Osmundae Rhizoma

Figure 1 A photograph of Osmundae Rhizoma

A. Osmundae Rhizoma

B. Cut surface of Osmundae Rhizoma (1 rhizome, 2 frond bases)

C. Lateral view of frond bases D. Magnified image of cut surface of frond base

1. NAMES

Official name: Osmundae Rhizoma

Chinese name: 紫萁貫眾

Chinese phonetic name: Ziqiguanzhong

2. SOURCE

Osmundae Rhizoma is the dried rhizome and frond bases of *Osmunda japonica* Thunb. (Osmundaceae). The rhizome and frond bases are collected in spring and autumn, washed clean, rootlets removed, then dried under the sun to obtain Osmundae Rhizoma.

3. DESCRIPTION

Conical, subfusiform or cylindrical, slightly curved, obtuse at apex, occasionally branched, relatively acute at bottom, 5-19 cm long, 25-93 mm in diameter. Rhizome diageotropic or obliquely grown, ramenta-free, sometimes bearing remnants of dark and hard rootlets at the lower side, densely covered remains of frond bases at the upper side. Fracture surface brown in colour, leaf-trace vascular bundles arranged in rings at the outer part, with a relatively broad and light-brown rhizome at the centre. Frond bases oblong, obliquely upward, 2.9-9.2 cm long, 2.1-9.3 mm in diameter, externally brown or brownish-black, slightly convex at dorsal surface, obtusely round at margins, auricled wings easily fallen off, usually absent or dilacerated. Cut surface oblong or crescent-shaped, almost hollow at centre, U-shaped meristele often detached from the outside tissues. Texture hard, uneasily broken. Odour slight; taste sweet and slightly astringent (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Frond base: Epidermis mostly broken and fallen off, consisting of 1 layer of cells, brownish-yellow in colour; underneath 2-18 layers of subrounded parenchymatous cells. Hypodermis occupying about 3/7 of frond base, consisting of 8-23 layers of brown, subrounded or polygonal, lignified sclerenchymatous cells forming a ring around the frond base, cell walls highly thickened; some containing oily substances; sclerenchymatous cells at two sides with even thicker cell walls and more dark brown in colour. Parenchyma of cortex occupying about 3/7 of frond base; parenchymatous cells subrounded or polygonal, containing numerous starch granules. Endodermis narrow and distinct. Meristele occupying about 1/7 of frond base,

amphicribral, U-shaped. Xylem arranged in U-shape, composed of 1-5 layers of tracheids, cells polygonal in shape, cell wall relatively thick. Phloem being a relative thin layer surrounding the xylem, scattered with yellowish-brown secretory cells. Secretory cells scattered within phloem, polygonal or subrounded in shape, yellowish-brown in colour before staining. Sclerenchyma consists of 1-10 layers of sclerenchymatous cells, subrounded or polygonal, forming a zone in the depressed side of the U-shaped meristele, cell walls usually thick and more dark brown in colour at two sides of meristele. Starch granules numerously found in the parenchymatous cells of hypodermis [Fig. 2(i)].

Rhizome: Sclerenchyma consists of 3-7 layers of sclerenchymatous cells, subrectangular or oblong in shape, tightly packed. Parenchyma of cortex underneath sclerenchyma consists of 1-2 layers of parenchymatous cells, polygonal or subrounded in shape; parenchyma locating at the centre of rhizome consisting of 3-12 layers of parenchymatous cells, polygonal and irregular in shape, usually broken at the centre. Meristele occupying 4/5 of rhizome, consisting of 7-13 subrounded or U-shaped meristeles arranged closely in a ring, amphicribral, surrounded by endodermis. Phloem inside the meristele, narrow, consisting of 1 layer of cells. Xylem occupying the whole meristele, well-developed, tightly packed and distinct, cells polygonal and with relative thick cell walls [Fig. 2(ii)].

Powder

Colour dark brown. Sclerenchymatous cells numerous, orange-brown to dark brown, fibre-like, in groups or singly scattered; cells subrounded or polygonal in shape, extremely thickened cell wall, cell cavity rarely observed. Tracheids scalariform, colourless to pale yellowish, 13-51 μm in diameter, 2-11 in groups. Parenchymatous cells abundant, two types: first type hexagonal in shape; second type subrectangular or subpolygonal in shape; cell wall colourless to yellowish, cell wall thin to slightly thickened; containing numerous starch granules; black and cruciate-shaped under the polarized microscope. Fibre orange-brown to brown in colour, 12-53 μm in diameter; bright orange under the polarized microscope. Secretory cells occasionally observed, yellowish-brown, subrounded to rounded, containing brown secretion. Xylem cells yellow, hexagonal to polygonal in shape, cell wall relatively thick. Starch granules numerously found; cluster of starch granules composed of 10-230 units; single starch granules subrounded or sub-oblong in shape, 4-15 μm in diameter; black and cruciate-shaped under the polarized microscope (Fig. 3).

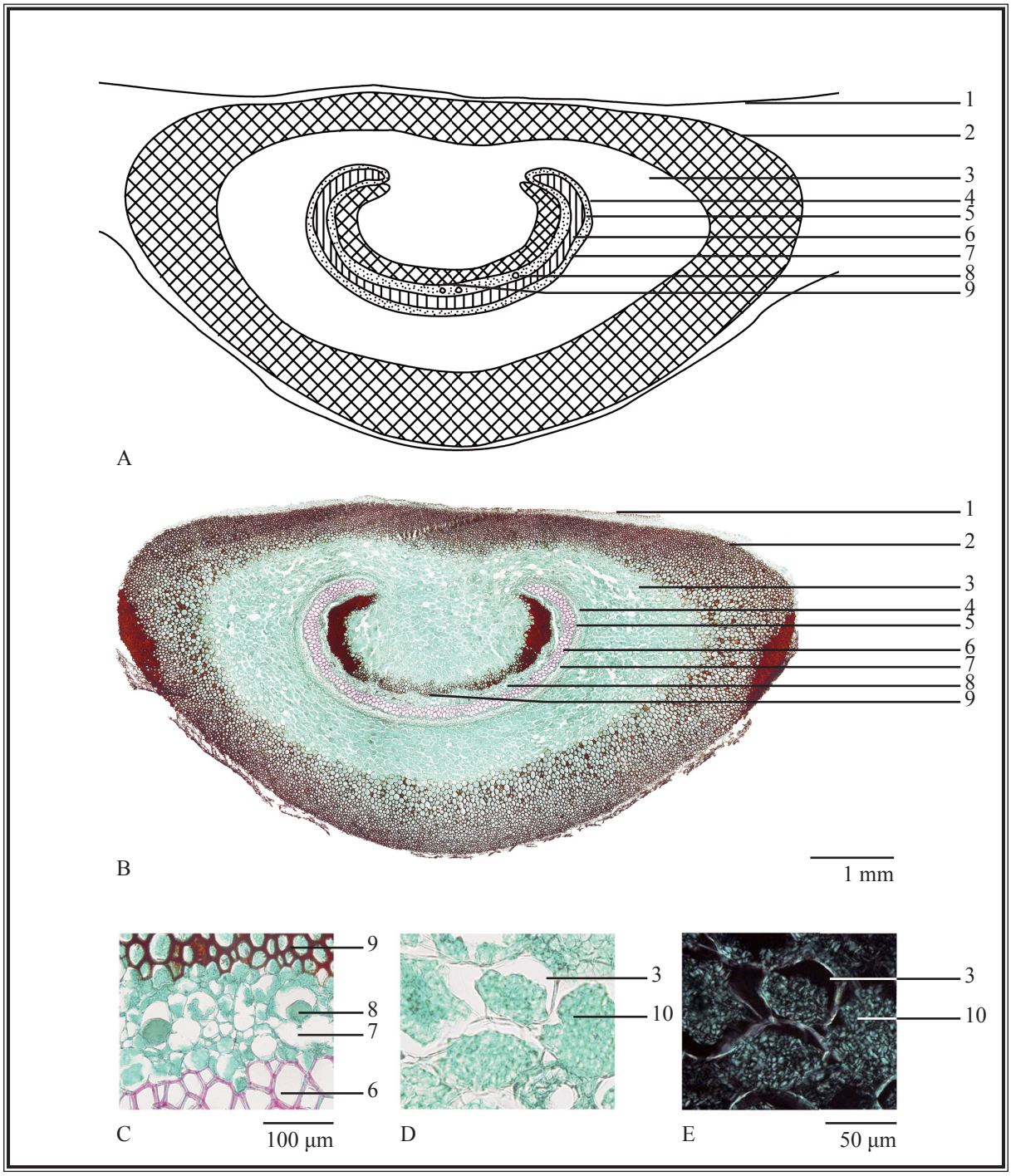


Figure 2 (i) Microscopic features of transverse section of frond base of Osmundae Rhizome

A. Sketch B. Section illustration C. Section magnified
D. Starch granules in parenchyma (under the light microscope)
E. Starch granules in parenchyma (under the polarized microscope)

1. Epidermis 2. Hypodermis 3. Parenchyma of cortex 4. Endodermis
5. Meristele 6. Xylem 7. Phloem 8. Secretory cells 9. Sclerenchyma
10. Starch granules

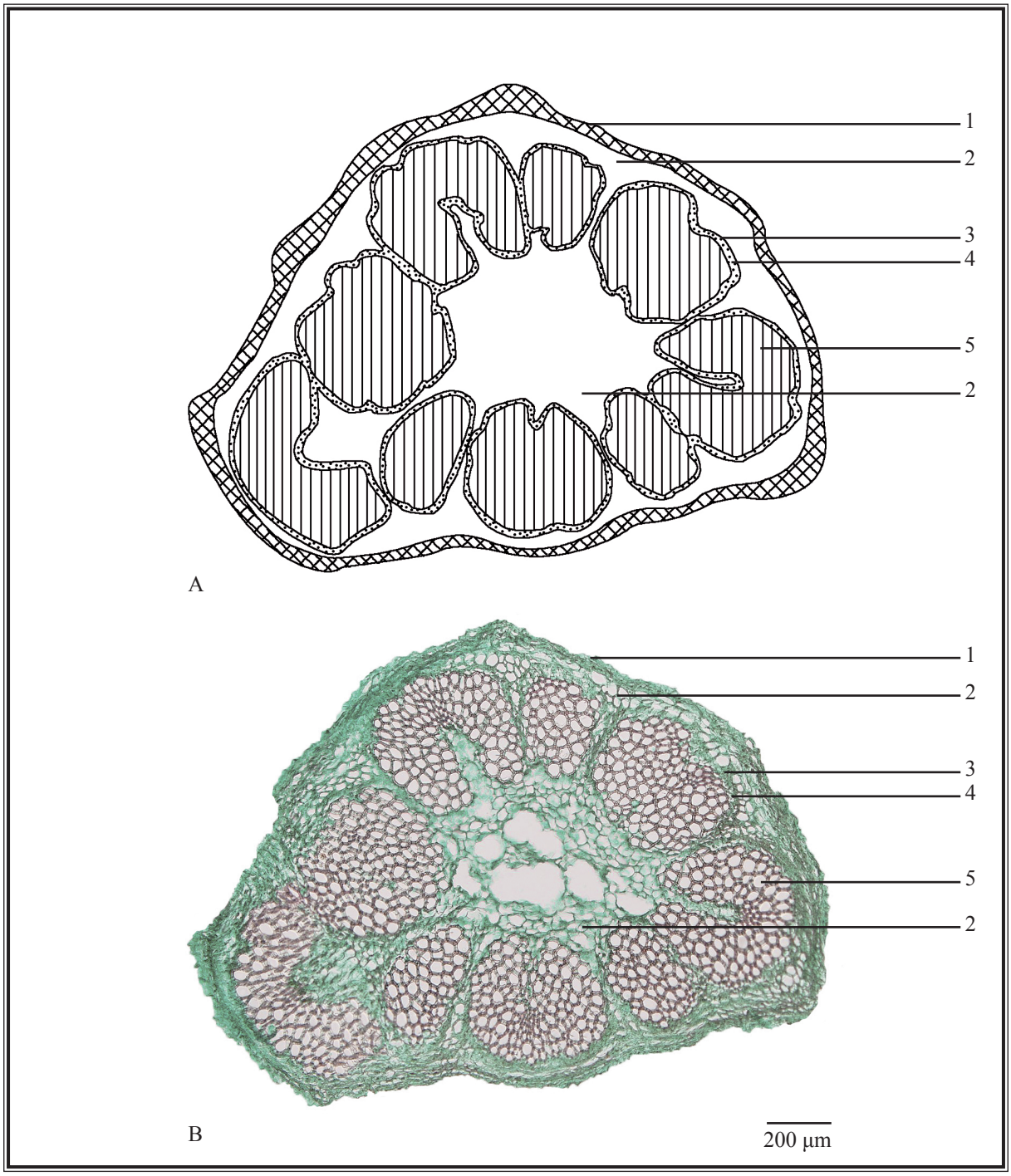


Figure 2 (ii) Microscopic features of transverse section of rhizome of Osmundae Rhizoma

A. Sketch B. Section illustration

1. Sclerenchyma 2. Parenchyma of cortex 3. Meristele
4. Phloem 5. Xylem

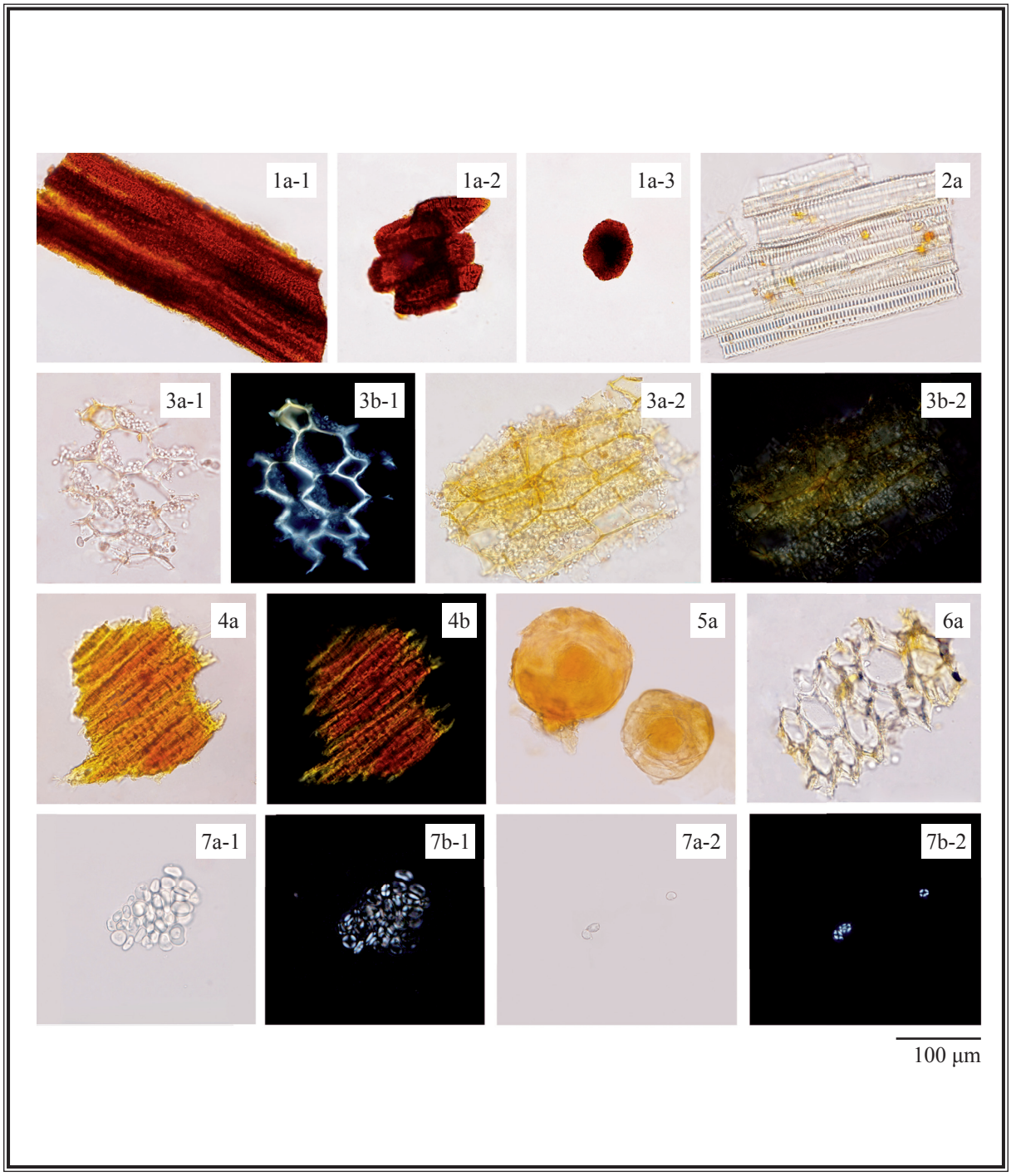


Figure 3 Microscopic features of powder of Osmundae Rhizoma

1. Sclerenchymatous cells (1-1 fibre-like, 1-2 in groups, 1-3 singly scattered)
2. Scalariform tracheids
3. Parenchymatous cells containing starch granules (3-1 hexagonal, 3-2 subrectangular)
4. Fibres 5. Secretory cells 6. Xylem cells
7. Starch granules (7-1 cluster form, 7-2 single form)

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

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

Standard solutions

Protocatechuic acid standard solution

Weigh 2.5 mg of protocatechuic acid CRS (Fig. 4) and dissolve in 5 mL of methanol.

Protocatechuic aldehyde standard solution

Weigh 2.5 mg of protocatechuic aldehyde CRS (Fig. 4) and dissolve in 5 mL of methanol.

Developing solvent system

Prepare a mixture of petroleum ether (60 - 80ºC), ethyl acetate and formic acid (5:5:0.1, v/v).

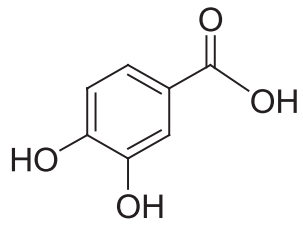
Test solution

Weigh 20.0 g of the powdered sample and place it in a 250-mL round-bottomed flask, then add 150 mL of ethanol (60%) and 1.5 mL hydrochloric acid. Reflux the mixture for 1 h. Cool down to room temperature. Filter and transfer the filtrate to a 250-mL round-bottomed flask. Evaporate the filtrate to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 30 mL of water. Transfer the extract to a 250-mL separating funnel. Extract the aqueous layer for two times each with 30 mL of ethyl acetate. Combine the ethyl acetate extracts. Adjust the pH to 7.0 with water. Transfer the extract to a 100-mL round-bottomed flask. Evaporate the extract to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 5 mL of ethyl acetate and add 0.5 g silica gel. Evaporate the extract to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 5 mL of ethyl acetate. Load the sample extract to the solid phase extraction (SPE) column containing silica gel packing (50 μm particle size, 20 mL, 5 g). Add 20 mL of ethyl acetate to the SPE column. Collect the eluate and transfer the eluate to 50-mL round-bottomed flask. Evaporate the eluate to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in a mixture of 1 mL of methanol and 10 μL of glacial acetic acid.

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 protocatechuic acid standard solution (2 μL), protocatechuic aldehyde standard solution (4 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. Examine the plate under UV light (254 nm). Calculate the R_f values by using the equation as indicated in Appendix IV (A).

(i)



(ii)

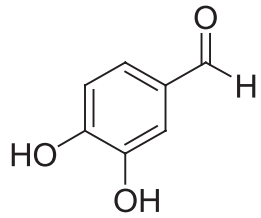


Figure 4 Chemical structures of (i) protocatechuic acid and (ii) protocatechuic aldehyde

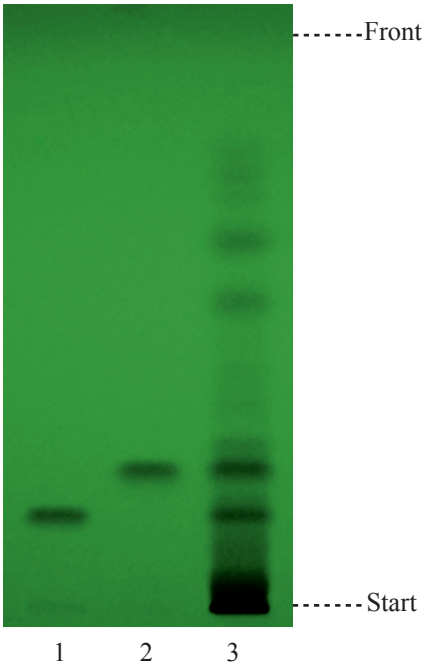


Figure 5 A reference HPTLC chromatogram of Osmundae Rhizoma extract observed under UV light (254 nm)

1. Protocatechuic acid standard solution 2. Protocatechuic aldehyde 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 protocatechuic acid and protocatechuic aldehyde (Fig. 5).

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

Standard solutions

Protocatechuic acid standard solution for fingerprinting, Std-FP (20 mg/L)

Weigh 0.2 mg of protocatechuic acid CRS and dissolve in 10 mL of 0.1% formic acid.

Protocatechuic aldehyde standard solution for fingerprinting, Std-FP (20 mg/L)

Weigh 0.2 mg of protocatechuic aldehyde CRS and dissolve in 10 mL of 0.1% formic acid.

Test solution

Weigh 1.0 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 20 mL of water. Sonicate (180 W) the mixture for 30 min. Centrifuge at about 2330 × *g* for 15 min. Transfer the supernatant to a 100-mL separating funnel. Extract for three times each with 20 mL of ethyl acetate. Combine the ethyl acetate extracts. Evaporate the combined extracts to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 1 mL of 0.1% formic acid. Filter through a 0.2-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (290 nm) and a column (2.1 × 50 mm) packed with ODS bonded silica gel (1.7 μm particle size). The column temperature is maintained at 25ºC during the separation. The flow rate is about 0.4 mL/min. Programme the chromatographic system as follows (Table 1) –

Table 1 Chromatographic system conditions

Time (min)	0.1% Formic acid (%, v/v)	Formic acid: Acetonitrile (0.1:99.9, v/v) (%, v/v)	Elution
0 – 8	100	0	isocratic
8 – 10	100 → 98	0 → 2	linear gradient
10 – 30	98 → 90	2 → 10	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 2 μL of protocatechuic acid Std-FP and protocatechuic aldehyde Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of protocatechuic acid and protocatechuic aldehyde should not be more than 5.0%; the RSD of the retention times of protocatechuic acid and protocatechuic aldehyde peaks should not be more than 2.0%; the column efficiencies determined from protocatechuic acid and protocatechuic aldehyde peaks should not be less than 2000 and 3000 theoretical plates respectively.

The *R* value between peak 1 and the closest peak; and 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 protocatechuic acid Std-FP, protocatechuic aldehyde Std-FP and the test solution (2 μL each) into the UHPLC system and record the chromatograms. Measure the retention times of protocatechuic acid and protocatechuic aldehyde peaks in the chromatograms of protocatechuic acid Std-FP, protocatechuic aldehyde Std-FP and the retention times of the seven characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify protocatechuic acid and protocatechuic aldehyde peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of protocatechuic acid Std-FP and protocatechuic aldehyde Std-FP. The retention times of protocatechuic acid and protocatechuic aldehyde 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 seven characteristic peaks of Osmundae Rhizoma extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the seven characteristic peaks of Osmundae Rhizoma extract

Peak No.	RRT	Acceptable Range
1 (protocatechuic acid)	0.51	± 0.03
2	0.66	± 0.04
3 (marker, protocatechuic aldehyde)	1.00	-
4	1.70	± 0.03
5	2.11	± 0.08
6	2.63	± 0.10
7	3.23	± 0.12

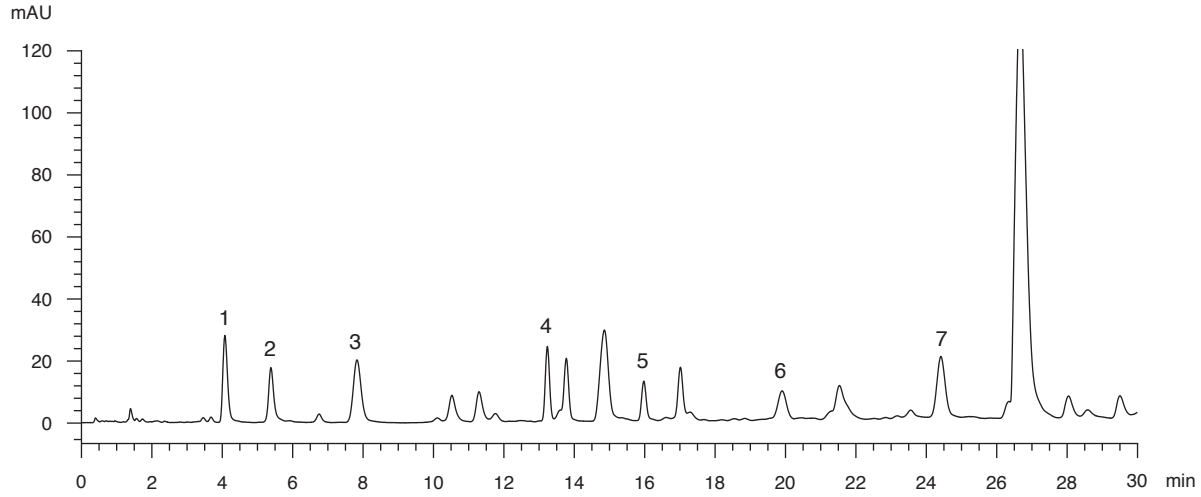


Figure 6 A reference fingerprint chromatogram of Osmundae Rhizoma extract

For positive identification, the sample must give the above seven 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 XVI)*: meet the requirements.

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

5.6 Ash *(Appendix IX)*

Total ash: not more than 5.5%.
Acid-insoluble ash: not more than 3.5%.

5.7 Water Content *(Appendix X)*

Oven dried method: not more than 10.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (cold extraction method): not less than 4.0%.
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 protocatechuic acid and protocatechuic aldehyde standard stock solution, Std-Stock (20 mg/L for protocatechuic acid and 40 mg/L for protocatechuic aldehyde)

Weigh accurately 0.2 mg of protocatechuic acid CRS and 0.4 mg of protocatechuic aldehyde CRS, and dissolve in 10 mL of ethanol (60%).

Mixed protocatechuic acid and protocatechuic aldehyde standard solution for assay, Std-AS

Measure accurately the volume of the mixed protocatechuic acid and protocatechuic aldehyde Std-Stock, dilute with ethanol (60%) to produce a series of solutions of 0.1, 0.5, 1, 2, 5 mg/L for protocatechuic acid and 1, 2, 4, 8, 10 mg/L for protocatechuic aldehyde.

Test solution

Weigh accurately 0.5 g of the powdered sample and place it in a 100-mL round-bottomed flask, then add 45 mL of ethanol (60%) and 4.5 mL of hydrochloric acid. Reflux the mixture for 1 h. Cool down to room temperature. Filter and transfer the filtrate to a 100-mL volumetric flask. Wash the residue with 3 mL of ethanol (60%). Repeat the extraction one more time. Combine the extracts and make up to the mark with ethanol (60%). Filter through a 0.2-μm PTFE filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (260 nm) and a column (2.1 × 50 mm) packed with ODS bonded silica gel (1.7 μm particle size). The flow rate is about 0.4 mL/min. Programme the chromatographic system as follows (Table 3) –

Table 3 Chromatographic system conditions

Time (min)	0.1% Formic acid (%, v/v)	Formic acid: Acetonitrile (0.1:99.9, v/v) (%, v/v)	Elution
0 – 8	100	0	isocratic
8 – 10	100 → 98	0 → 2	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 1 μL of the mixed protocatechuic acid and protocatechuic aldehyde Std-AS (1 mg/L for protocatechuic acid and 4 mg/L for protocatechuic aldehyde). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of protocatechuic acid and protocatechuic aldehyde should not be more than 5.0%; the RSD of the retention times of protocatechuic acid and protocatechuic aldehyde peaks should not be more than 2.0%; the column efficiencies determined from protocatechuic acid and protocatechuic aldehyde peaks should not be less than 1500 and 2000 theoretical plates respectively.

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

Calibration curves

Inject a series of the mixed protocatechuic acid and protocatechuic aldehyde Std-AS (1 μL each) into the UHPLC system and record the chromatograms. Plot the peak areas of protocatechuic acid and protocatechuic aldehyde against the corresponding concentrations of the mixed protocatechuic acid and protocatechuic aldehyde Std-AS. Obtain the slopes, y-intercepts and the r^2 values from the corresponding 5-point calibration curves.

Procedure

Inject 1 μL of the test solution into the UHPLC system and record the chromatogram. Identify protocatechuic acid and protocatechuic aldehyde peaks (Fig. 7) in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed protocatechuic acid and protocatechuic aldehyde Std-AS. The retention times of protocatechuic acid and protocatechuic aldehyde 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 protocatechuic acid and protocatechuic aldehyde in the test solution, and calculate the percentage contents of protocatechuic acid and protocatechuic aldehyde in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 0.060% of the total content of protocatechuic acid ($C_7H_6O_4$) and protocatechuic aldehyde ($C_7H_6O_3$), calculated with reference to the dried substance.

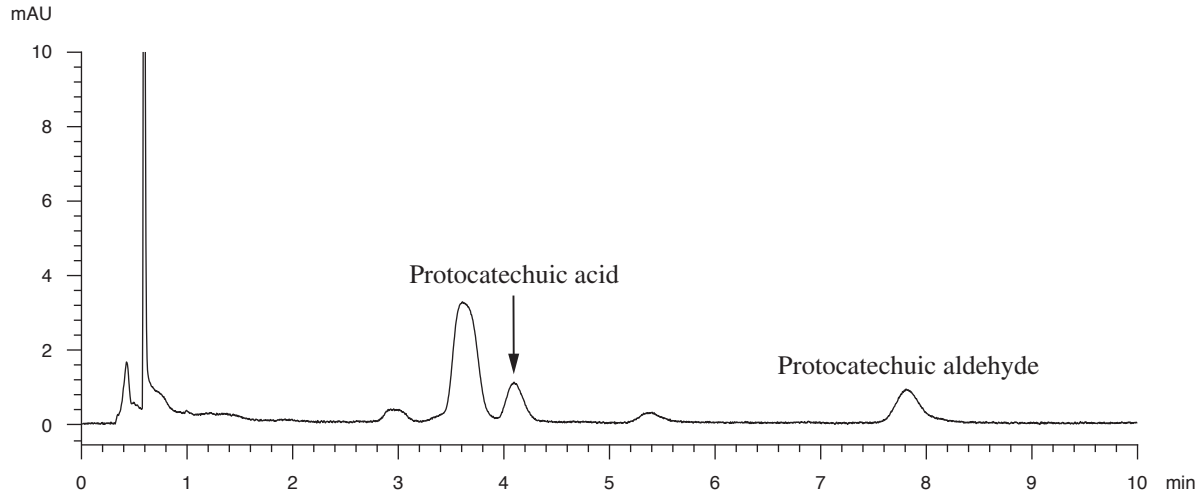


Figure 7 A reference assay chromatogram of Osmundae Rhizoma extract