Verbenae Herba

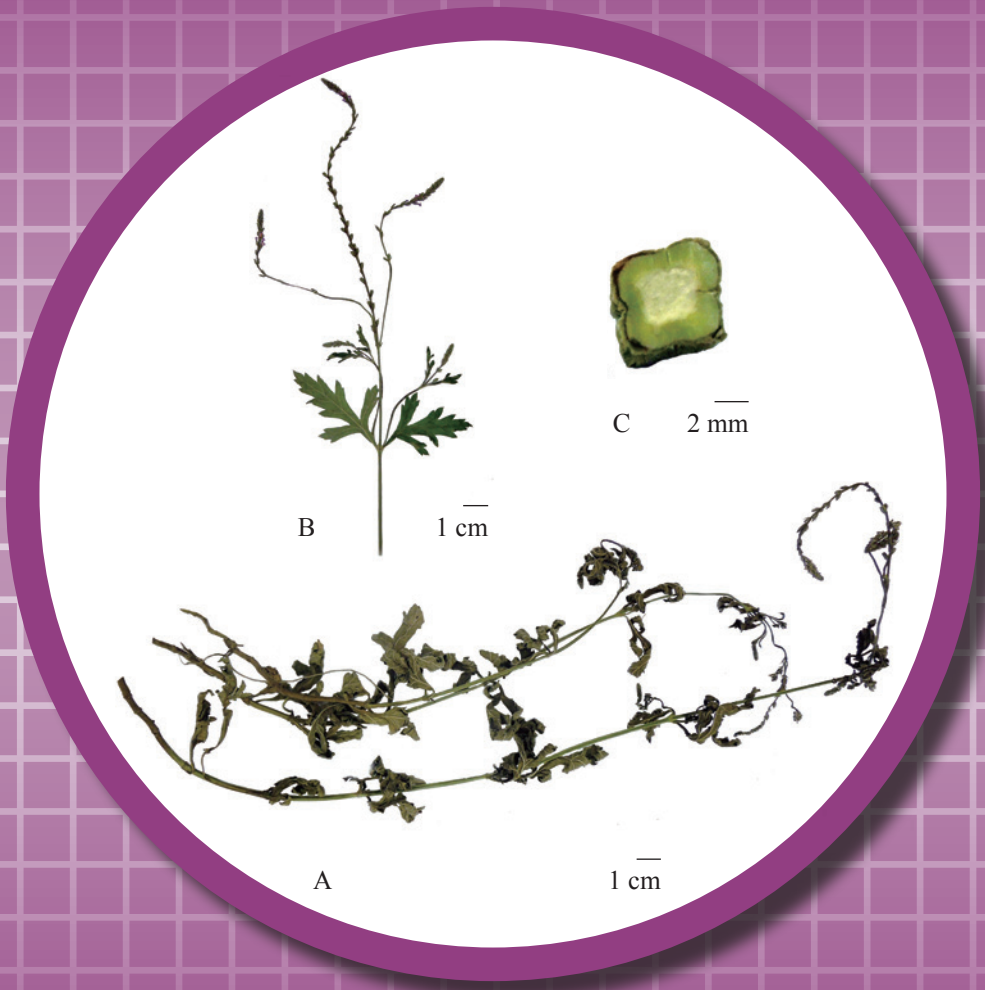


Figure 1 A photograph of Verbenae Herba

A. Verbenae Herba B. Leaf and inflorescence
C. Magnified image of transverse section of stem

1. NAMES

Official Name: Verbenae Herba

Chinese Name: 馬鞭草

Chinese Phonetic Name: Mabiancao

2. SOURCE

Verbenae Herba is the dried aerial part of *Verbena officinalis* L. (Verbenaceae). The aerial part is collected at flowering, foreign matter removed, then dried under the sun to obtain Verbenae Herba.

3. DESCRIPTION

Stem squared, much branched, with a longitudinal furrow at each side; externally brownish-green to brown, rough, hard and fragile, easily broken; fracture fibrous, with pith or hollow in the centre. Leaves opposite, greyish-green to brown; when intact flattened out, lamina ovate, obovate, or oblong, texture papery, margin coarsely serrate or sometimes pinnatified or 3-parted. Spikes long and slender, flower small, numerous. Odour slight; taste bitter (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

Transverse section

Stem: Epidermis consists of 1 layer of subsquare or rectangular cells, arranged orderly. Cortex relatively thin, with 4-5 layers of collenchymatous cells at angular regions. Cortex fibres in bundles, arranged in an interrupted ring, relatively large at angular regions. Phloem narrow. Cambium distinct. Xylem relatively broad, arranged in a ring; xylem vessels radically arranged. Pith broad, parenchymatous cells subrounded, occasionally broken or hollowed in the centre (Fig. 2).

Leaf: Upper epidermis consists of 1 layer of subsquare to rectangular cells. Palisade tissue consists of 1 layer of cells. Spongy tissue cells subrounded, arranged loosely. Vascular bundles collateral, xylem vessels radially arranged. Lower epidermis consists of 1 layer of irregularly shaped cells. Unicellular non-glandular and glandular scales rising from the epidermis, occasionally found (Fig. 2).

Powder

Colour yellowish-green. Epidermal cells of leaf with wavy and slightly curved walls, stomata anisocytic or anomocytic, with 3-5 subsidiary cells. Epidermal cells of stem irregularly rectangular or ovate, stomata anomocytic. Non-glandular hairs unicellular, apex acuminate. Glandular scales consist of multicellular head and unicellular stalk. Vessels bordered-pitted and scalariform, 10-53 μm in diameter. Fibres singly or in bundles, 11-74 μm in diameter, walls relatively thick; bluish-white under the polarized microscope. Pollen grains subrounded to subtriangular, 6-43 μm in diameter, surface smooth, with 3 germinal pores (Fig. 3).

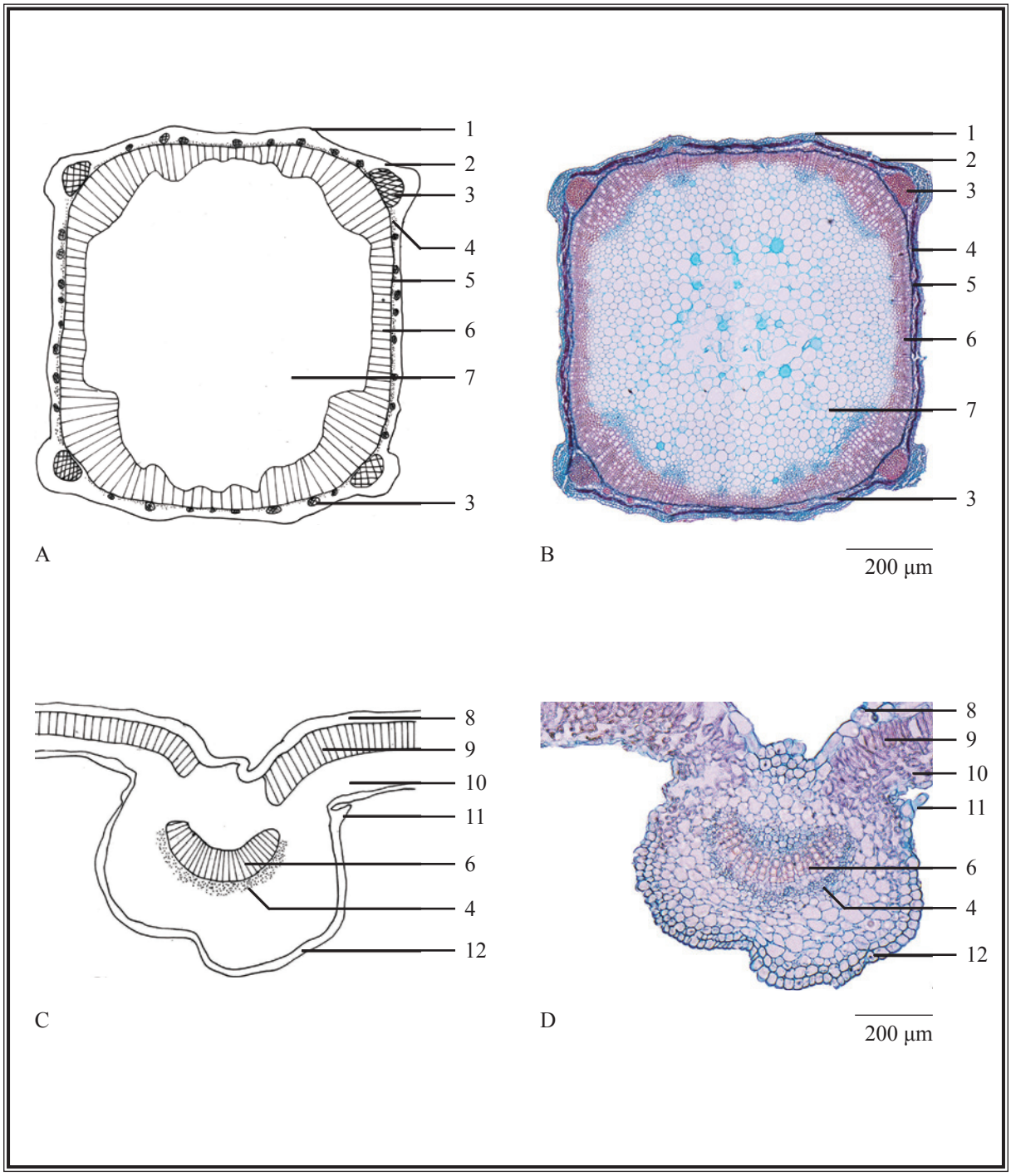


Figure 2 Microscopic features of transverse section of stem and leaf of Verbenae Herba

A. Sketch of stem B. Section illustration of stem C. Sketch of leaf D. Section illustration of leaf

1. Epidermis 2. Cortex 3. Cortex fibre bundle 4. Phloem 5. Cambium 6. Xylem 7. Pith
8. Upper epidermis 9. Palisade tissue 10. Spongy tissue 11. Unicellular non-glandular hair
12. Lower epidermis

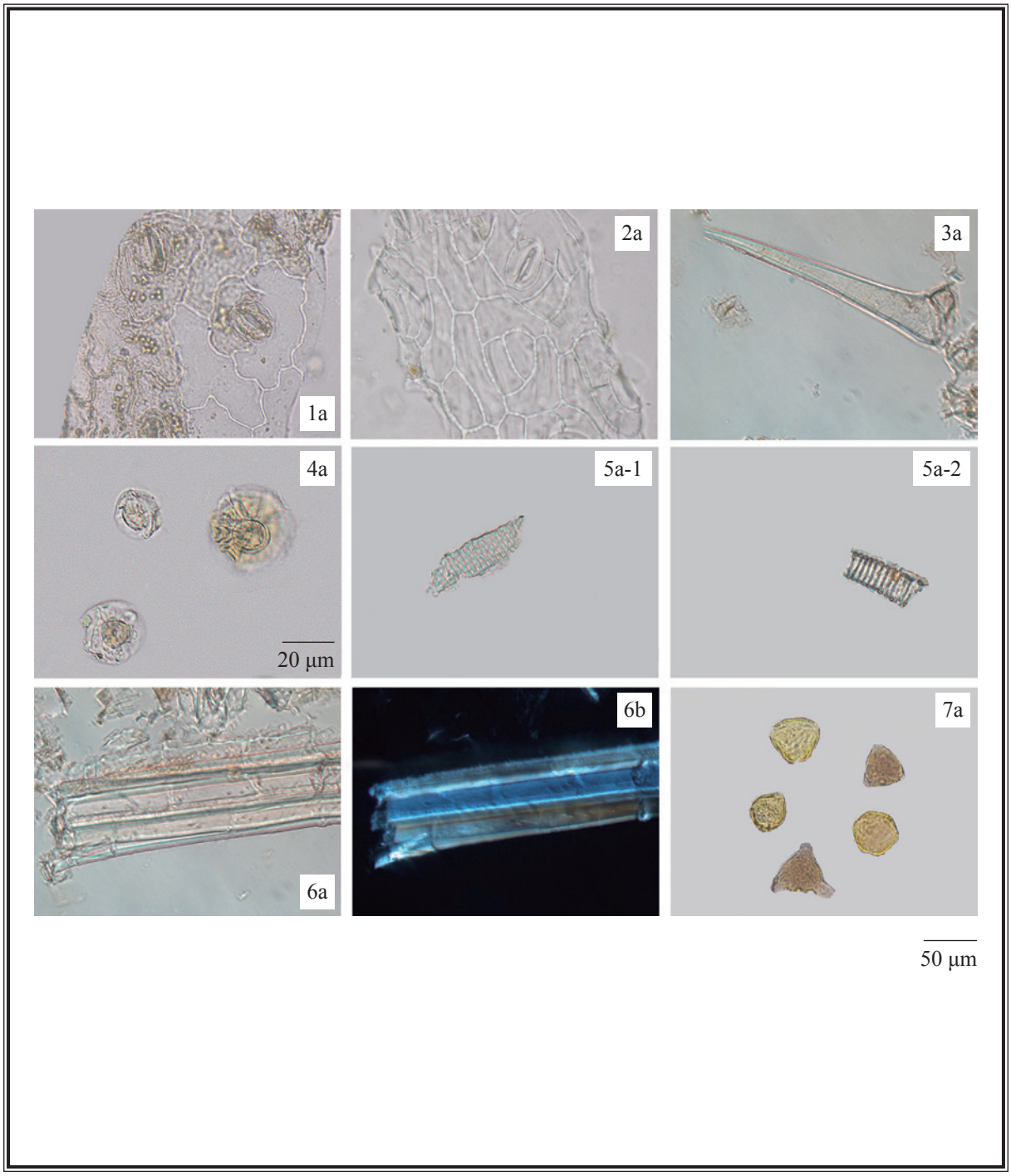


Figure 3 Microscopic features of powder of Verbenae Herba

1. Epidermal cells and stomata of leaf 2. Epidermal cells and stomata of stem
3. Unicellular non-glandular hair of leaf 4. Glandular scales of leaf
5. Vessel (5-1 bordered-pitted vessel, 5-2 scalariform vessel) 6. Fibre bundle 7. Pollen grains

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

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

Standard solutions

Hastatoside standard solution

Weigh 1.1 mg of hastatoside CRS (Fig. 4) and dissolve in 1 mL of methanol.

Verbenalin standard solution

Weigh 1.3 mg of verbenalin CRS (Fig. 4) and dissolve in 1 mL of methanol.

Developing solvent system

Prepare a mixture of ethyl acetate, water, formic acid and glacial acetic acid (8:2:1:1, v/v).

Spray reagent

Add slowly 5 mL of sulphuric acid to 95 mL of ethanol and dissolve 1 g of vanillin.

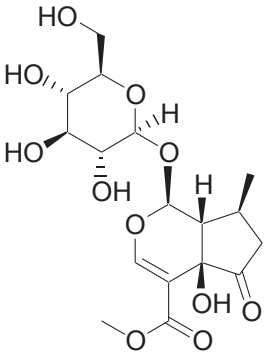
Test solution

Weigh 1.0 g of the powdered sample and place it in a 50-mL conical flask, then add 10 mL of methanol. Sonicate (350 W) the mixture for 30 min. 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 hastatoside standard solution (2 µL), verbenalin standard solution (2 µL) and the test solution (5 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 6 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 5 min). Examine the plate under visible light. Calculate the R_f values by using the equation as indicated in Appendix IV (A).

(i)



(ii)

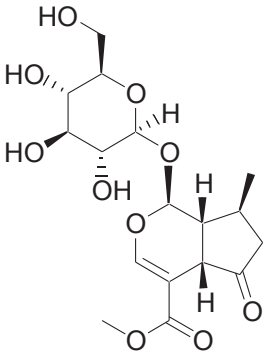


Figure 4 Chemical structures of (i) hastatoside and (ii) verbenalin

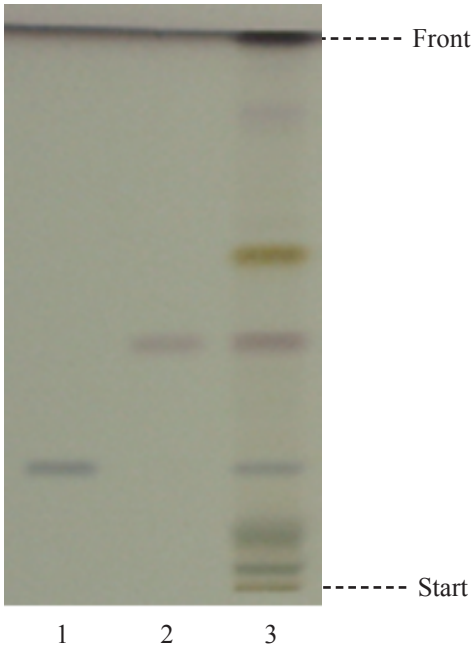


Figure 5 A reference HPTLC chromatogram of Verbenae Herba extract observed under visible light after staining

1. Hastatoside standard solution 2. Verbenalin 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 hastatoside and verbenalin (Fig. 5).

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

Standard solutions

Hastatoside standard solution for fingerprinting, Std-FP (220 mg/L)

Weigh 2.2 mg of hastatoside CRS and dissolve in 10 mL of methanol (80%).

Verbenalin standard solution for fingerprinting, Std-FP (200 mg/L)

Weigh 2.0 mg of verbenalin CRS and dissolve in 10 mL of methanol (80%).

Test solution

Weigh 1.0 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 20 mL of methanol (80%). Sonicate (240 W) the mixture for 45 min. Centrifuge at about 4000 × *g* for 5 min. Filter through a 0.45-μm nylon filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (230 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size). The column temperature is maintained at 30°C during the separation. The flow rate is about 1.0 mL/min. Programme the chromatographic system as follows (Table 1) –

Table 1 Chromatographic system conditions

Time (min)	0.05% Phosphoric acid (%, v/v)	Acetonitrile (%, v/v)	Elution
0 – 10	90	10	isocratic
10 – 30	90 → 80	10 → 20	linear gradient
30 – 60	80 → 60	20 → 40	linear gradient

System suitability requirements

Perform at least five replicate injections, each using 5 μL of hastatoside Std-FP and verbenalin Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of hastatoside and verbenalin should not be more than 5.0%; the RSD of the retention times of hastatoside and verbenalin peaks should not be more than 2.0%; the column efficiencies determined from hastatoside and verbenalin peaks should not be less than 15000 and 25000 theoretical plates respectively.

The *R* value between peak 1 and the closest peak; and the *R* value between peak 2 and the closest peak in the chromatogram of the test solution should not be less than 1.5 (Fig. 6).

Procedure

Separately inject hastatoside Std-FP, verbenalin Std-FP and the test solution (5 µL each) into the HPLC system and record the chromatograms. Measure the retention times of hastatoside and verbenalin peaks in the chromatograms of hastatoside Std-FP, verbenalin Std-FP and the retention times of the four characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify hastatoside and verbenalin peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of hastatoside Std-FP and verbenalin Std-FP. The retention times of hastatoside and verbenalin 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 four characteristic peaks of Verbenae Herba extract are listed in Table 2.

Table 2 The RRTs and acceptable ranges of the four characteristic peaks of Verbenae Herba extract

Peak No.	RRT	Acceptable Range
1 (hastatoside)	0.83	± 0.03
2 (marker, verbenalin)	1.00	-
3	1.67	± 0.03
4	2.03	± 0.06

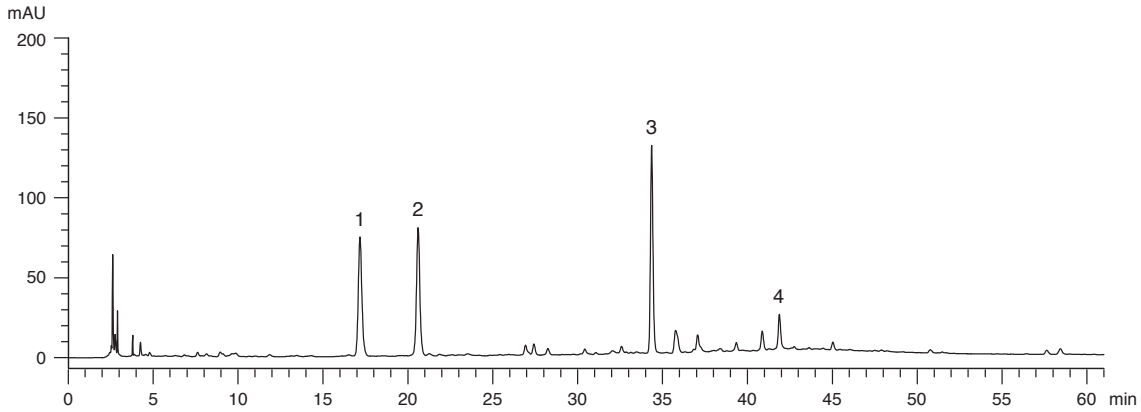


Figure 6 A reference fingerprint chromatogram of Verbenae Herba extract

For positive identification, the sample must give the above four 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 3.0%.

5.6 Ash *(Appendix IX)*

Total ash: not more than 12.0%.
Acid-insoluble ash: not more than 4.0%.

5.7 Water Content *(Appendix X)*

Oven dried method: not more than 10.0%.

6. EXTRACTIVES *(Appendix XI)*

Water-soluble extractives (hot extraction method): not less than 14.0%.
Ethanol-soluble extractives (hot extraction method): not less than 15.0%.

7. ASSAY

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

Standard solution
Mixed hastatoside and verbenalin standard stock solution for assay, Std-Stock (1350 mg/L for hastatoside and 1060 mg/L for verbenalin)
Weigh accurately 13.5 mg of hastatoside CRS and 10.6 mg of verbenalin CRS, and dissolve in 10 mL of methanol (80%).

Mixed hastatoside and verbenalin standard solution for assay, Std-AS

Measure accurately the volume of the mixed hastatoside and verbenalin Std-Stock, dilute with methanol (80%) to produce a series of solutions of 2.7, 13.5, 67.5, 135, 270 mg/L for hastatoside and 10.6, 53, 106, 212, 424 mg/L for verbenalin.

Test solution

Weigh accurately 0.1 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 15 mL of methanol (80%). Sonicate (240 W) the mixture for 45 min. Centrifuge at about 4000 × *g* for 5 min. Transfer the supernatant to a 100-mL round-bottomed flask. Repeat the extraction for one more time. Combine the supernatants and evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol (80%). Transfer the solution to a 10-mL volumetric flask and make up to the mark with methanol (80%). Filter through a 0.45-μm nylon filter.

Chromatographic system

The liquid chromatograph is equipped with a DAD (238 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 μm particle size). The column temperature is maintained at 30°C during the separation. The flow rate is about 1.0 mL/min. The mobile phase is a mixture of 0.05% phosphoric acid and acetonitrile (90:10, v/v). The elution time is about 28 min.

System suitability requirements

Perform at least five replicate injections, each using 5 μL of the mixed hastatoside and verbenalin Std-AS (67.5 mg/L for hastatoside and 106 mg/L for verbenalin). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of hastatoside and verbenalin should not be more than 5.0%; the RSD of the retention times of hastatoside and verbenalin peaks should not be more than 2.0%; the column efficiencies determined from hastatoside and verbenalin peaks should not be less than 10000 theoretical plates.

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

Procedure

Inject 5 µL of the test solution into the HPLC system and record the chromatogram. Identify hastatoside and verbenalin peaks in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed hastatoside and verbenalin Std-AS. The retention times of hastatoside and verbenalin 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 hastatoside and verbenalin in the test solution, and calculate the percentage contents of hastatoside and verbenalin in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 0.71% of the total content of hastatoside ($C_{17}H_{24}O_{11}$) and verbenalin ($C_{17}H_{24}O_{10}$), calculated with reference to the dried substance.