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Determination of Oleanolic Acid and Ursolic Acid in *Oldenlandia diffusa* and Its Substitute Using High Performance Liquid Chromatography

ZHITAO LIANG, ZHIHONG JIANG, DAVID WANGFUN FONG AND ZHONGZHEN ZHAO*

School of Chinese Medicine, Hong Kong Baptist University, Kowloon, Hong Kong, China

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ABSTRACT

An improved quality evaluation method for *Oldenlandia diffusa* (Willd.) Roxb. and its substitute was established by a well-defined high performance liquid chromatographic (HPLC) method. Firstly, a complete separation of oleanolic acid and ursolic acid was successfully achieved by optimizing the mobile phase systems based on previous reports. The extraction method was optimized and the less toxic solvent was selected for extraction. The systematic survey of herbal markets has shown that *O. corymbosa* (L.) Lam are widely available and used indiscriminately as *O. diffusa*. Twenty-seven samples of *O. diffusa* and seventeen samples of *O. corymbosa* were analyzed. The results showed that the contents of oleanolic acid and ursolic acid in *O. diffusa* were generally lower by almost two times than those in *O. corymbosa*. Moreover, the contents of oleanolic acid and ursolic acid between the fresh and dry samples were compared. The introduction of quality evaluation method could be useful for the species identity and quality evaluation of *O. diffusa* and its substitute.

Key words: *Oldenlandia diffusa*, *Oldenlandia corymbosa*, oleanolic acid, ursolic acid, HPLC

INTRODUCTION

The herb of *Oldenlandia diffusa* (Willd.) Roxb. (Family Rubiaceae), a well known folk medicine in China, has been used for the treatment of tonsillitis, sore throat, appendicitis and urethral infection since the Qing Dynasty. In recent years it has generated a great deal of interest because it is widely used in the treatment of hepatitis and malignant tumors of the liver[1]. According to the Chinese Pharmacopoeia, *O. diffusa* has been recorded as an ingredient of many Chinese patent medicines[2]. However, a systematic market investigation has shown that in the wholesale and food markets of Hong Kong, Macau and Guangdong province, *O. corymbosa* (L.) Lam has also been used indiscriminately as *O. diffusa*[3]. Based on fifteen samples collected from the same herbal market over an eight-month period, only five of the fifteen batches were *O. diffusa* and the other ten were *O. corymbosa*. Although in the previous study, both the water extracts of fresh Herba *Oldenlandiae diffusae* and Herba *Oldenlandiae corymbosae* had anti-tumor effects on implanted subcutaneous tumors induced by sarcoma-180 cells[4], confusions in the markets had led to a growing concern about the difference of the two herbs.

Oleanolic acid and ursolic acid can be found in *O. diffusa* and several other plants[5-9]. Pharmacological studies have shown that the two compounds have protective effects for both acute chemically injured liver and chronic liver fibrosis and cirrhosis[10,11], inhibit tumor initiation and promotion, and induce tumor cell differentiation and apoptosis[12-15]. Since these bioactivities can be related to the medicinal functions of *O. diffusa*, oleanolic acid and ursolic acid have been suggested for use as marker compounds for the quality evaluation of *O. diffusa*[16-18]. However, we are not aware of any previous report on the systematic and comparative study on contents of these two compounds in the two herbs.

Oleanolic acid and ursolic acid are isomers with similar chemical structures and therefore are difficult to separate. In recent years, a number of methods have been investigated for the quantitative analysis of oleanolic acid and ursolic acid in a variety of raw materials. These methods include high-performance liquid chromatography (HPLC)[19-27], gas chromatography (GC)[28], cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC)[29], micellar electrokinetic capillary chromatography (MECC)[30] and nonaqueous capillary electrophoresis (NACE)[31]. These methods have various drawbacks such as time-consuming and tedious sample preparation, demanding pre-column deriva-

* Author for correspondence. Tel: +852-3411-2424;
Fax: +852-3411-2461; E-mail address: zzzhao@hkbu.edu.hk

tion for GC analysis, complex buffer systems, and low reproducibility in the CD-MEKC, MECC and NACE methods. HPLC with acidic aqueous buffer as the mobile phase is arguably the most popular method for the determination of oleanolic acid and ursolic acid (Table 1) but this method often results in incomplete resolution.

The present study focused on the development of a rapid and accurate method for the quantitative analysis of ursolic acid and oleanolic acid in a multiple samples of *O. diffusa* and *O. corymbosa*. A convenient and reliable RP-HPLC method was developed based on optimizing of previous reported methods and a series of validation method was studied.

MATERIALS AND METHODS

I. *Materials*

(I) *Plant Materials*

Twenty-seven samples of *O. diffusa* (Willd.) Roxb. and seventeen samples of *O. corymbosa* (L.) Lam were collected from herbal markets or from cultivation sites in China (Table 2). Five batches of *O. diffusa* (samples 23 to 27) and ten batches of *O. corymbosa* (samples 28 to 37) were collected from the same herbal market over an eight-month period. All samples were authenticated by the authors and the voucher specimens were deposited in the Bank of China (HK) Chinese Medicines Centre of Hong Kong Baptist University.

Table 1. Reported use of mobile phase systems and columns in literatures

Method	Mobile phase (v/v)	Flow Rate (mL/min)	Column	References
HPLC-UV	methanol-water （82:18）	1.0	Kromasi C-18 ODS (4.6 × 250 mm, 5 μm)	16
HPLC-UV	acetonitrile-20 mmol potassium phosphate (45:55, pH=7.84)	1.0	Nucledure C-18 ODS (4.6 × 150 mm, 5 μm)	17
HPLC-UV	methanol-20 mmol/L tetrabutyl ammonium bromide-triethylamine (90:10:0.02) (pH was adjusted to 6.9 with acetic acid)	0.3	HiQ sil C18 V (4.6 × 150 mm, 5 μm)	18
HPLC-UV	methanol-water- acetic acid - triethylamine (83:17: 0.04:0.02)	1.0	Shim-packCLC-ODS (4.6 × 150 mm, 5 μm)	9
HPLC-UV	acetonitrile-water (85:15)	0.6	C18 ODS *	5
HPLC-ELSD	deionized water-isopropyl alcohol-tetrahydrofuran -acetic acid (90:10:6:1) and methanol-isopropyl alcohol-tetrahydrofuran-acetic acid (90:10:6:1)	1.0	C18 ODS *	19
HPLC-UV	acetonitrile- water (containing phosphoric acid)	1.0	Spherisob C-18 ODS (4.6 × 250 mm, 5 μm)	7
HPLC-UV	methanol-water (containing 0.03% phosphoric acid) (92:8)	0.8	Hypersil BDS C18(4.6 × 250 mm, 5 μm)	8
HPLC-ELSD	methanol-water (87:13)	1.0	Alltima C-18 (4.6 × 250 mm, 5 μm)	20
HPLC-UV	methanol-water (containing 0.08% glacial acetic acid) (94:6)	0.6	C-18 ODS (4.6 × 250 mm, 5 μm) *	21
HPLC-UV	methanol- water (containing 1% acetic acid) (88:12)	0.6	Zorbax ODS (4.6 × 250 mm, 5 μm)	22
HPLC-UV	water-methanol-glacial acetic acid-triethylamine (10: 90: 0.03: 0.06)	0.6	ODS C18 column (4.6 × 250 mm, 5 μm)	23
HPLC-ELSD	methanol-water-acetic acid (260: 40: 0.15)	0.6	Kromasil C18 (4.6 × 250 mm, 5 μm)	24
HPLC-UV	acetonitrile-methanol-water-ammonium acetate (70: 16: 14: 0.5)	1.0	Shim-Pack C18 (4.6 × 150 mm, 5 μm)	25
HPLC-ELSD	methanol- water (containing 0.5% CH_3COONH_4) (90:10)	1.0	Lichrospher C18 (4.6 × 250 mm, 5 μm)	26
HPLC-UV	methanol-phosphate (89:11)	0.8	Zorbax80A Extend-C18 (4.6 × 250 mm, 5 μm)	27

* The detail was not found in the original literature

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Table 2. Contents of oleanolic acid and ursolic acid in various samples of *Oldenlandia diffusa* and *Oldenlandia corymbosa*

Sample No.		Source	Collecting time	Oleanolic acid*	Ursolic acid*
1	*Oldenlandia diffusa*	Yibin, Sichuan, China; field	2004. 08.01	0.489 ± 0.023	2.482 ± 0.033
2		Chongqing, Sichuan, China; market	2004. 07. 25	0.609 ± 0.047	2.627 ± 0.061
3		Yixing, Jiangsu, China; cultivated	2004. 08. 16	0.531 ± 0.018	2.450 ± 0.066
4		Nanjing, Jiangsu, China; market	2004. 08. 15	0.543 ± 0.0001	3.119 ± 0.062
5		Hefei, Anhui, China; market	2004. 08. 17	0.418 ± 0.010	2.021 ± 0.065
6		Fuan, Fujian, China; field	2005. 08. 21	0.773 ± 0.027	3.577 ± 0.039
7		Putian, Fujian, China; field	2005. 08. 21	0.610 ± 0.005	2.825 ± 0.068
8		Fuzhou, Fujian, China; field (batch0821)	2005. 08. 21	0.435 ± 0.011	2.079 ± 0.011
9		Fuzhou, Fujian, China; field (batch0716)	2006. 07. 16	0.403 ± 0.009	1.954 ± 0.051
10		Xiapu, Fujian, China; field	2006. 07. 16	0.560 ± 0.032	2.577 ± 0.022
11		Kunming, Yunnan, China; market	2004. 08. 24	0.549 ± 0.010	2.473 ± 0.040
12		Nanning, Guangxi, China; field	2005. 07. 04	0.408 ± 0.005	1.982 ± 0.050
13		Nanning, Guangxi, China; market	2005. 07. 04	0.387 ± 0.003	1.833 ± 0.035
14		Wuhan, Hubei, China; market	2006. 07. 08	0.488 ± 0.006	2.165 ± 0.030
15		Beijing, China; market	1999. 07. 08	0.743 ± 0.002	2.974 ± 0.039
16		Lushan, Jiangxi, China; field	2005. 10.18	0.479 ± 0.043	1.971 ± 0.004
17		Ruyuan, Guangdong, China; field	2004. 07. 09	0.526 ± 0.015	2.537 ± 0.032
18		Gaoyao, Guangdong, China; field	2004. 09. 02	0.386 ± 0.027	1.642 ± 0.056
19		Guangzhou, Guangdong, China; market	2005. 03. 24	0.462 ± 0.008	2.130 ± 0.027
20		Shenzhen, Guangdong, China; cultivated	2005. 10. 30	0.496 ± 0.019	2.274 ± 0.081
21		Luoding, Guangdong, China; field	2004. 08. 19	0.246 ± 0.008	1.339 ± 0.034
22		Luoding, Guangdong, China; market	2004. 08. 24	0.411 ± 0.026	1.755 ± 0.108
23		Luoding, Guangdong, China; market (batch: LD0517)	2005. 05. 17	0.656 ± 0.015	2.690 ± 0.028
24		Luoding, Guangdong, China; market (batch: LD0601)	2005. 06. 01	0.698 ± 0.014	2.985 ± 0.103
25		Luoding, Guangdong, China; market (batch: LD0616)	2005. 06. 16	0.559 ± 0.004	2.640 ± 0.055
26		Luoding, Guangdong, China; market (batch: LD0901)	2005. 09. 01	0.423 ± 0.015	2.180 ± 0.015
27		Luoding, Guangdong, China; market (batch: LD1015)	2005. 10. 15	0.583 ± 0.021	2.521 ± 0.093
		Mean ± SD		0.514 ± 0.119	2.363 ± 0.497
28	*Oldenlandia corymbosa*	Luoding, Guangdong; market (batch: LD0701)	2005. 07. 01	2.052 ± 0.087	8.754 ± 0.571
29		Luoding, Guangdong; market (batch: LD0715)	2005. 07. 15	1.904 ± 0.008	8.105 ± 0.027
30		Luoding, Guangdong; market (batch: LD0731)	2005. 07. 31	1.314 ± 0.038	5.576 ± 0.061
31		Luoding, Guangdong; market (batch: LD0815)	2005. 08. 15	1.705 ± 0.006	7.594 ± 0.161
32		Luoding, Guangdong; market (batch: LD0915)	2005. 09. 15	1.174 ± 0.059	5.356 ± 0.057
33		Luoding, Guangdong; market (batch: LD1001)	2005. 10. 01	2.451 ± 0.035	10.781 ± 0.089
34		Luoding, Guangdong; market (batch: LD1101)	2005. 11. 01	1.863 ± 0.021	8.331 ± 0.271
35		Luoding, Guangdong; market (batch: LD1115)	2005. 11. 15	1.585 ± 0.040	6.878 ± 0.170

Table 2. Continued

Sample No.	Source	Collecting time	Oleanolic acid*	Ursolic acid*
36	Luoding, Guangdong; market (batch: LD1201)	2005. 12. 01	1.864 ± 0.054	7.959 ± 0.003
37	Luoding, Guangdong; market (batch: LD1215)	2005. 12. 15	1.167 ± 0.060	6.645 ± 0.453
38	Shenzhen, Guangdong; cutivated	2005. 10. 30	1.751 ± 0.033	7.702 ± 0.565
39	Shenzhen, Guangdong; market	2006. 06. 26	1.580 ± 0.030	6.741 ± 0.113
40	Wutongzai, Hong Kong; field	2004. 08. 09	1.778 ± 0.005	7.806 ± 0.060
41	Hong Kong; market	2004. 10. 23	1.847 ± 0.0003	8.107 ± 0.169
42	Gaoyao, Guangdong; market	2004. 09. 02	1.478 ± 0.027	6.557 ± 0.187
43	Guangzhou, Guangdong; market	2004. 08. 26	1.900 ± 0.067	7.663 ± 0.257
44	Nanning, Guangxi; field	2005. 07. 04	1.328 ± 0.072	7.702 ± 0.565
	Mean ± SD		1.691 ± 0.333	7.544 ± 1.251

* mg/g, Mean ± SD (n = 2)

(II) *Chromatographic System*

A CREST 1875HTAG ultrasonic processor (CREST, USA) was used for sample extraction. An Agilent 1100 series HPLC system equipped with a G1311A Quaternary Pumps, a G1315A Dio-Array Detector, a G1322A Degasser and a G1313A autosampler was used. A Zorbax Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm, Agilent Technologies, USA) coupled with a C18 guard column (7.5 mm × 4.6 mm, 5 μm, Alltech Associates, USA) was used at room temperature. The mobile phase was a mixture of methanol-water (83:17 containing 0.2% NH_4OAc, pH6.74) at a flow-rate of 1.0 mL/min. The detection wavelength was set at 210 nm with a reference wavelength of 550 nm.

(III) *Chemicals and Reagents*

Analytical and HPLC grade methanol, HPLC grade acetonitrile, chloroform, ethyl acetate and acetone (Labscan, Bangkok, Thailand) and analytical grade ammonium acetate (Farco chemical supplies) were used. De-ionized water used in the mobile phase was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). Oleanolic acid standard was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China (Batch No. 110709-200304). The reference sample of ursolic acid was isolated and purified from the dried herb of *Oldenlandia diffusa* (Willd.) Roxb. in our laboratory and its structure was confirmed on the basis of HR-MS, ^{1}H-NMR and ^{13}C-NMR spectral data[32]. The purity of ursolic acid as determined by HPLC-UV was greater than 98%.

II. *Methods*

(I) *Preparation of Standard Solutions*

Oleanolic acid and ursolic acid were weighed and dissolved in methanol to produce stock solutions at concentrations of 503 μg/mL and 1354 μg/mL, respectively. The stock solutions were diluted in methanol to make standard solutions that remained stable for at least three days at 4°C. Aliquots of 0.1, 0.25, 0.5, 1.0, 2.0 and 4.0 mL of standard solutions were transferred to 10 mL volumetric flasks and made up to volume with methanol to give calibration solutions. Aliquots of 20 μL of calibration solutions were analyzed by HPLC. Calibration curves were constructed by plotting the peak areas of the standards against their respective concentrations.

(II) *Preparation of Sample Extracts*

Samples were ground into powder and passed through a 20-mesh (0.9 mm) sieve. Each 1.0 g of powdered sample was transferred into a 50 mL centrifuge tube and extracted twice with 25 mL of acetone by sonication for 60 min at room temperature. Supernatants were collected after centrifugation at 3000 rpm (Centrifuge 5810, Eppendorf Ltd, Germany) for 5 min, combined and dried under reduced pressure. The solid residues were dissolved in methanol and then transferred into a 25 mL volumetric flask and made to volume with methanol. The solution was then filtered through a 0.45 μm membrane and 20 μL aliquots were analyzed by HPLC.

RESULTS AND DISCUSSION

I. *Selection of Mobile Phase*

To find the optimal elution conditions, nine previously reported mobile phase systems composed of simple

reagents were investigated (Figure 1). Acidic aqueous buffers have been used as the mobile phase in most HPLC studies. However, we have found that these buffers, such as those containing phosphoric acid and acetic acid, may not be able to consistently and completely separate oleanolic acid and ursolic acid. With the mobile phase system consisting methanol, acetic acid, triethylamine and water, complete separation of chromatographic peaks was difficult to achieve even under various flow rates. Another system consisting of acetonitrile, methanol, ammonium acetate and water was investigated and the separation was improved. When this system was changed to methanol-0.2% ammonium acetate in water (83:17), a complete separation of oleanolic acid and ursolic acid was successfully achieved (Figure 2). The resolution could reach 1.65 and the number of theoretical plate for oleanolic acid and ursolic acid were 9558 and 10131, respectively.

II. *Evaluation of Extraction Method*

Previous studies mainly used chloroform and acetone as extraction solvents on a soxhlet apparatus[14-16]. However, chloroform is toxic and the soxhlet extraction method is time-consuming. The extraction conditions were optimized with reference to the oleanolic acid and ursolic acid contents. Using the soxhlet extraction method, the contents of oleanolic acid and ursolic acid in chloroform extracts were 0.630 ± 0.047 mg/g (Mean ± SD, n = 3) and 2.948 ± 0.040 mg/g, respectively. For the same raw materials and in acetone extracts the contents of oleanolic acid and ursolic acid were 0.643 ± 0.034 mg/g and 2.930 ± 0.060 mg/g, respectively. The results indicated minor difference between the two solvents on the yields of oleanolic acid and ursolic acid. Sonication did not significantly improve the extraction efficiency of oleanolic acid and ursolic acid but greatly reduced the time required. Using the same raw materials after sonication, the contents of oleanolic acid and ursolic acid in acetone extracts were 0.616 ± 0.011 mg/g and 2.937 ± 0.008 mg/g, respectively. Repeated extraction with the same solvents offered no further advantage. When samples were extracted in 25 mL acetone by sonication four times for 60 min at room temperature, oleanolic acid and ursolic acid could not be detected in the last two extracts. Overall it could be concluded that the extraction scheme as outlined above was optimized for the analysis.

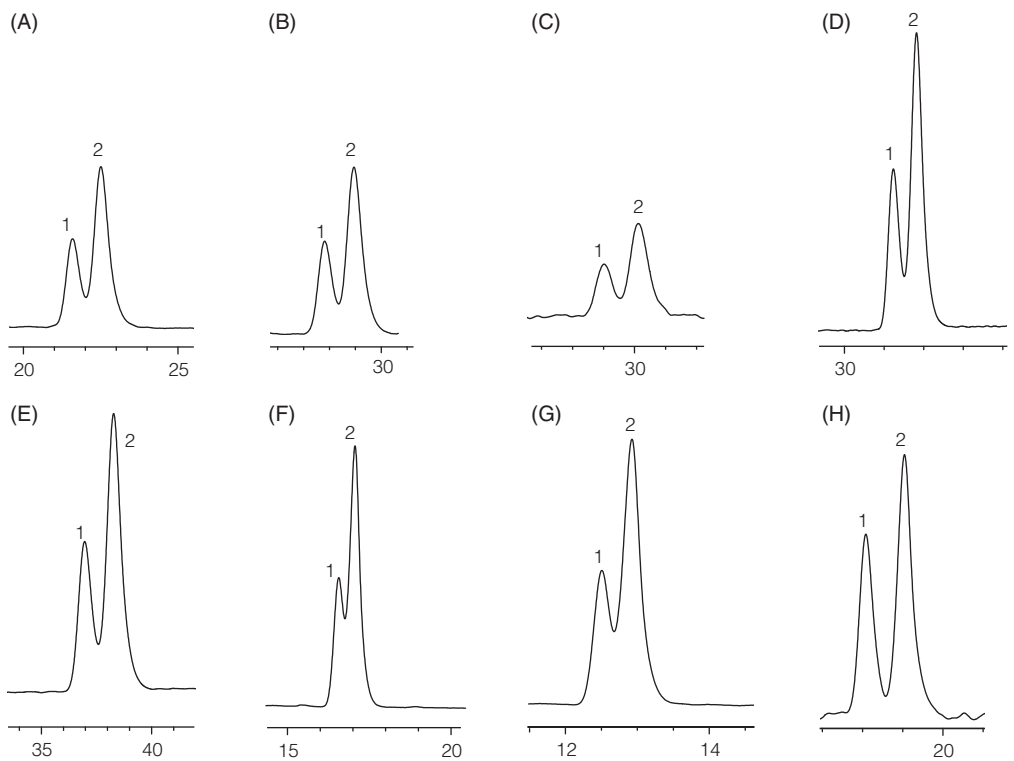

Figure 1. HPLC chromatograms of A (mobile phase: methanol-water 87:13, flow rate 1.0 mL/min, resolution 1.22); B (mobile phase: methanol-0.05% phosphoric acid in water 85:15, flow rate 1.0 mL/min, resolution 1.20); C (mobile phase: methanol- 0.5% acetic acid in water 88:12, flow rate 1.0 mL/min, resolution 1.16); D (methanol-water-acetic acid-triethylamine 85:15:0.04:0.02, flow rate 0.8 mL/min, resolution 1.10); E (methanol-water-acetic acid-triethylamine 85:15:0.04:0.02, flow rate 0.6 mL/min, resolution 1.13); F (acetonitrile-0.05% phosphoric acid in water 85:15, flow rate 1.0 mL/min, resolution 1.13); G (acetonitrile-water-phosphoric acid-triethylamine 85:15:0.03:0.05, flow rate 1.0 mL/min, resolution 1.18); and H (acetonitrile-methanol-water-ammonium acetate 70:10:20:0.2, flow rate 1.0 mL/min, resolution 1.53); Peak 1: oleanolic acid; Peak 2: ursolic acid.

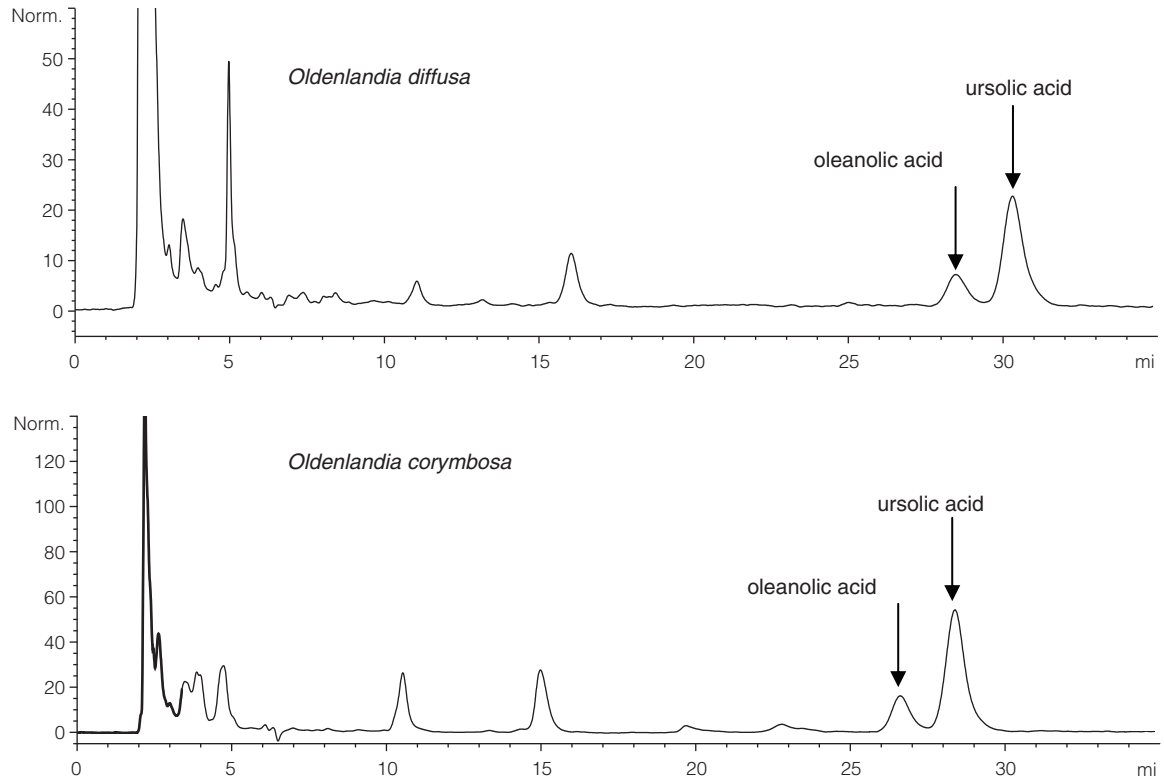

Figure 2. Typical HPLC chromatograms of *Oldenlandia diffusa* and *Oldenlandia corymbosa*

III. *Method Validation*

Satisfactory linearity for the analysis of each compound was obtained. Linearity was examined with a series of standard solution in the concentration range of 5.03-201.2 μg/mL for oleanolic acid and 13.54-541.6 μg/mL for ursolic acid. The linear regression equations of the calibration curves of oleanolic acid was calculated to be y = 9160.3x-1.21 with a correlation coefficient of R^2 = 0.9998 (n = 6), and for ursolic acid y = 8219.9x-26.176 with a correlation coefficient of R^2 = 0.9999 (n = 6).

Method precision was investigated by repeatedly analyzing the same set of sample solution, the values of relative standard deviations (RSDs) were 0.77% and 0.92% (n = 6) for oleanolic acid and ursolic acid, respectively.

Method reproducibility was evaluated by six replicated analyses of herbal samples. The RSD of the contents of oleanolic acid and ursolic acid in six replicated herbal samples was 1.81% and 0.87%, respectively.

Stability testing was performed on a sample solution after standing for 0, 2, 4, 6, 8, 12, 24, 48 and 72h. The results showed that the RSD of oleanolic acid and ursolic acid was 1.06% and 0.48% (n = 6) after 12h and 0.84% and 0.53% (n = 3) after three days, respectively. We conclude that oleanolic acid and ursolic acid are relatively stable in methanol for at least three days.

Recovery was studied in samples spiked with 50, 100 and 150% of the known contents of oleanolic acid and ursolic acid. Our results showed that the average recoveries of oleanolic acid and ursolic acid were 99.8% with an average RSDs of 3.44% (n = 9), and 100.4% with average RSDs of 2.60% (n = 9), respectively (Table 3).

The limit of detection, evaluated by a signal-to-noise ratio of about 3:1 for the standard solution, was determined to be 2.51 μg/mL and 6.77 μg/mL for oleanolic acid and ursolic acid, respectively.

IV. *Comparison of the Contents of Oleanolic Acid and Ursolic Acid in Oldenlandia diffusa and Its Substitutes*

Contents of oleanolic acid and ursolic acid were determined using the above analytical method (Table 3). Forty-four samples of *O. diffusa* and *O. corymbosa* collected from different geographic areas were studied. Oleanolic acid and ursolic acid were well separated by our HPLC method in the two species (Figure 2).

The average content of oleanolic acid and ursolic acid in seventeen batches of *O. corymbosa* was 1.691 ± 0.333 and 7.544 ± 1.251 mg/g (n = 17), respectively. These values were about two times higher than those in twenty-seven batches of *O. diffusa* which were 0.514 ± 0.119 and 2.363 ± 0.497 mg/g (n = 27), respectively (Table 3). Such results indicated that the herb of *O. corymbosa* may be used as *O. diffusa* for medicinal use. Howev-

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Table 3. Recovery of oleanolic acid and ursolic acid during extraction

Compound	Spiked (mg)	Level found (mg) (Mean ± SD)*	Recovery (%, Mean ± SD)	RSD (%)	Overall Recovery (%, Mean ± SD)
Oleanolic acid	0.159	0.158 ± 0.007	99.5 ± 4.25	4.27	99.8 ± 3.43 (RSD: 3.44 %)
	0.318	0.319 ± 0.012	100.4 ± 3.82	3.81	
	0.636	0.632 ± 0.024	99.4 ± 3.71	3.73	
Ursolic acid	0.537	0.540 ± 0.012	100.6 ± 2.26	2.25	100.4 ± 2.61 (RSD: 2.60 %)
	1.074	1.072 ± 0.035	99.8 ± 3.23	3.24	
	2.148	2.168 ± 0.070	100.9 ± 3.28	3.25	

*Standard deviation (n = 3).

Table 4. Contents of oleanolic acid and ursolic acid in dry and fresh samples of *Oldenlandia diffusa* and *Oldenlandia corymbosa*

Species	Source	Oleanolic acid*		Ursolic acid*	
		dry	fresh	dry	fresh
O. diffusa	Nanning, Guangxi; cultivated; collected in Aug 26, 2006	0.442 ± 0.011	0.354 ± 0.001	1.897 ± 0.080	1.909 ± 0.094
O. corymbosa	Lefu, Hong Kong; market; collected in Jul 03, 2006	1.966 ± 0.085	2.039 ± 0.122	8.414 ± 0.076	7.811 ± 0.429
O. corymbosa	Lefu, Hong Kong; market; collected in Jul 19, 2006	2.068 ± 0.055	2.369 ± 0.112	8.725 ± 0.173	8.776 ± 0.070
O. corymbosa	Jiulongcheng, Hong Kong; market; collected in Oct 16, 2006	2.142 ± 0.048	2.261 ± 0.002	9.450 ± 0.036	9.160 ± 0.030
O. corymbosa	Taizhong, Taiwan; market; collected in Oct 21, 2006	1.700 ± 0.078	1.877 ± 0.047	6.858 ± 0.100	6.957 ± 0.315
O. corymbosa	Huangdaxian, Hong Kong; market; collected in Jul 10, 2006	2.004 ± 0.016	2.049 ± 0.007	8.415 ± 0.218	7.827 ± 0.153
Average		1.720 ± 0.644	1.825 ± 0.741	7.293 ± 2.776	7.073 ± 2.648

* mg/g , Mean ± SD (n = 2)

er, it is interesting to note that in our previous studies the contents of iridoid glucosides, another prominent components of *O. diffusa*, were found to be much higher in *O. diffusa* than in *O. corymbosa*[32]. Because of these distinctions it is not recommended to use *O. corymbosa* and *O. diffusa* interchangeably until further supportive scientific data on the pharmacological effects of oleanolic acid, ursolic acid and iridoid glucosides are available. However, it can be fairly straightforward to chemically differentiate the two herbs based on the oleanolic acid and ursolic acid contents.

Fifteen batches of samples labeled as *O. diffusa* were collected in the same Guangdong herbal market in a period of eight months. Based on morphological features only five batches were authenticated as *O. diffusa* and the other ten batches were identified as *O. corymbosa*. Our morphological identification by herbal experts was completely in line with results obtained from this study. The average content of oleanolic acid and ursolic acid in the five batches of *O. diffusa* were 0.584 ± 0.106 mg/g and 2.603 ± 0.292 mg/g, respectively. As for *O. corymbosa*, the average content of oleanolic acid and ursolic acid in the ten batches was 1.708 ± 0.409 mg/g and 7.598 ± 1.596 mg/g, respectively.

In China, dry herb of *O. diffusa* was preferentially used as a medicinal material. However, the fresh state of *O. diffusa* has been used as an important herb tea ingredient in South China. Therefore, the contents of oleanolic acid and ursolic acid were determined for comparing dry and fresh *O. diffusa* and *O. corymbosa*. Here, the fresh samples were collected in the herbal markets, stored at -80°C to slow down any enzymatic activities, and then dried by vacuum freezing as tested fresh samples. The remaining fresh samples were dried with an drying oven under 60°C as tested dry samples. Using the developed methods, six batches of *O. diffusa* and *O. corymbosa* were analyzed (Table 4). The results showed that the average content of oleanolic acid and ursolic acid in the dry samples was 1.720 ± 0.644 mg/g (n = 6) and 7.293 ± 2.776 mg/g, respectively. As for the fresh samples, the average contents were 1.825 ± 0.751 mg/g and 7.073 ± 2.648 mg/g (n = 6). The results indicated minor differences of the contents of oleanolic acid and ursolic acid between the dry and fresh samples.

Here, a convenient method has been developed for the quantitative analysis of the contents of oleanolilc acid and ursolic acid in *O. diffusa* and its substitutes. The validation data indicated that this method is reliable. Satisfactory chromatographic separation of oleanolilc acid and ursolic acid could be obtained by using the mobile phase of methanol-0.2% ammonium acetate in water (83:17). The extraction efficiency of oleanolilc acid and ursolic acid was optimized by comparison with extraction solvents and methods.

Although oleanolic acid and ursolic acid were commonly used as marker compounds for the quality assessment of *O. diffusa* and its substitute, multi-samples analysis and comparison were carried out for the first time. The analytical results here showed that the levels of oleanolic acid and ursolic acid were in lower contents in *O. diffusa* than those of *O. corymbosa*. Based on the different contents of oleanolic acid and ursolic acid, it is easy to identify *O. diffusa* and its substitute of *O. corymbosa*. Furthermore, the herbs of *O. diffusa* and *O. corymbosa* should be used as independent herbal medicines.

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