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Antioxidant Activities and Xanthine Oxidase Inhibitory Effects of Extracts and Main Polyphenolic Compounds Obtained from *Geranium sibiricum* L.

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The antioxidant capacity and xanthine oxidase inhibitory effects of extracts and main polyphenolic compounds of *Geranium sibiricum* were studied in the present work. The antioxidant capacity was evaluated by ferric reducing antioxidant power, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, superoxide radical scavenging, nitric oxide scavenging, β-carotene−linoleic acid bleaching, and reducing power assays. Among the extracts and four fractions, the ethyl acetate fraction showed the highest phenolic content (425.36 ± 9.70 mg of gallic acid equivalent/g extracts) and the best antioxidant activity. The IC_{50} values of the ethyl acetate fraction were 0.93, 3.32, 2.06, 2.66, and 1.64 μg/mL in the DPPH radical scavenging, superoxide radical scavenging, nitric oxide scavenging, β-carotene−linoleic acid bleaching, and reducing power assays, respectively. Of the polyphenolic compounds separated from the ethyl acetate fraction, geraniin showed a higher activity than corilagin and gallic acid. The IC_{50} values ranged from 0.87 to 2.53 μM, which were even lower than the positive control (except for allopurinol). All test samples except for the petroleum ether fraction showed xanthine oxidase inhibitory effects. We conclude that *G. sibiricum* represents a valuable natural antioxidant source and is potentially applicable in the healthy food industry.

KEYWORDS: ***Geranium sibiricum*** **L.; polyphenolic compounds; antioxidant activity; xanthine oxidase**

INTRODUCTION

Geranium sibiricum L., which belongs to the family of Geraniaceae, is widely distributed in northeast China, Korea, Japan, and some European countries. It has been used for healing diarrhea, intestinal inflammation, eruptive skin disease, bacteria infection, and cancer in Korea and Bulgaria (*1*, *2*). Tubers of *G. sibiricum* are used as food in Russia and Turkey (*3*, *4*). A decoction of the roots of *G. sibiricum* is drunk to treat uterine cancer in Peru (*5*). In the folk medicine of China, *G. sibiricum* white spirit is used to treat rheumatoid arthritis. It is also used as a popular condiment for cooking meat, especially for chickens and ducks (*6*). Besides its main usage as food, the medical applications have provoked much interest. *G. sibiricum* has been used worldwide in folk medicine for healing intestinal inflammation, dermatitis, diarrhea, and cancer (*7*). Chemical constituent investigations indicated that it is rich in polyphenolic compounds, which have been considered as being responsible for the beneficial efficacies on human health (*8*). As the main substances of *G. sibiricum*, geraniin, corilagin, and gallic acid showed remarkable pharmacological activities including antiviral, hepatoprotective, and antihypertensive activities, etc. (*9*−*11*). Moreover, Sokmen et al. (*12*) demonstrated that the extracts of *Geranium sanguineum* (Geraniaceae) possessed strong antioxidant activity, which encouraged us to search for more antioxidants in the Geraniaceae family.

Reactive oxygen species (ROS) include superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), and peroxyl radicals ($ROO^{\bullet}$), etc. They are continuously generated during aerobic metabolism and exogenous sources such as UV radiation or environmental pollution. ROS are mainly produced by mitochondrial oxidative metabolism and cytochrome P450 systems in hepatocytes. However, when the ROS production is greater than the detoxification capacity of the cell, excessive ROS cause oxidative stress and even induce extensive damage to DNA, proteins, and lipids (*13*). It had been confirmed that uric acid, which causes gout, is formed from xanthine in the presence of xanthine oxidase (XOD). $O_2^{\bullet -}$, generated upon the formation of uric acid, has been found to cause oxidative damage of living tissues (*14*). Fortunately, antioxidants may help the body to protect itself from various types of oxidative damage, which are linked to diseases such as cancer, diabetes, cardiovascular disorders, and aging (*15*). Recently, searching for antioxidants from plants has been of major interest. However, scientific studies on

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antioxidant properties of wild plants are still rather scarce. The assessment of antioxidant activity of wild plants remains an interesting and useful task for finding new sources of natural antioxidants (*16*).

To the best of our knowledge, the antioxidant properties and XOD inhibitory effects of *G. sibiricum* extracts have not been evaluated yet. Therefore, the aim of the present study was to investigate the antioxidant properties of aqueous and ethanol extracts of *G. sibiricum*, different fractions of the ethanol extracts as well as three main polyphenolic components, namely, geraniin, corilagin, and gallic acid, by ferric reducing antioxidant power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, superoxide radical scavenging, nitric oxide (NO) scavenging, β-carotene−linoleic acid bleaching, and reducing power assays. Moreover, XOD inhibitory effects were further investigated.

MATERIALS AND METHODS

Chemicals. DPPH, β-carotene, linoleic acid, 1,3,5-tri(2-pyridyl)-2,4,6-triazine (TPTZ), lipopolysaccharide (LPS), Folin−Ciocalteu reagent, allopurinol, butylated hydroxytoluene (BHT), Tween 40, dimethyl sulfoximine (DMSO), and XOD were purchased from Sigma (St. Louis, MO). Silica gel was purchased from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China). Sephadex LH-20 for column chromatography was purchased from GE Healthcare (Bio-Sciences AB, Uppsala, Sweden). High-pressure liquid chromatography (HPLC) grade acetonitrile was purchased from J&K Chemical Ltd. (Beijing, China). Other reagents and chemicals including petroleum ether, ethyl acetate (EtOAc), *n*-butanol, and methanol were of analytical grade and purchased from Beijing Chemical Reagents Co. (Beijing, China). Deionized water was produced by a Millipore Direct-Q purification system (Millipore Corp., Bedford, MA).

Plant Materials. The plant *G. sibiricum* L. (Geraniaceae) was collected from the Botanical Garden of Northeast Forestry University and authenticated by Prof. Shaoquan Nie from the Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, People's Republic of China. A specimen (#054001010017001) was deposited in the Herbarium of this Key Laboratory. The materials were dried in the shade at room temperature.

Extraction, Fractionation, and Separation of Polyphenolic Compounds. The plant materials were ground into powder before use. The aqueous extracts were obtained as follows: Fifty grams of samples was refluxed by 400 mL of boiling water for 2 h, and the solvent was filtered and freeze-dried to obtain the extract powder (yield, 10.31%, w/w). For comparison, the samples were also soaked three times with 50% ethanol for 5 days at room temperature. The combined 50% ethanol extract was filtered through Whatman #2 filters and concentrated in vacuo to yield dried 50% ethanol extracts (yield, 15.80%, w/w). To obtain the fractions of polyphenolic compounds, the 50% ethanol extracts (150.0 g) were suspended in distilled water and partitioned successively with petroleum ether, EtOAc, and *n*-butanol. All of the extracts were concentrated in vacuo to obtain four fractions: petroleum ether fraction (3.81 g; yield, 2.54%), EtOAc fraction (32.60 g; yield, 21.73%), *n*-butanol fraction (35.82 g; yield, 33.13%), and aqueous residues (water fraction, 63.52 g; yield, 42.33%). The EtOAc fraction (30 g) was applied to column chromatography on silica gel (5 cm × 25 cm, 200−300 mesh, 180 g) with a stepwise gradient from 0 to 100% MeOH in EtOAc, resulting in 10 subfractions. Subfraction 2 (15% MeOH in EtOAc) was further separated on a Sephadex LH-20 column (2.5 cm × 60 cm, mobile phase 40% MeOH in water) combined with crystallization to derive compounds **1** (corilagin, 29.68 mg), **2** (geraniin, 8.73 mg), and **3** (gallic acid, 31.47 mg), respectively. Structures were assigned by comparing their spectroscopic data with literature (*17*, *18*).

HPLC Analysis of Corilagin, Geraniin, and Gallic Acid. Analytical HPLC was performed on a Jasco HPLC system (Jasco Inc., Easton, MD). All samples of *G. sibiricum* extracts were filtered through 0.25 μm filters before they were analyzed. They were separated on a HIQ Sil C18 V reversed-phase column (250 mm × 4.6 mm i.d., Kya Tech, Hachioji City, Japan). For the HPLC detection, the wavelength was 220 nm on the basis of full-wavelength scanning results. The mobile phase was acetonitrile−water (15/85, v/v). The flow rate was 1.0 mL/min, and the column temperature was kept at 30 °C. The retention times of gallic acid, corilagin, and geraniin were 3.9, 10.0, and 12.8 min, respectively. Those chromatographic peaks were confirmed with reference compounds in retention time and MS results.

Total Phenolic Content. Total phenolics were determined by using the Folin−Ciocalteu method (*19*) with little modification. Briefly, 40 μL of samples (0.05 mg/mL) was mixed with 1.8 mL of 10-fold-diluted Folin−Ciocalteu reagent. The mixture was allowed to stand for 5 min at room temperature. Then, the reaction was neutralized with 1.2 mL of saturated sodium carbonate (7.5%). The absorbance of the resulting blue color was measured at 765 nm with a UV−vis spectrophotometer (UNICO, Shanghai, China) after incubation for 90 min at room temperature. Quantification was done on the basis of the standard curve of gallic acid. Results were expressed in g gallic acid equivalent (GAE)/g extracts.

Total Antioxidant Capacity by FRAP Assay. The total antioxidant capacity of extracts and four fractions was determined by a total antioxidant capacity assay kit with the FRAP method (Beyotime Institute of Biotechnology, China). Plant extracts (5 μL) were allowed to react with 200 μL of the FRAP solution for 5 min at 37 °C in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 0.15 and 1.5 mM $FeSO_4$. Results are expressed in mM Fe(II)/g extracts and compared with those of ascorbic acid.

DPPH Radical Scavenging Assay. The ability of the extracts to scavenge DPPH free radicals was determined according to the method of Sokmen et al. (*12*) with little modification. Briefly, 10 μL of the sample (final concentrations were 0.5, 1, 5, 10, 25, 50, and 100 μg/mL, respectively) was mixed with ethanol (90 μL) and then added to 0.004% DPPH (200 μL) in ethanol. The mixture was vigorously shaken and then immediately placed in a UV−vis spectrophotometer (UNICO) to monitor the decrease in absorbance at 517 nm. Monitoring was continued for 70 min until the reaction reached a plateau. Ascorbic acid (Sigma−Aldrich), a stable antioxidant, was used as a synthetic reference. Pure ethanol was used as a control sample. The radical scavenging activities of samples, expressed as a percentage inhibition of DPPH, were calculated according to the formula: inhibition percentage (Ip) = [(AB − AA)/AB] × 100, where AB and AA are the absorbance values of the blank sample and of the tested samples checked after 70 min, respectively.

Superoxide Radical Scavenging Assay [Nitroblue Tetrazolium Chloride (NBT) Assay]. Superoxide radicals were generated by a modified method of Wang et al. (*20*). All solutions were prepared in 0.05 M phosphate buffer (pH 7.4). Twenty microliters of the test samples (final concentrations were 0.5, 1, 5, 10, 50, and 100 μg/mL), 180 μL of 0.6 mM NBT in buffer, and 20 μL of 3 mM hypoxanthine in 50 mM KOH were mixed in 96-well microplates. The reaction was started by adding 20 μL of XOD in buffer (1 unit in 10 mL of buffer) to the mixture. The reaction mixture was incubated at 37 °C for 30 min, and the absorbance at 560 nm was determined using an enzyme-linked immunosorbent assay reader (UNICO, United States). Trolox was used as a positive control. Three replicates were made for each test sample. The percent inhibition ratio (%) was calculated according to the following equation: % inhibition = [(rate of control reaction − rate of sample reaction)/rate of control reaction] × 100.

NO Scavenging Activity. The method of Wang et al. (*20*) was used to assay the scavenging activity of extracts or compounds on NO. The reaction solution (1 mL) containing 10 mM sodium nitroprusside in phosphate-buffered saline (PBS) (pH 7.0) was mixed with extracts or compounds (final concentrations were 0.5, 1, 5, 10, 50, and 100 μg/mL) followed by incubation at 37 °C for 1 h. A 0.5 mL aliquot was then mixed with 0.5 mL of Griess reagent. The absorbance at 540 nm was measured. The percent inhibition of NO generated was measured by comparison with the absorbance value of the negative control (10 mM sodium nitroprusside and PBS). Ascorbic acid was used as the positive control.

β-Carotene−Linoleic Acid Test. The antioxidant activity of the samples was determined using the β-carotene−linoleic acid test (*21*). Approximately 10 mg of β-carotene (type I, synthetic) was dissolved in chloroform (10 mL). The carotene−chloroform solution (0.2 mL) was pipetted into a boiling flask containing linoleic acid (20 mg) and 200 mg of Tween 40. Chloroform was removed using a rotary evaporator

(RE-52AA) at 40 °C for 5 min, and distilled water (50 mL) was added to the residue slowly with vigorous agitation to form an emulsion. A portion of the emulsion (5 mL) was added to a tube containing 0.2 mL of sample solution (final concentrations were 0.5, 1, 5, 10, 50, and 100 μg/mL), and the absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The tubes were placed in a water bath at 50 °C, and the oxidation of the emulsion was monitored spectrophotometrically by measuring the absorbance at 470 nm over a 60 min period. Control samples contained 200 μL of water instead. BHT, a stable antioxidant, was used as a synthetic reference. The antioxidant activity was expressed as inhibition percentage with reference to the control after a 60 min incubation using the following equation: AA = 100(DRC − DRS)/DRC, where AA = antioxidant activity, DRC = degradation rate of the control = [ln(a/b)/60], DRS = degradation rate in presence of the sample = [ln(a/b)/60], a = absorbance at time 0, and b = absorbance at 60 min.

Reduce Power Assay. The reducing power of all test samples was determined by the method prescribed by Wang et al. (*20*) with some modification. Briefly, 0.5 mL of ethanol containing different concentrations of sample (final concentrations were 0.5, 1, 5, 10, 50, and 100 μg/mL) was mixed with 0.5 mL of phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of potassium ferricyanide (1%). The reaction mixture was incubated at 50 °C for 20 min. After incubation, 0.5 mL of trichloroacetic acid (10%) was added and centrifuged (650*g*) for 10 min. From the upper layer, 0.5 mL of solution was mixed with 0.5 mL of distilled water and 0.1 mL of $FeCl_3$ (0.1%). The absorbance of all sample solutions was measured at 700 nm. An increased absorbance indicated increased reducing power.

Determination of XOD Inhibitory Activity. The measurement of the XOD inhibitory activity was carried out according to the method of Wang et al. (*20*) with slight modifications. First, 798 μL of 0.1 unit XOD in buffer (200 mM sodium pyrophosphate/HCl, pH 7.5) and 2 μL (500, 250, 50, 25, 5, and 2.5 μg/mL) of the test extracts or compounds in DMSO were mixed at 37 °C for 5 min. The control group contained no test agent. The reaction was started by adding 200 μL of 0.6 mM xanthine in double-distilled water to the mixture. The reaction mixture was incubated at ambient temperature. Finally, the absorption increments at 295 nm, which indicated the formation of uric acid, were determined every min up to 8 min. Allopurinol was used as a positive control. Three replicates were made for each test sample. The percent inhibition ratio (%) was calculated according to the following equation: % inhibition = [(rate of control reaction − rate of sample reaction)/rate of control reaction] × 100.

Statistical Analysis. All results are expressed as mean values ± standard deviations (SDs) (n = 3). The significance of difference was calculated by one-way analysis of variance, and values $p < 0.01$ were considered to be significant.

RESULTS AND DISCUSSION

Because different antioxidant compounds may act through different mechanisms, no single method can fully evaluate the total antioxidant capacity. For this reason, studying the complex antioxidant activities often uses a multimethod approach (*22*). Radical scavenging activities are very important due to the deleterious role of free radicals in biological systems. The effects of antioxidants on DPPH radical, superoxide radical, and NO scavenging are actual electron transfer-based assays, which measure the capacity of an antioxidant to reduce an oxidant, resulting in a color change. The models of these radical scavenging assays are widely used for evaluating antioxidant activity in a relatively short time (*23*). Besides electron transfer, a hydrogen atom transfer reaction is another antioxidant capacity-based assay. Hydrogen atom transfer reaction-based assays are methods in which antioxidant and substrate compete for thermally generated $ROO^{\bullet}$ through the decomposition of azo compounds. These assays include β-carotene bleaching, inhibition of low-density lipoprotein oxidation assays, etc. (*24*). In the β-carotene bleaching assay, the antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from the oxidation of linoleic acid (*25*). The reducing power assay (ferricyanide method) measures the amount of peroxide produced during the initial stages of oxidation, which is the primary product of oxidation (*24*). Therefore, a comparison of different methods could help the investigators to comprehend the antioxidant activity of samples deeply and completely. In the present work, FRAP, DPPH radical scavenging, superoxide radical scavenging, NO scavenging, β-carotene−linoleic acid bleaching, and reducing power assays were used to study the antioxidant activities of extracts obtained from *G. sibiricum* adequately.

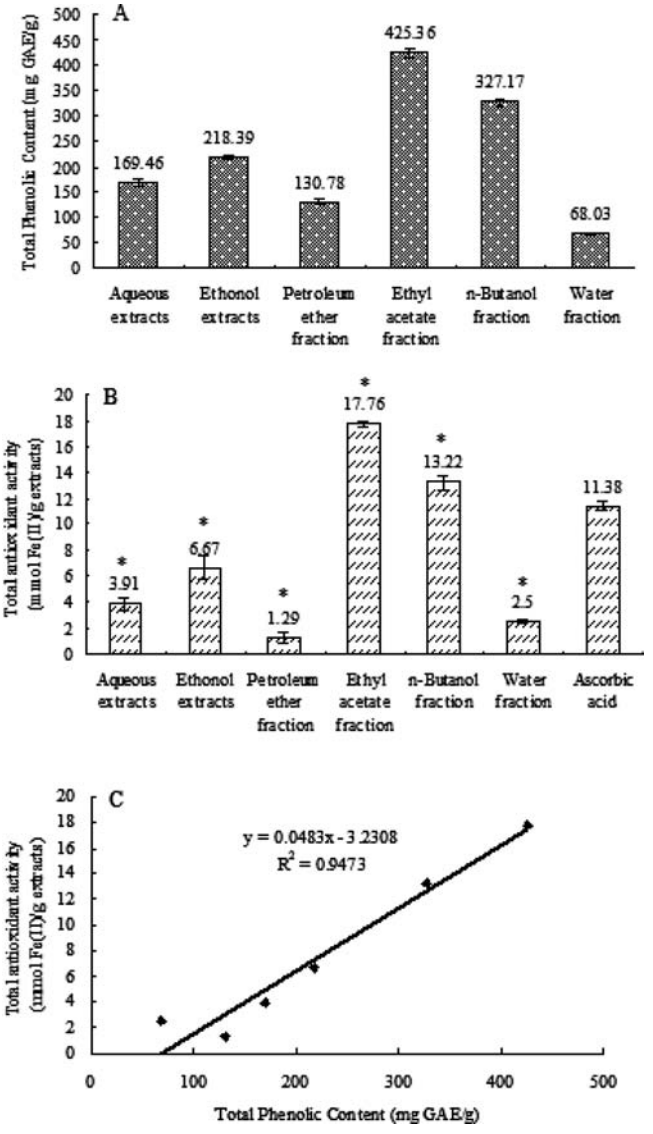

Figure 1. Contents of total phenolic (**A**), total antioxidant capacity by the FRAP assay (**B**), and correlation between total phenolic content and total antioxidant capacity (**C**) of the extracts and four fractions of *G. sibiricum* (n = 3). The symbols * indicate significant differences $p < 0.01$ with respect to the positive control (ascorbic acid).

Total Phenolic Contents and Total Antioxidant Capacity by FRAP Assay of *G. sibiricum* Extracts. The total phenolic contents and total antioxidant capacity by FRAP assay of aqueous and ethanol extracts as well as petroleum ether, EtOAc, n-butanol, and water fractions from *G. sibiricum* are shown in **Figure 1**. As shown in **Figure 1A**, the content of phenolic compounds in the EtOAc fraction of *G. sibiricum* was more (425.36 ± 9.71 mg GAE/g extracts) than those of aqueous extracts (169.46 ± 6.78 mg GAE/g extracts), ethanol extracts (218.39 ± 3.55 mg GAE/g extracts), petroleum ether (130.78 ± 5.24 mg GAE/g extracts), n-butanol (327.17 ± 6.41 mg GAE/g extracts), and water

Table 1. DPPH Radical Scavenging Activity, NO Scavenging Activity, Superoxide Radical Scavenging Activity, β-Carotene−Linoleic Acid Bleaching Activity, Reducing Power, and XOD Inhibitory Activity of the Extracts and Four Fractions of *G. sibiricum*[a]

	IC_{50} (μg/mL)					
sample	DPPH	superoxide radical (NBT)	NO	β-carotene−linoleic acid	reducing power[b]	XOD inhibition
aqueous extracts	2.92 D	6.34 D	6.11 C	4.58 E	6.17 C	266.14 D
ethanol extracts	2.46 E	5.18 E	4.58 D	4.01 E	5.79 D	342.27 B
petroleum ether fraction	48.34 A	91.66 A	58.43 A	70.16 A	66.20 A	>500 A
EtOAc fraction	0.93 G	3.32 F	2.06 F	2.66 F	1.64 F	198.85 E
n-butanol fraction	1.37 F	3.35 F	2.75 E	7.02 D	2.14 E	314.02 C
water fraction	18.33 B	29.71 B	24.32 B	39.87 B	25.59 B	321.39 C
positive control	9.5 C (ascorbic acid)	21.54 C (Trolox)	1.03 G (ascorbic acid)	10.47 C (BHT)	0.96 G (Trolox)	1.72 F (allopurinol)

[a] The letters (A−F) indicate significant differences at a significance level of $p < 0.01$. [b] The IC_{50} value (μg/mL) of reducing power assay is the effective concentration at which the absorbance was 0.5 for reducing power.

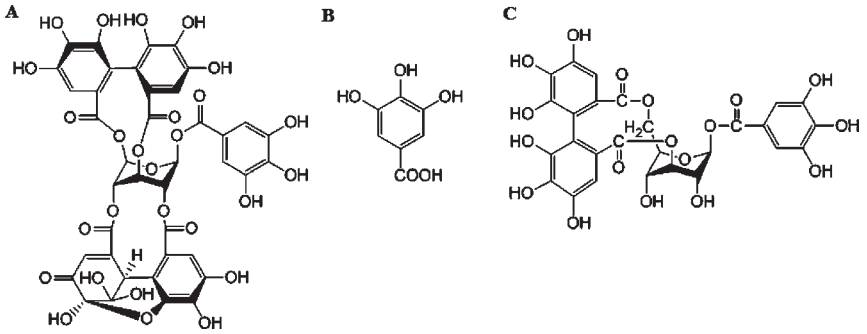

Figure 2. Chemical structures of the main polyphenolic compounds isolated from the EtOAc fraction of *G. sibiricum* (**A**, geraniin; **B**, gallic acid; and **C**, corilagin).

fractions (68.03 ± 1.49 mg GAE/g extracts) ($p < 0.01$). The phenolic content of the ethanol extracts (218.39 ± 3.55 mg GAE/g extracts) was higher than the reported contents of some other Geraniaceae species with antioxidant activities, such as methanol extracts of *Geranium macrorrhizum* (25.9 ± 0.2 mg GAE/g extracts) (*34*). The phenolic contents in extracts of *G. sibiricum* were also higher than those of many other plant species with well-known antioxidant activities, such as 16 varieties of *Helichrysum methanolic* extracts (ranged from 66.74 to 160.63 mg GAE/g extracts) and 54 different extracts of nine Asteraceae plants (ranged from 2.9 ± 0.1 to 404.3 ± 27.3 mg GAE/g extracts) (*26*, *27*).

The FRAP assay was often used to measure the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the presence of antioxidants. This assay was commonly used for the analysis of total antioxidant activity of plant extracts (*28*). The reducing ability of the EtOAc fraction [17.76 ± 0.21 mmol Fe(II)/g extracts] and *n*-butanol fraction [13.22 ± 0.21 mmol Fe(II)/g extracts] was higher than the other test extracts and fractions (**Figure 1B**). The FRAP values of EtOAc and *n*-butanol fractions were significantly higher than those of ascorbic acid [11.38 ± 0.31 mmol Fe(II)/g extracts].

A positive linear correlation between the total antioxidant activity (FRAP assay) and the phenolic content was observed (**Figure 1C**). The high correlation coefficient ($R^2 = 0.9473$) indicated that phenolics were one of the main components responsible for the antioxidant behavior of *G. sibiricum*. This statistically significant correlation was in agreement with the findings of Oktay et al. (*19*), who also found a strong relationship between the antioxidant capacity and the phenolic content.

Antioxidant Activities and XOD Inhibitory Effects of Extracts and Fractions from *G. sibiricum*. As shown in **Table 1**, among all extracts and fractions, the EtOAc fraction showed the best antioxidant activities. The IC_{50} values of the EtOAc fraction were 0.93, 3.32, and 2.06 μg/mL against DPPH radical, superoxide radical, and NO radical, and the IC_{50} values were 2.66 and 1.64 μg/mL in the β-carotene−linoleic acid and reducing power assays. Furthermore, IC_{50} values of different positive controls (ascorbic acid, Trolox, and BHT) were 9.5, 21.54, and 10.47 μg/mL in the DPPH, superoxide radical, and β-carotene-linoleic acid assays, which were higher than those of EtOAc and *n*-butanol fractions.

Until now, only a few authors have reported the antioxidant activities of plants of the Geraniaceae family. Some extracts showed stronger $O_2^{\bullet -}$ scavenging abilities than *G. sibiricum*, for example, the EtOAc fraction from the MeOH extract of *G. sanguineum* (IC_{50} value of 2.95 μg/mL) ($p < 0.01$). Meanwhile, more extracts showed much weaker antioxidant abilities, for example, MeOH extracts of *G. sanguineum* in the DPPH and superoxide radical scavenging assays (IC_{50} values of 13.86 and 26.0 μg/mL, respectively). Furthermore, the radical scavenging ability of 2.5 mg/mL MeOH extracts of *G. macrorrhizum* was only 91.7%, which was much lower than that of ethanol extracts of *G. sibiricum* at 100 μg/mL. Similarly, the inhibition ratio of 100 μg/mL ethanol extracts of *G. sibiricum* (97.48%) was much higher than 2 mg/mL MeOH extracts of *G. sanguineum* (88−89%) in the β-carotene−linoleic acid test system ($p < 0.01$) (*12*, *25*). Comparing these data, we conclude that *G. sibiricum* has a high potential for applications as a natural antioxidant for human health.

In the XOD inhibition test, the EtOAc fraction showed the strongest inhibitory effects (IC_{50} value of 198.85 μg/mL). However, it was still weaker than that of allopurinol (IC_{50} value of 1.72 μg/mL). It was quite interesting that both aqueous extracts (IC_{50} value of 266.14 μg/mL) and the water fraction (IC_{50} value of 321.39 μg/mL) showed a stronger XOD inhibition activity than ethanol extracts (IC_{50} value of 342.27 μg/mL) ($p < 0.01$). This result was contrary to that of the superoxide radical scavenging assay in the present study.

There were some reports indicating that the strength of inhibition of XOD among the polyphenolic was considerably different

from that of the inhibitory effects on the $O_2^{\bullet-}$ generation from a hypoxanthine−XOD system. Some compounds (e.g., cornusiin A and epigallocatechin), which strongly inhibited the generation of $O_2^{\bullet-}$, showed weak inhibition of XOD, while some other compounds (e.g., ellagic acid), which weakly inhibited $O_2^{\bullet-}$ generation, strongly inhibited XOD (*29*). It may be concluded that the inhibition of $O_2^{\bullet-}$ generation by *G. sibiricum* extracts was due to the radical scavenging activity of polyphenolic compounds instead of their inhibitory activity upon XOD (*14*).

In general, the EtOAc fraction of *G. sibiricum* extracts showed a higher phenolic content, better antioxidant activity, and stronger inhibition activity toward XOD. This implies that there were abundant antioxidant compounds in the EtOAc fraction. Thus, the EtOAc fraction was further investigated in this study to isolate polyphenolic compounds.

Isolation and Identification of Main Polyphenolic Compounds from *G. sibiricum* Extracts. Three main polyphenolic compounds were purified from the EtOAc fraction of *G. sibiricum*. Their structures were identified by using IR, ^{1}H and ^{13}C nuclear magnetic resonance (NMR), and MS analysis. As shown in **Figure 2**, three main polyphenolic compounds were determined as geraniin (**A**), gallic acid (**B**), and corilagin (**C**). As shown in **Table 2**, none of these three polyphenolic compounds were found in the petroleum ether fraction. The content of corilagin in the *n*-butanol fraction was higher than that in all of the other five test samples. Overall, the EtOAc fraction was found to be rich in all of these three components, with geraniin (15.32 ± 0.36 mg/g extracts), gallic acid (13.9 ± 0.33 mg/g extracts), and corilagin (1.82 ± 0.17 mg/g extracts). It could be inferred from our results that there was a positive correlation between the contents of these three compounds and the antioxidant activity. The higher activity of the EtOAc fraction could be attributed to the higher contents of geraniin, gallic acid, and corilagin.

Antioxidant Activities and XOD Inhibitory Activities of Main Polyphenolic Components from *G. sibiricum* Extracts. As shown in **Table 3** and **Figure 3**, the DPPH free radical scavenging activity of geraniin was superior to those of corilagin, gallic acid, and the positive control, ascorbic acid. The IC_{50} values were 0.99, 2.25, 7.44, and 46.06 μM, respectively. The activities of geraniin, corilagin, and gallic acid found in the superoxide radical scavenging assay (NBT assay) were similar to those of the DPPH assay. Geraniin exhibited the best superoxide scavenging activity, with a IC_{50} value of 2.53 μM, which was even lower than that of Trolox (46.08 μM). Geraniin (1.32 μM) also showed a stronger NO scavenging activity than corilagin, gallic acid, and ascorbic acid. As for β-carotene−linoleic acid assay, the activities of these polyphenolic components can be ranked as geraniin > corilagin > gallic acid > BHT ($p < 0.01$). In the reducing power assay, this order was changed to geraniin = Trolox > corilagin > gallic acid ($p < 0.01$). All of these results indicated that geraniin had the best antioxidant activity, even better than three positive controls (ascorbic acid, Trolox, and BHT). Moreover, at the concentration of 100 μM, the inhibition ratios of geraniin against DPPH, superoxide radical, NO, and β-carotene−linoleic acid were all above 100% (**Figure 3**).

Some polyphenolic compounds with chemical structures capable of capturing free radicals possess superior antioxidant activity as compared to known antioxidants, such as vitamins A and E. Moreover, polyphenolic compounds are rich in hydroxyl groups, which could readily stabilize ROS (*30*). As shown in **Figure 2**, there are plenty of hydroxyl groups that exist in geraniin, corilagin, and gallic acid. The hydroxyl groups could easily inactivate radicals and finally lead to strong antioxidant activities of these compounds.

It has been reported that polymeric polyphenolics have a higher antioxidant activity than simple monomeric phenolics because of the high molecular weights, the proximity of many aromatic rings, and the hydroxyl groups (*31*). The strong antioxidant activity of geraniin obtained in the present study indicates that geraniin was the major antioxidant in *G. sibiricum* extracts.

In contrast to the antioxidant activities, gallic acid showed a better XOD inhibitory activity than geraniin and corilagin, with IC_{50} values of 105.41, 129.88, and 222.89 μM, respectively. However, all of the compounds showed weaker XOD inhibition than allopurinol (8.95 μM).

As a powerful inhibitor of XOD, the structure of allopurinol is similar to xanthine. Allopurinol could be oxidized to give oxyallopurinol, which binds tightly to the active site of XOD and causes inhibition. Hence, allopurinol has been called a "suicide substrate" or "suicide product" of XOD (*32*). It is interesting to note that the chemical structure of gallic acid has some analogies to the structure of the A ring of xanthine and uric acid. Therefore, like allopurinol, gallic acid may act as a suicide substrate for XOD. On the other hand, the chemical structures of gallic acid include three hydroxyls and one carboxyl. When it comes in the protein pocket, it may act as a plug and efficiently inhibits uric acid formation. Different from gallic acid, the structure of corilagin and geraniin seemed too bulky, and the existence of glycosyl groups and galloyl groups may prevent the compounds from contacting XOD, finally resulting in weaker XOD inhibitory activities. Similar findings were reported by Chang et al. (*33*) and Nagno et al. (*34*).

Hatano et al. (*29*) studied the XOD inhibitory activity of low molecular weight polyphenols (ellagic acid, valoneic acid

Table 2. Contents of Main Polyphenolic Components in the Extracts and Four Fractions of *G. sibiricum* ($n = 3$)[a]

	contents (mg/g extracts)		
sample	geraniin	gallic acid	corilagin
aqueous extracts	6.93 ± 0.21	3.27 ± 0.26	1.68 ± 0.44
ethanol extracts	7.52 ± 0.47	3.82 ± 0.20	1.73 ± 0.19
petroleum ether fraction			
EtOAc fraction	15.32 ± 0.36	13.9 ± 0.33	1.82 ± 0.17
n-butanol fraction	9.35 ± 0.28	0.4 ± 0.01	2.69 ± 0.12
water fraction	0.12 ± 0.02	0.2 ± 0.03	0.49 ± 0.02

[a] Values are means ± SD of three determinations. Data in the same column indicate significant differences ($p < 0.01$).

Table 3. DPPH Radical Scavenging Activity, Superoxide Radical Scavenging Activity, NO Scavenging Activity, β-Carotene−Linoleic Acid Bleaching Activity, Reducing Power, and XOD Inhibitory Activity of Main Polyphenolics Isolated from the EtOAc Fraction of *G. sibiricum*[a]

	IC_{50} (μM)					
compound	DPPH	superoxide radical (NBT)	NO	β-carotene−linoleic acid	reducing power	XOD inhibition
geraniin	0.99 D	2.53 D	1.32 D	1.50 D	0.87 C	129.88 B
corilagin	2.25 C	14.27 C	7.2 B	7.97 C	2.68 B	222.89 A
gallic acid	7.44 B	38.85 B	9.08 A	17.39 B	7.52 A	105.41 C
positive control	46.06 A (ascorbic acid)	46.08 A (Trolox)	3.47 C (ascorbic acid)	47.97 A (BHT)	0.88 C (Trolox)	8.95 D (allopurinol)

[a] The letters (A−F) indicate significant differences at a significance level of $p < 0.01$.

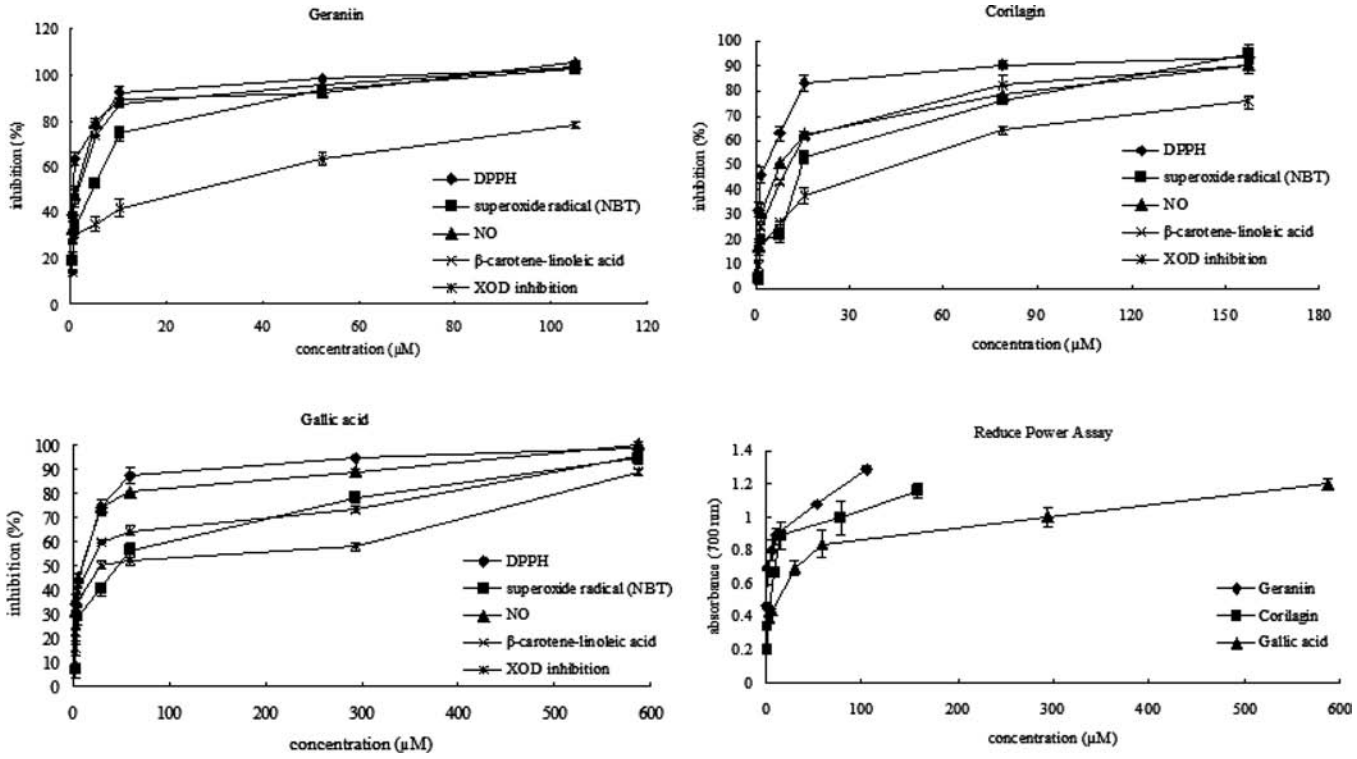

Figure 3. Antioxidant activities of three main components (geraniin, corilagin, and gallic acid) in *G. sibiricum*. Values of each curve are means ± SDs ($n = 3$); $p < 0.01$.

dilactone, and gallic acid, etc.) and some ellagitannins (e.g., corilagin, gemin D, casuarinin, and geraniin). Gallic acid showed a stronger XOD inhibitory activity than geraniin and corilagin, which was in accordance with our results. However, the IC_{50} value of gallic acid (24 μM) in their test was lower than that in our assay (105.41 μM). Besides the solvent and method, the content of samples in the mixture could account for this difference of activity. Taking into account the above results, we conclude that gallic acid could be used as a preventive and therapeutic agent for XOD-related diseases, hyperuricemia, gout, etc (*33*).

In conclusion, the results in the present study provide strong evidence for the antioxidant activities of *G. sibiricum*. Therefore, geraniin, corilagin, and gallic acid obtained from the EtOAc fraction of *G. sibiricum* extracts may be developed as natural antioxidants for the food industry and other fields. Further studies on the antioxidant activity in vivo are needed to support this point of view.

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