Clematidis Radix et Rhizoma

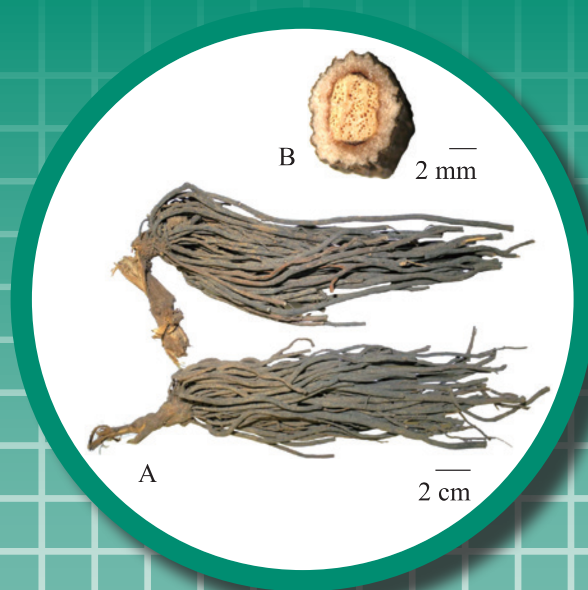

Figure 1 (i) A photograph of the dried root and rhizome of *Clematis chinensis* Osbeck

A. Root and rhizome B. Magnified transverse section of root

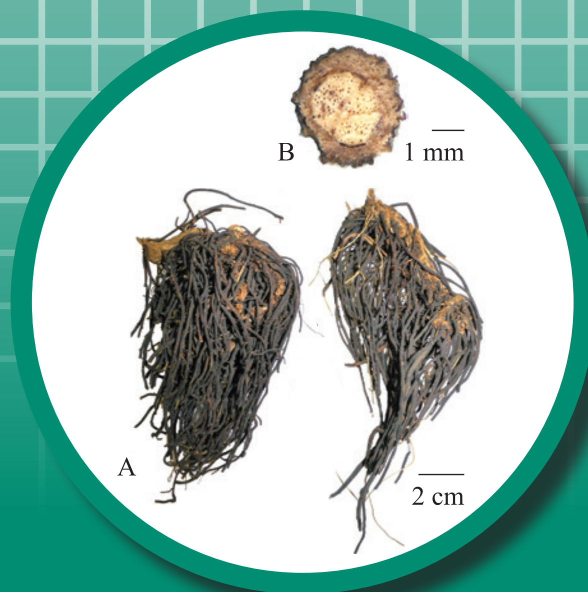

Figure 1 (ii) A photograph of the dried root and rhizome of *Clematis manshurica* Rupr.

A. Root and rhizome B. Magnified transverse section of root

1. NAMES

Official Name: Clematidis Radix et Rhizoma

Chinese Name: 威靈仙

Chinese Phonetic Name: Weilingxian

2. SOURCE

Clematidis Radix et Rhizoma is the dried root and rhizome of *Clematis chinensis* Osbeck or *Clematis manshurica* Rupr. (Ranunculaceae). The root and rhizome are collected in autumn, soil removed, then dried under the sun to obtain Clematidis Radix et Rhizoma.

Part I Dried root and rhizome of *Clematis chinensis* Osbeck

3. DESCRIPTION

Roots slender-cylindrical, slightly curved, 5-30 cm long, 1-5 mm in diameter. Externally greyish-brown or brown, with longitudinal fine wrinkles, sometimes showing yellowish-white wood when bark exfoliated. Texture hard and fragile, easily broken; fracture showing a relatively thick bark layer, wood slightly squared and yellowish-white, usually with cleft between bark and wood. Rhizomes unevenly cylindrical, 1.5-3.5 cm long (occasionally up to 10 cm), 2.5-25 mm in diameter. Externally pale brownish-grey, crowned by remnants of stem, with numerous rootlets at the lower part. Texture relatively tough; fracture fibrous. Odour slight; taste bland [Fig. 1 (i)].

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Root: The outer walls of epidermal cells thickened, brownish-black. Cortex broad, consisting of parenchytmatous cells, the cells contain with starch granules. Exodermis and endodermis distinct. Phloem fibre in bundles, or group with stone cells mainly present on the outer side of phloem. Cambium distinct. Xylem appearing diarch, completely lignified, subsquare (Fig. 2).

Powder

Colour greyish-brown or brown. Starch granules numerous, single starch granules subrounded, 4-19 µm in diameter, hilum pointed; black and cruciate-shaped under the polarized microscope; compound starch granules numerous, composed of 2-6 units. Epidermal cells subrectangular in surface view, 36-154 µm long, 20-48 µm wide, outer periclinal walls dark brown. Vessels bordered-pitted, 11-60 µm in diameter, bordered pits dense or sparse. Fibres scattered singly or in bundles, long fusiform, tapering towards the end or tail-like, 11-45 µm in diameter, walls thickened and lignified, pit canals relatively dense (Fig. 3).

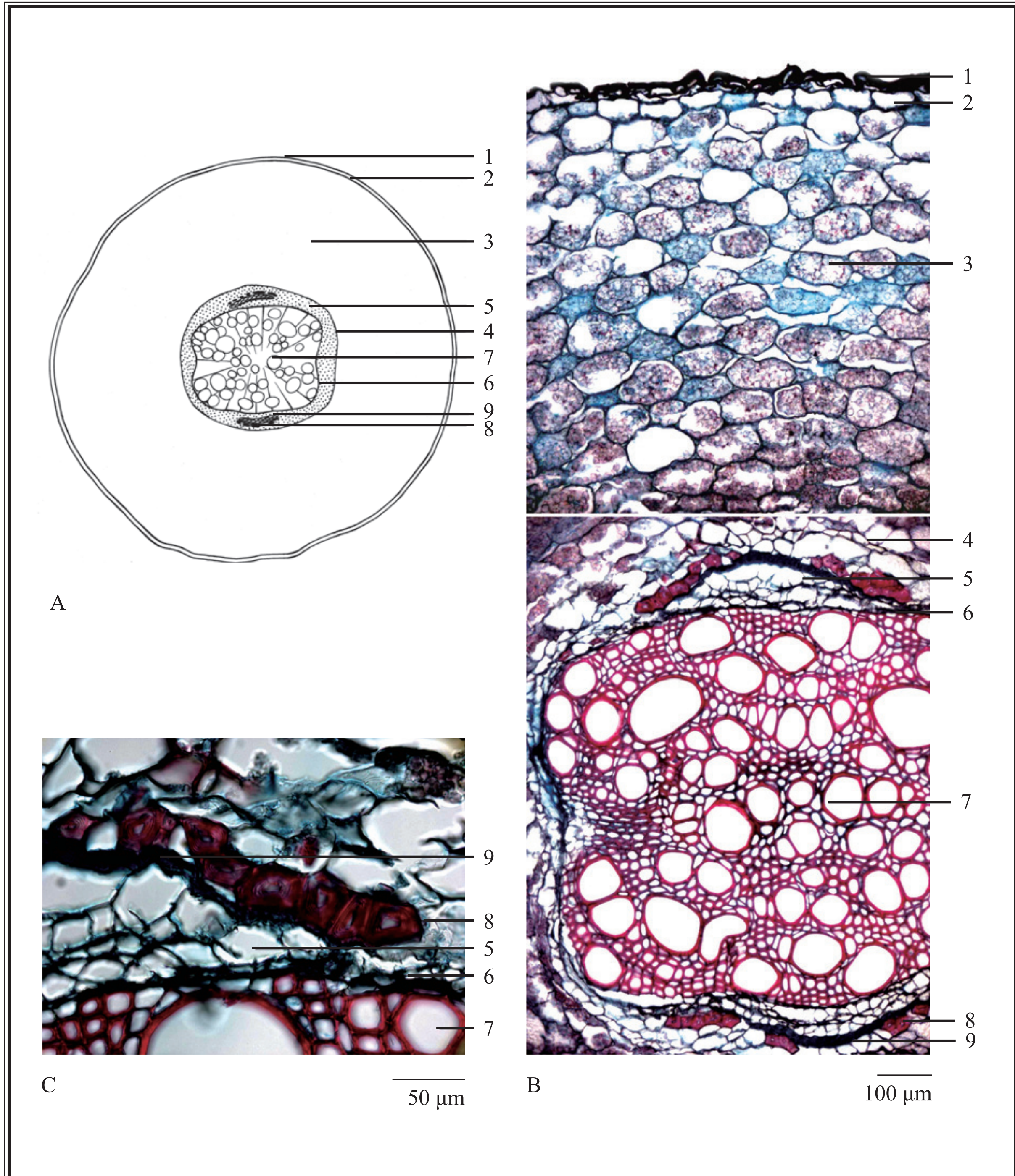

Figure 2 Microscopic features of transverse section of root of *Clematis chinensis* Osbeck

A. Sketch B. Section illustration C. Phloem fibre bundles and stone cells

1. Epidermis 2. Exodermis 3. Cortex 4. Endodermis 5. Phloem 6. Cambium
7. Xylem 8. Stone cells 9. Phloem fibre

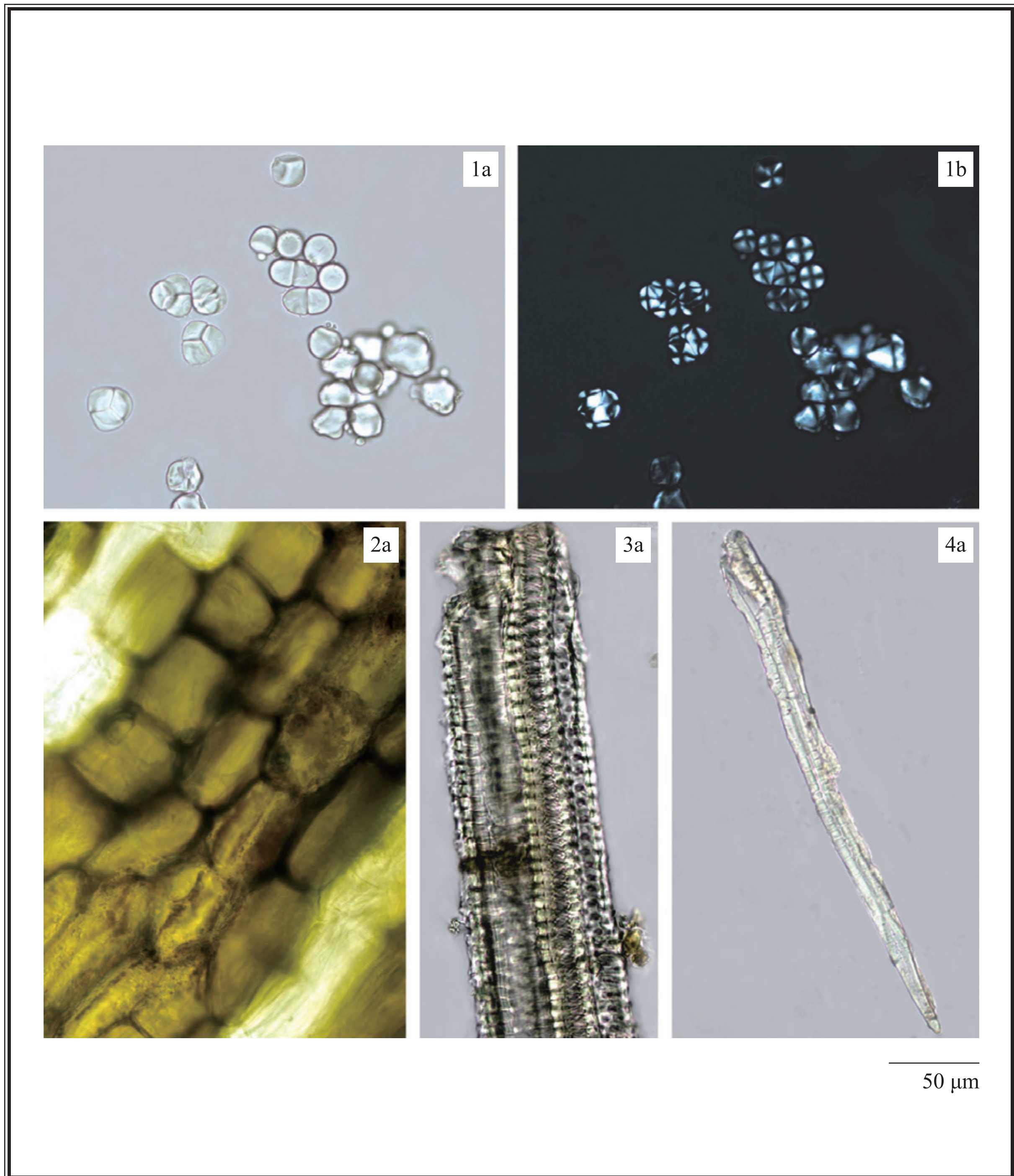

Figure 3 Microscopic features of powder of dried root and rhizome of *Clematis chinensis* Osbeck

1. Starch granules 2. Epidermal cells 3. Bordered-pitted vessels 4. Fibre

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

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

Standard solutions

Hederagenin standard solution

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

Oleanolic acid standard solution

Weigh 1.0 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 (5:3:0.1, v/v).

Spray reagent

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

Test solution

Weigh 1.0 g of the powdered sample and place it in a cellulose extraction thimble. Place the cellulose extraction thimble in soxhlet extractor. Add 100 mL of ethyl acetate to a 250-mL round-bottomed flask. Perform the soxhlet extraction for 2 h. Collect and dry the residue in air. Transfer the dried residue to a 50-mL conical flask and add 25 mL of methanol. Sonicate (100 W) the mixture for 30 min. Filter and transfer the filtrate to a 250-mL round-bottomed flask. Repeat the sonication for two more times. Combine the filtrates. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 30 mL of hydrochloric acid (7.3%, w/v). Reflux the mixture for 2 h. Cool down to room temperature. Transfer the solution to a 250-mL separating funnel. Extract for three times each with 30 mL of ethyl acetate. Combine the ethyl acetate extracts and transfer to a 250-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol. Transfer the solution to a 10-mL volumetric flask and make up to the mark with methanol. Filter through a 0.45-μm PTFE 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 hederagenin standard solution, oleanolic acid standard solution and the test solution (5 µL each) 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 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 visible light. Calculate the R_f values by using the equation as indicated in Appendix IV (A).

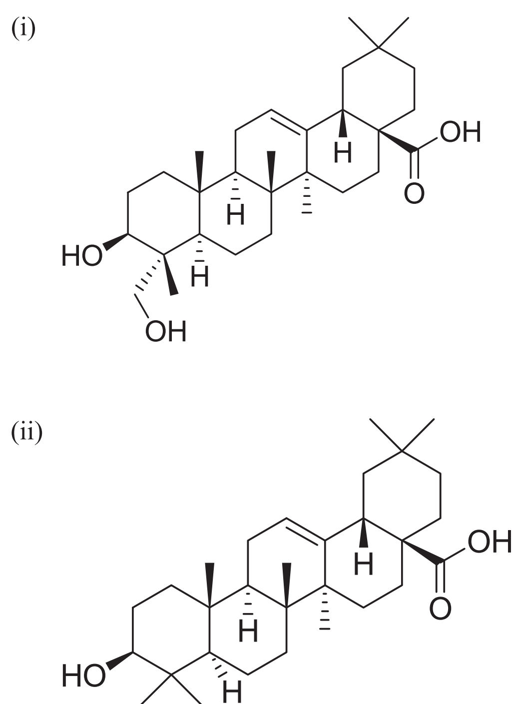

Figure 4 Chemical structures of (i) hederagenin and (ii) oleanolic acid

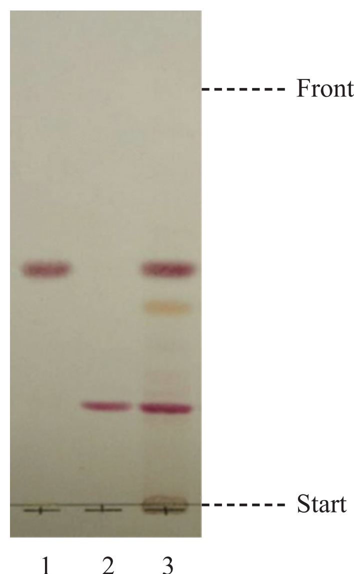

Figure 5 A reference HPTLC chromatogram of dried root and rhizome of *Clematis chinensis* Osbeck extract observed under visible light after staining

1. Oleanolic acid standard solution 2. Hederagenin 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 hederagenin and oleanolic acid (Fig. 5).

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

Standard solutions

Hederagenin standard solution for fingerprinting, Std-FP (220 mg/L)

Weigh 2.2 mg of hederagenin CRS and dissolve in 10 mL of methanol.

Oleanolic acid standard solution for fingerprinting, Std-FP (240 mg/L)

Weigh 1.2 mg of oleanolic acid CRS and dissolve in 5 mL of methanol.

Test solution

Weigh 0.5 g of the powdered sample and place it in a 50-mL conical flask, then add 25 mL of methanol (75%). Sonicate (100 W) the mixture for 30 min. Filter and transfer the filtrate to a 50-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 30 mL of hydrochloric acid (7.3%, w/v). Reflux the mixture for 2 h. Cool down to room temperature. Transfer the solution to a 250-mL separating funnel. Extract for three times each with 30 mL of ethyl acetate. Combine the ethyl acetate extracts and transfer to a 250-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure

in a rotary evaporator. Dissolve the residue in methanol. Transfer the solution to a 10-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 (205 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size). The column temperature is maintained at 30ºC 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)	0.1% Phosphoric acid (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 25	60	40	isocratic
25 – 45	60 → 10	40 → 90	linear gradient
45 – 60	10	90	isocratic

System suitability requirements

Perform at least five replicate injections, each using 10 µL of hederagenin Std-FP and oleanolic acid Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of hederagenin and oleanolic acid should not be more than 5.0%; the RSD of the retention times of hederagenin and oleanolic acid peaks should not be more than 2.0%; the column efficiencies determined from hederagenin and oleanolic acid peaks should not be less than 200000 and 150000 theoretical plates respectively.

The *R* value between peak 3 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 1.0 (Fig. 6).

Procedure

Separately inject hederagenin Std-FP, oleanolic acid Std-FP and the test solution (10 µL each) into the HPLC system and record the chromatograms. Measure the retention times of hederagenin and oleanolic acid peaks in the chromatograms of hederagenin Std-FP, oleanolic acid Std-FP and the retention times of the four characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify hederagenin and oleanolic acid peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of hederagenin Std-FP and oleanolic acid Std-FP. The retention times of hederagenin and oleanolic 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 dried root and rhizome of *Clematis chinensis* Osbeck extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the four characteristic peaks of dried root and rhizome of *Clematis chinensis* Osbeck extract

Peak No.	RRT	Acceptable Range
1	0.54	± 0.03
2	0.57	± 0.03
3 (marker, hederagenin)	1.00	-
4 (oleanolic acid)	1.19	± 0.03

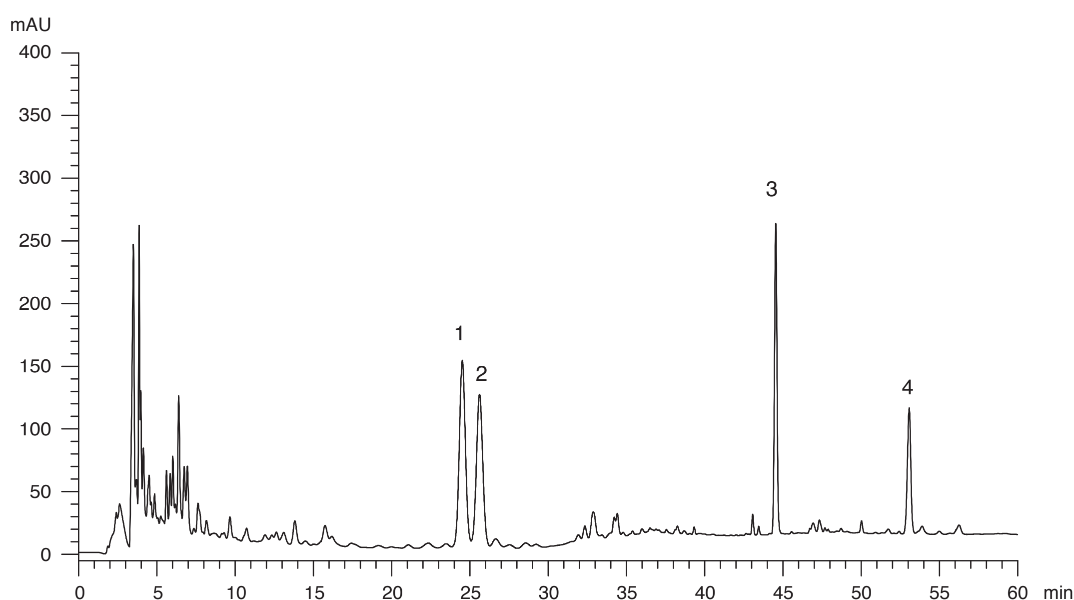

Figure 6 A reference fingerprint chromatogram of dried root and rhizome of *Clematis chinensis* Osbeck extract

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 *(Appendix VII)*: meet the requirements.

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

5.5 Foreign Matter *(Appendix VIII)*: not more than 4.0%.

5.6 Ash *(Appendix IX)*

Total ash: not more than 9.0%.
Acid-insoluble ash: not more than 5.0%.

5.7 Water Content *(Appendix X)*

Oven dried method: not more than 15.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (hot extraction method): not less than 19.0%.
Ethanol-soluble extractives (hot extraction method): not less than 5.0%.

7. ASSAY

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

Standard solution

Mixed hederagenin and oleanolic acid standard stock solution, Std-Stock (2000 mg/L for hederagenin and 2340 mg/L for oleanolic acid)

Weigh accurately 10.0 mg of hederagenin CRS and 11.7 mg of oleanolic acid CRS, and dissolve in 5 mL of methanol.

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

Measure accurately the volume of the mixed hederagenin and oleanolic acid Std-Stock, dilute with methanol to produce a series of solutions of 200, 500, 1000, 1500, 2000 mg/L for hederagenin and 117, 234, 585, 1170, 1755 mg/L for oleanolic acid.

Test solution

Weigh accurately 1.0 g of the powdered sample and place it in a cellulose extraction thimble. Place the cellulose extraction thimble in soxhlet extractor. Add 100 mL of ethyl acetate to a 250-mL round-bottomed flask. Perform the soxhlet extraction for 2 h. Collect and dry the residue in air. Transfer the dried residue to a 50-mL conical flask and add 25 mL of methanol. Sonicate (100 W) the mixture for 30 min. Filter and transfer the filtrate to a 250-mL round-bottomed flask. Repeat the sonication for two more times. Combine

the filtrates. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 30 mL of hydrochloric acid (7.3%, w/v). Reflux the mixture for 2 h. Cool down to room temperature. Transfer the solution to a 250-mL separating funnel. Extract for three times each with 30 mL of ethyl acetate. Combine the ethyl acetate extracts and transfer to a 250-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol. Transfer the solution to a 10-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 (205 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)	0.2% Phosphoric acid (%, 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 5 µL of the mixed hederagenin and oleanolic acid Std-AS (1000 mg/L for hederagenin and 585 mg/L for oleanolic acid). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of hederagenin and oleanolic acid should not be more than 5.0%; the RSD of the retention times of hederagenin and oleanolic acid peaks should not be more than 2.0%; the column efficiencies determined from hederagenin and oleanolic acid peaks should not be less than 2400 and 10000 theoretical plates respectively.

The *R* value between hederagenin 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 hederagenin and oleanolic acid Std-AS (5 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of hederagenin and oleanolic acid against the corresponding concentrations of the mixed hederagenin and oleanolic acid Std-AS. Obtain the slopes, y-intercepts and the r^2 values from the corresponding 5-point calibration curves.

Procedure

Inject 5 μL of the test solution into the HPLC system and record the chromatogram. Identify hederagenin and oleanolic acid peaks in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed hederagenin and oleanolic acid Std-AS. The retention times of hederagenin 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 hederagenin and oleanolic acid in the test solution, and calculate the percentage contents of hederagenin and oleanolic acid in the sample by using the equations as indicated in Appendix IV (B).

Limits

The dried root and rhizome of *Clematis chinensis* Osbeck contains not less than 0.54% of hederagenin ($C_{30}H_{48}O_4$) and not less that 0.39% of oleanolic acid ($C_{30}H_{48}O_3$), calculated with reference to the dried substance.

Part II Dried root and rhizome of *Clematis manshurica* Rupr.

3. DESCRIPTION

Roots slender-cylindrical, 5-20 cm long, 1-3 mm in diameter. Externally brownish-black; fracture showing wood subrounded or slightly squared, whitish. Rhizomes irregularly lumpy, with numerous rootlets. Taste pungent [Fig. 1 (ii)].

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Root: The outer walls of epidermal cells thickened, brownish-black. Cortex broad, consisting of parenchytmatous cells, the cells contain with starch granules. Exodermis and endodermis distinct. Phloem fibre in bundles, lignified stone cells absent on the outer side of phloem. Cambium distinct. Xylem appearing diarch, completely lignified, subsquare (Fig. 7).

Powder

Colour brown. Starch granules numerous, single starch granules subrounded, 3-16 µm in diameter, hilum pointed; black and cruciate-shaped under the polarized microscope; compound starch granules composed of 2-6 units. Epidermal cells subrectangular in surface view, 36-111 µm long, 14-40 µm wide, outer periclinal walls dark brown. Vessels bordered-pitted, 12-58 µm in diameter, bordered pits dense or sparse. Fibres scattered singly or in bundles, long fusiform, tapering towards the end or tail-like, 11-42 µm in diameter, walls thickened and lignified, pit canals relatively dense (Fig. 8).

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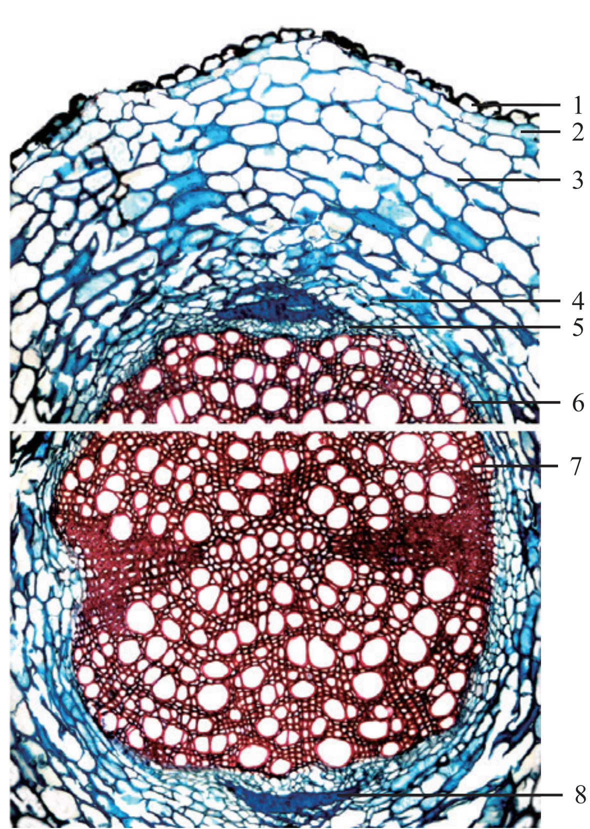
A



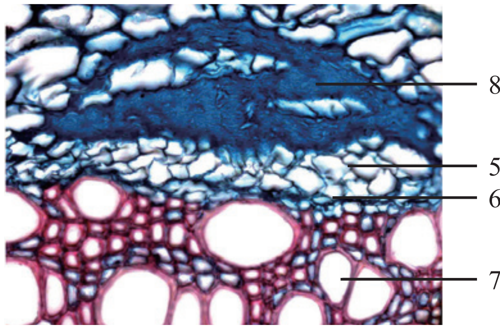


Figure 7 Microscopic features of transverse section of root of *Clematis manshurica* Rupr.

A. Sketch B. Section illustration C. Phloem fibre

1. Epidermis 2. Exodermis 3. Cortex 4. Endodermis 5. Phloem 6. Cambium 7. Xylem 8. Phloem fibre

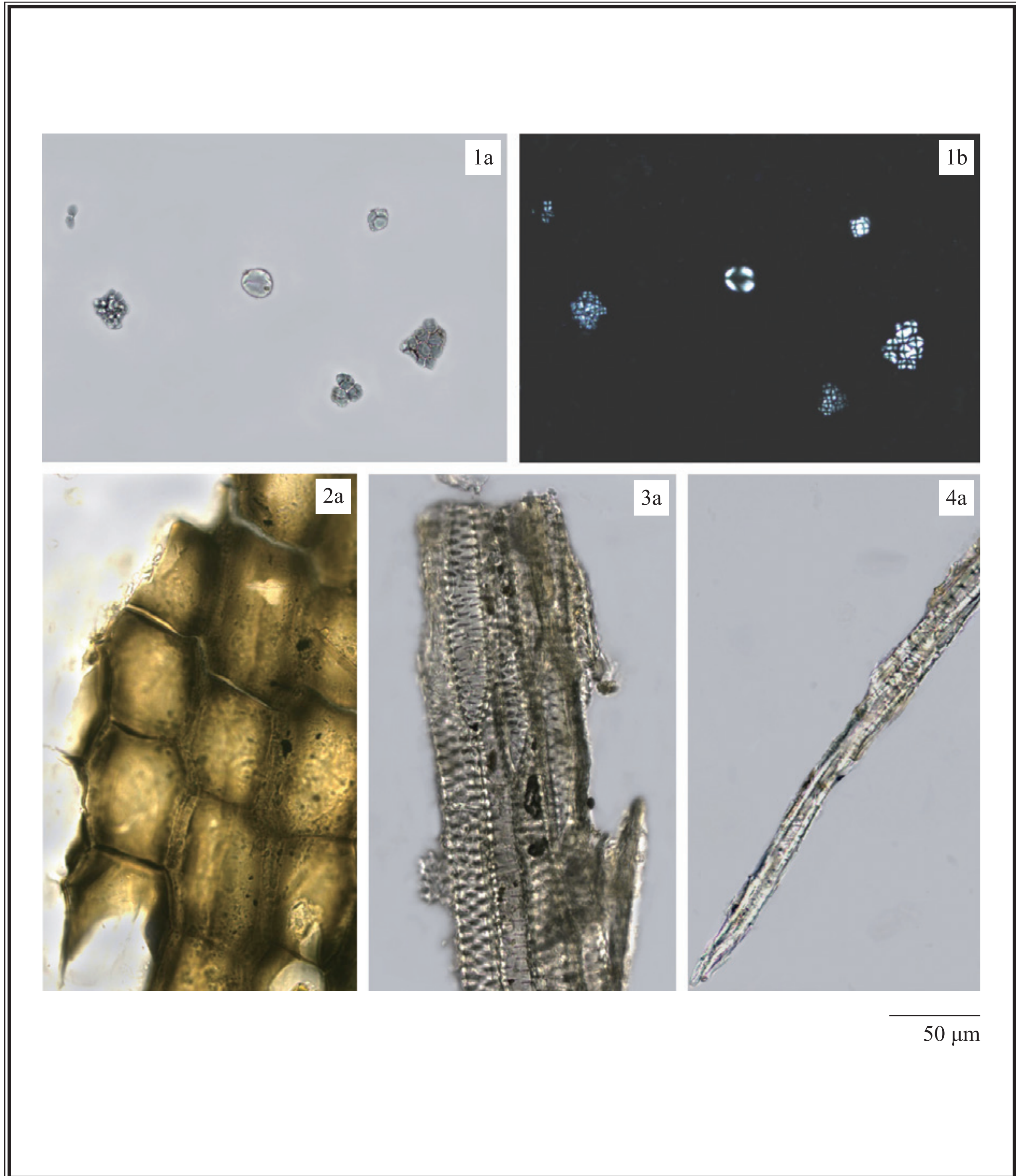


Figure 8 Microscopic features of powder of dried root and rhizome of *Clematis manshurica* Rupr.

1. Starch granules 2. Epidermal cells 3. Bordered-pitted vessels 4. Fibre

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

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

Standard solution

Oleanolic acid standard solution

Weigh 1.0 mg of oleanolic acid CRS (Fig. 9) and dissolve in 1 mL of methanol.

Developing solvent system

Prepare a mixture of cyclohexane, ethyl acetate and formic acid (5:3:0.1, v/v).

Spray reagent

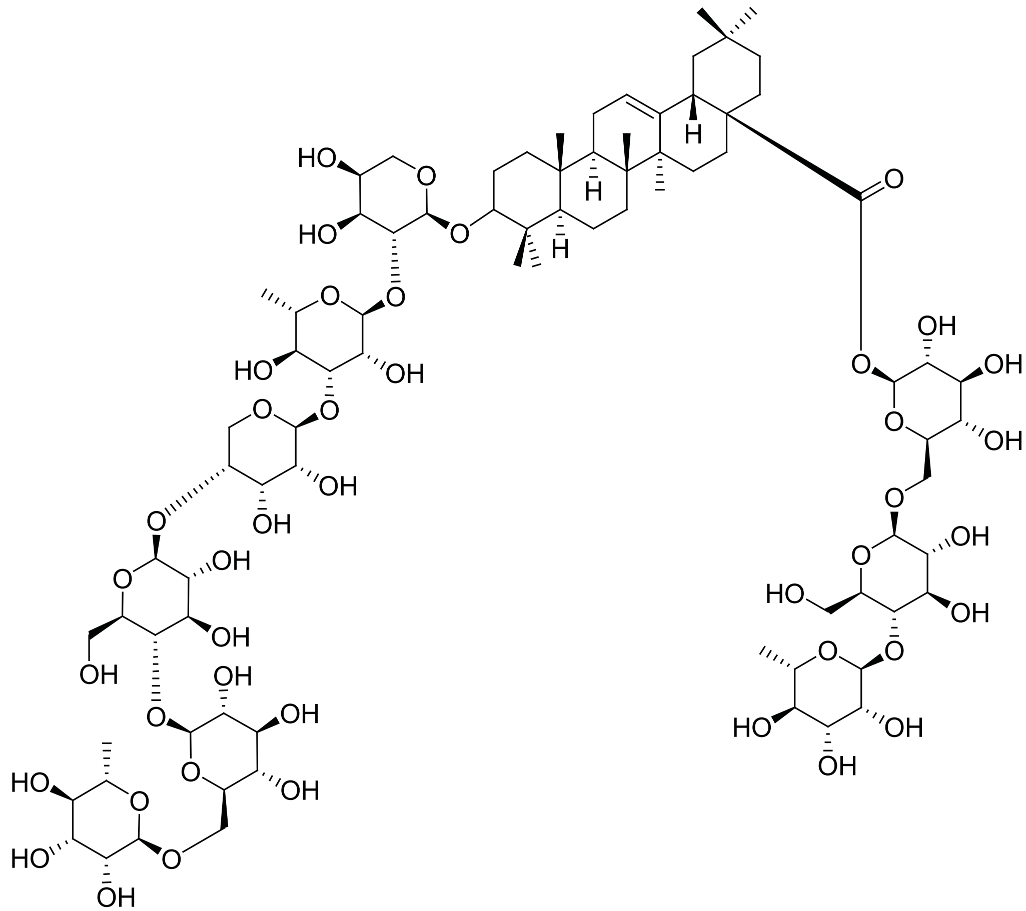
Add slowly 10 mL of sulphuric acid to 90 mL of ethanol.

Test solution

Weigh 1.0 g of the powdered sample and place it in a cellulose extraction thimble. Place the cellulose extraction thimble in soxhlet extractor. Add 100 mL of ethyl acetate to a 250-mL round-bottomed flask. Perform the soxhlet extraction for 2 h. Collect and dry the residue in air. Transfer the dried residue to a 50-mL conical flask and add 25 mL of methanol. Sonicate (100 W) the mixture for 30 min. Filter and transfer the filtrate to a 250-mL round-bottomed flask. Repeat the sonication for two more times. Combine the filtrates. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 30 mL of hydrochloric acid (7.3%, w/v). Reflux the mixture for 2 h. Cool down to room temperature. Transfer the solution to a 250-mL separating funnel. Extract for three times each with 30 mL of ethyl acetate. Combine the ethyl acetate extracts and transfer to a 250-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol. Transfer the solution to a 10-mL volumetric flask and make up to the mark with methanol. Filter through a 0.45-µm PTFE 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 oleanolic acid standard solution and the test solution (5 µL each) 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 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 visible light. Calculate the R_f value by using the equation as indicated in Appendix IV (A).



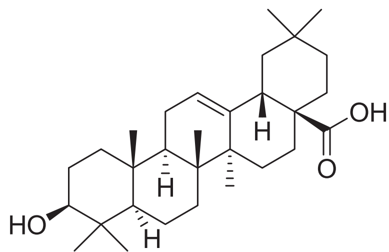


Figure 9 Chemical structures of (i) clematichinenoside AR and (ii) oleanolic acid

Figure 10 A reference HPTLC chromatogram of dried root and rhizome of *Clematis manshurica* Rupr. extract observed under visible light after staining

1. Oleanolic acid standard solution 2. Test solution

For positive identification, the sample must give spot or band with chromatographic characteristics, including the colour and the R_f value, corresponding to that of oleanolic acid (Fig. 10).

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

Standard solution

Clematichinenoside AR standard solution for fingerprinting, Std-FP (1000 mg/L)

Weigh 1.0 mg of clematichinenoside AR CRS (Fig. 9) and dissolve in 1 mL of methanol.

Test solution

Weigh 0.3 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 10 mL of methanol. Sonicate (100 W) the mixture for 30 min. Centrifuge at about 3000 × *g* for 5 min. Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (205 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 4) –

Table 4 Chromatographic system conditions

Time (min)	Acetonitrile (%, v/v)	Water (%, v/v)	Elution
0 – 40	20 → 35	80 → 65	linear gradient
40 – 60	35	65	isocratic

System suitability requirements

Perform at least five replicate injections, each using 10 µL of clematichinenoside AR Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak area of clematichinenoside AR should not be more than 5.0%; the RSD of the retention time of clematichinenoside AR peak should not be more than 2.0%; the column efficiency determined from clematichinenoside AR peak should not be less than 80000 theoretical plates.

The *R* value between peak 3 and the closest peak in the chromatogram of the test solution should not be less than 1.0 (Fig. 11).

Procedure

Separately inject clematichinenoside AR Std-FP and the test solution (10 µL each) into the HPLC system and record the chromatograms. Measure the retention time of clematichinenoside AR peak in the chromatogram of clematichinenoside AR Std-FP and the retention times of the four characteristic peaks (Fig. 11) in the chromatogram of the test solution. Identify clematichinenoside AR peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of clematichinenoside AR Std-FP. The retention times of clematichinenoside AR 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 dried root and rhizome of *Clematis manshurica* Rupr. extract are listed in Table 5.

Table 5 The RRTs and acceptable ranges of the four characteristic peaks of dried root and rhizome of *Clematis manshurica* Rupr. extract

Peak No.	RRT	Acceptable Range
1	0.61	± 0.03
2	0.91	± 0.03
3 (marker, clematichinenoside AR)	1.00	-
4	1.19	± 0.03

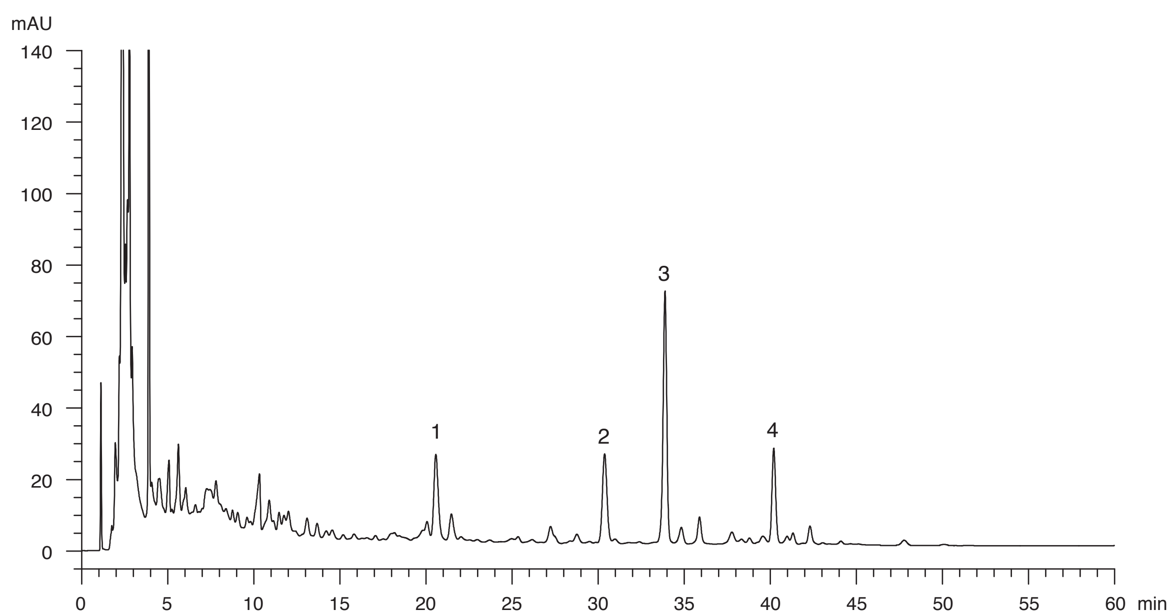


Figure 11 A reference fingerprint chromatogram of dried root and rhizome of *Clematis manshurica* Rupr. extract

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. 11).

5. TESTS

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

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 4.0%.

5.6 Ash *(Appendix IX)*

Total ash: not more than 9.0%.
Acid-insoluble ash: not more than 5.0%.

5.7 Water Content *(Appendix X)*

Oven dried method: not more than 15.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (hot extraction method): not less than 19.0%.
Ethanol-soluble extractives (hot extraction method): not less than 5.0%.

7. ASSAY

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

Standard solution

Oleanolic acid standard stock solution, Std-Stock (2340 mg/L)

Weigh accurately 11.7 mg of oleanolic acid CRS and dissolve in 5 mL of methanol.

Oleanolic acid standard solution for assay, Std-AS

Measure accurately the volume of the oleanolic acid Std-Stock, dilute with methanol to produce a series of solutions of 117, 234, 585, 1170, 1755 mg/L for oleanolic acid.

Test solution

Weigh accurately 1.0 g of the powdered sample and place it in a cellulose extraction thimble. Place the cellulose extraction thimble in soxhlet extractor. Add 100 mL of ethyl acetate to a 250-mL round-bottomed flask. Perform the soxhlet extraction for 2 h. Collect and dry the residue in air. Transfer the dried residue to a 50-mL conical flask and add 25 mL of methanol. Sonicate (100 W) the mixture for 30 min. Filter and transfer the filtrate to a 250-mL round-bottomed flask. Repeat the sonication for two more times. Combine the filtrates. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 30 mL of hydrochloric acid (7.3%, w/v). Reflux the mixture for 2 h. Cool down to room temperature. Transfer the solution to a 250-mL separating funnel. Extract for three times each with 30 mL of ethyl acetate. Combine the ethyl acetate extracts and transfer to a 250-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol. Transfer the solution to a 10-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 (205 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 6) –

Table 6 Chromatographic system conditions

Time (min)	0.2% Phosphoric acid (%, 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 5 µL of oleanolic acid Std-AS (585 mg/L). The requirements of the system suitability parameters are as follows: the RSD of the peak area of oleanolic acid should not be more than 5.0%; the RSD of the retention time of oleanolic acid peak should not be more than 2.0%; the column efficiency determined from oleanolic acid peak should not be less than 10000 theoretical plates.

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 curve

Inject a series of oleanolic acid Std-AS (5 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of oleanolic acid against the corresponding concentrations of oleanolic acid Std-AS. Obtain the slope, y-intercept and the r^2 value from the 5-point calibration curve.

Procedure

Inject 5 µL of the test solution into the HPLC system and record the chromatogram. Identify oleanolic acid peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of oleanolic acid Std-AS. The retention times of oleanolic acid peaks from the two chromatograms should not differ by more than 5.0%. Measure the peak area and calculate the concentration (in milligram per litre) of oleanolic acid in the test solution, and calculate the percentage content of oleanolic acid in the sample by using the equations as indicated in Appendix IV (B).

Limits

The dried root and rhizome of *Clematis manshurica* Rupr. contains not less than 0.47% of oleanolic acid ($C_{30}H_{48}O_3$), calculated with reference to the dried substance.