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Original Article

Quantitative determination of multi-class bioactive constituents for quality assessment of ten *Anoectochilus*, four *Goodyera* and one *Ludisia* species in China

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ABSTRACT

Objective: To establish multi-class bioactive constituents' determination of ten *Anoectochilus*, four *Goodyera* and one *Ludisia* species, and provide reference for the improvement of their quality control.
Methods: HPLC-ELSD and phenol–sulphuric acid methods were used for the quantitative determination of lactone glycosides (kinsenoside and its diastereoisomer, goodyeroside A) and polysaccharides, respectively, while an efficient iHPLC–MS/MS method was established for rapid determination of other minor constituents in ten *Anoectochilus* species and five related species.
Results: The contents of kinsenoside, goodyeroside A, polysaccharides and flavonoids varied notably almost in all tested samples, including both wild plants and tissue cultures. In particular, kinsenoside was the major lactone glycoside in *A. roxburghii*, *A. formosanus*, *A. xingrenensis*, *A. nandanensis*, *A. brevilabris* and *A. burmannicus*, whereas goodyeroside A was the predominant constituent in *A. lylei*, *A. longilobus*, *A. elatus*, *A. zhejiangensis*, *G. schlechtendaliana*, *G. biflora*, *G. yangmeishanensi*, *G. repens* and *Ludisia discolor*.
Conclusion: Our present study suggested that *A. lylei*, *A. longilobus*, *A. elatus*, *A. zhejiangensis*, *Ludisia discolor* and *Goodyera* species cannot be used as alternatives for *A. roxburghii*, and goodyeroside A may be reasonably used as a diagnostic marker for distinguishing *A. roxburghii* from *A. lylei*, *A. longilobus*, *A. elatus* and *A. zhejiangensis*, *Goodyera* and *Ludisia* species. The established method thus could be potentially used for the quality evaluation and control of *Anoectochilus* and some related species.

1. Introduction

The genus *Anoectochilus* (Orchidaceae) comprises a total of 43 species of perennial herbs, most of which is native to China and India, through South and Southeast Asia to Australia and the southwest Pacific islands (Pridgeon, Cribb, Chase, & Rasumussen, 2003; Qu, Huang, Feng, & Hu, 2015). In China, there are 20 *Anoectochilus* species (nine endemic), which is distributed mostly in Fujian, Yunnan, Guangxi, Guangdong, Guizhou and Taiwan Provinces (Chen & Shui, 2010; Chen, Gale, Cribb, & Ormerod, 2009; Hu, Tian, & Dong, 2012; Qu et al., 2015; Tian, Liu, Cheng, Hu, & Jiang, 2014; Wu, Tian et al., 2017; Wu, Zhang et al., 2017; Zheng et al., 2018, 2019). Among them, *A. brevilabris*, *A. elatus* and *A. papillosus* are three new records of *Anoectochilus* species (Orchidaceae) in China, which were recently discovered by our group (Zheng et al., 2018; Wu et al., 2017; Zheng et al., 2019). In China, almost all *Anoectochilus* species are used as folk medicine. Among those *Anoectochilus* species, *A. roxburghii* (Jinxianlian in Chinese) has been considered as the most famous and popular medicinal and edible *Anoectochilus* species in China. The fresh or dried whole plant of *A. roxburghii* has been primarily used for the treatment of diabetes, hepatitis, hypertension, tuberculosis hemoptysis, fever, rheumatism and rheumatoid arthritis, pleurodynia and snake bike in China (Huang, 2006; Ye, Shao, Xu et al., 2017; Ye, Shao, Zhang, 2017; Zeng et al., 2017). Previous pharmacological studies showed that *A. roxburghii* possessed hepatoprotective, antidiabetic, vascular protective, antioxidant, antibacterial, anticancer and immunostimulatory activities (Cai et al., 2010; Cui et al., 2013; Huang, Lu, Su,

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Fan, & Yu, 2007; Lin et al., 2017; Liu et al., 2014; Zhang et al., 2007, 2015). In China, *A. roxburghii* has been developed into various products with different dosage forms, including Jinxianlian Spray (Li, Zhou, Liu, Zheng, & Guan, 2012), Jinxianlian Oral Liquid (Liu & Li, 2008) and Jinxianlian Capsules (Chen, Xia, Zhang, Huang, & Xiao, 2010), which have been clinically practiced to treat Hand-foot-and-mouth disease, chronic hepatitis and hyperuricemia.

A. roxburghii is officially designated as the only source of Jinxianlian in the "Standards of Chinese Medicinal Materials of Fujian Province in China" (2006 edition) (Huang, 2006). In folk, other *Anoectochilus* species are also referred to as Jinxianlian in local herbal markets due to the similar traditional medicinal efficacies, including clearing heat and detoxification, expelling wind and eliminating dampness (Ye, Shao, Xu et al., 2017; Ye, Shao, Zhang, 2017). In addition, some clinical applications of *Goodyera* and *Ludisia* species are similar to those of *A. roxburghii*, such as their utilization in the treatments of tuberculosis hemoptysis, rheumatism, rheumatoid arthritis and snake bite etc (Ai, 2013). However, except *A. roxburghii* and *A. formosanus*, there are few reports on the chemical constituents of other *Anoectochilus*, *Goodyera* and *Ludisia* species. Due to their close morphological appearances, it is so difficult to distinguish exactly one from each other. The medicinal materials of Jinxianlian in local herbal markets are often mixed with the officially recorded species (Standards of Chinese Medicinal Materials of Fujian Province in China, 2006 edition), and some other alternatives or even false ones (Wang, Cai, Zhao, Zhu, & Zhang, 2012; Yin, Xu, Fan, Ma, & Lin, 2016; Zhang, Wan, & Wu, 2005). Therefore, it is an important issue to comprehensively evaluate the active components of *Anoectochilus*, *Goodyera,* and *Ludisia* species, so as to ensure the quality and clinical efficacy of Jinxianlian. The market demand of *A. roxburghii* has been increasing year by year due to its unique medicinal and edible properties. The excessive consumption thus led to the sharp decrease of the wild resources of *A. roxburghii*. Therefore, it is very important to find proper substitutes for the official recorded species of Jinxianlian from *Anoectochilus* species or other similar genera.

Previous phytochemical investigations revealed that *A. roxburghii* mainly contains three types of bioactive components, including lactones glycoside, flavonoids, and polysaccharides (Yin et al., 2016; Zheng, Pan, Cai, Yuan, & Cao, 2013). Among these components, 3-(*R*)-3-β-*D*-glucopyranosyloxybutanolide (kinsenoside, Fig. 1), one of the principle active constituents in *Anoectochilus* species (e.g. *A. roxburghii*, *A. formosanus*, *A. chapaensis* and *A. koshunensis*) (Ito & Kasa, 1993; Zhang et al., 2017), exhibits hepatoprotective, antihyperglycemic, antioxidant, anti-flammatory, and antihyperliposis activities (Du, Irino, Furusho, Hayashi, & Shoyama, 2008; Hsiao, Wu, Lin, & Lin, 2011; Liu et al., 2014; Wu et al., 2007; Zhang, Cai, Ruan, Pi, & Wu, 2007), whereas 3-(*S*)-3-β-*D*-glucopyranosyloxybutanolide (goodyeroside A, Fig. 1), the epimer of kinsenoside, is a characteristic component isolated from the *Goodyera* species (Du, Sun, & Chen, 2000). Pharmacological studies showed that, in contrast to kinsenoside, goodyeroside A had no activities for anti-hyperliposis, anti-hyperglycemic and anti-autoimmune hepatitis purposes (Du et al., 2008; Song, 2019; Xiang et al., 2016). Interestingly, goodyeroside A was also reported and isolated from *A. roxburghii* (Xiang et al., 2016). Thus, we speculate that the contents of kinsenoside and goodyeroside A can be used as a key index to reflect the quality of *A. roxburghii* and other *Anoectochilus* species. Polysaccharides, another category of bioactive constituents in *A. roxburghii*, displayed hepatoprotective, antidiabetic and vascular protective effects (Lin et al., 2017; Zeng et al., 2017; Zhang, Liu, Liu, Li, & Yi, 2015). In addition, flavonoids in *A. roxburghii* were also reported to be responsible for various pharmacological activities, including antioxidant and hypoglycemic activities (He, Wang, Guo, Yang, & Xiao, 2006; Liu et al., 2014; Wu, Tian et al., 2017; Wu, Zhang et al., 2017). It also contains other minor constituent, such as gastrodin, which exhibited antioxidant, anti-inflammatory, and neuroprotective activities (Yang et al., 2013; Zhao et al., 2012). Up to now, several HPLC, HPLC-UV-MS/MS, or UPLC-Q-TOF-MS/MS methods have been reported for the analysis of kinsenoside and flavonoids in *A. roxburghii* (Huang et al., 2007; Li et al., 2017; Xu et al., 2017; Zhang et al., 2015). However, a systematic method on the assessment of these multi-class bioactive constituents of *A. roxburghii* and its related species has not been well defined yet.

Therefore, in this study, we performed comprehensively quantitative determination of multi-class bioactive components in *A. roxburghii* and 14 related species (Fig. 2) collected from different geographical locations in China, based on combined multiple methods for quantitative analysis. HPLC-ELSD method was used for the quantitative determination of lactone glycosides (kinsenoside and goodyeroside A), and phenol–sulphuric acid methods was used for the quantitative determination of polysaccharides, while an efficient iHPLC–MS/MS method was established for rapid determination of other minor constituents in ten *Anoectochilus*, four *Goodyera* and one *Ludisia* species.

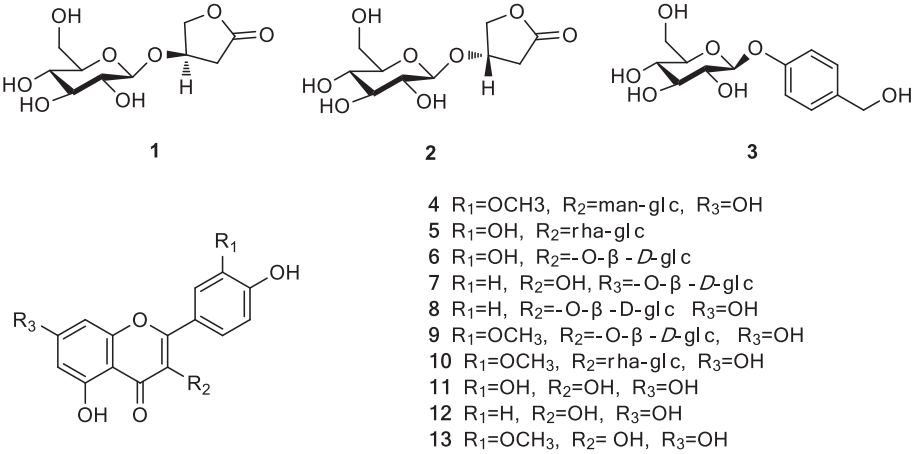

Fig. 1. Chemical structures of compounds **1**–**13**.**1**. kinsenoside; **2**. gooderoside A; **3**. gastrodin; **4**. isorhamnetin-3-*O*-β-*D*-neohespeidoside; **5**. rutin; **6**. isoquercitrin; **7**. kaempferol-7-*O*-β-*D*-glucoside; **8**. kaempferol-3-*O*-β-*D*-glucoside; **9**. isorhamnetin-3-*O*-β-*D*-glucoside; **10**. narcissin; **11**. quercetin; **12**. kaempferol; **13**. isorhamnetin.

Fig. 2. Ten *Anoectochilus*, four *Goodyera* and one *Ludisia* specie. 1. *A. roxburghii* (Jinxianlian); 2. *A. roxburghii* (Jinxianlian gong); 3. *A. formosanus*; 4. *A. xingrenensis*; 5. *A. lylei*; 6. *A. longilobus*; 7. *A. nandanensis*; 8. *A. burmannicus*; 9. *A. brevilabris*; 10. *A. elatus*; 11. *A. zhejiangensis*; 12. *G. schlechtendaliana*; 13. *G. biflora*; 14. *G. repens*; 15. *G. yangmeishanensis*; 16. *L. discolor*.

2. Materials and methods

2.1. Chemicals and materials

Methanol (HPLC grade) and acetonitrile (HPLC grade) were procured from Merck Millipore (Darmstadt, Germany). Formic acid (HPLC grade) was procured from Tedia (Fairfield, OH, USA). Ultrapure water was prepared using a Milli-Q system (Millipore, MA, USA). Other organic solvents (analytical grade) were procured from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). The standard compound of kinsenoside (purity > 98%) was procured from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Goodyeroside A standard (purity > 98%) was isolated from *A. zhejiangensis*.

The standard compounds (purity > 98%) of kaempferol-3-*O*-β-*D*-glucoside, isorhamnetin-3-*O*-β-*D*-neohespeidoside, isorhamnetin-3-*O*-β-*D*-glucoside, rutin, isoquercitrin, kaempferol-7-*O*-β-*D*-glucoside, narcissin, isorhamnetin, kaempferol and quercetin were procured from Chengdu MUST Bio-technology Co., Ltd. (Chengdu, China). The standard compound of gastrodin (purity > 98%) was procured from Shanghai Yuanye Bio-technology Co., Ltd (Shanghai, China). The chemical structures of analytes are shown in Fig. 1.

A total of 31 batches of samples from ten *Anoectochilus* species, four *Goodyera* and one *Ludisia* species were collected from Fujian, Yunnan, Guangxi, Guangdong, Jiangxi, Guizhou and Zhejiang Provinces in China and authenticated by associate professor Yan-Bin Wu of Fujian University of Traditional Chinese Medicine (Fig. 2, Table 1). All voucher specimens were deposited in School of Pharmacy, Fujian University of Traditional Chinese Medicine.

2.2. Instrumentation and analytical conditions

2.2.1. HPLC- ELSD conditions

The HPLC-ELSD was performed using an Agilent 1260 HPLC system (Agilent Technologies, USA), equipped with a binary solvent delivery system, autosampler, and an Evaporative Light Scattering

Table 1
Collected crude materials of ten *Anoectochilus*, four *Goodyera* and one *Ludisia* species.

Samples no.	Species	Codes	Locations of collection
1	*A. roxburghii*	AR-1	Qingyuan, Guangdong
2		AR-2	Laibin, Guangxi
3		AR-3	Nanning, Guangxi
4		AR-4	Ganzhou, Jiangxi
5		AR-5	Qingyuan, Guangdong
6		AR-6	Nanning, Guangxi
7		AR-7	Sanming, Fujian
8		AR-8	Nanjing, Fujian
9		AR-9	Nanjing, Fujian
10		AR-10	Nanjing, Fujian
11		AR-11	Nanjing, Fujian
12		AR-12	Nanjing, Fujian
13	*A. formosanus*	AF-1	Nanjing, Fujian
14		AF-2	Nanjing, Fujian
15		AF-3	Nanjing, Fujian
16		AF-4	Nanjing, Fujian
17		AF-5	Nanjing, Fujian
18	*A. xingrenensis*	AX-1	Xingyi, Guizhou
19		AX-2	Pingbian, Yunnan
20	*A. lylei*	AL-1	Jinghong, Yunnan
21	*A. nandanensis*	AN-1	Nandan, Guangxi
22	*A. longilobus*	LO-1	Malipo, Yunnan
23	*A. brevilabris*	AB-1	Malipo, Yunnan
24	*A. burmannicus*	BU-1	Jinghong, Yunnan
25	*A. elatus*	AE-1	Jinghong, Yunnan
26	*A. zhejiangensis*	AZ-1	Guigang, Guangxi
27	*G. schlechtendaliana*	GS-1	Lishui, Zhejiang
28	*G. biflora*	GB-1	Pingbian, Yunnan
29	*G. yangmeishanensis*	GY-1	Hechi, Guangxi
30	*G. repens*	GR-1	Nujiang, Yunnan
31	*L. discolor*	LD-1	Nanning, Guangxi

Note: AR 1–7 are wild samples collected from difference provinces and cities (AR 1–4, Chinese name "Jinxianlian"; AR 5–7, Chinese name "Jinxianlian gong" without obvious leaf veins as indicated in Fig. 2); AR 8–12 and AF 1–5 are tissue-cultured samples purchased from difference companies; Samples 18–31 are wild samples collected from difference provinces and cities.

Detector (ELSD) system. The chromatographic separation was accomplished based on a previously reported method (Wei et al., 2020) with slight modifications. The chromatography was performed on an AQ-C_{18} column (250 mm × 4.6 mm, 5 μm) and a isocratic elution system of ultrapure water containing 0.5% acetic acid as the mobile phase was adopted. The flow rate was set at 0.6 mL/min, while the column and the ELSD spray chamber temperature was maintained at 30 °C and 70 °C, respectively, and the nitrogen flow rate was 1.5 L/min.

2.2.2. iHPLC-MS/MS conditions

iHPLC was performed using an Agilent 1290 infinity HPLC system (Agilent Technologies, USA), equipped with a binary solvent delivery system with autosampler. The chromatography was achieved on an ACE 3 C_{18}-PFP (3.0 mm × 150 mm, 3.0 μm) column. The mobile phase is consisted as (A) 0.1% formic acid in methanol and (B) 0.1% formic acid in water. The eluting conditions was programmed as follows: 10% (A) in 0–2 min, 10%–50% (A) in 2–5 min, 50%–55% (A) in 5–15 min, 55%–85% (A) in 15–20 min and 85%–95% (A) in 20–22 min, at a flow rate of 0.4 mL/min. The column and autosampler temperature were maintained at 30 °C and 4 °C, respectively. The injection volume was 1 μL.

The MS analysis was performed using an Agilent 6470 tandem mass spectrometer (Agilent Technologies, USA) connected to an AJS electrospray source interface in negative ion mode. Quantitative determination was carried out using multiple reaction monitoring (MRM) mode. The ion source parameters were as follows: drying gas and sheath gas temperature 350 °C, drying gas flow rate10.0 L/min, sheath gas, flow rate11.0 L/min, capillary voltage 3.5 kV, nebulizer pressure 40 psi, respectively. The optimized compound dependent MRM parameters for precursor-to-product ion transition of each analyte including collision energy (CE), declustering potential (DP) and cell exit potential (CXP) were presented in Table 2.

2.2.3. Extraction and sample preparation

The plant materials were dried and grounded into powder and, 0.5 g of each powder was sonicated in 50 mL of 70% methanol for 45 min. Then, the extraction solutions were filtered and concentrated. The residues were dissolved with 10 mL methanol in a volumetric flask and sonicated for 5 min, and then filtered through a 0.22 μm PTFE syringe filter for iHPLC-MS/MS analysis. In addition, 0.1 g of each powder was sonicated in 20 mL pure water for 45 min. Then, the extract was filtered through a 0.22 μm PTFE syringe filter for HPLC-ELSD analysis.

The total polysaccharides of each sample was extracted according to the method of Chinese Pharmacopeia (Chinese Pharmacopoeia Committee, 2010). Each of the dried samples was extracted with water (1:100) in a water bath at 90 °C for 2 h. The extraction process was repeated twice. The extraction solutions were combined and filtered, and then the filtrate was centrifuged at 4500 rpm for 10 min. The supernatant (2 mL) was precipitated with 10 mL ethanol and then incubated at 4 °C for 12 h. Polysaccharide precipitates were collected by centrifugation, and subsequently washed with ethanol and acetone successively.

2.2.4. Preparation of standard solutions

The eleven reference compounds were prepared accurately in HPLC grade methanol. The concentrations were: gastrodin, 2.20 mg/mL; isorhamnetin-3-*O*-β-*D*-neohespeidoside, 1.52 mg/mL; rutin, 1.86 mg/mL; isoquercitrin, 1.84 mg/mL; narcissin, 1.72 mg/mL; kaempferol-3-*O*-β-*D*-glucoside, 2.20 mg/mL; kaempferol-7-*O*-β-*D*-glucoside, 1.59 mg/mL; isorhamnetin-3-*O*-β-*D*-glucoside, 2.02 mg/mL; quercetin, 2.02 mg/mL; kaempferol, 1.06 mg/mL; isorhamnetin, 2.48 mg/mL. Stock solutions were then mixed and diluted with methanol to provide the mixed standard stock solution. The mixed standard solution was serially diluted with methanol to yield a series of working standard solutions. The internal standard (IS: puerarin) solution was prepared at a concentration of 1.37 μg/mL. All stock solutions were stored at 4 °C before use.

3. Results and discussion

3.1. Optimization of extraction conditions

In order to optimize the sample preparation, different extraction solvents (water, 50% EtOH, 70% EtOH, 100% EtOH, 50% MeOH, 70% MeOH, 100% MeOH), extraction methods (ultrasonication and reflux) and extraction time (30, 45, 60 and 90 min) were investigated. The results suggested that ultrasonication using pure water for 45 min showed the best extraction efficiency for kinsenoside, whereas ultrasonication using 70% MeOH for 45 min worked better for the other compounds.

3.2. Optimization of MS conditions

Eleven analytes were optimized by continuous infusion into the mass spectrometer for the optimization of compound dependent parameters. MS spectra were recorded in negative ionization modes. Then, DP, CE and CXP were optimized to get the maximum sensitivity of precursor to product ions. The optimized MRM parameters for eleven analytes and internal standard were listed in Table 2. The optimized iHPLC-MS/MS method in MRM mode

Table 2
Optimized compound dependent MRM parameters for analytes and internal standard (IS).

Analytes	RT/min	Precursor ion	Product ion	DP/V	CE/eV	CXP/V
gastrodin	4.5	331.2	123.1	150	30	5
IS	7.2	415.0	267.1	100	10	5
isorhamnetin-3-*O*-β-*D*-neohespeidoside	9.0	623.2	299.2	150	60	5
rutin	10.1	609	300.1	85	50	5
isoquercitrin	10.4	463.2	300.1	110	40	5
kaempferol-7-*O*-β-*D*-glucoside	11.2	447.2	285.1	120	25	5
kaempferol-3-*O*-β-*D*-glucoside	12.0	447.2	283.9	120	40	5
isorhamnetin-3-*O*-β-*D*-glucoside	12.5	477.2	314.2	110	30	5
narcissin	12.6	623.3	315.1	110	30	5
quercetin	18.5	301.1	151.1	130	20	5
kaempferol	20.4	285.0	117.0	90	80	5
isorhamnetin	20.8	315.1	300.1	150	20	5

RT: retention time; DP: declustering potential; CE: collision energy; CXP: cell exit potential; IS: internal standard.

was applied to determine the content of eleven analytes in ten *Anoectochilus* species and five similar plants.

3.3. Qualitative analysis of targeted analytes

In the MRM chromatogram of samples, targeted analytes were identified as gastrodin (peak 1), isorhamnetin-3-*O*-β-*D*-neohespeidoside (peak 2), rutin (peak 3), isoquercitrin (peak 4), kaempferol-7-*O*-β-*D*-glucoside (peak 5), kaempferol-3-*O*-β-*D*- glucoside (peak 6), isorhamnetin-3-*O*-β-*D*-glucoside (peak 7), narcissin (peak 8), quercetin (peak 9), kaempferol (peak 10) and isorhamnetin (peak 11), respectively by comparison with their retention times and MS spectra of the authentic standard compounds (Figs. 3–5). The $[M-H]^-$ quasi-molecular ions and the characteristic fragment ions are listed in Table 2.

3.4. Method validation of quantitative analysis

The internal standard method was used to determine the contents of eleven active compounds in ten *Anoectochilus* species

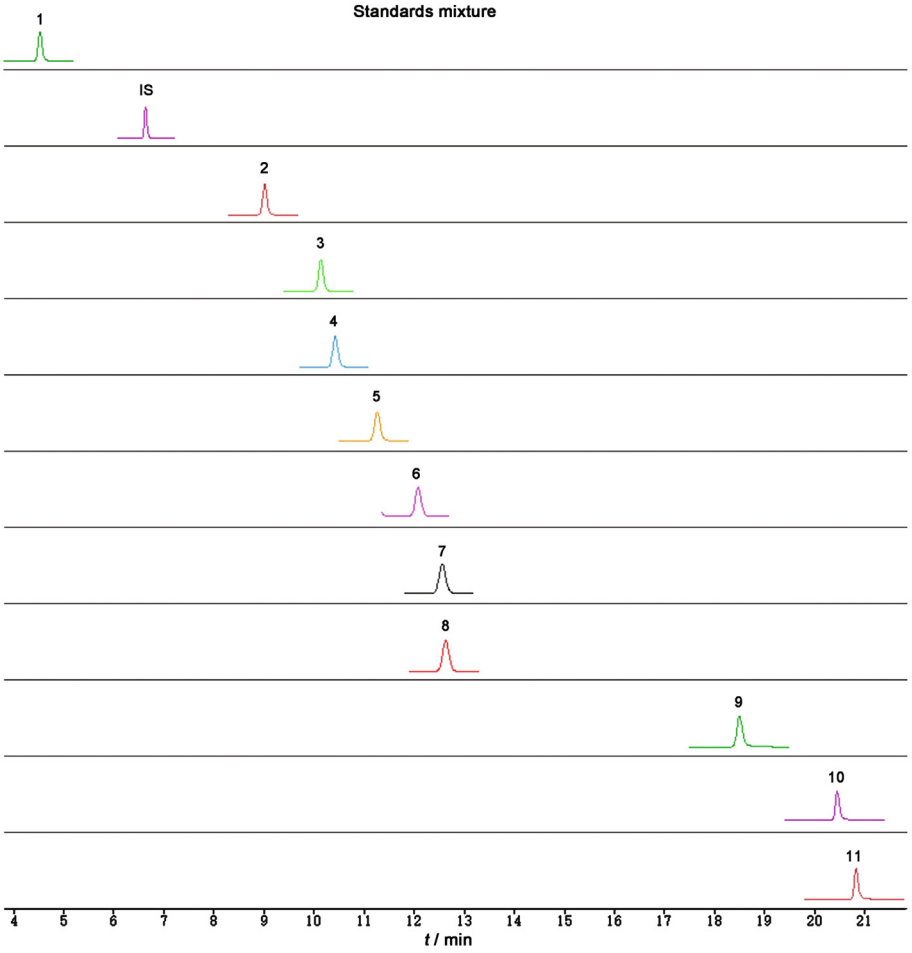

Fig. 3. MRM chromatogram of standards mixture obtained by iHPLC-MS/MS. 1. gastrodin; 2. isorhamnetin-3-*O*-β-*D*-neohespeidoside; 3. rutin; 4. isoquercitrin; 5. kaempferol-7-*O*-β-*D*-glucoside; 6. kaempferol-3-*O*-β-*D*-glucoside; 7. isorhamnetin-3-*O*-β-*D*-glucoside; 8. narcissin; 9. quercetin; 10. kaempferol; 11. isorhamnetin; IS. puerarin.

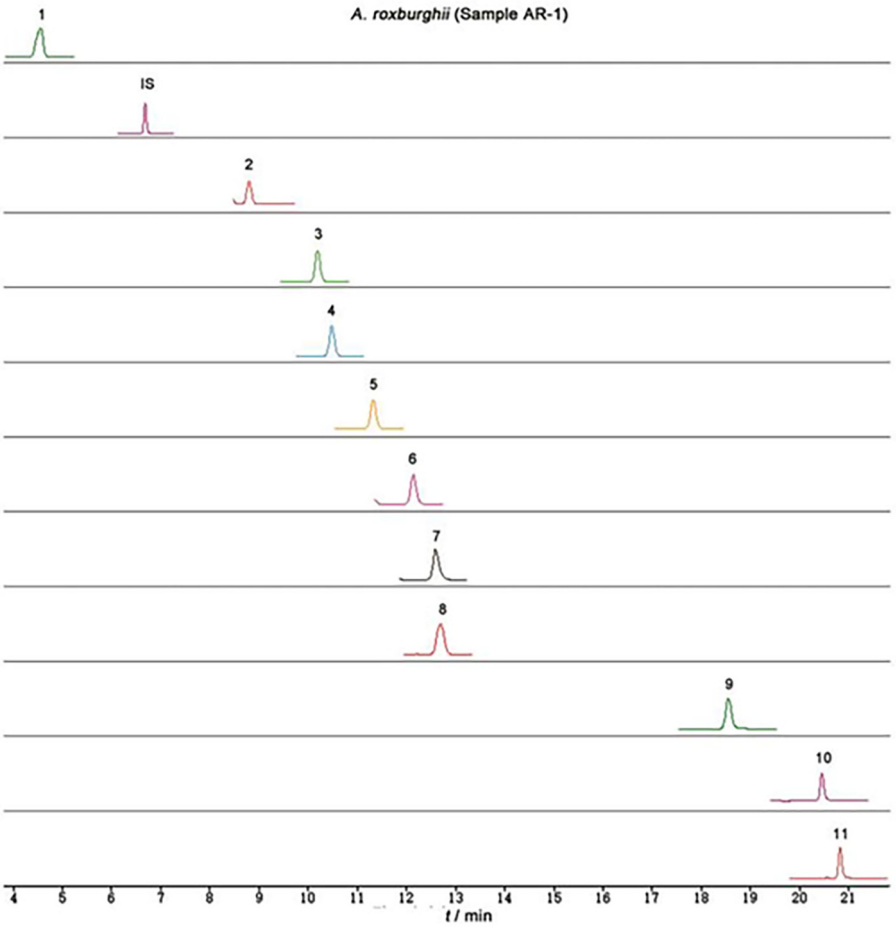

Fig. 4. MRM chromatogram of *A. roxburghii* (sample AR-1) obtained by iHPLC-MS/MS.

and five similar generas. The calibration curves were achieved by plotting the analytes-to-IS peak area ratios versus the theoretical concentration of each analyte using a 1/x weighting. Good linear relationships were gained, and the correlation coefficients (r^2) ranges from 0.9954 to 0.9997 within the test ranges. The limit of detection (LOD) and limit of quantification (LOQ) of eleven analytes were ascertained at a signal to baseline noise peak ratio high of 3 and 10, respectively. All results were shown in Table 3. The established method showing higher sensitivity than the previous reports (Zheng et al., 2013) with overall LOD and LOQ of no more than 12.5 ng/ml and 25 ng/ml, respectively.

The precision of the established method was evaluated by intra-day and inter-day variability. The intra-day and inter-day variation was assessed by analyzing a standard solution containing eleven analytes with IS for six times within a day and by repeating the experiments on three consecutive days. The relative standard deviation (RSD) of the eleven analytes for intra and inter-day precisions were in the range of 0.78%–1.88% and 0.82%–2.32%, respectively (Table 3). Stability was determined at room temperature, and the same sample solutions were analyzed within 24 h. The stability RSD values of the eleven analytes were in the range of 1.36%–2.72% (Table 3). The accuracy of the established method was evaluated by recovery test. The recoveries were carried out by spiking accurately known amounts of the eleven analytical standards (50%, 100% and 150%) into a sample, and then extracted and analyzed under this proposed method. The spiked samples were analyzed in three replicates at each level. The average recoveries were calculated by the formula: recovery (%) = (observed amount − original amount)/spiked amount × 100%. The average recoveries of the eleven analytes were between 96.85% and 102.43%, with RSD values less than 2.53% (Table 3), indicating that the developed method was accurate.

3.5. Quantitative analysis of different samples

Four types of constituents, including lactone glycosides, polysaccharides, flavonoids, and phenolic glycosides were quantified in 10 species of *Anoectochilus* and five species of other similar genera listed in Table 4. In previous studies, HPLC- UV or HPLC-ELSD methods have been used for the quantitative analysis of kinsenoside in *A. roxburghii* (Jin, Zhang, Piao, Gao, & Lian, 2018; Luo et al., 2018; Wan & Xiong, 2014; Zhang et al., 2017). Interestingly, the epimer of kinsenoside, goodyeroside A, was also reported in *A. roxburghii* and *Goodyera* species (Wei et al., 2020). However, the content of goodyeroside A in *A. roxburghii* and other *Anoectochilus* species have not been reported yet. Due to the lack of chromophores in kinsenoside and goodyeroside A, no ultraviolet absorption can be detected, thus limiting the ultraviolet detection for these two epimers, whereas HPLC-ELSD becomes a better choice for kinsenoside and goodyeroside A detection. Wei et al. reported that kinsenoside and goodyeroside A can be separated by HPLC-ELSD with AQ-C_{18} column, whereas both compounds were eluted as one chromatographic peak on amino (NH_2) column due to their extremely similar polarity (Wei et al., 2020). In this study, HPLC-ELSD with AQ-C_{18} column was therefore used for determining the content of kinsenoside and goodyeroside A in *Anoectochilus*, *Goodyera* and *Ludisia* species (Fig. 6). Among those different *Anoectochilus* species, the contents of kinsenoside varied

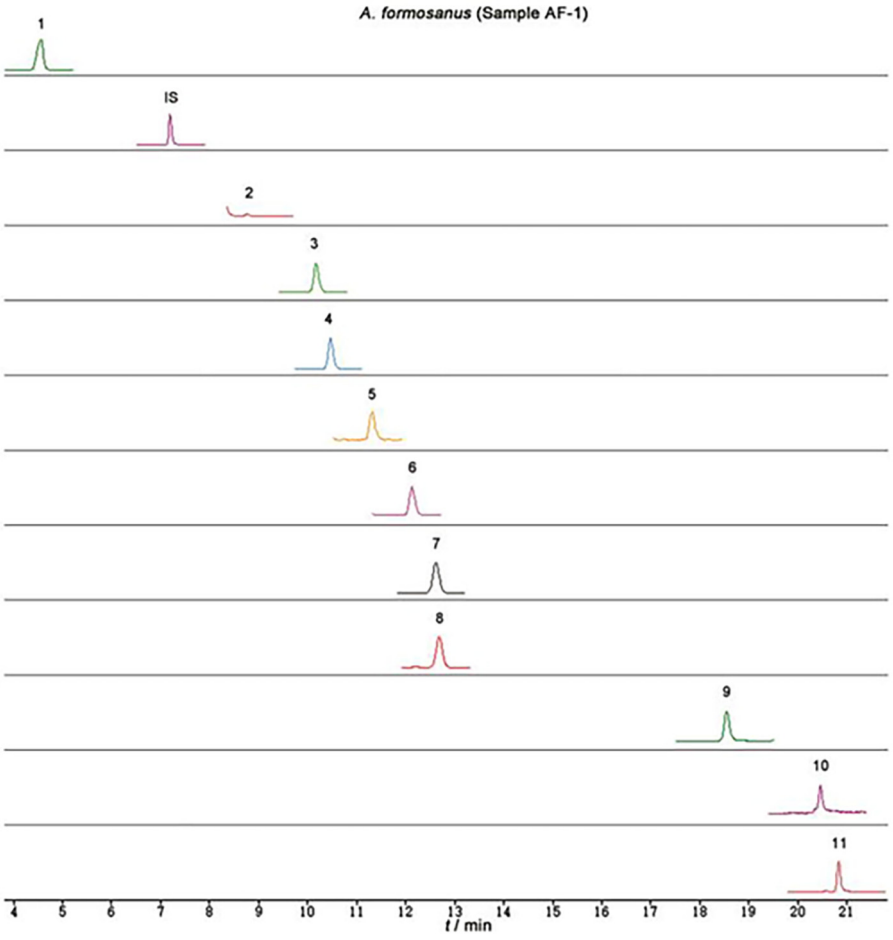

Fig. 5. MRM chromatogram of *A. formosanus* (sample AF-1) obtained by iHPLC-MS/MS.

Table 3
Method validation parameters for 11 analytes.

Analytes	Regression equations	r^2	Test range/ ($\mu g \cdot mL^{-1}$)	LOD [a]/ ($ng \cdot mL^{-1}$)	LOQ [a]/ ($ng \cdot mL^{-1}$)	Precision RSD/%		Stability RSD / % (n = 6)	Recovery (n = 9)	
						Intraday (n = 6)	Interday (n = 9)		Mean	RSD/%
1	$y = 0.111582x - 679325.1$	0.9991	50–10000	0.5	1	1.70	1.16	1.85	97.08	1.67
2	$y = 3.553065 \times 10^{-4}x + 0.008147$	0.9995	5–1000	0.5	1	1.56	1.42	1.56	98.43	1.20
3	$y = 4.367182 \times 10^{-4}x - 0.023675$	0.9996	500–100000	1.25	2.5	1.23	2.05	2.55	100.85	1.48
4	$y = 5.924533 \times 10^{-4}x - 0.005947$	0.9954	50–10000	0.5	1	1.34	1.56	1.54	97.85	2.53
5	$y = 6.877874 \times 10^{-4}x + 9.009652 \times 10^{-4}$	0.9978	5–1000	0.5	1	1.03	1.33	1.36	98.53	1.52
6	$y = 2.414413 \times 10^{-4}x + 2.002145 \times 10^{-5}$	0.9991	5–1000	0.5	1	0.95	1.85	2.72	99.14	2.03
7	$y = 5.783505 \times 10^{-4}x + 0.005276$	0.9994	500–100000	0.25	0.5	0.78	0.82	2.22	98.63	1.90
8	$y = 9.648908 \times 10^{-4}x + 0.025206$	0.9993	500–100000	1.25	2.5	1.58	2.25	2.58	102.04	2.17
9	$y = 0.001213x + 0.00757$	0.9984	50–10000	2.5	5	1.88	2.32	1.94	102.43	2.06
10	$y = 2.415004 \times 10^{-5}x + 0.001221$	0.9992	50–10000	12.5	25	0.94	2.15	1.43	96.85	1.42
11	$y = 0.006636x + 0.226556$	0.9992	50–10000	0.5	1	1.12	1.34	2.02	100.19	2.35

Note: 1: gastrodin; 2: isorhamnetin-3-*O*-β-*D*-neohespeidoside; 3: rutin; 4: isoquercitrin; 5: kaempferol-7-*O*-β-*D*-glucoside; 6: kaempferol-3-*O*-β-*D*-glucoside; 7; isorhamnetin-3-*O*-β-*D*- glucoside; 8: narcissi; 9: quercetin; 10: kaempferol; 11: isorhamnetin

[a] LOD and LOQ were determined on the basis of signal to noise ratio 3:1 and 10:1, respectively.

notably, ranging from 3.38 to 229.17 mg/g. Even in different batches of *A. roxburghii* samples, the contents of kinsenoside also varied remarkably, ranging from 57.10 to 164.92 mg/g. In addition, it is noteworthy that most of the tissue-cultured samples of *A. roxburghii* and *A. formosanus* produced higher amounts of kinsenoside than wild samples of *A. roxburghii*. Therefore, tissue-cultured *A. roxburghii* and *A. formosanus* could be considered as an ideal botanical source for kinsenoside production. Furthermore, in our study, kinsenoside was also detected in *A. xingrenensis*, *A. lylei*, *A. nandanensis*, *A. longilobus*, *A. brevilabris*, *A. burmannicus*, *A. elatus*, *G.*

Table 4
Contents of investigated bioactive constituents in 10 *Anoectochilus*, four *Goodyera* and one *Ludisia* species.

Samples	Analytes [a]														
	1/ (mg·g^{-1})	2/ (mg·g^{-1})	3/ (μg·g^{-1})	4/ (μg·g^{-1})	5/ (μg·g^{-1})	6/ (μg·g^{-1})	7/ (μg·g^{-1})	8/ (μg·g^{-1})	9/ (μg·g^{-1})	10/ (μg·g^{-1})	11/ (μg·g^{-1})	12/ (μg·g^{-1})	13/ (μg·g^{-1})	14/ (μg·g^{-1})	15/ (mg·g^{-1})
AR-1	105.90	– [b]	82.48	1.78	345.72	67.74	2.44	3.57	14.44	1017.5	166.31	23.11	28.63	1671.24	69.40
AR-2	78.96	– [b]	204.93	4.29	533.74	222.16	9.79	17.05	119.39	2285.18	395.65	37.24	70.38	3694.87	76.51
AR-3	57.10	1.69	127.30	2.48	268.16	71.13	10.62	4.36	18.84	965.86	183.27	22.74	34.48	1581.94	22.70
AR-4	89.44	– [b]	77.43	0.82	55.17	120.88	5.21	8.65	119.14	978.95	347.12	33.25	58.5	1727.69	81.30
AR-5	118.80	– [b]	106.91	2.71	41.09	12.74	0.19	2.41	19.28	624.89	9.32	1.2	9.45	723.28	94.06
AR-6	129.12	– [b]	153.61	1.89	22.61	7.71	0.38	1.18	21.58	910.43	6.11	0.65	8.24	980.78	93.41
AR-7	60.34	– [b]	261.60	0.11	24.61	19.13	1.79	11.43	69.51	548.77	6.10	2.11	19.89	703.45	48.18
AR-8	110.42	– [b]	416.21	0.75	154.01	22.9	0.55	0.21	1.27	373.55	26.59	0.64	3.98	584.45	173.30
AR-9	164.92	– [b]	99.20	0.9	28.58	48.97	1.76	1.63	6.08	149.99	26.83	7.13	3.58	275.45	231.13
AR-10	138.89	– [b]	206.66	0.37	166.32	19.29	0.2	0.29	0.46	284.34	27.84	1.6	4.34	505.05	234.40
AR-11	143.72	– [b]	248.74	0.8	347.86	64.16	0.93	1.27	4.02	519.92	60.61	7.28	7.65	1014.50	197.60
AR-12	152.67	– [b]	238.89	1.6	44.23	85.39	0.8	1.4	17.79	805.92	41.85	6.76	17.34	1023.08	160.40
AF-1	145.32	– [b]	686.20	– [b]	4.9	38.63	0.06	0.7	63.78	17.37	2.53	– [c]	3.97	131.94	76.00
AF-2	154.25	– [b]	304.36	– [b]	5.15	55.03	0.01	0.84	78.52	16.95	3.03	– [c]	5.23	164.76	150.82
AF-3	158.10	– [b]	405.39	– [b]	4.8	99.77	0.05	1.59	79.04	10.07	5.13	0.22	6.25	206.92	122.22
AF-4	152.92	– [b]	269.98	– [b]	6.67	59.83	0.36	0.88	62.66	16.38	12.43	0.57	9.76	169.54	75.86
AF-5	229.17	– [b]	331.64	– [b]	5.28	70.7	0.01	0.90	88.57	16.55	5.57	– [c]	7.33	194.91	51.03
AX-1	112.44	– [b]	378.37	0.75	27.26	17.03	0.45	5.42	56.27	1118.74	8.21	2.42	11.88	1248.43	95.90
AX-2	126.82	– [b]	580.64	0.27	9.86	2.61	0.09	5.14	40.87	833.73	1.05	0.69	5.53	899.84	77.81
AL-1	6.23	91.23	461.41	0.25	751.76	159.17	37.69	26.92	21.13	657.53	191.9	202.05	19.55	2067.95	95.03
AN-1	121.91	17.57	233.76	0.03	27.37	25.39	0.54	1.19	25.05	595.37	72.74	2.99	14.58	765.25	82.50
LO-1	2.09	169.39	353.61	2.87	127.81	31.84	0.13	2.79	115.36	1807.28	15.31	1.47	50.77	2155.63	79.30
AB-1	47.73	5.59	129.55	0.71	14.87	3.17	0.10	2.35	28.77	1779.94	0.89	0.63	17.77	1849.20	58.42
BU-1	74.19	5.95	785.60	0.1	13.26	5.15	0.13	4.28	143.67	672.77	0.45	– [c]	4.64	844.45	14.66
AE-1	3.38	111.08	153.49	0.14	114.84	6.06	0.06	1.44	25.88	1144.03	6.56	1.04	16.65	1316.7	112.96
AZ-1	10.20	83.26	134.25	2.34	11.27	1.85	0.18	0.85	7.52	491.05	0.72	– [c]	1.75	517.53	82.40
GS-1	84.50	145.79	25.25	0.01	– [c]	1.29	0.08	0.06	– [c]	81.74	74.34	8.50	5.98	172.00	58.19
GB-1	75.73	119.26	1336.72	– [c]	6.45	11.56	0.4	3.51	273.10	16.2	2.46	0.73	14.26	328.68	19.20
GY-1	20.50	137.10	2037.81	0.16	179.17	9.05	0.29	3.02	7.48	276.7	3.20	1.17	2.67	482.91	41.92
GR-1	14.81	150.61	1169.51	0.40	7.03	12.7	2.53	27.15	0.47	22.26	4.05	9.95	1.14	87.68	39.78
LD-1	22.77	79.26	4074.80	0.90	1934.81	41.47	0.29	9.50	17.24	1342.73	5.83	3.30	3.58	3359.65	43.44

Note: 1: kinsenoside; 2: gooderoside A; 3: gastrodin; 4: isorhamnetin-3-*O*-β-*D*-neohespeidoside; 5: rutin; 6: isoquercitrin; 7: kaempferol-7-*O*-β-*D*-glucoside; 8: kaempferol-3-*O*-β-*D*-glucoside; 9: isorhamnetin-3-*O*-β-*D*-glucoside; 10: narcissin; 11: quercetin; 12: kaempferol; 13: isorhamnetin; 14: total flavonoids; 15: total polysaccharides

[a] Each value represents for three samples (n = 3);
[b] not detected;
[c] Under detection limit.

biflora, *G. yangmeishanensis*, *G. repens* and *L. discolor* for the first time. Among those *Goodyera* and *Ludisia* species, the highest content of kinsenoside was found in *G. schlechtendaliana* (84.50mg/g).

Goodyeroside A, the epimer of kinsenoside, was found to be abundant in *Goodyera* and *Ludisia* species, ranging from 79.26 to 150.61 mg/g. As shown in Table 4, the content of goodyeroside A in *Goodyera* and *Ludisia* species was significantly higher than that of kinsenoside. Moreover, goodyeroside A was first isolated from *A. zhejiangensis*, and detected in *A. lylei*, *A. longilobus*, *A. elatus*, *A. nandanensis*, *A. brevilabris* and *A. burmannicus* for the first time in this study, but could not be detected in *A. formosanus* and *A. xingrenensis* (Fig. 6). Notably, the content of goodyeroside A in *A. lylei*, *A. longilobus*, *A. elatus* and *A. zhejiangensis* (91.23 mg/g, 169.39 mg/g, 111.08 and 83.26 mg/g, respectively) is much higher than that of kinsenoside. Therefore, these findings of the present study suggested that *A. lylei*, *A. longilobus*, *A. elatus*, *A. zhejiangensis*, *Ludisia discolor* and *Goodyera* species cannot be used as alternatives for *A. roxburghii*, and goodyeroside A thus can be reasonably used as a diagnostic marker for distinguishing *A. roxburghii* from *A. lylei*, *A. longilobus*, *A. elatus*, *A. zhejiangensis*, and other *Goodyera* and *Ludisia* species.

The total polysaccharide contents of all the tested samples were determined by the phenol-sulphuric acid method (Chinese Pharmacopoeia Committee, 2015) with *D*-glucose as standard. Polysaccharides were also found to be abundant in all samples, except in *G. biflora* and *A. burmannicus* (sample GB-1 and BU-1). The tissue-cultured *A. roxburghii* contained the highest contents of total carbohydrate (sample AR-10, 234.40 mg/g), which was 10-fold higher than that of the wild *A. roxburghii* (sample AR-3, 22.70 mg/g). This may be attributed to rich sugar source of the tissue culture medium for *A. roxburghii*.

The established iHPLC-MS/MS method was applied to the simultaneous quantitative analysis of ten flavonoids and gastrodin in ten *Anoectochilus* species and five related species. As showed in Table 4, flavonoid compounds were present in all tested samples, and the total flavonoid contents were ranged from 87.68 to 3694.87 μg/g. The huge variability of the flavonoids contents among these *Anoectochilus* species and the other related samples may derive from inter- or intra- specific differences, and different growing environment, climatic variables and time harvesting. However, it was clearly observed that the total flavonoid contents in the wild samples of *A. roxburghii* with obvious leaf veins were significantly higher than those of tissue-cultured samples of *A. roxburghii* and *A. formosanus*. Moreover, among these detected flavonoids, narcissin was the predominant constituents, with higher contents in *Anoectochilus* species, except in *A. formosanus* and *A. lylei*. It was clearly observed that tissue-cultured samples of *A. formosanus* had the lowest quantity of narcissin and rutin (10.07–17.37 μg/g and 4.80–6.67 μg/g, respectively), and no detectable amount of isorhamnetin-3-*O*-β-*D*-neohespeidoside was found in this species. In addition, among the detected flavonoids, only *A. lylei* contained the highest amounts of rutin in *Anoectochilus* species. Therefore, narcissin, rutin and isorhamnetin-3-*O*-β-*D*-neohespeidoside may be used as diagnostic markers for distin-

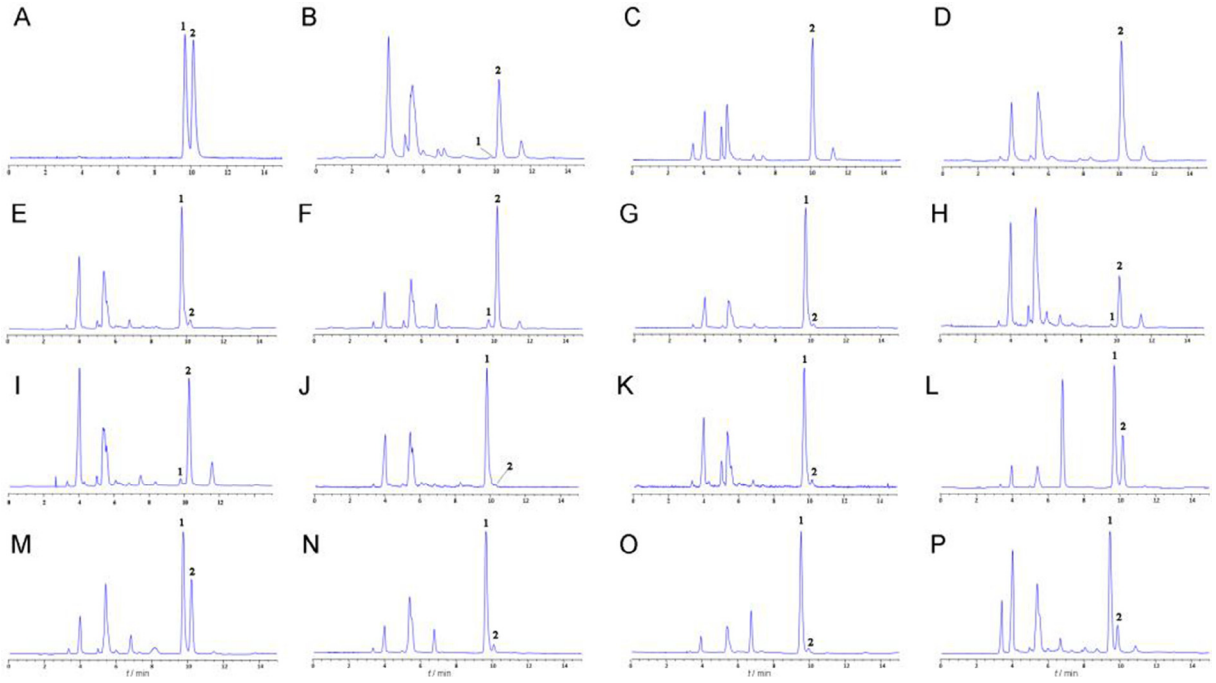

Fig. 6. HPLC-ELSD chromatograms of ten *Anoectochilus*, four *Goodyera* and one *Ludisia* species on AQ-C_{18} column. A) standards mixture of kinsenoside and goodyeroside; B) *A. roxburghii*; C) *A. formosanus*; D) *A. xingrenensis*; E) *A. lylei*; F) *A. nandanensis*; G) *A. longilobus*; H) *A. brevilabris*; I) *A. burmannicus*; J) *A. elatus*; K) *A. zhejiangensis*; L) *G. schlechtendaliana*; M) *G. biflora*; N) *G. yangmeishanensis*; O) *G. repens*; P) *L. discolor*. 1. kinsenoside; 2. goodyeroside A.

guishing *A. formosanus* and *A. lylei* from other *Anoectochilus* species plants. Furthermore, the contents of quercetin also varied remarkably in different batches of *A. roxburghii* samples, ranging from 6.10 to 395.65 μg/g. It is noteworthy that the contents of quercetin in wild *A. roxburghii* samples AR 5–7 (Chinese name Jinxianlian Gong, without obvious leaf veins as indicated in Fig. 2) were extremely low, ranging from 6.10 to 9.32 μg/g, which might be correlated with their morphological differences. In addition, except *A. roxburghii* and *A. formosanus*, gastrodin was detected in all the other tested samples for the first time, ranging from 25.25 to 4074.80 μg/g. This bioactive phenolic glycoside has much higher yields in four samples, including *G. biflora*, *G. yangmeishanensi*, *G. repens* and *Ludisia discolor*, which suggested that these four species might be considered as an ideal botanical source of gastrodin.

In order to control the quality of Jinxianlian, our results indicated that quantitative determination of lactone glycosides and flavonoids in these medicinal materials would become necessary. What's more, in term of the difficulty in authenticating the origins of Jinxianlian by conventional morphological and histological identification, the contents of lactone glycosides and flavonoids could be used as valuable and practical chemical evidence. Finally, our established methods should support the comprehensive quality evaluation of *Anoectochilus*, *Goodyera* and *Ludisia* species, using lactone glycosides and flavonoids (kinsenoside, goodyeroside A and narcissin) as indicative constituents.

4. Conclusion

This is the first study on the qualitative and quantitative determination of multi-class bioactive components in ten species of *Anoectochilus* and five species of other similar genera. In *Anoectochilus* species, kinsenoside was detected in *A. xingrenensis*, *A. lylei*, *A. nandanensis*, *A. longilobus*, *A. brevilabris*, *A. burmannicus* and *A. elatus* for the first time. Moreover, goodyeroside A was also detected in *A. zhejiangensis*, *A. lylei*, *A. longilobus*, *A. elatus*, *A. nandanensis*, *A. brevilabris* and *A. burmannicus* for the first time. Remarkable differences were observed in the contents of those multi-class bioactive constituents in all samples, including both wild plants and tissue cultures. Our results indicate that kinsenoside was the predominant constituent in *A. roxburghii*, *A. formosanus*, *A. xingrenensis*, *A. nandanensis*, *A. brevilabris* and *A. burmannicus*, whereas goodyeroside A was the predominant constituent in *A. lylei*, *A. longilobus*, *A. elatus*, *A. zhejiangensis*, *G. schlechtendaliana*, *G. biflora*, *G. yangmeishanensis*, *G. repens* and *L. discolor*. In addition, kinsenoside was found to be abundant especially in tissue-cultured *A. formosanus* and its contents in tissue cultures were generally higher than that of wild *A. roxburghii* samples. The contents of polysaccharides were also found higher in tissue cultures, whereas flavonoids were much richer in wild *A. roxburghii* samples with obvious leaf veins.

Some *Anoectochilus* species, such as *A. lylei*, *A. longilobus*, *A. elatus* and *A. zhejiangensis* possess high content of goodyeroside A, while the content of kinsenoside is very low, thus indicating that these *Anoectochilus* species cannot be considered as the desirable alternatives for the authentic origin of Jinxianlian (*A. roxburghii*). Our results may provide some favorable chemical evidence for the comprehensive utilization of these medicinal resources. The developed method could be potentially used for the quality evaluation and control of *Anoectochilus* and some related species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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