

Radix Achyranthis Bidentatae



Figure 1 A photograph of Radix Achyranthis Bidentatae

Radix Achyranthis Bidentatae

1. NAMES

Official Name: Radix Achyranthis Bidentatae

Chinese Name: 牛膝

Chinese Phonetic Name: Niuxi

2. SOURCE

Radix Achyranthis Bidentatae is the dried root of *Achyranthes bidentata* Bl. (Amaranthaceae). The root is collected in the winter time when the aerial part has withered. Following removal of rootlets and soil, the roots are tied up into a small bundle, sun-dried until they become wrinkled externally, cut evenly at the summit, then dried under the sun thoroughly to obtain Radix Achyranthis Bidentatae.

3. DESCRIPTION

Slender and cylindrical, straight or slightly curved, 13-90 cm long, 4-10 mm in diameter. Outer surface greyish-yellow or pale brown, with slightly twisted and fine longitudinal wrinkles, transverse lenticels, and sparsely distributed rootlet scars. Texture hard and fragile, softened when moistened, easily broken; fracture even, pale yellowish-brown, slightly keratose and oily. Xylem of vascular bundles in the center relatively large, yellowish-white; xylem in the outside small, in many scattered and spotted vascular bundles, arranged in 2-4 whorls. Odour slight; taste slightly sweet to somewhat bitter and astringent (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification (Appendix III)

Transverse section

The cork consisting of several rows of cells. Cortex parenchyma cells elongated and arranged in rows tangentially. Vascular bundles arranged in 2-4 interrupted whorls, relatively small in the outermost whorl, but relatively large inward, sometimes having only one to several vessels. Cambium nearly in a ring in the outermost whorl. Xylem mainly consists of vessels and small

xylem fibres; the central xylem aggregated into 2-3 groups. Parenchyma cells contain crystal sand of calcium oxalate (Fig. 2).

Powder

Colour pale brown to greyish-white. Crystal sand of calcium oxalate triangular, arrow-pointed, subsquare or irregular in shape, scattered in the parenchyma cells, polychrome when observed under a polarized microscope. Bordered-pitted and reticulate vessels 8-93 μm in diameter. Fibres mostly in bundles, walls slightly thickened, pits sparse, pit apertures oblique-porous, crisscross or V-shaped. Cork cells subsquare, subrectangular, subround or polygonal (Fig. 3).

4.2 Physicochemical Identification

Procedure

Weigh 1.0 g of the powdered sample and put into a 50-mL centrifugal tube, then add 10 mL of dichloromethane. Sonicate (560 W) the mixture for 30 min. Centrifuge at about $3000 \times g$ for 5 min. Filter through a 0.45- μm RC filter. Transfer 0.5 mL of the filtrate to a test tube. Cautiously add about 0.5 mL of sulphuric acid along the inner wall of the test tube. Allow to stand for 20 min. A reddish-brown or yellowish-brown ring is observed at the interface of the two solvent layers.

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

Standard solution

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 dichloromethane and ethyl acetate (5:1, v/v).

Spray reagent

Mix 1 mL of dilute sulphuric acid (50%, v/v) and 10 mL of *p*-hydroxybenzaldehyde in methanol (2%, w/v). Freshly prepare the reagent.

Radix Achyranthis Bidentatae

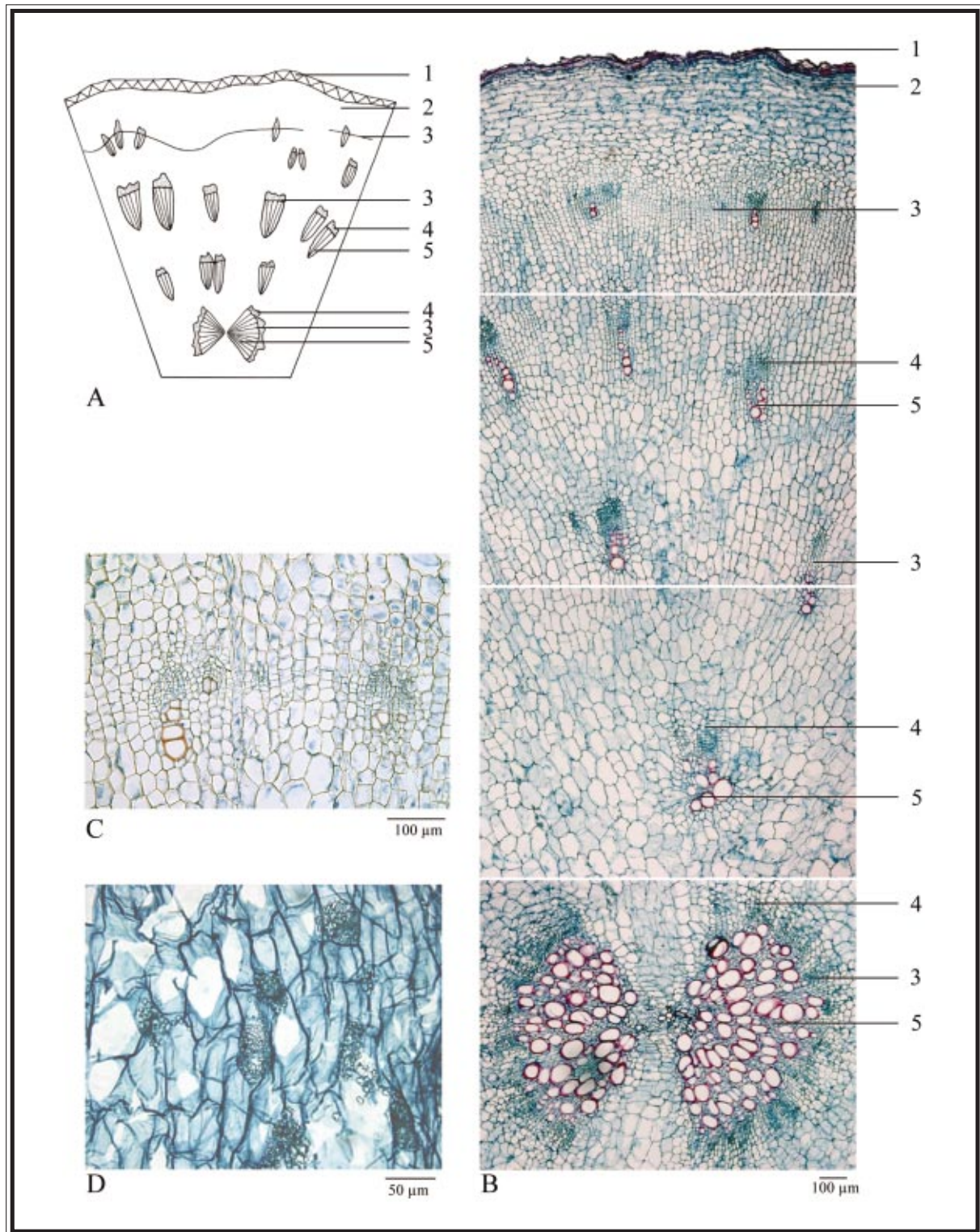


Figure 2 Microscopic features of transverse section of *Radix Achyranthis Bidentatae*

A. Sketch B. Section illustration C. Vascular bundles in the outermost whorl
D. Crystal sand of calcium oxalate

1. Cork 2. Cortex 3. Cambium 4. Phloem 5. Xylem

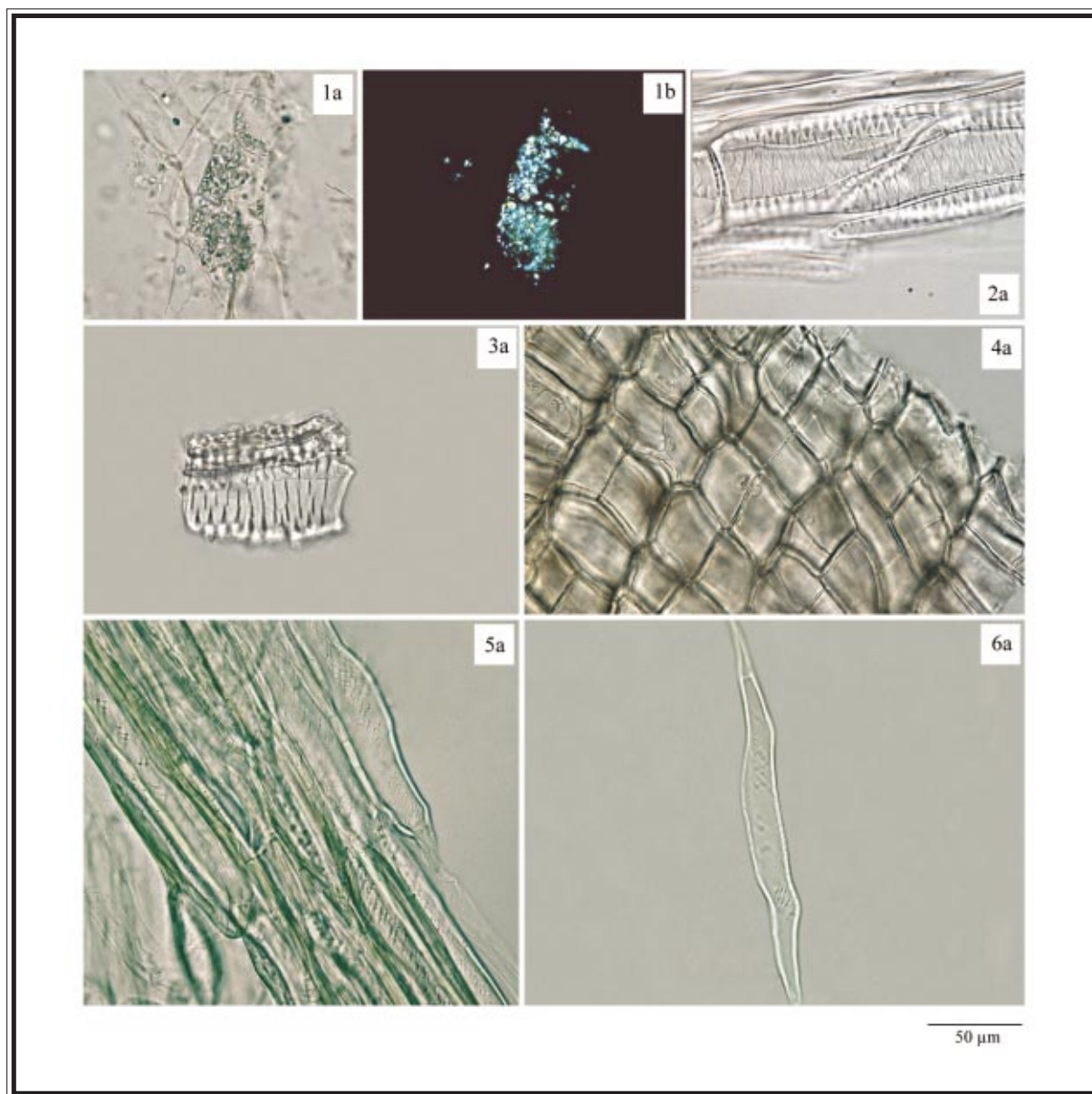


Figure 3 Microscopic features of powder of *Radix Achyranthis Bidentatae*

1. Crystal sand of calcium oxalate
2. Bordered-pitted vessels
3. Reticulate vessel
4. Cork cells
5. Xylem fibres in a bundle
6. Xylem fibre

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

Test solution

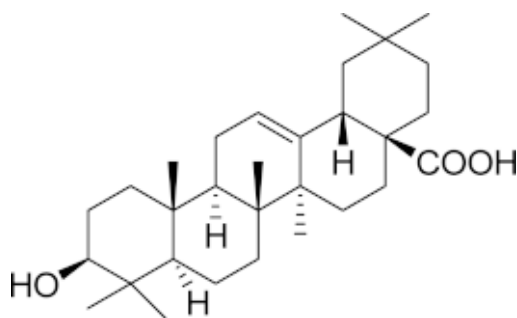
Weigh 1.0 g of the powdered sample and put into a 100-mL round-bottomed flask, then add 30 mL of ethanol (75%) and 3 mL of hydrochloric acid. Reflux the mixture for 1.5 h. Cool down to room temperature. Filter and evaporate the filtrate to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 20 mL of water. Transfer the solution to a separatory funnel. Extract with 100 mL of dichloromethane. Collect the dichloromethane layer and evaporate to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 1 mL of methanol.

Procedure

Carry out the method by using a HPTLC silica gel F_{254} plate and a freshly prepared developing solvent system as described above. Apply separately oleanolic acid standard solution (4 μ L) and the test solution (1 μ L) to the plate. Develop over a path of about 4 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 100°C until the spots or bands become visible (about 10 min). Examine the plate under visible light. Calculate the R_f value by using the equation as indicated in Appendix IV(A).

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.

(i)



(ii)

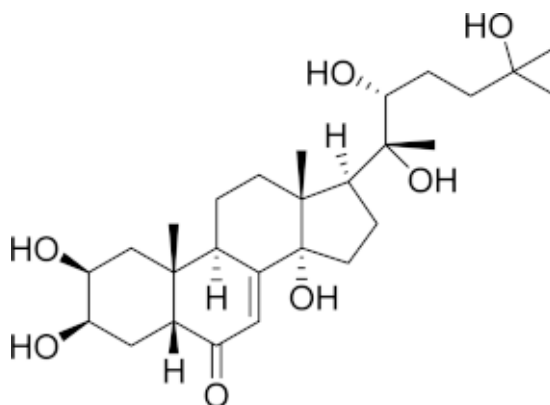


Figure 4 Chemical structures of (i) oleanolic acid and (ii) ecdysterone

4.4 High-Performance Liquid Chromatographic Fingerprinting (Appendix XII)

Standard solution

Ecdysterone standard solution for fingerprinting, Std-FP (30 mg/L)
Weigh 3.0 mg of ecdysterone CRS (Fig. 4) and dissolve in 100 mL of methanol.

Test solution

Weigh 1.0 g of the powdered sample and put into a 50-mL centrifugal tube, then add 10 mL of methanol. Sonicate (560 W) the mixture for 30 min. Centrifuge at about 3000 × g for 5 min. Filter the supernatant through a 0.45-μm RC filter.

Chromatographic system

The liquid chromatograph is equipped with a detector (270 nm) and a column (3.9 × 300 mm) packed with ODS bonded silica gel (4 μm particle size). The flow rate is about 0.8 mL/min. Programme the chromatographic system as follows –

Time (min)	Water (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 5	95	5	isocratic
5 – 40	95 → 55	5 → 45	linear gradient

System suitability requirements

Perform at least five replicate injections each with 10 μL of ecdysterone Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak area of ecdysterone should not be more than 5.0%; the RSD of the retention time of ecdysterone peak should not be more than 2.0%; the column efficiency determined from ecdysterone peak should not be less than 20000 theoretical plates.

The *R* value between peak 2 and peak 3 in the chromatogram of the test solution should not be less than 1.0 (Fig. 5).

Procedure

Separately inject ecdysterone Std-FP and the test solution (10 μL each) into the HPLC system and record the chromatograms. Measure the retention time of ecdysterone peak in the chromatogram of ecdysterone Std-FP and the retention times of the five characteristic peaks (Fig. 5) in the chromatogram of the test solution. Under the same HPLC conditions, identify ecdysterone peak in the chromatogram of the test solution by comparing its retention time with that in the

chromatogram of ecdysterone Std-FP. The retention times of ecdysterone 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 five characteristic peaks of Radix Achyranthis Bidentatae extract are listed in Table 1.

Table 1 The RRTs and acceptable ranges of the five characteristic peaks of Radix Achyranthis Bidentatae extract

Peak No.	RRT	Acceptable Range
1 (marker, ecdysterone)	1.00	-
2	1.03	±0.03
3	1.04	±0.03
4	1.30	±0.04
5	1.32	±0.03

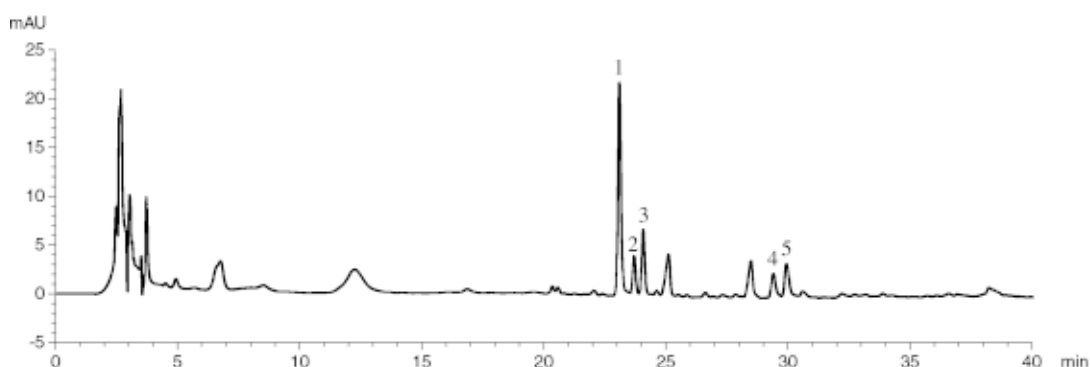


Figure 5 A reference fingerprint chromatogram of Radix Achyranthis Bidentatae extract

For positive identification, the sample must give the above five characteristic peaks with RRTs falling within the acceptable range of the corresponding peaks in the reference fingerprint chromatogram (Fig. 5).

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*): not more than 400 mg/kg.

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

5.6 Ash (*Appendix IX*)

Total ash: not more than 9.0%.

Acid-insoluble ash: not more than 1.5%.

5.7 Water Content (*Appendix X*): not more than 16.0%.

6. EXTRACTIVES (*Appendix XI*)

Water-soluble extractives (cold extraction method): not less than 59.0%.

Ethanol-soluble extractives (cold extraction method): not less than 54.0%.

7. ASSAY

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

Standard solution

Oleanolic acid standard stock solution, Std-Stock (1000 mg/L)

Weigh accurately 10.0 mg of oleanolic acid CRS and dissolve in 10 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 1, 10, 50, 100, 400 mg/L for oleanolic acid.

Test solution

Weigh accurately 0.2 g of the powdered sample and put into a 50-mL centrifugal tube, then add 10 mL of ethanol (70%). Sonicate (560 W) the mixture for 30 min. Centrifuge at about $3000 \times g$ for 8 min. Filter the supernatant through a 0.45- μ m RC filter. Repeat the extraction twice each with 10 mL of ethanol (70%). Combine the filtrate to a 50-mL volumetric flask. Make up to the mark with ethanol (70%). Pipette 10 mL of the solution into a 50-mL test tube. Add 1 mL of 6 M HCl. Mix and warm the solution in a water bath (75°C) for 22 h. Cool down to room temperature. Transfer the solution to a 50-mL round-bottomed flask and rinse the residual liquid for three times, each with 5 mL of ethanol (70%). Combine

the extracts. 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 RC filter.

Chromatographic system

The liquid chromatograph is equipped with a detector (208 nm) and a column (3.9 × 300 mm) packed with ODS bonded silica gel (4 μm particle size). The flow rate is about 1.0 mL/min. The mobile phase is a mixture of 0.0012 M HCl and acetonitrile (2:8, v/v). The elution time is about 15 min.

System suitability requirements

Perform at least five replicate injections each with 10 μL of oleanolic acid Std-AS (100 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 6000 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

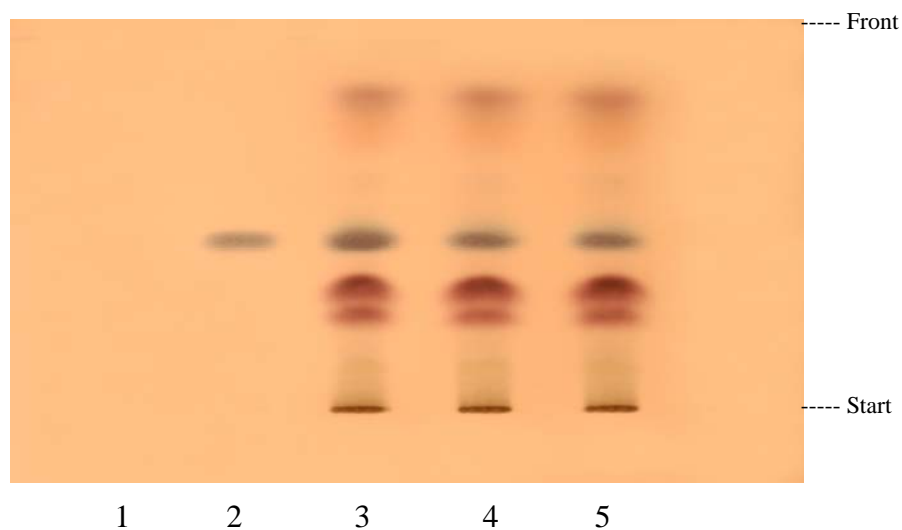
Inject a series of oleanolic acid Std-AS (10 μL each) into the HPLC system and record the chromatograms. Plot the peak areas of oleanolic acid against the corresponding concentrations of oleanolic acid Std-AS. Obtain the slope, y-intercept and the *r*² value from the 5-point calibration curve.

Procedure

Inject 10 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 indicated in Appendix IV(B).

Limits

The sample contains not less than 1.1% of oleanolic acid (C₃₀H₄₈O₃), calculated with reference to the dried substance.



Lane	Sample	Results
1	Blank (Methanol)	Negative
2	Standard (Oleanolic acid)	Oleanolic acid positive
3	Spiked sample (Sample plus oleanolic acid)	Oleanolic acid positive
4	Sample (Radix Achyranthis Bidentatae)	Oleanolic acid positive
5	Sample duplicate (Radix Achyranthis Bidentatae)	Oleanolic acid positive

Figure 1 TLC results of Radix Achyranthis Bidentatae extract observed under visible light after staining