Gleditsiae Fructus Abnormalis

Figure 1 A photograph of Gleditsiae Fructus Abnormalis

A. Gleditsiae Fructus Abnormalis

B. Magnified transverse and longitudinal sections of sterile fruit

1. NAMES

Official Name: Gleditsiae Fructus Abnormalis

Chinese Name: 豬牙皂

Chinese Phonetic Name: Zhuyazao

2. SOURCE

Gleditsiae Fructus Abnormalis is the dried sterile fruit of *Gleditsia sinensis* Lam. (Fabaceae). The sterile fruit is collected in autumn, foreign matter removed, then dried under the sun to obtain Gleditsiae Fructus Abnormalis.

3. DESCRIPTION

Cylindrical, slightly flattened and curved, 5-11 cm long, 7-15 mm wide. Externally purplish-brown, covered with greyish-white and waxy powders, showing fine warts and linear or reticulate fissures, becoming lustrous after being rubbed away. Apex with a beak-shaped remnants of the style, base with a scar of the fruit stalk. Texture hard and fragile, easily broken. Fracture brownish-yellow, lax in the centre, with pale green or pale brownish-yellow filiform substance, occasionally containing incompletely grown seeds. Odour slight and irritating; taste sweet and followed by pungent (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Exocarp consists of 1 layer of cells, cells subsquare, densely arranged and covered with cuticle. Mesocarp mainly composed of parenchymatous cells, scattered with fibre bundles, cells at the inner part mostly dilapidated; an interrupted annular band composed of stone cells located on the outer side, another interrupted annular band consists of 1 to several layers of sclerenchymatous and pitted cells (lignified sclerenchymatous cell) located on the inner side, cells subsquare, subrounded or subpolygonal, walls with distinct pits and striations. The inner of pitted cells band composed of parenchymatous cells, cells radially elongated or subrounded, several long cells and short cells mostly alternately arranged, sometimes cells dilapidated. Endocarp indistinct. Vascular bundles located in the mesocarp, vascular bundles and fibre bundles well developed in dorsal and ventral suture (Fig. 2).

Powder

Colour brownish-yellow. Stone cells numerous, scattered singly or in groups, nearly colourless or pale yellow, subrounded, subsquare, oblong or irregular in shape, 15-51 μm in diameter, striations visible, pit canals distinct, lumens often relatively small. Fibres frequently in bundles, 10-31 μm in diameter, walls slightly lignified, often surrounded by subsquare sclerenchymatous cells contain prisms of calcium oxalate, forming crystal fibres; yellowish-white under the polarized microscope. Sclerenchymatous cells scattered singly or in groups, subrectangular or suboblong, walls relatively unevenly thickened with distinct pit canals, lumens relatively small. Lignified parenchymatous cells extremely numerous, subrectangular, pits relatively large and distinct, walls slightly thickened, lumens relatively large. Epidermal cells of pericarp yellowish-brown or reddish-brown, polygonal or rounded-polygonal in surface view, walls relatively thickened, with slightly granular cuticle striations on surface, stomata anomocytic, rounded or long-rounded, subsidiary cells 7-10, arranged annularly. Clusters of calcium oxalate relatively small, 4-12 μm in diameter; bright white under the polarized microscope (Fig. 3).

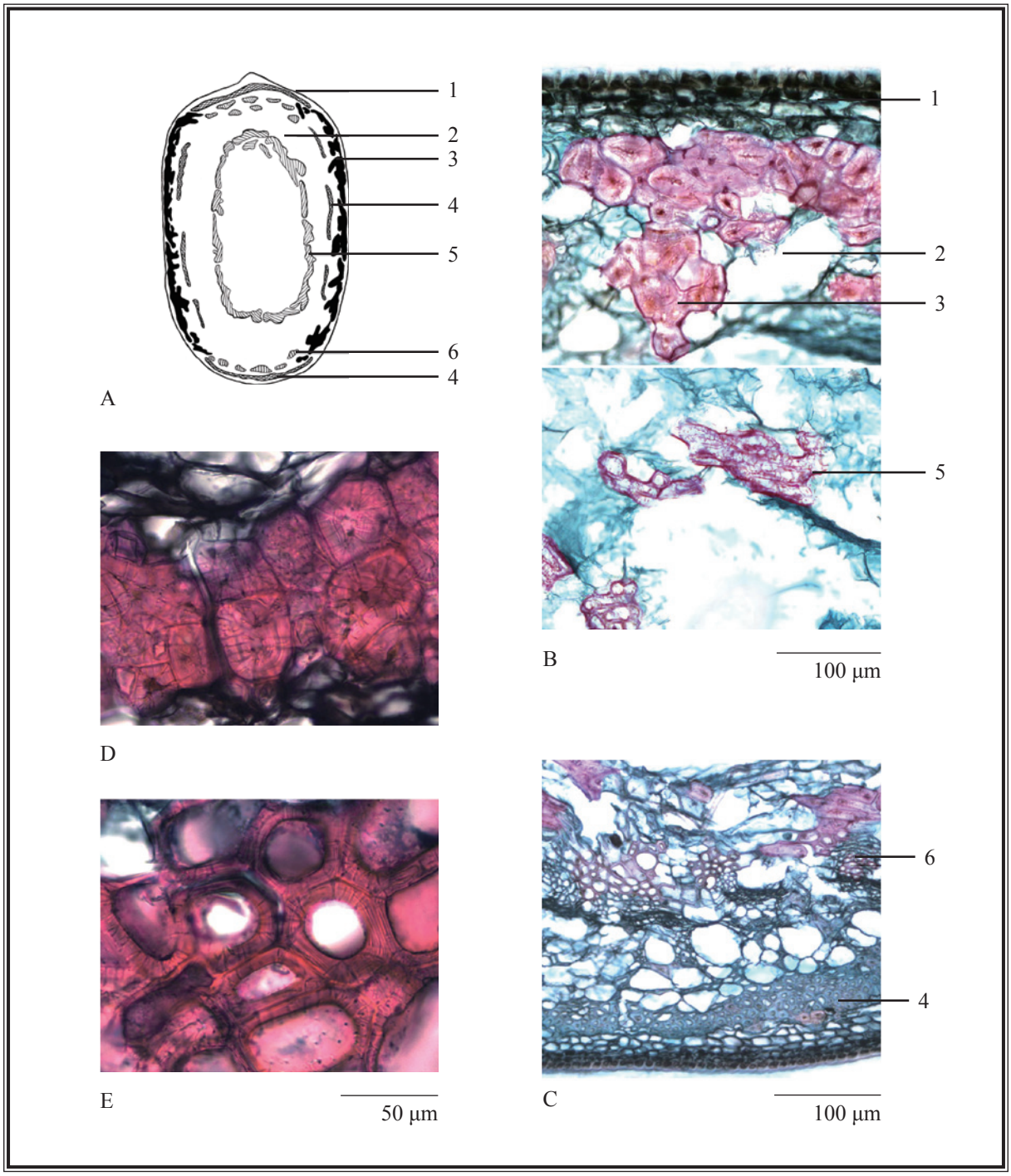


Figure 2 Microscopic features of transverse section of Gleditsiae Fructus Abnormalis

A. Sketch B. Section illustration C. Dorsal suture D. Stone cells E. Pitted cells

1. Exocarp 2. Mesocarp 3. Stone cell 4. Fibre 5. Pitted cell band 6. Vascular bundle

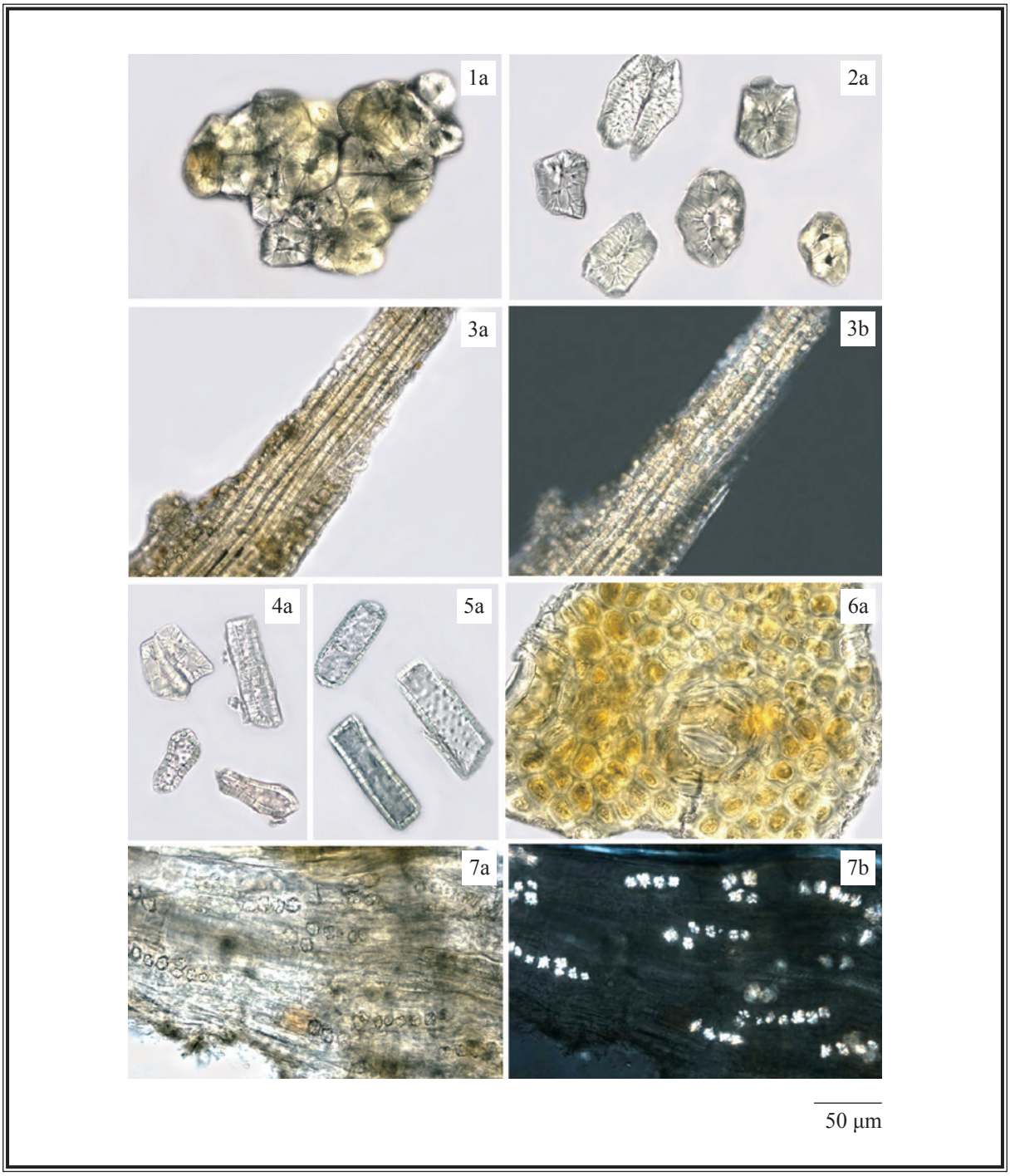


Figure 3 Microscopic features of powder of Gleditsiae Fructus Abnormalis

1. Stone cells in group 2. Stone cells 3. Crystal fibres
4. Sclerenchymatous cells 5. Lignified parenchymatous cells
6. Epidermal cells of pericarp 7. Clusters of calcium oxalate

a. Features under the light microscope b. Features under the polarized microscope

4.2 Thin-Layer Chromatographic Identification *[Appendix IV(A)]*

Standard solutions

Echinocystic acid standard solution

Weigh 0.5 mg of echinocystic acid CRS (Fig. 4) and dissolve in 1 mL of methanol.

Oleanolic acid standard solution

Weigh 0.5 mg of oleanolic acid CRS (Fig. 4) and dissolve in 1 mL of methanol.

Developing solvent system

Prepare a mixture of cyclohexane, ethyl acetate and formic acid (5:2.5:0.1, v/v).

Spray reagent

Add slowly 10 mL of sulphuric acid to 90 mL of ethanol.

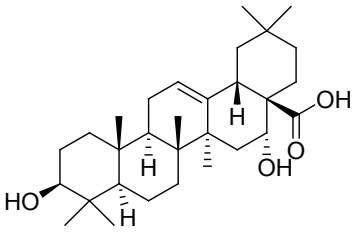
Test solution

Weigh 0.5 g of the powdered sample and place it in a 250-mL round-bottomed flask, then add 25 mL of methanol. Reflux the mixture for 30 min. Cool down to room temperature. Transfer the solution to a 50-mL centrifuge tube. Centrifuge at about 2700 × *g* for 5 min. Pipette 10 mL of the supernatant to a 250-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 8 mL of ethanol (50%) and 2 mL of hydrochloric acid. Reflux the mixture for 30 min. Cool down to room temperature. Transfer the solution to a 15-mL centrifuge tube. Centrifuge at about 2700 × *g* for 5 min and discard the supernatant. Combine the residues, then add 20 mL of methanol. Transfer the solution to a 25-mL volumetric flask. Make up to the mark with methanol. Filter through a 0.45-μm PTFE filter.

Procedure

Carry out the method by using a TLC silica gel F_{254} plate, a twin trough chamber and a freshly prepared developing solvent system as described above. Apply separately echinocystic acid standard solution, oleanolic acid standard solution and the test solution (4 μL each) to the plate. Before the development, add the developing solvent to one of the troughs of the chamber and place the TLC plate in the other trough. Cover the chamber with a lid and let equilibrate for about 15 min. Carefully tilt the chamber to allow sufficient solvent to pass from the trough containing the solvent to the other containing the TLC plate for development. Develop over a path of about 7 cm. After the development, remove the plate from the chamber, mark the solvent front and dry in air. Spray the plate evenly with the spray reagent and heat at about 105ºC until the spots or bands become visible (about 5-10 min). Examine the plate under UV light (366 nm). Calculate the R_f values by using the equation as indicated in Appendix IV (A).

(i)



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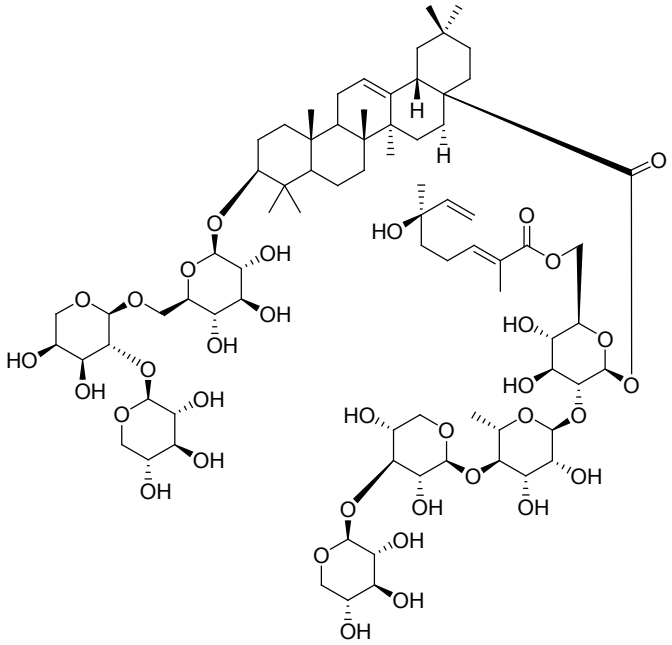

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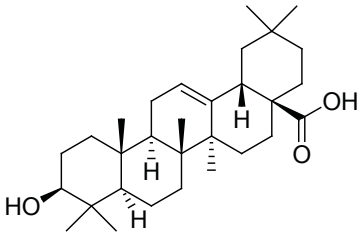

Figure 4 Chemical structures of (i) echinocystic acid (ii) gleditsioside A and (iii) oleanolic acid

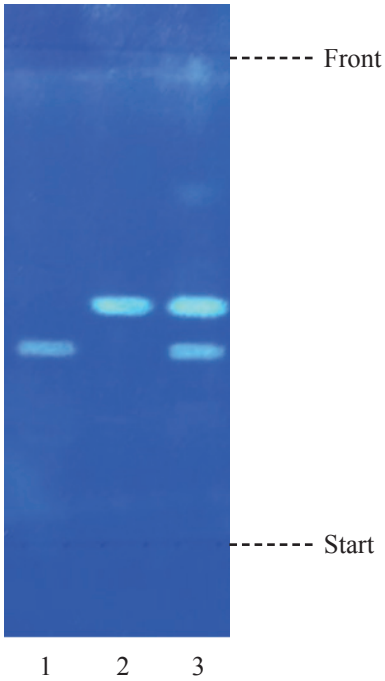

Figure 5 A reference TLC chromatogram of Gleditsiae Fructus Abnormalis extract observed under UV light (366 nm) after staining

1. Echinocystic acid standard solution 2. Oleanolic acid standard solution 3. Test solution

For positive identification, the sample must give spots or bands with chromatographic characteristics, including the colour and the R_f values, corresponding to those of echinocystic acid and oleanolic acid (Fig. 5).

4.3 High-Performance Liquid Chromatographic Fingerprinting *(Appendix XII)*

Standard solution

Gleditsioside A standard solution for fingerprinting, Std-FP (200 mg/L)

Weigh 1.0 mg of gleditsioside A CRS (Fig. 4) and dissolve in 5 mL of methanol.

Test solution

Weigh 0.1 g of the powdered sample and place it in a 50-mL conical flask, then add 10 mL of methanol (25%). Sonicate (100 W) the mixture for 30 min. Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (210 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size). The flow rate is about 1.0 mL/min. Programme the chromatographic system as follows (Table 1) –

Table 1 Chromatographic system conditions

Time (min)	Acetonitrile (%, v/v)	Water (%, v/v)	Elution
0 – 60	25 → 40	75 → 60	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 μL of gleditsioside A Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak area of gleditsioside A should not be more than 5.0%; the RSD of the retention time of gleditsioside A peak should not be more than 2.0%; the column efficiency determined from gleditsioside A peak should not be less than 100000 theoretical plates.

The *R* value between peak 4 and the closest peak in the chromatogram of the test solution should not be less than 1.0 (Fig. 6).

Procedure

Separately inject gleditsioside A Std-FP and the test solution (10 μL each) into the HPLC system and record the chromatograms. Measure the retention time of gleditsioside A peak in the chromatogram of gleditsioside A Std-FP and the retention times of the four characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify gleditsioside A peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of gleditsioside A Std-FP. The retention times of gleditsioside A peaks from the two chromatograms should not differ by more than 2.0%. Calculate the RRTs of the characteristic peaks by using the equation as indicated in Appendix XII.

The RRTs and acceptable ranges of the four characteristic peaks of Gleditsiae Fructus Abnormalis extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the four characteristic peaks of Gleditsiae Fructus Abnormalis extract

Peak No.	RRT	Acceptable Range
1	0.60	± 0.04
2	0.79	± 0.03
3	0.94	± 0.03
4 (marker, gleditsioside A)	1.00	-

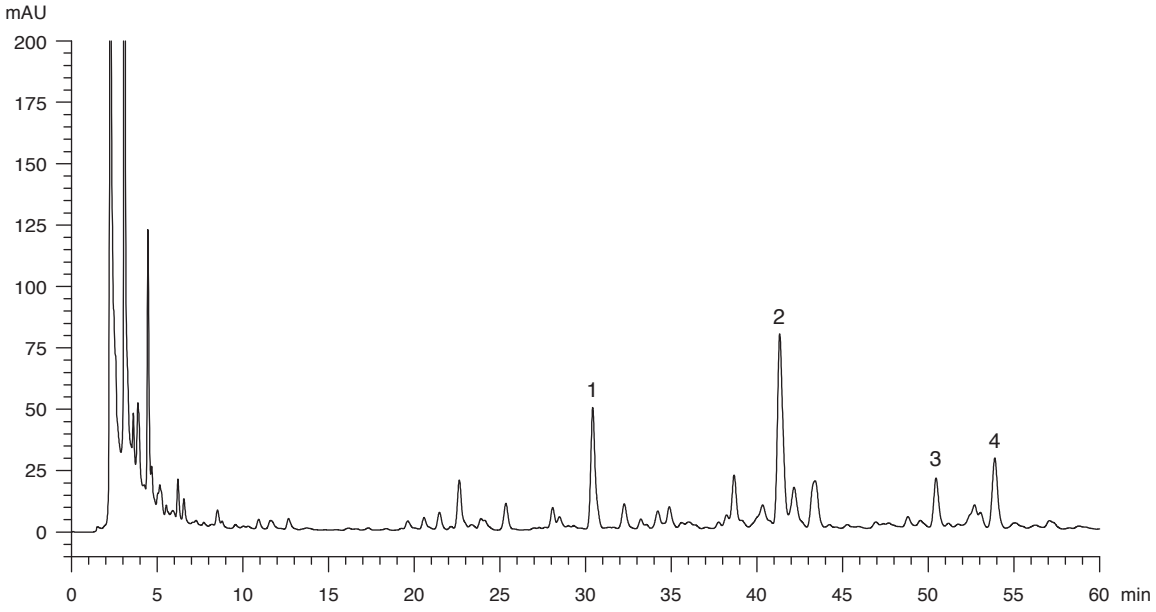

Figure 6 A reference fingerprint chromatogram of Gleditsiae Fructus Abnormalis extract

For positive identification, the sample must give the above four characteristic peaks with RRTs falling within the acceptable range of the corresponding peaks in the reference fingerprint chromatogram (Fig. 6).

5. TESTS

5.1 Heavy Metals *(Appendix V)*: meet the requirements.

5.2 Pesticide Residues *(Appendix VI)*: meet the requirements.

5.3 Mycotoxins – Aflatoxins *(Appendix VII)*: meet the requirements.

5.4 Sulphur Dioxide Residues *(Appendix XVII)*: meet the requirements.

5.5 Foreign Matter *(Appendix VIII)*: not more than 1.0%.

5.6 Ash *(Appendix IX)*

Total ash: not more than 4.5%.
Acid-insoluble ash: not more than 0.5%.

5.7 Water Content *(Appendix X)*

Oven dried method: not more than 14.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (cold extraction method): not less than 35.0%.
Ethanol-soluble extractives (cold extraction method): not less than 23.0%.

7. ASSAY

Carry out the method as directed in Appendix IV (B).

Standard solution

Mixed echinocystic acid and oleanolic acid standard stock solution, Std-Stock (400 mg/L for echinocystic acid and 500 mg/L for oleanolic acid)

Weigh accurately 4.0 mg of echinocystic acid CRS and 5.0 mg of oleanolic acid CRS, and dissolve in 10 mL of methanol.

Mixed echinocystic acid and oleanolic acid standard solution for assay, Std-AS

Measure accurately the volume of the mixed echinocystic acid and oleanolic acid Std-Stock, dilute with methanol to produce a series of solutions of 20, 40, 120, 160, 240 mg/L for echinocystic acid and 25, 50, 150, 200, 300 mg/L for oleanolic acid.

Test solution

Weigh accurately 0.5 g of the powdered sample and place it in a 250-mL round-bottomed flask, then add 25 mL of methanol. Reflux the mixture for 30 min. Cool down to room temperature. Transfer the solution to a 50-mL centrifuge tube. Centrifuge at about 2700 × *g* for 5 min. Pipette 10 mL of the supernatant to a 250-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 8 mL of ethanol (50%) and 2 mL of hydrochloric acid. Reflux the mixture for 30 min. Cool down to room temperature. Transfer the solution to a 15-mL centrifuge tube. Centrifuge at about 2700 × *g* for 5 min and discard the supernatant. Combine the residues, then add 20 mL of methanol. Transfer the solution to a 25-mL volumetric flask. Make up to the mark with methanol. Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (210 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size). The flow rate is about 1.0 mL/min. Programme the chromatographic system as follows (Table 3) –

Table 3 Chromatographic system conditions

Time (min)	Water (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 10	35	65	isocratic
10 – 20	35 → 15	65 → 85	linear gradient
20 – 30	15	85	isocratic

System suitability requirements

Perform at least five replicate injections, each using 10 µL of the mixed echinocystic acid and oleanolic acid Std-AS (120 mg/L for echinocystic acid and 150 mg/L for oleanolic acid). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of echinocystic acid and oleanolic acid should not be more than 5.0%; the RSD of the retention times of echinocystic acid and oleanolic acid peaks should not be more than 2.0%; the column efficiencies determined from echinocystic acid and oleanolic acid peaks should not be less than 13000 and 35000 theoretical plates respectively.

The *R* value between echinocystic acid peak and the closest peak; and the *R* value between oleanolic acid peak and the closest peak in the chromatogram of the test solution should not be less than 1.5.

Calibration curves

Inject a series of the mixed echinocystic acid and oleanolic acid Std-AS (10 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of echinocystic acid and oleanolic acid against the corresponding concentrations of the mixed echinocystic acid and oleanolic acid Std-AS. Obtain the slopes, y-intercepts and the r^2 values from the corresponding 5-point calibration curves.

Procedure

Inject 10 µL of the test solution into the HPLC system and record the chromatogram. Identify echinocystic acid and oleanolic acid peaks in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed echinocystic acid and oleanolic acid Std-AS. The retention times of echinocystic acid and oleanolic acid peaks in the chromatograms of the test solution and the Std-AS should not differ by more than 5.0%. Measure the peak areas and calculate the concentrations (in milligram per litre) of echinocystic acid and oleanolic acid in the test solution, and calculate the percentage contents of echinocystic acid and oleanolic acid in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 3.1% of the total content of echinocystic acid ($C_{30}H_{48}O_4$) and oleanolic acid ($C_{30}H_{48}O_3$), calculated with reference to the dried substance.