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Harpagoside Variation Is Positively Correlated with Temperature in *Scrophularia ningpoensis* Hemsl.

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Supporting Information

ABSTRACT: *Scrophularia ningpoensis* Hemsl. is an important Chinese medicinal herb with a history of domestication of over 1000 years. Phytochemical variation of *S. ningpoensis* in response to environmental gradients remains an attractive topic with both practiceal and theoretical significances. In the current study, HPLC fingerprinting and four major bioactive compounds of *S. ningpoensis*, that is, harpagoside, angroside C, acteoside, and cinnamic acid, were determined to explore its correlations with climatic, geographic, and soil factors. The present data confirmed the approximate three-group pattern of phytochemical differentiation among the five production regions, the population of Zhejiang (ZJ), the population of Hubei (HB), and the rest three populations of Chongqing, Hunan, and Shaanxi (CQ, HN, and SX). Harpagoside, the dominant bioactive compound of *S. ningpoensis*, contributed most to the phytochemical differentiation and displayed a significant positive correlation with monthly and annual average temperature and negative correlations with altitude and latitude. It was concluded that harpagoside variation was strongly positively correlated with environmental changes of temperature.

KEYWORDS: *Scrophularia ningpoensis*, Scrophulariaceae, phytochemical variation, harpagoside, heat response, Radix Scrophulariae

INTRODUCTION

It is established that secondary metabolites, which underlie the pharmaceutical quality of medicinal plants, are determined by genetic and environmental factors and their interactions.[1,2] A variety of environmental factors, such as season, altitude, radiation, and soil nutrition, have been proven to significantly modify the secondary metabolite profile in seed plants.[3−6] For example, both flavonoids and phenolic acids in flowering heads of *Matricaria chamomilla* cv. *bona* were reported to be positively correlated with altitude,[7] and supplemental light of selected wavelengths could be strategically used to enhance the nutritional value and growth of baby leaf lettuce grown under white light.[8] Nevertheless, our knowledge of the complex interactions between plants and their habitats related to the dynamics of secondary metabolites remains fairly limited. Except for the recent study of Seemann et al.[9] on the effects of different environmental factors on the variation of sesquiterpene lactone contents in different wild-growing plants of *Arnica montana*, previous research has mainly focused on the response of a specific compound or the specific effects of one particular biotic or abiotic stressor and do not reflect the complexities of the habitat.[7,10−13] Additional investigations into the metabolic profile of plant populations of one species inhabiting varied environments are therefore necessary to understand the complex relationships between secondary metabolites and the habits in which they are produced.

Scrophularia ningpoensis Hemsl. (Scrophulariaceae), an eastern Asian relative of *Scrophularia nodosa* L., is endemic to China, where it has a history of domestication of more than 1000 years. The dried root, known as Radix Scrophulariae, is a famous Chinese traditional medicinal herb. The iridoids and phenylpropanoid glycosides are considered to be the main bioactive components, with anti-inflammatory, antimicrobial, antitumor, and hepatoprotective activities.[14−17] Harpagoside, the major bioactive iridoid glycoside, has been widely used in the clinical treatment of pain in the joints and lower back for its neuroprotective and anti-inflammatory activities.[16,18] The Chinese Pharmacopeia requires a minimum content of 0.05% (m/m) of this compound in Radix Scrophulariae.[19] The phenylpropanoid glycosides, angroside C and acteoside, derived from the roots, are reported to have potential antioxidative activity.[20] Additionally, cinnamic acid was also identified as a differentiation-inducer against tumors[21] and mutations.[22] Because of these efficacies and bioactivities, *S. ningpoensis* has been an important medicinal plant used both in Chinese medicinal formulas and in daily health products. Despite its long history of massive cultivation, much remains to be learned about the origin of phytochemical variation in *S. ningpoensis*, which is not only of theoretical significance in understanding the biological functions of metabolites and their evolution but also critical for quality control of Radix Scrophularia in good agricultural practice (GAP).

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Table 1. Detailed Information of All Sampling Sites

sample	cultivation region	altitude (m)	longitude	latitude
ZJ1[a]	Pan'an County, Zhejiang Province	538	E 120.74028°	N 29.03917°
ZJ2	Pan'an County, Zhejiang Province	526	E 120.60917°	N 28.99028°
ZJ3	Pan'an County, Zhejiang Province	510	E 120.79972°	N 29.17972°
ZJ4[a]	Pan'an County, Zhejiang Province	401	E 120.42694°	N 29.15694°
ZJ5	Pan'an County, Zhejiang Province	389	E 120.37306°	N 28.96806°
ZJ6*	Pan'an County, Zhejiang Province	365	E 120.49083°	N 29.03556°
ZJ7*	Pan'an County, Zhejiang Province	312	E 120.40389°	N 29.13861°
ZJ8	Pan'an County, Zhejiang Province	325	E 120.63194°	N 29.04500°
ZJ9	Pan'an County, Zhejiang Province	368	E 120.52944°	N 28.95583°
ZJ10	Pan'an County, Zhejiang Province	454	E 120.53889°	N 29.24194°
ZJ11[a]	Pan'an County, Zhejiang Province	378	E 120.54944°	N 29.18500°
ZJ12	Pan'an County, Zhejiang Province	402	E 120.56528°	N 29.02000°
CQ1[a]	Mt. Jinfo, Chongqing Municipality	1324	E 107.19683°	N 29.04880°
CQ2[a]	Mt. Jinfo, Chongqing Municipality	1151	E 107.19521°	N 29.06810°
CQ3	Mt. Jinfo, Chongqing Municipality	1172	E 107.19252°	N 29.06474°
CQ4	Mt. Jinfo, Chongqing Municipality	1153	E 107.19501°	N 29.06763°
CQ5[a]	Mt. Jinfo, Chongqing Municipality	1263	E 107.21356°	N 29.04925°
CQ6	Mt. Jinfo, Chongqing Municipality	1282	E 107.21504°	N 29.04980°
CQ7[a]	Mt. Jinfo, Chongqing Municipality	1112	E 107.18855°	N 29.08265°
CQ8	Mt. Jinfo, Chongqing Municipality	1140	E 107.19219°	N 29.08742°
CQ9	Mt. Jinfo, Chongqing Municipality	1114	E 107.19051°	N 29.08597°
CQ10[a]	Mt. Jinfo, Chongqing Municipality	1135	E 107.19092°	N 29.08516°
CQ11	Mt. Jinfo, Chongqing Municipality	1104	E 107.18934°	N 29.08612°
CQ12	Mt. Jinfo, Chongqing Municipality	1114	E 107.19100°	N 29.08437°
CQ13	Mt. Jinfo, Chongqing Municipality	1106	E 107.18747°	N 29.08266°
HB1[a]	Enshi City, Hubei Province	1433	E 110.21123°	N 30.82830°
HB2[a]	Enshi City, Hubei Province	1440	E 110.20068°	N 30.82660°
HB3	Enshi City, Hubei Province	1431	E 110.20383°	N 30.82817°
HB4[a]	Enshi City, Hubei Province	1593	E 110.14430°	N 30.79780°
HB5	Enshi City, Hubei Province	1606	E 110.14426°	N 30.79748°
HB6[a]	Enshi City, Hubei Province	1579	E 110.14980°	N 30.79520°
HB7	Enshi City, Hubei Province	1572	E 110.14970°	N 30.79548°
HB8	Enshi City, Hubei Province	1569	E 110.15093°	N 30.79616°
HB9[a]	Enshi City, Hubei Province	1580	E 110.15291°	N 30.79610°
HB10	Enshi City, Hubei Province	1596	E 110.15338°	N 30.79632°
HB11	Enshi City, Hubei Province	1578	E 110.15427°	N 30.79847°
HN1[a]	Longshan County, Hunan Province	915	E 109.72237°	N 29.59915°
HN2	Longshan County, Hunan Province	891	E 109.72143°	N 29.59951°
HN3	Longshan County, Hunan Province	832	E 109.72211°	N 29.59954°
HN4[a]	Longshan County, Hunan Province	832	E 109.66335°	N 29.53149°
HN5	Longshan County, Hunan Province	842	E 109.72127°	N 29.59881°
HN6[a]	Longshan County, Hunan Province	844	E 109.72187°	N 29.59846°
HN7	Longshan County, Hunan Province	1107	E 109.72166°	N 29.59873°
HN8[a]	Longshan County, Hunan Province	1106	E 109.66425°	N 29.53143°
HN9	Longshan County, Hunan Province	1106	E 109.66441°	N 29.53151°
HN10	Longshan County, Hunan Province	1101	E 109.66193°	N 29.52918°
HN11[a]	Longshan County, Hunan Province	1086	E 109.66195°	N 29.52982°
HN12	Longshan County, Hunan Province	1095	E 109.66142°	N 29.53008°
SX1[a]	Zhenping County, Shaanxi Province	994	E 109.44833°	N 31.84680°
SX2	Zhenping County, Shaanxi Province	996	E 109.44623°	N 31.84456°
SX3	Zhenping County, Shaanxi Province	998	E 109.44624°	N 31.84575°
SX4[a]	Zhenping County, Shaanxi Province	1023	E 109.47593°	N 31.89290°
SX5	Zhenping County, Shaanxi Province	1229	E 109.43641°	N 31.89957°
SX6	Zhenping County, Shaanxi Province	1204	E 109.43703°	N 31.89976°

Table 1. Continued

sample	cultivation region	altitude (m)	longitude	latitude
SX7[a]	Zhenping County, Shaanxi Province	1176	E 109.43800°	N 31.90200°
SX8[a]	Zhenping County, Shaanxi Province	1013	E 109.50629°	N 31.74870°
SX9	Zhenping County, Shaanxi Province	1013	E 109.50477°	N 31.74986°
SX10	Zhenping County, Shaanxi Province	1011	E 109.50543°	N 31.74869°
SX11	Zhenping County, Shaanxi Province	1074	E 109.48661°	N 31.74588°
SX12	Zhenping County, Shaanxi Province	1081	E 109.45527°	N 31.75263°
SX13[a]	Zhenping County, Shaanxi Province	1184	E 109.43600°	N 31.76100°

[a] Sample site where soil analyses were performed.

It was proposed that global climate change influenced overall ecosystem functions, including the secondary metabolic profiles of plants.[23] We reported a considerably consistent pattern and degree of differentiation between phytochemical and genetic variations of wild and cultivated populations of *S. ningpoensis*.[24] Our previous study also implied that the local environment may play an important role in the phytochemical variation in cultivated populations (unpublished data). This raised our interest to test whether the environmental parameters influence the phytochemical diversity in cultivated *S. ningpoensis*, and, if so, which is/are the most contributive one(s). Therefore, we enlarged our sampling to include five main regions of cultivation of *S. ningpoensis* in China with more plants, as well as the corresponding climatic and geographic data and soil samples in the same growth year, aiming to (1) further investigate the secondary metabolic variation of cultivated Radix Scrophulariae among geographical regions and (2) assess the effects of different environmental variables on phytochemical variation in terms of both the overall chromatographic fingerprint and four single major compounds of Radix Scrophulariae. Consequently, suggestions can be proposed for GAP to enhance the productivity and quality of the herb medicine.

MATERIALS AND METHODS

Sampling. Five major cultivation regions for *S. ningpoensis* throughout eastern and central China were included for sampling in December 2007. More than 10 batches of plant samples from each region were collected and dried uniformly in drying ovens at 50 °C preceding pulverization. The identity of the plants was authenticated by one of the authors (C.F.). Voucher specimens have been deposited in the Herbarium of Zhejiang University (HZU). Five sites where the plants were collected in each region were randomly selected for soil analysis. Geographic coordinates and altitude were recorded using a global positioning system (see details in Table1 and Figure 1).

Phytochemical Analyses with HPLC. The phytochemical diversity of Radix Scrophulariae was presented in terms of variations of both overall chromatographic fingerprints and major single compounds. Chromatographic fingerprinting has been well accepted as a holistic approach representing the complex chemical composition of herbal medicines.[25,26] The present methods followed those in Yang et al.[24] with minor modification. An accurately weighed root powder (1.0 g) was ultrasonically extracted with 50 mL of 70% methanol for 30 min and brought to a constant volume after filtration by filter paper. The solutions were filtered through a 0.22 μm membrane before HPLC analysis. Chromatograms were generated on a Varian Prostar 210 HPLC system, consisting of a ProStar 335 diode array detector, a manual injector, a column oven, and a data system (Galaxie Workstation). The chromatographic separation was performed on an Agilent HC-C18 column (250 mm × 4.6 mm, 5 μm) using a gradient of acetonitrile (A) and 1% aqueous acetic acid (B). The gradient program was performed as follows: 0−5 min, 5−15% A; 5−15 min, 15−25% A; 15−30 min, 25−30% A; 30−50 min, 30−65% A; 50−60 min, 65% A, with flow rate of 1 mL min^{-1}, column temperature of 35 °C, and wavelength of 280 nm. The four major compounds presented on the chromatograms of Radix Scrophulariae, including an iridoid glucoside (harpagoside), two phenylpropanoid glycosides (angroside C and acteoside), and an organic acid (cinnamic acid), were identified and quantified by corresponding reference compounds.

Soil Analyses. The collected soil samples were transported to the laboratory and air-dried before screening through 1 mm mesh sieve. The following attributes of each sample were analyzed: pH, cationic exchange capacity (CEC), electrical conductivity (EC), content of organic matter (OM%), content of available nitrogen (N), Olsen phosphorus, and available potassium. Six microelements, Fe, Mn, Cu, Zn, Mo, and Se, were also detected by an Agilent ICP-MS G3271A. All soil analyses were performed according to the method of Lu.[27]

Climatic Data. Annual and monthly average temperature, precipitation, hours of sunshine, and relative humidity in 2007 were respectively acquired from the weather stations nearest the sampling sites, which are available from local weather bureaus or the China Meteorogical Data Sharing Service System on the Web site of the China Meteorological Administration.[28]

Statistical Analyses. We examined the environmental correlations with both chromatographic fingerprints and the four single compounds based on comparisons within each set of the parameters. In our present study, 31 characteristic peak areas comprising the fingerprint of each plant sample were extracted from the chromatograms. The variation of chromatographic fingerprints was computed via Cosine similarity comparison and principal component analysis (PCA) using SPSS for Windows 16.0. Screened by Cosine similarity calculation, 10 representative batches of samples from each region were selected to produce five regional model fingerprints using the Median Clustering method for the individual comparison of Cosine similarity. The chromatographic data set of the 10 representative samples was further subject to PCA. One-way ANOVA (post hoc multiple comparisons) were performed to determine the statistical significance of content differences of the four major chemicals among the samples from different regions using SPSS 16.0. The test of homogeneity of variances was performed for the data before post hoc multiple comparisons, if equal variances are assumed, selected LSD as post hoc test method; otherwise, selected Tamhane T2. Furthermore, ordination and PLS regression analysis were carried out using Canoco 4.0 and Simca-P 12.0 software packages, respectively, to indicate correlation and contribution of the environmental factors with/to chromatographic fingerprints of Radix Scrophulariae. Pearson correlation analysis was applied to test the correlations between the four major components and environmental parameters using SPSS 16.0. The soil characters and environmental differentiation were also analyzed via ANOVA and PCA, respectively.

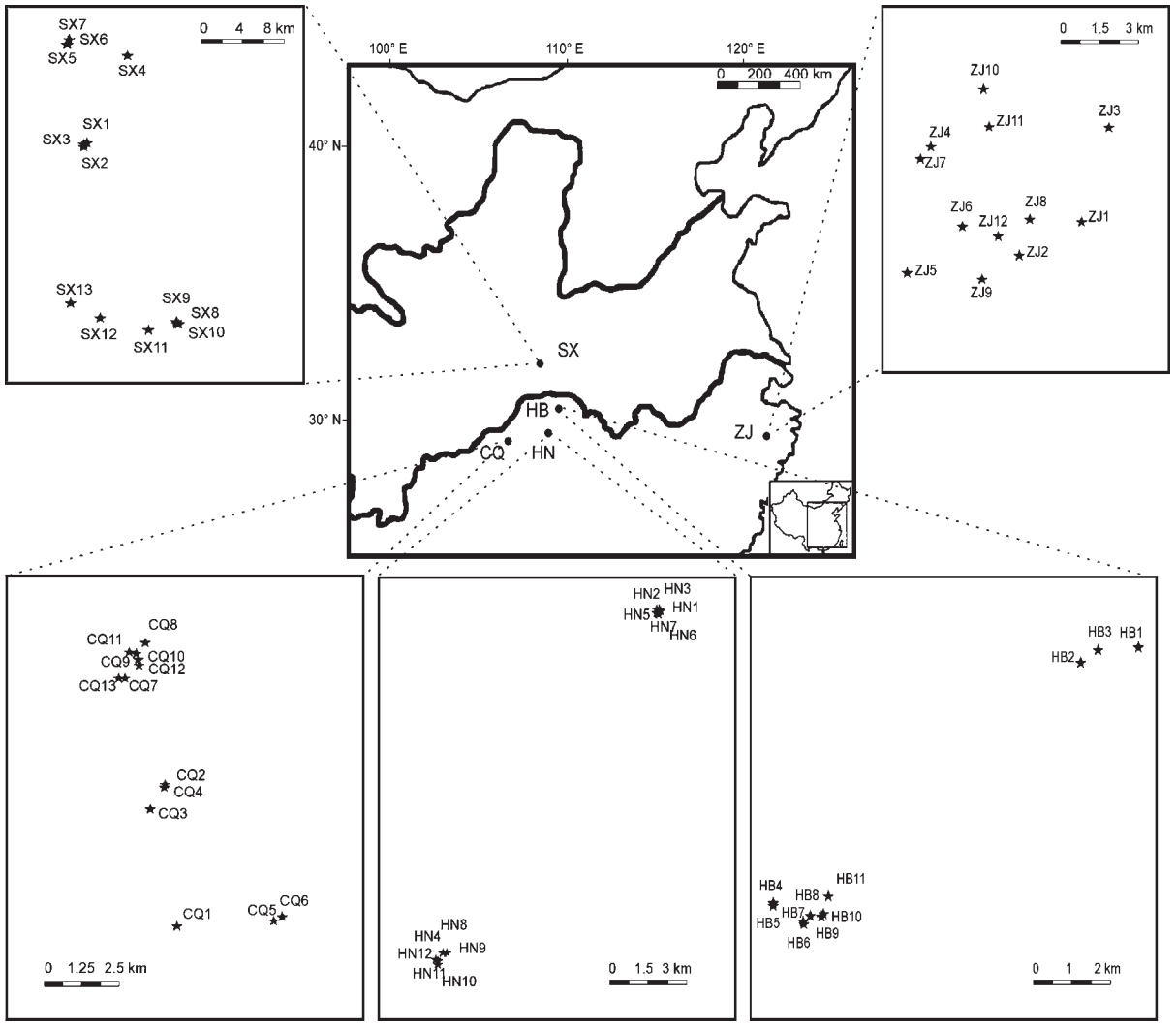

Figure 1. Sample localities of *S. ningpoensis*.

RESULTS

Climatic, Geographic, and Soil Characteristics of the Cultivation Regions. All of the regions where the cultivated samples were collected are located in mountainous areas, basically along the Yangtze River basin, with a warm, humid semitropical climate. The annual rainfall in these regions in 2007 varied from 1016 to 1845 mm, and the average yearly temperature ranged from 10.3 to 17.5 °C. Obvious distinctions in geographic factors exist between the sites, especially in altitude: Zhejiang (ZJ) is at the lowest elevation, whereas Hubei (HB) is at the highest (Table 1). These distinctions result in climatic differences among regions (see monthly climatic details in Supporting Information Figure 1S). The sampling sites in ZJ had the highest monthly and annual average temperature throughout the year, whereas those in HB were the opposite. The maximum precipitation and sunshine hours in those regions also appeared in different months, and the relative humidity in Shaanxi (SX) was distinctly lower than in other places. The soil was slightly acid in most sites, with pH values from 4.71 to 7.36, and both extremes occurred in the samples from HB. None of the regions were deficient in nutrients, and the soil in Hubei province exhibited a distinctive high concentration of available nitrogen (Table 2). Among the 13 soil traits tested, one-way ANOVA indicated that only four, that is, content of organic matter (OM%), available nitrogen (N), and Olsen phosphorus and zinc concentrations, differed significantly among the five regions (Table 2). PCA of climatic and soil factors showed that the environment of the Chongqing (CQ) site was closest to the Hunan (HN) site, whereas the other three regions are distributed in the three corners of the plot, respectively (Supporting Information Figure 2S). The determinant bringing about these results is temperature, although SX is distributed at the bottom because of its lower relative humidity.

Phytochemical Variation of Radix Scrophulariae. Most samples showed general consistency with their regional model fingerprint in the Cosine similarity analysis (>0.90) (Table 3). However, the highest mean similarity of 0.98 was presented in the ZJ population, with a small deviation of 0.017, indicating stable chemical profiles in this population, whereas the CQ and SX populations exhibited the lowest similarities of 0.918 and 0.908, respectively, with great deviation (0.121 and 0.109, respectively). Moderate values were generated in the HB and HN populations (0.944 ± 0.052 and 0.946 ± 0.040, respectively). When the five regional models were compared to

Table 2. Main Traits of Soil Samples from the Five Cultivation Regions of *S. ningpoensis*

trait	ZJ	CQ	HB	HN	SX	signif[a]
pH (1:5)	5.7 ± 0.5	6.2 ± 0.7	6.2 ± 1.1	5.3 ± 0.2	6.8 ± 0.8	ns
EC (μs cm^{-1}) (1:5)	56.60 ± 25.49	52.72 ± 23.55	77.40 ± 27.10	46.44 ± 16.72	76.34 ± 60.65	ns
CEC (cmol kg^{-1})	15.71 ± 7.99	13.93 ± 7.35	19.07 ± 1.56	12.39 ± 2.15	15.27 ± 2.51	ns
N (mg kg^{-1})	77.46 ± 31.49 b[b]	83.13 ± 37.65 b	183.73 ± 46.50 a	123.81 ± 32.34 b	111.82 ± 22.10 b	**
P (mg kg^{-1})	49.11 ± 20.61 ab	14.48 ± 5.44 b	50.77 ± 31.65 ab	53.01 ± 6.96 a	17.22 ± 6.26 b	***
K (mg kg^{-1})	235.36 ± 128.37	181.24 ± 96.91	191.36 ± 50.59	224.69 ± 40.95	100.94 ± 55.54	ns
OM (%)	1.84 ± 0.99 b	2.01 ± 1.01 b	4.55 ± 0.91 a	3.01 ± 0.98 b	2.98 ± 0.57 b	**
Mn (mg kg^{-1})	20.121 ± 13.331	19.441 ± 11.856	8.946 ± 3.921	14.884 ± 6.043	14.757 ± 10.784	ns
Fe (mg kg^{-1})	18.636 ± 11.835	11.642 ± 5.31	19.059 ± 6.427	13.581 ± 5.265	29.411 ± 13.467	ns
Cu (mg kg^{-1})	0.883 ± 0.342	1.051 ± 0.334	1.041 ± 0.164	1.178 ± 0.247	1.171 ± 0.428	ns
Zn (mg kg^{-1})	1.408 ± 0.466 b	1.101 ± 0.603 b	1.132 ± 0.386 b	2.344 ± 0.466 a	1.12 ± 0.554 b	***
Se (mg kg^{-1})	0.103 ± 0.025	0.083 ± 0.032	0.082 ± 0.007	0.086 ± 0.005	0.112 ± 0.030	ns
Mo (mg kg^{-1})	0.015 ± 0.008	0.004 ± 0.001	0.019 ± 0.017	0.014 ± 0.005	0.064 ± 0.056	ns

[a] ns, insignificant by ANOVA analysis; **, significant at the level of 0.01;***, significant at the level of 0.001. [b] Lower case letters represent significant differences revealed by post hoc multiple comparison (N = 5).

Table 3. Comparisons of Cosine Similarity between Each Plant Sample and Corresponding Regional Fingerprints of Radix Scrophulariae

sample	similarity to M_{ZJ}[a]	sample	similarity to M_{CQ}[b]	sample	similarity to M_{HB}[c]	sample	similarity to M_{HN}[d]	sample	similarity to M_{SX}[e]
ZJ1	0.997	CQ1	0.939	HB1	0.937	HN1	0.901	SX1	0.954
ZJ2	0.971	CQ2	0.982	HB2	0.903	HN2	0.995	SX2	0.982
ZJ3	0.983	CQ3	0.973	HB3	0.820	HN3	0.850	SX3	0.976
ZJ4	0.996	CQ4	0.920	HB4	0.937	HN4	0.997	SX4	0.913
ZJ5	0.950	CQ5	0.979	HB5	0.915	HN5	0.920	SX5	0.629
ZJ6	0.983	CQ6	0.960	HB6	0.988	HN6	0.953	SX6	0.920
ZJ7	0.991	CQ7	0.981	HB7	0.943	HN7	0.951	SX7	0.897
ZJ8	0.993	CQ8	0.599	HB8	0.992	HN8	0.956	SX8	0.998
ZJ9	0.979	CQ9	0.984	HB9	0.988	HN9	0.951	SX9	0.993
ZJ10	0.946	CQ10	0.962	HB10	0.964	HN10	0.966	SX10	0.958
ZJ11	0.979	CQ11	0.993	HB11	0.993	HN11	0.961	SX11	0.742
ZJ12	0.993	CQ12	0.951			HN12	0.946	SX12	0.864
		CQ1	0.709					SX13	0.979
mean ± SD	0.980 ± 0.017	mean ± SD	0.918 ± 0.121	mean ± SD	0.944 ± 0.052	mean ± SD	0.946 ± 0.040	mean ± SD	0.908 ± 0.109

[a] Model fingerprint of the ZJ population. [b] Model fingerprint of the CQ population. [c] Model fingerprint of the HB population. [d] Model fingerprint of the HN population. [e] Model fingerprint of the SX population.

the main model (M_{50}), the HB model fingerprint showed the lowest value (0.936), followed by that of ZJ (0.953), and the other three exhibited high and close similarity (CQ, 0.983; HN, 0.988; SX, 0.994). Hierarchical clustering of the five populations showed a trend similar to the Cosine similarity to M_{50} (Supporting Information Figure 3S). Furthermore, the PCA plot demonstrated three groups, the ZJ population in the third quadrant, the HB population in the fourth quadrant, and most of the rest (the CQ, HN, and SX populations) in the middle of the upper quadrants (Figure 2). The loading values of PCA indicated that harpagoside was the most contributive variable on the first axis.

We further quantified the four main bioactive components, that is, harpagoside, angroside C, acteoside, and cinnamic acid. The first three bioactive chemicals were present in significantly different amounts among regions as determined by one-way ANOVA ($p \leq 0.001$) (Figure 3). Both the average content of harpagoside and angroside C were highest in the ZJ population (3.674 and 3.507 mg g^{-1}, respectively) and lowest in the HB population (1.037 and 1.632 mg g^{-1}, respectively). The HB population also showed the lowest average content of acteoside (0.593 mg g^{-1}). No significant regional variation in cinnamic acid was detected.

Correlations between Chemical Variations of Radix Scrophulariae and Environmental Variations. Twenty-five observations available with both environmental and phytochemical data were applied to explore the correlations between environment and chemical diversity of Radix Scrophulariae. The PCA projection generated from the chromatographic fingerprints of the 25 samples led to three groups, the ZJ population, the HB population, and the other three largely overlapped populations (CQ, HN, and SX) (Figure 4A). Harpagoside was still the variable with the greatest loading value on the first axis. The ordination analysis suggested obvious environmental gradients (Figure 4B). Specifically, the first axis mainly presents decreasing altitude and increasing temperature gradients from left to right.

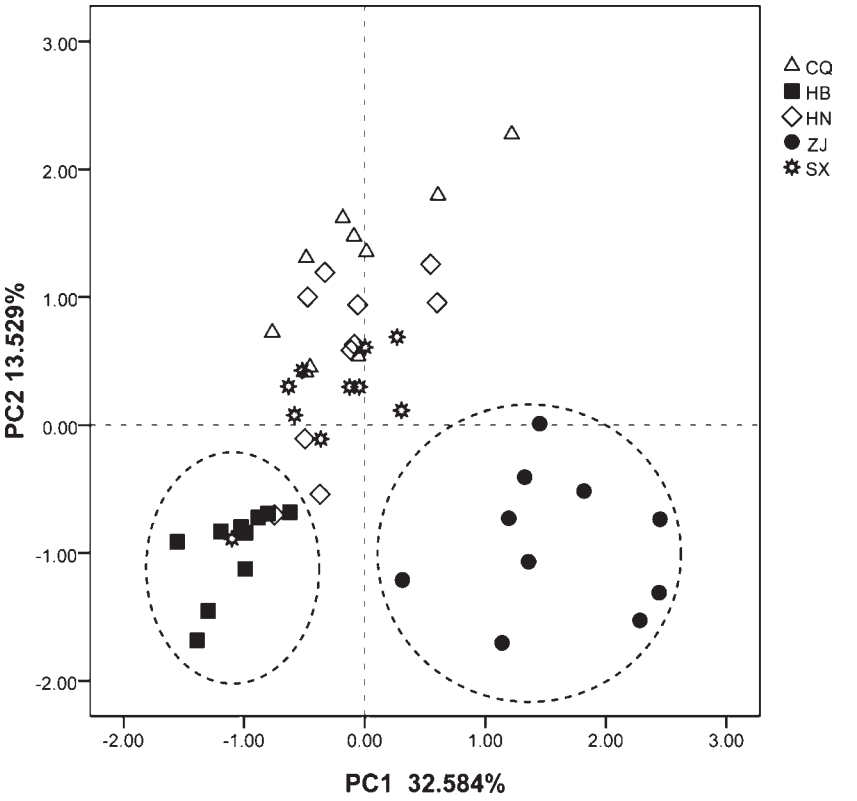

Figure 2. PCA plot for chromatographic fingerprints of 50 samples of *S. ningpoensis* from the five studied regions.

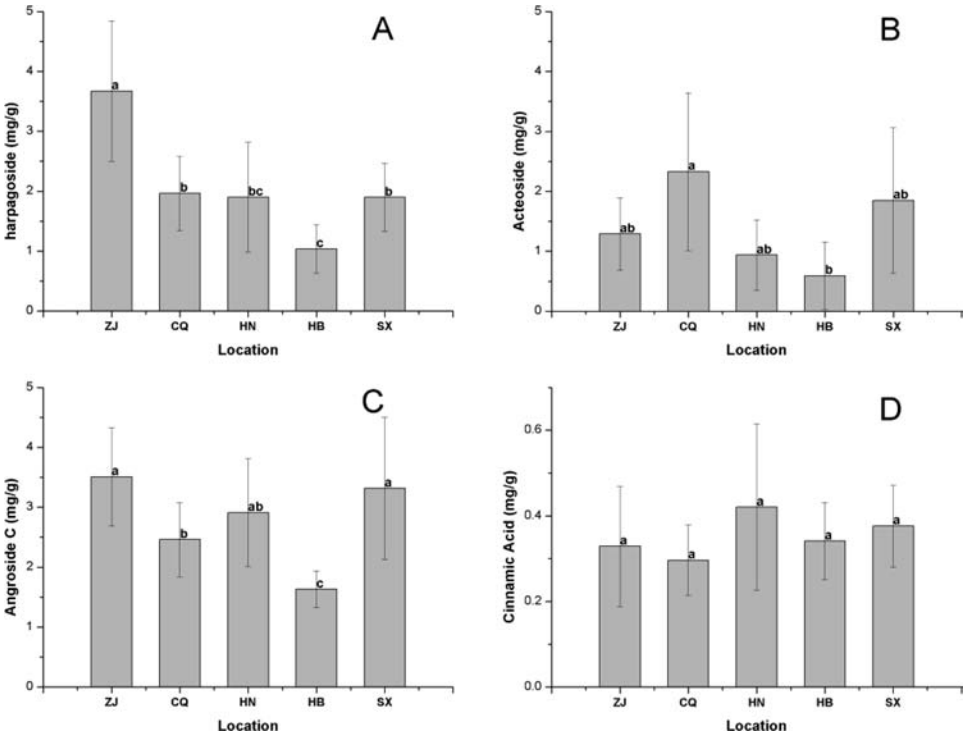

Figure 3. Content comparisons of harpagoside (A), acteoside (B), angroside C (C), and cinnamic acid (D) among Radix Scrophulariae from different regions. (Error bars indicate standard deviations. Lowercase letters indicate significance at the level of 95% resulted from post hoc multiple comparison of either LSD or Tamhane T2.)

All hours of sunshine indices except two (Sun_8 and Sun_9) showed the same trend in temperature parameters. We observed that the average temperature in July (T_7), altitude, hours of sunshine in July (Sun_7), and relative humidity (RH) in July (RH_7) ranked

among the top four factors and produced a correlation coefficient above 0.8 on the first axis (result unshown). On the other hand, RH and precipitation (Pr) occupy the main ranks on the second axis and display a descending gradient from the bottom up, except for precipitation in July (Pr_7), but none of those variables presented a correlation coefficient above 0.7; that is, those factors are less important than the former ones. Interestingly, the PCA three-cluster pattern based on chromatographic fingerprint data in Figure 4A is generally coincident with the variations of the above top important environmental factors, of which most values in the CQ, HN, and SX populations were between those in the ZJ and HB populations (Supplemented information Figure 2S).

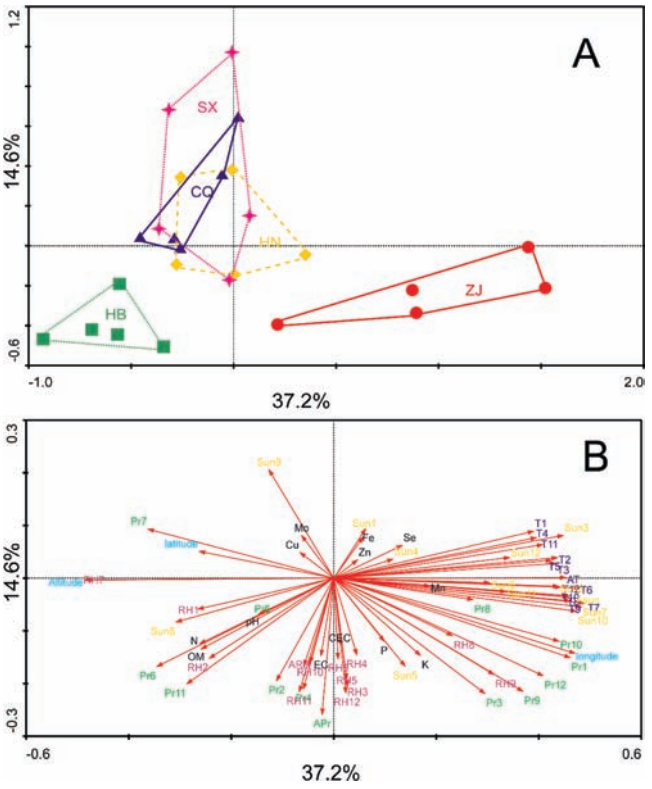

Figure 4. Correlation between chromatographic fingerprints of 25 samples of *S. ningpoensis* and the environmental factors of the corresponding sampling sites: (A) PCA plot of chromatographic fingerprints; (B) ordination plot of all environmental factors contributing to the variations (or distribution pattern) of chromatographic fingerprints.

In addition, on the basis of the contents of the four single compounds, Pearson correlation analysis revealed only harpagoside and angroside C were significantly correlated with a number of the environmental factors ($P < 0.05$), including climatic factors (average temperature and hours of sunshine), geographical factors (altitude, latitude, and longitude) and soil factors (contents of organic matter and available nitrogen) (see details in Table 4). Among those, factors of average temperature, hours of sunshine, and longitude demonstrated significant positive correlations, particularly all factors of monthly and annual average temperature, which were valued at greatest correlation coefficients. The content of harpagoside was significantly correlated with more environmental factors with higher values of correlation coefficient (most over 0.65) than for angroside C (most below 0.55), notwithstanding the similar trend of environmental effects. To disclose the determining factor(s), the environmental variables with significant correlation above 0.6 were subject to analysis of partial least-squares (PLS) regression, which is a powerful tool for searching for key factors with an algorithm eliminating distinct collinearity among numerous variables that may result in poor stability of the model.[29,30] The PLS model constructed for harpagoside fits the data well ($R^2 = 0.612$) and exhibits good predictivity ($Q^2 > 0.5$). The average temperatures in July, September, June, and October (T_7, T_9, T_6, and T_{10}) were computed to be the successive key factors affecting the content of harpagoside in Radix Scrophulariae. With regard to angroside C, the most important variable was precipitation in November (Pr_{11}), whereas both the fitness ($R^2 = 0.424$) and predictability ($Q^2 = 0.337$) were poor.

Table 4. Pearson Correlations between Harpagoside and Angroside C in Radix Scrophulariae and Environmental Factors [a]

factor	harpagoside	angroside C	factor	harpagoside	angroside C	factor	harpagoside	angroside C	factor	harpagoside	angroside C
T_1	0.666**	0.435*	Sun_1	ns	ns	Pr_1	0.714**	ns	RH_1	ns	−0.467*
T_2	0.731**	0.451*	Sun_2	0.681**	0.406*	Pr_2	ns	ns	RH_2	ns	−0.506**
T_3	0.740**	0.410*	Sun_3	0.727**	0.520**	Pr_3	0.431**	ns	RH_3	ns	ns
T_4	0.661**	0.473*	Sun_4	ns	ns	Pr_4	ns	−0.419*	RH_4	ns	ns
T_5	0.705**	0.457*	Sun_5	ns	ns	Pr_5	ns	ns	RH_5	ns	ns
T_6	0.754**	0.486*	Sun_6	0.448*	ns	Pr_6	−0.586**	−0.560**	RH_6	ns	ns
T_7	0.772**	0.455*	Sun_7	0.769**	ns	Pr_7	−0.651**	ns	RH_7	−0.748**	−0.489*
T_8	0.744**	0.405*	Sun_8	ns	−0.645**	Pr_8	ns	0.434*	RH_8	ns	ns
T_9	0.755**	ns	Sun_9	ns	ns	Pr_9	0.562**	ns	RH_9	0.504**	ns
T_{10}	0.751**	ns	Sun_{10}	0.726**	0.401*	Pr_{10}	0.723**	ns	RH_{10}	ns	ns
T_{11}	0.693**	0.450*	Sun_{11}	ns	0.549**	Pr_{11}	−0.426*	−0.653**	RH_{11}	ns	−0.478*
T_{12}	0.746**	ns	Sun_{12}	0.444*	0.538**	Pr_{12}	0.646**	ns	RH_2	ns	ns
AT	0.743**	0.438*	ASun	0.697**	0.423*	APr	ns	ns	ARH	ns	ns
altitude	−0.746**	−0.533**	longitude	0.686**	ns	latitude	−0.514**	ns			
OM	−0.506**	ns	N	−0.487*	ns						

[a] Abbreviations: T, average monthly temperature of each month; AT, annual average temperature in 2007; Sun, monthly total hours of sunshine; ASun, total hours of sunshine in 2007; RH, average monthly relative humidity; ARH, annual average relative humidity in 2007; Pr, monthly precipitation; APr, annual average precipitation in 2007; the subscript Arabic numbers stand for month; OM, content of organic matter; N, available nitrogen. *, significant at the level of 0.05; **, significant at the level of 0.01; ns, not significant. Acteoside and cinnamic acid were omitted due to no significant correlation with any of the tested environmental factors.

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DISCUSSION

Our present results of the Cosine similarity (Table 3), hierarchical dendrogram (Figure 3S), PCA (Figures 2 and 4A), and ANOVA of the contents of the single compounds consistently support the similar pattern of chemical differentiation of Radix Scrophulariae among the five regions. Chemical variation of Radix Scrophulariae in the ZJ population differed most from that in the HB population, whereas the CQ, HN, and SX populations resembled each other and were almost equally differentiated from the ZJ and HB populations. This differentiation pattern basically coincides with that in our earlier study, although the HN population was not included and many fewer samples in the HB, CQ and SX populations were analyzed previously.[24] The four single compounds analyzed responded differently to the environmental gradients. Harpagoside was the detected metabolite most varied in content among the five regions (Figure 3) and significantly correlated with most environmental factors with greatest coefficients (Table 4) and was also the variable with the greatest loading value, resulting in PCA clustering based on overall chemical fingerprints (Figures 2 and 4A). Therefore, harpagoside is proposed to be a reasonable representative of the chemical variation of *S. ningpoensis* and consequently be associated with environmental variations.

The correlation analyses suggest that climate variables, especially temperature, produced a significantly greater effect on the overall chemical profile and the contents of harpagoside and angroside C in Radix Scrophulariae. All temperature parameters (annual and monthly average temperature) displayed pronounced positive correlation with chemical variations of Radix Scrophulariae in terms of overall chromatographic profile and single compounds, particularly harpagoside (Figure 4B; Table 4). Despite few reports on the effect of temperature factors on plant secondary metabolism in natural environments, an array of similar work has been carried out in controlled conditions. A temperature-controlled greenhouse experiment showed that a relatively higher temperature significantly enhanced the contents of a number of phenolic acid, flavonols, and anthocyanins in the fruit of strawberry (*Fragaria* × *ananassa*).[31] However, some plant metabolites were also reduced by an increase of temperature, whereas others were promoted.[32] The difference of the content changes of different metabolites may be attributed to dynamics of secondary metabolism responding to temperature fluctuation. For example, the shift from B-ring *o*-diphenolic flavonols (quercetin and its derived glycosides) to B-ring monophenolic flavonols (kaempferol and its derived glycosides) was triggered by a decrease of 5 °C in day temperature in heads of *Arnica montana* L. cv. *arbo* cultured in climate chambers.[33] This phenolic change was further speculated to deal with cold tolerance via capability of scavenging free radicals resulting from cold damage. Accordingly, the chemical variation of Radix Scrophulariae might be related to heat tolerance.

The pattern of chemical differentiation of Radix Scrophulariae also corresponds to the altitudinal gradient; that is, the ZJ and HB populations were located at the lowest and highest altitudes, respectively, and the other three were at intervening sea levels (Table 1). This altitudinal effect was further supported by a strong negative correlation between chemical variation and altitude (Figure 4B; Table 4). The effect of altitudinal variation on chemical compounds was also demonstrated in several other plants.[7,13,34] Spitaler et al.[13] assessed altitudinal variation of phenolics in *A. montana* L. cv. *arbo* and proposed that temperature and ultraviolet (UV)-B radiation may be the two key determining factors. Temperature was further confirmed to be the key factor, which triggered shifts in the phenolic composition in *Arnica* grown at higher altitudes.[33] Considering the negative correlation between temperature and altitude, the significant negative correlation between harpagoside and altitude of Radix Scrophulariae could be further evidence to support the proposal that harpagoside was positively correlated with temperature.

Latitudinal gradient may also influence plant chemical variation. A positive correlation between latitude and chemical diversity was detected in juniper (*Juniperus communis*) needles.[35] Concentrations of total anthocyanidin and delphinidin in *Vaccinium myrtillus* fruits varied significantly across latitude, with higher values from northern latitudes, whereas another anthocyanidin, cyanidin, was opposite.[36] Therefore, different plants or even different metabolites might differ in their responses to latitudinal variation. Our results indicated a negative correlation between harpagoside content and latitude (Table 4). The factor of latitude comprises a series of other environmental factors, among which temperature tends to fall with the growth of latitude. Although there is no research dissecting the contribution of those factors to the latitudinal effect on plant chemical variation so far, the latitudinal change of temperature is not contrary to the positive correlation between harpagoside variation and temperature in *S. ningpoensis*.

Furthermore, it appears that the climatic factors influencing harpagoside variation of Radix Scrophulariae are unevenly distributed across the growth year. July is the month when the corresponding climatic parameters displayed the greatest impact on the content of harpagoside (Table 4), indicating July might be the month when the plants of *S. ningpoensis* are most sensitive to environmental changes. This result is basically consistent with the study of seasonal variations on the content of harpagoside in *S. scorodonia* L., which demonstrated the percentage of harpagoside was highest during the maximum development of the plant, especially in July.[37] The summer months, July and August, presented the highest temperature in all five regions in 2007 (Supporting Information Figure 1S). The phenological observations showed that the plant of *S. ningpoensis* rapidly expands root tubers and emerges flower buds successively in July and then begins to bloom in August.[38] The tolerance to high temperature in July and August therefore is crucial to the plant's survival and successful propagation of *S. ningpoensis*. The significant growth of harpagoside in the summer is probably related to such a process of heat tolerance. Our ongoing investigation of the monthly dynamics of metabolites through common garden experiments may facilitate further explaining the function of harpagoside in response to the environmental change of temperature.

Our results confirmed the approximately three-group pattern of chemical differentiation of Radix Scrophulariae among the five regions, the ZJ population, the HB population, and the remaining three populations (CQ, HN, and SX). Harpagoside appeared to be representative of the chemical variation of *S. ningpoensis* and displayed significant positive correlations with monthly and annual average temperature and negative correlations with altitude and latitude. We concluded that the harpagoside variation was strongly positively correlated with environmental changes of temperature. Relatively higher average temperature and lower altitude were recommended for the cultivation of *S. ningpoensis* for GAP. Out result supports harpagoside as a marker compound for quality control of Radix Scrophulariae adopted in

China Pharmacopeia. The present research also provides a new comprehensive model for evaluating the chemical diversity of herbal medicines and its correlation with environmental variables.

■ ASSOCIATED CONTENT

Supporting Information. Figures 1S−3S. This material is available free of charge via the Internet at http://pubs.acs.org.

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