Alpiniae Katsumadai Semen

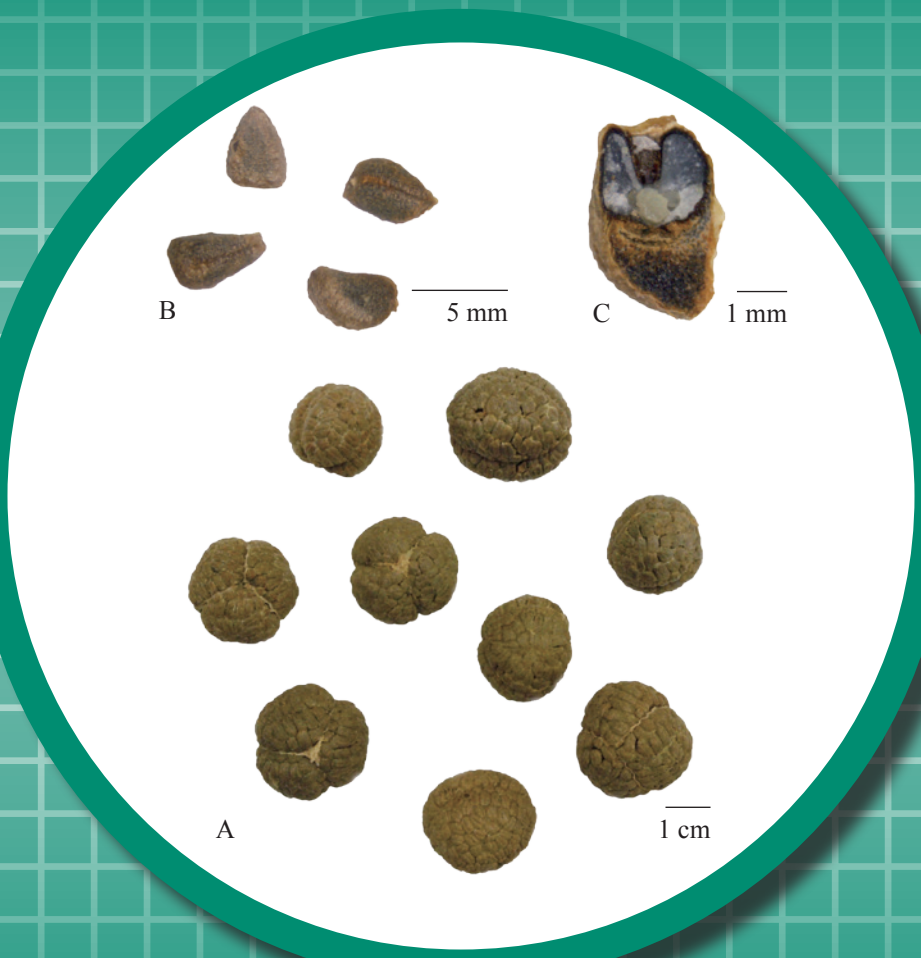


Figure 1 A photograph of Alpiniae Katsumadai Semen

A. Alpiniae Katsumadai Semen B. Magnified seeds
C. Magnified longitudinal section of the seed

1. NAMES

Official Name: Alpiniae Katsumadai Semen

Chinese Name: 草豆蔻

Chinese Phonetic Name: Caodoukou

2. SOURCE

Alpiniae Katsumadai Semen is the dried almost ripe seed of *Alpinia katsumadai* Hayata (Zingiberaceae). The fruit is collected in summer and autumn, dried under the sun until almost dry, or dipped into boiling water followed by drying under the sun until semi-dried, then the pericarp removed and the seed masses dried under the sun to obtain Alpiniae Katsumadai Semen.

3. DESCRIPTION

Seed masses subspherical, with 3 obvious shallow furrows, 11-29 mm in diameter. Externally greyish-brown to yellowish-brown, with yellowish-white or pale brown septa in the middle dividing the seed mass into 3 groups, each bearing numerous seeds stuck together. Seeds ovoid-polyhedral, 1-4 mm in diameter, covered with pale brown membranous aril; raphe in the form of a longitudinal furrow, with a hilum at one end; when cutting in the longitudinal section, testa extending along the raphe into the inner part occupying about half of the surface area; endosperms greyish-white. Texture hard. Odour aromatic; taste pungent and slightly bitter (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Epidermal cells of testa subrounded, aril occasionally found. Hypodermis consists of 1-3 layers of tangentially elongated cells. Pigment layer consists of several layers of cells, boundaries indistinct, with subrounded oil cells scattered. Endotesta consists of 1 layer of palisade sclerenchymatous cells, with heavily thickened inner and lateral walls; lumen small, containing silica bodies. Perisperm cells filled with tiny starch granules, some contain small prisms of calcium oxalate. Endosperm cells contain aleurone grains (Fig. 2).

Powder

Colour yellowish-brown. Endotesta cells orange-yellow to yellowish-brown, polygonal or subrounded in surface view, walls thickened, containing silica bodies; palisade-like in lateral view, lumens on one side, containing silica bodies; orange-yellow or brown under the polarized microscope. Epidermal cells of testa colourless or pale yellow, strip-shaped in surface view; bright yellow under the polarized microscope. Hypodermal cells colourless or pale yellow, usually adhere epidermal cells of testa on the lower layer. Pigment cells yellowish-brown to reddish-brown, with indistinct boundaries; oil cells often scattered in pigment cells, subrounded, 11-59 μm in diameter. Perisperm cells rectangular or polygonal, filling with tiny starch granules, some contain prisms of calcium oxalate of 1-13 μm in diameter; pale white under the polarized microscope (Fig. 3).

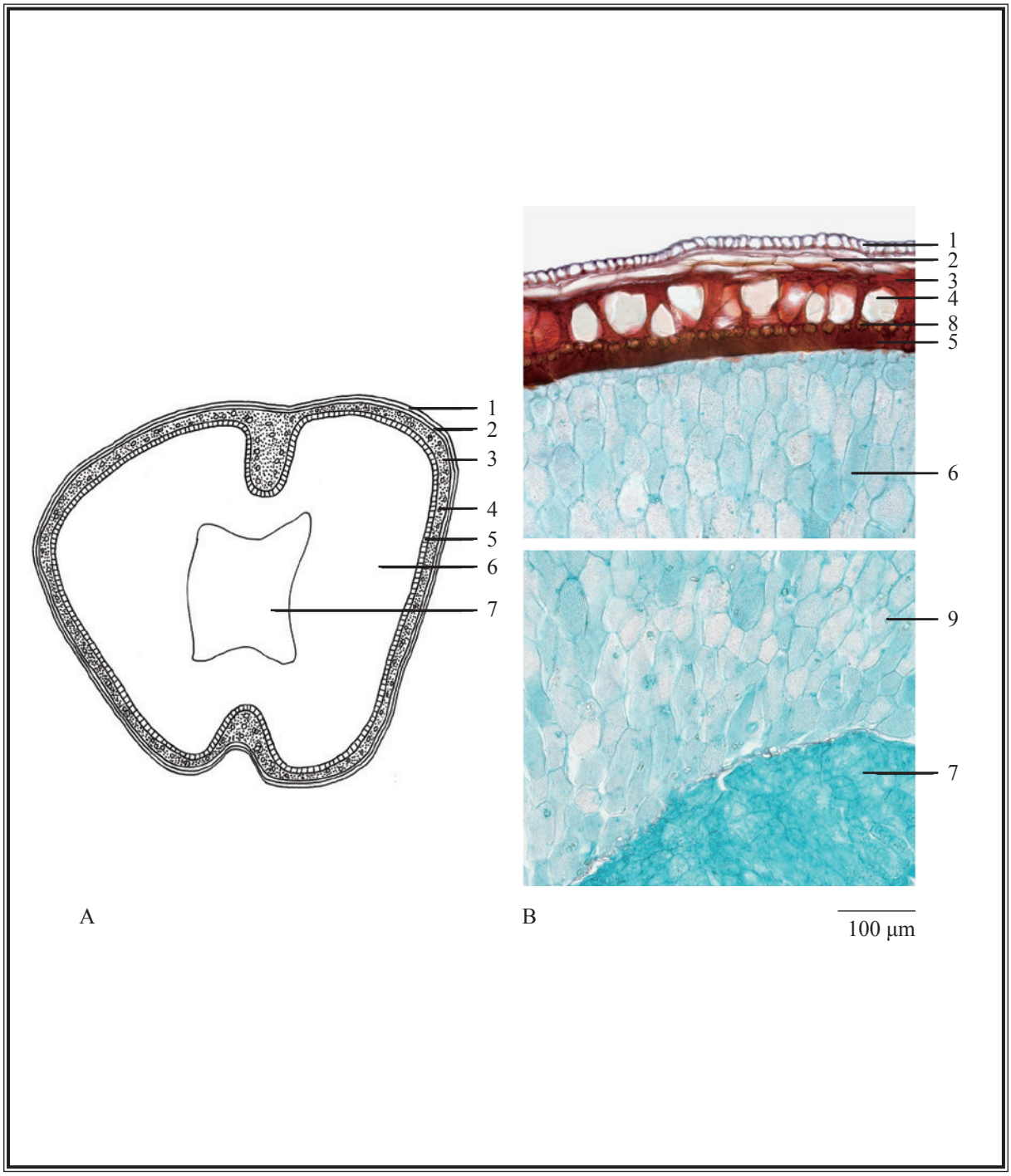


Figure 2 Microscopic features of transverse section of Alpiniae Katsumadai Semen

A. Sketch B. Section illustration

1. Epidermis of testa 2. Hypodermis 3. Pigment layer 4. Oil cell 5. Endotesta
6. Perisperm 7. Endosperm 8. Silica body 9. Prism of calcium oxalate

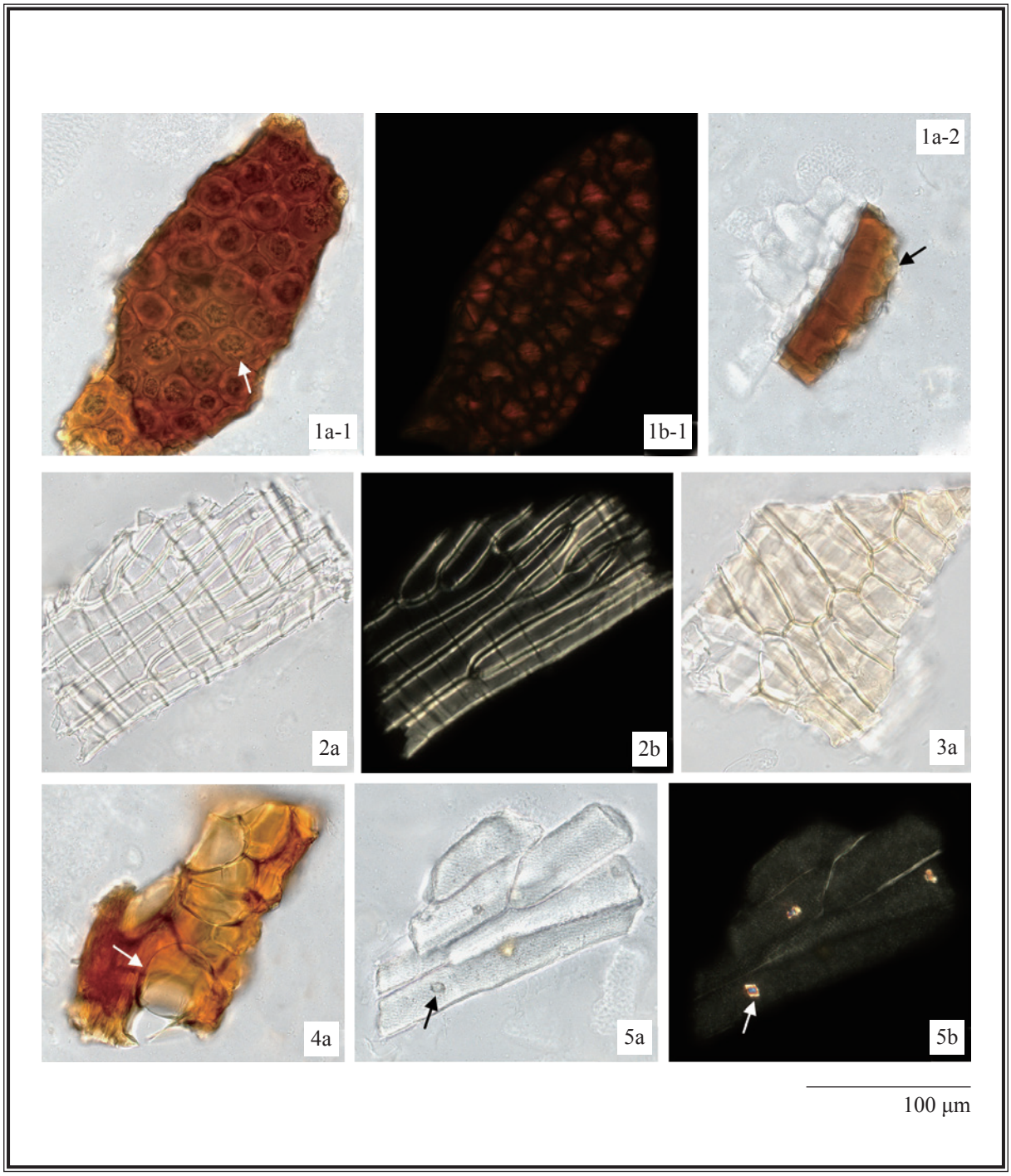


Figure 3 Microscopic features of powder of Alpiniae Katsumadai Semen

1. Endotesta cells contain silica bodies (1-1 in surface view, 1-2 in lateral view, silica body ⟶)
2. Epidermal cells of testa 3. Hypodermal cells 4. Pigment cells (oil cells ⟶)
5. Perisperm cells (prisms of calcium oxalate ⟶)

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

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

Standard solutions

Alnustone standard solution

Weigh 2.0 mg of alnustone CRS (Fig. 4) and dissolve in 1 mL of methanol.

Alpinetin standard solution

Weigh 2.0 mg of alpinetin CRS (Fig. 4) and dissolve in 1 mL of methanol.

Cardamonin standard solution

Weigh 2.0 mg of cardamonin CRS (Fig. 4) and dissolve in 1 mL of methanol.

Pinocembrin standard solution

Weigh 2.0 mg of pinocembrin CRS (Fig. 4) and dissolve in 1 mL of methanol.

Developing solvent system

Prepare a mixture of *n*-hexane, ethyl acetate and methanol (15:4:1, v/v).

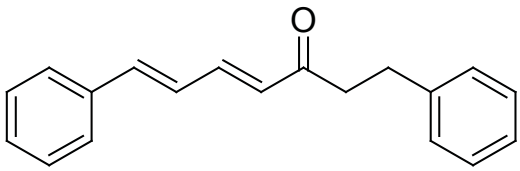
Test solution

Weigh 1.0 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 5 mL of methanol. Sonicate (140 W) the mixture for 5 min. Centrifuge at about 2800 × *g* for 10 min. Filter through a 0.45-µm PTFE filter.

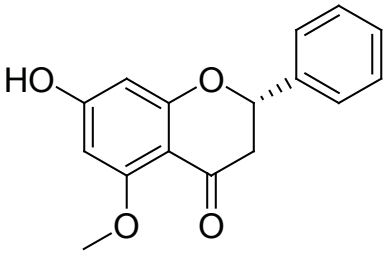
Procedure

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 alnustone standard solution (1.5 µL), alpinetin standard solution (1.5 µL), cardamonin standard solution (1.5 µL), pinocembrin standard solution (1.5 µL) and the test solution (3 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 7 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).

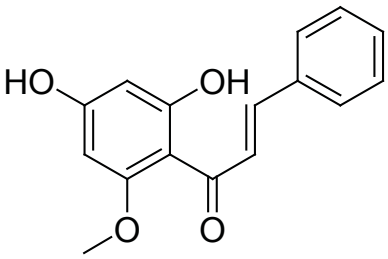
(i)



(ii)



(iii)



(iv)

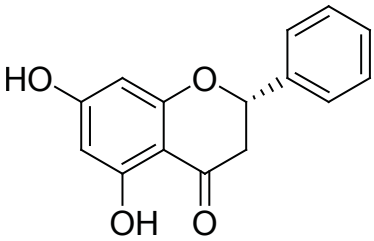


Figure 4 Chemical structures of (i) alnustone (ii) alpinetin (iii) cardamonin and (iv) pinocembrin

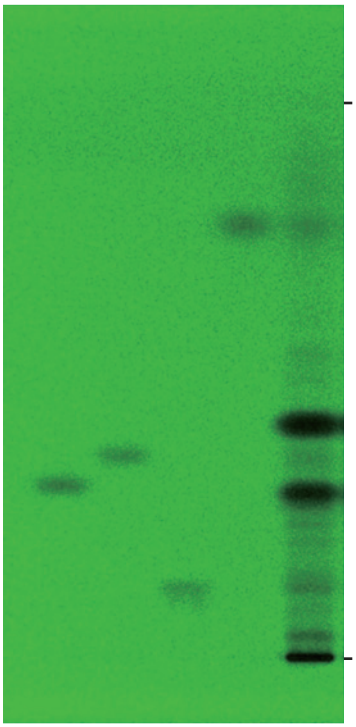


Figure 5 A reference HPTLC chromatogram of Alpiniae Katsumadai Semen extract observed under UV light (254 nm)

1. Cardamonin standard solution 2. Pinocembrin standard solution
3. Alpinetin standard solution 4. Alnustone standard solution 5. 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 alnustone, alpinetin, cardamonin and pinocembrin (Fig. 5).

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

Standard solutions

Alnustone standard solution for fingerprinting, Std-FP (50 mg/L)

Weigh 0.5 mg of alnustone CRS and dissolve in 10 mL of methanol.

Alpinetin standard solution for fingerprinting, Std-FP (50 mg/L)

Weigh 0.5 mg of alpinetin CRS and dissolve in 10 mL of methanol.

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

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

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

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

Test solution

Weigh 0.5 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 20 mL of methanol. Sonicate (270 W) the mixture for 30 min. Centrifuge at about 4000 × *g* for 10 min. Transfer the supernatant to a 50-mL volumetric flask. Repeat the extraction for one more time. Wash the residue with methanol. Centrifuge at about 4000 × *g* for 10 min. Combine the supernatants 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 (260 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 µm particle size). The column temperature is maintained at 40ºC during the separation. The flow rate is about 0.8 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 – 45	60 → 48	40 → 52	linear gradient
45 – 60	48 → 0	52 → 100	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 µL of alnustone Std-FP, alpinetin Std-FP, cardamonin Std-FP and pinocembrin Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of alnustone, alpinetin, cardamonin and pinocembrin should not be more than 5.0%; the RSD of the retention times of alnustone, alpinetin, cardamonin and pinocembrin peaks should not be more than 2.0%; the column efficiencies determined from alnustone, alpinetin, cardamonin and pinocembrin peaks should not be less than 50000, 10000, 10000 and 10000 theoretical plates respectively.

The *R* value between peak 1 and the closest peak; the *R* value between peak 2 and the closest peak; the *R* value between peak 5 and the closest peak; and the *R* value between peak 7 and the closest peak in the chromatogram of the test solution should not be less than 1.0 (Fig. 6).

Procedure

Separately inject alnustone Std-FP, alpinetin Std-FP, cardamonin Std-FP, pinocembrin Std-FP and the test solution (10 µL each) into the HPLC system and record the chromatograms. Measure the retention times of alnustone, alpinetin, cardamonin and pinocembrin peaks in the chromatograms of alnustone Std-FP, alpinetin Std-FP, cardamonin Std-FP, pinocembrin Std-FP and the retention times of the seven characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify

alnustone, alpinetin, cardamonin and pinocembrin peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of alnustone Std-FP, alpinetin Std-FP, cardamonin Std-FP and pinocembrin Std-FP. The retention times of alnustone, alpinetin, cardamonin and pinocembrin 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 Alpiniae Katsumadai Semen extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the seven characteristic peaks of Alpiniae Katsumadai Semen extract

Peak No.	RRT	Acceptable Range
1 (alpinetin)	0.49	± 0.03
2 (marker, pinocembrin)	1.00	-
3	1.35	± 0.04
4	1.39	± 0.04
5 (cardamonin)	1.63	± 0.03
6	1.70	± 0.04
7 (alnustone)	2.56	± 0.11

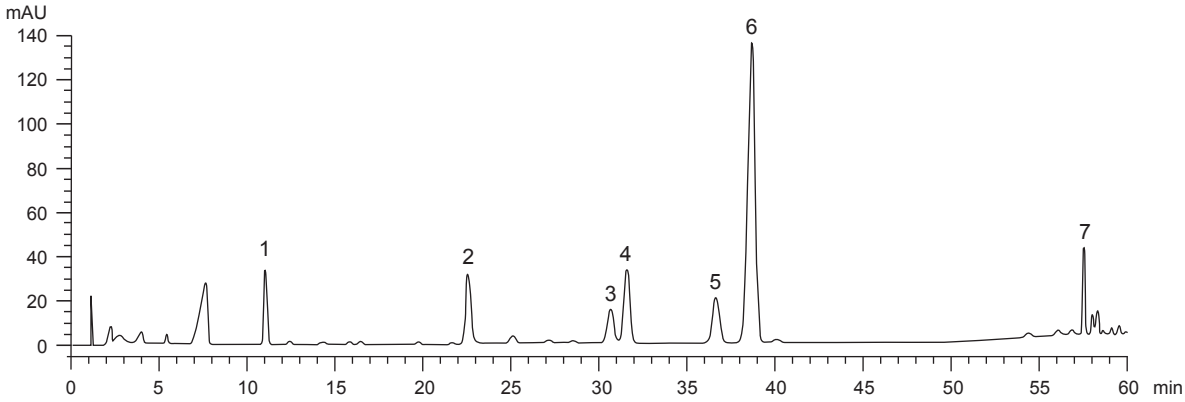


Figure 6 A reference fingerprint chromatogram of Alpiniae Katsumadai Semen extract

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 – Aflatoxins *(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 3.5%.
Acid-insoluble ash: not more than 1.5%.

5.7 Water Content *(Appendix X)*

Toluene distillation method: not more than 13.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (cold extraction method): not less than 6.0%.
Ethanol-soluble extractives (cold extraction method): not less than 10.0%.

7. ASSAY

7.1 Assay of Alnustone, Alpinetin, Cardamonin and Pinocembrin

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

Standard solution
Mixed alnustone, alpinetin, cardamonin and pinocembrin standard stock solution, Std-Stock (1000 mg/L for alnustone, 1000 mg/L for alpinetin, 1500 mg/L for cardamonin and 1500 mg/L for pinocembrin)
Weigh accurately 5.0 mg of alnustone CRS, 5.0 mg of alpinetin CRS, 7.5 mg of cardamonin CRS and 7.5 mg of pinocembrin CRS, and dissolve in 5 mL of methanol.
Mixed alnustone, alpinetin, cardamonin and pinocembrin standard solution for assay, Std-AS
Measure accurately the volume of the mixed alnustone, alpinetin, cardamonin and pinocembrin Std-Stock, dilute with methanol to produce a series of solutions of 10, 20, 50, 100, 200 mg/L for alnustone, 10, 20, 50, 100, 200 mg/L for alpinetin, 15, 30, 75, 150, 300 mg/L for cardamonin and 15, 30, 75, 150, 300 mg/L for pinocembrin.

Test solution

Weigh accurately 0.5 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 20 mL of methanol. Sonicate (270 W) the mixture for 30 min. Centrifuge at about 4000 × *g* for 10 min. Transfer the supernatant to a 50-mL volumetric flask. Repeat the extraction for one more time. Wash the residue with methanol. Centrifuge at about 4000 × *g* for 10 min. Combine the supernatants 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 (300 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 µm particle size). The column temperature is maintained at 40ºC during the separation. The flow rate is about 0.8 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 – 45	60 → 48	40 → 52	linear gradient
45 – 60	48 → 0	52 → 100	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 10 µL of the mixed alnustone, alpinetin, cardamonin and pinocembrin Std-AS (50 mg/L for alnustone, 50 mg/L for alpinetin, 75 mg/L for cardamonin and 75 mg/L for pinocembrin). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of alnustone, alpinetin, cardamonin and pinocembrin should not be more than 5.0%; the RSD of the retention times of alnustone, alpinetin, cardamonin and pinocembrin peaks should not be more than 2.0%; the column efficiencies determined from alnustone, alpinetin, cardamonin and pinocembrin peaks should not be less than 50000, 10000, 10000 and 10000 theoretical plates respectively.

The *R* value between alnustone peak and the closest peak; the *R* value between alpinetin peak and the closest peak; the *R* value between cardamonin peak and the closest peak; and the *R* value between pinocembrin 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 alnustone, alpinetin, cardamonin and pinocembrin Std-AS (10 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of alnustone, alpinetin, cardamonin and pinocembrin against the corresponding concentrations of the mixed alnustone, alpinetin, cardamonin and pinocembrin Std-AS. Obtain the slopes, y-intercepts and the r^2 values from the corresponding 5-point calibration curves.

Procedure

Inject 10 µL of the test solution into the HPLC system and record the chromatogram. Identify alnustone, alpinetin, cardamonin and pinocembrin peaks in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed alnustone, alpinetin, cardamonin and pinocembrin Std-AS. The retention times of alnustone, alpinetin, cardamonin and pinocembrin 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 alnustone, alpinetin, cardamonin and pinocembrin in the test solution, and calculate the percentage contents of alnustone, alpinetin, cardamonin and pinocembrin in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 1.4% of the total content of alpinetin ($C_{16}H_{14}O_4$), cardamonin ($C_{16}H_{14}O_4$) and pinocembrin ($C_{15}H_{12}O_4$); and not less than 0.31% of alnustone ($C_{19}H_{18}O$), calculated with reference to the dried substance.

7.2 Assay of Volatile Oil

Weigh accurately 75 g of the powdered sample and place it in a 1000-mL round-bottomed flask. Add 500 mL of water and a few glass beads, shake and mix well. Carry out the method as directed in Appendix XIII (Method A).

Limits

The sample contains not less than 1.0% (v/w) of volatile oil.