Figure 1  A photograph of Chrysanthemi Indici Flos

A. Chrysanthemi Indici Flos
B. Magnified image of capitulum (top view)
C. Magnified image of capitulum (lateral view)
1. NAMES

Official Name: Chrysanthemi Indici Flos

Chinese Name: 野菊花

Chinese Phonetic Name: Yejuhua

2. SOURCE

Chrysanthemi Indici Flos is the dried capitulum of *Chrysanthemum indicum* L. (Asteraceae). The capitulum is collected at the early stage of flowering in autumn and winter, then dried under the sun or dried after steaming to obtain Chrysanthemi Indici Flos.

3. DESCRIPTION

Subspherical, 3-6 mm in diameter, brownish-yellow. Involucre consists of 3-5 layers of bracts, the outer bracts ovate or strip-shaped, the middle part of the outer surface greyish-green to pale brown, often covered with white hairs, margins membranaceous, 2-5 mm long; the middle bracts triangular ovate, 4-7 mm long; the inner bracts elongated-elliptic, membranaceous, 4-8 mm long, hairs absent on the outer surface. The base of involucre sometimes with remnant of pedicel. Ligulate florets arranged in 1 whorl, yellow, crumpled and rolled; tubular florets numerous, deep yellow. Texture fluffy and soft, light in weight. Odour aromatic; taste bitter (Fig.1).

4. IDENTIFICATION

4.1 Microscopic Identification *(Appendix III)*

**Powder**

Colour brownish-yellow. Non-glandular hairs arms slightly circuitous or T-shaped, apical cell unequally furcated, walls slightly thickened, sometimes thickened only on one side, 1-5 cells uniseriate arranged at the base, 20-40 μm in diameter. Glandular hairs consist of a 4- to 6-celled head, without stalk, cells biseriate and overlapping, oval in surface view and obtuse triangular in lateral view, 40-60 μm in diameter. Pollen grains numerous, yellow, subglobular to ellipsoid, 18-35 μm in diameter, with 3 germinal pores; outer layer of exine thicker than the inner layer, with columns visible inside the outer layer, and inverse reticulate and spiny protuberance on the surface, spines 3-10 μm long. Epidermal cells of ovary wall elongated, composed of two types...
of cells, first type arranged horizontally forming a broad line, second type located vertically on the side. Fragments of stigma showing papillae, with the cells elongated at the apex, border cells villiform. Epidermal cells of filament rectangular to subrectangular, 10-18 μm in diameter. Epidermal cells of bract polygonal in surface view, anticlinal walls straight or sinuous, with longitudinal cuticular striations, stomata infinitive, rounded or oval, 18-25 μm in diameter, subsidiary cells 3-6. Clusters of calcium oxalate occasionally visible, small; polychromatic under the polarized microscope. Spiral vessels occasionally visible (Fig. 2).
Figure 2  Microscopic features of powder of Chrysanthemi Indici Flos

5. Fragments of stigma showing papillae  6. Epidermal cells of filament
7. Epidermal cells of bract (7-1 epidermal cells of bract, 7-2 epidermal cells of bract with stoma →)

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

**Standard solutions**

*Chlorogenic acid standard solution*
Weigh 2.0 mg of chlorogenic acid CRS (Fig. 3) and place it in a 5-mL amber glass volumetric flask. Make up to the mark with methanol (70%).

*3,5-Dicaffeoylquinic acid standard solution*
Weigh 2.0 mg of 3,5-dicaffeoylquinic acid CRS (Fig. 3) and place it in a 5-mL amber glass volumetric flask. Make up to the mark with methanol (70%).

*4,5-Dicaffeoylquinic acid standard solution*
Weigh 2.0 mg of 4,5-dicaffeoylquinic acid CRS (Fig. 3) and place it in a 5-mL amber glass volumetric flask. Make up to the mark with methanol (70%).

*Luteolin 7-O-β-D-glucoside standard solution*
Weigh 2.0 mg of luteolin 7-O-β-D-glucoside CRS (Fig. 3) and place it in a 10-mL amber glass volumetric flask. Make up to the mark with methanol (70%). Sonicate to dissolve the standard.

**Developing solvent system**
Prepare a mixture of *n*-butyl acetate, formic acid and water (28:13:10, v/v). Shake well and use the upper layer.

**Spray reagent**
Weigh 1 g of aluminium trichloride and dissolve in 100 mL of ethanol.

**Test solution**
Weigh 1.0 g of the powdered sample and place it in a 50-mL conical flask wrapped in aluminium foil, then add 10 mL of methanol (70%). Sonicate (180 W) the mixture for 30 min. Filter the mixture.

**Procedure**
Carry out the method by using a HPTLC silica gel F254 plate and a freshly prepared developing solvent system as described above. Apply separately chlorogenic acid standard solution (2 μL), 3,5-dicaffeoylquinic acid standard solution (2 μL), 4,5-dicaffeoylquinic acid standard solution (2 μL), luteolin 7-O-β-D-glucoside standard solution (1 μL) and the test solution (2 μL) to the plate. 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. Spray the plate evenly with the spray reagent and heat at about 100°C (about 5 min). Examine the plate under UV light (366 nm). Calculate the $R_f$ values by using the equation as indicated in Appendix IV (A).
Figure 3  Chemical structures of (i) chlorogenic acid (ii) 3,5-dicaffeoylquinic acid (iii) 4,5-dicaffeoylquinic acid and (iv) luteolin 7-O-β-D-glucoside
Figure 4  A reference HPTLC chromatogram of Chrysanthemi Indici Flos extract observed under UV light (366 nm) after staining

1. Chlorogenic acid standard solution
2. Luteolin 7-O-β-D-glucoside standard solution
3. 4,5-Dicaffeoylquinic acid standard solution
4. 3,5-Dicaffeoylquinic acid 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 chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside (Fig. 4).
4.3 High-Performance Liquid Chromatographic Fingerprinting *(Appendix XII)*

**Standard solutions**

*Chlorogenic acid standard solution for fingerprinting, Std-FP (5 mg/L)*

Weigh 0.5 mg of chlorogenic acid CRS and place it in a 100-mL amber glass volumetric flask. Make up to the mark with methanol (70%).

*3,5-Dicaffeoylquinic acid standard solution for fingerprinting, Std-FP (5 mg/L)*

Weigh 0.5 mg of 3,5-dicaffeoylquinic acid CRS and place it in a 100-mL amber glass volumetric flask. Make up to the mark with methanol (70%).

*4,5-Dicaffeoylquinic acid standard solution for fingerprinting, Std-FP (5 mg/L)*

Weigh 0.5 mg of 4,5-dicaffeoylquinic acid CRS and place it in a 100-mL amber glass volumetric flask. Make up to the mark with methanol (70%).

*Luteolin 7-O-β-D-glucoside standard solution for fingerprinting, Std-FP (5 mg/L)*

Weigh 0.5 mg of luteolin 7-O-β-D-glucoside CRS and place it in a 100-mL amber glass volumetric flask. Make up to the mark with methanol (70%). Sonicate to dissolve the standard.

**Test solution**

Weigh 0.1 g of the powdered sample and place it in a 50-mL conical flask wrapped in aluminium foil, then add 25 mL of methanol (70%). Sonicate (180 W) the mixture for 30 min. Filter and transfer the filtrate to a 25-mL amber glass volumetric flask. Make up to the mark with methanol (70%). Filter through a 0.45-µm PTFE filter.

**Chromatographic system**

The liquid chromatograph is equipped with a DAD (334 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. Programme the chromatographic system as follows (Table 1) –

**Table 1** Chromatographic system conditions

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0.1% Phosphoric acid (% v/v)</th>
<th>Methanol (% v/v)</th>
<th>Elution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 15</td>
<td>75</td>
<td>25</td>
<td>isocratic</td>
</tr>
<tr>
<td>15 – 20</td>
<td>75 → 56</td>
<td>25 → 44</td>
<td>linear gradient</td>
</tr>
<tr>
<td>20 – 40</td>
<td>56 → 55</td>
<td>44 → 45</td>
<td>linear gradient</td>
</tr>
<tr>
<td>40 – 45</td>
<td>55 → 45</td>
<td>45 → 55</td>
<td>linear gradient</td>
</tr>
<tr>
<td>45 – 60</td>
<td>45</td>
<td>55</td>
<td>isocratic</td>
</tr>
</tbody>
</table>
System suitability requirements

Perform at least five replicate injections, each using 10 µL of chlorogenic acid Std-FP, 3,5-dicaffeoylquinic acid Std-FP, 4,5-dicaffeoylquinic acid Std-FP and luteolin 7-O-β-D-glucoside Std-FP. The requirements of the system suitability parameters are as follows: the RSD of the peak areas of chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside should not be more than 5.0%; the RSD of the retention times of chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside peaks should not be more than 2.0%; the column efficiencies determined from chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside peaks should not be less than 4000, 45000, 25000 and 15000 theoretical plates respectively.

The R value between peak 1 and the closest peak; the R value between peak 3 and the closest peak; the R value between peak 4 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 1.5 (Fig. 5).

Procedure

Separately inject chlorogenic acid Std-FP, 3,5-dicaffeoylquinic acid Std-FP, 4,5-dicaffeoylquinic acid Std-FP, luteolin 7-O-β-D-glucoside Std-FP and the test solution (10 µL each) into the HPLC system and record the chromatograms. Measure the retention times of chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside peaks in the chromatograms of chlorogenic acid Std-FP, 3,5-dicaffeoylquinic acid Std-FP, 4,5-dicaffeoylquinic acid Std-FP, luteolin 7-O-β-D-glucoside Std-FP and the retention times of the seven characteristic peaks (Fig. 5) in the chromatogram of the test solution. Identify chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside peaks in the chromatogram of the test solution by comparing its retention time with that in the chromatograms of chlorogenic acid Std-FP, 3,5-dicaffeoylquinic acid Std-FP, 4,5-dicaffeoylquinic acid Std-FP and luteolin 7-O-β-D-glucoside Std-FP. The retention times of chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-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 seven characteristic peaks of Chrysanthemi Indici Flos extract are listed in Table 2.
Table 2  The RRTs and acceptable ranges of the seven characteristic peaks of Chrysanthemi Indici Flos extract

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>RRT</th>
<th>Acceptable Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (chlorogenic acid)</td>
<td>0.59</td>
<td>± 0.03</td>
</tr>
<tr>
<td>2 (3,4-dicaffeoylquinic acid)</td>
<td>0.95</td>
<td>± 0.03</td>
</tr>
<tr>
<td>3 (marker, 3,5-dicaffeoylquinic acid)</td>
<td>1.00</td>
<td>-</td>
</tr>
<tr>
<td>4 (luteolin 7-O-β-D-glucoside)</td>
<td>1.08</td>
<td>± 0.03</td>
</tr>
<tr>
<td>5 (4,5-dicaffeoylquinic acid)</td>
<td>1.19</td>
<td>± 0.03</td>
</tr>
<tr>
<td>6 (linarin)</td>
<td>1.81</td>
<td>± 0.03</td>
</tr>
<tr>
<td>7 (luteolin)</td>
<td>1.89</td>
<td>± 0.03</td>
</tr>
</tbody>
</table>

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. 5).

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 Foreign Matter (*Appendix VIII*): not more than 2.0%.

5.5 Ash (*Appendix IX*)

Total ash: not more than 8.5%.

Acid-insoluble ash: not more than 2.0%.
5.6 Water Content (Appendix X)

Oven dried method: not more than 14.0%.

6. EXTRACTIVES (Appendix XI)

Water-soluble extractives (hot extraction method): not less than 31.0%.
Ethanol-soluble extractives (hot extraction method): not less than 28.0%.

7. ASSAY

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

Standard solution

* Mixed chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside standard stock solution, Std-Stock (800 mg/L each) *

Weigh accurately 0.8 mg of chlorogenic acid CRS, 0.8 mg of 3,5-dicaffeoylquinic acid CRS, 0.8 mg of 4,5-dicaffeoylquinic acid CRS and 0.8 mg of luteolin 7-O-β-D-glucoside CRS and place them in a 1-mL amber glass volumetric flask. Make up to the mark with methanol (70%). Sonicate to dissolve the standards.

* Mixed chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside standard solution for assay, Std-AS *

Measure accurately the volume of the mixed chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside Std-Stock, dilute with methanol (70%) to produce a series of solutions of 0.25, 0.5, 1, 5, 10 mg/L for chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside. Store in amber glass volumetric flasks.

Test solution

Weigh accurately 0.2 g of the powdered sample and place it in a 50-mL conical flask wrapped in aluminium foil, then add 30 mL of methanol (70%). Sonicate (180 W) the mixture for 30 min. Filter and transfer the filtrate to a 100-mL amber glass volumetric flask. Repeat the extraction for two more times. Combine the solutions and make up to the mark with methanol (70%). Filter through a 0.45-µm PTFE filter.
Chromatographic system

The liquid chromatograph is equipped with a DAD (334 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. Programme the chromatographic system as follows (Table 3) –

Table 3  Chromatographic system conditions

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<td>44 → 45</td>
<td>linear gradient</td>
</tr>
</tbody>
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System suitability requirements

Perform at least five replicate injections, each using 10 µL of the mixed chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside Std-AS (1 mg/L each). The requirements of the system suitability parameters are as follows: the RSD of the peak areas of chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside should not be more than 5.0%; the RSD of the retention times of chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside peaks should not be more than 2.0%; the column efficiencies determined from chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside peaks should not be less than 4000, 45000, 25000 and 15000 theoretical plates respectively.

The R value between chlorogenic acid peak and the closest peak; the R value between 3,5-dicaffeoylquinic acid peak and the closest peak; the R value between 4,5-dicaffeoylquinic acid peak and the closest peak; and the R value between luteolin 7-O-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 chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside Std-AS (10 µL each) into the HPLC system and record the chromatograms. Plot the peak areas of chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside against the corresponding concentrations of the mixed chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside 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 chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside peaks in the chromatogram of the test solution by comparing their retention times with those in the chromatogram of the mixed chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside Std-AS. The retention times of chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-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 chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside in the test solution, and calculate the percentage contents of chlorogenic acid, 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and luteolin 7-O-β-D-glucoside in the sample by using the equations as indicated in Appendix IV (B).

Limits

The sample contains not less than 0.38% of the total content of chlorogenic acid (C_{16}H_{18}O_{9}), 3,5-dicaffeoylquinic acid (C_{25}H_{24}O_{12}) and 4,5-dicaffeoylquinic acid (C_{25}H_{24}O_{12}); and not less than 0.035% luteolin 7-O-β-D-glucoside (C_{21}H_{20}O_{11}), calculated with reference to the dried substance.