

Determination of oleanolic acid and ursolic acid contents in *Ziziphora clinopodioides* Lam. by HPLC method

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ABSTRACT

A simple, precise, rapid and accurate, binary-phase high performance liquid chromatographic method has been developed for the determination of oleanolic acid and ursolic acid contents in the *Ziziphora clinopodioides* Lam. with short run time. Chromatographic separation is achieved by using HPLC system consisting of a Shimadzu LC-6AD and Kromasil C₁₈ column (150 × 4.6 mm, 10 μm, with pre-column), the mobile phase consists of methanol and 0.03 M phosphate buffer (pH = 3, 90:10). Detection wavelength is 214 nm. The speed of flow is 0.5 ml/min. The specimen handing quantity is 10 μl. The oleanolic acid's linearity range is 0.4 ~ 1.2 mg/ml ($r = 0.9996$). The ursolic acid's linearity range is 0.6 ~ 1.8 mg/ml ($r = 0.9996$), and the linear relationship is accurate. The average recovery ($n = 6$) of oleanolic acid is 99.5% (RSD = 1.19%) and ursolic acid is 102.3% (RSD = 1.25%). The content of oleanolic acid and ursolic acid in *Ziziphora clinopodioides* are 0.76 mg/g and 1.176 mg/g, respectively. The developed HPLC method can therefore be applied to both *in vitro* studies of oleanolic acid and ursolic acid formulations as well as drug estimation in biological samples.

Key words: HPLC, oleanolic acid, ursolic acid, *Ziziphora clinopodioides* Lam

INTRODUCTION

Oleanolic acid and ursolic acid are the common constituents of plants. These two triterpenes may occur as aglycones of saponins and free acids. Reports on their wide-ranging occurrence in one of the most important groups of medicinal plants, the family Lamiaceae, usually also describe the isolation of free oleanolic acid and ursolic acid besides other compounds.^[1] Both oleanolic acid and ursolic acid have many important pharmacological effects, which are rather similar because of the closeness of their chemical structures. The literature furnishes numerous data on their anti-inflammatory,^[2] hepatoprotective,^[3] antitumor,^[4] anti-HIV,^[5] antimicrobial,^[6] antifungal,^[7] gastroprotective,^[8] hypoglycemic^[9] and antihyperlipidemic^[10] properties. They are relatively non-toxic and have been used in cosmetics and some health products.^[3] Unfortunately, insufficient information is available concerning the distributions of

oleanolic acid and ursolic acid in the *Ziziphora clinopodioides* Lam, because the published quantitative investigations generally extended to only a few species, and mainly qualitative data are to be found only with the presence of these compounds.

Ziziphora clinopodioides Lam is a traditional Uygur medicinal plant, which is a semi-perennial shrub-like plant that grows on low hills, grassland and arid slopes and is widely distributed in China, Mongolia, Turkey, Kazakhstan and Kyrgyzstan.^[11] It is mainly used for the treatment of heart disease, high blood pressure, asthma hyperhidrosis, palpitation insomnia, edema, cough, bronchitis, lung abscess and other diseases. The results of animal experiments show that it can significantly prolong the survival time of hypoxic-mouse model for its good effect against myocardial ischemia and hypoxia. So far, the research on the *Ziziphora clinopodioides* focuses on the chemical constituents of volatile oil, as well as antibacterial activities. In preliminary work, we have also studied the stability of the volatile oil,^[12] and the different parts of polar solvent extraction in antioxidant activities, but HPLC method used for the determination of oleanolic acid and ursolic acid content has not been reported.

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MATERIALS AND METHODS

Plant material and reagents

The plant (whole plant) used for the present study is collected from Wulabo of Urumqi, Xinjiang province. Plant materials are further identified by Yonghe Li, a Pharmacist from Chinese medicine hospital of Xinjiang. Oleanolic acid and ursolic acid are obtained from National Institute for The Control of Pharmaceutical and Biological Products, 110709-200304110742-200516. HPLC grade Methanol supplied by USA, Fisher Scientific, 201-796-7100. Potassium phosphate monobasic of analytical grade is obtained from Tianjin, Tianxin chemical reagents company. All reagent solutions and buffers are prepared with water from Millipore Q3 ultra-pure water system (Millipore, USA).

Instrumentation and chromatographic conditions

The HPLC system consists of a Shimadzu LC-6AD, SPD-20AVP variable wavelength UV detector and Shimadzu CBM-20A station for data analysis. The analytical column is Kromasil C₁₈ column (4.6 × 150 mm, 10 μm, with pre-column). The mobile phase is methanol-0.03M phosphate buffer (pH = 3, 90:10). The flow rate is 0.5 ml/min. The effluent is monitored for UV absorption at 214 nm. The injection volume is 10 μl. All separations are performed at ambient temperature.

Sample preparation

Powder samples (10.0 g) are extracted by methanol (100 ml) for 30 min ultrasonic extraction, filtered, dried in water bath, dissolved with methanol and transferred to a 10 ml measuring flask, and finally, have the volume to the calibration using methanol and shaken evenly before filtering in a 0.45 μm membrane filter.

Standard solution preparation

A standard solution containing 2.0 mg/ml of oleanolic acid and 3.0 mg/ml of ursolic acid is prepared by dissolving in methanol.

RESULTS

Optimization of the chromatographic conditions

LC parameters are optimized by investigating the influence of the mobile phase, column temperature and detection wavelength. The initial separation is carried out on an Agilent Zorbax Extend C₁₈ reserved-phase column with mixtures of methanol and water while the mobile phase with a gradient elution method. Because the peak shapes are unsatisfactory, a small amount of phosphate buffer is added to the mobile phase in order to suppress the ionization of these compounds, sharpen peak shapes and improve analytical sensitivity and resolution. The optimum

mobile phase was composed of methanol and phosphate buffer (pH = 3, 90:10), with a flow rate of 0.5 ml/min, and detection wavelength of 214 nm. Retention time for oleanolic acid and ursolic acid are 21.93 and 23.38 min, respectively. The results are shown in Figures 1 and 2. An HPLC chromatograph of oleanolic acid and ursolic acid in standard solution is shown in Figure 1, an HPLC chromatograph of oleanolic acid and ursolic acid in sample solution is given in Figure 2.

Specification curve

Oleanolic acid standard curve: $y = 5138012.5x - 110328.8$ (x is the concentration of oleanolic acid and y is the peak area integral value of oleanolic acid). The oleanolic acid's linearity range was 0.4-1.2 mg/ml, with a mean correlation coefficient $r = 0.9996$.

Ursolic acid standard curve: $y = 3693315.3x - 113635.2$ (x is the concentration of ursolic acid and y is the peak area integral value of ursolic acid). Ursolic acid's linearity range

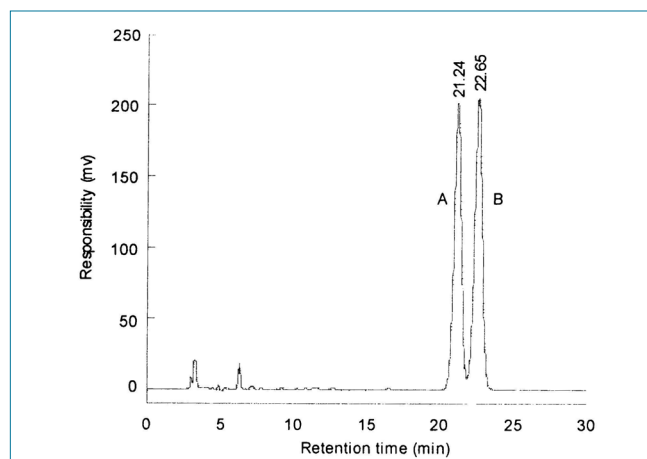


Figure 1: Standard solution chromatogram (A: Oleanolic acid, B: Ursolic acid)

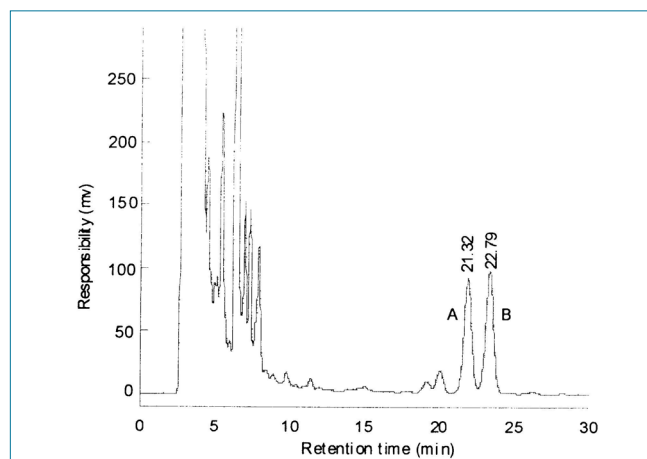


Figure 2: *Ziziphora clinopodioides* sample solution chromatogram (A: Oleanolic acid, B: Ursolic acid)

was 0.6-1.8 mg/ml, with a mean correlation coefficient $r = 0.9996$.

Precision

Take the oleanolic acid and the ursolic acid, mix with 10 μ l standard comparison solution, and determine according to the above chromatographic condition. Handle the specimen six times. Determine the oleanolic acid and the ursolic acid peak area integral values. RSD% is 1.53 and 1.13%, respectively. The results obtained confirm a good precision of the method developed. The results are shown in Table 1.

Table 1: Precision conclusion

| No | Oleanolic acid sample peak area integral value | Ursolic acid sample peak area integral value |
|---------|--|--|
| 1 | 4436286 | 4842616 |
| 2 | 4602612 | 4962482 |
| 3 | 4581736 | 4947798 |
| 4 | 4624223 | 4996164 |
| 5 | 4615594 | 4993141 |
| 6 | 4596357 | 4949598 |
| RSD (%) | 1.53 | 1.13 |

Table 2: Stability conclusion

| Time (h) | Oleanolic acid peak area integral value | Ursolic acid peak area integral value |
|----------|---|---------------------------------------|
| 0 | 3884202 | 4233366 |
| 2 | 3828496 | 4151307 |
| 4 | 3841187 | 4125055 |
| 6 | 3794021 | 4089668 |
| 8 | 3741026 | 4063406 |
| RSD (%) | 1.40 | 1.58 |

Stability

Take the identical sample to supply the test solution for 0, 2, 4, 8, 24 h, respectively, and determine the oleanolic acid and the ursolic acid peak area integral values. RSD % is 1.40 and 1.58% respectively. The experimental results indicate that samples are stable in 24 h. The results are shown in Table 2.

Reproducibility

Take *Ziziphora clinopodioides* Lam powder 10.0 g, according to "2.2" strip by the same method. Determine the oleanolic acid and the ursolic acid peak area integral values according to the above chromatograph condition, RSD % is 1.86% and 1.62 % respectively. The experiment results indicate that the accuracy is good. The results are shown in Table 3.

Recovery

Take 5.0 g *Ziziphora clinopodioides* Lam of the known content for each group and add the 3.80 mg oleanolic acid standard solution and 5.92 mg ursolic acid standard solution and calculate the spotting recovery rate. According to "2.2" strip by the same method and computation content using standard curve method, the average recovery ($n = 6$) of

Table 3: Reproducibility conclusion

| No. | Oleanolic acid peak area integral value | Ursolic acid peak area integral value |
|---------|---|---------------------------------------|
| 1 | 3840722 | 4294788 |
| 2 | 3781915 | 4203736 |
| 3 | 3692671 | 4176719 |
| 4 | 3748147 | 4175203 |
| 5 | 3895012 | 4227854 |
| 6 | 3798748 | 4308687 |
| RSD (%) | 1.86 | 1.62 |

Table 4: Recovery rate of oleanolic acid conclusion

| Sampling quantity (g) | Sample content A (mg) | Addition B (mg) | Determination C (mg) | Recovery (%) | Average (%) | RSD (%) |
|-----------------------|-----------------------|-----------------|----------------------|--------------|-------------|---------|
| 5.00 | 3.80 | 3.80 | 7.64 | 101.1 | | |
| 5.00 | 3.80 | 3.80 | 7.59 | 99.7 | | |
| 5.00 | 3.80 | 3.80 | 7.51 | 97.6 | 99.5 | 1.19 |
| 5.00 | 3.80 | 3.80 | 7.58 | 99.5 | | |
| 5.00 | 3.80 | 3.80 | 7.56 | 98.9 | | |
| 5.00 | 3.80 | 3.80 | 7.61 | 100.2 | | |

Recovery rate = $[(C-A)/B] \times 100\%$

Table 5: Recovery rate of ursolic acid conclusion

| Sampling quantity (g) | Sample content A (mg) | Addition B (mg) | Determination C (mg) | Recovery (%) | Average (%) | RSD (%) |
|-----------------------|-----------------------|-----------------|----------------------|--------------|-------------|---------|
| 5.00 | 5.88 | 5.92 | 12.01 | 103.5 | | |
| 5.00 | 5.88 | 5.92 | 11.93 | 102.1 | | |
| 5.00 | 5.88 | 5.92 | 11.83 | 100.5 | 102.3 | 1.25 |
| 5.00 | 5.88 | 5.92 | 11.95 | 102.5 | | |
| 5.00 | 5.88 | 5.92 | 12.03 | 103.8 | | |
| 5.00 | 5.88 | 5.92 | 11.87 | 101.2 | | |

Recovery rate = $[(C-A)/B] \times 100\%$

oleanolic acid is 99.5% (RSD = 1.19%) and of ursolic acid is 102.3% (RSD = 1.25%). The results are shown in Tables 4 and 5.

Quantification of oleanolic acid and ursolic acid in sample

The standard addition and recovery experiments are conducted to determine the accuracy of the present method for the quantification of oleanolic acid and that of ursolic acid in *Ziziphora clinopodioides* Lam samples. The content of oleanolic acid and ursolic acid in *Ziziphora clinopodioides* Lam are 0.76 mg/g and 1.176 mg/g, respectively.

DISCUSSION

A simple and sensitive HPLC method is developed for the determination of oleanolic acid and ursolic acid contents in *Ziziphora clinopodioides* Lam. This easy method provided excellent sensitivity, accuracy and precision, with relatively short retention time for both oleanolic acid and ursolic acid. This developed HPLC method can therefore be applied to both *in vitro* studies of oleanolic acid and ursolic acid formulations as well as drug estimation in biological samples.

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