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Analysis of three related Chinese endemic *Curcuma* herbs by HPLC and *in vitro* antioxidant activity

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Abstract The rhizomes of *Curcumalonga* are used as herbs in traditional Chinese medicine (TCM) for antioxidant activity and treatment of a variety of ailments, while *C. sichuanensis* and *C. chuanhuangjiang* are similar to *C. longa* in morphology, curcuminoid contents which were commonly used as substitutes in TCM. In this study, two methods (HPLC and *in vitro* antioxidant activity) were combined to identify the three related *Curcuma* species with population samples to evaluate suitable germplasm resource, quality control, construction of good agricultural practice base (GAP Base). The contents of curcuminoids in both *C. longa* and *C. chuanhuangjiang* were much higher than that in *C. sichuanensis*. The antioxidant properties of curcuminoid extracts were investigated *in vitro* for their hydroxyl free radical and 1,1-diphenyl-2-picrylhydrazyl radical scavenging activities. The extracts from *C. longa* and *C. chuanhuangjiang* showed higher scavenging capacities than from *C. sichuanensis*. Both the curcuminoid contents and their scavenging capacities were linked to the geographic locations of the three *Curcuma* species. The cities of Leshan (*C. longa*) and Jianyang (*C. chuanhuangjiang*) are ideal regions for GAP Base construction.

Keywords *Curcuma*, Herbs, HPLC, Curcuminoids, Antioxidant

Introduction

Curcuminoids are mainly composed of curcumin, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC), which can be extracted from the roots of *Curcuma* species. In a variety of studies, these extracted compounds have been reported to hold certain medicinal qualities, including antioxidant, anti-cancer, anti-Alzheimer's, and anti-tumor effects [1–3]. The rhizomes of *C. longa* are referred to as Radix Curcumae in traditional Chinese medicine (TCM) and have been officially recorded as folk medicine in the Pharmacopoeia of the People's Republic of China (Chinese Pharmacopoeia) [4]. However, the species *C. sichuanensis* and *C. chuanhuangjiang* frequently appear as substitutes or fakes of *Curcuma longa* in agricultural cultivation, herb trading

and TCM in China for their similar growth habits and morphological features in both their vegetative and reproductive organs [5–7].

From a biochemical perspective, various methods such as HPLC have been employed to measure the curcuminoid content of *Curcuma* species, and only a few previous studies have focused on analyzing and comparing the compounds' content diversity among *Curcuma* species, though no population level sampling was involved [8–10]. The pharmacological effects of *Curcuma* species in TCM are influenced by the diversity of curcuminoids in population level samples from different geographic location [1,11].

The evaluation of the antioxidant activities of natural substances *in vitro*has received increased attention in biology, medicine and food chemistry in recent years [12–14]. The earliest study of the antioxidant activity of curcuminoids was published in 1975 [15]. The curcuminoids act as scavengers of oxygen free radicals [16,17]. Here, we investigate the ·OH and DPPH scavenging activity of curcuminoid extracts from different species and localities as a method of measuring their antioxidant effects.

In this study, 20 samples (including population samples) of related *Curcuma* species were evaluated by HPLC and *in vitro* antioxidant assays to identify these species in germplasm resources and to select ideal *Curcuma* germplasm resources, to understand best agricultural practices (GAP) base for *Curcuma* cultivation in agriculture, then to meet the quality of Radix Curcumae in TCM.

Material and Methods

Plant Samples

Twenty samples from three *Curcuma* species and their collection locations were included in the study shown in Table 1. Sichuan province is the main geo-herbalism habitat of *C. longa*, *C. sichuanensis* and *C. chuanhuangjiang* in China [18]. Notably, *C. sichuanensis* and *C. chuanhuangjiang* are endemic to Sichuan province, while *C. chuanhuangjiang* is exclusively located in Jianyang [5]. The 20 *Curcuma* samples were cultivated on the Sichuan Agricultural University farm in Sichuan, China.

Table 1 The sampled *Curcuma* species used in this study, their geographic locations. The GAP (Good Agricultural Practice base) is a base selected specifically for the cultivation of special herbal species to guarantee the quality of herbs used in TCM.

Number	Species	Location	Number	Species	Location
C1	*C. longa*	Dayi, Sichuan	C11	*C. longa*	Shawan, Leshan,Sichuan
C2	*C. longa*	Longquan, Chengdu, Sichuan	C12	*C. longa*	Muchuan, Leshan, Sichuan
C3	*C. longa*	Qianwei, Leshan, Sichuan	C13	*C. longa*	Yibin, Sichuan
C4	*C. longa*	Shuangliu, Sichuan	C14	*C. longa*	Medicinal Botanical Garden, Guangxi
C5	*C. longa*	Qianwei, Leshan, Sichuan	C15	*C. sichuanensis*	GAP bases, Chongzhou, Sichuan
C6	*C. longa*	Xinjing, Sichuan	C16	*C. sichuanensis*	Chongzhou, Sichuan
C7	*C. longa*	Muchuan, Leshan, Sichuan	C17	*C. sichuanensis*	Yibin, Sichuan
C8	*C. longa*	Qianwei, Leshan, Sichuan	C18	*C. sichuanensis*	Weiyuan, Sichuan
C9	*C. longa*	Yibin, Sichuan	C19	*C. sichuanensis*	Chongzhou, Sichuan
C10	*C. longa*	Ziyang, Sichuan	C20	*C. chuanhuangjiang*	Jianyang, Sichuan

Reference standards

Curcumin (No: 110823-200603), DMC (No: MUST-10042905) and BDMC (number: MUST-11041307) were acquired from the Chinese National Institute of Food and Drug Control Agency and used as reference standards (purity >98%).

HPLC

Reference standard solutions and samples

The three reference standards were separately dissolved into 50 ml of ethanol and stored at 4 ℃ for antioxidant analysis using HPLC. The concentrations of the reference standards were 102 μg/ml curcumin, 106 μg/ml DMC, and 104 μg/ml BDMC.

The 20 *Curcuma* samples were air-dried and pulverized into a fine powder before filtration through an 80 μm mesh screen, after which 0.1 g of the powder was extracted using the optimized extraction procedure (see below) until the suspension was colorless. The supernatant solution was then diluted to 50 ml in a volumetric flask and filtered through a 0.45 μm mesh membrane.

Curcuminoid extraction

To efficiently extract curcuminoids from the *Curcuma* samples, we optimized the variables in the extraction procedure. The variables included the extraction ultrasonication, extraction solvent (50% -100% ethanol), extraction time (30-90 min) and material-to-liquid ratio (5:1-30:1 g/ml). The final optimized extraction procedure was as follows: 20:1 g/ml (material-to-liquid ratio), 60 minute extraction time, and 10 ml of 70% ethanol in an ultrasonic bath (KQ-300DV) at 25 kHz and 200 W.

HPLC conditions and methodology validation

HPLC analysis was conducted on an HPLC system (Daojin, Japan LC-10AVP) under the optimized conditions as follows: column temperature at 30 ℃, volume injection of 10 μl, and peak monitoring at 422 nm. The chromatographic column was DiMa C18 (4.6 × 150 mm, 5 μm). The mobile phase consisted of acetonitrile (CH_3CN) and 0.1% phosphoric acid diluted in pure water with a flow rate of 0.9 ml/min. A gradient elution procedure was used, with the eluent consisting of 50% acetonitrile for 0-5 min, 50-62% acetonitrile for 5-7 min, and 62-50% acetonitrile for 7-10 min.

Across both short (same day) and long (three days) time scales, the linearity, reproducibility, stability and average recovery rates were evaluated three times to validate the HPLC methodology with reference standards. The linearity analysis was performed using five different concentrations (10, 20, 30, 40 and 50 μg/ml) of reference standard solutions. Each concentration was measured in triplicate. The relationship between concentration (x) and its corresponding peak area ratio (y) is expressed by the equation $y = ax + b$.

The three reference standards were mixed together, extracted and analyzed using the above-described methods. Samples were analyzed six times on the same day and reanalyzed twice per day for three consecutive days to examine both the short and long term variation. To ensure reproducibility, each sample was extracted and analyzed in five replicate experiments. To test stability, the same sample solution was analyzed every 6 h for 48 h at room temperature. The relative standard deviation (RSD) was introduced to show the results of the same day and multiple day analyses as well as their reproducibility and stability. The recovery rate was also used to examine the accuracy of the HPLC methodology validation.

Antioxidant activity

Instrumentation and samples

The antioxidant analysis was performed on a UV-1750 Spectrophotometer (Daojin, Japan). Vitamin C (Vc) was used as positive reference in antioxidant analysis. Each sample was tested three times, and the average results are presented. The value of the reference standards scavenging and IC_{50} were calculated to inform the antioxidant analysis of *Curcuma* extracts in this study.

1g powdered sample of *Curcuma* radix was extracted until the suspension was colorless (see Extraction procedure of curcuminoids). The supernatant solution was diluted to 100 ml and filtered through 0.45 μm mesh membrane for antioxidant analysis.

Antioxidant evaluation

The Fenton reaction was used to evaluate ·OH scavenging [19].The mixtures for testing the ability of each compound to scavenge·OH included 1 ml extracts, 2 ml of $FeSO_4$ solution (6 mmol/l), and 2 ml of H_2O_2 (6 mmol/l), added to 2 ml of 2-hydroxybenzoic acid (6 mmol/l) and incubated for 30 min. The UV absorbance of the samples at 510 nm was then recorded.

DPPH scavenging was analyzed using the procedures described in previous studies [20]. A mixture containing 0.05 ml of the curcuminoid extract solutions and 5 ml of DPPH (0.03 g/l, dissolved in methanol) was incubated for 30 min in the dark and its UV absorbance at 510 nm was then recorded.

The .OH and DPPH scavenging effects were calculated as follows:

$$\cdot\mathrm{OH}(\%) = \frac{A_0 - (A_i - A_j)}{A_0} \times 100$$

$$\mathrm{DPPH}(\%) = \frac{A_0 - A_i}{A_0} \times 100$$

Where A_0 is the absorbance of distilled water, A_i is the absorbance of the mixture, and A_j is the absorbance of the mixture with distilled water added instead of 2-hydroxybenzoic acid.

Results and Discussion

HPLC

Methodology validation

The HPLC method established in this study was validated according to the following parameters: the optimized HPLC method showed linearity within a range of 10 μg/ml-50 μg/ml, with a regression coefficient of 0.99 for curcumin, DMC and BDMC. The calibration curves showed good linear relationships within relatively wide dynamic ranges (Table 2).

Table 2: Calibration curves of the reference standards

Curcuminoids	Regression	Dynamic (μg/ml)	r^2
curcumin	$y = 1.0 \times 10^3x + 0.4203$	6.00-180.00	0.9999
DMC	$y = 9.5 \times 10^5x - 0.054$	6.00-180.00	0.9999
BDMC	$y = 1.0 \times 10^4x - 2709$	6.00-180.00	0.9999

Note: In the regression equation y = ax+b, 'x' is the concentration of pure curcumin (μg/ml); 'y' is the peak; and 'r' refers to the correlation coefficient.

The quantitative method presented here exhibited satisfactory accuracy with the overall average recoveries from three tests ranging from 98.0% to 99.0%. The RSDs obtained from the analysis of curcumin, BDMC and DMC were 0.2%, 0.3%, and 0.4% within the same day and 2.7%, 2.7%, and 3.1% over three days, respectively, showing that the sample solution was stable for at least for three days. The method exhibited good reproducibility with an RSD of less than 5.0% and showed extreme stability within 48 h with an RSD of less than 1.03%. The average recovery values and their RSDs are presented in Table 3.

Table 3: Average recovery values and RSD (relative standard deviation) of reference standards

Curcuminoids	Sample concentration (μg/ml)	Added standards (μg/ml)	Recovered amounts (μg/ml)	Average recovery (%)	RSD (%)
BDMC	2.66	6	8.58	99	0.305
DMC	2.69	6	8.51	98	1.457
curcumin	10.76	6	16.6	99	0.339

Degree of separation and quantitative analysis of curcuminoids

The degree of separation and quantitative analysis of both reference standards and the twenty *Curcuma* extracts were tested under the optimized conditions at 422 nm (Figs. 1 and 2). The peaks were monitored at 422 nm with high sensitivity, and the gradient elution results showed that separation occurred when the samples were subjected to 50% acetonitrile for 10 minutes.

The contents of curcuminoids varied among the different population samples and within different *Curcuma* species (Table 4). Samples collected from Leshan had much higher curcuminoid content relative to other species and localities of the same species. Among these related species, the composites contained 4.23 to 37.26 mg/g curcuminoids, 0.19 to 22.30 mg/g curcumin, 0.06 to 8.90 mg/g DMC, and 0.02 to 9.58 mg/g BDMC.

C. *Longa* (C11), which was collected from Shawan, Leshan city, Sichuan Province, contained the highest curcuminoid (37.26 mg/g) and curcumin (22.3 mg/g) amounts. *C. chuanhuangjiang* and13 out of 14 *C. longa* samples had curcumin contents greater than 1% (w/w) and thus met the requirements of the Chinese Pharmacopoeia [21]. The curcumin content was less than 1% in the *C. sichuanensis* samples, which did not meet the criteria of the Chinese Pharmacopoeia. Therefore, *C. sichuanensis* is not a suitable source of Radix Curcumae for TCM. *C. chuanhuangjiang*, an endemic species in Jianyang, Sichuan Province, exhibited the second highest curcuminoid (36.55 mg/g) and curcumin (17.61 mg/g) content and thus could be an alternative source of Radix Curcumae.

Table 4: Contents (mg/g) of extracts and their antioxidant effects (%)

No.	Location	Curcumin	DMC	BDMC	CUR	·OH	DPPH
C1	Dayi, Chongzhou, Sichuan	10.76	2.69	2.66	16.12	54.85	59.15
C2	Longquan, Chengdu, Sichuan	11.20	3.16	3.67	18.03	54.99	69.24
C3	Qianwei, Leshan, Sichuan	17.37	4.53	4.71	26.60	65.57	88.53
C4	Shuangliu, Sichuan	13.78	2.94	2.28	18.99	62.33	51.78
C5	Qianwei, Leshan, Sichuan	10.64	5.18	5.38	21.20	62.94	62.13
C6	Xinjing, Chengdu,Sichuan	19.50	5.63	6.40	31.54	58.76	44.94
C7	Muchuan, Leshan, Sichuan	12.78	3.18	2.93	18.88	67.79	70.51
C8	Qianwei, Leshan, Sichuan	16.01	4.24	4.43	24.67	62.13	78.43
C9	Yibin, Sichuan	8.09	1.78	1.55	11.42	59.03	43.70
C10	Ziyang, Sichuan	17.37	4.53	4.71	26.60	54.92	43.60
C11	Shawan, Leshan, Sichuan	22.30	6.83	8.13	37.26	70.55	76.75
C12	Muchuan, Leshan, Sichuan	19.62	5.18	5.10	29.90	61.52	70.12
C13	Yibin, Sichuan	16.90	8.62	9.58	35.11	59.70	19.32
C14	Medicinal Botanical Garden, Guangxi	4.67	1.96	0.77	7.40	60.11	42.16
C15	GAP bases, Chongzhou, Sichuan	2.72	1.06	0.44	4.23	41.91	40.57
C16	Chongzhou, Sichuan	3.14	1.20	0.51	4.85	41.85	40.18
C17	Yibin, Sichuan	5.04	2.09	0.86	7.99	47.04	36.27
C18	Weiyuan, Sichuan	2.79	1.15	0.41	4.35	52.76	18.00
C19	Chongzhou, Sichuan	4.08	1.64	0.86	7.57	49.12	36.88
C20	Jianyang, Sichuan	17.61	8.90	10.04	36.55	75.20	67.52

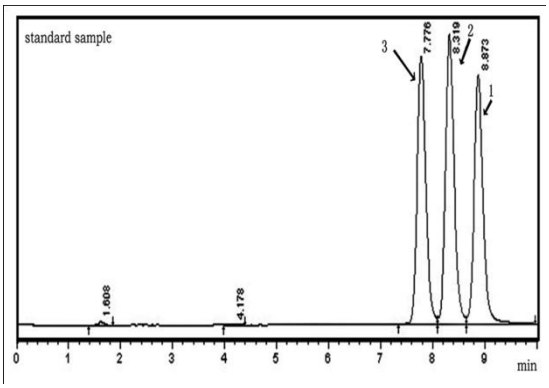

Figure 1: HPLC chromatogram of reference standards in ethanol solvent monitored at 422 nm. 1= curcumin, 2 = DMC, 3 = BDMC

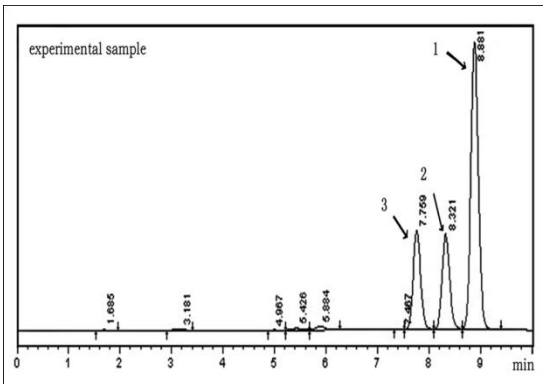

Figure 2: Typical HPLC chromatogram of the extracts in ethanol solvent monitored at 422 nm. 1= curcumin, 2 = DMC, 3 = BDMC

Antioxidant effects

Antioxidant effects of reference standards

The reference standards exhibited ideal scavenging activities. The scavenging effects (%) are presented in Figs. 3 and 4. When the concentration was in the range of 1-8 μg/ml, the ·OH scavenging ability was high. At concentrations ranging from 1-8.25 μg/ml, the DPPH scavenging effect was generally high.

.OH and DPPH scavenging effects

The curcuminoid extracts displayed strong scavenging capabilities for ·OH and DPPH (Table 4). The ·OH scavenging effect was 49.12%-75.2%, with a mean value of 58.15%. The 12 extracts, including 11 from *C. longa* samples and one from *C. chuanhuangjiang*, showed scavenging activity of over 58.15%. Of the 20 samples, *C. chuanhuangjiang* had the highest ·OH scavenging effect (75.2%); *C. longa* ranged from 54.85% to 70.55%, and *C. sichuanensis* ranged from 41.85% to 52.76%. Sample C11, which was collected from Shawan, Leshan city, Sichuan province, exhibited the highest ·OH scavenging effect (70.55%) among *C. longa* samples.

The DPPH scavenging effect ranged from 18.00% to 88.53%, with a mean of 52.99% among the twenty samples. Eight *C. longa* extracts and one *C. chuanhuangjiang* extract showed good scavenging activities that were above the mean value. All the samples of *C. longa* collected from Leshan, Sichuan, displayed high antioxidant activities. The C3 extract from *C. longa* (obtained in Qianwei, Leshan, Sichuan) exhibited the highest scavenging effect of 88.53%. *C. chuanhuangjiang* exhibited a scavenging effect of 67.52% among the 20 samples. The DPPH scavenging effects of extracts from five of the *C. sichuanensis* population samples were below the mean value, ranging from 18.00%-40.18%.

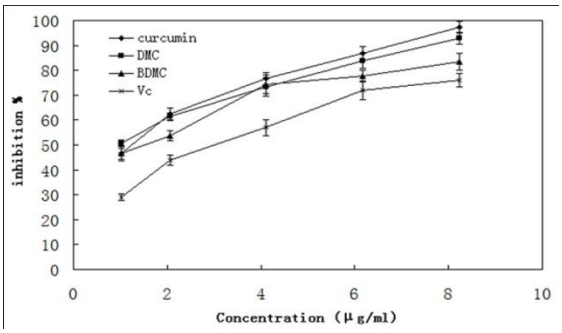

Figure 3: The .OH scavenging effects (%) of the three curcuminoid reference standards. Vitamin C (Vc) was used as a positive reference.

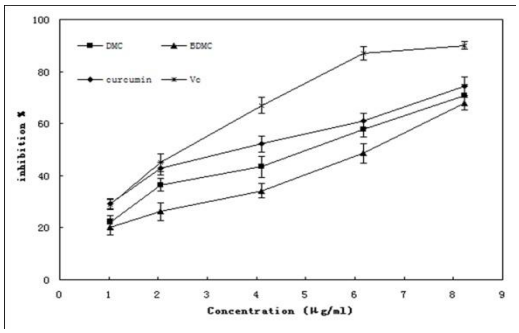

Figure 4: DPPH scavenging effects (%) of the three curcuminoid reference standards. Vitamin C (Vc) was used as a positive reference

Conclusion

The three methods presented here provided increased insight into *Curcuma* germplasm resource selection, GAP base construction, and qualitycontrol in TCM.*C. sichuanensis* was easily identified as a fake herbal species based on the results from HPLC and antioxidant. *C. chuanhuangjiang* is a potential germplasm resource due to its high curcuminoid content and antioxidant scavenging activity. The cities of Leshan (*C. longa*) and Jianyang (*C. chuanhuangjiang*) are ideal regions for GAP base construction based on compound contents and their antioxidant capabilities. The quality of Radix Curcumae in TCM was greatly influenced by different *Curcuma* species and their different geographic locations within species.

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