A validated UV-HPLC method for determination of chlorogenic acid in *Lepidogrammitis drymoglossoides* (Baker) Ching, Polypodiaceae

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ABSTRACT

Background: *Lepidogrammitis drymoglossoides* (Baker) Ching (*L. drymoglossoides*), a member of the Polypodiaceae family, was used in the treatment of numerous diseases. However, none of the potential ingredients and the quality control methods concerning this plant medicine was pronounced. **Objective:** To identify chlorogenic acid (CGA) from *L. drymoglossoides* and develop a high performance liquid chromatography (HPLC) assay of CGA. **Materials and Methods:** UV, TLC, and HPLC were utilized to identify the phytochemicals of *L. drymoglossoides* and determine the CGA content, respectively. The HPLC conditions were as following: a Phenomenex Luna C18 (2) (250 × 4.6 mm *i.d.*; 5 μm particle size; 100 Å pore size) column; the mobile phase of the mixture of acetonitrile and 0.5% aqueous phosphoric acid (11.5:88.5 v/v); the flow rate of 1.0 mL/min and determination wavelength of 327 nm. **Results:** The proposed HPLC method has been developed and validated. The calibration curve was $y = 28328x + 16610$ ($R^2 = 0.9997$). The intra-day and inter-day precision and intermediate precision were validated with the RSD less than 5%. The mean recovery rate of the method ranged from 95% to 104%, with the RSD less than 5%. The LOD and LQD values were 0.049 and 0.132 mg/L, respectively. The content of CGA in *L. drymoglossoides* approximately reached 0.24% (v/v) by the proposed extraction and determination methods. **Conclusion:** The assay method was simple, convenient, and accurate to the quantification of CGA and can be used for the quality control of the herb.

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INTRODUCTION

Lepidogrammitis drymoglossoides is a member of the Polypodiaceae family, which is widely distributed in southern and western China, especially in Guangdong, Guangxi, and Guizhou provinces. The medicinal plants are extensively used in the treatment of pulmonary tuberculosis, diarrhea, fever, and inflammatory diseases[1-3] by the community of Chinese ethnics Miao, Yao, and Tong. However, little is known concerning the phytochemistry of the species, especially the bioactive ingredients, and yet no method has been proposed for the standardization of this herb, which limit the exploitation and commercialization of these medicinal plants.

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Currently, the pharmaceutical analytical methods based on the determination of one or several phytochemicals are commonly utilized in the quality control[4,5] of raw drug materials or commercial drugs. To establish an analytical method of assessing the quality of *L. drymoglossoides*, the identification of certain natural products of secondary metabolism in the raw materials is indispensable. On the basis of the reports involving the phytochemistry of other common Polypodiaceae spices—*Pyrrosla lingua* (Thunb)[6] and *Lepisorus thunbergianus* (Kaulf) Ching,[7] chlorogenic acid (CGA) was found in high quantity. Thus, it credits the hypothesis that CGA is an important natural product present in *L. drymoglossoides*. In addition, because of the resemblance between the pharmaceutical properties of CGA (anti-inflammation,[8] antifungal activity,[9] and antioxidant[10]) and the efficacy of *L. drymoglossoides*, the hypothesis seemed more convincing. Eventually, our initial investigation on the phytochemicals of *L. drymoglossoides* verified the hypothesis.

For the quality control of various phytochemical plants and drugs, CGA was commonly employed as a biomarker such as Chinese proprietary medicines *Yi Qiao Jie Du* tablets[11] and *Flos Chrysanthemi Indici* suppository.[12] CGA was also employed as a marker in quality monitoring during the postharvesting treatments of Brazilian coffees.[13] The initial discovery of CGA in *L. drymoglossoides* facilitates the development of an efficient analysis method. In this study, the proposed method has been validated in terms of specificity, linearity, limits of detection and quantity, precision, accuracy, and robustness, according to guidelines from ICH.

MATERIALS AND METHODS

Plant material

The whole grass of *L. drymoglossoides* was obtained from Hunan Pharmaceuticals Co., Ltd. (the raw materials were collected in the area of Shaoguan, approximately 1000 m, Guangdong province, China, in October 2009). The species were identified by Dr. Jinping Li, a Botanist from School of Pharmaceutical Sciences, Central South University (China). Voucher specimens were deposited at the herbarium of the School of Biological Science and Technology under number CSU 091023. The whole grass of the plant was powdered by an electrical blender, sieved through a #24 mesh sieve and dried in a drying oven under the temperature of 60°C before extraction.

Chemicals and reagents

Chlorogenic acid standard reference (purity > 98.0%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). High performance liquid chromatography (HPLC)-grade acetonitrile was supplied by American Tedia Company (Fairfield, OH, USA). All the other solvents are analytically pure and obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Ultrapure water with a resistivity greater than 18 mΩwas collected from a certified Millipore Milli-Q system (Bedford, MA, USA).

Sample preparation for identification

A sample (2.0 g) of *L. drymoglossoides* powder was weighed and sonicated with 25 mL aqueous ethanol (50% v/v). The solution was filtrated and diluted into different concentrations prior to the UV spectra test. The reference solution for the UV test was also prepared by dissolving standard CGA in aqueous ethanol (50% v/v) to obtain a concentration of 1 mg/mL. For the test of TLC and HPLC, methanol was used as the solvent. Two grams of plant powder was weighed and extracted by 25 mL methanol for 30 min sonication extraction. The resulting solution was filtrated and concentrated to 1 mL. The concentration of the total CGA in the reference solution was also 1 mg/mL.

Identification of chlorogenic acid

Different concentrations of reference and test solutions were detected under a UV-Visible Spectrophotometer (UV-2550, Shimadzu, Japan) at the range from 200 to 400 nm. The spectra of the both solutions were obtained and analyzed. Two kinds of isolation solvents [acetic acid and isopropanol–formic acid (10:0.5 v/v)] were used in TLC. An aliquot (20 μL) of the solutions were developed on silicon G thin-layer plates, and finally the spots on the plates were determined under the wavelength of 365 nm. HPLC analysis was carried out on a chromatography system equipped with a high-precision pump (Shimadzu model LC-10-ATVP, Kyoto, Japan), a UV detector (Shimadzu model LC-10-ATVP) operating at 327 nm, and a Phenomenex Luna C18 (2) (250 × 4.6 mm *i.d.*; 5 μm particle size; 100 Å pore size). Chromatographic data were recorded and integrated in the class VP computer software. The mixture of acetonitrile and 0.5% aqueous phosphoric acid (11.5:88.5 v/v) was used as the mobile phase with the flow rate at 1.0 mL/min. The solutions were filtered through a 0.45 μm pore size filter (Millipore, Bedford, USA) prior to injection (injection volume is 10 μL).

Sample preparation for high performance liquid chromatography analysis

A sample (0.2 g) of dry plant powder was weighed accurately and transferred to a conical flask. Forty-five milliliters of aqueous methanol (50:50 v/v) was added to the powder and sonicated at room temperature in an ultrasonic bath for 40 min. The resulting mixture was filtered, and the residue was washed with 10 mL of 50% aqueous methanol. The two filtrates were combined and condensed to 25 mL approximately by a rotary evaporator at the temperature of 50 °C. After cooling, the liquid was transferred into a 50 mL volumetric flask and diluted with 50% aqueous methanol to the volume. An aliquot was refiltered through a 0.45 μm Millipore filter and 10 μL of the Millipore filtrates were assayed by a HPLC system.

The development and validation of high performance liquid chromatography analysis

During the initial study, all the HPLC conditions were investigated to obtain the best separation, and the optimization conditions of the analysis separation had been depicted in the section of "identification of CGA".

For the chromatographic method, developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all interferences. Therefore, the extraction mixtures obtained from the sample were analyzed and the analyte peak (CGA) was evaluated for peak purity and resolution from the nearest eluting peak.

A stock solution of standard was prepared by accurately

weighing 7.84 mg of CGA and diluting in a 100 mL volumetric flask by 100 mL of 50% aqueous methanol obtained a final concentration of 78.4 mg/L. Five additional standard solutions with concentrations of 54.88, 39.20, 23.52, 7.84, and 3.92 mg/L were prepared by appropriated dilution of the stock solution. The above six standard solutions (injecting volume: 10μL) was injected thrice into the chromatograph to obtain the peak values. The calibration curve was drawn by plotting the peak area against the concentration of the CGA standard.

To verify precision of the method, the results obtained from six repeated experiments on real samples, at a target concentration, on one day (intra-day) and on three consecutive days (inter-day) were analyzed. Intermediate precision was determined by analyzing the same samples in the same laboratory in triplicate, but by another analyzer and different instruments. Intermediate precision analysis was carried out on a Shimadzu Prominence LC-20A instrument equipped with a model SIL-20A autosampler, a model SPD-20A UV-VIS detector and a LC-20AT pump. The separation was performed on the same Phenomenex analytical column as described above. The sample solution employed in the repeatability test was re-analyzed in triplicate under the above laboratory conditions.

The accuracy of the method was evaluated by the percentage of recovery. The samples were spiked with three different amounts of standard CGA before extraction and each level of addition was executed in triplicate. The average content of CGA was obtained by analysis of the six samples with no addition of standard in parallel.

The limits of detection (LOD) and the limits of quantification (LOQ) were calculated on the basis of signal-to-noise ratios (S/N) of 3 and 10, respectively. LOD and LOQ were finally obtained by injecting a series of diluted solutions with known concentration.

Robustness was investigated by carrying out assays under slightly altered chromatographic conditions involving different flow rates of the mobile phase, different compositions of the mobile phase, and different temperatures of the column oven.

RESULTS AND DISCUSSION

Ultraviolet and Thin-Layer Chromatography for identification

To identify the compound of CGA in *L. drymoglossoides*, a simple UV spectrum test was firstly designed in the identification procedure. The sample and standard solutions with different concentrations were detected under the UV-VIS spectrophotometer, and the resulting spectra of different concentrations showed a slight difference. The spectrogram with the optimized UV absorption curve is shown in Figure 1. Due to the various interferences in the sample solutions, the absorption curve of the sample shows a large inconsistency with the curve of CGA standard. However, a mild and low peak can be distinguished around the wavelength ranging from 315 nm to 335 nm, which is consistent with the CGA standard. TLC is a traditional analysis method employed to identify the ingredients contained in the herb with more reliability.[14] The results of TLC are shown in Figures 2a and b, respectively. The isolation time and temperature were investigated to obtain a better separation (the RF values) and clear spots. The best isolation time for separation with the solvent of acetic acid is 15 min (RF = 0.66), 10 min for separation with isopropanol:formic acid (10:0.5 v/v) as the solvent

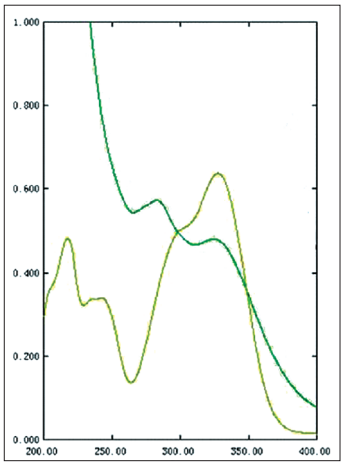

Figure 1: The spectrum of UV scanning: the green curve stands for *L. drymoglossoides* extracts and the yellow curve represents the standard CGA solvent

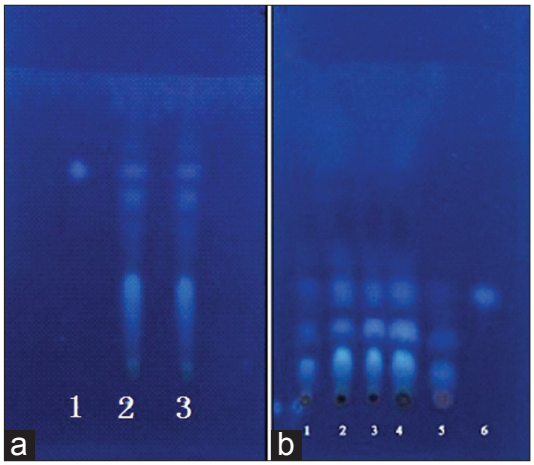

Figure 2: TLC chromatograms of two different isolation solvents (a) Isopropanol–formic acid (10:0.5 v/v), lane 1: CGA standard, lanes 2 and 3: *L. drymoglossoides* extracts (b) Acetic acid: lanes 1 to 5: *L. drymoglossoides* extracts, lane 6: CGA standard

(RF = 0.29), and both of the isolations was performed at the temperature of 25°C.

High performance liquid chromatography analysis for the analyte

The methods of CA determination are various, such as ultraviolet spectroscopy,[15] thin layer chromatography,[16] liquid chromatography,[17] ^{1}H NMR spectroscopy,[18] mass spectroscopy,[19,20] chemiluminescent,[21,22] etc. Among these methods, UV assay and thin layer chromatography are widely used because of their simplicity and rapidness. However, the UV detection method has some disadvantages such as low accuracy and stability. Moreover, our preliminary investigations upon this method also indicated these disadvantages. ^{1}H NMR spectroscopy, mass spectroscopy, and chemiluminescent methods are accurate and sensitive, but too expensive and require highly complicated instruments, which most laboratories are in defect of. However, HPLC is the legal method for the determination of CGA in many herbs in the regulation of Chinese Pharmacy.[23] This method is not only in possession of good accuracy, sensitivity, and stability, but also unnecessary of high complicated instruments. Therefore, we employed the HPLC method here to quantify the CGA in *L. drymoglossoides*. This method was equally essential to confirm the existence of CGA in *L. drymoglossoides* by comparing the retention time and peak purity between sample and standard reference.

The preliminary investigations were directed toward obtaining a perfect profile of chromatography of the extraction matrix. Thus, the following factors were assessed: the detection wavelength, the type of column, the flow rate, and the composition of the mobile phase. Our studies indicated that the type of columns and the composition of the mobile phase gave a significant impact to the separation of the CGA peak and its adjacent peaks. Only when the separation was carried out on the Phenomenex Luna C18 (2) column and acetonitrile–0.5% aqueous phosphoric acid (11.5:88.5 v/v) was employed as the mobile phase, an ideal Gaussian curve of the peak was found with the theoretical plate number larger than 10,000, a slight tailing (T = 1.125) and a high resolution (R = 2.597) between the CGA peak and its adjacent peaks. The chromatograms of the standard and the sample under the optimized conditions were shown in Figure 3. The retention time and shape of the CGA peak between the two chromatograms showed a precise resemblance and no interference was found. Therefore, the identification of natural product CGA in *L. drymoglossoides* was confirmed.

Validation of the high performance liquid chromatography analysis method

Validation of specificity of the analytical method was

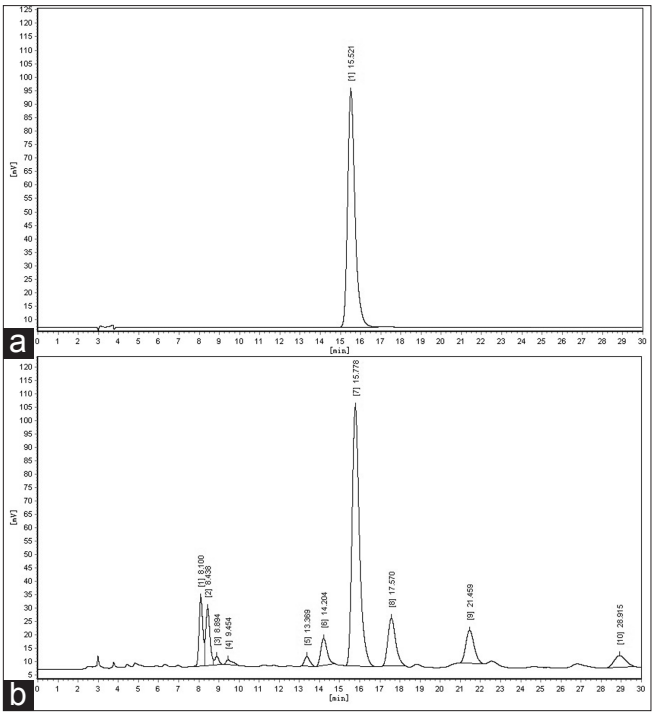

Figure 3: Chromatograms of (a) CGA standard and (b) *L. drymoglossoides* extracts. The mobile phase consisted of acetonitrile and 0.5% aqueous phosphoric acid (11.5:88.5 v/v). The retention time of analyte is 15.521 min of (a) and 15.778 min of (b)

confirmed by the resolution value (R is apparently larger than 1.5) of the targeted peak, and the comparison between the chromatograms of the standard analyte and of the extracts assayed. From the profile of the extracts spiked with the standard, no additional peak was obtained, which indicates the purity of the targeted peak. The calibration curve for CGA constructed with the UV detector set at 327 nm was linear in the range 78.4–3.92 mg/L. The regression equation and correlation coefficient (R^2) were derived as $y = 28328x + 16610$ ($R^2 = 0.9997$).

The results obtained from the analysis of intra-day precision were 9.75 ± 0.21 mg/L (RSD% = 2.2) for the six repeated injections of the sample. The results of intra-day precision on two consecutive days were 10.04 ± 0.24 mg/L (RSD% = 2.4) and 9.31 ± 0.19 mg/L (RSD% = 2.0), respectively, which are shown in Table 1. The RSD% of the inter-precision on three consecutive days valued 3.8. According to the average CGA concentration of sample solution, the content of CGA in *L. drymoglossoides* can approximately reach 0.24% (w/w). The results of intermediate precision using different analysts, different instruments, and on different days indicated that these parameters did not have any significant effect on the variation of results. The reproducibility of the methods, an important step in precision validation, which focused more on the bias in results, rather than on determining the differences, would be further investigated.

Table 1: Intra-day and inter-day precision

Days	CGA content (mg/L)						Mean ± SD (intra-day)	RSD% (intra-day)	RSD% (inter-day)
	A1	A2	A3	A4	A5	A6			
1	9.87	9.81	9.57	9.43	9.92	9.97	9.75 ± 0.21	2.2	3.8
2	9.90	10.23	9.78	9.84	10.37	10.14	10.04 ± 0.24	2.4	
3	9.33	9.27	9.41	9.37	8.97	9.52	9.31 ± 0.19	2.0	

RSD% = ((SD/Mean) × 100)

Table 2: Accuracy of the method

Theoretical contents of CGA in sample (mg/L)	Addition (mg/L)	Theoretical amount (mg/L)	Amount recovery (mg/L)	Recovery %	RSD %
9.88	7.84	17.72	16.92	95.49	1.2
			17.29	97.57	
			16.97	95.77	
9.88	9.88	19.76	20.13	101.88	3.2
			19.27	97.53	
			18.90	95.66	
9.88	11.92	21.8	21.28	97.63	3.1
			22.53	103.36	
			22.36	102.58	

Mean ± SD (n = 3); RSD% = ((SD/Mean) × 100)

The analysis of accuracy is shown in Table 2. Six parallel sample solutions of *L. drymoglossoides* were prepared and each CGA content was determined according to the regression equation, the theoretical quantity of CGA in the extracted matrix (9.88 mg/L) was equivalent to the average of CGA contents in the six samples (RSD% = 2.27). The three different amounts of standard CGA added to the samples were 80%, 100%, and 120% of the quantity of CGA in the extracted matrix, with the determined RSD% of 1.17, 3.24, and 3.07, respectively.

The LOD and LOQ values of the developed method were 0.049 and 0.132 mg/L, respectively. No significant change in the resolution of CGA was caused during the variation in the composition of acetonitrile and 0.5% aqueous phosphoric acid within the range from 12:88 to 11:89, the change of detecting wavelength from 326 nm to 328 nm by 1 nm, and the alteration of the flow rate (0.9, 1.0, and 1.1 mL/min). Therefore, the tests of robustness revealed that the method was insensitive to minor changes under the conditions evaluated.

CONCLUSION

For the first time, the natural compound of CGA was identified in the crude material of *L. drymoglossoides* by the simple analysis methods of UV, TLV, and HPLC. After the optimization of chromatographic conditions for the determination of CGA, the HPLC quantification method was found to be specific and suitable for CGA analysis in *L. drymoglossoides* because of its good specificity, sensitivity, accuracy, and precision. The assay is surprisingly essential to guarantee the quality and effectiveness of this ethnical herb in China.

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