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Rapid analysis of the bioactive components in *Saxifraga stolonifera*, an edible and medicinal herb with anti-tumor effects, by HPLC-DAD, ESI/MS^n

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Summary

Saxifraga stolonifera is an edible and herbaceous plant, which has been demonstrated to have anti-tumor effects in vivo and in vitro. The aim of this paper is to determine the main bioactive components in *S. stolonifera,* and their distribution in different parts of *S. stolonifera* and in *S. stolonifera* that was cultivated in different places in China using a high-performance liquid chromatography-diode array detector and electrospray ionization/ion trap mass spectrometry (HPLC-DAD-ESI/MS^n). Four main components were identified and three were quantified. The contents of gallic acid, protocatechuic acid and bergenin had significant differences not only between the roots and stems-leaves of the plant, but also among different cultivated varieties of *S. stolonifera*. The experiment showed that the method used here exhibited good repeatability and recovery. Therefore, the results provide reliable data for research and development in the future on the level and distribution of the three bioactive components of *S. stolonifera*.

Keywords: gallic acid; protocatechuic acid; bergenin; HPLC-DAD; *Saxifraga stolonifera*

Introduction

Saxifraga stolonifera Curtis is an herb that belong to the *Saxifragaceae* family. This family is nearly cosmopolitan in distribution. The vast majority of the genera and species are found in the Northern Hemisphere, particularly in mountainous areas, with centres of diversity in western North America, East Asia and the Himalayas and Europe (Soltis, 2007). In East Asia, *S. stolonifera* is generally considered a decorative horticultural plant, and it is sometimes used as a medicine. In China, *S. stolonifera* is used as traditional medicine to treat many diseases, such as tympanitis, haemorrhoids and phthisis bulbi.

Recently, *S. stolonifera* has also been found to have pharmacological benefits, including anti-inflammatory effects, beneficial effects for prostate hyperplasia, liver function protection, and anticancer effects. It was found that saxifragin isolated from *S. stolonifera* has anti-inflammatory effects via the inhibition of NF-ϰB involved caspase-1 activation (Cheon et al., 2015). Researchers found that saxifragin can suppress the lipopolysaccharide (LPS)-induced production level of nitrous oxide (NO), PGE2, and pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6, in vitro and in vivo. In addition, injections, tablets, and suppositories made from 100% of the whole plant *S. stolonifera* have positive effects on prostate hyperplasia (Chen et al., 2003). Nakagiri et al. (2001) demonstrated that *S. stolonifera* or isolation of *S. stolonifera* protects liver function. Their group found that increased level of alanine aminotransferase (AAT) and glutamic oxaloacetic transaminase (GOT) in animals that were treated with LPS were reduced after treatment with *S. stolonifera*. It was demonstrated that quercetin isolated from *S. stolonifera* exhibited a strong inhibitory effect on human gastric cancer cell line (BGC-823) cells in a time- and dose-dependent manner (Chen et al., 2008). Moreover, Ju found that extracts of *S. stolonifera* have positive effects on benign thyroid tumours (Longtao, 2008). In addition, the intake of *S. stolonifera* by drinking a decoction of dried leaves had antitumor effects on gastric cancers (Nagata et al., 2016).

The structures of gallic acid, protocatechuic acid, and bergenin are identified (Chen et al., 2008) and presented in Fig. 2. Gallic acid, protocatechuic acid, and bergenin are phenolic acids, and bergenin is a gallic acid derivative as well as a c-glycoside, having the simplest structure in nature. In recent years, some reports have clarified that *S. stolonifera* has many biologically active components, including quercetin, quercetin-3-O-rhamnoside (Luo et al., 1988), saxifragin (Morita et al., 1974), kaempferol, bergenin (Luo et al., 1988), gallic acid (Luo et al., 1988), protocatechuic acid (Luo et al., 1988) and chlorogenic acid (Aoyagi et al., 1995). Gallic acid has many important biological activities such as anti-inflammatory effects (Luo et al., 1988), antitumour effects (Kawada et al., 2001), apoptosis-inducing effect (Ohno et al., 1999), antibacterial activities (Chanwitheesuk et al., 2007), anti-melanogenic and antioxidant properties (Kim, 2007), anticancer effects (Faried et al., 2007), and cardioprotective effects (Priscilla et al., 2009). Further, protocatechuic acid has biological properties such as anti-ageing effects (Zhang et al., 2011), anti-inflammatory effects (Min et al., 2010), antioxidant properties (Shi et al., 2006), antihepatotoxicity (Liu et al., 2002), antitumour effects (Tseng et al., 1998, 2000) and anti-neurotoxicity (An et al., 2006). Bergenin's biological activities include antioxidant properties (Nazir et al., 2011), antihepatotoxic activity (Kim et al., 2000), anti-inflammatory effects (Swarnalakshmi et al., 1984), antimicrobial activities (Prithiviraj et al., 1997), electrocatalytical properties (Zhuang et al., 2008) and neuroprotective effects (Takahashi et al., 2003). Therefore, it is meaningful to determine the composition and pharmacological activities of *S. stolonifera* and to find more rational uses of *S. stolonifera* as a source of crude drugs to provide medicines to alleviate and treat many diseases and conditions.

Despite the many studies that have analysed the structure and pharmacological activity of the components that have been isolated from *S. stolonifera*, the distributions and differences in gallic acid, protocatechuic acid and bergenin contents between *S. stolonifera* stem-leaves and roots have rarely been examined. The aim of this study was to evaluate the gallic acid, protocatechuic acid and bergenin contents in *S. stolonifera* and to establish a method for determining the gallic acid, protocatechuic acid and bergenin contents in *S. stolonifera*. The gallic acid, protocatechuic acid and bergenin contents in the samples was assayed by reversed-phase high-performance liquid chromatography (HPLC) with DAD detection.

Materials and methods

Chemicals and materials

The gallic acid standard was purchased from Targetmol (Targetmol. Co. Ltd, USA, CAS:149-91-7). Both the protocatechuic acid and bergenin standards were purchased from Tokyo Chemical Industry (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan, CAS: 99-50-3,477-90-7). The solvents used for the extraction of *S. stolonifera* samples were analytical-grade anhydrous alcohol (Qiangsheng

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Chemical Industry, Jiangsu, China) and Milli-Q distilled water (Millipore Australia Pty. Ltd., North Ryde, New South Wales, Australia), whereas those used for the HPLC analysis were HPLC-grade acetonitrile (Merck KgaA, Darmstadt, Germany) and Milli-Q distilled water.

Equipment

The Shimadzu HPLC system (Shimadzu Inc., LC-20A, Japan) consists of a computer-controlled system with upgraded LC-20A software and an SCL-10A VP system controller. Chromatographic separation was achieved using a J&K CHEMICA HPLC-C18 column (2.1 69 × 150 mm, 5 μm). Other accessories include two LC-20AT Shimadzu Liquid Chromatography Pumps, an RF-20A high-sensitivity fluorescence detector, an SPD M20A Diode Array Detector, and a CTO-20A Column Oven.

HPLC chromatographic conditions

The mobile phase consisted of acetonitrile (A) and 0.4% glacial acetic acid (B). The gradient program was as follows: A. 0-30.0 min, 5%-20%, B. 0-30.0 min, 95%-80%. The column temperature was maintained at 30 °C. The flow rate was 1.0 mL/min, and the injection volume was 20 μL.

Preparation of the standard sample solution

Standard samples of gallic acid 1 mg, protocatechuic acid 1 mg, and bergenin 2 mg were dissolved in a 1 mL 70% methanol solution, respectively (i.e., 1.0 mg/mL, 1.0 mg/mL and 2 mg/mL, respectively). For UV spectroscopy analysis, the standard gallic acid, protocatechuic acid and bergenin solution were all diluted with 70% methanol solution into five concentrations as follows: standard gallic acid: 10, 200, 300, 400, and 450 μg/mL; standard protocatechuic acid: 2, 50, 100, 150, and 180 μg/mL; and standard bergenin: 100, 300, 600, 800, and 1,860 μg/mL. All the standard samples were measured in the 200-400 nm UV absorption spectra range.

Sample preparation

Eight sample groups were used in this experiment, and they included the entire *S. stolonifera*, the above-ground part (the leaves and branches of *S. stolonifera*) and the below-ground part (the roots of *S. stolonifera*). The *S. stolonifera* herb that was cultivated in Suzhou (China) was purchased from a Guangliangji Chinese medicine store, and the other four *S. stolonifera* herbs cultivated in different places in China were purchased from Sichuan, Fujian, Suqian, and Shuyang. Both the above-ground part and the below-ground part were washed with water and then placed in a vacuum freeze-drying machine for 3 days, after which they were ground into fine powder. The dried *S. stolonifera* powder was filtered by a sieve. All the samples were then stored in polyethylene bags in the freezer at -20 °C for further investigation.

Preparation of the sample solution

Each sample (200 mg) was added into a 5 mL 70% methanol solution (the other 30% was ultrapure water), and the solutions were then extracted by ultrasound for 90 min with the temperature set at 75 °C. Finally, all the extracts were filtered through a 0.45-μm membrane for the HPLC analysis.

Analytic conditions

The sample solution was analysed by HPLC using a Shimadzu LC-20A HPLC-DAD with a C18 reversed phase column and a Metaguard column (4.6 mm Metasil AQ 5U C18 120A). The sample solution was filtered through a 0.45-μm nylon membrane filter (Millipore), and 20 μL was injected into the liquid chromatography column. The flow rate was set at 1.0 mL·min-1. The temperature of the column oven was set at room temperature. The mobile phase consisted of solvent B (acetonitrile) and solvent A (Milli-Q distilled water) at 70/30 (v/v). The spectra were recorded in the 200 to 400 nm range, and the absorbance of the effluent was monitored at 275 nm.

Electrospray ionization/ion trap mass spectrometry

Mass spectrometric analysis for the determination of gallic acid, protocatechuic acid and bergenin was performed using a TSQ quantum ultra-triple-quadrupole mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), which was equipped with an electro-spray ionization (ESI) interface in the negative mode. The following are the parameters of the mass spectrometer: sheath gas flow rate of 40 (arbitrary units); auxiliary gas flow rate of 10 (arbitrary units); spray voltage of 2500 V; vaporizer temperature of 350 °C; and capillary temperature of 350 °C. Helium was used as the collision gas for collision-induced dissociation (CID).

Quantification method

The quantification of gallic acid, protocatechuic acid and bergenin was performed using the external standard method. Three standard samples of gallic acid, protocatechuic acid and bergenin at various concentration levels were injected into the HPLC-DAD system, and the peak areas corresponding to the standard sample concentrations were observed, from which the calibration curves were created. The levels of the three main components in S. *stolonifera* were calculated with the calibration curves.

Results and discussion

Chromatographic and spectral characteristics

The HPLC chromatogram and TIC of the entire plant were identified by HPLC-DAD-ESI/MS^n, which are presented in Fig. 1A and B. As shown in Fig. 1 and Tab. 1, there are four main components in *S. stolonifera*. According to their molecular weight and MS/MS spectra of these four components (Fig. 1C), we could identity the potential compounds. The molecular weight of these four components (1-4) are 170, 314, 154 and 328, and, the molecular formula are $C_7H_6O_5$, $C_{13}H_{16}O_9$, $C_7H_6O_4$, and $C_{14}H_{16}O_9$, respectively, which may be gallic acid, norbergenin, protocatechuic acid, and bergenin, respectively.

The standard curve and of gallic acid, protocatechuic acid and bergenin

According to the four main active components identified by mass spectrometry, three of the main components gallic acid, protocatechuic acid and bergenin were qualitatively and quantitatively analysed in this study by HPLC-DAD.

As shown in Fig. 2, the HPLC-DAD spectra of the three standard samples gallic acid, protocatechuic acid and bergenin were compared in detail with the retention time and UV spectra of three main components in *S. stolonifera* (Fig. 2A). The results showed that the peak times of the three standard compounds on HPLC-DAD were 9.258, 15.317 and 19.582 min, respectively, and there were three corresponding absorption peaks on the HPLC-DAD spectra of *S. stolonifera*, the retention time of these absorption peaks was about the same as that of standard compounds. Fig. 2B showed the comparison of UV spectra of three standard samples with the three peaks of *S. stolonifera*. It could be found that the retention times and UV absorption spectra of the three peaks were the same as that of the

three standard samples. This fully proved the identification results of HPLC-DAD-ESI/MS^n, the three absorption peaks with retention time at 9.258, 15.317 and 19.582 min are gallic acid, protocatechuic acid and bergenin, respectively (Fig. 2C).

In order to quantitatively analyze the three main active components in *S. stolonifera*, standard solutions of the three compounds were injected into the HPLC column (Fig. 3) with the concentrations as follows. Gallic acid: 10, 200, 300, 400, and 450 µg/mL; protocatechuic acid: 2, 50, 100, 150, and 180 µg/mL; and bergenin: 100, 300, 600, 800, and 1860 µg/mL. The regression equations and correlation coefficients (r) were calculated by plotting the peak-area (y) vs. concentration (x, µg/mL) as follows: the correlation coefficients (r) of gallic acid were $y = 0.10139 + 0.04775x$, $R = 0.99734$. The correlation coefficients (r) of protocatechuic acid were $y = 0.08249 + 0.03626x$, $R = 0.9955$. The correlation coefficients (r) of bergenin were $y = 0.95934 + 0.00596x$, $R = 0.98215$ (x: sample concentration; y: peak area).

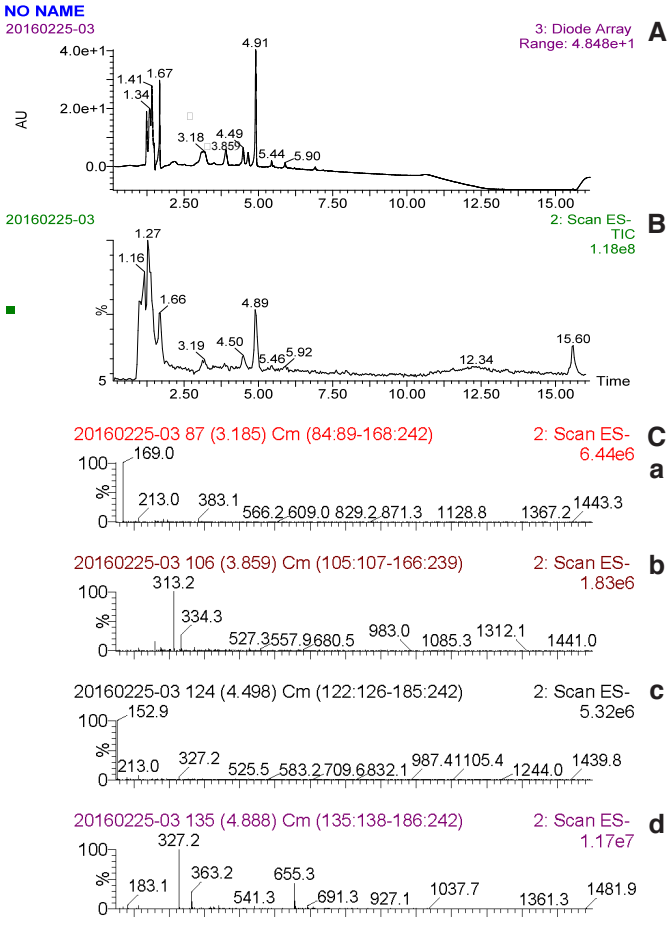

Fig. 1: Chromatography profile (275 nm) (A), the total ion chromatography (TIC) (B) and mass spectrometry (C) of *S. stolonifera* by HPLC-DAD-MS/ES$^-$. In Fig. 1 A, peak 1, 2, 3 and 4 respresent four components in *S. stolonifera*. MS/ES$^-$ spectra (a, b, c and d) of the four components in *S. stolonifera* are corresponding to 1, 2, 3 and 4 in Fig. 1 A and B, respectively.

The reproducibility of the HPLC for the determination of gallic acid, protocatechuic acid and bergenin in *S. stolonifera*

The sample extraction solution of *S. stolonifera* was measured by HPLC-DAD five times, and the content of gallic acid, protocatechuic acid, and bergenin was calculated. As shown in Tab. 2, the values of the repeated measurements of gallic acid were 0.339, 0.357, 0.336, 0.339, and 0.337 mg/g, with an average of 0.342 mg/g (±0.009 mg/g) and an RSD = 2.570%. In addition, the values of repeated measurements of protocatechuic acid were 0.077, 0.87, 0.078, 0.078, and 0.087 mg/g, with an average of 0.081 mg/g (±0.005 mg/g) and an RSD = 6.037%. Furthermore, the values of repeated measurements of bergenin were 2.260, 2.701, 2.204, 2.294, and 2.581 mg/g, with an average of 2.409 mg/g (±0.218 mg/g) and an RSD = 9.052%. These results indicate that the HPLC determination of the gallic acid, protocatechuic acid and bergenin in *S. stolonifera* shows good repeatability.

Extraction recovery of gallic acid, protocatechuic acid and bergenin in *S. stolonifera*

To determine the accuracy of the HPLC results, an ethanol extraction of *S. stolonifera* was used as a test sample solution. The sample solution was divided into three parts, and the content of gallic acid, protocatechuic acid, and bergenin in these samples were determined. The content of gallic acid, protocatechuic acid, and bergenin were calculated by the linear equation produced by the standard samples; 70, 50, and 1500 µg of gallic acid, protocatechuic acid, and bergenin standard samples were added to the test sample solution before blending as part of the recovery study. The experimental results showed that the extraction recovery rate for gallic acid, protocatechuic acid, and bergenin in *S. stolonifera* were 102.491%±6.516%, 99.685%±10.485%, and 105.050%±10.197%, respectively (Tab. 3). These results show high recovery considering the complexity of the analyses. The recovery tests of gallic acid, protocatechuic acid and bergenin in *S. stolonifera* exhibit very high precision.

The difference in gallic acid, protocatechuic acid and bergenin contents between cultivated *S. stolonifera* varieties

This experiment tested the five varieties of *S. stolonifera* cultivated in different locations in China. The gallic acid, protocatechuic acid and bergenin content in different varieties of cultivated *S. stolonifera* were investigated by longitudinal comparison. The results are shown in Tab. 4 and Fig. 4. Here it can be seen that there is a significant difference ($p < 0.05$) in gallic acid and protocatechuic acid content and no significant difference in bergenin content between the five cultivated varieties. On a dry-weight basis, *S. stolonifera* that was cultivated in Fujian, Suzhou, and Suqian contained the highest concentrations of gallic acid, protocatechuic acid, and bergenin (0.538, 0.616, and 15.314 mg/g, respectively). Meanwhile, *S. stolonifera* cultivated in Suqian contained the lowest concentration of gallic

Tab. 1: MS/MS analysis of methanol extract of *S. stolonifera*.

Peak	RT	[M-H]$^-$	Molecular weight	Tentative identification	Molecular formula
1	3.185	169.0	170.0	gallic acid	$C_7H_6O_5$
2	3.859	313.2	314.2	norbergenin	$C_{13}H_{16}O_9$
3	4.498	152.9	154.02	protocatechuic acid	$C_7H_6O_4$
4	4.888	327.2	328.07	bergenin	$C_{14}H_{16}O_9$

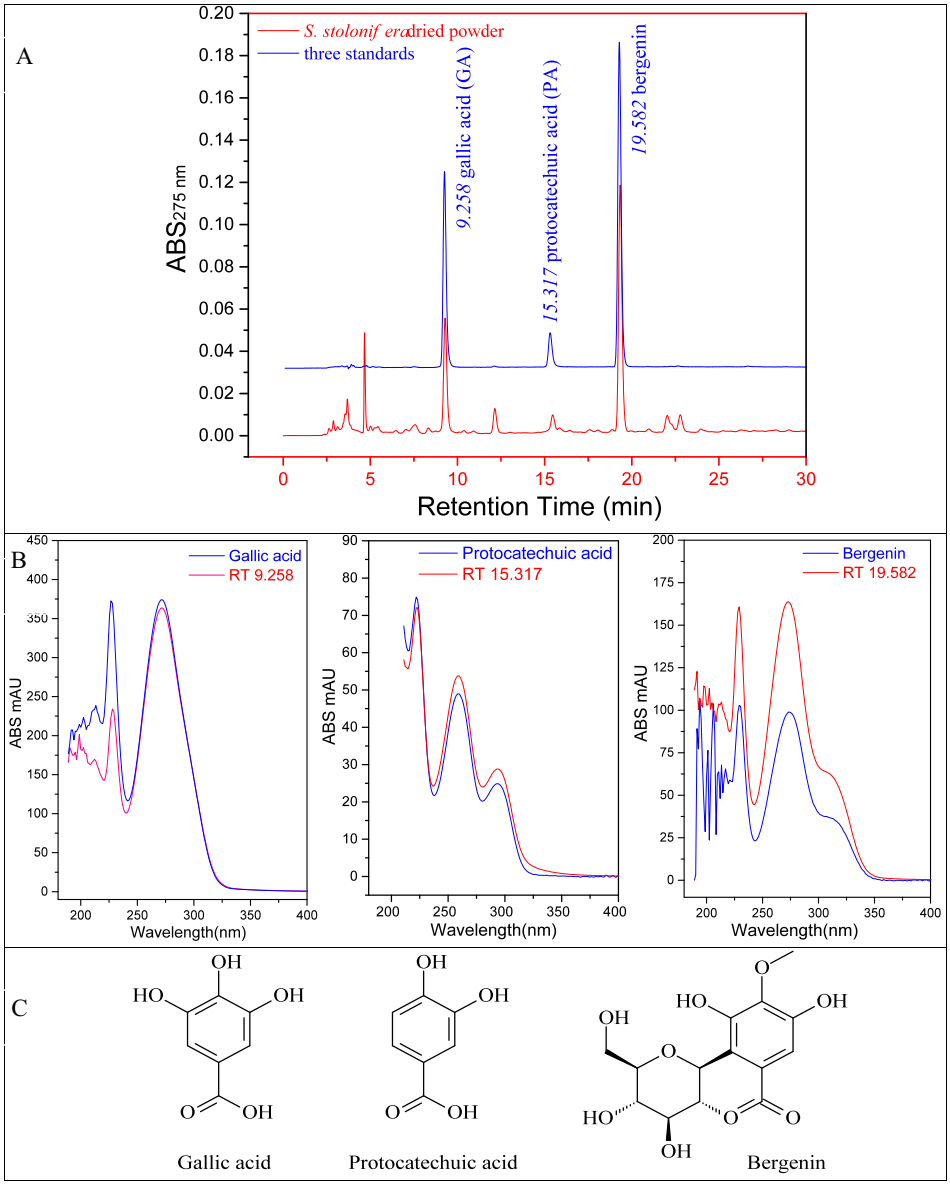

Fig. 2: HPLC-DAD patterns of three standards (GA, PA and bergenin) and the main components in *S. stolonifera* (A). UV spectra (B) and their molecular structures (C) of three standards and three peaks in *S. stolonifera*. RT: retention time of three peaks in HPLC-DAD.

acid (0.191 mg/g), and *S. stolonifera* cultivated in Shuyang contained the lowest concentration of protocatechuic acid (0.171 mg/g) and bergenin (9.605 mg/g). The results therefore indicate that *S. stolonifera* cultivated in Fujian, Suzhou, and Suqian have the greatest potential for the commercial extraction of gallic acid, protocatechuic acid and bergenin, respectively.

The Distribution of gallic acid, protocatechuic acid and bergenin in the *S. stolonifera* cultivated in Suzhou

The roots, stem-leaves and whole plant of *S. stolonifera* that was cultivated in Suzhou were extracted using 70% anhydrous alcohol to analyse the distribution of gallic acid, protocatechuic acid and bergenin in the stem-leaves and roots. The extracts were analysed using HPLC and DAD, and the results are shown in Fig. 5. There were significant differences ($p < 0.05$) in gallic acid, protocatechuic acid and bergenin content between the different parts of *S. stolonifera*. The gallic acid level in the roots was as high as 0.199 mg/g, whereas the stem-leaves contained much less gallic acid (less than one-fifth of the content in the roots). Conversely, the content of protocatechuic acid and bergenin in the roots was as high as 0.090 mg/g and 1.799 mg/g, respectively, whereas stem-leaves contained 0.554 mg/g and 14.463 mg/g, respectively.

Conclusion

Our research demonstrated that there are four main components in *S. stolonifera*, gallic acid, norbergenin, protocatechuic acid and bergenin. Three main components were quantitatively analysed, and the results showed that the contents of gallic acid, protocatechuic acid and bergenin in the stem-leaves and roots of *S. stolonifera* have obvious differences. The content of gallic acid in the roots (0.199 mg/g) was much higher than that in the stem-leaves (0.037 mg/g). In addition, the content of protocatechuic acid and bergenin in the roots (0.090 mg/g and 1.799 mg/g, respectively) were much lower than those in the stem-leaves (0.554 mg/g and14.463 mg/g, respectively).

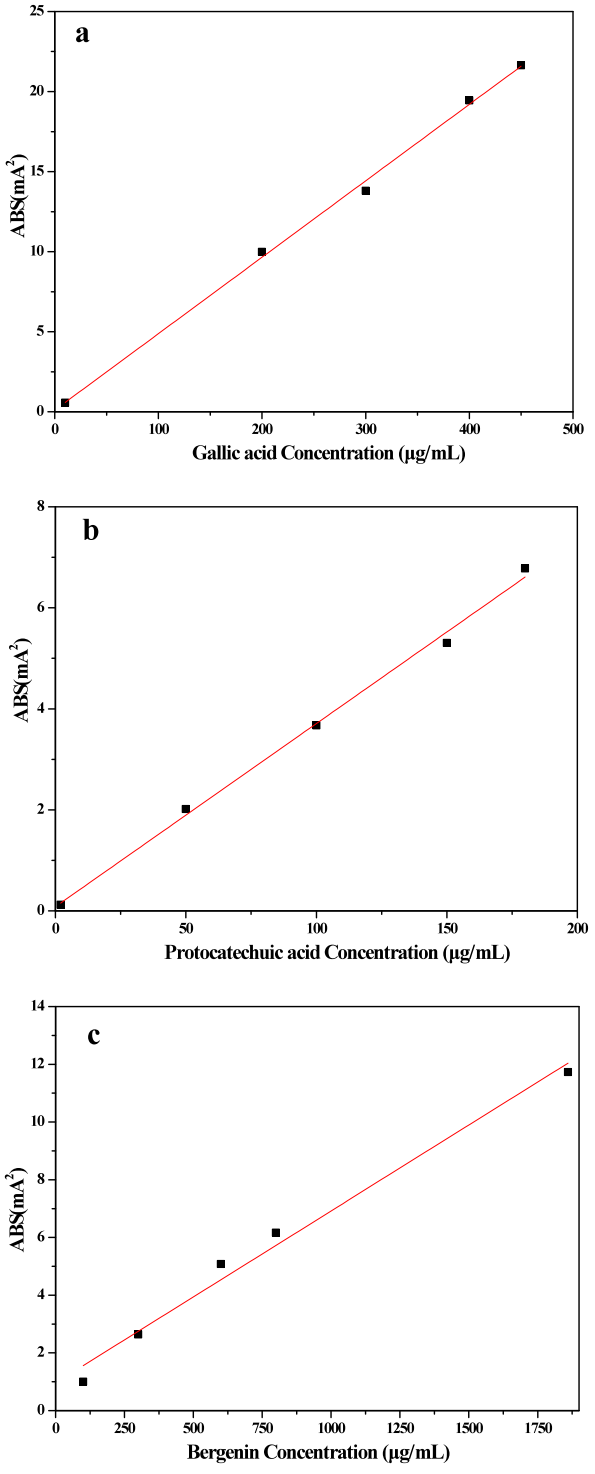

Fig. 3: Standard curves of samples concentration and their absorption at 275 nm. a, b and c represent the curves of standard gallic acid, protocatechuic acid and bergenin, respectively.

Furthermore, *S. stolonifera* that was cultivated in different locations in China has differences in the content of these three bioactive components. *S. stolonifera* cultivated in Suqian contained the lowest concentration of gallic acid (0.191 mg/g). *S. stolonifera* that was cultivated in Shuyang contained the lowest concentration of protocatechuic acid (0.171 mg/g) and bergenin (9.605 mg/g). Moreover, the method used in this study for determining the gallic acid, protocatechuic acid, and bergenin content exhibited good repeatability (RSD 2.570%, 6.037% and 9.052%) and recovery (102.491%, 99.685% and 105.050%, respectively). The results provide reliable data for research and development in the future on the level and distribution of gallic acid, protocatechuic acid and bergenin in *S. stolonifera*.

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Author contributions

Yu-Qing Zhang conceived this study; Meng Zhang and Dong Liu constructed the database and performed the statistical analysis and wrote the paper. Yu-Qing Zhang revised the paper.

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Tab. 2: Reproducibility of gallic acid, protocatechuic acid, and bergenin of the HPLC analysis.

Sample	Values of repeated measurements (n = 5, mg/g)					Mean	SD	RSD
gallic acid	0.339	0.357	0.336	0.339	0.337	0.342	0.009	2.570
protocatechuic acid	0.077	0.087	0.078	0.078	0.087	0.081	0.005	6.037
bergenin	2.260	2.701	2.204	2.294	2.581	2.409	0.218	9.052

Tab. 3: The recoveries of gallic acid, protocatechuic acid and bergenin in *S. stolonifera*.

Sample	Sample content (μg)	Dosage (μg)	Theoretical value (μg)	Measured value (μg)	Recovery (%)	SD	RSD (%)
gallic acid	136.668±0.009	70	206.668	211.92±0.040	102.491%	0.067	6.516
protocatechuic acid	32.532±0.005	50	82.532	82.32±0.023	99.685%	0.105	10.485
bergenin	963.776±0.218	1500	2463.776	2590.992±0.768	105.050%	0.107	10.197

Values represent means ± SD (n = 5).

Tab. 4: The difference in gallic acid, protocatechuic acid and bergenin content between *S. stolonifera* varieties.

Sample resource area	gallic acid (mg/g) ±SD	protocatechuic acid (mg/g) ±SD	bergenin (mg/g) ±SD
Sichuan in western China	0.347 ± 0.014^{b}	0.422 ± 0.056^{b}	11.381 ± 0.373^{c}
Shuyang in eastern China	0.287 ± 0.014^{c}	0.171 ± 0.008^{e}	9.605 ± 0.099^{d}
Suqian in eastern China	0.191 ± 0.016^{e}	0.362 ± 0.056^{c}	15.314 ± 0.832^{a}
Fujian in southern China	0.538 ± 0.046^{a}	0.217 ± 0.014^{d}	13.841 ± 0.515^{b}
Suzhou in eastern China	0.212 ± 0.011^{d}	0.616 ± 0.035^{a}	14.137 ± 0.805^{ab}

Values represent means ± SD (n = 8). The superscript letters of the same column of data are completely different, indicating that the difference is significant ($P < 0.05$), and any of the same letters indicates that the difference is not significant ($P > 0.05$).

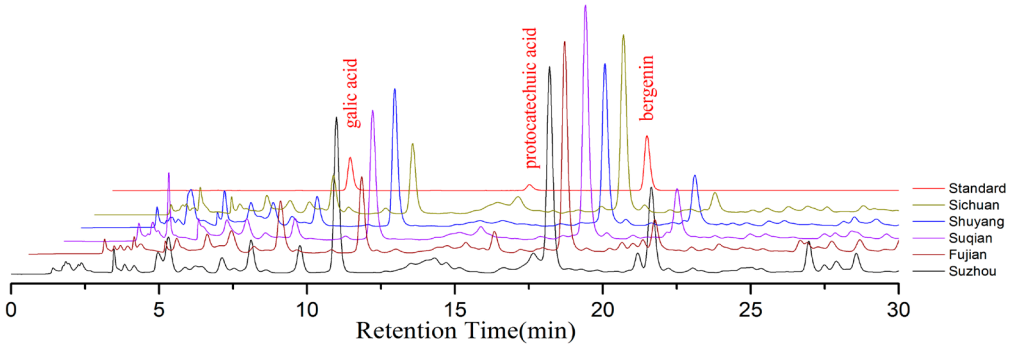

Fig. 4: The difference in content and HPLC pattern of GA, PA and bergenin in *S. stolonifera* cultivated in five different locations in China.

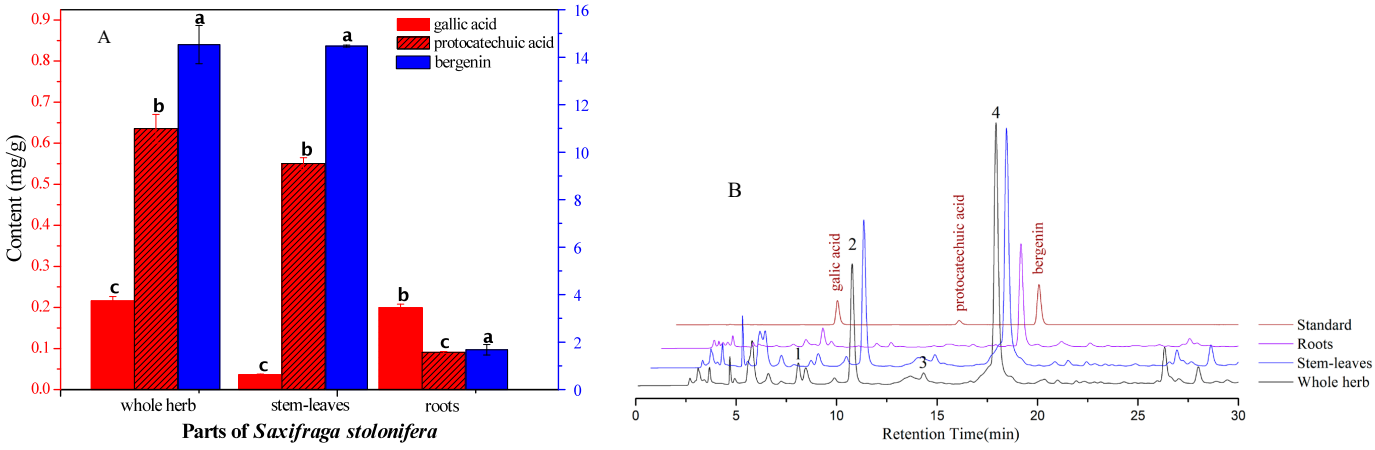

Fig. 5: (A) The distribution of gallic aicd, protocatechuic acid and bergenin in different parts of *S. stolonifera* (n = 8). (B) HPLC chromatogram of the standard sample, alcohol extract of the stem-leaves, roots of *S. stolonifera* and the whole herb. The superscript letters of the same column of data are completely different, indicating that the difference is significant ($P < 0.05$), and any of the same letters indicates that the difference is not significant ($P < 0.05$).

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