by offline NMR experiments. Moreover, the chemotaxonomic value of the radical scavengers was discussed.

EXPERIMENTAL

Materials and Chemicals

The aerial part of *Neo-T. siphonanthum* was purchased from Bozhou, Anhui province, in November 2006 and was identified by Prof. Juanhua Xu, College of Pharmaceutical Sciences, Zhejiang University.

Methanol used for analytical HPLC was of chromatographic grade (Merck, Darmstadt, Germany). All aqueous solutions used in the experiments were prepared with pure water produced by a Milli-Q water system (Millipore, Bedford, MA). Other analytical-grade chemicals were purchased from the Chemical Reagent Factory of Hunan Normal University (Changsha, Hunan, China). Deuterated dimethyl sulfoxide (DMSO-d_6) used for NMR was bought from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). DPPH (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), multiwell plates (Greiner Bio-One GmbH, Frickenhausen, Germany), and a multiwell plates reader (Bio-Tek Instruments, Winooski, VT) were used in the antioxidant activity experiments. Caffeic acid, chlorogenic acid, ferulic acid, quercetin, luteolin, luteolin-7-*O*-β-D-glucopyranoside, genkwanin, and genkwanin-4′-*O*-β-D-lutinoside used for the standards were isolated previously by our research group from *Taraxacum mongolicum* and identified by ultraviolet (UV), MS, one-dimensional (1D) NMR, and two-dimensional (2D) NMR experiments.[16]

Sample Preparation

Fresh plants of *Neo-T. siphonanthum* were dried at 50°C for 3 days before grinding. About 10 g of the powder was extracted by refluxing with methanol (100 mL) for three times (1.5 h for each time). All the filtrates were combined and concentrated to dryness under reduced pressure by rotary evaporation at 45°C. The residues were then diluted to 100 mL, stored at 4°C, and then brought to room temperature before analysis. The solution was filtered through a 0.45-μm polytetrafluoroethylene (PTEE) filter, and 20 μl of the sample solution was injected for HPLC analysis.

Off-Line DPPH Free Radical Scavenging Activity

The quenching of free radicals by *Neo-T. siphonanthum* methanolic extract was assayed spectrophotometrically at 517 nm against the absorbance of the stable free DPPH radical. The free radical scavenging efficiency of the compounds was determined by decoloration of the DPPH radical. In brief, reaction mixtures contained various concentrations of the test compounds, which were dissolved in DMSO and DPPH (0.4 mg/mL)

dissolved in methanol. Negative and positive controls of the experiment were established by a methanolic solution of DPPH and quercetin (free radical scavenger), respectively. The absorbance was measured at 517 nm after incubating the mixture at 37°C for 30 min. The antiradical activity was expressed as IC_{50} (antiradical dose required to cause a 50% inhibition), which was calculated by the following formula: $[(A_{blank} - A_{sample})/A_{sample}] \times 100$, where A_{blank} is the absorbance of the DPPH radical solution and A_{sample} is the absorbance of the DPPH radical solution after the addition of the sample.[17]

Instrumental

HPLC Conditions

The analytical HPLC setup consisted of two LC-8A pumps, a Prominence SPD-M20A DAD performing the wavelength scanning from 190 to 800 nm, a manual injection valve with a 20-μl loop, and an LC Solution workstation (Shimadzu, Kyoto, Japan). The target compounds were separated by using a reverse-phase Zorbax SB C_{18} (250 mm × 4.6 ID, 5 μm, Agilent Technologies, Wilmington, DE) column and a security guard C_{18} ODS (4.0 × 3.0 mm ID) from Phenomenex (Torrance, CA). The mobile phase consisted of 0.1% aqueous acetic acid (solvent A) and methanol (solvent B) in a gradient elution mode. The gradient program was as follows: 20% B to 50% B (10 min), 50% B isocratic (10 min), 50% B to 60% B (10 min), 60% B to 70% B (5 min), 70% B isocratic (15 min). Total run time was 50 min. The flow rate was kept at 1.0 mL/min, while the ambient temperature was controlled at 20°C by an air conditioner. Spectra were recorded from 200 to 400 nm (peak width 0.2 min and data rate 1.25 s^{-1}) while the chromatogram was acquired at 254 nm.

HPLC-DAD-RSD-ESI-MS/MS Instrumentation

An online HPLC-DAD-RSD-MS/MS method has been described for a rapid screening and identification of radical scavengers by using a methanolic solution of DPPH stable free radicals.[14] The analytical HPLC analysis was conducted on an Acquity UPLC system (Waters Corp., Milford, MA). The stationary phase and the elution gradient were the same as described in the HPLC analysis. The crude extract was dissolved in methanol and injected into the HPLC system. Before being delivered into the system the solvent was filtered through a 0.45-μm PTEE filter and degassed using vacuum. The flow rate was 1.0 mL/min at 20°C. The column eluent was split at a ratio of 4:1 using an adjustable high-pressure stream splitter (Supelco Port, Bellefonte, PA); the lower one was introduced into a Micromass Quattro micro mass spectrometer with an ESI interface, while a Masslynx

4.1 data system was equipped with a QuanLynx program. Negative ion mode for ESI-MS was selected. Mass detection was performed in full scan mode for m/z in the range 100–800. MS/MS product ions were produced by collision-induced dissociation (CID) of the selected precursor ions in the collision cell of the triple-quardrupole mass spectrometer. The following settings were applied to the instrument: Capillary and cone voltage were 2500 V and 40 V, respectively; nebulizer nitrogen gas flow rate was 500 L/h; and the ionization sources worked at 120°C. The desolvation temperature was 450°C. The larger flow was again split into two streams using an adjustable high-pressure stream splitter. One part (0.2 mL/min) was used for the radical scavenging detection. The length of the capillary used for the postcolumn reaction was adjusted to achieve a reaction time of 0.6 min. The antioxidants reacted post-column with the DPPH radical at a concentration of 50 mg/L in methanol. The flow of the DPPH radical solution was set to 0.4 mL/min. The DPPH radical-scavenging detection chromatogram is detected as a negative peak at 517 nm with a variable-wavelength PC300 detector and the chromatogram was accordingly recorded on a model SCJS-3000 workstation (Tianjin Scientific Instrument Ltd., Tianjin, China). The other part (0.6 mL/min) was continuously monitored by a 2487 dual-wavelength UV detector in the range of 200–400 nm, and the chromatograms were recorded at 254 nm.

Preparative HPLC Experiments

The preparative HPLC experiments were performed on a self-assembled instrument, which was composed of a P3000 delivery pump, a 2PB00C sample injection pump, a UV3000 variable wavelength detector with detection monitored at 254 nm, and an SCJS-3000 ChemStation. A preparative column (500 mm × 80 mm ID) packed with 5 μm ODS C_{18}(Fuji, Japan) was used for the preparative separation. The flow rate was 25 mL/min at 20°C.

HSCCC Separation

HSCCC, first invented by Ito,[18] is a support-free liquid–liquid chromatographic technique with no solid support matrix, and separation is based on fast partitioning effects of the compounds between two immiscible liquid phases. This method has been successfully applied to separation and isolation of natural metabolites.[19–22]

The preparative HSCCC was performed on a seal-free high-speed countercurrent chromatography by Prof. Qizhen Du (Institute of Food and Biological Engineering, Zhejiang Gongshang University, Hangzhou, China). The apparatus was equipped with one PTFE layer coil column with an average ID of 2.6 mm and a total volume of 420 mL. The column

revolves around its own axis at the angular velocity in the same direction. The revolution speed of the apparatus could be regulated between 0 and 1000 rpm. The revolution radius or the distance between the holder axis and central axis of the centrifuge was 8 cm, and the β value of the coils from the inner layer to the outer layer is 0.50–0.79;. $\beta = r/R$, where r is the distance from the coil to the holder shaft and R is the revolution radius or the distance between the holder axis and central axis of the centrifuge. The solvent was pumped into the tubing with an FMI pump (Zhejiang Instrument Factory, Hangzhou, China). The effluent was continuously monitored with a variable-wavelength PC300 detector at 254 nm and the chromatogram with a model SCJS-3000 workstation (Tianjin Scientific Instrument Ltd., Tianjin, China). A manual sample injection valve with a 10-mL loop was adjusted to the system. The suitable solvent systems were evaluated by HPLC according to the partition coefficients.

HSCCC was performed as follows: The multilayer coiled column was first entirely filled with the upper phase. The lower mobile phase was then pumped into the inlet of the column at the flow rate of 1.5 mL/min, while the apparatus was run at 800 rpm. After hydrodynamic equilibrium was reached, indicated by a clear mobile phase eluting at the tail outlet, a sample dissolved in 10 mL of the upper phase was injected into the injection valve. The effluent from the outlet of the column was continuously monitored with a UV detector at 254 nm and the peak fractions were collected manually according to the chromatographic profile. After target compounds were eluted, the centrifuge was stopped and the column contents were fractionated by continuously eluting the column with the mobile phase. The effluent was collected for purity analysis.

Preparative Isolation of Radical Scavenging Compounds

The extraction and isolation procedures were guided by the HPLC–DAD–RSD–ESI–MS/MS screening assay. The pulverized material of *Neo-T. siphonanthum* (5.0 kg) was exhaustively extracted with methanol under reflux for 8 hr and concentrated under reduced pressure to give a brown syrup (509 g). A mass of 500 g syrup was suspended in water and was carried out by open column chromatography over C_{18} (2.0 kg, 40 μm, Elite Co., Dalian, China) using H_2O–MeOH (1:0–0:1) mixtures as gradient elution solvent. Four main fractions were collected (F_A, 10% methanol elution; F_B, 30% methanol elution; F_C, 50% methanol elution; F_D, 70% methanol elution) according to the thin-layer chromatography. F_A was further purified by preparative HPLC. In the preparative HPLC, a linear gradient elution of solvents A (0.1% aqueous acetic acid) and B (methanol) was used. Gradient solution started with 20% B for 5 min, reaching 25% after

30 min, and then 25% B for 25 min to afford pure compounds 1 (7 mg), 2 (9 mg), 3 (38 mg), and 4 (6 mg). F_B was chromatographed over Sephadex LH-20, eluting with MeOH to give four subfractions (F_{B-1}–F_{B-4}). Subfraction F_{B-2} and F_{B-4} were recrystallized to achieve compounds 11 (10 mg) and 6 (15 mg), respectively. Subfraction F_{B-1} was performed by HSCCC with a two-phase solvent system composed of ethyl acetate–*n*-butanol–water (2:1:3, v/v/v) to obtain four compounds, compounds 5 (9 mg), 7 (18 mg), 8 (11 mg), and 9 (8 mg). Subfraction F_{B-3} was also subjected to HSCCC with a biphasic solvent system composed of *n*-hexane–*n*-butanol–water (1:1:2, v/v) to give two compounds, compounds 10 (12 mg) and 12 (10 mg). F_C was separated by Sephadex LH-20, and then the eluent, mainly consisting of compound 14, was recrystallized with MeOH to give 18 mg of 14. F_D was separated by preparative HPLC, and the elution run with 50% B for 30 min, linear gradient from 50% B to 70% B in 30 min, which remained at 70% B for 20 min, and then was left to reach 90% B for 25 min, affording pure compounds 13 (9 mg), 15 (6 mg), 16 (10 mg), 17 (6 mg), and 18 (7 mg). The chromatographic purities of all separated compounds were determined by HPLC as higher than 97% for all individual compounds.

Characterization of Target Compounds

Characterization of the target compounds was accomplished by their spectroscopic spectra, mass data, and NMR spectra. The NMR experiments were performed on a Varian INOVA-400 NMR spectrometer (Varian Corporation, Palo Alto, CA). The reference compound TMS was used as the internal standard for the determination of chemical shifts.

RESULTS AND DISCUSSION

DPPH Radical Scavenging Activity of Methanolic Extract

DPPH is a free radical compound and has been used widely to test the free radical scavenging ability of various samples. In the present study, the antioxidant activity of the *Neo-T. siphonanthum* methanolic extract was assessed by the ability to scavenge DPPH radical, and its IC_{50} value was 58.3 μg/mL. The result implies that there are abundant radical scavengers present in the methanolic extract of *Neo-T. siphonanthum*.

Selection of Suitable Chromatographic Conditions

The separation of all compounds in a complex extract is one of the challenging tasks in analytical HPLC. According to our preliminary test, the methanolic extract of *Neo-T. siphonanthum* contained abundant

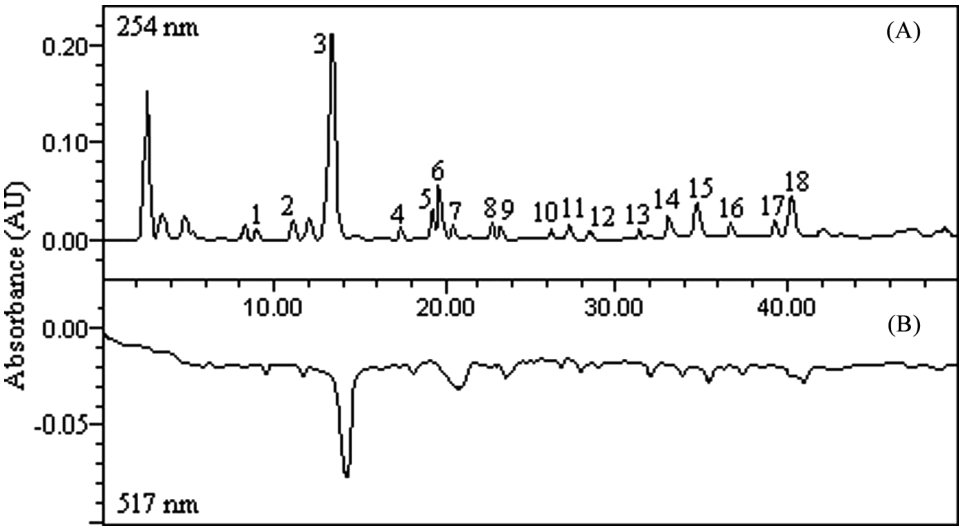

FIGURE 1 (A) HPLC-UV (254 nm) and (B) DPPH radical scavenging detection profile of *Neo-T. siphonanthum* methanolic extract. Peaks 1–18, see Figure 2.

flavonoids and phenolic acids. For HPLC–ESI–MS/MS, the compositions of the mobile phase were limited, where phosphate buffer solutions and extreme pH conditions should be avoided for preventing hydrolysis during the sample process. In the course of optimizing the conditions of separation, the system conditions including the mobile phase (methanol–acetic acid, acetonitrile–water, and different concentrations of acetic acid in water were compared to get the most suitable mobile phase), gradient program (gradient time, gradient shape, and initial composition of the mobile phase), column temperature, and detection wavelength (relatively higher absorption) were investigated. The final results showed that best resolution and shortest analysis time were achieved when a gradient elution mode composed of solvent A 0.1% acetic acid and solvent B methanol was used in the following linear gradient combination: 20% B to 50% B (10 min), 50% B isocratic (10 min), 50% B to 60% B (10 min), 60% B to 70% B (5 min), 70% B isocratic (15 min). Total run time was 50 min. The flow rate was 1.0 mL/min, the column temperature was set at 20°C, and 254 nm was selected as the detection wavelength. Under the optimum gradient elution, all the compounds in methanolic extract of *Neo-T. siphonanthum* reached baseline separation (Figure 1A).

Online HPLC–DAD–RSD–ESI–MS/MS Method

The online coupling of separation and activity determination techniques can be used for a rapid and selective assessment of radical scavenging

substances in complex mixtures, particularly plant extract with a minimum of sample preparation. The more rapid is the absorbance decrease, the stronger will be the ability of a specific compound to be hydrogen donating, and so to be potenct as an antioxidant agent.

The methanolic extract of *Neo-T. siphonanthum* has been assessed for the free radical scavenging activity in online HPLC–DAD–RSD–ESI–MS/MS assay. As shown in Figure 1, at least 18 eluted constituents were detected and gave positive peaks on the UV detector (254 nm) and negative peaks on the DPPH quenching chromatogram (517 nm). The retention times and UV and MS/MS data of the antioxidants obtained by HPLC–DAD–RSD–ESI–MS/MS are presented in Table 1.

Structure Elucidation of Antioxidants

The combination of UV and MS/MS spectra allowed tentative identification of target compounds in crude extracts. Compounds 1–4 and 6, with similar UV spectra, which comprised the strong caffeoyl absorption bands with maxima around 220–226, 246–252 (sh), 296–300 (sh), and 320–330 nm, could be identified as caffeic acid or its esters.[23] Compound 3 gave an $[M\text{-}H]^-$ ion at m/z 179 and a characteristic fragment at m/z 135 due to the loss of CO_2, and corresponded to caffeic acid. The MS analysis of compound 6 showed an $[M\text{-}H]^-$ ion at m/z 193, a major fragment in MS^2 at m/z 149 (loss of CO_2), and a characteristic fragmentation in MS^2 at m/z 178 (loss of a methyl moiety), all of which suggested compound 6 was methyl caffeic acid. On the basis of the evidence and by comparison with the HPLC retention time of standard compound, compound 6 was ascribed to ferulic acid. Compound 1 showed the loss of a tartaric acid moiety (132 atomic mass unit) and a peak at m/z 179 in the MS^2 experiment, which corresponded to caftaric acid. However, the absolute configuration of compound 1 could not be confirmed only by MS/MS experiment. Compounds 2 and 4 presented an $[M\text{-}H]^-$ ion at m/z 353 in accordance with a molecular formula $C_{16}H_{18}O_9$, and the fragmentation in the MS^2 experiment produced a caffeic acid (m/z 179) as base peak and quinic acid moiety (m/z 191) as a weak secondary fragment ion peak, which was suggested that compounds 2 and **4** were isomers of monocaffeoylquinic acid. Compound 2 was unambiguously identified as chlorogenic acid by comparison of its liquid chromatography (LC)–DAD–MS/MS spectra with those of the standard compound. Moreover, compound 4 produced a dehydrated quinic acid moiety (m/z 173) in the MS^2 experiment, which was the characteristic fragmentation behavior of 4-*O*-caffeoylquinic acid.[24]

Compounds 5, 7–15, and 17 had two maximum absorption bands at 250–270 nm and 340–370 in UV spectra, which were the typical spectra of flavonoid derivatives. In the hydrolysis experiment, the major aglycones

TABLE 1 Spectral Data of the Main Antioxidants Acquired by HPLC–DAD–RSD–ESI–MS/MS and Offline NMR From Methanolic Extract of *Neo-T. siphonanthum*

		UV by HPLC-DAD	Ion full scan MS		^{1}H NMR signal	
	t_R (min)	(λ_{max}, nm)	$[M-H]^-$	MS/MS	Chemical shift (ppm) multiplicity (coupling constant J (Hz))	Substance
					Caffeic acid derivatives	
1	9.06	219, 300 sh, 325	311	149, 179	7.64 (1H, d, J=16.0 Hz, H-7′), 7.15 (1H, d, J=2.0 Hz, H-2′), 7.05 (1H, dd, J=8.4, 2.0 Hz, H-6′), 6.88 (2H, d, J=8.2 Hz, H-5′), 6.37 (1H, d, J=16.0 Hz, H-8′), 5.40 (1H, d, J=2.4 Hz, H-2), 4.75 (1H, d, J=2.4 Hz, H-3)	*trans*-Caftaric acid
2	11.14	220, 244, 300 sh, 330	353	179, 191	7.43 (1H, d, J=16.0 Hz, H-7′), 7.03 (1H, d, J=2.0 Hz, H-2′), 6.98 (1H, dd, J=8.4, 2.0 Hz, H-6′), 6.76 (2H, d, J=8.2 Hz, H-5′), 6.15 (1H, d, J=16.0 Hz, H-8′), 5.10 (1H, ddd, J=10.1, 9.8, 4.6 Hz, H-3), 3.95 (1H, m, H-5), 3.57 (1H, m, H-4), 2.04 (1H, m, H-6a), 2.00 (2H, m, H-2), 1.81 (1H, m, H-6b)	Chlorogenic acid
3	13.23	219, 243, 300 sh, 332	179	135	7.14 (1H, d, J=16.0 Hz, H-7), 7.02 (1H, d, J=2.0 Hz, H-2), 6.95 (1H, dd, J=8.0, 2.0 Hz, H-6), 6.76 (1H, d, J=8.0 Hz, H-5), 6.15 (1H, d, J=16.0 Hz, H-8)	Caffeic acid
4	17.28	220, 239, 304 sh, 325	353	173, 179, 191	7.60 (1H, d, J=16.0 Hz, H-7′), 7.04 (1H, d, J=2.0 Hz, H-2′), 6.95 (1H, dd, J=8.4, 2.0 Hz, H-6′), 6.77 (2H, d, J=8.2 Hz, H-5′), 6.30 (1H, d, J=16.0 Hz, H-8′), 4.90 (1H, m, H-5), 4.73 (1H, m, H-3), 4.27 (1H, m, H-4), 2.40 (2H, m, H-6a), 2.14 (2H, m, H-2)	4-*O*-caffeoylquinic acid
6	19.72	219, 233, 300 sh, 321	193	178, 149	7.49 (1H, d, J=16.0 Hz, H-7), 7.27 (1H, d, J=1.6 Hz, H-2), 7.09 (1H, dd, J=8.4, 2.0 Hz, H-6), 6.80 (1H, d, J=8.0 Hz, H-5), 6.35 (1H, d, J=16.0 Hz, H-8), 3.82 (3H, s, 3-OCH_3)	Ferulic acid
					Flavonoid derivatives	
5	19.28	257, 356	433	301	7.60 (1H, dd, J=8.4, 2.0 Hz, H-6′), 7.50 (1H, d, J=2.0 Hz, H-2′), 6.86 (1H, J=8.4 Hz, H-5′), 6.43 (1H, d, J=2.0 Hz, H-8), 6.20 (1H, d, J=2.0 Hz, H-6), 5.28 (1H, d, J=5.2 Hz, H-1”), 3.2–4.0 (5H, m)	Quercetin-3-*O*-α-D-arabinopyranoside
7	20.26	258, 357	463	301	7.56 (1H, dd, J=8.4, 2.0 Hz, H-6′), 7.49 (1H, d, J=2.0 Hz, H-2′), 6.87 (1H, J=8.4 Hz, H-5′), 6.40 (1H, d, J=2.0 Hz, H-8), 6.19 (1H, d, J=2.0 Hz, H-6), 5.43 (1H, d, J=7.5 Hz, H-1”), 3.1–3.9 (6H, m)	Quercetin-3-*O*-β-D-glucopyranoside

8	22.95	258, 355	433	301	7.57 (1H, dd, J=8.4, 2.0 Hz, H-6′), 7.49 (1H, d, J=2.0 Hz, H-2′), 6.87 (1H, J=8.4 Hz, H-5′), 6.45 (1H, d, J=2.0 Hz, H-8), 6.21 (1H, d, J=2.0 Hz, H-6), 5.58 (1H, d, J=1.2 Hz, H-1”), 3.2–4.2 (5H, m)	Quercetin-3-*O*-α-D-arabinofuranoside
9	23.48	256, 358	447	301	7.43 (1H, d, J=2.0 Hz, H-2′), 7.37 (1H, dd, J=8.0, 2.0 Hz, H-6′), 6.96 (1H, J=8.0 Hz, H-5′), 6.45 (1H, d, J=2.2 Hz, H-8), 6.26 (1H, d, J=2.2 Hz, H-6), 5.45 (1H, s, H-1”), 3.2–4.2 (4H, m), 0.89 (3H, d, J=5.4 Hz, CH_3)	Quercetin-3-*O*-α-L-rhamnoside
10	26.29	255, 268, 339	445	285	7.90 (1H, dd, J=8.4, 2.0 Hz, H-6′), 7.51 (1H, d, J=2.0 Hz, H-2′), 7.49 (1H, d, J=8.0 Hz, H-5′), 7.01 (1H, d, J=2.0 Hz, H-8), 6.83 (1H, s, H-3), 6.82 (1H, d, J=2.0 Hz, H-6), 5.80 (1H, d, J=7.2 Hz, H-1″), 3.35–4.65 (5H, H-2″-H-6″)	Luteolin-3′-*O*-β-D-glucopyranoside
11	27.41	253, 268, 345	445	285	12.96 (1H, br s, 5-OH), 7.44 (1H, dd, J=8.4, 2.0 Hz, H-6′), 7.42 (1H, d, J=2.0 Hz, H-2′), 6.91 (1H, d, J=8.0 Hz, H-5′), 6.77 (1H, d, J=2.0 Hz, H-8), 6.72 (1H, s, H-3), 6.45 (1H, d, J=2.0 Hz, H-6), 5.07 (1H, d, J=7.2 Hz, H-1″), 3.73 (1H, dd, J=10.0, 2.8 Hz, H-6″a), 3.51 (1H, t, J=8.5 Hz, H-3″), 3.42 (1H, dd, J=10.0, 2.8 Hz, H-6″b), 3.39 (1H, t, J=8.5 Hz, H-2″), 3.28 (1H, t, J=8.5 Hz, H-4″), 3.19 (1H, m, H-5″)	Luteolin-7-*O*-β-D-glucopyranoside
12	28.56	255, 268, 342	445	285	12.95 (1H, br s, 5-OH), 7.55 (1H, d, J=2.0 Hz, H-2′), 7.51 (1H, dd, J=8.4, 2.0 Hz, H-6′), 7.23 (1H, d, J=8.0 Hz, H-5′), 6.82 (1H, s, H-3), 6.50 (1H, d, J=2.0 Hz, H-8), 6.20 (1H, d, J=2.0 Hz, H-6), 4.89 (1H, d, J=7.2 Hz, H-1″), 3.11–3.79 (6H, H-2″-H-6″)	Luteolin-4′-*O*-β-D-glucopyranoside
13	31.42	254, 370	301	–	12.45 (1H, br s, 5-OH), 7.68 (1H, d, J=2.0 Hz, H-2′), 7.54 (1H, dd, J=8.4, 2.0 Hz, H-6′), 6.89 (1H, J=8.4 Hz, H-5′), 6.41 (1H, d, J=2.0 Hz, H-6), 6.20 (1H, d, J=2.0 Hz, H-8)	Quercetin
14	33.17	267, 333	591	445, 283	12.89 (1H, br s, 5-OH), 8.04 (2H, dd, J=9.0, 2.0 Hz, H-6′ and H-2′), 7.15 (2H, dd, J=9.0, 2.0 Hz, H-3′ and H-5′), 6.92 (1H, s, H-3), 6.80 (1H, d, J=2.0 Hz, H-8), 6.47 (1H, d, J=2.0 Hz, H-6), 5.07 (1H, d, J=7.2 Hz, H-1″), 4.58 (1H, br s, H-1‴), 3.88 (3H, s, 7-OCH_3), 3.20–3.60 (6H, m, H-2″ to H-6″), 3.20–3.60 (3H, m, H-2‴ to H-6‴), 2.51 (1H, d, J=6.0 Hz, H-5‴), 1.10 (3H, d, J=6.0 Hz, H-6‴)	Genkwanin-4′-*O*-β-D-rutinoside

(*Continued*)

TABLE 1 Continued

		UV by HPLC-DAD	Ion full scan MS		^{1}H NMR signal	
t_R (min)		(λ_{max}, nm)	$[M-H]^-$	MS/MS	Chemical shift (ppm) multiplicity (coupling constant J (Hz))	Substance
15	34.75	254, 349	285	–	12.94 (1H, br s, 5-OH), 7.41 (1H, dd, J = 8.4, 2.0 Hz, H-6′), 7.39 (1H, d, J = 2.0 Hz, H-2′), 6.89 (1H, d, J = 8.4 Hz, H-5′), 6.64 (1H, s, H-3), 6.44 (1H, d, J = 2.0 Hz, H-8), 6.19 (1H, d, J = 2.0 Hz, H-6)	Luteolin
16	36.75	233, 289, 324 sh	317	299	7.08 (1H, d, J = 2.0 Hz, H-2′), 7.03 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 6.96 (1H, d, J = 8.0 Hz, H-5′), 5.99 (1H, d, J = 2.0 Hz, H-8), 5.96 (1H, d, J = 2.0 Hz, H-6), 5.09 (1H, d, J = 11.2 Hz, H-2), 4.64 (1H, d, J = 11.2 Hz, H-3), 3.86 (3H, s, 4′-OCH_3)	(2 R,3 R)-(+)-4′-*O*-methyldihydro-quercetin
17	39.29	256, 351	283	269	12.89 (1H, br s, 5-OH), 8.00 (2H, dd, J = 9.0, 2.0 Hz, H-6′ and H-2′), 7.08 (2H, dd, J = 9.0, 2.0 Hz, H-3′ and H-5′), 6.84 (1H, s, H-3), 6.48 (1H, d, J = 2.0 Hz, H-8), 6.18 (1H, d, J = 2.0 Hz, H-6), 3.84 (3H, s, 7-OCH_3)	Genkwanin
18	40.32	230, 291,325 sh	331	313	7.09 (1H, d, J = 2.0 Hz, H-2′), 7.03 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 6.98 (1H, d, J = 8.0 Hz, H-5′), 6.06 (1H, d, J = 2.0 Hz, H-8), 6.04 (1H, d, J = 2.0 Hz, H-6), 5.09 (1H, d, J = 11.2 Hz, H-2), 4.67 (1H, d, J = 11.2 Hz, H-3), 3.87 (3H, s, 4′-OCH_3), 3.86 (3H, s, 7-OCH_3)	(2 R,3 R)-(+)-4′,7-di-*O*-methyldihydro-quercetin

of flavonoids were identified as quercetin, luteolin, and genkwanin by comparison of retention times and UV and mass spectra with those of the standard compounds. Compounds 5 and 7–9 produced a prominent ion at m/z 301 in MS^2, which allowed the identification of these compounds as quercetin-*O*-glycoside, and the characteristic UV absorption showed that the 3-position in compounds 5 and 7–9 was glycosylated. Compounds 5 and 8 showed the same $[M-H]^-$ ion at m/z 433, which corresponded to the loss of one pentose moiety. Compound 7 showed an $[M-H]^-$ ion at 463, which can reveal a quercetin glycoside with a hexose moiety. The MS spectrum of compound 9 was characterized by a negative ion signal at m/z 447; thus, compound 9 could be tentatively identified as quercetin-3-*O*-rhamnoside. Therefore, compounds 5 and 7–9 only differed in the category of saccharide moiety. Compounds 10–12 showed the same $[M-H]^-$ ion at 445 with the same fragmentation pattern (m/z 285), which corresponds to the loss of a hexose molecule. Compound 11 had already been identified as luteolin-7-*O*-*β*-D-glucopyranoside by comparison with the standard compound. Compounds 10 and 12 were tentatively identified as luteolin-3′-hexoside and luteolin-4′-hexoside, respectively, according to the comparison of their relative retention times in HPLC and characteristic UV and MS spectra with those of compound 11.[24] Compound 14 presented an $[M-H]^-$ ion at 591, and the MS^2 revealed the loss of 308 atomic mass unit, which resulted from a disaccharide moiety composed of a hexose and a deoxyhexose unit (rutinose). Consequently, this compound might be genkwanin-4′-*O*-rutinoside, which was testified by injecting the standard compound.

Compounds 16 and 18 had their maximum UV absorption around 290 and 325 (sh) nm, which suggested that these two compounds were flavanone analogues.[25] The characteristic fragment $[M\text{-}H\text{-}18]^-$ in the MS^2 experiment was due to the loss of water from the molecules. All these clearly indicated that compounds 16 and 18 were dihyroflavonol derivatives.

Compounds could not be exactly assigned by UV and MS/MS experiments because the availability of standard compounds is limited. For unambiguous identification, further studies are required by using standard compounds or their offline NMR spectra, which can determine the category of the substituent groups such as glycosyl and the position for isomers by analyzing their distinctive spin–spin coupling patterns in ^{1}H-NMR spectra and the correlation signal in two-dimensional (2D) NMR. The structure of all the antioxidants were finally established by comparison their spectral data with literatures: *trans*-caftaric acid (1), chlorogenic acid (2), caffeic acid (3), 4-*O*-caffeoylquinic acid (4), quercetin-3-*O*-α-D-arabinopyranoside (5), ferulic acid (6), quercetin-3-*O*-*β*-D-glucopyranoside (7), quercetin-3-*O*-α-D-arabinofuranoside (8), quercetrin-3-*O*-α-L-rhamnoside (9), luteolin-3′-*O*-*β*-D-glucopyranoside (10), luteolin-7-*O*-*β*-D-glucopyranoside (11), luteolin-4′-*O*-*β*-D-glucopyranoside (12), quercetin (13), genkwanin-4′-*O*-*β*-D-rutinoside

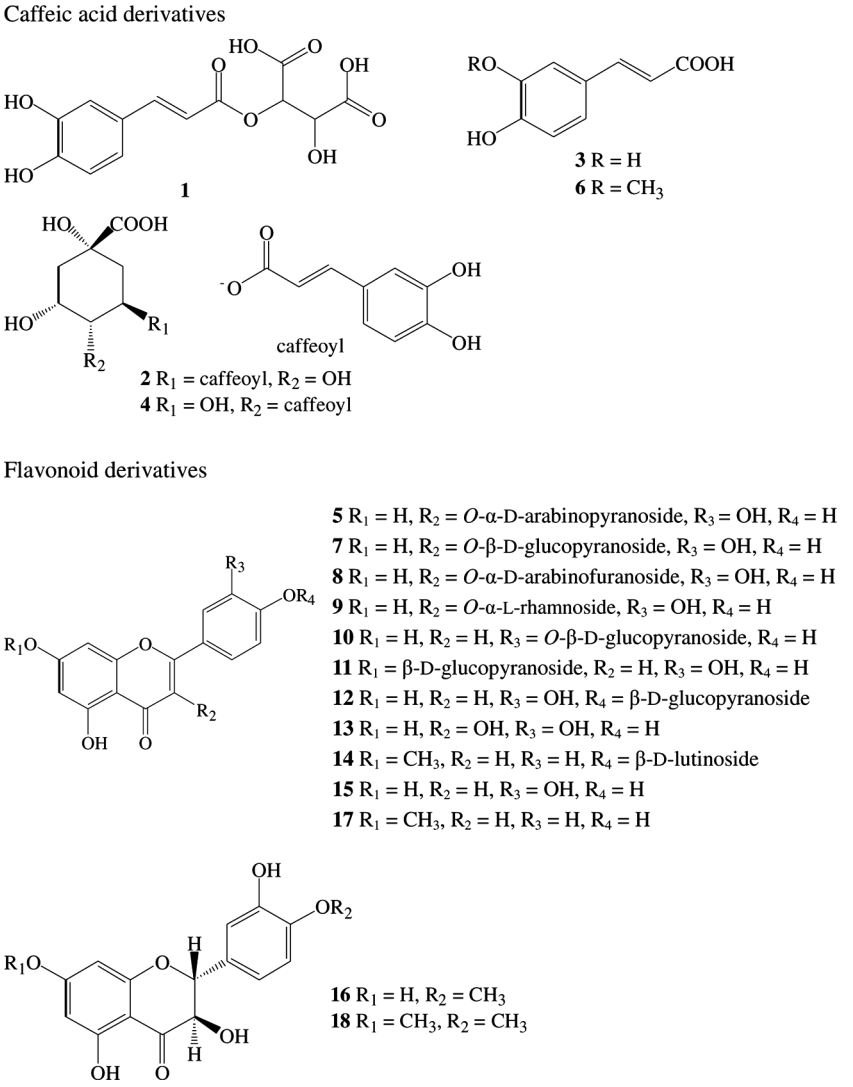

FIGURE 2 Structures of the identified radical scavengers.

(14), luteolin (15), (2R,3R)-(+)-4′-*O*-methyldihydroquercetin (16), genkwanin (17), and (2R,3R)-(+)-4′,7-di-*O*-methyldihydroquercetin (18) (Figure 2). All of the isolated compounds have been reported to have significant DPPH radical scavenging activity.[26–31]

CHEMOTAXONOMIC SIGNIFICANCE

All the compounds were isolated from this species for the first time. Among them, compound 3 was found to be the major metabolite, which

has ever been found as the major compound of many *Taraxacum* species.[32–35] Furthermore, compounds 2, 6, 11, 13, and 15 have also been found to be present in many *Taraxacum* species.[15,29–34] On the other hand, compounds 1, 10, 12, 14, and 17 are comparatively rare, and compounds 1, 10, and 12 have only been yielded in *Taraxacum officinale*,[24,36] while compounds 14 and 17 have ever achieved from *Taraxacum mongolicum*.[15] The preceding results were very interesting and suggested that the genera *Neo-Taraxacum* and *Taraxacum* may related chemosystematically. However, compounds 4–5, 7–9, 16, and 18, to the best of our knowledge, have not been found in the *Taraxacum* genus, which has been described from some plants of family Asteraceae.[37–43] Therefore, the isolation of compounds 4–5, 7–9, 16, and 18 could have a chemotaxonomic significance for this genus.

CONCLUSION

This work is the first report on the identification of radical scavengers from *Neo-T. siphonanthum*. An on-line HPLC–DAD–RSD–ESI–MS/MS coupled with an offline NMR experiment has been developed for the rapid screening and identification of radical scavengers from methanolic crude extracts of *Neo-T. siphonanthum*. Eighteen radical scavengers were screened and characterized. The hyphenated techniques used herein provided a rapid and selective detection of natural antioxidants in complex matrices with simple operation. Furthermore, the chemotaxonomic analysis suggested that the genera *Neo-Taraxacum* and *Taraxacum* might relate chemosystematically. Therefore, the results of the present study may be helpful for further elucidation of the medicinal potential of *Neo-T. siphonanthum*.

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