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Antioxidative extracts and phenols isolated from Qinghai–Tibet Plateau medicinal plant *Saxifraga tangutica* Engl.

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ABSTRACT

Saxifraga tangutica Engl., is a medicinal herb that grows on the Qinghai–Tibet Plateau. Extracts and phenols from the Qinghai population have been subjected to antioxidative assays against DPPH radical-scavenging and reducing power (FRAP). The 50% ethanol extract showed strong antioxidative activity against DPPH and FRAP, with $IC_{50} \pm SEM$ [μg/mL] values of 9.38 ± 0.46 and 15.46 ± 0.52, respectively. The antioxidative activity-guided fractionations were performed according to the DPPH and FRAP screening results. Fourteen fractions from the 50% ethanol extract showed dissimilar antioxidative activity against DPPH and FRAP of $8.16 \pm 0.76 \sim 38.42 \pm 0.58$ μg/mL and $13.22 \pm 0.68 \sim 61.47 \pm 0.49$ μg/mL. The chemical assay-guided separation of the active fractions (fractions 3, 6, 7 and 8) led to eight phenols: protocatechuic aldehyde (**1**), ethyl gallate (**2**), rhododendrin (**3**), *p*-hydroxyacetophenone (**4**), rhododendrol (**5**), protocatechuic acid ethyl ester (**6**), frambinone (**7**) and ethylparaben (**8**). All phenols are reported here for the first time from *S. tangutica* Engl. Protocatechuic aldehyde (**1**), ethyl gallate (**2**), rhododendrin (**3**) and protocatechuic acid ethyl ester (**6**) showed strong antioxidative activities ($IC_{50} \pm SEM$ [mM] between 8.79 ± 0.15 and 4.25 ± 0.47 and between 6.15 ± 0.48 and 2.83 ± 0.49) against DPPH and FRAP.

1. Introduction

Over the length of the Qinhai–Tibet Plateau in central Asia (primarily in West China, including Qinhai, Tibet, West of Sichuan, Northern Yunnan, and Western Gansu Province), numerous wild type plants are used as alternative medical treatments (Wasner et al., 2001; Xie et al., 2007). Most of the species are used in local traditional medicine and are employed to treat hepatitis and tumors, influenza, osteoarthritis and purgation. These include species belonging to the following genera: *Saxifraga*, *Lycium* and *Rheum* (Chen et al., 2008; Ren et al., 2012; Wang and Ren, 2009). These species are currently in high demand and their marketing in herbalists and popular markets is increasing.

Saxifrage genus, one of the typical alpine plants, distributes in high elevations above 2900 m in Qinghai–Tibet plateau. Approximately 200 species of the *Saxifraga* genus exist in the Qinhai–Tibet Plateau, and 32 species grow in Qinghai Province (Webb and Gornall, 1989). The literature reports the chemical composition of *Saxifraga stolonifera* and *Saxifraga melanocentra* (Chen et al., 2008; Zuo et al., 2005), and *Saxifraga cuneifolia* (Chevalley et al., 2000) collected from diverse mountains of the Qinhai–Tibet Plateau. The biological activities of phenols isolated from *Saxifraga* species, such as radical scavengers, antineoplastic, inhibition of HCVNS3 protease and antifilarial have been reported (Chevalley et al., 2000; Chen et al., 2008; Zuo et al., 2007; Singh et al., 2000). The aerial part of the *Saxifraga tangutica* Engl. (*S. tangutica*), collected from the Qinhai–Tibet Plateau is recommended and sold by herbalists to treat hepatitis and cholecystitis, as well as fever and influenza.

Frequently, *S. tangutica* has been used in traditional medicine to treat inappetence or cardiovascular disease associated with digestive problems (Yang, 1991). Here, we report the antioxidative properties of the extracts and isolated bioactive phenols of the *S. tangutica*, which were assayed against DPPH radical-scavenging and reducing power (FRAP).

2. Materials and methods

2.1. General reagents

Analytical grade ethanol, methanol (Shandong, China) and formic acid (Tianjin, China), HPLC grade methanol (Tianjin, China), HPLC grade formic acid (Fisher, USA), HPLC grade water from

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a Milli-Q system (Millipore, USA), analytical water from reverse osmosis equipment (Shanghai, China) were used. Chromatographic columns (including preparative columns and the analytical column) used reversed phase separation materials (Beijing, China and Waters, USA; respectively). Quercetin, glutathione and DPPH were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2. Structural identification of phenols and HPLC conditions

Nuclear magnetic resonance (NMR) spectra of the phenols in DMSO-d_6 were obtained in a Bruker Avance (600 MHz) DRX spectrometer, operating at 600 MHz for ^{1}H and 151 MHz for ^{13}C. TMS was employed as the internal standard. ESI-HRMS spectra were recorded on a Waters Q-TOF Premier™ mass spectrometer. HPLC separations were conducted using an industrial scale preparative LC system (CXTH, China) and a laboratorial scale preparative HPLC system consisting of two Waters 2525 binary gradient modules, a Waters 2498 UV/Visible detector and Waters Masslynx software V.4.1. An ACCHROM RP-C3 (10 μm, 100 × 250 mm) column working at a flow rate of 331 mL/min and an XAqua RP-C18 (5 μm, 20 × 250 mm) column working at a flow rate of 10 mL/min were used for separations.

2.3. Plant material

S. tangutica Engl. (Saxifragaceae) herb was collected at a location belonging to the state of Guoluo in central Qinhai–Tibet Plateau, Qinghai province, Maqin: altitude about 4200 m, 34°30′ north latitude, 99°54′ east longitude, during the flowering period (August, 2011) and was identified by Prof. Lijuan Mei at the Northwest Institute of Plateau Biology, Chinese Academy of Sciences (NWIPB). A voucher specimen (NWIPB-STE-2011-08-01) has been deposited at the Qinghai–Tibetan Plateau Museum of Biology (QPMB).

2.4. Preparation of extracts from S. tangutica

Extracts were obtained from the air-dried leaves and stems (200.0 g finely grinded of each collection) by extractions with distilled water, 50% ethanol aqueous solution and 95% ethanol aqueous solution under reflux (80 °C, 3 h for three times) to afford 0% ethanol, 50% ethanol and 95% ethanol extracts. The three extracts were tested for antioxidative activity and 50% ethanol extract showed highest antioxidative activity. Another 2.5 kg air-dried leaves and stems was extracted by 50% ethanol aqueous solution (80 °C, 3 h for three times) to afford approximately 75.0 L extracted solution.

The extracted solution was filtered by qualitative filter paper to remove the solid particles. The filter liquor was disposed with an alcohol-resistant industrial ultrafiltration membrane system (8000 MW, Dalian Institute of Chemical Physics, Chinese Academy of Sciences). The permeate liquor was collected and condensed by rotary evaporation at 60 °C in a vacuum to obtain a brown powder (84.2 g).

In order to verify existence of isolated ethyl ester phenols in *S. tangutica* Engl., 5.0 g of air-dried leaves and stems (finely grinded) was extracted by methanol (2 h for three times). The extracted solution was combined and filtered through 0.45 μm membranes to afford *S. tangutica* Engl. methanol sample solution.

2.5. Antioxidative activity-guided chromatographic separation of S. tangutica sample

The S. *tangutica* sample (brown powder, 84.2 g) was separated through an industrial level RP-C3 column with mobile phase A (0.2% formic acid aqueous solution) and mobile phase B (methanol). The linear gradient elution steps were as follows: 0–100 min, 5 ~ 95%B; 100–110 min, 95%B. Fourteen fractions were obtained by eight separations and were tested for antioxidant activity.

Fractions 3 (2274.4 mg), 6 (820.8 mg), 7 (3115.2 mg) and 8 (6787.2 mg) showed moderate antioxidation and were applied to the laboratory scale XAqua RP-C18 column with mobile phase A (0.2% formic acid aqueous solution) and mobile phase B (methanol). Isocratic elution procedure for fraction 3 was 8%B for 35 min, Fraction 6 was 25%B for 30 min, Fraction 7 was 29%B for 30 min, and Fraction 8 was 32%B for 30 min. Eight compounds were obtained and showed higher antioxidative activity.

2.6. Antioxidative activity

2.6.1. DPPH radical-scavenging assay

The DPPH-radical-scavenging potential of S. *tangutica* extracts, fractions 1 to 14 (10 mg) and eight phenols (2 mM) was examined by mixing with absolute ethanol and 1×10^{-3} mol/L (0.18 mL) DPPH. The obtained samples were properly mixed using ultrasonication and were incubated for 45 min. The obtained mixture was then monitored at 515 nm against a blank using a spectrophotometer (UV–vis Shanghai, China) and the absorbance was recorded when the reaction reached a steady state. All measurements were performed in triplicate. Quercetin (2 mM) was used as a standard. The radical scavenging activity was calculated using the formula: % inhibition = $(A_c(0) - A_A(t))/A_c(0) \times 100$, where $A_c(0)$ is the absorbance of the control at $t = 0$ min and $A_A(t)$ is the absorbance of the antioxidant at $t = 1$ h (Nile and Park, 2013).

2.6.2. FRAP assay (reducing power assay)

The Fe^{3+} reducing power of S. *tangutica* extracts, fractions 1 to 14 and eight phenols was determined as described previously with slight modifications. The reaction mixture contained 10 mL of S. *tangutica* extracts and fractions 1 to 14 (10 mg/fraction) and phenols (5 mM in 0.5% v/v dimethyl sulfoxide) in 3 mL of potassium ferricyanide solution (1 mM). The obtained mixture was incubated at 50 °C in a water bath for 20 min. After incubation, 0.5 mL of trichloroacetic acid (TCA) (10%) was added to terminate the reaction. The upper portion of the solution (1 mL) was mixed with distilled water (1 mL) and 0.1 mL $FeCl_3$ (0.01%). The reaction mixture was incubated at room temperature for 10 min, and the absorbance was measured using a spectrophotometer at 700 nm with a blank solution. All tests were performed in triplicate. A higher absorbance of the reaction mixture indicated greater reducing power. Glutathione (2 mM) was used as a reference compound (Nile and Park, 2014).

2.6.3. Statistical analysis

The results were expressed as the means ± SEM of the indicated number of experiments ($n \geq 3$). The statistical significance of the differences between means was established by ANOVA with the LSD tests. P values <0.05 were considered to indicate statistical significance.

3. Results and discussion

3.1. Identification of antioxidative active compounds in fractions 3, 6, 7 and 8

The industrial scale RP-C3 column separation of the *S. tangutica* sample is presented in Fig. 1, and the antioxidative activity screening results of *S. tangutica* extracts and *S. tangutica* sample fractions are summarized in Table 1. The antioxidative activity-guided isolation of the *S. tangutica* sample showed that fractions 3, 6, 7 and 8 had greater antioxidative activity. Fraction 3 (2274.4 mg) was purified by the XAqua RP-C18 preparative column and eluted with 8% isocratic methanol for 35 min to afford 1291.8 mg of compound **1**:

Table 1
Antioxidant activity of the tested *S. tangutica* extracts, *S. tangutica* PE fractions and isolated phenols.

Antioxidant activity (IC_{50} + SEM: extracts/fractions [μg/mL]; isolated phenols [mM])										
S. tangutica ethanol extracts										
	0%	50%	95%							
DPPH	17.95 ± 0.52	9.38 ± 0.46	13.00 ± 0.63							
FRAP	43.79 ± 0.36	15.46 ± 0.52	23.14 ± 0.46							
S. tangutica sample fractions										
	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	Fraction 7			
DPPH	26.16 ± 0.68	28.50 ± 0.58	8.16 ± 0.76	27.49 ± 0.44	30.83 ± 0.65	9.92 ± 0.43	10.97 ± 0.67			
FRAP	38.98 ± 0.33	46.72 ± 0.42	13.22 ± 0.68	60.75 ± 0.89	46.87 ± 0.43	15.76 ± 0.32	16.45 ± 0.49			
	Fraction 8	Fraction 9	Fraction 10	Fraction 11	Fraction 12	Fraction 13	Fraction 14			
DPPH	8.59 ± 0.40	29.48 ± 0.76	30.00 ± 0.31	38.42 ± 0.58	32.43 ± 0.44	29.90 ± 0.57	33.48 ± 0.48			
FRAP	16.06 ± 0.53	46.28 ± 0.64	44.90 ± 0.56	61.47 ± 0.49	50.27 ± 0.55	61.0 ± 0.43	57.58 ± 0.73			
Phenols from *S. tangutica*										
	1	2	3	4	5	6	7	8	QR	GT
DPPH	5.91 ± 0.28	4.25 ± 0.47	7.36 ± 0.42	11.69 ± 0.35	13.78 ± 0.62	8.79 ± 0.15	14.34 ± 0.55	10.25 ± 0.68	4.84 ± 0.22	–
FRAP	3.65 ± 0.56	2.83 ± 0.49	5.30 ± 0.68	7.70 ± 0.45	8.67 ± 0.50	6.15 ± 0.48	8.98 ± 0.66	6.64 ± 0.45	–	5.77 ± 0.14

Values are the mean ± SD (n = 3). (**1**) protocatechuic aldehyde; (**2**) ethyl gallate; (**3**) rhododendrin; (**4**) *p*-hydroxyacetophenone; (**5**) rhododendrol; (**6**) protocatechuic ethyl ester; (**7**) frambinone; (**8**) ethylparaben, QR: quercetin, GT: glutathione.

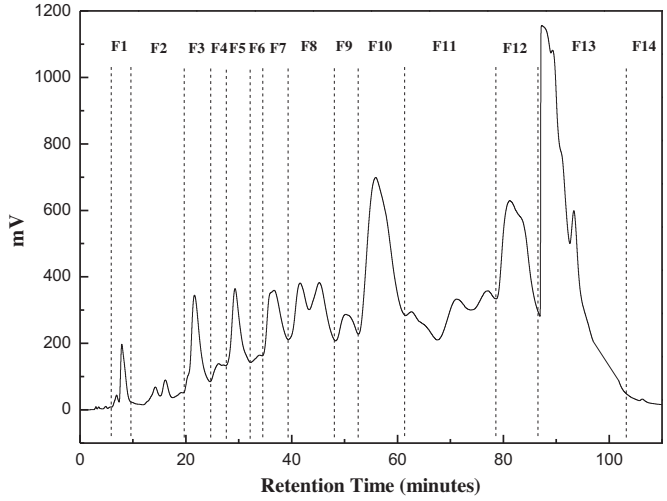

Fig. 1. The separation profiles (270 nm) of *S. tangutica* sample on a RP-C3 preparative column.

protocatechuic aldehyde ([M–H]$^-$ 137.15). The ^{1}H NMR and ^{13}C NMR data of compound **1** are in good agreement with the literature (Lee et al., 2011).

Fraction 6 (820.8 mg) was purified by the XAqua RP-C18 preparative column and eluted with 25% isocratic methanol for 30 min to yield 284.0 mg of compound **2**: ethyl gallate ([M–H]$^-$ 197.32) and 302.9 mg of **3**: rhododendrin ([M + H]$^+$ 328.67). The ^{1}H NMR and ^{13}C NMR data of these compounds are in good agreement with the literature (Choi et al., 1987; Zhang et al., 2009).

Fraction 7 (3115.2 mg) was purified by the XAqua RP-C18 preparative column and eluted with 29% isocratic methanol for 30 min to yield 894.0 mg of compound **4**: *p*-hydroxyacetophenone ([M–H]$^-$ 135.19) and 1336.4 mg of compound **5**: rhododendrol ([M + H]$^+$ 166.73). The ^{1}H NMR and ^{13}C NMR data of compounds **4** and **5** are in good agreement with the literature (Kwon and Lee, 2001; Parmar et al., 1991).

Fraction 8 (6787.2 mg) was purified by the XAqua RP-C18 preparative column and eluted with 32% isocratic methanol for 30 min to yield 1269.2 mg of compound **6**: protocatechuic acid ethyl ester ([M + H]$^+$ 182.85), 1500.0 mg of compound **7**: frambinone ([M + H]$^+$ 165.00), and 1533.9 mg of **8**: ethylparaben ([M–H]$^-$ 164.74). The ^{1}H NMR and ^{13}C NMR data of the three compounds are in good agreement with the literature (Reis et al., 2010; Tian et al., 2006; Viviano et al., 2011). The preparative chromatograms are summarized in Fig. 2. The purity characterization of the eight isolated phenols was conducted by total ion chromatography in the negative or positive ion mode (in Fig. 3). The HPLC column was an Xterra (5 μm, 4.6 × 100 mm, Waters, USA) with a gradient of 5 ~ 90% methanol (the other mobile phase was 0.2% formic acid aqueous solution) from 0 to 25 min. The actual existence rather than artifacts of compounds **2** (ethyl gallate), **6** (protocatechuic acid ethyl ester) and **8** (ethylparaben) was verified by ESI-HRMS spectra. The HPLC column was Xterra (5 μm, 4.6 × 100 mm, Waters, USA) with gradient of 5 ~ 90% methanol (the other mobile phase was 0.2% formic acid aqueous solution) from 0 to 25 min and isocratic elution of 90% methanol from 25 min to 30 min. Fig. 4A was total ion chromatography of *S. tangutica* Engl. methanol sample solution in the positive ion mode. The three compounds of ethyl gallate (compound **2**), protocatechuic acid ethyl ester (compound **6**) and ethylparaben (compound **8**) have been found with retention times of 9.7 min, 15.6 min and 17.1 min, respectively. Molecular ion peaks of compounds **2**, **6** and **8** are in Fig. 4B, C and D, respectively. Therefore, compounds **2**, **6** and **8** was proven to exist in *S. tangutica* Engl.

3.2. Antioxidant activity

The antioxidant activities of *S. tangutica* extracts and *S. tangutica* sample fractions, together with the isolated phenols, including protocatechuic aldehyde, ethyl gallate, rhododendrin, *p*-hydroxyacetophenone, rhododendrol, protocatechuic acid ethyl ester, frambinone and ethylparaben, were investigated for in vitro antioxidant activity against DPPH and FRAP; the results are presented in Table 1. The IC_{50} ± SEMs values of the *S. tangutica* extracts and the *S. tangutica* sample fractions are expressed as μg/mL, and the IC_{50} ± SEMs values of the isolated phenols are expressed as mM.

The *S. tangutica* extracts (distilled water extract, 50% ethanol extract and 95% ethanol extract) showed antioxidant activity in a concentration-dependent manner, and the highest antioxidative activity of the *S. tangutica* extracts was found against DPPH as compared to FRAP, in the 50% ethanol extracts (IC_{50} ± SEM [μg/mL]: 9.38 ± 0.46 and 15.46 ± 0.52, respectively).

To identify the antioxidative compounds, after the process of membrane pretreatment (removal of the polysaccharide, chlorophyll, and other biomacromolecules), antioxidative activity-guided isolation was performed on the *S. tangutica* sample. Fractions 3, 6, 7 and 8, which displayed antioxidative activity against DPPH and FRAP with IC_{50} ± SEM ranging from 8 to 11 μg/mL and 13 to 17 μg/mL, were further purified on an XAqua RP-C18 preparative

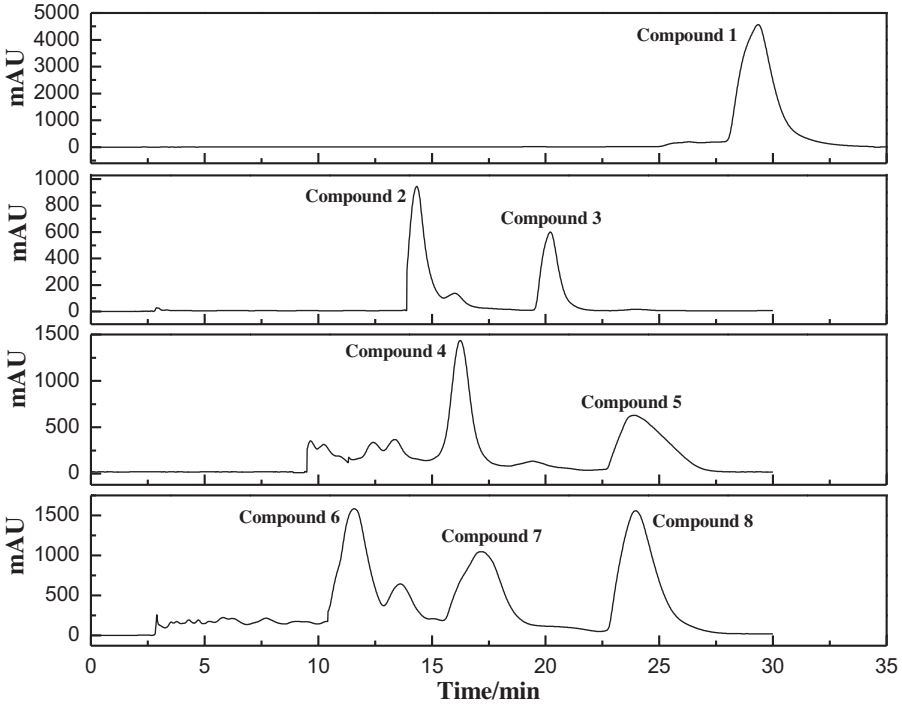

Fig. 2. Preparative chromatograms (270 nm) of active fractions 3, 6, 7 and 8.

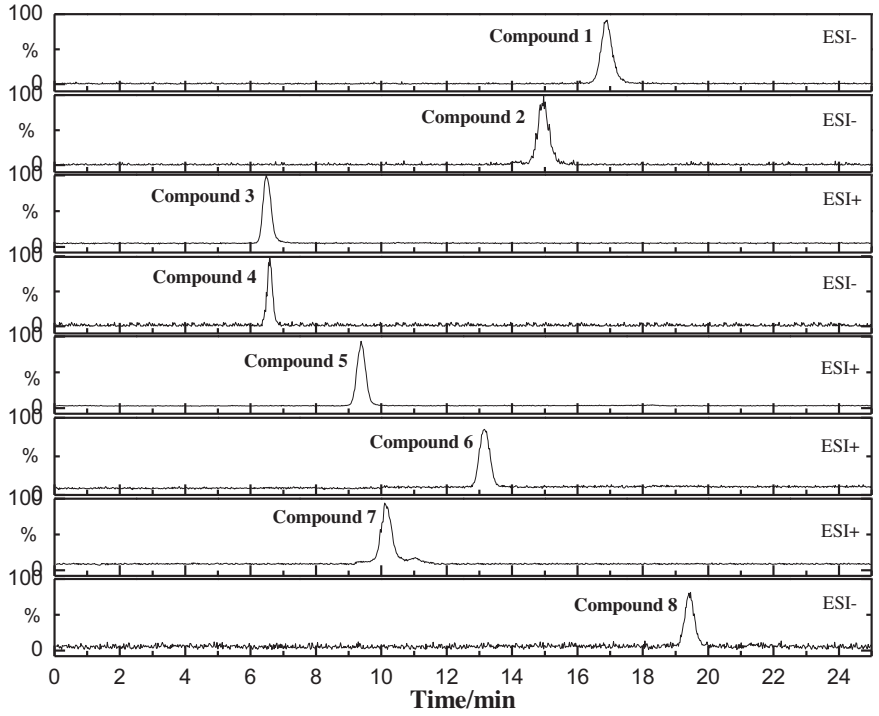

Fig. 3. Total ion chromatograms of the eight isolated phenols.

column affording: protocatechuic aldehyde (**1**), ethyl gallate (**2**), rhododendrin (**3**), *p* -hydroxyacetophenone (**4**), rhododendrol (**5**), protocatechuic acid ethyl ester (**6**), frambinone (**7**), ethylparaben (**8**) (Fig. 5).

Protocatechuic aldehyde (**1**, $IC_{50} \pm SEM$ [mM]: 5.91 ± 0.28 and 3.65 ± 0.56, respectively) and ethyl gallate (**2**, $IC_{50} \pm SEM$ [mM]: 4.25 ± 0.47 and 2.83 ± 0.49, respectively), showed the stronger antioxidative activity against DPPH and FRAP. Rhododendrin (**3**, $IC_{50} \pm SEM$ [mM]: 7.36 ± 0.42 and 5.30 ± 0.68, respectively) and protocatechuic acid ethyl ester (**6**, $IC_{50} \pm SEM$ [mM]: 8.79 ± 0.15 and 6.15 ± 0.48, respectively) displayed strong antioxidative activity against DPPH and FRAP. Although protocatechuic aldehyde, ethyl gallate, rhododendrin and protocatechuic acid ethyl ester were proven to have strong antioxidative activity against DPPH and FRAP, the antioxidant effect of compounds were in most cases due to the stimulation of the endogenous antioxidant defence system, which

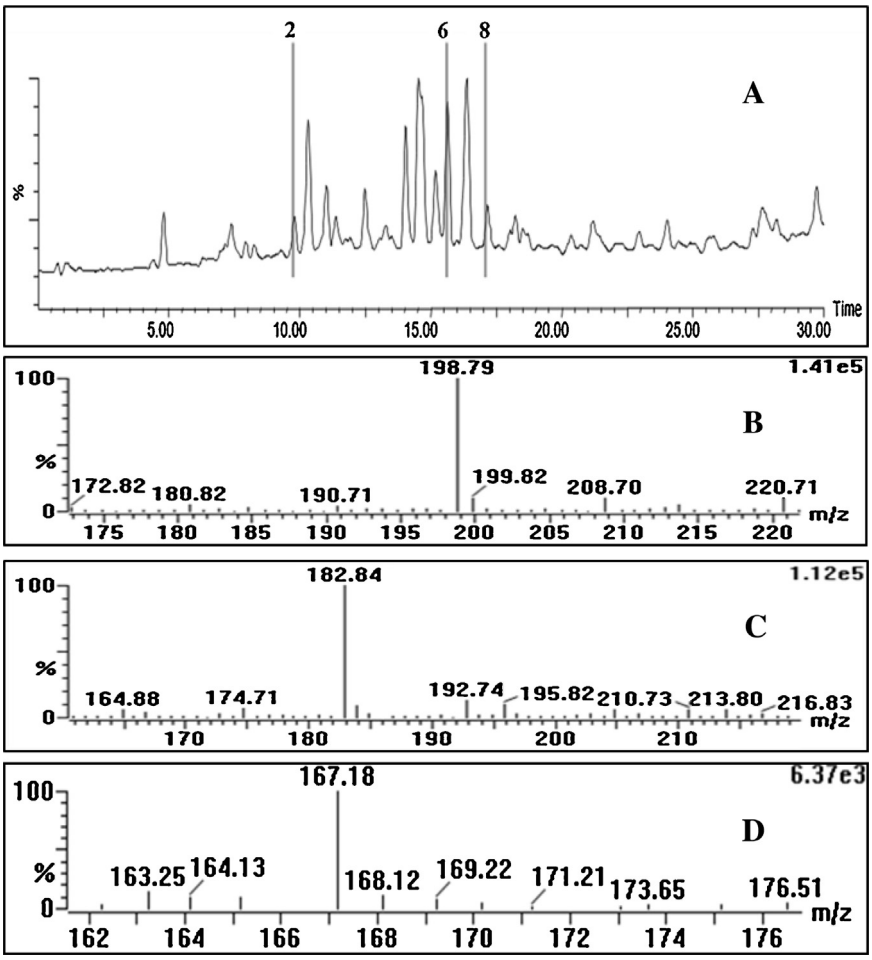

Fig. 4. Total ion chromatograms of the *S. tangutica* Engl. methanol sample solution (A), molecular ion peak of ethyl gallate (B), molecular ion peak of protocatechuic acid ethyl ester (C), molecular ion peak of ethylparaben (D).

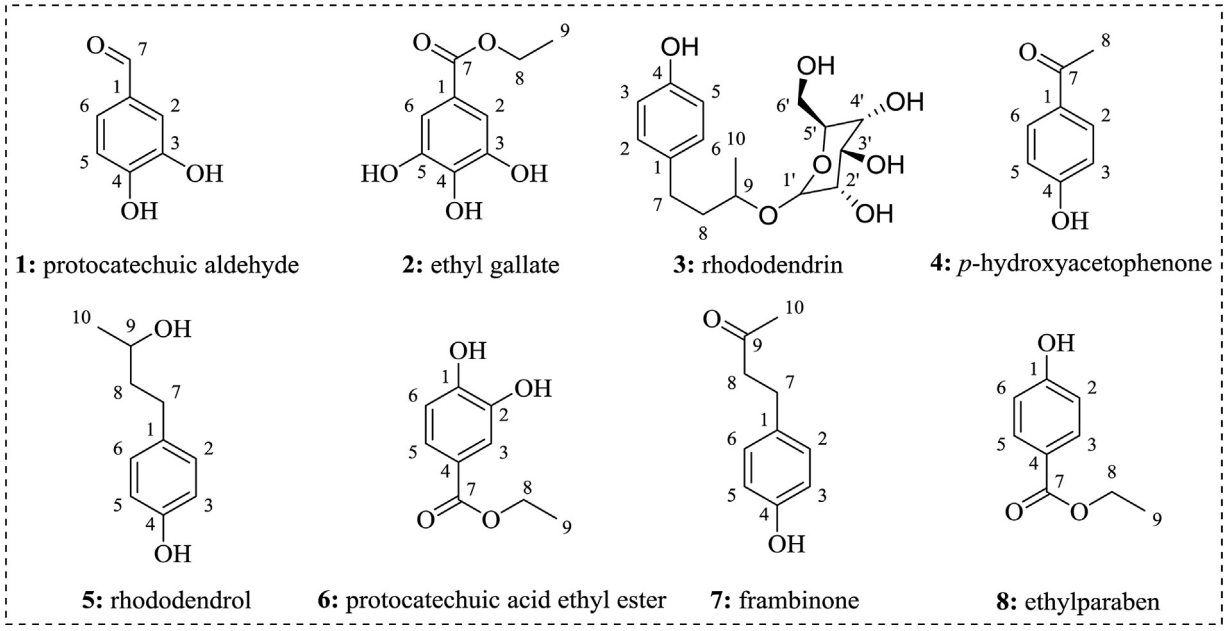

Fig. 5. Isolated phenols 1–8 from *S. tangutica* 50% ethanol extract.

includes the formation of phase II antioxidant enzymes and/or endogenous small antioxidant molecules such as glutathione, and all the compounds that stimulate the endogenous antioxidant system were able to react with cysteine in proteins in vivo whether they showed antioxidant activity in vitro or not. Therefore, there was still no direct correlation between antioxidant activity in vitro and prevention of oxidative stress.

Recently, the antioxidative activity of *S. stolonifera* methanol extract against DPPH scavenging activity was reported (Sohn et al., 2008). Some phenols isolated from *S. tangutica* were previously identified in other species of the *Saxifraga* genus, such as ethyl gallate (**2**) isolated from *S. cuneifolia* (Chevalley et al., 2000) and rhododendrin (**3**) identified from *S. melanocentra* (Zuo et al., 2005). Until now, no study on the isolation of phenols from *S. tangutica* has been reported.

Protocatechuic aldehyde (**1**) was isolated from *Salviae miltiorrhiza* (Xu et al., 2007), and *p*-hydroxyacetophenone (**4**) was reported as an antiviral constituent of *Artemisia morrionensis* (Huang et al., 2014). The phenolic compound rhododendrol (**5**) was obtained from the leaves of *Rhododendron aureum* (Kim et al., 2011). Protocatechuic acid ethyl ester (**6**) was isolated from *Drynariae rhizoma* phenolic extracts (Kang et al., 2014). Frambinone (**7**) and antimicrobial ethylparaben (**8**) were obtained from raspberry fruits (Koeduka et al., 2011) and roots of *Ranunculus ternatus* (Tian et al., 2006), respectively.

Protocatechuic aldehyde (**1**), *p*-hydroxyacetophenone (**4**), rhododendrol (**5**), protocatechuic acid ethyl ester (**6**), frambinone (**7**) and ethylparaben (**8**) are reported here for the first time from *Saxifraga* genus. The results supplement previous reports on *Saxifraga* genus antioxidative activity (Aoyagi et al., 1995).

4. Conclusions

The results of the present study showed that the *S. tangutica* extracts (distilled water, 50% ethanol extract and 95% ethanol extract) presented antioxidative activity. The 50% ethanol extract showed strong antioxidative activity against DPPH and FRAP compared with distilled water and 95% ethanol extract. The *S. tangutica* sample fractions 3, 6, 7 and 8 (fractions 1–14 from 50% ethanol extract) showed strong antioxidative activity against DPPH and FRAP. Eight phenols isolated from the four active fractions were identified, and the protocatechuic aldehyde (**1**) and ethyl gallate (**2**) showed the strongest antioxidative activity and rhododendrin (**3**) and protocatechuic acid ethyl ester (**6**) showed stronger antioxidative activity.

Acknowledgments

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