Differentiating *Curculigo Orchioides* Rhizoma and Curculigo glabrescens Rhizoma by a Unique New Compound

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Research

Keywords: Curculigo orchioides Rhizoma (COR), Curculigo glabrescens Rhizoma (CGR), identification, glabrescenin

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Abstract

Background: Market research found that *Curculigo glabrescens* Rhizoma (CGR) is the major counterfeit of the *Curculigo orchioides* Rhizoma (COR). *C. orchioides* Gaertn and *C. glabrescens* (Ridl.) Merr. belong to the same family and genus, with close plant relationships and high genetic similarity, and rhizomes of the herbs part have less distinguished characteristics, which make the identification difficult.

Methods: In order to distinguish COR from CGR more accurately and conveniently, HPLC method was used to establish a characteristic chromatogram of the two herbs, and a unique component in CGR was discovered for first time. Based on that, the characteristic component was systematically separated and purified.

Results: The unique component was a new neolignans and named glabrescenin, which could specifically distinguish COR from CGR. The HPLC method we used could establish a characteristic chromatography of CGR.

Conclusion: This study was conducive to the quality control of *Curculigo*, and could promote the correct application of genuine COR in clinical practice.

Background

Curculigo orchioides Rhizoma(COR) is the dried rhizome of *C. orchioides* Gaertn, which has a long history of using as herbal medicine in China. The first appearance of COR was in the ancient medical book “Lei Gong Pao Zhi Lun”, and it has been included in Pharmacopoeia of the People’s Republic of China (PPRC). COR has the traditional effects of nourishing the kidney, strengthening bones and muscles, dispelling cold and dampness [1]. COR and its preparations are widely used in clinical practice, and have pharmacological activities such as preventing osteoporosis [2–4], anti-tumor [5], anti-oxidation [6], anti-depression [7], neuroprotection [8], and improving learning ability [9]. In recent years, extensive research has been conducted on the chemical components of COR, including phenolic and their glycosides [10], triterpenoid glycosides [11], lignans and their glycoside s[1], and Cl-containing compounds, etc [12].

C. orchioides Gaertn is mostly wild, mainly distributed in Sichuan, Zhejiang, Guangdong and other places in China, there are no cultivation and planting bases, with limited resources and low yields. The formula of a hot-selling health care product contains COR, which is in great demand, and the wild resources of COR are gradually depleted. The imbalance between supply and demand has led to an increase of counterfeit. According to market research, *C. glabrescens* Rhizoma (CGR), which originated from Vietnam, is a major counterfeit. Furthermore, there are lack of pharmacological activities and clinical evidences of CGR, only a new compound crassifoside H and free radical scavenging activity have been reported [13]. The safety and effectiveness of CGR are still controversial. Nevertheless, it is difficult to identify accurately the COR and CGR. *C. orchioides* Gaertn and *C. glabrescens* (Ridl.) Merr. belong to the same family and genus, with close plant relationships and high genetic similarity [14], on the other hand, the plant identification characteristics of *C. glabrescens* (Ridl.) Merr. are smooth and glabrous on the back of

leaves. However, the appearance of the CGR is similar to the COR. Thus, it is necessary to establish a quick and accurate method to distinguish the two medicines.

The 2020 edition of the PPRC only includes COR, and stipulates that the content of curculigoside is not less than 0.10%. Preliminary research found that the existing standards of the PPRC could not accurately distinguish CGR and COR through microscopic characteristics, TLC, and curculigoside content. A chemical fingerprint/characteristic defines an unique pattern of a herb and reflects the presence of multiple chemical constituents. This approach follows the fundamental holistic theory of traditional Chinese medicines as major and minor chemical constituents are analyzed simultaneously in herbal sample [15, 16]. This method may contribute to evaluate herbal medicine, quality, safety and effectiveness. High performance liquid chromatography (HPLC) is the most popular analytical method in separation science and is regarded as one of the gold standard in the authentication of pharmaceutics and herbal medicines due to its good precision, sensitivity and reproducibility [17]. Compared with traditional morphological and microscopic identification methods, the HPLC method is relatively faster and more accurate.

Based on that, in this study, in order to distinguish COR and CGR more accurately and conveniently, HPLC method was used to analyze their components. A characteristic component from CGR was discovered for the first time, and was systematically separated and purified. The structure was identified as 5-(3',4'-dihydroxyphenyl)-1-(4"-hydroxyphenyl) pentane-1,4-dione, it was a new neolignans and named glabrescenin (Fig. 3). Therefore, the HPLC method we used could establish a characteristic chromatography of CGR, which can distinguish between COR and CGR. The novel compound and characteristic chromatography discovered in this study could effectively identify CGR, and provided technical reference and support for the correct medication, market norms and healthy development of COR.

Methods And Materials

Chemicals and solvents

Analytical grade methanol, chloroform, ethyl acetate, absolute ethanol (99.5% w/w), phosphoric acid were purchased from Chengdu Chron Chemicals Co., Ltd. (Chengdu, China). HPLC-grade methanol, acetonitrile were purchased from Thermo Fisher Scientific (Shanghai, China). Curculigoside (> 99%) were obtained from National Institutes for Food and Drug Control (Chengdu, China). Ultra-pure water was purified by Milli-Q Reference (Millipore, Beijing, China). For column chromatography (CC) silica gel (200–300 mesh) were purchased from Qingdao Marine Chemical Co., Ltd. (Qingdao, China) and Sephadex LH-20 were purchased from Ge Healthcare Bio Sciences AB (Uppsala, Sweden)

Plant materials

Six dried samples were collected from Sichuan Institute for Food and Drug Control (Chengdu, China) (Fig. 1). The collected samples were authenticated by comparing their macroscopic and microscopic

characteristics with the descriptions mentioned in the PPRC. Three samples were authenticated as COR, whereas three samples were authenticated as CGR by Prof. Minru Jia (Chengdu University of Traditional Chinese Medicine). Voucher specimens were kept at the Sichuan Institute for Food and Drug Control.

HPLC analysis

1.0 g COR and CGR powder was accurately weighed respectively, accurately added 50 mL of methanol, weighed it, heated to reflux for 2 h. The mixture was cooled down to room temperature and weighed it again, methanol were added to make up the lost weight, shook and filtered through filter paper. Took 20 mL of the filtrate and evaporated to dryness, dissolved the residue with methanol, transferred to a 10 mL volumetric flask, then added methanol to the mark to obtain the sample solution. The solution was filtered through a 0.22 μm membrane filter before injection into the HPLC system.

The sample solution of COR and CGR were analyzed by Agilent 1260 High Performance Liquid Chromatography (Agilent Technologies, CA, USA) equipped with a Zorbax Eclipse Plus C18 analytical column (4.6 mm × 150 mm, 5 μm) and a guard column. The temperature was set at 30 ℃, the injection volume was 10 μL, and the detection wavelength was set to 285 nm. A binary elution at a flow rate of 1.0 mL/min was employed using an aqueous phase of 0.1% formic acid as solvent A and acetonitrile as solvent B, the gradient elution procedure was A:B = 21:79, detection time was 18 minutes.

Extraction and isolation

The air-dried, powdered CGR (5.0 Kg) were extracted with 100 L 70% EtOH under reflux conditions for 3 h, three times to give a crude extract. The extract was suspended in H_2O (2.0 L) and extracted with petroleum ether (PE, 60 ~ 90℃, 4 × 2.0 L), ethyl acetate(AcOEt, 4 × 2.0 L) and n-butanol (n-BuOH, 4 × 2.0 L) successively, to yield a PE soluble fraction (23.6 g), an AcOEt soluble fraction (59.6 g) and a n-BuOH soluble fraction (104.6 g). The part of AcOEt soluble (55.6 g) was subjected to silica gel CC eluted with CH_2Cl_2-MeOH (50:1–1:1), to yield 13 fractions (1–13). Fractions 6 were further subjected on silica gel CC using a stepwise gradient elution of CH_2Cl_2-AcOEt (50:1–1:1) to afford nine subfractions (F1-F9). F4 was submitted to Sephadex LH-20 CC developed with PE-CHC_{l3}- MeOH (5:5:1) to give portions F4.2, which was further purified by preparative Reversed-phase HPLC (LC-20, Shimadzu, Kyoto, Japan) (MeOH-H_2O, 30:70, v/v, wavelength 210 nm) to obtain compound 1(32.1 mg). Deuterated DMSO was used to dissolve compound 1, and its structure was identified by ^{1}H NMR, ^{13}C NMR and 2D NMR (Bruker Ascend 600 NMR spectrometer, Bruker Corporation, Switzerland).

Results

HPLC characteristic peak

The chemical feature can describe and evaluate the medicine materials as a whole. The HPLC method has good precision, sensitivity and reproducibility, it can quickly and specifically identify different herbs

based on the overall chemical composition. The HPLC chromatograms of the COR and CGR are illustrated in Fig. 2. The identities of the peaks were confirmed with the retention time and ultra-violet spectra (285 nm) of the chemical markers. The main chemical composition of COR and CGR were similar. The overall peak area of CGR were significantly lower than that of COR. As anticipated, curculigoside (peak 1),the indicator component in COR was significantly higher than that in CGR, too. Notably, CGR has a unique compound (peak 2) through the HPLC chromatograms, which was not found in COR. Therefore, this unique compound was specifically separated, purified and the structure was identified by modern spectroscopy techniques.

Structure elucidation of new compound

As shown in Table 1, ^{1}H NMR spectrum of compound 1 exhibited signals for seven aromatic protons, and among them a para-substituted benzene ring proton protons at δ_H 7.83 (2H, d, J = 8.8 Hz,H-2", 6") and 6.83 (2H, d, J = 8.7 Hz, H-3", 5"), that was the compound had two aromatic rings; 3 methylene protons at δ_H 3.59(2H, s, H-5), 3.09 (2H, t, J = 6.6, 6.0 Hz, H-2) and 2.75 (2H, t, J = 6.6, 6.1 Hz, H-3). The ^{13}C NMR spectrum showed 17 carbon signals. In addition to the carbon signals corresponding to the ^{1}H NMR, the signals in the ^{13}C NMR spectrum ascribed to two ketone carbonyl carbon at δ_C 207.6(C-4) and 196.6(C-1), three oxygen-linked aromatic carbons at δ_C 162.1(C-4"), 144.0(C-3'), 145.1(C-4'), together with three methylene carbons at δ_C 48.5 (C-5), 35.8 (C-3) and 31.6 (C-2) (Table 1). Compared with the ^{1}H and ^{13}C NMR spectrum data of compound breviscapin C [18], compound 1 was supposed to be the same type.

Table 1

The ^{1}H and ^{13}C NMR data for compound 1 (DMSO-d_6, ^{1}H NMR 600 MHz, ^{13}C NMR 150 MHz, δ in ppm, J in Hz)

NO.	δ (H)	δ (C)
1		196.6
2	3.09 (2H, t, J = 6.6, 6.0 Hz)	31.6
3	2.75 (2H, t, J = 6.6, 6.1 Hz)	35.8
4		207.6
5	3.59 (2H, s)	48.5
1'		125.6
2'	6.60 (1H, d, J = 2.1 Hz)	116.9
3'		145.1
4'		144.0
5'	6.67 (1H, d, J = 8.0 Hz)	115.5
6'	6.46 (1H, dd, J = 8.0, 8.0 Hz)	120.3
1"		128.1
2", 6"	7.83 (2H, d, J = 8.8 Hz)	130.4
3", 5"	6.83 (2H, d, J = 8.7 Hz)	115.2
4"		162.1

In order to further confirm the structure of compound 1, a 2D NMR experiment was performed (Fig. 4). The chemical shift assignments of the carbon atoms were established from direct ^{1}H-^{13}C correlations in the HMQC spectrum. The ^{1}H-^{1}H COSY correlation of H-2/H-3, H-5'/H-6', H-2"/H-3", H-5"/H-6" had also been confirmed. Long range ^{1}H-^{13}C correlations in HMBC spectrum (Fig. 4) were observed between protons at δ_H 3.09 (H-2), 2.75 (H-3) and δ_C 196.6 (C-1), 207.6 (C-4), which confirmed that C-2 and C-3 were connected to the ketone carbonyl carbon respectively; one protons at δ_H 3.59 (H-5) correlated with one carbon at δ_C 207.6 (C-4), indicating ketone carbonyl carbon with C-5; one aromatic proton at δ_H 6.60 (H-2') correlated with one carbon at δ_C 48.5(C-5), one aromatic proton at δ_H 6.67 (H-5') correlated with two carbons at δ_C 125.6 (C-1') and 145.1 (C-3'), one aromatic proton at δ_H 6.46 (H-6') correlated with three carbons at δ_C 48.5 (C-5), 116.9 (C-2') and 144.0 (C-4'), one aromatic proton at δ_H 7.83 (H-2") correlated with three carbons at δ_C 196.6 (C-1), 162.1 (C-4") and 130.4 (C-6"), one aromatic proton at δ_H 6.83 (H-5") correlated with three carbons at δ_C 128.1 (C-1"), 115.2 (C-3") and 162.1 (C-4"), another aromatic proton at δ_H 7.83 H-6" correlated with one carbon at δ_C 196.6 (C-1) (Fig. 4). From the above mentioned evidence,

compound 1 was determined to be 5-(3',4'-dihydroxyphenyl)-1-(4"-hydroxyphenyl) pentane-1,4-dione and named glabrescenin (Fig. 3).

Discussion

Correct species identification is crucial for ensuing the quality, safety and efficacy of a medicinal herb. The substitution and wrong identification occurs in clinical practice, when the medicinal herbs have similar morphological characteristics or names [19]. In the market, CGR is the major counterfeit of COR. Nevertheless, it is difficult to identify COR and CGR accurately. *C.* orchioides Gaertn and *C.* glabrescens (Ridl.) Merr. belong to the same family and genus, with close plant relationships and high genetic similarity, and the appearance of the CGR is similar to the COR. In our study, based on the HPLC characteristic chromatograms of COR and CGR, the new neolignans compound, glabrescenin, was separated and purified from CGR for the first time. It could specifically identify COR and CGR, providing a simple and friendly idea for the identification and quality control of *Curculigo* herbs, which is conducive to the market standard of COR. Through the analysis, we suggested that PPRC could add this check item and stipulate glabrescenin shall not be detected in COR, so as to promote the correct application of genuine COR in clinical practice. However, there is no systematic study on the chemical composition of CGR, and there are some controversies in the botanical classification of *Curculigo*, warranting further investigation.

Conclusion

The novel compound and characteristic chromatography discovered in this study could effectively identify CGR, and provided technical reference and support for the correct medication, market norms and healthy development of COR.

Abbreviations

COR *Curculigo orchioides* Rhizoma; CGR:*Curculigo glabrescens* Rhizoma; HPLC:High performance liquid chromatography; NMR:Nuclear magnetic resonance; HMBC:Heteronuclear multiple bond correlation; COSY:Correlation spectroscopy.

Declarations

Acknowledgement

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Authors' contributions

YSL, YPG, and LG conceived and designed the study. YSL and YPG wrote the main manuscript text. JP and LG supervised the study. YSL, JRL collected and analyzed the data. JP, XHB, JZ, FL, JL and WZ provided the technical support and advices for the study. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Competing interests

All authors declared no competing interests.

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Figures

Figure 1

Curculigo orchioides Rhizoma (COR) (a) and Curculigo glabrescens Rhizoma (CGR) (b)

Figure 1

Curculigo orchioides Rhizoma (COR) (a) and Curculigo glabrescens Rhizoma (CGR) (b)

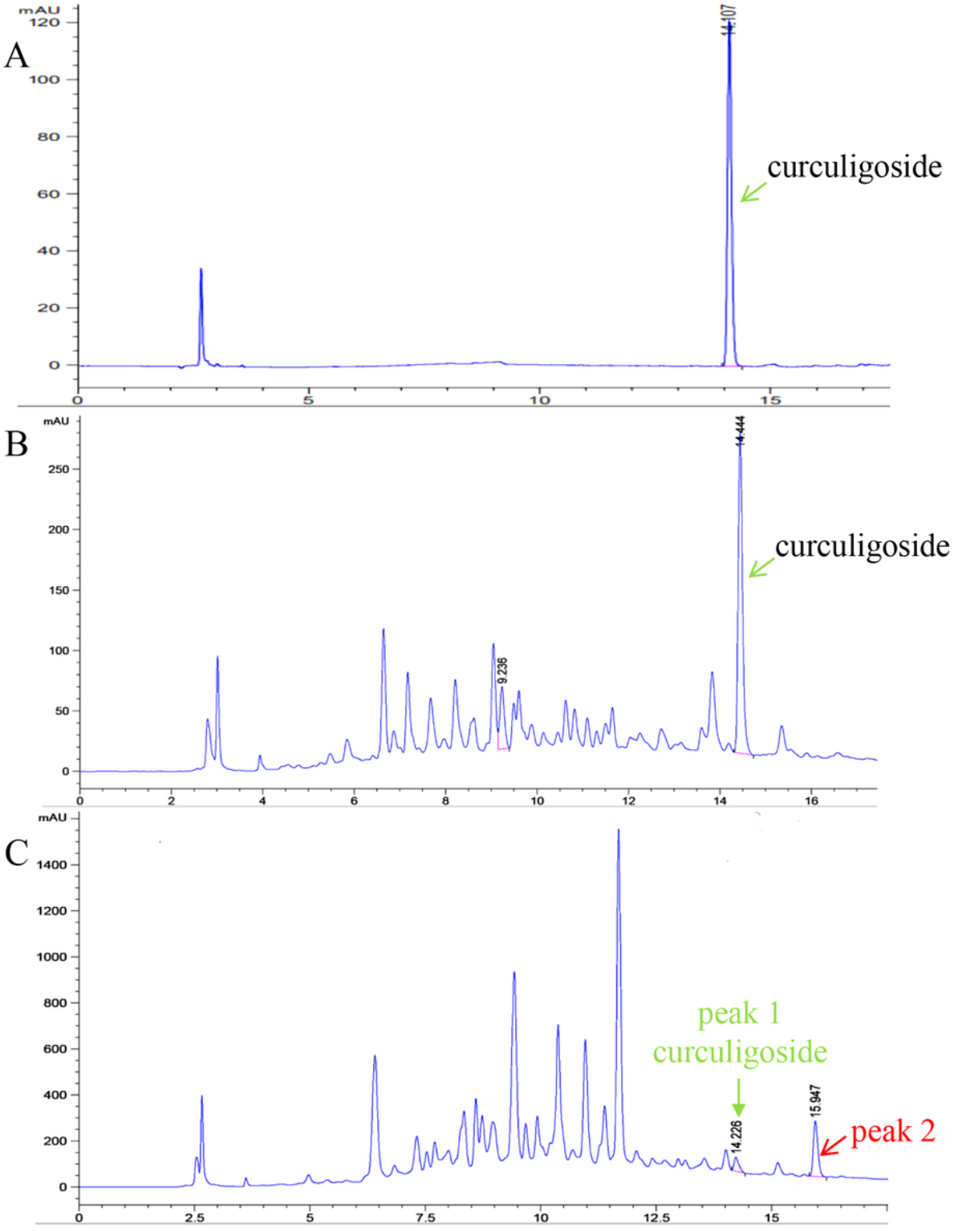
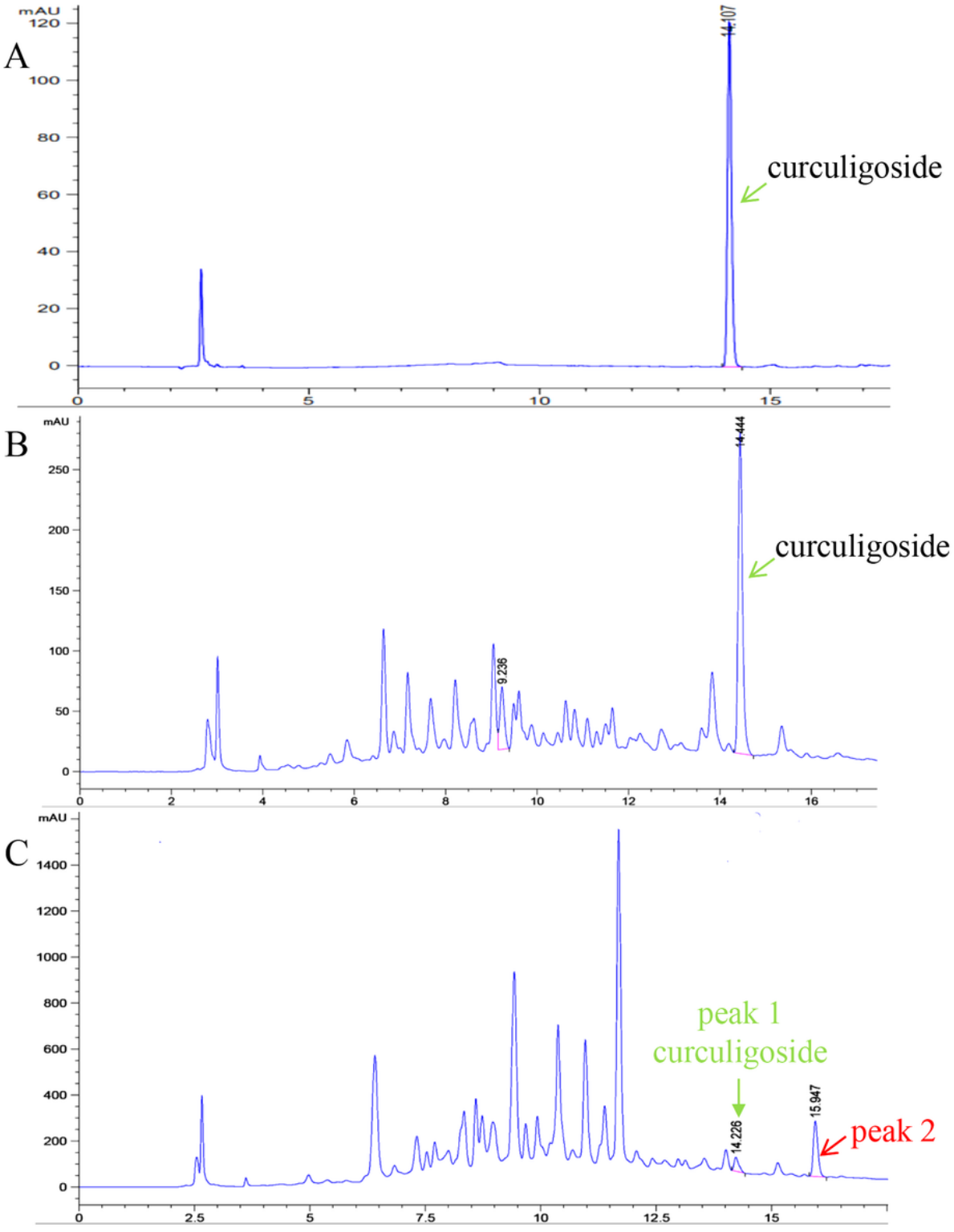


Figure 2

HPLC chromatogram of curculigoside (a), COR (b) and CGR (c) samples. Peak 1: curculigoside. Peak 2: the characteristic compound



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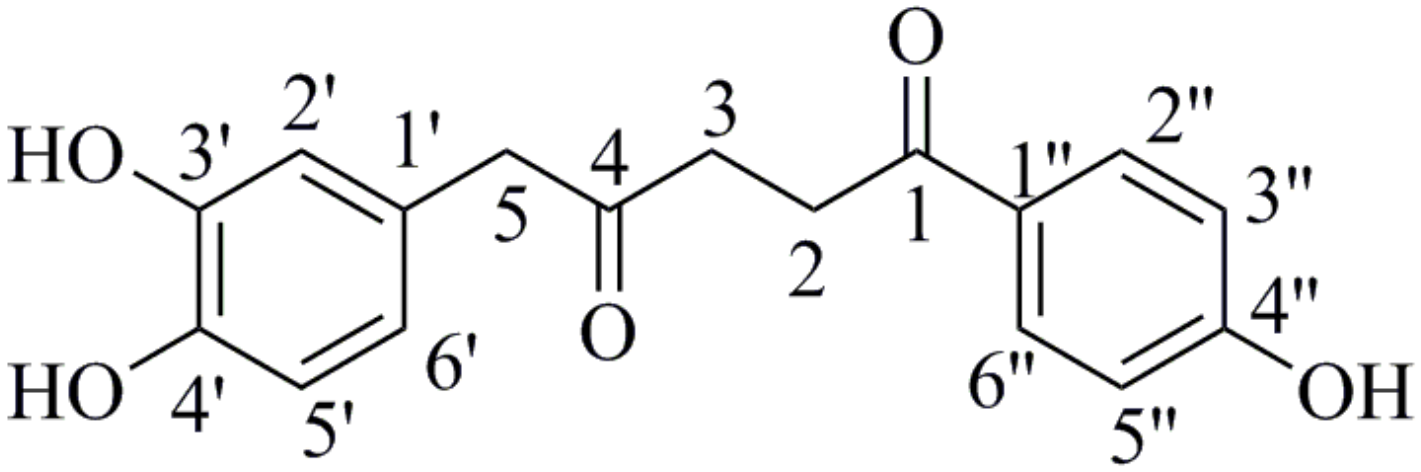
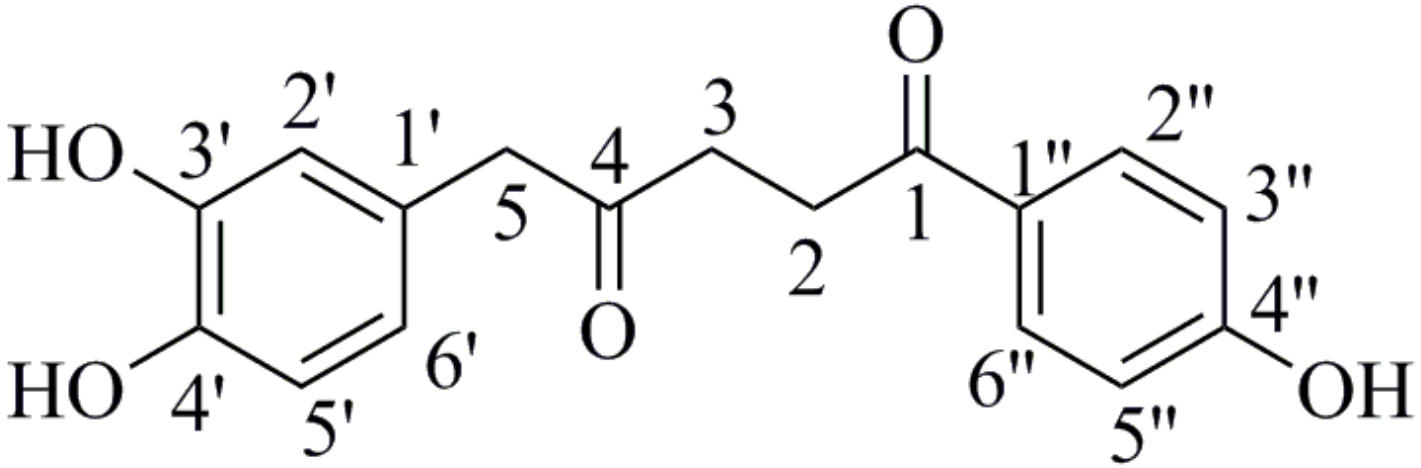


Figure 3

Chemical structure of compound 1



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Chemical structure of compound 1

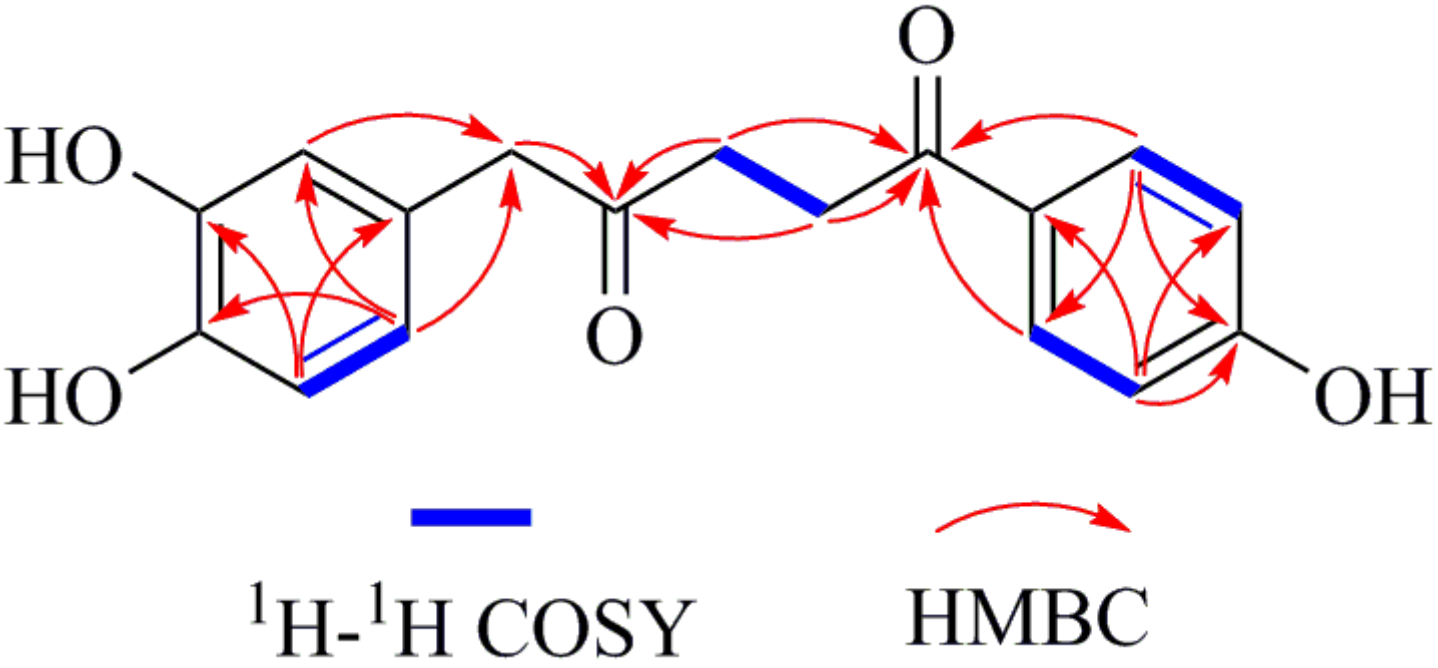

Figure 4

1H-1H COSY and key HMBC correlations of compound 1

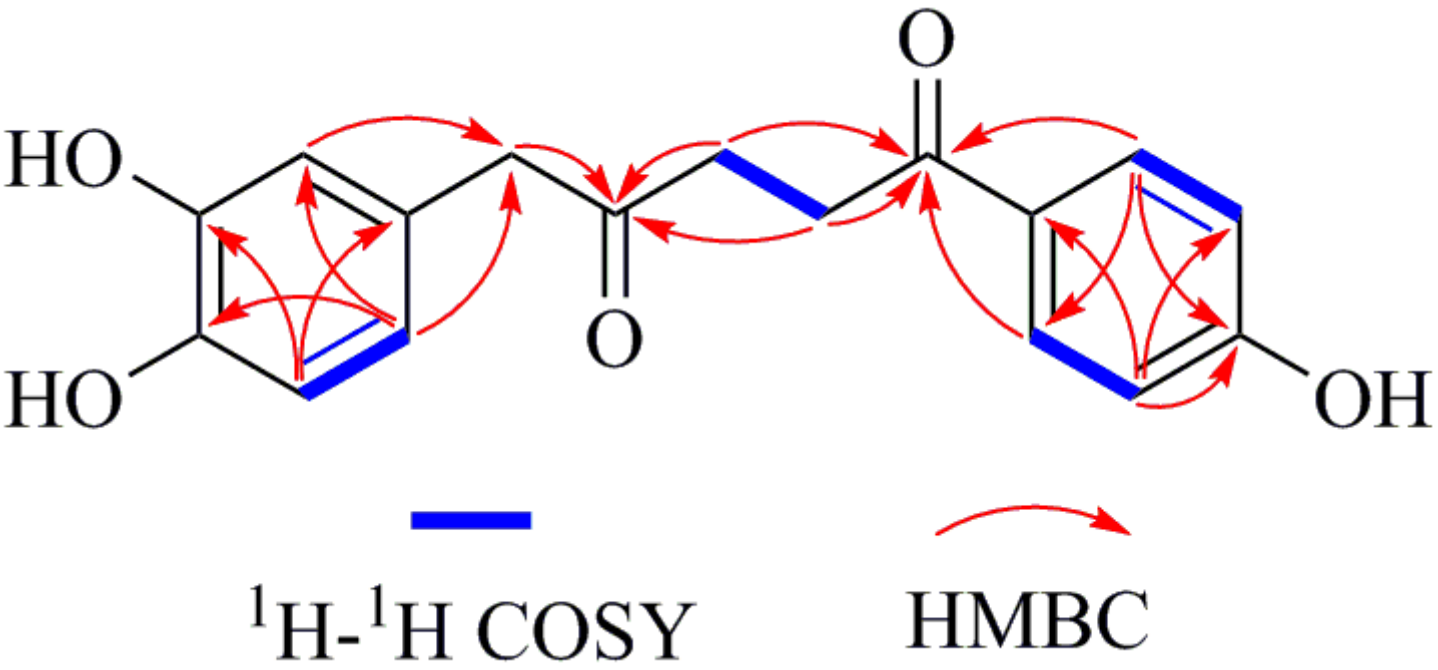

Figure 4

1H-1H COSY and key HMBC correlations of compound 1

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