Figure 1  A photograph of Melicopes Pteleifoliae Caulis

A. Melicopes Pteleifoliae Caulis
B. Magnified image of slice of stem
1. NAMES

Official Name: Melicopes Pteleifoliae Caulis

Chinese Name: 三叉苦

Chinese Phonetic Name: Sanchaku

2. SOURCE

Melicopes Pteleifoliae Caulis is the dried stem of *Melicope pteleifolia* (Champ. ex Benth.) T. G. Hartley (Rutaceae). The stem is collected throughout the year, cut into segments when fresh, then dried under the sun to obtain Melicopes Pteleifoliae Caulis.

3. DESCRIPTION

Cylindrical, 10-50 mm in diameter, mostly cut into segments or thick slices. Externally bark greyish-brown to brown with dense brown lenticels, easily rubbed off. Wood yellowish-white with concentric annulations, with the pith in central. Texture hard, uneasily broken. Fracture yellowish-white. Odour rancid; taste bitter (Fig. 1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

**Transverse section**

Cork consists of 5-10 or more layers of subsquare cells, slightly elongated tangentially, some containing brown pigment, easily fallen off during transverse section processing. In young stem, epidermis consists of 1 layer of subsquare cells. Cortex relative narrow, scattered with secretory cavities, fibre bundles and stone cells arranged in an interrupted ring in the innermost area of the cortex. Phloem relatively narrow. Xylem broad, ray consists of 1-2 rows of cells. Pith distinct, scattered with clusters of calcium oxalate (Fig. 2).
Melicopes Pteleifoliae Caulis

**Powder**

Colour pale yellow. Cork cells subpolygonal, 12-25 μm in diameter, some containing brown pigment. Vessels mainly bordered-pitted, 30-70 μm in diameter, pit fine and arranged densely. Stone cells scattered singly or in groups, subsquare or irregular in shape, 25-50 μm in diameter; bright white or polychromatic under the polarized microscope. Xylem fibres in bundles, walls relatively thin; bright white under the polarized microscope. Clusters of calcium oxalate scattered, 8-20 μm in diameter; polychromatic under the polarized microscope (Fig. 3).
Figure 2  Microscopic features of transverse section of Melicopes Ptelefoliae Caulis

A. Sketch    B. Section illustration    C. Clusters of calcium oxalate (under the light microscope)
D. Clusters of calcium oxalate (under the polarized microscope)

Figure 3  Microscopic features of powder of Melicopes Ptelefoliae Caulis


a. Features under the light microscope  b. Features under the polarized microscope
4.2 Thin-Layer Chromatographic Identification [Appendix IV(A)]

Standard solution

Evolitrine standard solution
Weigh 1.0 mg of evolitrine CRS (Fig. 4) and dissolve in 1 mL of methanol.

Developing solvent system
Prepare a mixture of cyclohexane, ethyl acetate, ethanol and triethylamine (9:3:0.5:0.5, v/v).

Test solution
Weigh 1.0 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 25 mL of methanol. Sonicate (160 W) the mixture for 30 min. Centrifuge at about 2000 × g for 5 min. Transfer the supernatant to a 100-mL round-bottomed flask. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in 1 mL of methanol.

Procedure
Carry out the method by using a HPTLC silica gel F254 plate, a twin trough chamber and a freshly prepared developing solvent system as described above. Apply separately evolitrine standard solution (2 μL) and the test solution (7 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 $R_f$ value by using the equation as indicated in Appendix IV (A).

![Figure 4 Chemical structure of evolitrine](image)
4.2 Thin-Layer Chromatographic Identification

**Standard solution**
Evolitrine standard solution (Fig. 4) Weigh 1.0 mg of evolitrine CRS (Fig. 4) and dissolve in 1 mL of methanol.

**Developing solvent system**
Prepare a mixture of cyclohexane, ethyl acetate, ethanol and triethylamine (9:3:0.5:0.5, v/v).

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**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 evolitrine standard solution (2 μL) and the test solution (7 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 \( R_f \) value by using the equation as indicated in Appendix IV (A).

**Figure 4**
Chemical structure of evolitrine

For positive identification, the sample must give spots or bands with chromatographic characteristics, including the colour and the \( R_f \) value, corresponding to those of evolitrine (Fig. 5).

4.3 High-Performance Liquid Chromatographic Fingerprinting (Appendix XII)

**Standard solution**
Evolitrine standard solution for fingerprinting, Std-FP (20 mg/L)
Weigh 0.2 mg of evolitrine 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 45 mL of methanol. Sonicate (100 W) the mixture for 30 min. Centrifuge at about 1200 × g for 10 min. Transfer the supernatant to a 250-mL round-bottomed flask. Repeat the extraction for one more time. Combine the supernatants. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol. Transfer the solution to a 10-mL volumetric flask and make up to the mark with methanol. Filter through a 0.45-μm PTFE filter.

**Figure 5**
A reference HPTLC chromatogram of Melicopes Pteleifoliae Caulis extract observed under UV light (254 nm)

1. Evolitrine 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 evolitrine (Fig. 5).
Chromatographic system

The liquid chromatograph is equipped with a DAD (243 nm) and a column (4.6 × 250 mm) packed with ODS bonded silica gel (5 µm particle size). The flow rate is about 0.8 mL/min.

Programme the chromatographic system as follows (Table 1) –

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0.1% Formic acid (%)</th>
<th>Acetonitrile (%)</th>
<th>Elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 10</td>
<td>85 → 70</td>
<td>15 → 30</td>
<td>linear gradient</td>
</tr>
<tr>
<td>10 – 15</td>
<td>70 → 65</td>
<td>30 → 35</td>
<td>linear gradient</td>
</tr>
<tr>
<td>15 – 25</td>
<td>65</td>
<td>35</td>
<td>isocratic</td>
</tr>
<tr>
<td>25 – 30</td>
<td>65 → 60</td>
<td>35 → 40</td>
<td>linear gradient</td>
</tr>
<tr>
<td>30 – 35</td>
<td>60 → 55</td>
<td>40 → 45</td>
<td>linear gradient</td>
</tr>
<tr>
<td>35 – 50</td>
<td>55 → 40</td>
<td>45 → 60</td>
<td>linear gradient</td>
</tr>
<tr>
<td>50 – 60</td>
<td>40 → 30</td>
<td>60 → 70</td>
<td>linear gradient</td>
</tr>
</tbody>
</table>

**System suitability requirements**

Perform at least five replicate injections, each using 20 µL of evolitrine Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak area of evolitrine should not be more than 5.0%; the RSD of the retention time of evolitrine peak should not be more than 2.0%; the column efficiency determined from evolitrine peak should not be less than 20000 theoretical plates.

The $R$ value between peak 4 and the closest peak in the chromatogram of the test solution should not be less than 1.0 (Fig. 6).

**Procedure**

Separately inject evolitrine Std-FP and the test solution (20 µL each) into the HPLC system and record the chromatograms. Measure the retention time of evolitrine peak in the chromatogram of evolitrine Std-FP and the retention times of the four characteristic peaks (Fig. 6) in the chromatogram of the test solution. Identify evolitrine peak in the chromatogram of the test solution by comparing its retention time with that in the chromatogram of evolitrine Std-FP. The retention times of evolitrine 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 four characteristic peaks of Melicopes Pteleifoliae Caulis extract are listed in Table 2.

Table 2  The RRTs and acceptable ranges of the four characteristic peaks of Melicopes Pteleifoliae Caulis extract

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>RRT</th>
<th>Acceptable Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.68</td>
<td>± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>0.72</td>
<td>± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>0.91</td>
<td>± 0.03</td>
</tr>
<tr>
<td>4 (marker, evolitrine)</td>
<td>1.00</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 6  A reference fingerprint chromatogram of Melicopes Pteleifoliae Caulis 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 XVI)*: meet the requirements.

5.5 **Foreign Matter** *(Appendix VIII)*: not more than 1.0%.

5.6 **Ash** *(Appendix IX)*

   Total ash: not more than 1.5%.
   Acid-insoluble ash: not more than 0.5%.

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 5.0%.

7. **ASSAY**

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

   **Standard solution**
   
   *Evolitrine standard stock solution, Std-Stock (100 mg/L)*
   
   Weigh accurately 2.0 mg of evolitrine CRS and dissolve in 20 mL of methanol.

   *Evolitrine standard solution for assay, Std-AS*
   
   Measure accurately the volume of the evolitrine Std-Stock, dilute with methanol to produce a series of solutions of 1.3, 1.5, 2, 3.5, 5 mg/L for evolitrine.

   **Test solution**
   
   Weigh accurately 0.2 g of the powdered sample and place it in a 50-mL centrifuge tube, then add 45 mL of methanol. Sonicate (100 W) the mixture for 30 min. Centrifuge at about 1200 × g for 10 min. Transfer the supernatant to a 250-mL round-bottomed flask. Repeat the extraction for one more time. Combine the supernatants. Evaporate the solvent to dryness at reduced pressure in a rotary evaporator. Dissolve the residue in methanol. Transfer the solution to a 10-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 a DAD (243 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. The mobile phase is a mixture of 0.1% formic acid and acetonitrile (60:40, v/v). The elution time is about 30 min.

System suitability requirements

Perform at least five replicate injections, each using 10 µL of evolitrine Std-AS (2 mg/L). The requirements of the system suitability parameters are as follows: the RSD of the peak area of evolitrine should not be more than 5.0%; the RSD of the retention time of evolitrine peak should not be more than 2.0%; the column efficiency determined from evolitrine peak should not be less than 10000 theoretical plates.

The R value between evolitrine peak and the closest peak in the chromatogram of the test solution should not be less than 1.5.

Calibration curve

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

Limits

The sample contains not less than 0.010% of evolitrine \((C_{13}H_{11}NO_3)\), calculated with reference to the dried substance.