**Determination of Echinocystic Acid in Extract of Spina Gleditsiae by HPLC**

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**Abstract.** A high performance liquid chromatography (HPLC) method for the determination of echinocystic acid in spina gleditsiae was established. Chromatographic conditions were as follows: The separation was performed on Dikma Kromasil C<sub>18</sub> column (250 mm×4.6 mm, 5 μm) with column temperature kept at 30℃. The mobile phase consisted of acetonitrile-0.1% phosphoric acid solution (45:55) at a flow rate of 1.0 mL/min with detection wavelength set at 215 nm. The sample input was 10 μL. The results showed that echinocystic acid had a good linear relationship in the concentration range of 3.16-63.4 μg/mL. The average recovery was 98.21% and RSD was 0.93% (n=9). The established method was proved to sensitive, accurate, simple, repeatable and applicable for the quality control of the extracts of spina gleditsiae.

1. **Introduction**
Spina gleditsiae, also known as acacia stinger, acacia stinger, is the dried thorn of gleditsia sinensis Lam. Modern pharmacological studies have shown that spina gleditsiae has many pharmacological effects such as anti-coagulation, anti-hepatic fibrosis, immune regulation and tumor inhibition. The total saponin in spina gleditsiae has the activity of removing phlegm and antibacterial. The content of echinocystic acid in methanol extract of spina gleditsiae was determined by high performance liquid chromatography (HPLC), so as to provide a basis for the quality evaluation of spina gleditsiae.

2. **Materials and Instruments**
HPLC, including four-element low pressure gradient pump, diode array detector, column temperature box and chromatographic workstation; Ultrasonic cleaning machine; Electronic balance; Ultrapure water; Chromatographic pure acetonitrile as flowing phase; echinocystic acid reference substance; Other analytical pure reagents.

3. **Methods and Results**

3.1. **Chromatographic conditions**
The separation was performed on Dikma Kromasil C<sub>18</sub> column(250 mm×4.6 mm, 5 μm) with column temperature kept at 30℃. The mobile phase consisted of acetonitrile-0.1% phosphoric acid solution (45:55) at a flow rate of 1.0 mL/min with detection wavelength set at 215 nm. The sample input was 10 μL.

3.2. **Preparation of reference solution**
The echinocystic acid was dissolved with 50% acetonitrile, which dried in phosphorus pentoxide vacuum dryer for 36 h, and a concentration of 63.2 g/mL of which was prepared.
3.3. Preparation of test solution
Methanol extract of spina gleditsiae: 5 kg dry spina gleditsiae powder was extracted with 95% ethanol reflux, and the extract was condensed by decompression. Then, the extract was dispersed in water and extracted successively with ether, chloroform and water saturated n-butanol. At last, silica column chromatography was carried out with about 25 g of the extract, and methanol was recrystallized to obtain spina gleditsiae extract.

Solution of the test: 0.2 g extract prepared above was accurately weighed and placed in a 25 ml measuring bottle. The solution should be filtered with a 0.45 μm micro-porous membrane filter before sampling.

3.4. System suitability test
The solution of the reference and test was injected into the chromatograph with a concentration of 10 μL under the chromatographic conditions described in 3.1. The results showed that the retention time of echinocystic acid was about 15 min, and it was completely separated from other components which had a separation degree greater than 1.5 from other chromatographic peaks. The number of theoretical plates on the peak of echinocystic acid was greater than 3000. The chromatographic figure is shown in figure 1.

![HPLC Figure](image)

Figure 1. HPLC
A. Reference substance; B. ethanol extract. 1. Echinocystic Acid

3.5. Standard curve
The echinocystic acid reference solutions of 0.5 ml, 1.0 ml, 2.0 ml, 5.0 ml, 8.0 ml and 10.0 ml were accurately absorbed and placed in a 10 mL volumetric bottle, and then methanol was added to the scale and mixed. The samples were taken separately with a value of 10 μL, which were determined in the chromatographic conditions described in 3.1. The standard curve was drawn with the concentration of echinocystic acid as the horizontal coordinate and the peak area as the vertical coordinate. The regression equation is Y=3.7152X+0.2873, r=0.9998. The results showed that echinocystic acid had a good linear relationship in the range of 3.16-63.4 μg/mL.

3.6. Precision test
In the chromatographic conditions described in 3.1, the peak area was determined by repeated injection of echinocystic acid reference solution for 6 times. RSD was 1.42%, which indicated that the precision of the instrument was good.

3.7. Reproducibility test
Six methanol extracts of the same batch of spina gleditsiae were prepared according to the method described in 3.3. Peak area was measured and echinocystic acid content was calculated, and RSD was calculated as 1.76% which indicated a good reproducibility.

3.8. Stability test
According to the chromatographic conditions above, the peak area of the test solution prepared in 3.2
was determined at 0, 1, 2, 4, 8, 16 and 24 h respectively. The RSD was 1.67%, which showed that the test solution had good stability within 24 h.

3.9. Sample recovery test
0.1g of nine methanol extracts of spina gleditsiae (echinocystic acid content was 0.28%) were weighed, to which echinocystic acid reference solutions (63.2 μg/mL) of 10, 12 and 15 mL were respectively added. Test solution was prepared by the method described in 3.3, and recovery rate was calculated according to the chromatographic conditions described in 3.1. The results are shown in Table 1.

| Sample(g) | Content (mg) | Added (mg) | Total (mg) | Recovery (%) | Average (%) | RSD(%) |
|-----------|--------------|------------|------------|--------------|-------------|--------|
| 0.1006    | 0.2816       | 0.6320     | 0.9136     | 97.82        |             |        |
| 0.1001    | 0.2803       | 0.6320     | 0.9123     | 98.24        |             |        |
| 0.1007    | 0.2820       | 0.6320     | 0.9140     | 98.15        |             |        |
| 0.1002    | 0.2806       | 0.7584     | 1.0390     | 100.08       |             |        |
| 0.1005    | 0.2814       | 0.7584     | 1.0798     | 98.33        | 99.21       | 1.00   |
| 0.1003    | 0.2808       | 0.7584     | 1.0092     | 97.02        |             |        |
| 0.9998    | 0.2799       | 0.9480     | 1.2089     | 98.13        |             |        |
| 0.1004    | 0.2811       | 0.9480     | 1.2091     | 97.41        |             |        |
| 0.1002    | 0.2806       | 0.9480     | 1.2191     | 99.23        |             |        |

3.10. Sample content determination
Three batches of methanol extracts of spina gleditsiae at different growth stages were taken. The test solution was prepared by 3.3 methods, and the chromatographic conditions were determined by 3.1. The content of echinocystic acid was calculated by external standard method. The results showed that the contents of echinocystic acid were 0.21%, 0.25% and 0.28%, and RSD was 0.92%, 1.05% and 1.16%.

4. Discussion

4.1. Detect wavelength selection
A diode array detector was used to conduct spectral scanning of echinocystic acid reference solution in the range of 200-380 nm. The results showed that echinocystic acid had the maximum absorption at 215 nm, so 215 nm was chosen as the measurement wavelength.

4.2. Solvent selection
Solvent screening tests were carried out with methanol, acetonitrile, 50% acetonitrile and other solvents. The results showed that when the sample was dissolved with 50% acetonitrile, the content of echinocystic acid was the highest, the peak shape was sharp and the separation degree was good. Therefore, 50% acetonitrile was finally used as the solvent.

4.3. Flow phase selection
Various flow phase systems of acetonitrile-phosphate solution in different proportions were investigated, including 50:50, 40:60, 35:65, 30:70 and 25:75. The results showed that the flow phase separation selected by this method was the best. Echinocystic acid was separated at baseline and the separation degree was above 1.5, which met the requirement of content determination.

4.4. Methanol extracts from spina gleditsiae at different growth stages
Methanol extracts from spina gleditsiae produced in the same area at different growth stages were detected to have different contents of echinocystic acid. The results showed that the content of echinocystic acid was different due to the development degree of spina gleditsiae.

5. Conclusions
The content of echinocystic acid in methanol extract of spina gleditsiae was determined by high performance liquid chromatography (HPLC). The results showed under the chromatographic conditions proposed in this study, echinocystic acid had a good linear relationship in the concentration
range of 3.16-63.4 μg/mL. Therefore, this method could be used to control the quality of echinocystic acid in spina gleidisiae.

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