Bletillae Rhizoma

Figure 1 A photograph of Bletillae Rhizoma

A. Bletillae Rhizoma B. Cut surface of tuber

1. NAMES

Official Name: Bletillae Rhizoma

Chinese Name: 白及

Chinese Phonetic Name: Baiji

2. SOURCE

Bletillae Rhizoma is the dried tuber of *Bletilla striata* (Thunb.) Reichb. f. (Orchidaceae). The tuber is harvested in summer and autumn, rootlets removed, washed clean, boiled or steamed until no dry core visible, dried to semi-dryness under the sun, peeled to remove the outer tissue (phelloderm), then dried again under the sun to obtain Bletillae Rhizoma.

3. DESCRIPTION

Irregularly oblate, mostly with 2-3 claw-like branches, 2.3-6.7 cm long, 0.6-2.5 cm thick. Externally yellowish-white, yellowish-brown to blackish-brown, with thin wrinkles, possessing several concentric rings and brown dotted rootlet scars, with raised stem scars at the upper part and a trace of joint to another tuber at the lower part. Texture hard, uneasily broken, cut surface whitish and horny. Odour slight; taste bitter and viscous on chewing (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Epidermis consists of 1 layer of subrounded or subrectangular cells, covered with cuticle. Parenchyma consists of irregular cells, scattered with numerous mucilage cells and gelatinous starch masses. Mucilage cells large, sometimes containing raphides of calcium oxalate. Stele scattered with numerous vascular bundles in closed collateral type, mainly single, occasionally linked or appeared side by side; fibres in bundles, hat-shaped, arranged at the outer side of vascular bundles, some with small cells containing silica bodies outside the fibre bundles (Fig. 2).

Powder

Colour pale yellowish-white to pale yellowish-brown. Fibres in bundles, 20-51 μm in diameter, walls lignified, with elliptical pits; bright white under the polarized microscope; sometimes with small cells contain silica bodies arranged on the outer side. Raphides of calcium oxalate scattered or present in mucilage cells, needle crystals slender, 21-78 μm long; polychromatic under the polarized microscope. Mucilage cells large, subrounded or elliptical, 98-325 μm in diameter. Epidermal cells irregular in shape, with walls sinuous, slightly thickened and lignified, with distinct pit canals. Gelatinous starch masses colourless, fairly abundant. Vessels mostly scalariform, 15-49 μm in diameter; spiral vessels rare (Fig. 3).

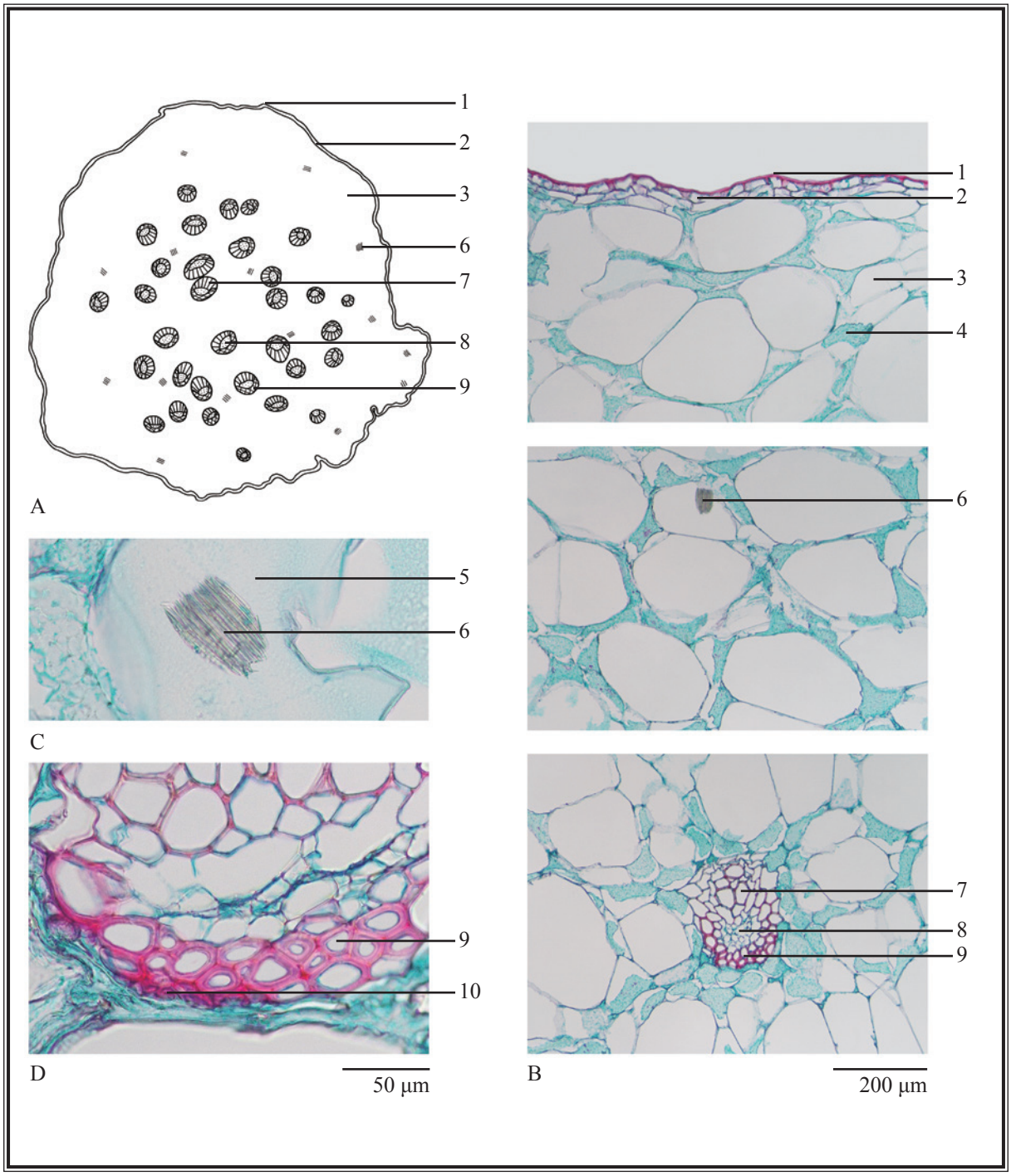

Figure 2 Microscopic features of transverse section of Bletillae Rhizoma

A. Sketch B. Section illustration C. Raphides of calcium oxalate in mucilage cell
D. Vascular bundle

1. Cuticle 2. Epidermis 3. Parenchyma 4. Gelatinous starch masses 5. Mucilage cell
6. Raphides of calcium oxalate 7. Xylem 8. Phloem 9. Fibres 10. Silica body

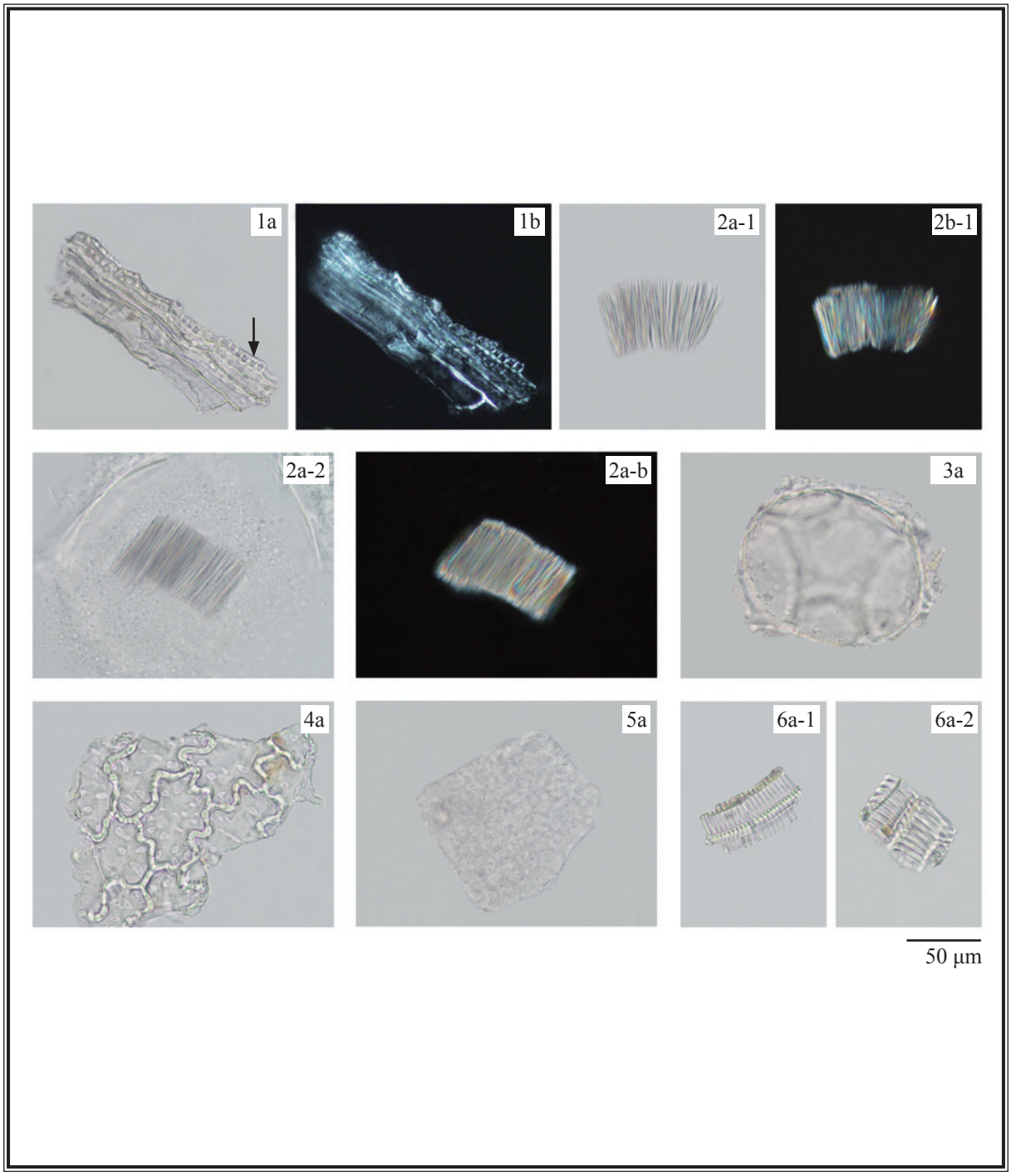

Figure 3 Microscopic features of powder of Bletillae Rhizoma

1. Fibre bundles with cells containing silica bodies on the side (silica bodies ⟶)
2. Raphides of calcium oxalate (2-1 scattered throughout the powder, 2-2 present in mucilage cell)
3. Mucilage cell 4. Epidermal cells 5. Gelatinous starch masses
6. Vessels (6-1 scalariform vessels, 6-2 spiral vessels)

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

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

Standard solution

Militarin standard solution

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

Developing solvent system

Prepare a mixture of *n*-butanol, ethyl acetate and water (4:1:5, v/v). Use the upper layer.

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 50-mL conical flask, then add 10 mL of methanol. Sonicate (400 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 1 mL of methanol. 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 militarin standard solution (5 μL) and the test solution (2 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 8 min). Examine the plate under visible light. Calculate the R_f value by using the equation as indicated in Appendix IV (A).

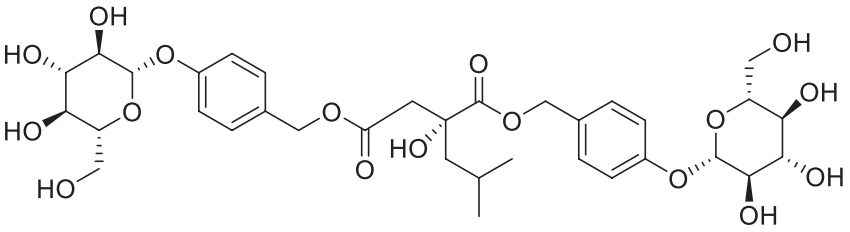

Figure 4 Chemical structure of militarin

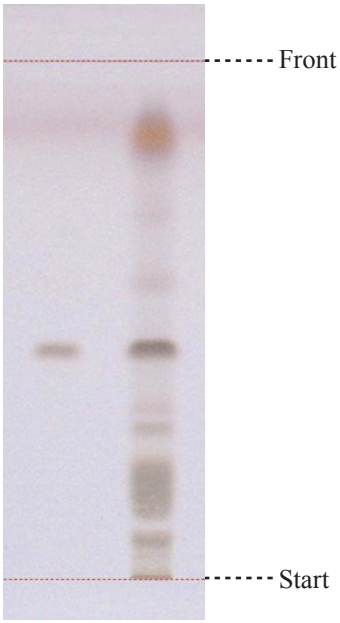

Figure 5 A reference HPTLC chromatogram of Bletillae Rhizoma extract observed under visible light after staining

1. Militarin 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 militarin (Fig. 5).

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

Standard solution

Militarin standard solution for fingerprinting, Std-FP (32 mg/L)

Weigh 0.32 mg of militarin CRS and dissolve in 10 mL of methanol (50%).

Test solution

Weigh 0.1 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 30 mL of methanol (50%). Sonicate (400 W) the mixture for 30 min. Centrifuge at about 3000 × *g* for 10 min. Transfer the supernatant to a 100-mL volumetric flask. Repeat the extraction for two more times. Combine the supernatants and make up to the mark with methanol (50%). Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (223 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)	Water (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 5	90	10	isocratic
5 – 20	90 → 75	10 → 25	linear gradient
20 – 30	75 → 60	25 → 40	linear gradient
30 – 60	60 → 45	40 → 55	linear gradient

System suitability requirements

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

The *R* value between peak 3 and the closest peak in the chromatogram of the test solution should not be less than 1.5 (Fig. 6).

Procedure

Separately inject militarin Std-FP and the test solution (10 μL each) into the HPLC system and record the chromatograms. Measure the retention time of militarin peak in the chromatogram of militarin Std-FP and the retention times of the three characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify militarin peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of militarin Std-FP. The retention times of militarin 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 three characteristic peaks of Bletillae Rhizoma extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the three characteristic peaks of Bletillae Rhizoma extract

Peak No.	RRT	Acceptable Range
1	0.78	± 0.03
2	0.82	± 0.03
3 (marker, militarin)	1.00	-

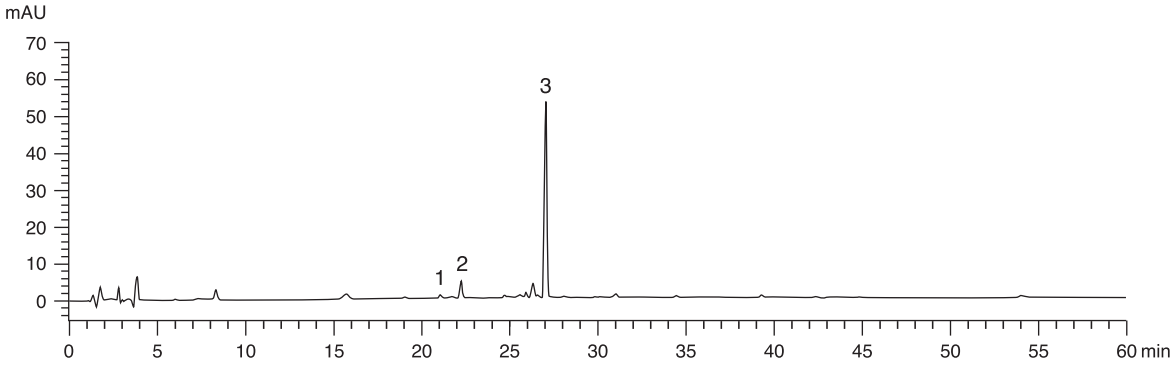

Figure 6 A reference fingerprint chromatogram of Bletillae Rhizoma extract

For positive identification, the sample must give the above three 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 XVI)*: not more than 400 mg/kg.

5.5 Foreign Matter *(Appendix VIII)*: not more than 3.0%.

5.6 Ash *(Appendix IX)*

Total ash: not more than 3.0%.
Acid-insoluble ash: not more than 1.0%.

5.7 Water Content *(Appendix X)*

Oven dried method: not more than 15.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (cold extraction method): not less than 9.0%.
Ethanol-soluble extractives (cold extraction method): not less than 6.0%.

7. ASSAY

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

Standard solution

Militarin standard stock solution, Std-Stock (500 mg/L)
Weigh accurately 5.0 mg of militarin CRS and dissolve in 10 mL of methanol (50%).
Militarin standard solution for assay, Std-AS
Measure accurately the volume of the militarin Std-Stock, dilute with methanol (50%) to produce a series of solutions of 4, 8, 16, 32, 64 mg/L for militarin.

Test solution

Weigh accurately 0.1 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 30 mL of methanol (50%). Sonicate (400 W) the mixture for 30 min. Centrifuge at about 3000 × *g* for 10 min. Transfer the supernatant to a 100-mL volumetric flask. Repeat the extraction for two more times. Combine the supernatants and make up to the mark with methanol (50%). Filter through a 0.45-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (223 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 – 5	90	10	isocratic
5 – 20	90 → 75	10 → 25	linear gradient
20 – 30	75 → 60	25 → 40	linear gradient
30 – 60	60 → 45	40 → 55	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 μL of militarin Std-AS (16 mg/L). The requirements of the system suitability parameters are as follows: the RSD of the peak area of militarin should not be more than 5.0%; the RSD of the retention time of militarin peak should not be more than 2.0%; the column efficiency determined from militarin peak should not be less than 100000 theoretical plates.

The *R* value between militarin peak and the closest peak in the chromatogram of the test solution should not be less than 1.5 (Fig. 7).

Calibration curve

Inject a series of militarin Std-AS (10 μL each) into the HPLC system and record the chromatograms. Plot the peak areas of militarin against the corresponding concentrations of militarin 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 militarin peak (Fig. 7) in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of militarin Std-AS. The retention times of militarin 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 militarin in the test solution, and calculate the percentage content of militarin in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 1.9% of militarin ($C_{34}H_{46}O_{17}$), calculated with reference to the dried substance.

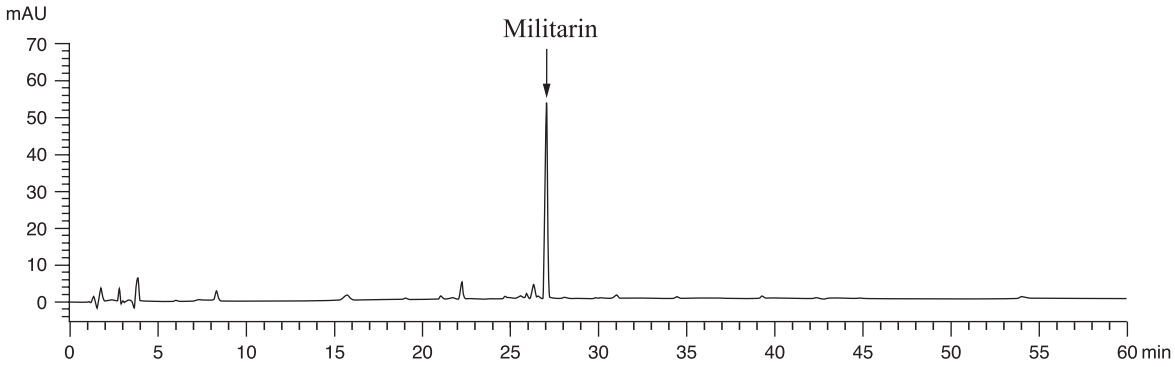

Figure 7 A reference assay chromatogram of Bletillae Rhizoma extract