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Antioxidant Activity of Chemical Constituents Isolated from *Pithecellobium clypearia*

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Abstract: Phytochemical investigation of the aqueous extract of *Pithecellobium clypearia* afforded one novel compound, clypearoside A (**1**), and one new stereoisomer, (-)-(2S,3S)-epigallocatechin-7-gallate (**2**) and four known ones (**3-6**). Their structures were elucidated on the basis of spectroscopic data, especially 2D NMR, HRESIMS and ECD spectra. Compounds **2-4** showed strong activity against DPPH (diphenylpicrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) with an IC_{50} value of 11.9-13.2 μg/mL (DPPH) and 4.5-6.4 μg/mL (ABTS).

Keywords: *Pithecellobium clypearia*; DPPH and ABTS radical scavenging assay; ECD spectra.

1. Introduction

The genus *Pithecellobium* (Leguminosae family) consists of about 100 species with many more subspecies, widely distributed in Asia and Tropical America. *Pithecellobium clypearia* Benth (Chinese patent medicine), well-known as a herbal medicine for the treatment of respiratory tract diseases in southern China, is recorded in the Pharmacopeia of China [1,2]. The aqueous extract of the leaves and stems of *P. clypearia* has been used for the treatment of upper respiratory tract infections, pharyngitis, laryngitis, acute tonsillitis, acute gastroenteritis, and bacterial dysentery [3,4]. Previous investigations of the chemical components of *P. clypearia* have indicated that flavanes are important secondary metabolites of this plant [5-7].

In order to clarify the active ingredients of *P. clypearia*, aqueous extracts of the leaves and stems of *P. clypearia* were subjected to phytochemical investigation resulting in the isolation of a novel ionone derivative (**1**) and one new stereoisomer of galloyl-substituted flavanol (**2**) (Fig. 1),

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together with four known compounds. This paper describes the details of the isolation and structure elucidation of the chemical constituents **1-6** (Fig. 1) including their physicochemical properties and spectral data, such as NMR, MS and ECD spectra. Furthermore, their antioxidant activity was also tested using a DPPH and ABTS radical scavenging assay.

2. Materials and Methods

2.1. General

Mass spectra were determined on a HRESI-MS: MicroTOF spectrometer (Bruker Daltonics, CA). NMR spectra were recorded on a Bruker ARX-300 (1D) and ARX-600 (2D) spectrometer with TMS as an internal standard in MeOD or dimethyl sulfoxide-d_6 (DMSO-d_6). Silica gel (200 -300 mesh, Qingdao Marine Chemical Co., China), Polyamide (200 -400 mesh, Qingdao Marine Chemical Co., China), MCI gel (CHP20P, 75 -150 μm, Mitsubishi Chemical Corporation, Japan), Sephadex LH-20 (25-100 μm, Green Herbs Science and Technology Development Co., Ltd. China) and reversed-phase C_{18} silica gel (ODS 60 -80 μm, Merck, Germany) were used for column chromatography and silica gel GF254 (Qingdao Marine Chemical Co., China) was used for TLC. In addition, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), diphenylpicrylhydrazyl (DPPH) and Trolox as a standard were all purchased from Sigma–Aldrich (Steinheim, Germany) in order to screen the antioxidant activities of the isolated compounds. All solvents were of industrial purity and distilled prior to use**.**

2.2. DPPH radical scavenging activity assay

The DPPH radical scavenging activity of compounds**1-6** was measured using a modified established method [8]. A series of different concentrations of the samples in ethanol (0.1 mL) were added to a 96-well plate followed by 0.2 mM DPPH (0.1 mL). The mixture plate was shaken vigorously and then immediately incubated in the dark for 30 min. The absorbance of the reaction solution was determined using a Bio-Tek microplate reader (Bio-Tek, Winooski,VT) at an absorbance of 517nm. All samples, standards, and controls were run in triplicate. Trolox, a stable antioxidant, was used as a positive reference. The DPPH radical-scavenging activity in percentage of sample was calculated as follows:

$$\text{DPPH scavenging activity}\ (\%) = \left(1-\frac{S - S_B}{C - C_B}\right)\times 100\%$$

Where S, S_B, C and C_B are the absorbancies of the sample, the blank sample, the control and the blank control, respectively.

2.3. ABTS radical scavenging activity assay

The antioxidant capacity of compounds**1-6** was also evaluated using an improved ABTS$^{\bullet+}$ decolourisation assay [9]. An ABTS radical cation (ABTS$^{\bullet+}$) was produced by reacting a 7mM stock solution of ABTS with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 16h before use. The ABTS$^{\bullet+}$ solution was diluted with ethanol, to an absorbance of 0.7±0.02 at 734nm. Then, an ethanolic solution (0.1mL) of the samples or Trolox standard at various concentrations was mixed with 0.15mL diluted ABTS$^{\bullet+}$ solution. After reaction at room temperature for 20 min, the absorbance was measured at 734 nm using a Bio-Tek microplatereader. The capability to scavenge ABTS$^{\bullet+}$ was calculated using the formula given below:

$$\text{ABTS}^{+}\ \text{scavenging activity}\ (\%) = \left(1-\frac{S - S_B}{C - C_B}\right)\times 100\%$$

Where S, SB, C and CB are the absorbancies of the sample, the blank sample, the control, and the blank control, respectively. Tests were performed in triplicate.

2.4. Plant material

The leaves and stems of *P. clypearia* were collected from Guangdong province, P. R. China, in June 2011, and authenticated by Prof. Jin-Cai Lu, Department of Pharmacognosy, Shenyang Pharmaceutical University. A voucher specimen (No. PCGD1106) is kept in the Nature Products Laboratory of Shenyang Pharmaceutical University, Shenyang, P. R. China.

2.5. Extraction and isolation

The leaves and stems of the *P. clypearia* (5.5 kg) were extracted with water (50 L) by refluxing (100 ℃, 2 h) three times to give 450 g of crude extract, which was subjected to the macroporous resin (0.20−0.80 mm, 10 kg) column chromatography involving elution with EtOH–H_2O (0:100, 20:80, 40:60, 60:40, 80:20 and 95:5, v/v) to yield six fractions 1−6. Fraction 4 (116 g) was further separated by silica gel column chromatography involving elution with a CH_2Cl_2–MeOH gradient system (100:1, 60:1, 30: 1, 20:1, 10:1 and 0: 1, v/v) to obtain five subfractions 4a–4e. Subfraction 4b (7 g) was subjected to polyamide column chromatography using MeOH–H_2O (0:100→100:0, v/v) and purified by ODS silica gel CC using MeOH–H_2O (30:70→100:0, v/v) to give a mixture of two major compounds, which was finally purified by semi-preparative HPLC (CH_3CN/H_2O 52:48, v/v) to yield compounds **5** (15 mg) and **6** (27 mg). Subfraction 4c (7 g) was subjected to further separation by silica gel column chromatography eluting with a CH_2Cl_2–MeOH gradient system (40:1, 20:1, 10:1 and 0:1, v/v) and then purified by ODS silica gel CC using MeOH–H_2O (60:40, v/v), followed by preparative HPLC MeOH–H_2O (70:30, v/v) to yield compounds **1** (6 mg) and **4** (7 mg). Subfraction 4d (20 g) was subjected to MCI gel column chromatography eluting with MeOH–H_2O (0:100→100:0, v/v) and then further purified by Sephadex LH-20 column chromatography eluting with MeOH:H_2O (1:1, v/v), followed by preparative HPLC using MeOH–H_2O (65:35, v/v) to yield compounds **2** (9 mg) and **3** (18 mg).

3. Results and Discussion

Using repeated silica gel column chromatography, ODS column chromatography, and preparative HPLC, we isolated the following compounds: clypearoside A (**1**); (-)-(2S,3S)-epigallocatechin-7-gallate (**2**); (-)-(2R,3R)-epigallocatechin-7-gallate (**3**) [10, 11]; (-)-(2S)-5,7,3',4',5'-pentahydroxyflavan-7-gallate (**4**) [7]; (-)-(2S)-5,7,3',4',5'-pentahydroxyflavan (**5**) [12]; and catechin (**6**) [13]. The structures of all these compounds are shown in Figure 1.

3.1. Structure elucidation

Compound **1** was obtained as colourless oil. The molecular formula was established as $C_{26}H_{38}O_{12}$ by HRESITOFMS, which showed a sodium adduct ion peak $[M+Na]^+$ at *m/z* 565.2273 (calculated 565.2255). The ^1H NMR spectrum (Table 1), showed the proton signals of a 1,3,4,5 tetrasubstituted benzene group at δ_H 6.96 (2H, s), a *trans* olefin group at δ_H 5.28 (1H, d, J = 15.6 Hz) and 5.42 (1H, dd, J = 15.6 Hz, 7.2 Hz), two methyl groups attached to the quaternary carbon at δ_H 0.67 (3H, s), 0.68 (3H, s), as well as two methyl groups in addition to the tertiary carbon at δ_H 1.15 (3H, d, J = 6.0 Hz), and 0.64 (3H, d, J = 6.0 Hz). In the ^{13}C NMR spectrum, the presence of 6 aromatic quaternary carbon signals at δ_C 109.0×2, 119.8, 139.0, 146.0×2, and 166.0 suggested that the structure of **1** contained an aromatic group and one ester group. In addition to the sugar part, the left 13 carbon

signals, including four methyls (δ_C 25.8, 24.7, 22.9, 16.8), two methylenes (δ_C 45.6, 39.9), three methines (δ_C 73.0, 65.3, 33.9) and two quaternary carbon signals (δ_C 76.5, 40.4), indicated that compound **1** was an ionone derivative [14]. Further confirmation of the planar skeleton structure of **1** was obtained from the HMBC experiment (Supporting Information). The HMBC spectrum exhibited correlations from δ_H 0.64 (13-Me) to δ_C 39.9 (C-4), 33.9 (C-5) and 76.5 (C-6), δ_H 1.15 (10-Me) to δ_C 130.6 (C-8) and 73.0 (C-9), δ_H 0.67 (11-Me) to δ_C 40.4 (C-1), 45.6 (C-2), 25.8 (C-12) and 76.5 (C-6), δ_H 0.68 (12-Me) to δ_C 40.4 (C-1), 45.6 (C-2), 24.7 (C-11) and 76.5 (C-6), indicating the planar structure of the monoterpene skeleton. The presence of a galloyl group was supported by these correlations from δ_H 6.96 (H-2″, 6″) to δ_C 119.8 (C-1″), 146.0 (C-3″, 5″), 139.0 (C-4″) and 166.0 (C-7″). Furthermore, the glycosidic site was established unambiguously by an HMBC experiment in which a long-range correlation between δ_H 4.18 (H-1′) and δ_C 73.0 (C-9) was observed (Fig. 2). The downfield shift for the methylene protons of C-6′ (δ_c 64.1) clearly supported the proposed structure indicating that the galloyl group was located at C-6′**.** Moreover, a long range correlation between the carbon signal at δ_C 166.0 (C-7″) and the proton signal at δ_H 4.52 (H-6′,) was observed in the HMBC spectrum (Figure 2).

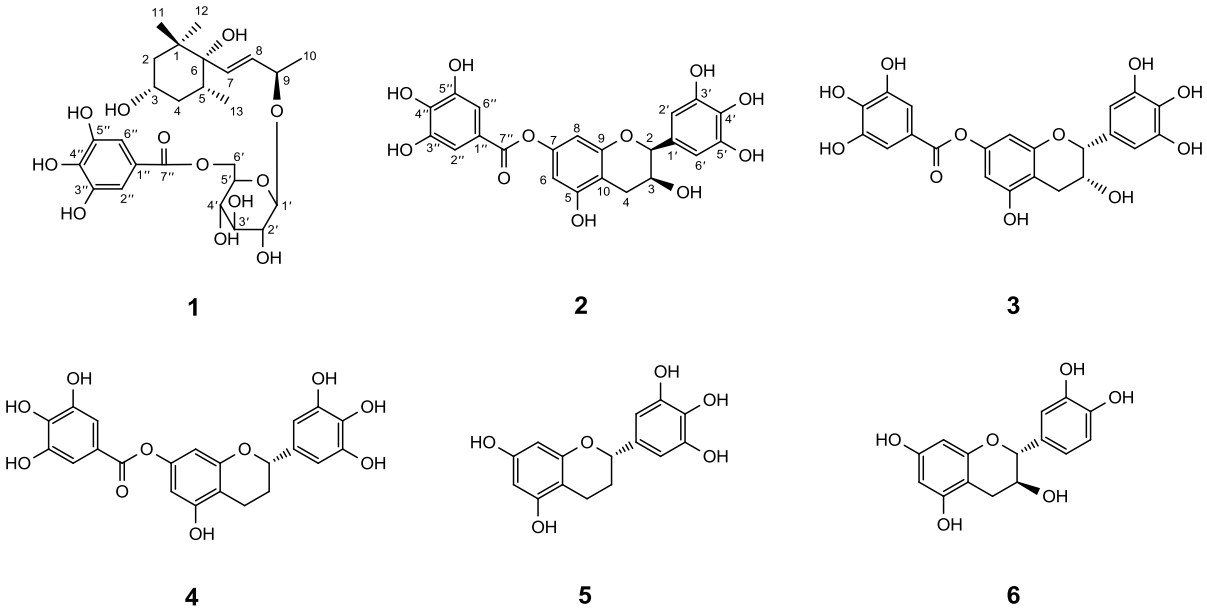

Figure 1. Structures of compounds **1**-**6**

The relative stereochemistry of **1** was completely established by the NOESY spectrum (Supporting Information), and significant NOESY correlation signals were observed at 11-Me/H-3, H-3/H-5 and H-5/H-7, indicating that the 11-Me, H-3, H-5 and H-7 were all on the same side of the cyclohexane-ring. In the sugar part, a proton signal attached to anomeric carbon atom at δ_H 4.18 (1H, d, J = 7.5 Hz) and the ^{13}C NMR signals of sugar showed the presence of a β-glucopyranosyl moiety (Table 1). The absolute configuration of glucose was determined by acid hydrolysis and comparison with an authentic sample [15]. The sugar unit was identified as β-D-glucopyranose, which was further confirmed by strong NOE signals between H-1glc and H-3glc, H-5glc [16] (Fig. 2). Consequently, the structure of compound **1** was established, and given the trivial name clypearoside A.

Compound **2** was obtained as an amorphous colourless powder. Its molecular formula was established as $C_{22}H_{18}O_{11}$ by HRESITOFMS and it, exhibited a pseudomolecular ion $[M+Na]^+$ at *m/z* 481.0744 (calculated 481.0741). In the ^{1}H NMR and ^{13}C NMR spectra of **2**, six aromatic proton signals at δ_H 6.19 (1H, d, J = 2.2 Hz), 6.12 (1H, d, J = 2.2 Hz), 6.27 (2H, s), 7.05 (2H, s) and the presence of 19 aromatic quaternary carbon signals at δ_C 156.6, 155.5, 150.2, 146.2 (4C), 139.6, 133.0, 129.9, 118.8, 109.5 (2C), 106.2 (2C), 106.0, 101.1, 100.8, and 164.9 suggested that **2** contained three

aromatic groups and one ester group. Signals at δ_H 4.58 (1H, d, J = 6.3 Hz), 3.88 (1H, m), 2.48 (1H, m), δ 2.68 (1H, m) and signals at δ_C 81.6, 66.2, and 27.5 indicated the presence of one methylene group and two oxygenated methine groups. Comparison of the ^{1}H NMR and ^{13}C NMR spectra of **2** with 7-O-galloyltricetiflavan [7], the appearance of one more oxygenated methine (δ_C 66.2) and the significant downfield shift of C-2 ($\Delta\delta$ = 81.6-79.1 = 2.5) indicated that **2** was a 7-O-galloyl-substituted flavanol. Further demonstration of the planar skeleton structure of **2** was supported by the HMBC experiment. The absolute configuration (2S, 3S) of **2** was elucidated on the basis of ECD spectra. The CD spectrum (Fig. 3) of **2** showed a negative Cotton effect at 280 nm, which is in contrast to the known compound **3** [10, 11] who showed a positive Cotton effect at the same wavelength [17] (Fig. 3). Thus, the structure and absolute configuration of **2** were elucidated, and **2** was given the name (-)-(2S, 3S)-epigallocatechin-7-gallate which is a new stereoisomer of **3**.

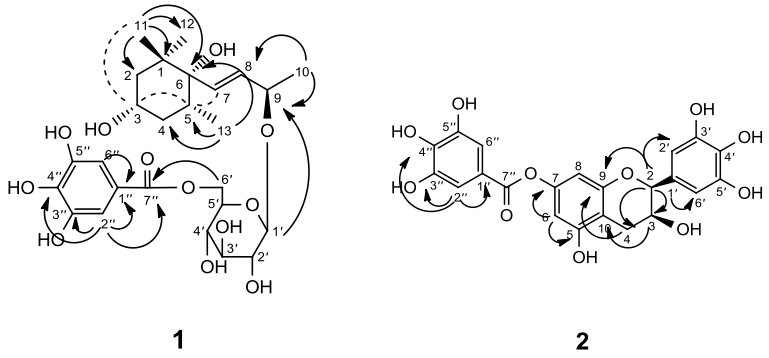

Figure 2. NOESY (···) and Key HMBC (→) correlations of compounds **1** and **2**

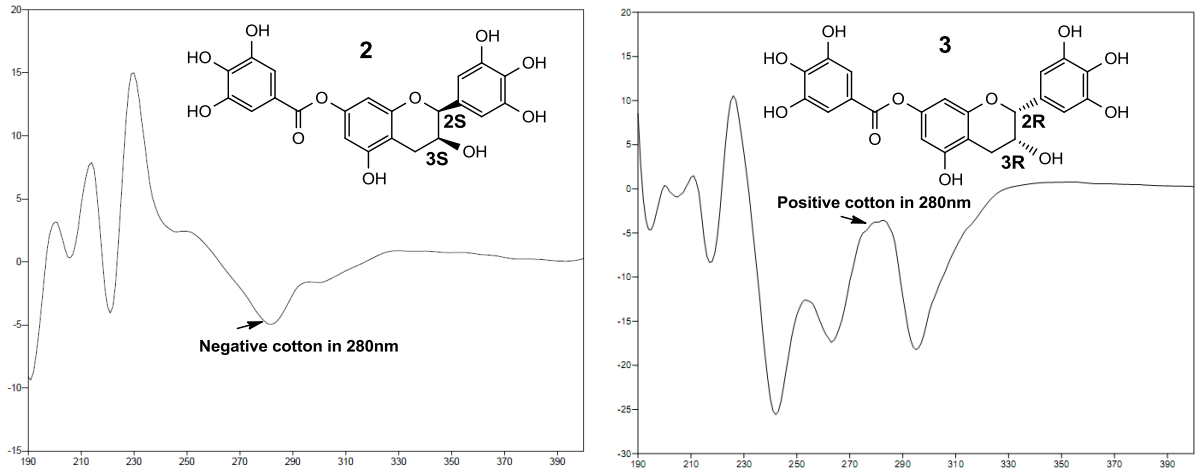

Figure 3. The ECD spectra of compounds **2** and **3**

Table 1. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) Spectroscopic Data of Compounds **1-2** (in DMSO-d_6, in ppm, J in Hz) and Diagnostic HMBC cross peaks.

No.	**1**			**2**		
	δ_H	δ_C	HMBC	δ_H	δ_C	HMBC
1		40.4				
2	δ 1.18 (1H, m), δ 1.45 (1H, m)	45.6	C-1, C-3, C-11	δ 4.58 (1H, d, J = 6.3 Hz)	81.6	C-3, C-4, C-9, C-2', C-6'
3	δ 3.64 (1H, m)	65.3	C-2, C-4	δ 3.88 (1H, m)	66.2	C-2, C-10, C-1'
4	δ 1.18 (1H, m), δ 1.45 (1H, m)	39.9	C-3, C-5	δ 2.48 (1H, m), δ 2.68 (1H, m)	27.5	C-2, C-3, C-9, C-10
5	δ 1.45 (1H, m)	33.9			156.6	
6		76.5		δ 6.19 (1H, d, J = 2.2 Hz)	100.8	C-5, C-7, C-8, C-10
7	δ 5.28 (1H, d, J = 15.6 Hz)	137.7	C-6, C-9		150.2	
8	δ 5.42 (1H, dd, J = 7.2,15.6 Hz)	130.6	C-6, C-9	δ 6.12 (1H, d, J = 2.2 Hz)	101.1	C-6, C-7, C-9, C-10
9	δ 4.30 (1H, m)	73.0	C-7, C-10		155.5	
10	δ 1.15 (3H, d, J = 6.0 Hz)	22.9	C-8, C-9		106.0	
11	δ 0.67 (3H, s)	24.7	C-1, C-2, C-6, C-12			
12	δ 0.68 (3H, s)	25.8	C-1, C-2, C-6, C-11			
13	δ 0.64 (3H, d, J = 6.0 Hz)	16.8	C-4, C-5, C-6			
1'	δ 4.18 (1H, d, J = 7.5 Hz)	99.3	C-9, C-3'		130.0	
2'	δ 3.03 (1H, m)	73.9	C-1', C-3'	δ 6.27 (1H, s)	106.2	C-2, C-1', C-3', C-4', C-5'
3'	δ 3.21 (1H, m)	77.3	C-2', C-4'		146.2	
4'	δ 3.12 (1H, m)	70.9	C-3', C-5', C-6'		133.0	
5'	δ 3.08 (1H, m)	74.5	C-3', C-4'		146.2	
6'	δ 4.25 (1H, m), δ 4.52 (1H, m)	64.1	C-4', C-5' C-7"	δ 6.27 (1H, s)	106.2	C-2, C-1', C-3', C-4', C-5'
1"		119.8			118.8	
2"	δ 6.96 (1H, s)	109.0	C-1", C-3", C-4", C-5", C-7"	δ 7.05 (1H, s)	109.5	C-1", C-3", C-4", C-5", C-7"
3"		146.0			146.2	
4"		139.0			139.6	
5"		146.0			146.2	
6"	δ 6.96 (1H, s)	109.0	C-1", C-3", C-4", C-5", C-7"	δ 7.05 (1H, s)	109.5	C-1", C-3", C-4", C-5", C-7"
7"		166.0			164.9	

3.2. Antioxidant activity

All the six compounds were tested for their antioxidant activity in a DPPH and ABTS radical scavenging assay using a modification of an established protocol [8, 9]. The clearance of compounds **1-6** showed a concentration dependence of 5-40 μg/mL. The IC_{50} values for compounds (**1-6**) are showed in Table 2. The assay involved a comparison with Trolox (IC_{50} = 17.9, 13.7 μg/mL in DPPH and ABTS) which was used as a positive control. Compounds **2-4** displayed stronger activities compared with the positive control (Figure 3). The different antioxidant activities of the test

compounds could be due to their structural differences. Compounds **2-4** with one additional galloyl group at C-7 were more active than compounds **5-6**, suggesting that the presence of more phenolic hydroxyl groups might increase the antioxidant activity.

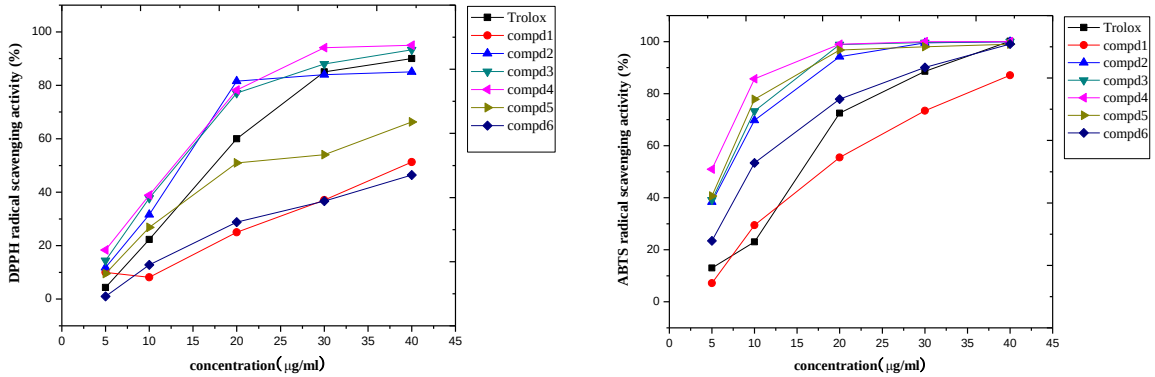

Figure 3. DPPH and ABTS radical scavenging activity of compounds **1-6** and **Trolox**

Table 2. Antioxidant activity of compounds **1-6** and **Trolox** (IC_{50}, μg/mL).

Experiment	Trolox	1	2	3	4	5	6
DPPH (IC_{50}, μg/mL)	17.9	45.8	12.1	11.9	13.2	21.9	42.0
ABTS (IC_{50}, μg/mL)	13.7	17.1	6.4	6.2	4.5	5.8	9.4

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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