Fritillariae Pallidiflorae Bulbus

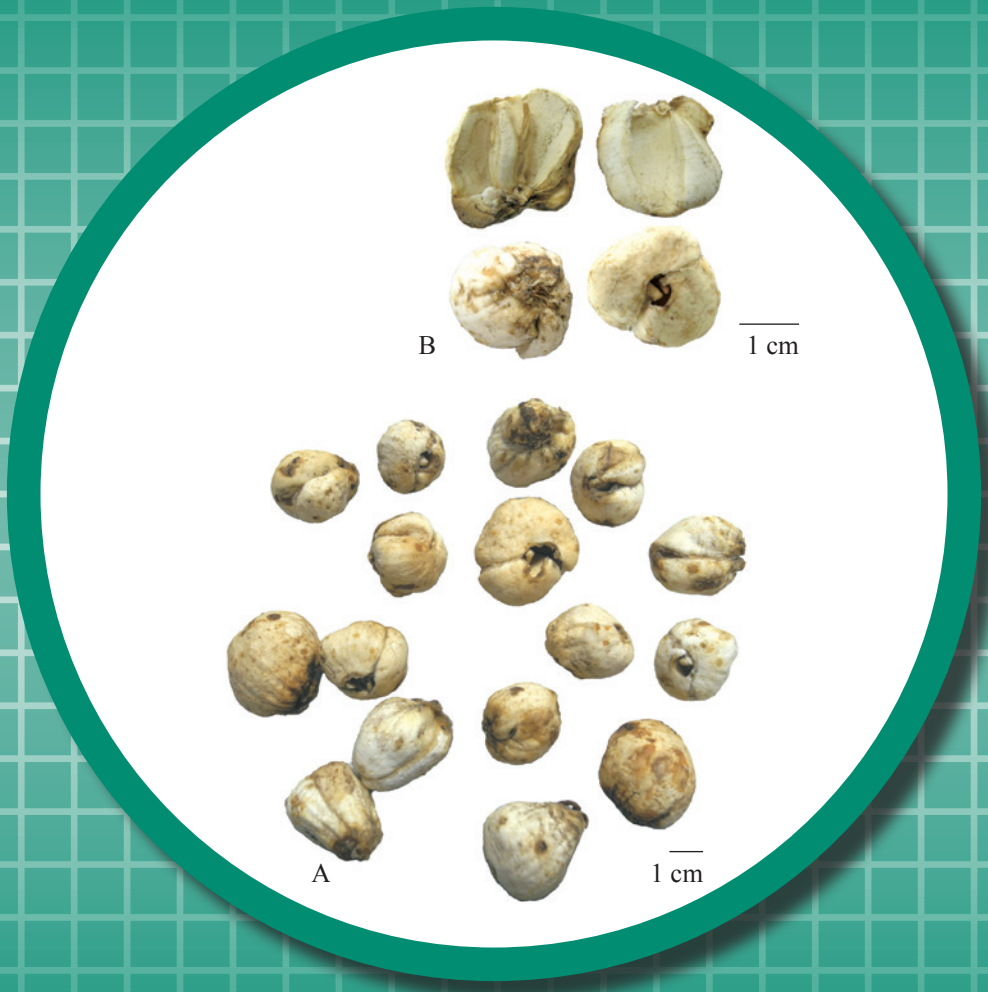


Figure 1 A photograph of Fritillariae Pallidiflorae Bulbus

A. Fritillariae Pallidiflorae Bulbus B. Magnified bulbs

1. NAMES

Official Name: Fritillariae Pallidiflorae Bulbus

Chinese Name: 伊貝母

Chinese Phonetic Name: Yibeimu

2. SOURCE

Fritillariae Pallidiflorae Bulbus is the dried bulb of *Fritillaria pallidiflora* Schrenk (Liliaceae). The bulb is collected from May to July, soil removed, dried under the sun, then fibrous root and the outer bark removed to obtain Fritillariae Pallidiflorae Bulbus.

3. DESCRIPTION

Conical, relatively large, 1.2-3.5 cm high, 10-30 mm in diameter. Externally pale yellowish-white to whitish, slightly rough. The outer scale leaves 2, cordate, plump and large, almost equal size and embraced together. Apex slightly acute, seldom crackable; base slightly concave, with remnants of stem and bud inside. Texture hard and fragile; fracture white, highly starchy. Odour slight; taste slightly bitter (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Outer epidermis of scale leaf consists of 1-5 layers of cells. Inner epidermis of scale leaf consists of 2-8 layers of cells, some showing nipple-like protuberances. Crystals of calcium oxalate present in epidermal cells. Parenchyma occupies the major portion of the scale leaf, filled with starch granules. Vascular bundle small, undeveloped, scattered in parenchyma (Fig. 2).

Powder

Colour whitish. Starch granules extremely numerous, mostly simple, broad-ovoid, triangular-ovoid, subconchoidal or irregular-ovoid, 6-60 μm in diameter, hilum indistinct, striations distinct; black and cruciate-shaped under the polarized microscope. Epidermal cells subrectangular in surface view, stomata obscurely visible, subrounded, 40-65 μm in diameter, with 4-7 subsidiary cells. Vessels spiral or reticulate, 15-50 μm in diameter. Crystals of calcium oxalate rare, finely fusiform, columnar or granular; bright white or polychromatic under the polarized microscope (Fig. 3).

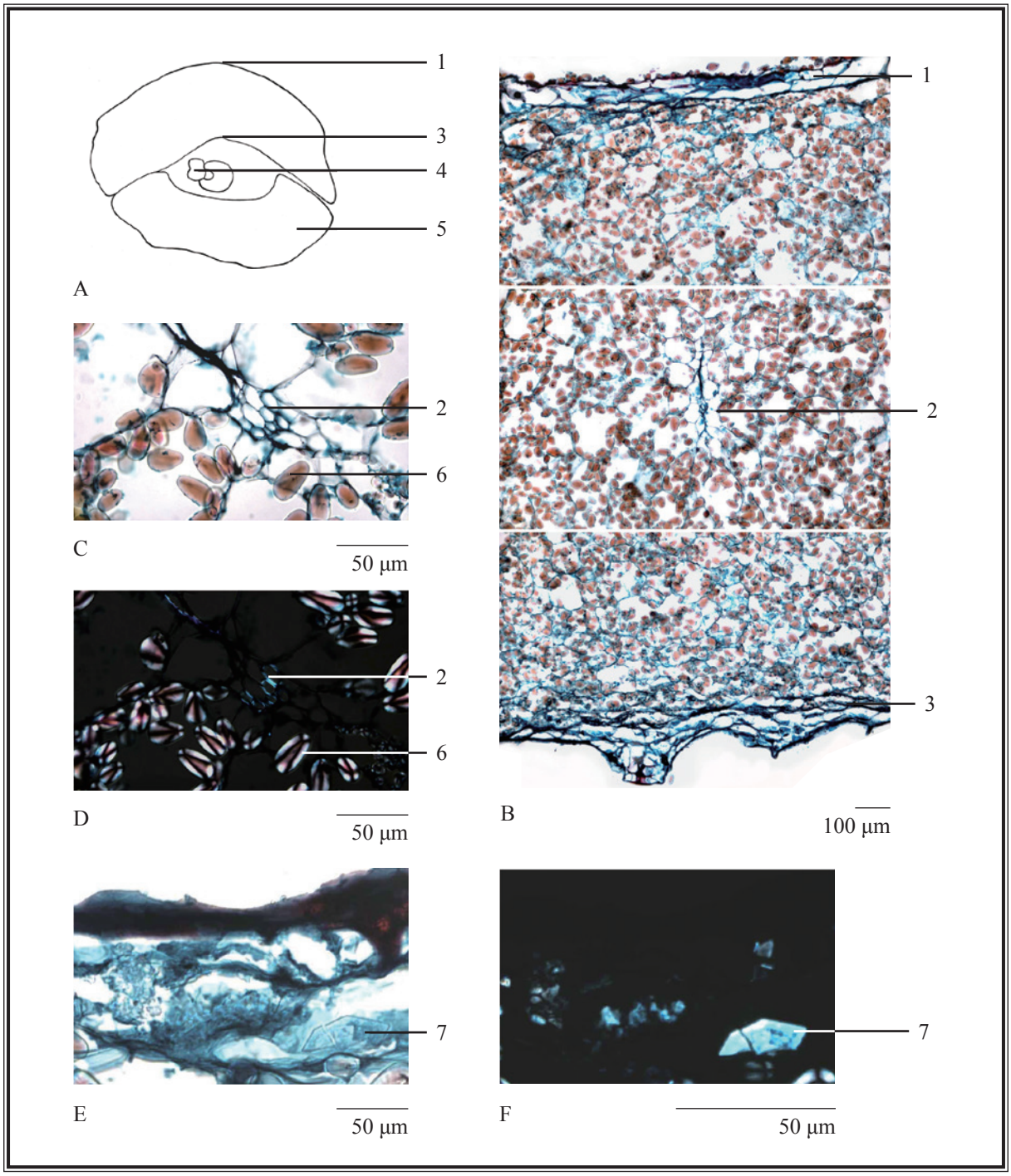


Figure 2 Microscopic features of transverse section of Fritillariae Pallidiflorae Bulbus

A. Sketch B. Section illustration C. Vascular bundle and starch granule
D. Vascular bundle and starch granule (under the polarized microscope)
E. Crystals of calcium oxalate F. Crystals of calcium oxalate (under the polarized microscope)

1. Outer epidermis 2. Vascular bundle 3. Inner epidermis 4. Bud 5. Scale leaf
6. Starch granule 7. Crystals of calcium oxalate

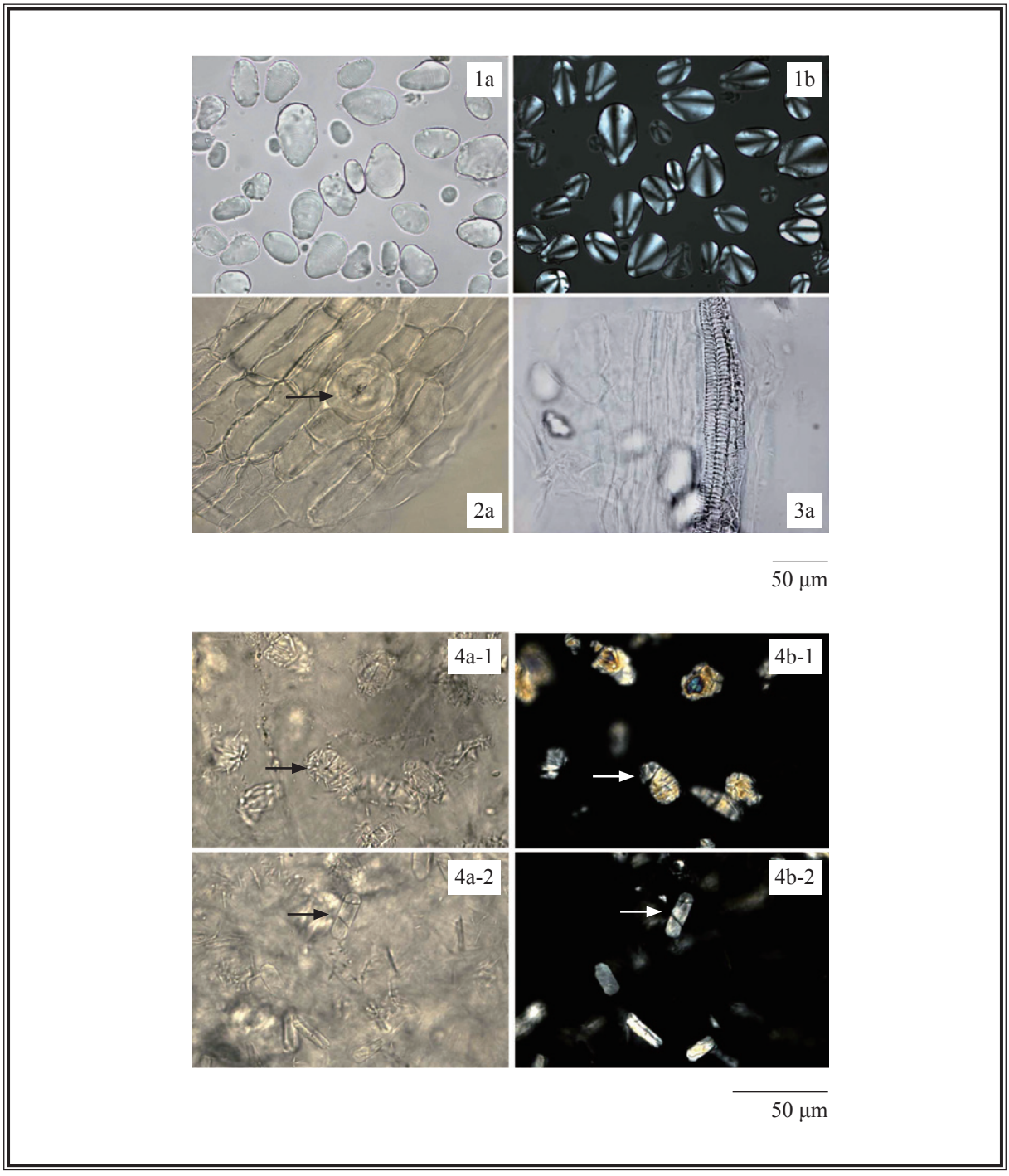


Figure 3 Microscopic features of powder of Fritillariae Pallidiflorae Bulbus

1. Starch granules 2. Epidermal cells with a stoma 3. Vessels 4. Crystals 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 solution

Imperialine standard solution

Weigh 1.0 mg of imperialine CRS (Fig. 4) and dissolve in 2 mL of dichloromethane.

Developing solvent system

Prepare a mixture of cyclohexane, ethyl acetate and diethylamine (12:8:3, v/v).

Spray reagent

Solution A

Weigh 0.85 g of bismuth oxynitrate and dissolve in a mixture of 10 mL of glacial acetic acid and 40 mL of water.

Solution B

Weigh 4 g of potassium iodide and dissolve in 10 mL of water.

Spray reagent 1

Transfer 5 mL of Solution A, 5 mL of Solution B and 20 mL of glacial acetic acid into a 100-mL volumetric flask and make up to the mark with water. Freshly prepare the reagent.

Spray reagent 2

Weigh 0.5 g of sodium nitrite and dissolve in 100 mL of ethanol (60%).

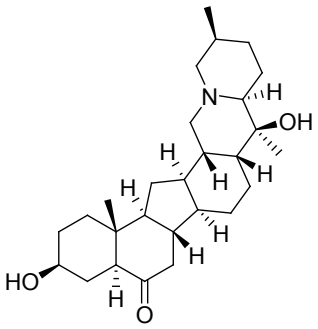
Test solution

Weigh 5.0 g of the powdered sample and place it in a 50-mL conical flask, then add 2 mL of ammonium hydroxide solution (25%, v/v) and 20 mL of dichloromethane. Allow to stand for 12 h. 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 dichloromethane.

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 imperialine 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 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 7.5 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 1 and the spray reagent 2, then dry in air. Examine the plate under visible light. Calculate the R_f value by using the equation as indicated in Appendix IV (A).

(i)



(ii)

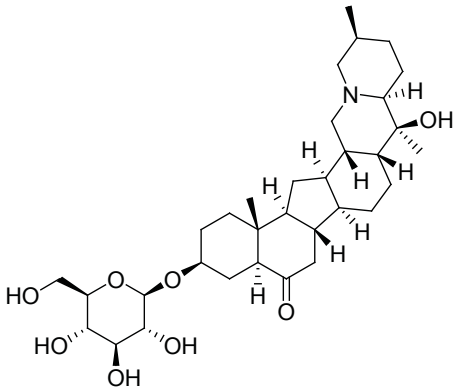


Figure 4 Chemical structures of (i) imperialine and (ii) imperialine-3-β-D-glucoside

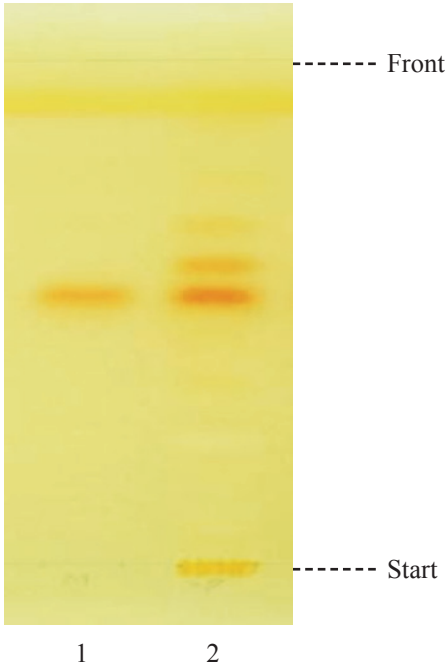


Figure 5 A reference HPTLC chromatogram of Fritillariae Pallidiflorae Bulbus extract observed under visible light after staining

1. Imperialine 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 imperialine (Fig. 5).

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

Standard solutions

Imperialine standard solution for fingerprinting, Std-FP (75 mg/L)

Weigh 0.75 mg of imperialine CRS and dissolve in 10 mL of methanol.

Imperialine-3-β-D-glucoside standard solution for fingerprinting, Std-FP (200 mg/L)

Weigh 2.0 mg of imperialine-3-β-D-glucoside CRS (Fig. 4) and dissolve in 10 mL of methanol.

Test solution

Weigh 0.6 g of the powdered sample and place it in a 250-mL round-bottomed flask, then add 1.5 mL of ammonium hydroxide solution (25%, v/v). Allow to stand for 2 h. Add 40 mL of a mixture of dichloromethane and methanol (4:1, v/v). Reflux the mixture at 80°C for 2 h. Cool down to room temperature. Filter and transfer the filtrate to a 100-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 5-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 an ELSD [drift tube temperature: 60°C; nebulizer gas (N_2) pressure: 3.5 bar] 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)	0.25% Triethylamine (%, v/v)	Triethylamine:Acetonitrile (0.25:99.75, v/v) (%, v/v)	Elution
0 – 20	65 → 58	35 → 42	linear gradient
20 – 40	58 → 36	42 → 64	linear gradient
40 – 50	36 → 10	64 → 90	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 20 µL of imperialine Std-FP and imperialine-3-β-D-glucoside Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of imperialine and imperialine-3-β-D-glucoside should not be more than 5.0%; the RSD of the retention times of imperialine and imperialine-3-β-D-glucoside peaks should not be more than 2.0%; the column efficiencies determined from imperialine and imperialine-3-β-D-glucoside peaks should not be less than 150000 and 60000 theoretical plates respectively.

The *R* value between peak 1 and the closest peak; and the *R* value between peak 5 and the closest peak in the chromatogram of the test solution should not be less than 1.0 (Fig. 6).

Procedure

Separately inject imperialine Std-FP, imperialine-3-β-D-glucoside Std-FP and the test solution (20 µL each) into the HPLC system and record the chromatograms. Measure the retention times of imperialine and imperialine-3-β-D-glucoside peaks in the chromatograms of imperialine Std-FP, imperialine-3-β-D-glucoside Std-FP and the retention times of the five characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify imperialine and imperialine-3-β-D-glucoside peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of imperialine Std-FP and imperialine-3-β-D-glucoside Std-FP. The retention times of imperialine and imperialine-3-β-D-glucoside 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 five characteristic peaks of Fritillariae Pallidiflorae Bulbus extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the five characteristic peaks of Fritillariae Pallidiflorae Bulbus extract

Peak No.	RRT	Acceptable Range
1 (marker, imperialine-3-β-D-glucoside)	1.00	-
2	1.40	± 0.03
3	1.51	± 0.03
4	1.54	± 0.05
5 (imperialine)	1.59	± 0.05

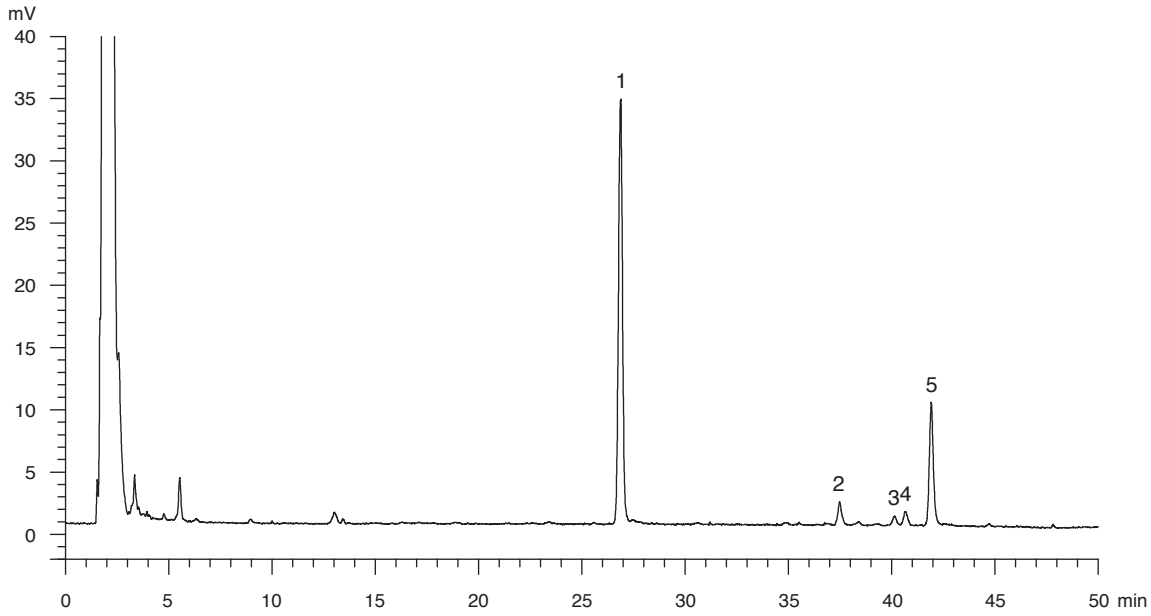


Figure 6 A reference fingerprint chromatogram of Fritillariae Pallidiflorae Bulbus extract

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. 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 1.0%.

5.6 Ash *(Appendix IX)*

Total ash: not more than 4.5%.
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 11.0%.
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

Mixed imperialine and imperialine-3-β-D-glucoside standard stock solution, Std-Stock (300 mg/L for imperialine and 800 mg/L for imperialine-3-β-D-glucoside)

Weigh accurately 1.5 mg of imperialine CRS and 4.0 mg of imperialine-3-β-D-glucoside CRS, and dissolve in 5 mL of methanol.

Mixed imperialine and imperialine-3-β-D-glucoside standard solution for assay, Std-AS

Measure accurately the volume of the mixed imperialine and imperialine-3-β-D-glucoside Std-Stock, dilute with methanol to produce a series of solutions of 37.5, 56.25, 75, 112.5, 150 mg/L for imperialine and 20, 50, 100, 150, 200 mg/L for imperialine-3-β-D-glucoside.

Test solution

Weigh accurately 0.6 g of the powdered sample and place it in a 250-mL round-bottomed flask, then add 1.5 mL of ammonium hydroxide solution (25%, v/v). Allow to stand for 2 h. Add 40 mL of the lower layer of a mixture of dichloromethane, methanol and water (8:2:1, v/v). Reflux the mixture at 80°C for 2 h. Cool down to room temperature. Filter and transfer the filtrate to a 100-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 5-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 an ELSD [drift tube temperature: 60°C; nebulizer gas (N_2) pressure: 3.5 bar] 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)	0.25% Triethylamine (%, v/v)	Triethylamine:Acetonitrile (0.25:99.75, v/v) (%, v/v)	Elution
0 – 30	58 → 36	42 → 64	linear gradient
30 – 40	36 → 20	64 → 80	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 20 µL of the mixed imperialine and imperialine-3-β-D-glucoside Std-AS (75 mg/L for imperialine and 100 mg/L for imperialine-3-β-D-glucoside). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of imperialine and imperialine-3-β-D-glucoside should not be more than 5.0%; the RSD of the retention times of imperialine and imperialine-3-β-D-glucoside peaks should not be more than 2.0%; the column efficiencies determined from imperialine and imperialine-3-β-D-glucoside peaks should not be less than 55000 and 25000 theoretical plates respectively.

The *R* value between imperialine peak and the closest peak; and the *R* value between imperialine-3-β-D-glucoside 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 imperialine and imperialine-3-β-D-glucoside Std-AS (20 µL each) into the HPLC system and record the chromatograms. Plot the natural logarithm of peak areas of imperialine and imperialine-3-β-D-glucoside against the natural logarithm of the corresponding concentrations of the mixed imperialine and imperialine-3-β-D-glucoside Std-AS. Obtain the slopes, y-intercepts and the r^2 values from the corresponding 5-point calibration curves.

Procedure

Inject 20 µL of the test solution into the HPLC system and record the chromatogram. Identify imperialine and imperialine-3-β-D-glucoside peaks in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed imperialine and imperialine-3-β-D-glucoside Std-AS. The retention times of imperialine and imperialine-3-β-D-glucoside 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 imperialine and imperialine-3-β-D-glucoside in the test solution by using the following equation –

Concentration of imperialine or imperialine-3-β-D-glucoside in the test solution = $e^{[Ln\,(A)-I]/m}$

Where A = the peak area of imperialine or imperialine-3-β-D-glucoside in the test solution,
I = the y-intercept of the 5-point calibration curve of imperialine or imperialine-3-β-D-glucoside,
m = the slope of the 5-point calibration curve of imperialine or imperialine-3-β-D-glucoside.

Calculate the percentage contents of imperialine and imperialine-3-β-D-glucoside in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 0.10% of the total content of imperialine ($C_{27}H_{43}NO_3$) and imperialine-3-β-D-glucoside ($C_{33}H_{53}NO_8$), calculated with reference to the dried substance.