Title: Isolation and identification of compounds from the ethanolic extract of flowers of the tea (*Camellia sinensis*) plant and their contribution to the antioxidant capacity

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Abstract

While beneficial health properties of tea leaves have been extensively studied, less attention has been given to that of flowers of the tea (*Camellia sinensis*) plant. In this work, the ethanolic extract and its ethyl acetate-soluble fraction (EEA) from the tea flowers were found to possess the potent antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging assay. The compounds present in EEA had comparatively strong DPPH scavenging activity and strongly contributed to the antioxidant activity of the tea flowers. From EEA, besides eight catechins, five flavonol glycosides were isolated and their structures were elucidated on the basis of mass spectrometry and nuclear magnetic resonance spectroscopy as myricetin 3-*O*-β-D-galactopyranoside, quercetin 3-*O*-β-D-galactopyranoside**,** kaempferol 3-*O*-β-D

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-galactopyranoside, kaempferol 3-*O*-β-D-glucopyranoside, and kaempferol 3-*O*-[α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside]. In addition, epigallocatechin gallate and epicatechin gallate were found as the major active components responsible for the antioxidant activity of tea flowers through the use of a combination of preparative liquid chromatography separation and DPPH assay.

Keywords: Tea (*Camellia sinensis*) flower; Catechins; Flavonol glycosides; Antioxidant activity; Active components

Introduction

Numerous in *vitro* and *vivo* studies reported beneficial health properties of tea leaves and their phenolic compounds (**Bushman, 1998; Trevisanato, 2000**). However, less attention has been given to these properties of flowers of the tea (*Camellia sinensis*) plant. This is due to the fact that people have been only picking the tender shoots from tea plants to manufacture tea since a long time. Some chemicals, such as ethephon and naphthalene acetic acid, have been employed to inhibit the blossoming of tea and promote the production and quality of tea (**Lin, Wu, & Lin, 2003**).

Compared to tea leaves and teas, tea flowers have similar chemical compositions and contain comparable amounts of total catechins but less caffeine (**Su, Chen, Lin, Hu, & Shao, 2000; Lin, Wu, & Lin, 2003**). Furthermore, floratheasaponins were firstly found in the tea flowers and showed potent inhibitory activities on serum triglyceride elevation and the release of β-hexosaminidase from RBL-2H3 cells (**Yoshikawa et al., 2005; Yoshikawa, Nakamura, Kato, Matsuhira, & Matsuda, 2007**). Moreover, the tea flower extracts exhibit strong hydroxyl radical scavenging effects in the Fenton

reaction system and nitric oxide suppressing effects in LPS-induced RAW 264.7 cells (**Lin, Wu, & Lin, 2003**). We have reported that the ethanolic extract from the tea flower possessed the stronger scavenging activity to hydroxyl radical and 2, 2-diphenyl-1- picrylhydrazyl (DPPH) radical than the water extract (**Yang, Xu, Jie, He, & Tu, 2007**). Now it is important to determine the major active components responsible for the antioxidant activity of tea flowers.

The aims of the present study were to ascertain the profile and identity of the antioxidative active compounds isolated from the ethanolic extract of the tea flowers using liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) techniques and elucidate their contribution to the total antioxidative activity.

Materials and methods

Chemicals

DPPH, ascorbic acid (AA) and the eight catechins, namely, catechin (C), epicatechin (EC), gallocatechin, epigallocatechin (EGC), gallocatechin gallate, epigallocatechin gallate (EGCG), catechin gallate, and epicatechin gallate (ECG), were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of the extracts from the tea flowers

The lyophilized and powdered tea flowers (from the Tea Resource Garden of Zhejiang University, Hangzhou, China) were extracted three times with EtOH: H_2O (70:30, v/v) under reflux for 120 min (solvent: tea flower powders = 10:1, v/w). The infusion was filtered with a 0.45 μm Millipore filter and concentrated under reduced

pressure to give the ethanolic extract of tea flowers (EE, yield 96.7 ± 10.4 g / 200 g lyophilized tea flowers). The EE (60 g) was further separated by liquid-liquid partitions using chloroform, ethyl acetate and *n*-butanol successively to give chloroform-soluble fraction (EEC, 3.6 g), ethyl acetate-soluble fraction (EEA, 7.3 g), *n*-butanol-soluble fraction (EEB, 12.4 g), and the final residue fraction (EER, 32.5 g) respectively.

Assay for DPPH radical scavenging activity

The DPPH scavenging activity was determined as described by **Yang et al.** (**2007**). Therefore for each assay, 0.8 mL of 0.001 mol/L of DPPH in methanol was mixed with 2.4 mL of the test sample dissolved in methanol. The mixture was then vortexed vigorously and kept for 30 min at room temperature in the dark. The OD_1 was measured at 517 nm (HITACHI U-3210 spectrophotometer, Japan). A control sample containing the same amount of methanol and DPPH radical was prepared and measured at the same wavelength (OD_0). The absorbance of the sample dissolved in 3.2 mL methanol was recorded as OD_2. This activity is given as DPPH scavenging rate and is calculated according to the following equation:

$$DPPH\ scavenging\ rate\,[\%] = \frac{[OD_0 - (OD_1 - OD_2)]}{OD_0} \times 100$$

HPLC-MS analysis and HPLC separation for the evaluation of the major antioxidant components

HPLC-ESI-MS analysis of EEA was performed in an LCMS-2010 A system (Shimadzu Cooperation, Tokyo, Japan) equipped with a 2.0 mm × 150 mm i.d., 5 μm particle size, UG120 C-18 reversed-phase column (Shiseido Co. Ltd., Japan). A total of 10 μL of the sample solutions was analyzed using gradient elution with the solvent A [acetonitrile / acetic acid / water (6:1:193, v/v/v)] and the solvent B [acetonitrile / acetic

acid / water (60:1:139, v/v/v)], at a flow rate of 0.2 ml/min at 35 °C. The elution was performed using a linear gradient from solvent A to solvent B in 45 min followed by an isocratic step of solvent B for 15 min. Uv-vis spectra were recorded between 200 and 600 nm for each chromatographic peak. Negative or positive iononization mode was applied in full scan range *m/z* 200-900. Optimized electrospray operating conditions were: dry gas 1.5 L/min, capillary voltage 1.5 kV, dry temperature 250 ºC.

The HPLC separation of EEA was performed using a *JASCO* system (Japan Spectroscopic Co. Ltd., Japan) equipped with a 10 mm× 250 mm, 5 μm particle size, UG120Å C-18 reversed-phase column (Shiseido Co. Ltd., Japan). Similar chromatographic conditions as described above were used, but the flow rate was set to 4.7 mL/min.

Isolation of flavonol glycosides from EEA and NMR identification

The EEA (2.0 g) was subjected to a Biotage Flash40 chromatography system (ODS C-18 40 mm ID×15.0 cm, column volume= 188 mL), and the compounds were eluted in order of decreasing polarity with a methanol gradient [MeOH-H_2O (1:99→5:95→10:90→20:80→30:70→50:50→75:25, v/v) →MeOH] using the double column volumes for every gradient step. From the 16 fractions obtained, fraction 12 was further purified by preparative HPLC using a reversed-phase C-18 column and the mobile phase of MeCN: [F_3CCOOH-H_2O, 0.1:99.9, v/v] (13:87, v/v) to give five flavonol glycosides. The chemical structures of these compounds were elucidated by NMR (JEOL JNM-LA 500 FT-NMR) using ^{1}H, ^{13}C, COSY, HSQC, and HMBC.

Statistical analysis

All experiments were performed in triplicate. The data are presented as mean ±

S.D. Mean values were compared using the Tukey test at $p < 0.05$. A probability level of 5% ($p < 0.05$) was considered as significant. The data were processed using the SAS statistical package (ver. 8.01).

Results and discussion

The inhibiting abilities of the extracts of the tea flowers on DPPH were ranked by 50%-inhibition concentrations (IC_{50}). The lower IC_{50} value implies the higher antioxidant activity. Upon regression analysis of scavenging rate (%) and the natural logarithm of the extracts concentration, a good linear relationship was observed between these two parameters, and the regression equations and correlation coefficients are listed in **Table 1**. With regression equations derived, it was easy to calculate the IC_{50} values of each sample. Comparing the IC_{50} values of each sample and AA as a positive control, the order of DPPH scavenging ability was AA > EEA > EEB > EE > EEC > EER (**Table 1**). This indicates that EEA exhibited the strongest scavenging activity to DPPH among the extracts. **Fig. 1 A** shows that the DPPH radical-scavenging activity of EE was dose-dependently increased at the concentrations of 5 to 200 μg/mL. To evaluate the contribution of each fraction to the antioxidant ability of EE, the DPPH assay of each fraction at corresponding concentration in 5 to 200 μg/mL of EE was performed (**Fig. 1 B**). It suggests that EEA also had the most contribution to the antioxidant activity of the ethanol extract of tea flowers. These results propose that EEA contained the major active components responsible for the antioxidant activity of the tea flowers. It was worthwhile to further identify its components and their contribution to the antioxidant activity in the tea flower extract.

The EEA was separated into 21 fractions by HPLC (**Fig. 2A**). To elute all compounds, the last fraction numbered as fraction 22 was obtained by washing the column another 15 min isocratically with methanol. The 22 fractions were concentrated to the same volume of 2.4 ml and the DPPH scavenging rate of each fraction was obtained by photometric assay. **Fig. 2B** points out that Frs. 12 and 16 had the most potential DPPH scavenging activity. Frs. 12 and 16 were identified as the two catechins, EGCG and ECG respectively on the basis of the HPLC-ESI-MS evidences and the authentic compounds. This indicates that EGCG and ECG were the major active components responsible for the antioxidant activity of the tea flowers.

To ascertain the profile of the active compounds in EEA, besides the eight catechins (**Fig. 3**, C, EC, gallocatechin, ECG, gallocatechin gallate, EGCG, catechin gallate and ECG) identified by LC-MS data and authentic compounds, the five flavonol glycosides, namely, myricetin 3-*O*-β-D-galactopyranoside (**1**), quercetin 3-*O*-β-D-galactopyranoside (**2**)**,** kaempferol 3-*O*-β-D-galactopyranoside (**3**), kaempferol 3-*O*-β-D-glucopyranoside (**4**) were identified by comparison of their ^{1}H-NMR (data not shown) and ^{13}C-NMR data (**Table 2**) with reported values (**Fossen, Froystein, & Andersen, 1998; Nørbæk, & Kondo, 1999; Wada, He, Hashimoto, Watanabe, & Sugiyama, 2000; Yan, Murphy, Hammond, Vinson, & Neto, 2002; Loizzo et al, 2007**). Furthermore, compound **5** was identified based on the DQF-COSY, HSQC, and HMBC spectra, and the connectivities among each moiety were elucidated. On the HMBC, cross peaks between 1^{IV}-H/C-$6'''$(δ68.56), and $1'''$-H/C-3 (δ135.47) revealed that compound **5** was kaempferol 3- *O*-[α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside]. In this similar manner, the chemical structures of other compounds were confirmed.

Although two other unknown compounds were found in the EEA extract, their antioxidantive activity was 1/20 less than those of the compounds so far identified.

Tea (*Camellia sinensis*) is one of the most widely consumed beverages in the world. Some experimental and epidemiological studies have linked the drinking of tea to reduction in the risk of cancer and cardiovascular disease (**Yang, Chung, Yang, Chhabra, & Lee, 2000**). These beneficial effects are believed to be mainly due to the antioxidant activity of phenolic compounds. Whereas catechins are the most abundant polyphenols in green tea, the typical pigments in black tea are theaflavins, thearubigins and theabrownins, which are derived from the oxidation of catechins and their gallates during fermentation stage of black tea processing (**Wan, 2003**). The occurrence of five catechins, namely, EGCG, EGC, C, EC and ECG in tea flower in tea flowers had been demonstrated by **Lin, et al.** (**2003**). In the present study, eight catechins (**Fig. 3**) were detected in tea flowers by LC-MS identification. Furthermore, EGCG and ECG were established as the major active components responsible for the antioxidant activity of tea flowers by HPLC separation and DPPH assay (**Fig. 2**). In addition, myricetin, quercetin, and kaempferol mono-, diglycosides were identified in tea flower by NMR (**Fig. 3**). These flavonol glycosides were demonstrated to contribute importantly to the color of green tea (**Wan, 2003**). These imply that the tea flowers may exhibit the similar beneficial health properties to that of tea leaves. Some scholars proposed that tea flowers might be suitable for making alternative tea beverages (**Lin, Wu, & Lin, 2003**). At present, there has been a drinking beverage from black tea leaves scented with tea flowers in China. There is need for more detailed studies to promote tea flowers as well-accepted agricultural products.

Abbreviations

AA, Ascorbic acid; C, Catechin; DPPH, 2, 2-Diphenyl-1-picrylhydrazyl; EC, Epicatechin; ECG, Epicatechin gallate; EE, Ethanolic extract of tea flowers; EEA, Ethyl acetate-soluble fraction of EE; EEB, *n*-butanol-soluble fraction of EE; EEC, Chloroform-soluble fraction of EE; EER, Residue fraction of EE; EGC, Epigallocatechin; EGCG, Epigallocatechin gallate; IC_{50}, 50%- inhibiting concentrations.

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Legends to figures:

Figure 1 DPPH scavenging activities of EE (A) and its fractions (EEC, EEA, EEB, and EER) (B).

(A) The shadow rectangle indicates that DPPH radical-scavenging activity of EE was dose-dependently increased at the concentrations of 5 to 200 μg/mL. (B) Each line shows the DPPH-scavenging rate of each fraction at the corresponding concentrations (calculated by the proportion of each fraction in EE). Data are expressed as means ± S.D. of (n=3). Means with different letters are significantly different ($p < 0.05$). (●— EE, ◆— EEC, ■— EEA, ▲— EEB, ✕— EER).

Figure 2 Chromatogram of EEA (A) and free radical scavenging effects of the fractions obtained from EEA (0.75 mg) by HPLC separation (B).

Data are expressed as means ± S.D. of (n=3).

Figure 3 Chemical structures of catechins and flavonol glycosides isolated from EEA.

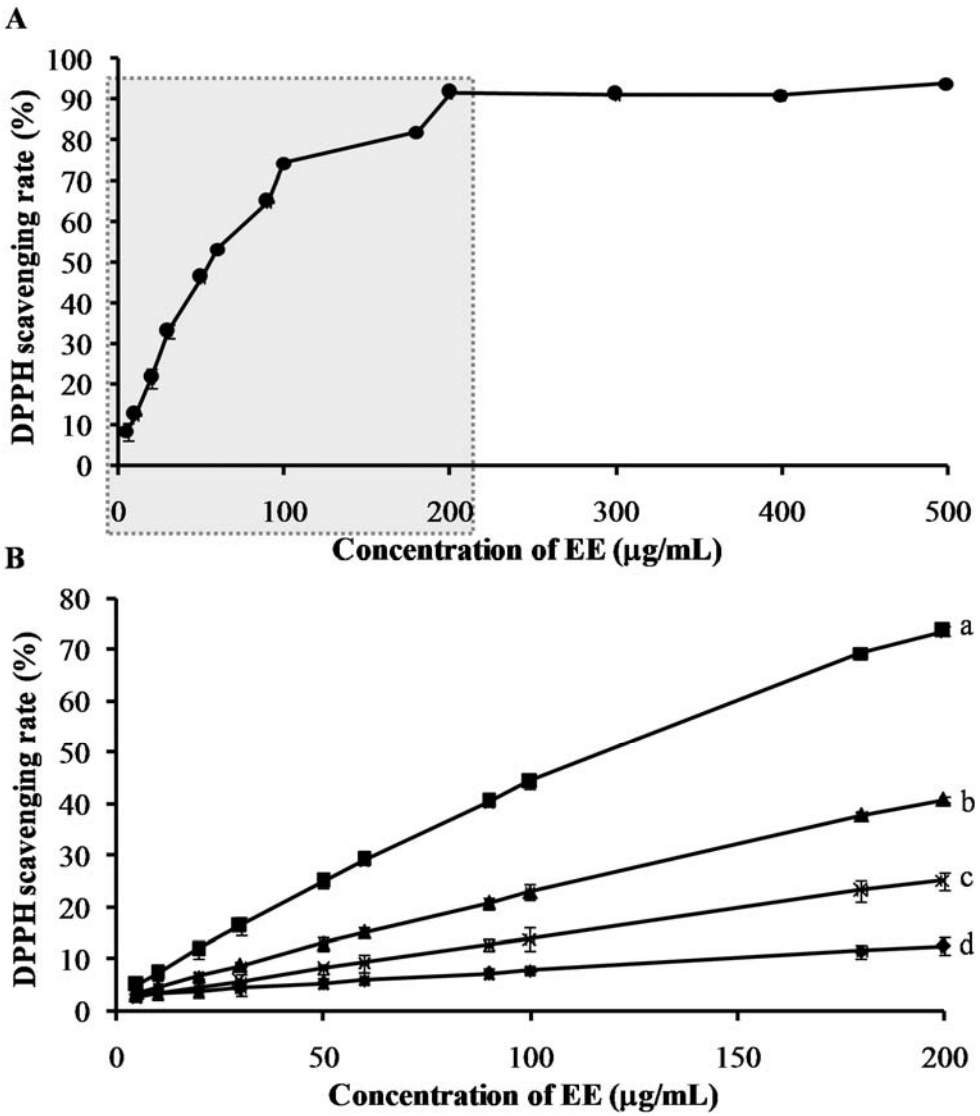


Fig. 1 Ziyin YANG

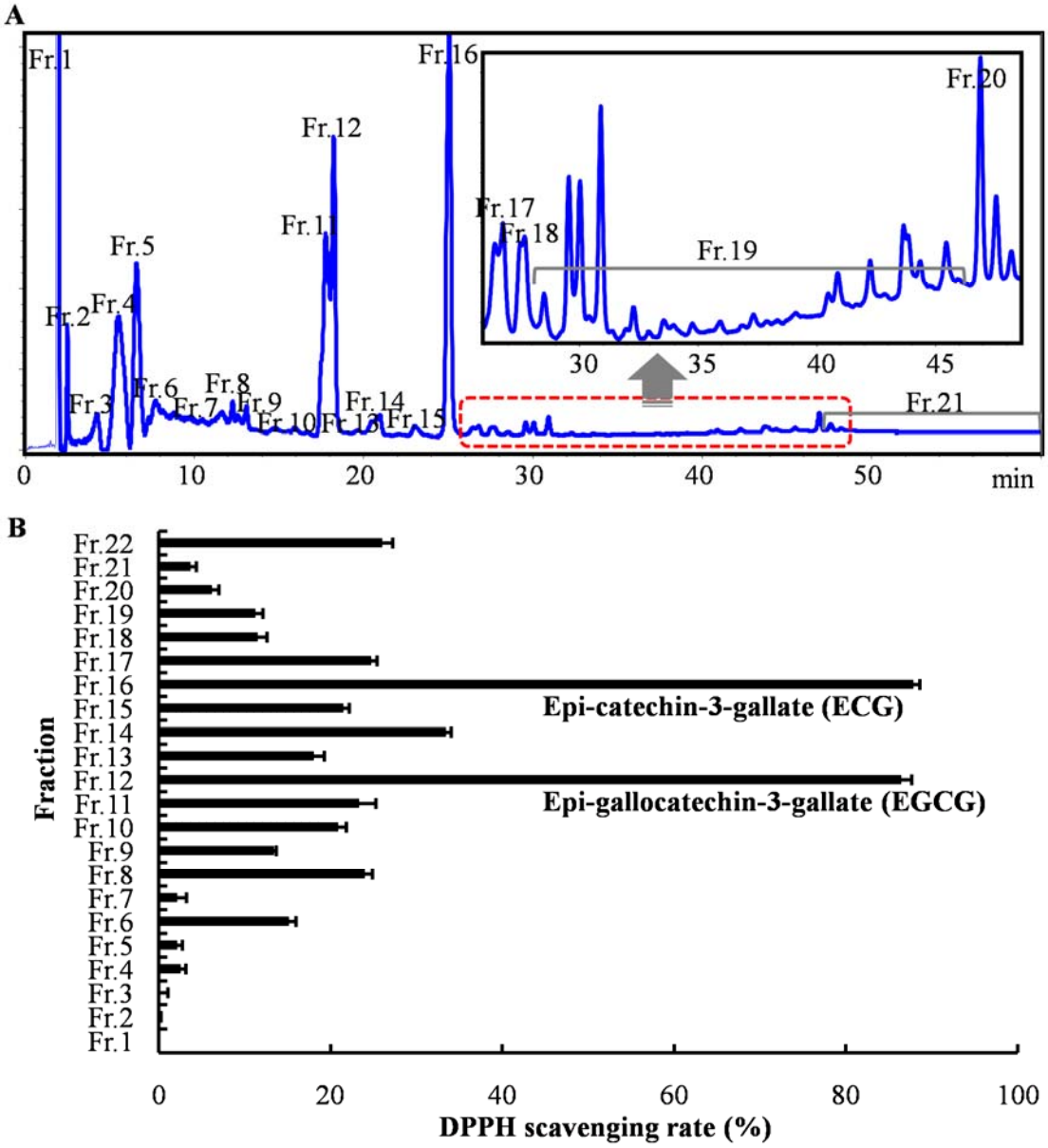
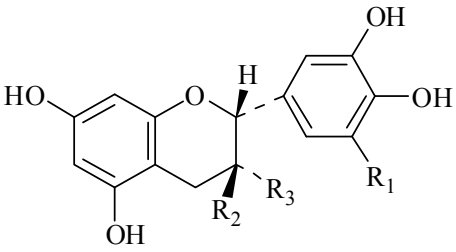
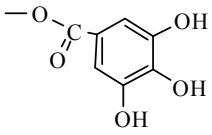


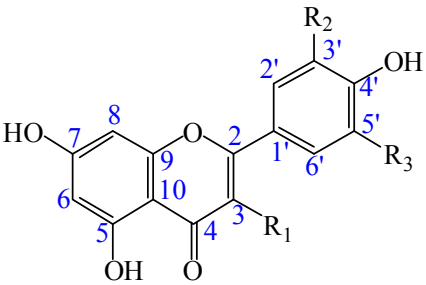
Fig. 2 Ziyin YANG



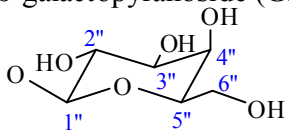
Gallate=



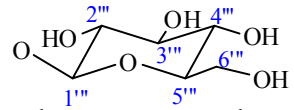
	R_1	R_2	R_3
Epi-catechin	H	H	OH
Catechin	H	OH	H
Epi-gallocatechin	OH	H	OH
Gallocatechin	OH	OH	H
Epi-catechin-3-gallate	H	H	gallate
Catechin-3-gallate	H	gallate	H
Epi-gallocatechin-3-gallate	OH	H	gallate
Gallocatechin-3-gallate	OH	gallate	H



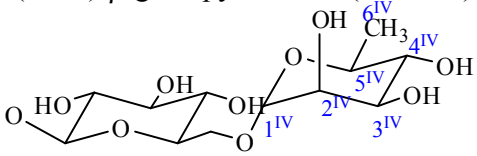
β-galactopyranoside (Gal)



β-glucopyranoside (Glc)



α-rhamnopyranosyl (1➝6)-β-glucopyranoside (Rha-Glc)



	R_1	R_2	R_3
Myricetin 3-β- D-galactopyranoside (1)	Gal	OH	OH
Quercetin 3-β- D-galactopyranoside (2)	Gal	OH	H
Kaempferol 3-β- D-galactopyranoside (3)	Gal	H	H
Kaempferol 3- β- D-glucopyranoside (4)	Glc	H	H
Kaempferol 3-*O*-[α-L-rhamnopyranosyl -(1-6)-β-D-glucopyranoside] (5)	Rha-Glc	H	H

Fig. 3 Ziyin YANG

Table 1. DPPH-scavenging activity of the extracts of tea flowers [a]

sample[a]	Regression equation	R^2	Linear range (μg/mL)	IC_{50} (μg/mL)
AA [c]	*y = 35.8x - 27.6*	0.976**	2.5-30	8.8
EE	*y = 23.8x – 42.0*	0.931 *	5.0-200	47.6
EEC	*y = 22.4x - 41.6*	0.924 *	5.0-200	59.6
EEA	*y = 29.8x - 21.8*	0.974 **	2.5-40	11.1
EEB	*y = 24.0x – 42.0*	0.933 *	5.0-200	45.9
EER	*y = 24.1x - 75.6*	0.923*	20.0-500	183.6

[a] *y*, scavenging rate (%); *x*, natural logarithm values of corresponding concentrations of the extracts of tea flowers. [b]* $p < 0.05$; ** $p < 0.01$. [c] AA was used as a control.

Table 2. ^{13}C NMR data for compound 1-5 (Chemical shifts in δ ppm from TMS, 125 MHz) [a]

Position	1	2	3	4	5
2	158.38	157.80	156.48	159.14	159.45
3	135.10	135.22	133.19	135.46	135.47
4	179.40	178.99	177.36	179.53	179.38
5	162.50	161.46	161.16	163.04	162.89
6	99.96	100.21	99.02	99.93	100.02
7	166.17	165.60	165.35	165.99	166.02
8	94.73	95.43	93.81	94.79	94.97
9	158.71	158.47	156.17	158.51	158.51
10	105.47	105.19	103.51	105.74	105.64
1'	121.68	122.63	120.82	122.80	122.74
2'	109.97	116.42	130.91	132.28	132.36
3'	146.38	144.93	115.09	116.10	116.15
4'	138.20	149.03	160.06	161.54	161.42
5'	146.38	117.67	115.09	116.10	116.15
6'	109.97	123.28	130.91	132.28	132.36
Gal					
1''	105.60	104.44	101.83		
2''	73.49	72.57	71.23		
3''	75.09	74.09	73.15		
4''	70.01	69.40	67.85		
5''	77.21	76.37	75.75		
6''	62.23	61.35	60.15		
Glc					
1'''				104.09	104.54
2'''				75.73	75.72
3'''				78.02	77.14
4'''				71.35	72.04
5'''				78.38	78.08
6'''				62.61	68.56
Rha					
1^{IV}					102.38
2^{IV}					71.41
3^{IV}					73.86
4^{IV}					72.28
5^{IV}					69.72
6^{IV}					17.88

[a] Solvents: compound 3 was dissolved in DMSO-d_6; compound 1, 2, 4, and 5 were dissolved in CD_3OD.