Ilicis Cornutae Folium

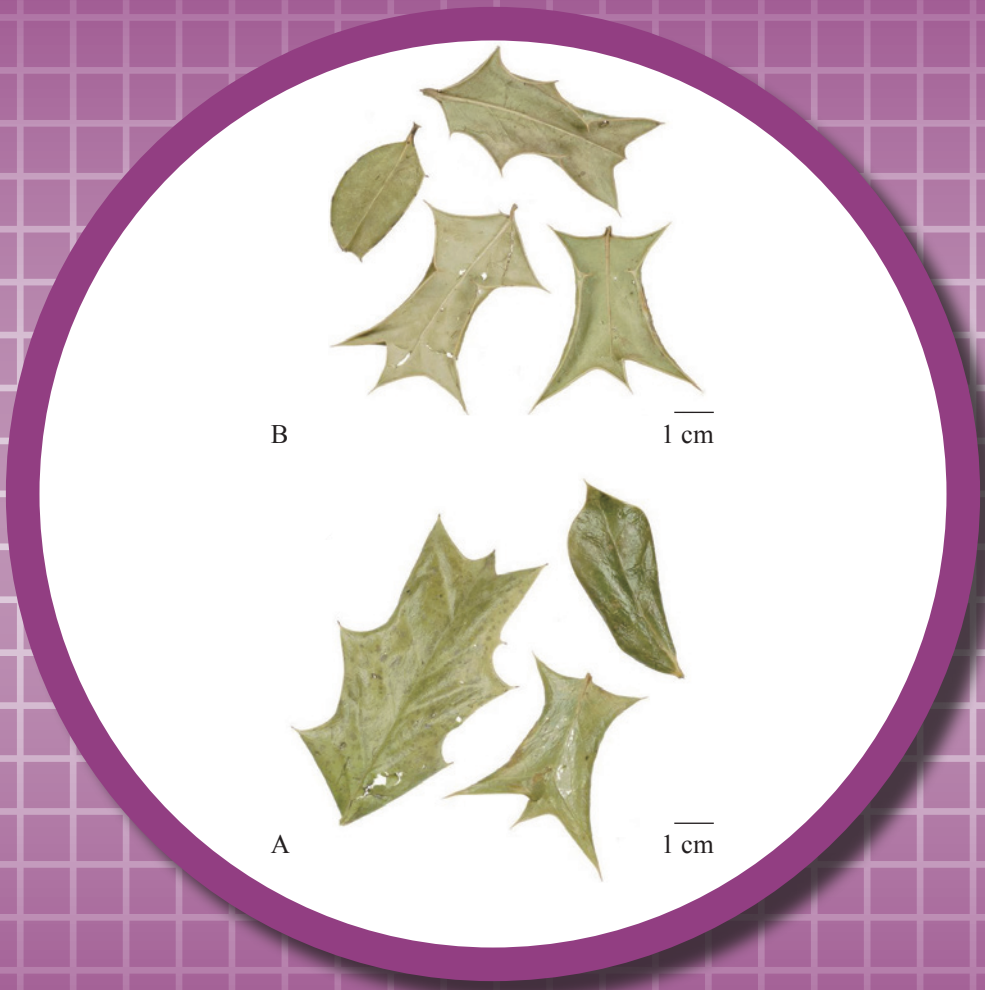


Figure 1 A photograph of Ilicis Cornutae Folium

A. Upper surface of leaves B. Lower surface of leaves

1. NAMES

Official Name: Ilicis Cornutae Folium

Chinese Name: 枸骨葉

Chinese Phonetic Name: Gouguye

2. SOURCE

Ilicis Cornutae Folium is the dried leaf of *Ilex cornuta* Lindl. ex Paxt. (Aquifoliaceae). The leaf is collected in autumn, foreign matter removed, then dried under the sun to obtain Ilicis Cornutae Folium.

3. DESCRIPTION

Subrectangular, oblong-rectangular, occasionally oblong, 2.6-10.0 cm long, 1.1-4.1 cm wide, with short petiole. Apex with 3 relatively large and hard spines, the apical one usually recurved; base truncate or broadly cuneata, sometimes each of both sides with 1-3 spines, edges slightly recurved; the oblong blade usually free from spines or only with 1 spine at apex. Upper surface yellowish-green to greenish-brown, lustrous; lower surface greyish-yellow to greyish-green. Veins pinnate, middle vein concave above. Texture coriaceous, hard and thick. Odour slight; taste slightly bitter (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Leaf: Upper epidermal cells subsquare, thick-walled, covered with a thick cuticle; unicellular non-glandular hairs rising from the midrib occasionally found. Palisade tissue consists of 2-4 layers of cells, located on the inner side of the upper epidermis; spongy tissue arranged loosely. One to several layers of collenchymatous cells located on the inner side of the upper and lower epidermis of the midrib. Vascular bundle of midrib collateral, with lignified fibre bundles located above and below the vascular bundle. Clusters of calcium oxalate scattered in the parenchyma, occasionally found near lower epidermis. Lower epidermal cells relatively small. Stone cells occasionally scattered in the collenchyma inside the lower epidermis of midrib and outside the lignified fibre bundles below the phloem [Fig. 2 (i)].

Leaf margin: Collenchymatous cells, stone cells and lignified fibre bundles sequentially occur inside the epidermis of the leaf margin. Several layers of collenchymatous cells found at the margin near the petiole. Stone cells occasionally found in the collenchyma inside the epidermis of the leaf margin [Fig. 2 (i)].

Petiole: Epidermal cells subsquare, thick-walled, covered with a thick cuticle. Several layers of collenchymatous cells located inside the epidermis. Vascular bundles collateral, the one in the middle of the midrib relatively large, one small bundle located on either side of the middle bundle, sometimes with groups of fibres beneath the vascular bundle. Parenchyma scattered with clusters of calcium oxalate [Fig. 2 (ii)].

Powder

Colour greyish-green to greenish-brown. Upper epidermal cells subsquare or polygonal, walls straight or slightly wavy, thick, pit canals distinct in surface view. Lower epidermal cells polygonal or irregular in shape, smaller than upper epidermis, walls slightly thin, stomata anomocytic, arranged densely. Non-glandular hairs occasionally found, unicellular. Collenchymatous cells subsquare, subrounded, polygonal or irregular in shape, with thick walls. Stone cells scattered singly or in groups, subrounded, polygonal, fusiform, ovate or irregular in shape, 6-27 μm in diameter, walls slightly thin, striations sometimes present, pit canals distinct; bright white under the polarized microscope. Clusters of calcium oxalate 10-33 μm in diameter, angles blunt; polychromatic under the polarized microscope. Fibres scattered singly or in bundles of two or more, 4-22 μm in diameter; bright white under the polarized microscope (Fig. 3).

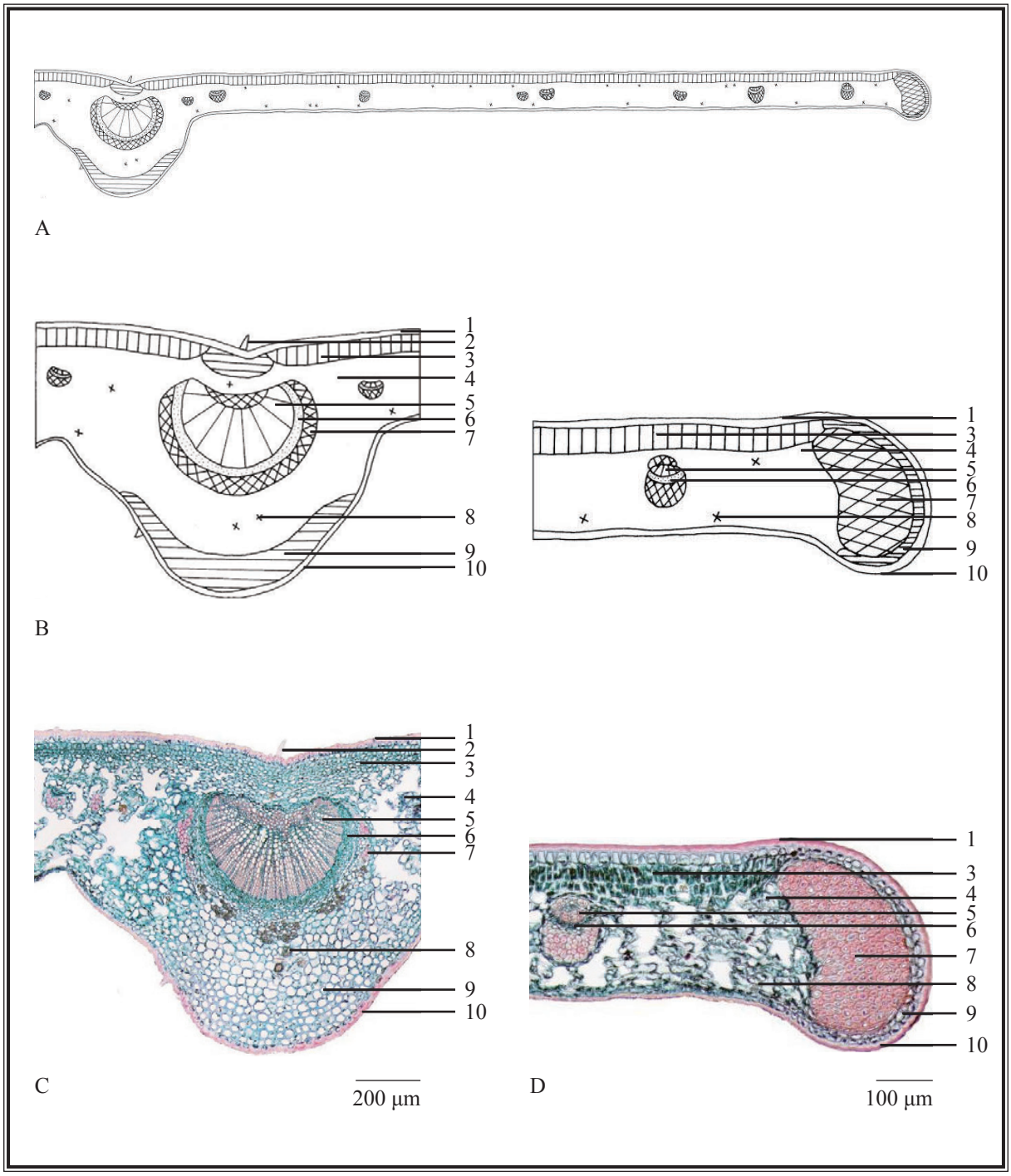


Figure 2 (i) Microscopic features of transverse section of Ilicis Cornutae Folium

A. Sketch B. Magnified image of sketch C. Section illustration of leaf
D. Section illustration of leaf margin

1. Upper epidermis 2. Non-glandular hair 3. Palisade tissue 4. Spongy tissue 5. Xylem 6. Phloem
7. Fibre bundles 8. Clusters of calcium oxalate 9. Collenchyma 10. Lower epidermis

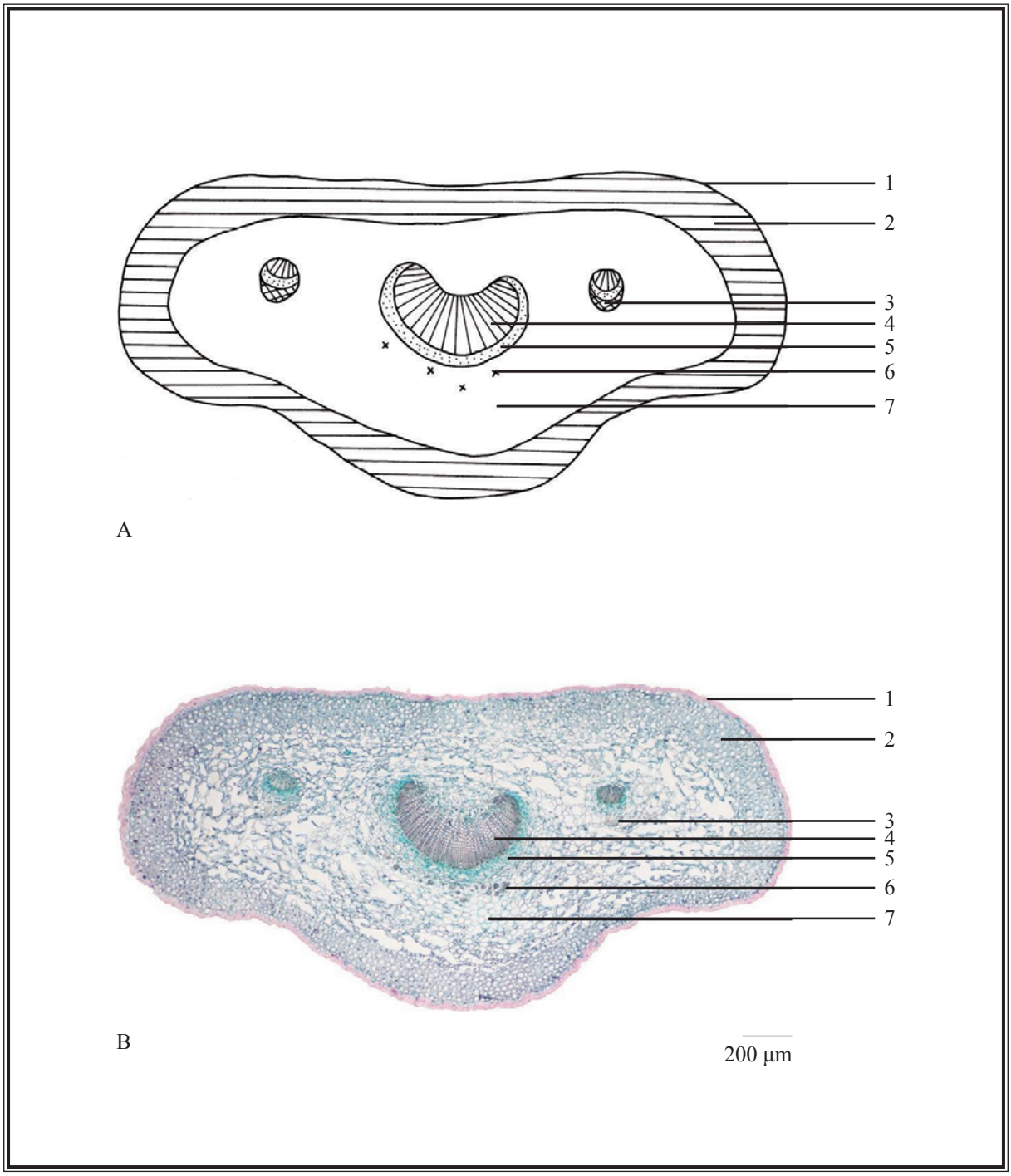


Figure 2 (ii) Microscopic features of transverse section of petiole of Ilicis Cornutae Folium

A. Sketch B. Section illustration

1. Epidermis 2. Collenchyma 3. Fibre bundles 4. Xylem 5. Phloem 6. Clusters of calcium oxalate 7. Parenchyma

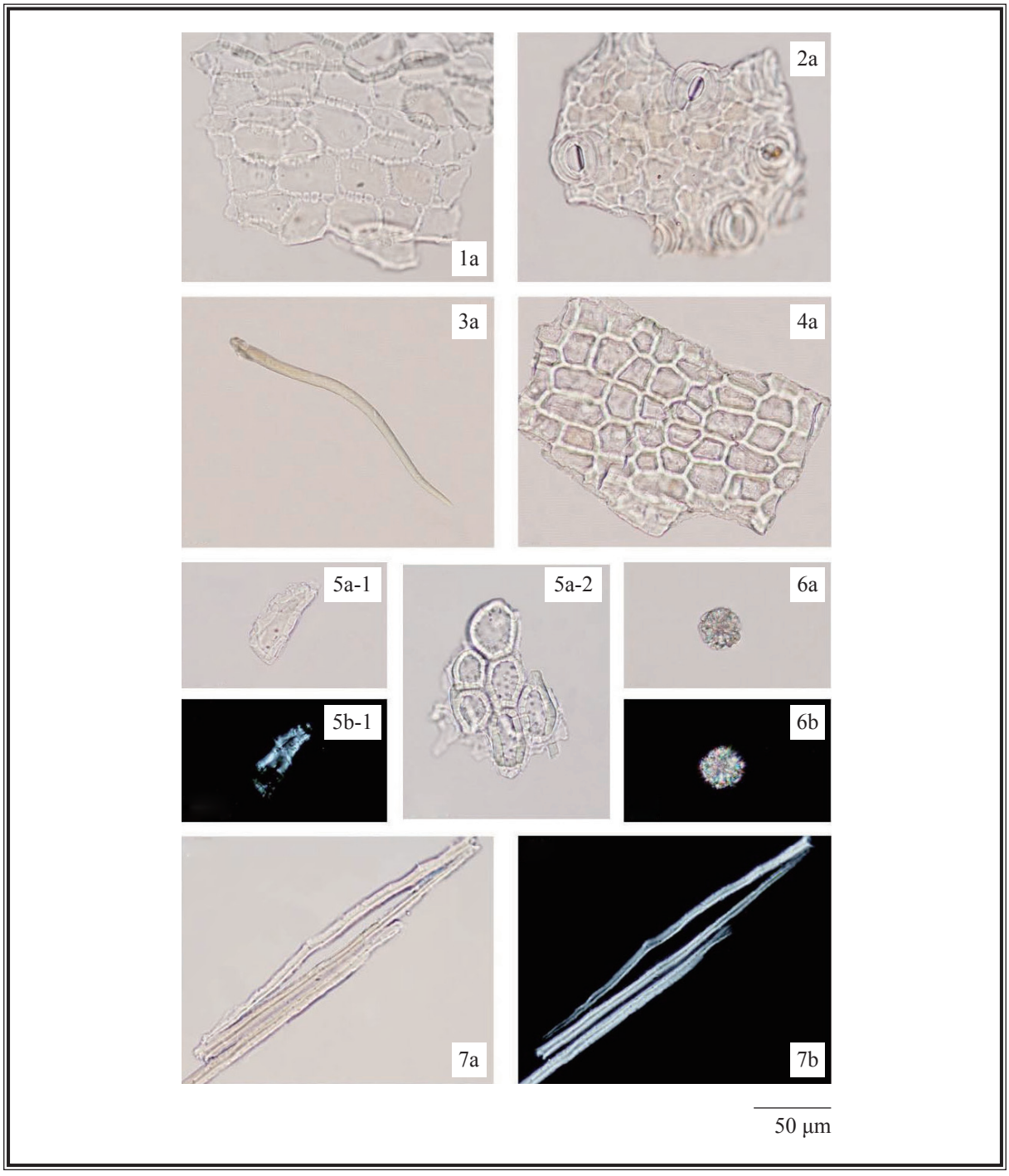


Figure 3 Microscopic features of powder of Ilicis Cornutae Folium

1. Upper epidermal cells 2. Lower epidermal cells with stomata 3. Non-glandular hair
4. Collenchymatous cells 5. Stone cells 6. Cluster of calcium oxalate 7. Fibres

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

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

Standard solutions

Lupeol standard solution

Weigh 1.0 mg of lupeol CRS (Fig. 4) and dissolve in 1 mL of methanol.

Ursolic acid standard solution

Weigh 1.0 mg of ursolic acid CRS (Fig. 4) and dissolve in 1 mL of methanol.

Developing solvent system

Prepare a mixture of *n*-hexane, dichloromethane and methanol (5:5:1, v/v).

Spray reagent

Add slowly 80 mL of sulphuric acid to 20 mL of ethanol and dissolve 0.5 g of vanillin.

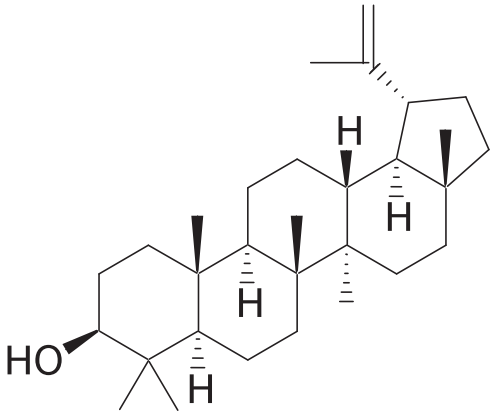
Test solution

Weigh 1.0 g of the powdered sample and place it in a 50-mL conical flask, then add 10 mL of methanol. Sonicate (400 W) the mixture for 30 min. Filter through a 0.45-μm nylon filter.

Procedure

Carry out the method by using a HPTLC silica gel F_{254} plate, a twin trough chamber and a freshly prepared developing solvent system as described above. Apply separately lupeol standard solution (2 μL), ursolic acid standard solution (2 μL) and the test solution (5 μL) to the plate. Before the development, add the developing solvent to one of the troughs of the chamber and place the HPTLC 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 HPTLC plate for development. Develop over a path of about 8 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 2 min). Examine the plate under visible light. Calculate the R_f values by using the equation as indicated in Appendix IV (A).

(i)



(ii)

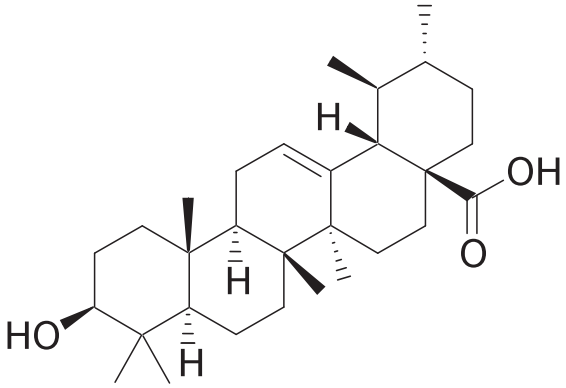


Figure 4 Chemical structures of (i) lupeol and (ii) ursolic acid

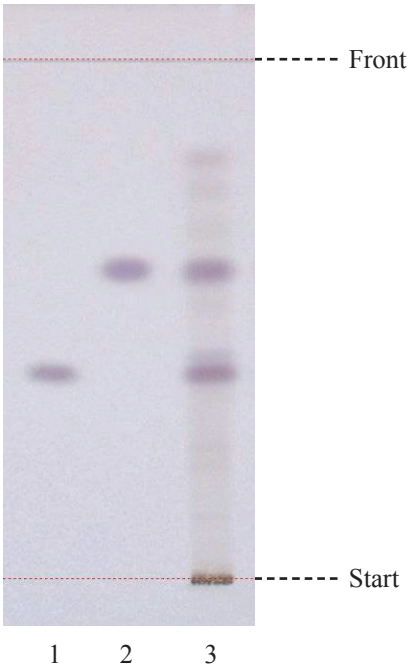


Figure 5 A reference HPTLC chromatogram of Ilicis Cornutae Folium extract observed under visible light after staining

1. Ursolic acid standard solution 2. Lupeol 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 lupeol and ursolic acid (Fig. 5).

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

Standard solutions

Lupeol standard solution for fingerprinting, Std-FP (70 mg/L)

Weigh 0.7 mg of lupeol CRS and dissolve in 10 mL of methanol.

Ursolic acid standard solution for fingerprinting, Std-FP (120 mg/L)

Weigh 1.2 mg of ursolic acid CRS and dissolve in 10 mL of methanol.

Test solution

Weigh 0.2 g of the powdered sample and place it in a 100-mL round-bottomed flask, then add 25 mL of methanol. Reflux the mixture for 30 min. Cool down to room temperature. Filter and transfer the filtrate to a 100-mL round-bottomed flask. Repeat the extraction for one more time. Wash the residue with 5 mL of methanol. Combine the solutions. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol. Transfer the

solution to a 25-mL volumetric flask and 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 (202 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (3.5 μm particle size). The column temperature is maintained at 35ºC during the separation. 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)	0.01% Phosphoric acid (%, v/v)	Elution
0 – 27	71	29	isocratic
27 – 31	71 → 80	29 → 20	linear gradient
31 – 36	80 → 88	20 → 12	linear gradient
36 – 60	88	12	isocratic

System suitability requirements

Perform at least five replicate injections, each using 10 μL of lupeol Std-FP and ursolic acid Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of lupeol and ursolic acid should not be more than 5.0%; the RSD of the retention times of lupeol and ursolic acid peaks should not be more than 2.0%; the column efficiencies determined from lupeol and ursolic acid peaks should not be less than 90000 and 20000 theoretical plates respectively.

The *R* value between peak 2 and the closest peak; and the *R* value between peak 4 and the closest peak in the chromatogram of the test solution should not be less than 1.5 (Fig. 6).

Procedure

Separately inject lupeol Std-FP, ursolic acid Std-FP and the test solution (10 μL each) into the HPLC system and record the chromatograms. Measure the retention times of lupeol and ursolic acid peaks in the chromatograms of lupeol Std-FP, ursolic acid Std-FP and the retention times of the four characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify lupeol and ursolic acid peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of lupeol Std-FP and ursolic acid Std-FP. The retention times of lupeol and ursolic acid peaks in the chromatograms of the test solution and the corresponding Std-FP 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 Ilicis Cornutae Folium extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the four characteristic peaks of Ilicis Cornutae Folium extract

Peak No.	RRT	Acceptable Range
1	0.71	± 0.04
2 (marker, ursolic acid)	1.00	-
3	1.21	± 0.03
4 (lupeol)	2.19	± 0.05

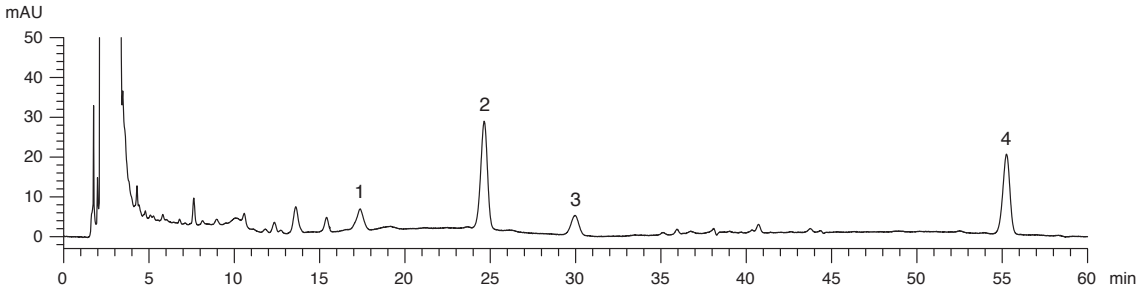


Figure 6 A reference fingerprint chromatogram of Ilicis Cornutae Folium extract

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 (except cadmium should not be more than 6.0 mg/kg).

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

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

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

5.5 Foreign Matter *(Appendix VIII)*: not more than 13.0%.

5.6 Ash *(Appendix IX)*

Total ash: not more than 7.0%.
Acid-insoluble ash: not more than 1.5%.

5.7 Water Content *(Appendix X)*

Oven dried method: not more than 8.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (hot extraction method): not less than 15.0%.
Ethanol-soluble extractives (hot extraction method): not less than 18.0%.

7. ASSAY

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

Standard solution

Mixed lupeol and ursolic acid standard stock solution, Std-Stock (600 mg/L for lupeol and 1000 mg/L for ursolic acid)

Weigh accurately 15.0 mg of lupeol CRS and 25.0 mg of ursolic acid CRS, and dissolve in 25 mL of methanol.

Mixed lupeol and ursolic acid standard solution for assay, Std-AS

Measure accurately the volume of the mixed lupeol and ursolic acid Std-Stock, dilute with methanol to produce a series of solutions of 18, 36, 72, 144, 288 mg/L for lupeol and 30, 60, 120, 240, 480 mg/L for ursolic acid.

Test solution

Weigh accurately 0.2 g of the powdered sample and place it in a 100-mL round-bottomed flask, then add 25 mL of methanol. Reflux the mixture for 30 min. Cool down to room temperature. Filter and transfer the filtrate to a 100-mL round-bottomed flask. Repeat the extraction for one more time. Wash the residue with 5 mL of methanol. Combine the solutions. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol. Transfer the solution to a 25-mL volumetric flask and 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 (202 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (3.5 µm particle size). The column temperature is maintained at 35ºC during the separation. The flow rate is about 1.0 mL/min. Programme the chromatographic system as follows (Table 3) –

Table 3 Chromatographic system conditions

Time (min)	Acetonitrile (%, v/v)	0.01% Phosphoric acid (%, v/v)	Elution
0 – 27	71	29	isocratic
27 – 31	71 → 80	29 → 20	linear gradient
31 – 36	80 → 88	20 → 12	linear gradient
36 – 60	88	12	isocratic

System suitability requirements

Perform at least five replicate injections, each using 10 µL of the mixed lupeol and ursolic acid Std-AS (72 mg/L for lupeol and 120 mg/L for ursolic acid). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of lupeol and ursolic acid should not be more than 5.0%; the RSD of the retention times of lupeol and ursolic acid peaks should not be more than 2.0%; the column efficiencies determined from lupeol and ursolic acid peaks should not be less than 90000 and 20000 theoretical plates respectively.

The *R* value between lupeol peak and the closest peak; and the *R* value between ursolic 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 lupeol and ursolic acid Std-AS (10 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of lupeol and ursolic acid against the corresponding concentrations of the mixed lupeol and ursolic 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 lupeol and ursolic acid peaks in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed lupeol and ursolic acid Std-AS. The retention times of lupeol and ursolic 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 lupeol and ursolic acid in the test solution, and calculate the percentage contents of lupeol and ursolic acid in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 1.7% of the total content of lupeol ($C_{30}H_{50}O$) and ursolic acid ($C_{30}H_{48}O_3$), calculated with reference to the dried substance.

8. CAUTION

This CMM should be used after proper processing (such as decoction).