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Characterization and determination of the major constituents in Belamcandae Rhizoma by HPLC–DAD–ESI-MS^n

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ABSTRACT

Belamcandae Rhizoma, derived from the rhizome of *Belamcanda chinensis* (L.) DC., has been used as traditional Chinese medicine for the treatment of coughing and pharyngitis. However, there have been few studies dealing with the systematic analysis of the bioactive constituents in Belamcandae Rhizoma. In this work, high performance liquid chromatography–diode array detection–electrospray ionization multiple-stage mass spectrometry (HPLC-DAD-ESI-MS^n) combined with liquid chromatography–time of flight-mass spectrometry (HPLC–TOF/MS) was established for profiling and characterization of multi-constituent in Belamcandae Rhizoma. The ESI-MS^n fragmentation behaviors of the authentic references were proposed for aiding the structural identification of components in the extract. Thirty-five flavonoids, including 30 isoflavones and five xanthones, were identified or tentatively identified by comparing their retention times, UV and MS spectra with those of authentic compounds or literature data. Twelve of the identified compounds (neomangiferin, mangiferin, tectoridin, iristectorin B, iristectorin A, iridin, tectorigenin, iristectorigenin A, irigenin, irisflorentin, irilone and dichtomitin) were determined by HPLC–DAD using a C_{18} column. The results indicated that the developed analysis method could be employed as a rapid, effective technique for structural characterization of chemical constituents in herbal medicine. This work is expected to provide comprehensive information for the quality evaluation of Belamcandae Rhizoma, which would be a valuable reference for the further study and development of this herb and related medicinal products.

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1. Introduction

Belamcanda chinensis (L.) DC. (*Iridaceae*) is a perennial herbaceous plant widely distributed in China, Korea, Japan, India and eastern Russia. Belamcandae Rhizoma (Shegan in Chinese), derived from the rhizome of *Belamcanda chinensis* (L.) DC., has been used as traditional Chinese medicine for the treatment of coughing and pharyngitis [1]. Phytochemical and pharmacological studies demonstrated that isoflavones were the major bioactive constituents of Belamcandae Rhizoma, e.g. tectoridin, iridin, tectorigenin, irigenin and irisflorentin [2,3]. Isoflavones have shown pharmacological actions of estrogenic and antioxidative effects, and possess high potential to prevent the diseases such as cancer, atherosclerosis, osteoporosis, hyperlipidemia and cardiovascular diseases [4,5].

Quality control analysis of the active components is important for the safe and effective use of herbal medicines. However, this goal remains challenging because of the diversity and complexity of the chemical compounds present in these herbal matrices. Utilizing an HPLC–APCI–MS^n method to evaluate the quality of Belamcandae Rhizoma through establishing chromatographic fingerprint and simultaneous determination of seven phenolic compounds has been reported recently [6]. However, there have been few studies dealing with the systematic analysis of isoflavones in Belamcandae Rhizoma. And the ESI-MS^n fragment pathways of these isoflavones have not been investigated thoroughly for the lack of appropriate authentic standards.

The application of hyphenated techniques such as GC/MS, CE/MS, LC/MS and MS/MS (MS^n) to on-line structural characterization has played more and more significant role in the analysis of herbal medicine. High performance liquid chromatography coupled with DAD and electrospray ionization tandem mass spectrometry (HPLC–DAD–ESI-MS^n) is a powerful analytical tool for the analysis of the known compounds and elucidation of unknown compounds in herbal extracts. HPLC–DAD–ESI-MS^n presents high sensitivity and can provide abundant information

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on the molecular mass and structural features [7–9]. High performance liquid chromatography/time-of-flight mass spectrometry (HLPC–TOF/MS) possesses the benefits of increased resolution, accurate mass measurement and high full-scan capability. The combination of LC–ESI-MS^n and LC–TOF/MS, giving complementary information for structure confirmation, has been a novel and powerful tool for the analysis of the secondary metabolites profiles of herbal medicines.

In our study, an HPLC–DAD–ESI-MS^n was developed to identify the major constituents in Belamcandae Rhizoma. TOF/MS was applied together to further confirm the structure characterizations. As a result, a total of 35 flavonoids, including 30 isoflavones and five xanthones, were identified or tentatively characterized. In addition, quantification of 12 bioactive components in Belamcandae Rhizoma was performed with HPLC–DAD. This study provides full-scale qualitative and quantitative information for the quality evaluation of Belamcandae Rhizoma, which would be a valuable reference for the further study and development of this herb and its related medicinal products.

2. Experiment

2.1. Reagents and materials

HPLC grade acetonitrile was supplied by Tedia (Fairfield, OH, USA), and water was purified by a Milli-Q purification system (Bedford, MA, USA). Methanol used for sample preparation was of HPLC grade (Jiangsu Hanbon Sci.&Tech. Co. Ltd., Jiangsu, China). Analytical grade acetic acid was purchased from Nanjing Chemical Factory (Nanjing, China).

The rhizomes of *Belamcanda chinensis* were collected in Nanjing, China, in April 2008 and were authenticated by Professor Minjian Qin. The voucher specimen (BC-08-0420) was deposited at the Herbarium of Medicinal Plants of China Pharmaceutical University.

Twelve reference substances including neomangiferin (**F1**), mangiferin (**F2**), tectoridin (**F3**), iristectorin B (**F4**), iristectorin A (**F5**), iridin (**F6**), tectorigenin (**F7**), iristectorigenin A (**F8**), irigenin (**F9**), irisflorentin (**F10**), irilone (**F11**) and dichtomitin (**F12**) were isolated and purified from the rhizome of *Belamcanda chinensis* (L.) DC. in our laboratory [10–12]. Their structures were identified by spectroscopic methods (UV, IR, MS, ^{1}H NMR and ^{13}C NMR) and shown in Fig. 1. The purity of each compound was determined to be over 98% by HPLC. The samples of the herb and chemicals for analysis were stored in the refrigerator at −20 °C.

2.2. Standard solutions and sample preparation

Each accurately weighed standard was dissolved and diluted with methanol to provide a series of standard solutions. The solutions were stored at 4 °C.

A challenge in analyzing herbs is the complexity of the sample matrices. Efficient sample preparation can improve extraction and enrich the target analytes. Ultrasound-assisted extraction, as a simple, fast and inexpensive extraction method, has been widely utilized in extracting flavonoids in herbal medicines [13,14]. In this study, the dry powder (0.1 g) of Belamcandae Rhizoma was sonicated with 10 ml methanol for 30 min. The extracted solution was then centrifuged at 2500 rpm at room temperature for 10 min. The supernatant was filtered through a 0.45 μm membrane filter prior to HPLC analysis.

2.3. HPLC–DAD–ESI/MS analysis

The HPLC system consisted of an Agilent 1100 series HPLC with diode array detector equipped with quaternary pump, vacuum degasser, autosampler, column heater–cooler (Agilent Corporation, MA, USA). The chromatographic separation was carried out on an Agilent Zorbax SB-C_{18} column (250 mm × 4.6 mm, 5 μm) with an Alltech Associates C_{18} guard column (7.5 mm × 4.6 mm, 5 μm). The column temperature was maintained at 25 °C. The mobile phase consisted of water–acetic acid (100:0.4, v/v) (A) and acetonitrile (B). The gradient elution program was as follows: 8–17% (B) in 0–15 min, 17–20% (B) in 15–35 min, 20% (B) in 35–40 min, 20–26% (B) in 40–43 min, 26–40% (B) in 43–65 min, and 40–50% (B) in 65–75 min. The flow rate was 1.0 ml/min and the sample volume injected was 10 μl. The detection wavelength was set at 269 nm for all the tested compounds. By solvent splitting, 0.2 ml/min portion of the column effluent was delivered into the ion source of the mass spectrometer.

The above HPLC system was interfaced with an Agilent 1100 Series LC/MSD Trap SL (Agilent, Santa Clara, CA, USA). The same chromatographic conditions were used during the HPLC–ESI-MS^n analysis. The optimized mass spectrometry operating conditions were as follows: negative and positive ionization mode, scan spectra from m/z 100 to 1000, nitrogen flow rate of 10 l/min, drying gas temperature of 350 °C, nebulizer pressure 50 psi, capillary voltage 3000 V. All the data analysis was applied by Agilent 1100 series MSD Trap software 4.2 (Agilent Technologies, USA).

TOF mass spectrometer (Agilent Corp., Santa Clara, CA, USA) was applied for the accurate mass determination. The TOF/MS analysis was performed in positive ion mode using full scan mode and the mass range was set at 100–1000 Da. The conditions of the ESI source were: drying gas (N_2) flow rate, 10.0 l/min; drying gas temperature, 330 °C; nebulizer, 35 psig; the applied fragmentors, 120 V; capillary, 3000 V; skimmer, 60 V; OCT RF V, 250 V. All the acquisition and data analysis were processed by Agilent LC–MS TOF software version A.01.00 and Applied Biosystems/MDS-SCIEX Analyst QS software (Frankfurt, Germany) with accurate mass application-specific additions from Agilent MSD TOF software.

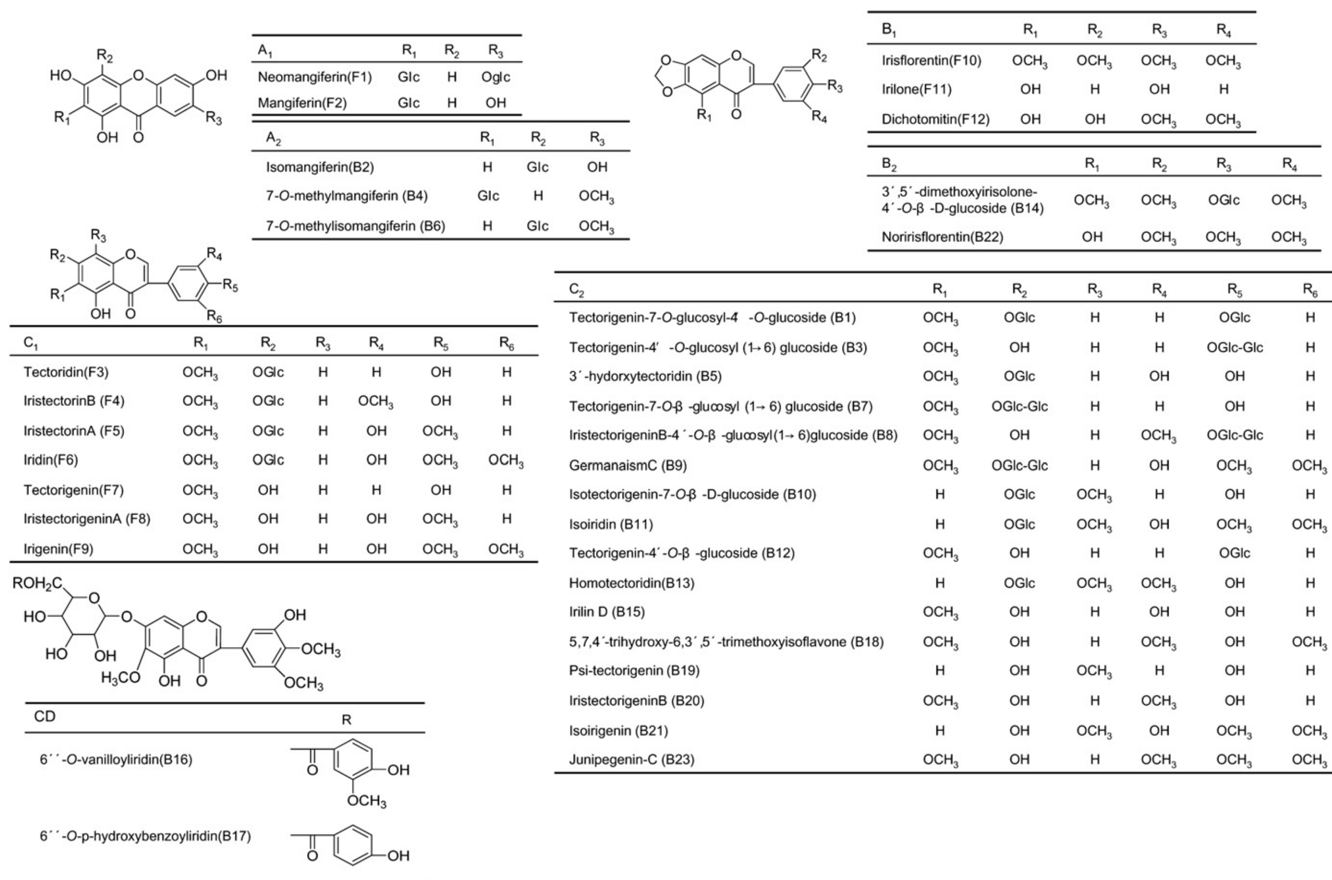
2.4. Identification of flavonoid compounds

Identification of constituents in Belamcandae Rhizoma extract was carried out by HPLC–DAD–MS^n analysis, and further confirmed by TOF/MS. When an authentic reference was available, the compound was identified by comparing its retention time, the UV and MS spectra with those of the standards. Otherwise, the structures of compounds were proposed mainly based on the mass spectra. The obtained structural information and proposed fragmentation patterns were helpful for the structural identification of constituents.

2.5. Validation of quantitative analysis

The mixed standard stock solution containing 12 reference compounds was prepared and diluted to a series of appropriate concentrations for the construction of calibration curves. The calibration curve of individual constituents was performed with at least six appropriate concentrations. The limits of detection (LOD) and quantification (LOQ) were determined at a signal-to-noise (S/N) ratio of 3 and 10, respectively.

Intra-day variations and inter-day variations were chosen to determine the precision of the developed method. The intra-day variability was performed in triplicate on the same sample extracted within 1 day, while the inter-day precision was carried out in triplicate in another independent sample extracted for consecutive 3 days. The recovery was utilized to evaluate the accuracy of this method. Suitable amounts of 12 standards (about 50% of the content) were added to Belamcandae Rhizoma sample and then processed and analyzed as described in Section 2.2. An unspiked sample was prepared and analyzed simultaneously for comparison. Three replicates were performed for the recovery test.



A_1	R_1	R_2	R_3
Neomangiferin(F1)	Glc	H	Oglc
Mangiferin(F2)	Glc	H	OH

A_2	R_1	R_2	R_3
Isomangiferin(B2)	H	Glc	OH
7-*O*-methylmangiferin (B4)	Glc	H	OCH_3
7-*O*-methylisomangiferin (B6)	H	Glc	OCH_3

B_1	R_1	R_2	R_3	R_4
Irisflorentin(F10)	OCH_3	OCH_3	OCH_3	OCH_3
Irilone(F11)	OH	H	OH	H
Dichotomitin(F12)	OH	OH	OCH_3	OCH_3

B_2	R_1	R_2	R_3	R_4
3′,5′-dimethoxyirisolone-4′-*O*-β -D-glucoside (B14)	OCH_3	OCH_3	OGlc	OCH_3
Noririsflorentin(B22)	OH	OCH_3	OCH_3	OCH_3

C_1	R_1	R_2	R_3	R_4	R_5	R_6
Tectoridin(F3)	OCH_3	OGlc	H	H	OH	H
IristectorinB (F4)	OCH_3	OGlc	H	OCH_3	OH	H
IristectorinA (F5)	OCH_3	OGlc	H	OH	OCH_3	H
Iridin(F6)	OCH_3	OGlc	H	OH	OCH_3	OCH_3
Tectorigenin(F7)	OCH_3	OH	H	H	OH	H
IristectorigeninA (F8)	OCH_3	OH	H	OH	OCH_3	H
Irigenin(F9)	OCH_3	OH	H	OH	OCH_3	OCH_3

C_2	R_1	R_2	R_3	R_4	R_5	R_6
Tectorigenin-7-*O*-glucosyl-4′-*O*-glucoside (B1)	OCH_3	OGlc	H	H	OGlc	H
Tectorigenin-4′-*O*-glucosyl (1→6) glucoside (B3)	OCH_3	OH	H	H	OGlc-Glc	H
3′-hydorxytectoridin (B5)	OCH_3	OGlc	H	OH	OH	H
Tectorigenin-7-*O*-β -glucosyl (1→6) glucoside (B7)	OCH_3	OGlc-Glc	H	H	OH	H
IristectorigeninB-4′-*O*-β -glucosyl(1→6)glucoside (B8)	OCH_3	OH	H	OCH_3	OGlc-Glc	H
GermanaismC (B9)	OCH_3	OGlc-Glc	H	OH	OCH_3	OCH_3
Isotectorigenin-7-*O*-β -D-glucoside (B10)	H	OGlc	OCH_3	H	OH	H
Isoiridin (B11)	H	OGlc	OCH_3	OH	OCH_3	OCH_3
Tectorigenin-4′-*O*-β -glucoside (B12)	OCH_3	OH	H	H	OGlc	H
Homotectoridin(B13)	H	OGlc	OCH_3	OCH_3	OH	H
Irilin D (B15)	OCH_3	OH	H	OH	OH	H
5,7,4′-trihydroxy-6,3′,5′-trimethoxyisoflavone (B18)	OCH_3	OH	H	OCH_3	OH	OCH_3
Psi-tectorigenin (B19)	H	OH	OCH_3	H	OH	H
IristectorigeninB (B20)	OCH_3	OH	H	OCH_3	OH	H
Isoirigenin (B21)	H	OH	OCH_3	OH	OCH_3	OCH_3
Junipegenin-C (B23)	OCH_3	OH	H	OCH_3	OCH_3	OCH_3

CD	R
6′′-*O*-vanilloyliridin(B16)	
6′′-*O*-p-hydroxybenzoyliridin(B17)	

Fig. 1. Chemical structures of compounds identified in the extracts of Belamcandae Rhizoma.

2.6. Nomenclature

The fragments observed in this study are presented in Scheme 1. As shown in Scheme 1a, fragment ions containing intact A and B rings of isoflavone skeleton are designated according to a widely accepted nomenclature system developed by Ma et al. [15]. The $^{i,j}A^-$ and $^{i,j}B^-$ represent fragment ions containing intact A and B rings, the superscripts *i* and *j* indicate the C-ring bonds of isoflavone skeleton that have been broken. For C-glycosidic isoflavones (Scheme 1b), based on the description by Li and Claeys [16], the ions produced by the cleavage of hexose are termed $^{i,j}{}_6X$. The superscripts *i* and *j* indicate the C-bonds of sugar ring that have been broken.

3. Results and discussion

3.1. Optimum conditions for HPLC–DAD–ESI-MS^n analysis

To achieve a satisfactory separation of chemical compounds in Belamcandae Rhizoma, different HPLC parameters including column temperature, detection wavelength and mobile phases were examined. It was suggested that a column temperature of 25 °C and detection wavelength of 269 nm provided good separation and strong UV absorption for the constituents. Furthermore, the mobile phase was optimized through comparisons of different solvents, solvent ratio and gradient profile. Compared with other solvent systems, acetonitrile–water system presented more powerful separation ability for investigated compounds. By adding acetic acid in mobile phase, the chromatographic separations were greatly improved with sharper peak and better peak symmetry. And the separation was gradually improved with the increasing concentration of acetic acid. As a result, a solvent system consisting of water–acetic acid (100:0.4, v/v) (A) and acetonitrile (B) was ultimately selected as mobile phase system. The HPLC profiles of a mixed standard solution and Belamcandae Rhizoma extract are shown in Fig. 2a and b.

The MS analysis in positive and negative ion modes were also studied and compared. The results showed that the positive ion mode was more selective and sensitive for methylenedioxy isoflavones, while the negative ion mode was preferred for hydroxylated isoflavones, as was in agreement with the literatures [17]. Moreover, the ion signals of some components investigated were only observed in positive or negative ion mode. Therefore, to obtain complimentary information for the structural analysis of isoflavones by mass spectrometry, the combined application of positive and negative ion modes appeared to be necessary.

3.2. Tandem mass spectrometry of authentic standards

The dominant fragmentation pathways of 12 authentic standards were studied. Most of the authentic compounds, exhibiting $[M-H]^-$ and/or $[M+H]^+$ ions of sufficient abundance, were subjected to MS^n analysis. The obtained MS^n data (Table 1), were applied for the structural identification of compounds with similar fragmentation patterns.

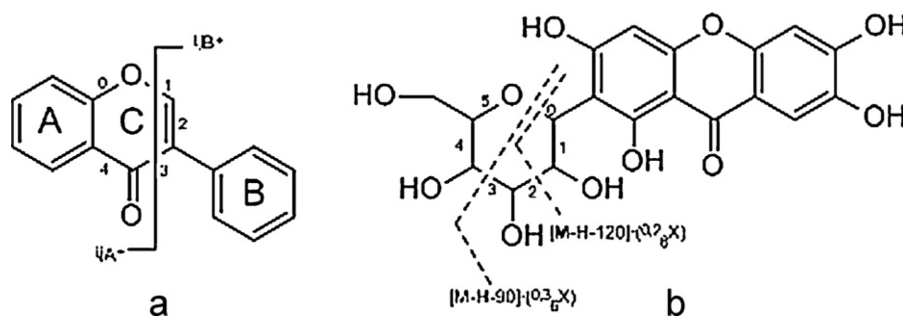
The ESI-MS^n data of isoflavone glycosides (**F3–6**) displayed some common features, such as the neutral losses of CH_3 (15 Da), CO (28 Da), and simultaneous or successive loss of CH_3 and CO (43 Da). CH_3 radical elimination explained the presence of a methoxy group, and CO loss is due to the contraction of C ring [18]. In MS^2 experiment, the loss of a glucose residue from pseudomolecular ion was the predominant fragmentation, which was demonstrated as characteristic ions of O-glycosides. The diagnostic Retro–Diels–Alder

Table 1

MS^n product ions and fragmentations obtained from authentic compounds in this study.

No.	$[M+H]^+$ (m/z)	$[M-H]^-$ (m/z)	Fragment ions (m/z)		Fragment ions (m/z)		Identification
			Common fragment pathways	Other fragment ions (m/z)	Common fragment pathways	Other fragment ions (m/z)	
Xanthones							
F1	585	583	$[M+H-H_2O]^+$ $[M+H-C_3H_6O_3]^+$ $[M+H-C_4H_8O_4]^+$ $[M+H-C_4H_8O_4-CO]^+$ $[M+H-C_3H_6O_3-CO]^+$	$[M+Na-C_4H_8O_4]^+/487$ $[M+H-C_6H_{10}O_5]^+/423$ $[M+H-C_3H_6O_3\times 2]^+/405$ $[M+H-C_4H_8O_4\times 2]^+/345$	$[M-H-H_2O]^-$ $[M-H-C_3H_6O_3]^-$ $[M-H-C_4H_8O_4]^-$	$[M-H-C_3H_6O_3-CH_3]^-/478$ $[M-H-C_4H_8O_4-CH_3]^-/448$ $[M-H-C_3H_6O_3-CH_3\times 2]^-/463$ $[M-H-C_4H_8O_4-CH_3\times 2]^-/433$ $[M-H-C_6H_{10}O_5]^-/421$ $[M-H-C_3H_6O_3-C_4H_8O_4]^-/373$	Neomangiferin
F2	423	421		$[M+H-C_4H_8O_4-CO-CH_3]^+/260$		$[M-H-C_3H_6O_3-CO]^-/303$ $[M-H-C_4H_8O_4-CO]^-/273$ $[M-H-C_4H_8O_4-CH_3-CO]^-/258$	Mangiferin
Isoflavone glycosides							
F3	463	461	$[M+H-CO]^+$ $[M+H-CH_3]^+$	$[M+H-C_6H_{10}O_5-CH_3]^+/286$ $[M+H-C_6H_{10}O_5-CH_3-CO]^+/258$	$[M-H-CO]^-$ $[M-H-CH_3]^-$	$[M-H-C_6H_{10}O_5-CH_3]^-/284$ $[M-H-C_6H_{10}O_5-CH_3-CO]^-/256$	Tectoridin
F4	493	491	$[M+H-CH_3-CO]^+$ $[M+H-C_6H_{10}O_5]^{+1,3}A^+$	$[M+H-C_6H_{10}O_5-CH_3]^+/316$ $[M+H-C_6H_{10}O_5-CH_3-CO]^+/288$	$[M-H-CH_3-CO]^-$ $[M-H-C_6H_{10}O_5]^-$	$[M-H-C_6H_{10}O_5-CH_3]^-/314$ $[M-H-C_6H_{10}O_5-CH_3-CO]^-/286$	Iristectorin B
F5	493	491		$[M+H-C_6H_{10}O_5-CH_3]^+/346$ $[M+H-C_6H_{10}O_5-CH_3-CO]^+/318$		$[M-H-C_6H_{10}O_5-CH_3]^-/344$ $[M-H-C_6H_{10}O_5-CH_3-CO]^-/316$	Iristectorin A
F6	523	521		$[M+H-C_6H_{10}O_5-CH_3]^+/346$ $[M+H-C_6H_{10}O_5-CH_3-CO]^+/318$		$[M-H-C_6H_{10}O_5-CH_3]^-/344$ $[M-H-C_6H_{10}O_5-CH_3-CO]^-/316$	Iridin
Isoflavone aglycones							
F7	301	299	$[M+H-CO]^+$ $[M+H-CH_3]^+$	–	$[M-H-CO]^-$ $[M-H-CH_3]^-$	ND[a]	Tectorigenin
F8	331	329	$[M+H-CH_3-CO]^{+1,3}A^+$	$[M+H-CH_3\times 2]^+/301$ $[M+H-CH_3\times 2-CO]^+/273$	$[M-H-CH_3-CO]^-$	$[M-H-CH_3\times 2]^-/299$ $[M-H-CH_3\times 2-CO]^-/271$	Iristectorigenin A
F9	361	359		$[M+H-CH_3\times 2]^+/331$ $[M+H-CH_3\times 2-CO]^+/303$		$[M-H-CH_3\times 2]^-/329$ $[M-H-CH_3\times 2-CO]^-/301$	Irigenin
Isoflavones aglycones with methylenedioxy group							
F10	387	ND[a]	$[M+H-CO]^+$ $[M+H-CH_3]^+$	–	ND[a]		Irisflorentin
F11	299	297	$[M+H-CH_3\times 2]^+$ $[M+H-CH_3-CO]^+$	$[M+H-H_2O]^+/281$	$[M-H-CO]^-/269$		Irilone
F12	359	357	$[M+H-CH_3\times 2-CO]^{+1,3}A^+$	$[M+H-CH_3-H_2O]^+/326$ $[M+H-CH_3-H_2O-CO]^+/298$ $[M+H-CH_3-H_2O\times 2-CO]^+/270$	$[M-H-CH_3]^-/342[M-H-CH_3\times 2]^-/327[M-H-CH_3\times 2-CO]^-/299$		Dichotomitin

[a] The ion signals of this component investigated were not detected in this ion mode.



Scheme 1. Nomenclature adopted for various retrocyclization fragments observed in this study.

(RDA) ions were only observed in positive ion mode, which were very helpful for the structural determination of the A- and B-ring substitution patterns. The MS^3 spectra of $[M-H-162]^-$ ion was very simple (Table 1), and exhibited a significant ion resulting from a methyl radical loss as base peak. The fragmentation pathways of iristectorin B (**F4**) in positive ion mode are proposed in Scheme 2a. Similar to the isoflavone glycosides, fragment ions derived from RDA fragment and loss of CH_3 and/or CO were abundant and characteristic for isoflavone aglycones (**F7–9**). Compounds **F10–12** were isoflavone aglycones with methylenedioxy group. As shown in Table 1, all these compounds generated $[M+H-CO]^+$, $[M+H-CH_3]^+$, $[M+H-CH_3 \times 2]^+$, $[M+H-CH_3-CO]^+$ and $[M+H-CH_3 \times 2\text{-}CO]^+$ ions. Besides, the RDA fragmentations involving the 1 and 3 bonds ($^{1,3}A^+$) were also predominant.

C-glycosidic xanthones neomangiferin (**F1**) and mangiferin (**F2**) were also analyzed by ESI-MS^n. In the negative ion mode full mass spectrum, the C-glycosidic xanthones only showed the $[M-H]^-$ ions. In ESI-MS^2 spectra, xanthones gave different fragmentation patterns from those of the O-glucosides. For example, ions of $^{0,3}{}_6X^-$ ($[M-H-90]^-$) at m/z 331 and $^{0,2}{}_6X^-$ ($[M\text{-}H\text{-}120]^-$) at m/z 301, resulting from cross-ring cleavages in sugar moiety, were highly abundant in the mass spectra of **F2** (Fig. 3). $[M-H-90]^-$ and $[M-H-120]^-$ were demonstrated as characteristic ions of C-flavonoids [19]. But the characteristic ion $[M-H-162]^-$ of O-glycosides were not observed for C-glycosidic xanthones. These results would be very helpful for differencing C-glycosides and O-glycosides. In MS^3 analysis of the $^{0,2}{}_6X^-$ ion of **F2**, two ions attributing to the elimination of CO and successive loss of CH_3 were also produced. The fragmentation pattern of C-glycosidic xanthone **F2** is proposed and presented in Scheme 2b.

3.3. Identification of constituents in Belamcandae Rhizoma by HPLC–DAD–ESI-MS^n

The HPLC–DAD chromatogram and HPLC–MS total ion chromatograms of Belamcandae Rhizoma extract were shown in Fig. 2. A total of 35 constituents were identified or tentatively identified, including 30 isoflavones and five xanthones. Their chemical structures are provided in Fig. 1, and their ESI-MS^n data are shown in Table 2. Table 3 lists the TOF/MS information of the identified compounds, including the accurate mass measurements, retention times (t_R), formula, experimental and theoretical masses and errors.

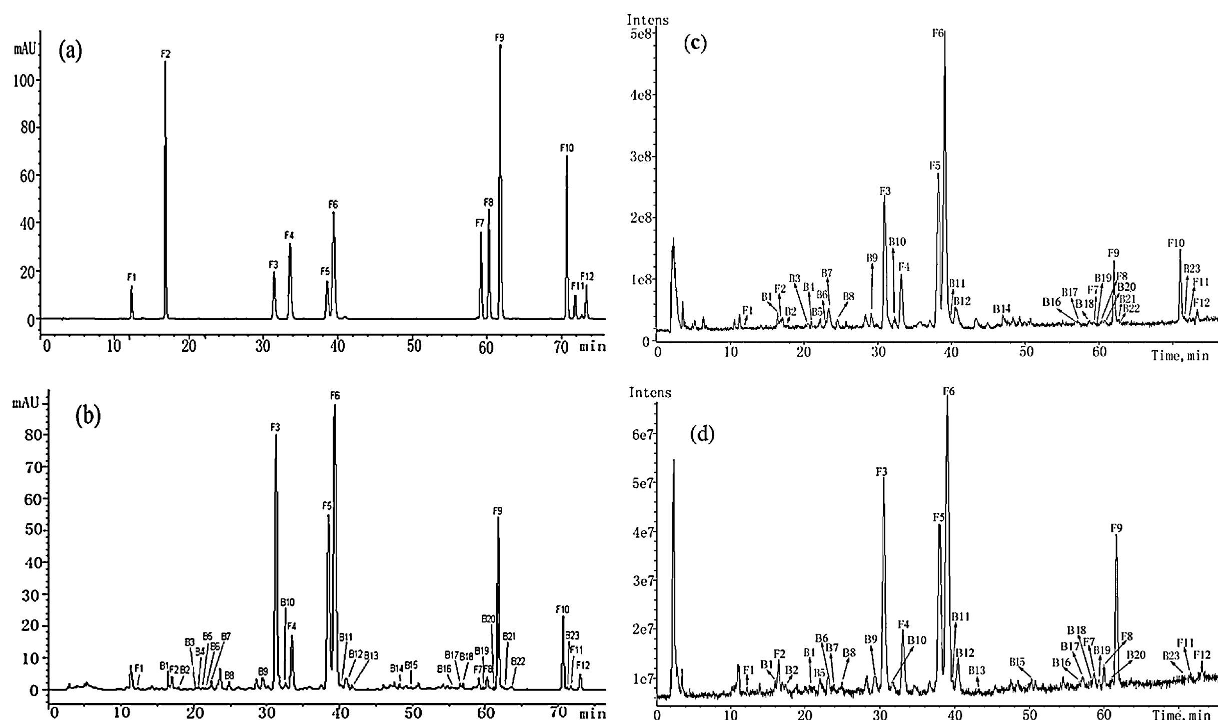


Fig. 2. LC–DAD–ESI-MS^n chromatograms of the authentic standards and Blamcandae Rhizoma extract. (a) UV chromatogram of the 12 authentic standards at 269 nm; (b) UV chromatogram of Belamcandae Rhizoma extract at 269 nm; (c) TIC chromatograms of Belamcandae Rhizoma extract in positive ion mode; (d) TIC chromatograms of Belamcandae Rhizoma extract in negative ion mode.

Table 2
MS data for characterization of compounds in Belamcandae Rhizoma extract by HPLC–DAD–ESI-MS^n.

No.	t_R (min)	Maximum absorption wavelength λ_{max} (nm)	Reference	[M+H]$^+$ (m/z)	[M−H]$^-$ (m/z)	(+)ESI-MS^n fragment ions (m/z)	(−)ESI-MS^n fragment ions (m/z)	Identification
F1	12.01	244, 258, 318, 360	25	585	583	MS^2[585]: 567, 495, 467, 465, 437, 423 MS^3[423]: 405, 345, 303	MS^2[583]: 565, 493, 478, 463, 448, 421 MS^3[421]: 463, 433, 403, 373, 301	Neomangiferin[c]
B1	16.47	212(sh), 266	26	625	623	MS^2[625]: 463, 301 MS^3[301]: 286, 258, 183	MS^2[623]: 461, 299 MS^3[299]: 284, 256, 181	Tectorigenin-7-*O*-glucosyl-4′-*O*-glucoside
F2	16.85	242, 260, 320, 370	25	423	421	MS^2[423]: 405, 333, 303 MS^3[303]: 275, 260	MS^2[421]: 403, 331, 301 MS^3[301]: 273, 258	Mangiferin[c]
B2	17.69	244, 258, 318, 364	39	423	421	MS^2[423]: 405, 333, 303 MS^3[303]: 275, 260	MS^2[421]: 403, 331, 301 MS^3[301]: 273, 258	Isomangiferin
B3	20.61	220, 276, 326(sh)	29	625		MS^2[625]: 301 MS^3[301]: 286, 258, 183	ND[a]	Tectorigenin-4′-*O*-glucosyl (1→6)glucoside
B4	21.11	240, 258, 320, 362	39	459[b]	435	MS^2[435]: 419, 347, 317 MS^3[317]: 302, 274	MS^2[435]: 417, 345, 315 MS^3[315]: 300, 272	7-*O*-methylmangiferin
B5	22.25	360, 320, 350	30	479	477	MS^2[479]:461, 452, 439, 407, 397, 395,391, 361, 348, 329, 317, 302, 228 MS^3[317]: 302, 299, 284, 271, 257, 229, 186, 183, 168	MS^2[477]: 433, 357, 315 MS^3[315]: 300, 255	3′-Hydorxytectoridin
B6	23.20	242, 260, 320, 362	40	437	435	MS^2[435]: 419, 347, 317 MS^3[317]: 302, 274	MS^2[435]: 417, 345, 315 MS^3[315]: 300, 272	7-*O*-methylisomangiferin
B7	24.01	216(sh), 266	16	625	623	MS^2[625]: 301 MS^3[301]: 286, 258, 183	MS^2[623]: 461, 299 MS^3[299]: 284, 256	Tectorigenin-7-*O*-β-glucosyl (1→6)glucoside
B8	24.55	214(sh), 266	31	655	653	MS^2[655]: 331, 316 MS^3[331]: 316, 301, 298, 287, 273, 168	MS^2[653]: 329, 314 MS^3[329]: 314, 301, 286	Iristectorigenin B-7-*O*-β-glucosyl (1→6)glucoside
B9	29.11	220(sh), 267	32	685	683	MS^2[685]: 361, 346 MS^3[361]: 346, 333, 331, 168	MS^2[683]: 359, 344 MS^3[359]: 344, 331, 329	Germanaism C
F3	30.80	214(sh), 266, 334(sh)	14	463	461	MS^2[463]: 448, 435, 420, 301 MS^3[301]: 286, 258, 183	MS^2[461]: 446, 433, 418, 299 MS^3[299]: 284, 256	Tectoridin[c]
B10	32.09	214(sh), 265, 358(sh)	32	463	461	MS^2[463]: 448, 301 MS^3[301]: 286, 258, 168	MS^2[461]: 446, 299, 284 MS^3[299]: 284, 256, 240, 212	Isotectorigenin-7-*O*-β-D-glucoside
F4	33.15	214(sh), 268, 290(sh)	16	493	491	MS^2[493]: 478, 465, 450, 331 MS^3[331]: 316, 301, 288, 270, 265, 237, 252, 183, 168	MS^2[491]: 476, 463, 448, 329, 314 MS^3[329]: 314, 299, 296	Iristectorin B[c]
F5	37.97	220(sh), 266, 292(sh)	16	493	491	MS^2[493]: 478, 465, 450, 331 MS^3[331]: 316, 301, 288, 270, 265, 183, 168	MS^2[491]: 476, 463, 448, 329, 314 MS^3[329]: 314, 299, 286, 242	Iristectorin A[c]
F6	38.84	216(sh), 267, 332	16	523	521	MS^2[523]: 508, 495, 490, 361, 346 MS^3[361]: 346, 331, 328, 318, 310, 243, 198, 183, 168	MS^2[521]: 506, 495, 488, 359 MS^3[359]: 344, 316, 286, 273, 257, 193	Iridin[c]
B11	39.71	224(sh), 266	32	523	521	MS^2[523]: 361, 346 MS^3[361]: 346, 328, 331, 310, 256, 270, 258, 230, 178	MS^2[521]: 506, 488, 359 MS^3[359]: 344, 316, 286, 273, 257, 193	Isoiridin

Table 2 (*Continued*)

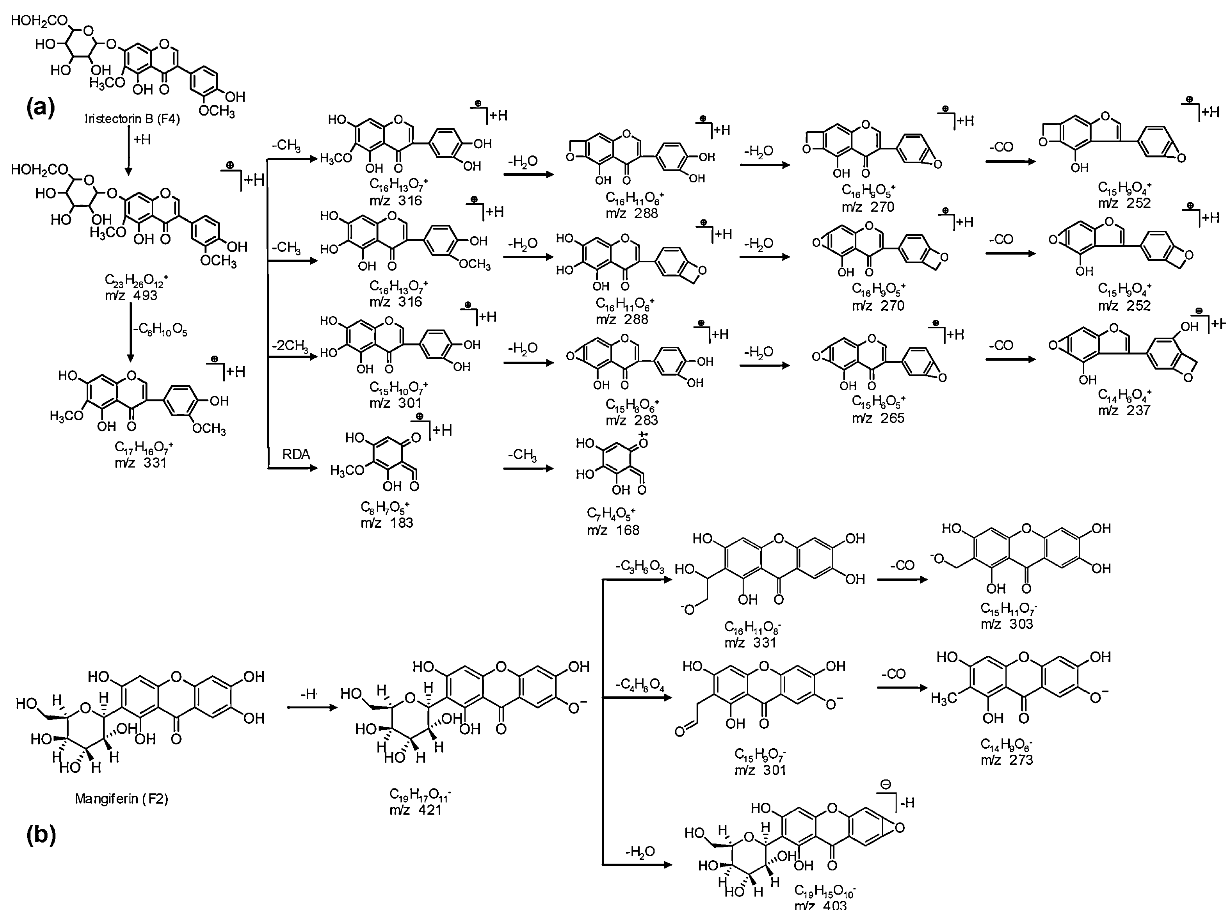
No.	t_R (min)	Maximum absorption wavelength λ_{max} (nm)	Reference	[M+H]+ (m/z)	[M−H]− (m/z)	(+)ESI-MS^n fragment ions (m/z)	(−)ESI-MS^n fragment ions (m/z)	Identification
B12	40.32	216(sh), 266, 342(sh)	34	463	461	MS^2[463]: 448, 301 MS^3[301]: 286, 258, 168	MS^2[461]: 446, 299, 284 MS^3[299]: 284, 256	Tectorigenin-4′-glucoside
B13	43.23	214(sh), 260	33		491	ND[a]	MS^2[491]: 476, 329, 314, 299 MS^3[329]: 314, 299, 286, 271	Homotectoridin
B14	48.31	220(sh), 262, 328(sh)	37	535	ND[a]	MS^2[535]: 373 MS^3[373]: 358, 340, 322, 256, 163, 148	ND[a]	3′,5′-Dimethoxyirisolone-4-*O*-β-D-glucoside
B15	50.22	222(sh), 266	32	ND[a]	315	ND[a]	MS^2[315]: 300, 233 MS^2[300]: 285, 272, 256, 244, 201, 191, 173	Irilin D
B16	56.73	220(sh), 266, 360(sh)	35	673	671	MS^2[673]: 361 MS^3[361]: 346, 331, 328, 314, 313, 310, 295, 282, 183	MS^2[673]: 359 MS^3[359]: 344, 329, 301	6″-*O*-vanilloyliridin
B17	57.15	220(sh), 266, 340(sh)	35	643	641	MS^2[643]: 361,331,282 MS^3[361]: 346, 331, 328, 313, 310, 295, 282, 183	MS^2[673]: 359 MS^3[359]: 344, 329, 301	6″-*O*-p-hydroxybenzoyliridin
B18	58.70	222(sh), 270, 330(sh)	32	361	359	MS^2[361]: 346, 328, 310, 301, 286, 273, 255 MS^3[346]: 331, 328, 310, 207, 197, 184, 179, 169	MS^2[359]: 344, 329, 327, 299 MS^3[344]: 329, 314, 299, 186, 180	5,7,4′-trihydroxy-6,3′,5′-trimethoxyisoflavone
B19	59.69	214(sh), 266, 340(sh)	32	301	299	MS^2[301]: 286, 260 MS^3[286]: 258, 256, 212, 168,112	MS^2[299]: 284, 217, 265, 239, 224, 201, 175 MS^3[284]: 215, 212	Psi-tectorigenin
F8	60.29	218(sh), 268, 294(sh)	16	331	329	MS^2[331]: 316, 301, 288 MS^3[316]: 301, 298, 288, 273, 245, 194, 131	MS^2[329]: 314, 299, 286, 236 MS^3[314]: 299, 284, 271, 256	Iristectorigenin A[c]
B20	61.04	225(sh), 268	34	331	329	MS^2[331]: 316, 301, 288 MS^3[316]: 301, 298, 288, 273, 245, 194, 131	MS^2[329]: 314, 301, 286, 236 MS^3[314]: 299, 284, 271, 256	Iristectorigenin B
F9	61.78	220(sh), 267	15	361	359	MS^2[361]: 346, 331, 328, 310 MS^3[346]: 331, 328, 310, 303, 282, 183	MS^2[359]: 344, 329, 325, 316, 311, 277, 268 MS^3[344]: 329, 325, 316, 285, 301, 261, 211, 191	Irigenin[c]
B21	62.27	224(sh), 268, 330(sh)	34	361	ND[a]	MS^2[361]: 346, 331, 328, 310 MS^3[346]: 331, 328, 310, 303, 282, 183	ND[a]	Isoirigenin
B22	62.58	222(sh), 272, 330(sh)	38	373	ND[a]	MS^2[373]: 358, 343, 340, 312, 256, 220, 165 MS^3[358]: 343, 340, 322, 312, 255, 195	ND[a]	Noririsflorentin
B23	71.10	220(sh), 270, 342(sh)	36	375	373	MS^2[375]: 360, 329, 319, 313 MS^3[360]: 345, 342, 329, 183	MS^2[373]: 358, 343, 329, 315, 301 MS^3[358]: 343, 315, 328, 191	Junipegenin-C
F11	71.92	218(sh), 272, 340(sh)	15	299	297	MS^2[299]: 281, 243, 185, 145, 123, 91 MS^3[185]: 167	MS^2[297]: 279, 269, 217 MS^3[217]: 175, 156	Irilone[c]
F12	73.26	224(sh), 274, 336(sh)	14	359	357	MS^2[359]: 344, 326, 298 MS^3[299]: 284, 270, 256, 198, 242, 225	MS^2[357]: 342, 327, 315, 255, 271 MS^3[342]: 327, 314, 299, 204, 180, 163	Dichotomitin[c]

sh: shoulder peak.

[a] The ion signals of this component investigated were not detected in this ion mode.

[b] This fragment ion was [M+Na]+.

[c] Authentic standards.



Scheme 2. The proposed fragmentation pathways of isoflavones iristectorin B (**F4**) (a) and xanthone mangiferin (**F2**) (b).

3.3.1. Identification of isoflavones

Isoflavones were identified as the major constituents in the Belamcandae Rhizoma. In this study, 16 isoflavone O-glucosides, nine aglycones and five isoflavones with methylenedioxy group were unambiguously or tentatively identified. By comparing retention times and the MS^n spectra with those of the authentic standards, 10 peaks were definitely characterized as tectoridin (**F3**), iristectorin B (**F4**), iristectorin A (**F5**), iridin (**F6**), tectorigenin (**F7**), iristectorigenin A (**F8**), irigenin (**F9**), irisflorentin (**F10**), irilone (**F11**) and dichotomitin (**F12**). For other peaks, the structures were tentatively characterized by comparing their UV spectrum, molecular weight and structural information from MS^n spectra with the literature and isolated compounds.

Compound **B1** and **B7** belonged to a group of isomeric isoflavone glycosides, since they had similar UV spectral and the same $[M+H]^+/[M-H]^-$ ion. In (−)ESI-MS/MS, the glycosidic bond of **B1** was easily cleaved to generate daughter ion of $[M-H-162]^-$ at m/z 461 and $[M-H-162-162]^-$ at m/z 299. The latter, representing the aglycone fragment, was identical to that of tectorigenin. **B1** was tentatively identified as a di-O-glucoside of

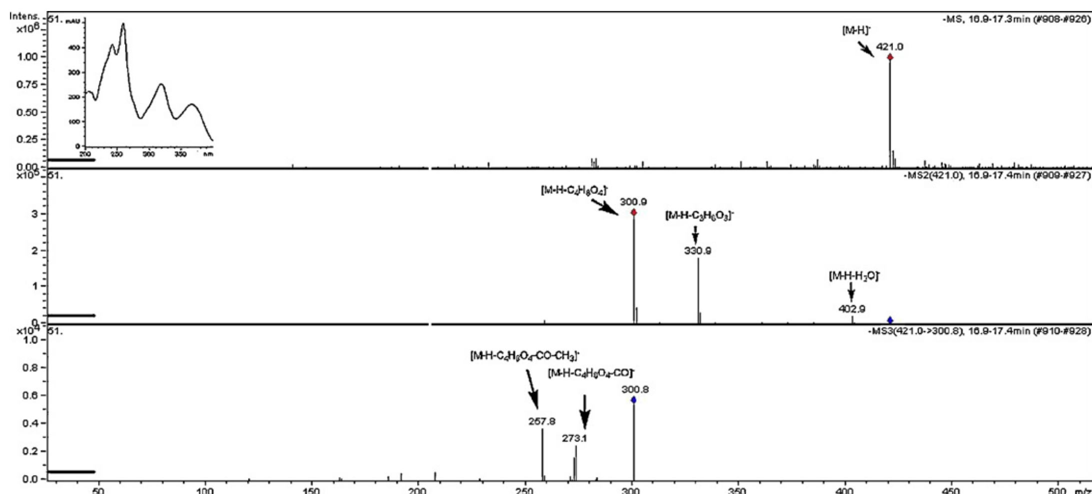


Fig. 3. The MS^n spectra of xanthone mangiferin (**F2**).

Table 3
HPLC–DAD–ESI–TOF/MS analysis for accurate mass measurements of constituents in Belamcandae Rhizoma.

No.	t_R (min)	Formula [M+H]+	Experimental mass (m/z) [M+H]+	Theoretical mass (m/z) [M+H]+	Error mDa	Error ppm	Compound
F1	12.98	$C_{25}H_{29}O_{16}$	585.1450	585.1457	0.6881	1.1760	Neomangiferin
B1	17.02	$C_{28}H_{33}O_{16}$	625.1763	625.1767	0.3880	0.6205	Tectorigenin-7-*O*-glucosyl-4′-*O*-glucoside
F2	17.54	$C_{19}H_{19}O_{11}$	423.0921	423.0924	0.2117	0.5005	Mangiferin
B2	18.61	$C_{19}H_{19}O_{11}$	423.0921	423.0927	0.5117	1.2096	Isomangiferin
B3	21.67	$C_{28}H_{33}O_{16}$	625.1763	625.1764	0.0880	0.1407	Tectorigenin-4′-*O*-glucosyl (1→6)glucoside
B4	22.40	$C_{20}H_{21}O_{11}$	437.1078	437.1083	0.4617	1.0562	7-*O*-methylmangiferin
B5	23.88	$C_{22}H_{23}O_{12}$	479.1184	479.1186	0.1969	0.4111	3′-Hydorxytectoridin
B6	24.81	$C_{20}H_{21}O_{11}$	437.1078	437.1084	0.5617	1.2850	7-*O*-methylisomangiferin
B7	25.33	$C_{28}H_{33}O_{16}$	625.1763	625.1768	0.4880	0.7806	Tectorigenin-7-*O*-β-glucosyl (1→6)glucoside
B8	26.52	$C_{29}H_{35}O_{17}$	655.1868	655.1872	0.3232	0.4934	Iristectorigenin B-4′-*O*-β-glucosyl(1→6)glucoside
B9	32.23	$C_{30}H_{37}O_{18}$	685.1974	685.1972	-0.2414	-0.3523	Germanaism C
F3	34.23	$C_{22}H_{23}O_{11}$	463.1234	463.1240	0.5116	1.0470	Tectoridin
B10	35.59	$C_{22}H_{23}O_{11}$	463.1234	463.1239	0.4116	0.8887	Isotectorigenin-7-*O*-β-D-glucoside
F4	36.42	$C_{23}H_{25}O_{12}$	493.1340	493.1343	0.2468	0.5006	Iristectorin B
F5	42.71	$C_{23}H_{25}O_{12}$	493.1340	493.1341	0.0468	0.0951	Iristectorin A
F6	43.15	$C_{24}H_{27}O_{13}$	523.1346	523.1342	-0.1178	-0.2431	Iridin
B11	44.90	$C_{24}H_{27}O_{13}$	523.1383	523.1381	-0.5178	-0.9632	Isoiridin
B12	45.39	$C_{22}H_{23}O_{11}$	463.1234	463.1238	0.3116	0.6728	Tectorigenin-4′-*O*-β-glucoside
B13	46.24	$C_{23}H_{25}O_{12}$	493.1340	493.1343	0.2468	0.5006	Homotectoridin
B14	49.28	$C_{25}H_{27}O_{13}$	535.1446	535.1449	0.2821	0.5272	3′,5′-Dimethoxyirisolone-4-*O*-β-D-glucoside
B15	51.53	$C_{16}H_{13}O_{7}$	317.0655	317.0660	0.4205	1.3264	Irilin D
B16	57.92	$C_{32}H_{33}O_{16}$	673.1763	673.1764	0.0880	0.1307	6″-*O*-vanilloyliridin
B17	58.38	$C_{31}H_{31}O_{15}$	643.1657	643.1680	2.2527	3.5025	6″-*O*-p-hydroxybenzoyliridin
B18	60.32	$C_{17}H_{17}O_{8}$	361.0917	361.0924	0.6057	1.6776	5,7,4′-Trihydroxy-6,3′,5′-trimethoxyisoflavone
F7	61.02	$C_{16}H_{13}O_{6}$	301.0706	301.0711	0.4352	1.4455	Tectorigenin
B19	61.74	$C_{16}H_{13}O_{6}$	301.0706	301.0713	0.6352	2.1098	Psi-tectorigenin
F8	61.98	$C_{17}H_{15}O_{7}$	331.0812	331.0819	0.6705	2.0251	Iristectorigenin A
B20	62.60	$C_{17}H_{15}O_{8}$	331.0812	331.0820	0.7704	2.3272	Iristectorigenin B
F9	63.56	$C_{18}H_{17}O_{8}$	361.0917	361.0930	1.2057	3.3393	Irigenin
B21	63.93	$C_{18}H_{17}O_{8}$	361.0917	361.0920	0.2057	0.5698	Isoirigenin
B22	64.12	$C_{19}H_{17}O_{8}$	373.0917	373.0922	0.4057	1.0876	Noririsflorentin
F10	72.11	$C_{20}H_{19}O_{8}$	387.1074	387.1090	1.5556	4.0187	Irisflorentin
B23	72.78	$C_{19}H_{19}O_{8}$	375.1074	375.1082	0.7556	2.0146	Junipegenin-C
F11	73.59	$C_{16}H_{11}O_{6}$	299.0550	299.0557	0.6852	2.2915	Irilone
F12	74.63	$C_{18}H_{15}O_{8}$	359.0761	359.0764	0.2558	0.7125	Dichotomitin

tectorigenin, tectorigenin-7-O-β-glucosyl-4′-O-β-glucoside [20]. Compared with **B1**, **B7** showed different fragment behaviors in (−)ESI-MS/MS. Its deprotonated ion directly lost 324 Da and generated daughter ion of $[M-H-162-162]^-$ at m/z 299, while $[M-H-162]^-$ ion was not observed. The observations supposed that **B7** was O-diglycosides and **B7** was tentatively deduced as tectorigenin-7-O-β-glucosyl(1→6)glucoside according to the previous report [12]. Compound **B3**, exhibiting no signal in (−)ESI-MS spectra, had the same molecular weight and fragment ions as B7 in positive mode. **B3**, an isomer of **B7**, was tentatively identified as tectorigenin-4′-O-glucosyl-glucoside [21].

Compound **B5** fragmented in the similar pathways as tectoridin. Its $[M-H]^-$ ion at m/z 477 and aglycone radical ion at m/z 315 were more 16 Da than those of tectoridin, suggesting **B5** had one more hydroxy group than tectoridin. This deduction was further confirmed by the elemental composition analysis by TOF/MS. The RDA fragment ion at m/z 181 indicated that the hydroxy group was attached to the B-ring. **B5** was plausibly characterized as 3′-hydroxytectoridin [22]. **B8** was characterized as an iristectorigenin B diglycoside, owing to the direct loss of 324 Da and an aglycone radical ion at m/z 329. By examining the known isoflavone glycosides in Belamcandae Rhizoma, **B8** was tentatively assigned as iristectorigenin B-7-O-β-glucosyl(1→6)glucoside [23]. By the similar method, other isoflavone glycosides **B9–13**, **B16** and **B17** were characterized. The results are seen in Table 2 [24,3,25].

Besides aglycones **F8–9**, other isoflavone aglycones were also detected in trace amounts. **F7** and **B19**, **F8** and **B20**, and **F9**, **B18** and **B21** were three groups of isomers, sharing identical molecular ions and fragment pathways. Since **F7**, **F8** and **F9** were unambiguously identified as above, **B18**, **B19**, **B20** and **B21** were tentatively characterized as 5,7,4′-trihydroxy-6,3′,5′-trimethoxyisoflavone, psi-tectorigenin, iristectorigenin B and isoirigeninbasis, respectively, on the basis of reported data [23,24,3]. **B15** and **B23** produced maximum UV absorption at about 266 nm and shoulder peaks at about 220 nm or 330 nm, suggesting that they belonged to isoflavones. By comparing the MS data and UV spectra with the literature [24,26], peaks **B15** and **B23** were respectively deduced as irilin D and junipegenin-C.

In this work, five isoflavones containing methylenedioxy were identified in the extract of Belamcandae Rhizoma. Compounds **F10**, **F11**, and **F12** were unequivocally identified as irisflorentin, irilone and dichotomitin by comparison with the reference standards. Both compounds **B14** and **B22** yielded diagnostic ions at $[M+H-CH_3]^+$, $[M+H-2CH_3-CO]^+$ and $[M+H-2CH_3]^+$. In MS^3 spectrum, **B14** exhibited high intensity of $[M+H-162]^+$ ion as well as fragment ions forming by the successive loss of CH_3 and CO. However, it was interesting to note that **B22** produced no MS^3 fragmentation under the same condition. This could be attributed to the low intensity of MS^2 product ions for this compound. In negative ion mode, no deprotonated ion signals of **B14** and **B22** were detected. By examining the known flavonoids in Belamcandae Rhizoma, two isoflavones, named 3′,5′-dimethoxyirisolone-4-O-β-D-glucoside and noririsflorentin, are consistent with the above data [27,28]. Therefore, **B14** and **B22**, were tentatively characterized as 3′,5′-dimethoxyirisolone-4-O-β-D-glucoside and noririsflorentin.

3.3.2. Identification of xanthones

As seen in Table 2, the UV data of xanthones were different from that of isoflavones. Xanthones presented strong absorption at wavelengths of about 240, 260, 320 and 360 nm. **F1** and **F2**, with t_R of 12.01 min and 16.85 min, respectively, could be unambiguously identified as neomangiferin and mangiferin with

Table 4
Linear regression data, LOD, LOQ, precision and recovery of 12 constituents in Belamcandae Rhizoma extract.

Analyte	Regression equation	R^2 ($n=6$)	Linear range (μg/ml)	LOD (ng/ml)	LOQ (ng/ml)	Precision R.S.D. (%)		Recovery ($n=3$)	
						Intra-day ($n=6$)	Inter-day ($n=3$)	Mean (%)	R.S.D. (%)
Neomangiferin	$y=28.17x+2.6826$	0.9999	0.27–55.0	15	70	1.1	3.54	98.8	3.12
Mangiferin	$y=20.763x-6.5106$	0.9990	0.35–70.4	17.5	87.5	2.15	2.8	96.75	4.01
Tectoridin	$y=94.188x-37.26$	0.9998	3.86–154.5	7.5	20	0.95	2.01	98.16	1.21
Iristectorin B	$y=82.508x-10.642$	0.9999	1.65–66.0	2.5	12.5	1.03	2.06	98.1	1.59
Iristectorin A	$y=49.802x-43.194$	0.9992	1.10–110.0	2.5	10	0.92	1.94	100.69	2.66
Iridin	$y=60.229x-11.295$	0.9999	1.06–159.0	15	80	1	2.16	98.84	2.49
Tectorigenin	$y=95.281x+25.323$	0.9999	0.27–108.5	5	20	1.16	2.6	98.82	3.13
Iristectorigenin A	$y=56.823x-4.839$	0.9999	0.16–16.2	27.5	42.5	1.11	2.27	101.39	2.93
Irigenin	$y=109.93x+5.0333$	0.9998	0.83–41.6	7.5	22.5	1.03	2.25	97.97	3.22
Irisflorentin	$y=123.9x-0.1943$	0.9993	0.23–11.7	7.5	30	0.96	2.19	97.97	2.76
Irilone	$y=146.93x-2.9187$	0.9992	0.11–4.4	2.5	27.5	1.98	2.35	102.86	3.87
Dictomitin	$y=18.202x-1.469$	0.9991	0.59–17.7	50	147.5	1.46	2.4	104.08	3.64

In the regression equation $y=ax+b$, x refers to the concentration (μg/ml), y indicates the peak area. R^2 refers to the correlation coefficient of regression equations.

corresponding standard compounds. Compound **B2** had identical molecule weight and fragmentation patterns to those of **F2**. **B2** was tentatively assigned as an isomer of mangiferin, isomangiferin. Compounds **B4** and **B6** showed similar fragmentation behaviors as that of mangiferin, thus they could be deduced as derivatives of mangiferin. The $[M-H]^-$ ions of **B4** and **B6** were both observed at m/z 435, 14 Da more than that of mangiferin. It could be presumed that a hydroxyl group was replaced by a methoxy group in structures of **B4** and **B6**. **B4** and **B6** were tentatively ascribed as 7-O-methylmangiferin and 7-O-isomethylmangiferin, two xanthones isolated from Belamcandae Rhizoma in previous studies [21,29].

3.4. Quantitative determination of constituents in Belamcandae Rhizoma by HPLC–DAD

Twelve peaks **F1–12** in chromatogram of Belamcandae Rhizoma with good resolution were quantified. They were generally considered as active components of this herb. Fig. 2a and b displayed representative HPLC profiles of standard substances and Belamcandae Rhizoma extract detected at 269 nm. Because of the great polarity difference of the analytes, gradient elution and the long HPLC run time were needed for baseline separation of the marker components.

As shown in Table 4, the proposed HPLC–DAD method provided a good precision for the determination of the analytes. The intra- and inter-day precisions (R.S.D. %) were found to be in the range of 0.92–2.15% and 1.94–3.54%, respectively. The recovery was between 96.75 ± 4.01% and 104 ± 3.64%. The linearity ranges, LODs and LOQs (in the range 10–147.5 ng/ml) and the coefficient of correlations (not <0.999) revealed a good sensitivity and linearity response for the developed method.

3.5. Quantitative application

The established quantification method was subsequently applied to comprehensive analysis and quality evaluation of Belamcandae Rhizoma, through simultaneous determination of 12 marker compounds. Twenty-six commercial crude drug samples

Table 5
The contents (mg/g) of 12 constituents in 26 commercial crude drug samples of Belamcandae Rhizoma ($n=3$).

Sample no.	Sources	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
S1	Yichang, Hubei	nd	29.98	111.98	11.84	45.48	8.51	22.51	6.33	4.66	0.25	0.05	2.64
S2	Wuhan, Hubei	0.69	13.17	97.64	7.82	18.82	12.54	13.09	5.16	4.47	0.71	tr	1.83
S3	Shimen, Hunan	tr	9.75	71.79	6.21	14.79	16.79	14.82	5.20	4.64	0.87	0.06	2.93
S4	Bozhou, Anhui	0.63	6.89	51.89	3.96	10.14	7.41	13.01	5.24	4.38	0.82	nd	nd
S5	Fuyang, Anhui	nd	7.86	72.96	5.53	10.79	12.51	11.60	4.00	3.01	0.73	tr	tr
S6	Tongling, Anhui	nd	3.86	70.69	4.97	10.79	9.80	10.06	3.85	2.95	0.62	tr	nd
S7	Anguo, Hebei	tr	14.39	69.83	6.74	14.26	20.30	3.99	2.59	2.38	0.83	0.04	3.70
S8	Handan, Hebei	tr	10.43	82.07	7.66	13.67	19.47	3.77	2.35	1.76	0.86	tr	2.12
S9	Anyang, Henan	0.84	22.41	121.80	11.02	47.95	5.85	40.62	12.07	7.35	tr	0.06	nd
S10	Kunming, Yunnan	1.21	20.66	97.89	12.85	54.85	8.61	22.57	8.16	4.91	tr	tr	nd
S11	Huanggang, Hubei	1.33	36.07	146.89	19.56	84.09	13.19	26.89	8.78	6.24	tr	tr	nd
S12	Xilinhaote, Neimenggu	1.24	36.06	123.14	14.87	61.68	10.16	31.31	7.71	5.90	tr	tr	nd
S13	Jinzhong, Shanxi	0.85	22.61	72.76	8.96	38.33	18.30	13.06	5.85	7.83	1.20	tr	11.28
S14	Taihe, Jiangxi	tr	12.72	66.33	5.24	13.21	14.49	10.23	4.12	3.03	0.99	tr	1.97
S15	Liuzhou, Guangxi	nd	6.56	61.12	5.57	13.03	15.32	11.73	4.17	3.35	0.78	tr	2.45
S16	Suqian, Jiangsu	0.70	31.50	77.44	7.20	26.23	37.40	14.20	7.13	13.52	3.25	0.26	16.65
S17	Taizhou, Jiangsu	tr	17.72	42.87	5.65	21.90	31.54	2.17	1.96	3.92	1.44	0.08	7.02
S18	Xuzhou, Jiangsu	0.70	17.95	27.84	4.17	28.08	23.58	10.01	6.33	12.47	2.68	0.33	17.96
S19	Nanjing, Jiangsu	0.62	8.99	12.16	3.46	33.91	37.61	0.09	1.17	4.46	1.47	tr	13.52
S20	Shanghai	0.56	19.75	16.10	4.34	26.09	39.17	0.95	1.59	6.86	1.76	tr	15.68
S21	Hangzhou, Zhejiang	0.64	14.68	8.90	2.66	20.12	26.70	0.82	1.49	7.02	2.00	nd	17.74
S22	Jinan, Shandong	0.80	16.69	11.14	3.10	23.17	28.15	1.27	1.55	6.17	1.45	nd	14.55
S23	Tianjin	0.53	17.56	9.43	2.73	23.39	32.71	0.54	1.32	6.23	1.66	tr	14.26
S24	Beijing	0.64	21.85	14.33	3.70	28.43	39.81	1.11	1.65	6.91	1.99	0.07	14.00
S25	Dalian, LiaoNing	0.69	22.23	17.62	4.81	30.87	39.06	1.19	1.55	5.79	1.55	tr	14.28
S26	Meihekoushi, JiLing	tr	10.36	8.36	2.02	17.59	18.51	5.61	4.80	11.51	2.01	tr	15.72

tr: trace, below the LOQ.
nd: not detected, below the LOD.

were analyzed and obtained from various provinces and cities in China. The quantitative analytical results are summarized in Table 5. The content of some flavonoids varied in different samples, which would influence the quality stability of this crude drug. Tectoridin (**F3**), with the contents ranged from 8.36 to 111.98 mg/g, was the most abundant in most samples. Mangiferin (**F2**), iristectorin A (**F5**), iridin (**F6**) and tectorigenin (**F7**) were also commonly present. The contents of neomangiferin (**F1**), irilone (**F11**) and dichtomitin (**F12**) are low. Different origins, sources, cultural manner, and harvest time affect the quality of herbal materials. Among crude drugs from different origins, the total contents of 12 flavonoids fell in the range of 96.49–343.04 mg/g. The highest total concentration was found in sample 11 from Huanggang of Hubei Province, which was traditionally regarded as origins of the indigenous Belamcandae Rhizoma. It is evident from a number of reports that flavonoids are major active compounds responsible for the biological activities of Belamcandae Rhizoma, and the result showed that isoflavones are rich in samples. As a result, flavonoids should be undoubtedly determined to comprehensively evaluate the quality of this herb and its preparations.

4. Conclusion

In this study, a powerful and reliable analytical method was developed for the qualitative and quantitative analysis of components in Belamcandae Rhizoma by using HPLC–DAD–ESI-MS^n. Thirty-five components including isoflavones and xanthones were successfully identified based on retention time, UV and MS spectra compared with those of authentic compounds or literature data. In addition, 12 components in Belamcandae Rhizoma were simultaneously determined by HPLC–DAD at 269 nm. The developed method could readily provide full-scale qualitative and quantitative information for the quality evaluation of Belamcandae Rhizoma, which would be a valuable reference for the further study and development of this herb and its related medicinal products.

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