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Comparative Authentication of Three “Snow Lotus” Herbs by Macroscopic and Microscopic Features

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ABSTRACT

“Snow lotus” is a famous Chinese Materia Medica derived from species of the genus *Saussurea* (Compositae). To differentiate three representative easily-confused snow lotus herbs, namely, *S. involucrata* (Kar. et Kir.) Sch.-Bip., *S. laniceps* Hand.-Mazz. and *S. medusa* Maxim., macroscopic features of the three herbs were systemically observed, and microscopic features were compared by using ordinary light microscopy, polarized light microscopy and scanning electron microscopy. The results indicate that, as for macroscopic identification, capitula situation and arrangement, and as for microscopic identification, pollen grains, non-glandular hairs, glandular hairs, cells of inner surface of the microdiodange, and epidermal cells of the corolla can be used to authenticate the three snow lotus herbs. Comprehensive table comparing the characteristics were presented in this study. Scanning electron microscopy has been found to provide a number of unique characteristics of pollen grains. Based on the observation of pollen grains, evolution sequence of the three species was speculated. The present method was proven to be efficient, convenient, simple and reliable, which was successfully applied to the authentication of three snow lotus herbs.

KEYWORDS

Snow lotus; *Saussurea*; ordinary light microscopy; polarized light microscopy; scanning electron microscopy
“Snow lotus”, a famous Chinese Materia Medica (CMM) derived from the dried aerial parts of species of the genus *Saussurea* (Compositae), has been widely used in Tibetan, Mongolian, Uighur and Chinese folk medicines (Health Bureau of Autonomous Region of Xizang, 1973; Liu, 1985; Meyer 1983; Xiao, 2007; Xie, 1994). One of the botanical origins of “snow lotus” is *S. involucrata* (Kar. et Kir.) Sch.-Bip. (Fig. 1), a plant growing in the mountains at heights of 4,000–4,300 m in the Tianshan and A’er Tai areas of China (Chen et al., 1999; Yi et al., 2010); this species has long been used for diminishing inflammation and facilitating blood circulation, e.g. treating rheumatoid arthritis, stomachache and dysmenorrhea in Uighur folk medicine (Chinese Pharmacopoeia Commitee, 2010; Jiang and Li, 1999; Liu and Shawuti, 1999; Xiao, 2007). Two other species of the same genus, *S. laniceps* Hand.-Mazz. and *S. medusa* Maxim. (Fig. 1), mainly distributed in the Qinghai-Tibet plateau at heights of 3500–5300 m, have also been used under the name “Snow Lotus”. These latter two species are prescribed for the treatment of pain and inflammatory conditions in Tibetan folk medicine (Commission of Chinese Ethnomedicine, 1984; Jiang and Li, 1999; Xiao, 2007).

The three species have been confused because of their shared folk names, morphological appearances and medicinal uses (Chen et al., 1999; Xiao, 2007; Yang, 1991). Moreover, *Saussurea* plants, once dried or processed for herbal use, can be easily damaged or broken apart during commercial distribution, thus morphological characters are usually not sufficient for their identification. For some proprietary Chinese medicines, snow lotus herbs are powdered. Powdered herbs are not only very difficult to identify by sight but also easily replaced by other materials. For these reasons, a variety of identification methods and characters are needed to accurately authenticate the three species of snow lotus herbs. Therefore, it is necessary to distinguish the three species clearly.

In our previous studies (Yi et al., 2009 a,b, 2010, 2012, 2014), the chemical composition and pharmacological activities of these three herbs have been
investigated. However, the chemical and pharmacological approaches, as used in that study, while accurate, involve much time and effort, as well as expensive experimental equipment and animals. Since the three herbs have similar clinical uses, their chemical profiles and pharmacological effects cannot be distinguished effectively. In comparison, observing microscopic features is an efficient, simple, economical, reliable, and effective means for identifying CMM plant material powders (An et al., 2009; Chu et al., 2009). With the use of ordinary light microscope and polarized light microscope, CMM can be successfully identified. The application and advantages of polarized light microscopy in observing CMM plant tissues are described in our previous publication (Tang et al., 2013).

Apart from using optical microscopes, comparing pollen grain characters under scanning electron microscope is also an effective and reliable approach for herbal identification (Wodehouse, 1928). Since pollen grains are single cells and spores, the pollen grain characters are indicative of relationships among plants (Wodehouse, 1935). The size, shape and color of the grains, the number and arrangement of the germinal apertures, and the sculpturing of the exines, are of high diagnostic value (Erdtman, 1943). It has been reported that scanning electron microscopy has been widely used to observe pollen grains of plants to distinguish different species (Çeter et al., 2013). The present study represents the first attempt to systemically observe and compare the macroscopic and microscopic features of powdered materials and pollen grains of the three medicinal species of *Saussurea*. The method used is convenient and reliable, and can be used to authenticate the three representative snow lotus herbs. This method can be widely applied to other *Saussurea* herbs and even to other confused CMM.
MATERIALS AND METHODS

Materials

Fifteen batches of samples, namely 5 batches each of *S. involucrata*, *S. laniceps* and *S. medusa*, were collected from different main production areas in China (Supporting Information Table S1). The voucher specimens of standard samples were authenticated by Dr. Hubiao Chen, School of Chinese Medicine, Hong Kong Baptist University (HKBU). The above materials were deposited in the Bank of China (Hong Kong) Chinese Medicines Centre of HKBU.

Reagents

Chloral hydrate and glycerin (Uni-chem, England) were used for mounting microscope slides. Chloral hydrate solution and dilute glycerin were prepared according to procedures described in Appendix XV B of the Pharmacopoeia of the People’s Republic of China (Chinese Pharmacopoeia Commitee, 2010). Purified
water was provided by a Millipore water purification system (Millipore, Bedford, MA).

**Apparatus**

Axioplan 2 and Axiophot 2 universal microscopes with reflector Axiophot Photo Module (Zeiss Group) were used for observing powdered samples; images were recorded with a Leica direct current (DC) camera. LEO1530 Field Emission Scanning Electron Microscope (Oxford Instrument) was used for observing pollen grains.

**Software**

Matrox inspector (Matrox Electronic Systems), Leica IM50 and SmartSEM® were used.

**Methods**

*Macroscopic Identification of Crude Drugs.*

Each sample was measured and examined with regard to appearance, surface texture, odor and taste. The color photographs were taken with a digital camera (Panasonic DMC-FZ7, Panasonic, Japan).

*Microscopic Identification of Powdered Crude Drugs.*

All herbal samples were powdered, passed through a 250 μm sieve, and treated with chloral hydrate solution for observation. At least 10 different slides of each powdered sample were examined under ordinary and polarized light microscope. Distinctive microscopic features observed were digitally recorded.

*Microscopic Identification of Pollen Grains.*

Pollen grains were removed from anthers of the herbarium materials, suspended in distilled water on coverslips glued to 12 mm specimen holders, and air-dried
(Andersen and Bertelsen, 1972). The specimens were coated with gold and observed with a field emission scanning electron microscope (SEM) operated at 20 kV. Palynological characters examined included equatorial and polar diameters, shapes and ornamentation.

RESULTS

S. involucrata

Macroscopic Characteristics.

Leaves oblong or broad-lanceolate, yellowish-green, apexes blunt or slightly acuminate, bases decurrent, margins serrate and ciliate. Bracteal leaves ovate or oval, margins sharply toothed, yellowish-white, texture membranous, 55-70 mm long and 20-70 mm wide. Involucral bracts hemispherical, surrounding capitula, 10 mm in diameter, lanceolate, in three to four layers; bracts of the outer layer oblong, 11 mm long and 5 mm wide, brownish-yellow and pubescent throughout; bracts of the middle and inner layers lanceolate, 15-18 mm long and 2 mm wide, yellowish-white and pubescent only on top. Capitula crowded at top of stem. All flowers tubular, corolla purplish-red, limb 9 mm long, tube 7 mm long, anther purple, stigma 2-lobed. Pappi grayish-white, in two layers, outer layer coarse and 3 mm long, inner layer feathery and 15 mm long. Odor with special fragrant, taste slightly bitter (Table 1; Fig. 1).

Microscopic Characteristics.

Optical Microscopy (Table 2; Fig. 2, 3):

Powder yellowish-green, with light fragrance.

1. Pollen grains: Subrounded, yellow, 23-52 μm in diameter, 3 germinal pores, warts on the outer surface.

2. Non-glandular hairs: Of three kinds. Unicellular (SI-2-1); linear and bulgy; cell walls smooth. Unicellular (SI-2-2); twisted and flat; cell walls uneven.
Multicellular (SI-2-3), comprised of 5-6 cells as a single stalk; cell walls smooth.

3. Glandular hairs: Rare. Mostly broken. Head with 5-6 single- or double-celled flat layers, colorless or pale yellow; stalk colorless, with interlocked cells in each layer.


5. Cells of inner surface of microdiodange: Rectangular, arranged orderly; sometimes pale yellow; cell walls thickened in longitudinal stripes and appeared bright blue under polarized light; outer peripheral walls irregularly prominent in lateral view.


7. Epidermal cells of corolla: Tip of limb (SI-7-1) with papillary protrusions; yellow. Other cells of limb (SI-7-2) tightly arranged in lines; pale yellow; anticlinal walls undulate. Tube cells (SI-7-3a) rectangular; arranged tightly in lines; colorless. Cells below the tube epidermis (SI-7-3b) contained crystals of calcium oxalate appearing bright blue under polarized light.

8. Epidermal cells of bract: Elongate spindle-shaped; colorless; cell walls appearing bright blue under polarizing light; pit canals dense.

9. Epidermal cells of bracteal leaf: Upper epidermis (SI-9-1), irregular in shape; colorless; anticlinal walls sinuous; lower epidermis (SI-9-2) similar to upper epidermis, but with deeply sinuous anticlinal walls; non-glandular hairs present on both upper and lower epidermal cells; stomata anomocytic.

10. Epidermal cells of leaf: Polygonal; pale brown; anticlinal walls slightly curved.

*Scanning Electron Microscopy (Fig. 4):* Pollen grains subrounded, 23-52 μm in diameter, 3 germinal pores, warts coarse and widely spaced, with reticular sculpturing on the surface.
<table>
<thead>
<tr>
<th></th>
<th><em>S. involucrata</em></th>
<th><em>S. laniceps</em></th>
<th><em>S. medusa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole plant</td>
<td>Sparsely covered with hairs</td>
<td>Usually with hairs, only on top</td>
<td>Entirely covered with hairs</td>
</tr>
<tr>
<td><strong>Leaves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shape</td>
<td>Oblong or broad-lanceolate</td>
<td>Oblanceolate or spatulate</td>
<td>Ovate or linear spatulate</td>
</tr>
<tr>
<td>Apex</td>
<td>Blunt or slightly acuminate</td>
<td>Acute or acuminate</td>
<td>Blunt or rounded</td>
</tr>
<tr>
<td>Base</td>
<td>Decurrent</td>
<td>Cuneate</td>
<td>Cuneate</td>
</tr>
<tr>
<td>Margin</td>
<td>Serrate and ciliate</td>
<td>Undulate</td>
<td>Entire or serrate</td>
</tr>
<tr>
<td>Surface</td>
<td>Sparsely covered with hairs</td>
<td>Both surfaces with hairs</td>
<td>Both surfaces with hairs</td>
</tr>
<tr>
<td><strong>Bracteal leaves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shape</td>
<td>Ovate or oval</td>
<td>Linear lanceolate</td>
<td>Linear lanceolate</td>
</tr>
<tr>
<td>Margin</td>
<td>Sharply toothed</td>
<td>Not observable</td>
<td>Not observable</td>
</tr>
<tr>
<td>Texture</td>
<td>Membranous</td>
<td>Not observable</td>
<td>Not observable</td>
</tr>
<tr>
<td>Surface</td>
<td>Sparsely hair-covered</td>
<td>Both surfaces with hairs</td>
<td>Both surfaces with hairs</td>
</tr>
<tr>
<td><strong>Involucral bracts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer</td>
<td>3-4, surrounding capitula</td>
<td>3-4</td>
<td>3</td>
</tr>
<tr>
<td>Shape</td>
<td>Lanceolate</td>
<td>Broadly campanulate</td>
<td>Narrow cylindrical</td>
</tr>
<tr>
<td>Size</td>
<td>11-18 mm × 2-5 mm</td>
<td>6-9 mm × 1-4 mm</td>
<td>10-11 mm × 2-4 mm</td>
</tr>
<tr>
<td><strong>Capitula</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crowded at top of stem</td>
<td>Some crowded at top of stem; in spikes</td>
<td>Some crowded at top of stem; in racemes</td>
</tr>
<tr>
<td><strong>Flowe</strong>rs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Limb</td>
<td>9 mm long</td>
<td>6-9 mm long</td>
<td>5 mm long</td>
</tr>
<tr>
<td>Tube</td>
<td>7 mm long</td>
<td>2-3 mm long</td>
<td>5 mm long</td>
</tr>
<tr>
<td>Anther</td>
<td>Purple</td>
<td>Light purple</td>
<td>Purple</td>
</tr>
<tr>
<td>Stigma</td>
<td>2-lobed</td>
<td>2-lobed</td>
<td>2-lobed</td>
</tr>
<tr>
<td><strong>Papp</strong>i</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Layer</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Color</td>
<td>Grayish-white</td>
<td>Gray</td>
<td>Grayish-white</td>
</tr>
<tr>
<td><strong>Odor</strong></td>
<td>Fragrant</td>
<td>Light</td>
<td>Light</td>
</tr>
<tr>
<td><strong>Taste</strong></td>
<td>Slightly bitter</td>
<td>Slightly bitter</td>
<td>Slightly bitter</td>
</tr>
</tbody>
</table>
Fig. 2. Characteristics of the powder of the three snow lotus herbs under optical microscope (I). 1. Pollen grains; 2. Non-glandular hairs; 3. Glandular hairs; 4. Pappi; 5. Cells of inner surface of microdiodange. Red arrows indicate the glandular hairs on epidermal cells of corolla. (Scale: 100 μm)
**S. laniceps**

*Macroscopic Characteristics.*

Top of plant covered with white hairs. Leaves crowded, oblanceolate or spatulate, apexes acute or acuminate, bases cuneate, margin undulate, upper surface with arachnoid hairs, lower surface with dense brown tomentum. Bracteal leaves linear lanceolate, both surfaces covered with white tomentum. Involucral bracts broadly campanulate, 15 mm in diameter, in 3-4 layers, outer layer lanceolate or linear lanceolate, 6 mm long and 1 mm wide, covered with white or brown hairs, inner layer lanceolate, apexes acuminate, 9 mm long and 4 mm wide, covered densely with long, dark brown hairs. Capitula arranged in paniculate spikes along upper stem. All flowers tubular, white, 8-12 mm long, limbs 3 times longer than tubes, anthers light purple, stigma 2-lobed. Pappi gray, 2 layers, outer layer coarse and short, inner layer feathery and long. Odor light, taste slightly bitter (Table 1; Fig. 1).

*Microscopic Characteristics.*

*Optical Microscopy (Table 2; Fig. 2, 3):*

Powder brownish-yellow, with light fragrance.

1. Pollen grains: Subrounded, pale yellow, 24-49 µm in diameter, 3 germinal pores; densely covered with perforate and verrucate warts, surrounded by reticulate sculpturing on the surface.

2. Non-glandular hairs: Abundant. Multicellular. Of two kinds. Comprised of 4-7 short cells (SL-2-1), with a single slender cell on top; cell connections shrunk and yellow; cell walls smooth. Mostly broken, comprised of more than 3 long cells, either curly (SL-2-2a) or straight (SL-2-2b); cell connections smooth and colorless; cell walls smooth.

3. Glandular hairs: Rare. Of two kinds. One kind (SL-3-1) frequently found on corolla epidermal cells, with single-celled head and stalk, head usually colorless and stalk brown. Another kind (SL-3-2) comprised of a yellow head with 5-6 single-celled
flat layers, and a stalk usually unicellular and colorless.


5. Cells of inner surface of microdiodange: Rectangular, arranged orderly; colorless or pale yellow; cell walls smooth, with no specific view under polarized light.


7. Epidermal cells of corolla: Tip of limb (SL-7-1) with papillary protrusions; brownish-yellow; with glandular hairs frequently found (SL-7-1a, 1b, 1c). Other cells of limb (SL-7-2) thin rectangular; pale yellow; anticlinal walls slightly curved. Tube cells (SL-7-3a) rectangular; arranged tightly in lines; pale yellow or brownish-yellow. Cells below the tube epidermis (SL-7-3b) contained dense prisms of calcium oxalate appearing bright blue or yellow under polarized light.

8. Epidermal cells of bract: Elongate spindle-shaped; colorless or pale yellow; both cell lumina and wall appeared bright blue under polarized light.

9. Epidermal cells of bracteal leaf: Irregular in shape; colorless or pale brown; anticlinal walls thin and wavy; stomata anomocytic.

10. Epidermal cells of leaf: Rectangular, arranged tightly in lines; pale brown; anticlinal walls straight.

Scanning Electron Microscopy (Fig. 4): Pollen grains subrounded, 24-50 μm in diameter, 3 germination pores, warts more crowded than those of *S. involucrata*, with reticular sculpturing on the surface smoother than that of *S. involucrata*. 
Fig. 3. Characteristics of the powder of the three snow lotus herbs under optical microscope (II). 6. Cells of stigma; 7. Epidermal cells of corolla; 8. Epidermal cells of bract; 9. Epidermal cells of bracteal leaf; 10. Epidermal cells of leaf. Red arrows indicate the glandular hairs on epidermal cells of corolla. (Scale: 100 μm)
S. medusa

Macroscopic Characteristics.

Whole plant cylindrical, covered with thick white or grayish-white long tomentum. Leaves ovate or linear speculate, apexes blunt or rounded, bases cuneate, margins entire or serrate, both surfaces covered with long white tomentum. Bracteal leaves linear lanceolate, both surfaces with long white tomentum. Involucral bracts narrow cylindrical, 5-7 mm in diameter, in 3 layers; bracts of outer layer ovate, acuminate, purple, covered with white or brown tomentum, 11 mm long and 2 mm wide; bracts of middle layer oblong, 10 mm long and 4 mm wide; bracts of inner layer oblanceolate, 11 mm long and 2 mm wide. Capitula sometimes densely crowded at top of stem, arranged in racemes. All flowers tubular, 10 mm long, limb as long as tube, tube purple or purplish-blue, anther purple, stigma 2-lobed. Pappi white or grayish-white, in 2 layers, outer layer coarse, inner layer feathery. Odor light, taste slightly bitter (Table 1; Fig. 1).

Microscopic Characteristics.

Optical Microscopy (Table 2; Fig. 2, 3):

Powder brownish-yellow, with little fragrance.

1. Pollen grains: Subrounded, pale yellow or colorless, 30-57 μm in diameter, 3 germinal pores, outer surface sculptured with dense spinules.

2. Non-glandular hairs: Abundant. Of three kinds. Comprised of 4-8 or more short cells (SM-2-1), with a single slender cell on top; cell connections shrunk and yellow; cell walls smooth. Mostly broken, comprised of more than 3 long and curly cells (SM-2-2); cell connections smooth and colorless; cell walls smooth. Of 3-5 cells, with yellowish-brown (SM-2-3a) or pale red (SM-2-3b) cell contents; cell connections slightly shrunken; cell walls with longitudinal and corrugated striations.

3. Glandular hairs: Found on corolla epidermal cells. Head unicellular, with brown
content; stalk too short to observe.


5. Cells of inner surface of microdiodange: Rectangular, arranged orderly; colorless; cell walls thickened in longitudinal stripes and appearing bright blue under polarized light; outer peripheral walls irregularly prominent in lateral view.


7. Epidermal cells of corolla: Tip of limb (SM-7-1) with papillary protrusions; brown or pale purple. Other cells of limb (SM-7-2) thin rectangular; brownish-yellow; with smooth anticlinal walls. Tube cells (SM-7-3a) slender spindle-shaped; colorless. Cells below the tube epidermis (SM-7-3b) with a few prisms of calcium oxalate appearing bright blue under polarized light.

8. Epidermal cells of bract: Spindle-shaped or rectangular; colorless, yellowish-brown or pale purple; both cell lumina and wall appearing bright blue under polarized light.

9. Epidermal cells of bracteal leaf: Irregular-shaped to rectangular; pale brown to brown; anticlinal walls smooth; stomata anomocytic.

10. Epidermal cells of leaf: Polygonal to rectangular; cell walls and lumina brown.

Scanning Electron Microscopy (Fig. 4): Pollen grains subrounded, 30-57 μm in diameter, 3 germinating pores, spinules dense and with blunt tips, outer surface with granular sculptures.
<table>
<thead>
<tr>
<th></th>
<th><strong>S. involucrata</strong></th>
<th><strong>S. lanceps</strong></th>
<th><strong>S. medusa</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Powder</strong></td>
<td>Color</td>
<td>Brownish-yellow</td>
<td>Brownish-yellow</td>
</tr>
<tr>
<td></td>
<td>Fragrance</td>
<td>Light</td>
<td>Light</td>
</tr>
<tr>
<td><strong>Pollen grains</strong></td>
<td>Shape</td>
<td>Subrounded</td>
<td>Subrounded</td>
</tr>
<tr>
<td></td>
<td>Color</td>
<td>Yellow</td>
<td>Pale yellow</td>
</tr>
<tr>
<td></td>
<td>Diameter (µm)</td>
<td>23-41-52</td>
<td>24-36-50</td>
</tr>
<tr>
<td></td>
<td>Germinal pores</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Ornamentation</td>
<td>Warts coarse; sculpture reticular</td>
<td>Warts dense; sculpture reticular</td>
</tr>
<tr>
<td><strong>Non-glandular</strong></td>
<td>Amount</td>
<td>Relatively less</td>
<td>Abundant</td>
</tr>
<tr>
<td><strong>hairs</strong></td>
<td>Description</td>
<td>1) Unicellular; cell linear and bulgy; cell walls smooth</td>
<td>1) Multicellular; top cell slender; cell connections shrunk and yellow; cell walls smooth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) Unicellular; cell flat and twisted; cell walls uneven</td>
<td>2) Multicellular; linear; cell connections smooth and colorless; cell walls smooth</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) 5-6 cells; cell walls smooth</td>
<td>3) Multicellular; cell connections slightly shrunk; cell walls striated</td>
</tr>
<tr>
<td><strong>Glandular</strong></td>
<td>Amount</td>
<td>Relatively more</td>
<td>Rare</td>
</tr>
<tr>
<td><strong>hairs</strong></td>
<td>Head</td>
<td>5-7 flat layers; colorless or pale yellow</td>
<td>1) Unicellular; cellless</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) 5-6 single-celled flat layers; yellow</td>
<td>2) Unicellular, colorless</td>
</tr>
<tr>
<td></td>
<td>Stalk</td>
<td>Cells interlocked in each layer; colorless</td>
<td>Unicellular</td>
</tr>
<tr>
<td><strong>Pappi</strong></td>
<td>Fiber width (µm)</td>
<td>9-14-18</td>
<td>9-12-17</td>
</tr>
<tr>
<td><strong>Cells of inner</strong></td>
<td>Cell wall</td>
<td>Thickened in longitudinal stripes; bright blue under polarized light; outer walls unregularly prominent in lateral view</td>
<td>Smooth; no specific color under polarized microscope</td>
</tr>
<tr>
<td>surface of microdiodange</td>
<td></td>
<td></td>
<td>Thickened in longitudinal stripes; bright blue under polarized light; outer walls unregularly prominent in lateral view</td>
</tr>
<tr>
<td>Stigmas</td>
<td>Description</td>
<td>Protrusions scale-like; tips blunt; brown</td>
<td>Protrusions scale-like; tips blunt; dark</td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------------------------------------------</td>
<td>------------------------------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Epidermal cells of corolla</td>
<td>Limb: Tip</td>
<td>Protrusions papillary; yellow</td>
<td>Protrusions papillary; brownish-yellow</td>
</tr>
<tr>
<td></td>
<td>Other cells</td>
<td>Anticinal walls curved; pale yellow</td>
<td>Anticinal walls curved; brownish-yellow</td>
</tr>
<tr>
<td></td>
<td>Tube: Shape</td>
<td>Rectangular</td>
<td>Rectangular</td>
</tr>
<tr>
<td></td>
<td>Color</td>
<td>Colorless</td>
<td>Pale yellow or brownish-yellow</td>
</tr>
<tr>
<td></td>
<td>Cells below epidermis</td>
<td>Prisms of calcium oxalate appeared bright blue under polarized light</td>
<td>Dense prisms of calcium oxalate appeared bright blue or yellow under polarized light</td>
</tr>
<tr>
<td>Epidermal cells of bract</td>
<td>Shape</td>
<td>Elongated spindle-shaped</td>
<td>Elongated spindle-shaped</td>
</tr>
<tr>
<td></td>
<td>Color</td>
<td>Colorless</td>
<td>Colorless or pale yellow</td>
</tr>
<tr>
<td></td>
<td>Under polarized light</td>
<td>Cell walls bright blue</td>
<td>Cell lumina and walls bright blue</td>
</tr>
<tr>
<td>Epidermal cells of bracteal leaf</td>
<td>Shape</td>
<td>Irregular</td>
<td>Irregular</td>
</tr>
<tr>
<td></td>
<td>Color</td>
<td>Colorless</td>
<td>Colorless or pale brown</td>
</tr>
<tr>
<td></td>
<td>Anticinal wall</td>
<td>Upper epidermis sinuous; lower epidermis deeply curved</td>
<td>Undulate</td>
</tr>
<tr>
<td></td>
<td>Stoma</td>
<td>Anomocytic</td>
<td>Anomocytic</td>
</tr>
<tr>
<td>Epidermal cells of leaf</td>
<td>Shape</td>
<td>Polygonal</td>
<td>Rectangular</td>
</tr>
<tr>
<td></td>
<td>Color</td>
<td>Pale brown</td>
<td>Pale brown</td>
</tr>
<tr>
<td></td>
<td>Anticinal wall</td>
<td>Slightly curved</td>
<td>Straight</td>
</tr>
</tbody>
</table>
DISCUSSION

Innovation Highlights

This is the first research that combines macroscopic identification, ordinary light microscopy, polarized light microscopy and scanning electron microscopy to thoroughly distinguish the three snow lotus herbs, *S. involucrata*, *S. laniceps*, and *S. medusa*. The most reliable microscopic characters for identification are listed in Table 2 and Fig. 5.

Differentiation of the 3 Species by Macroscopic Features

For macroscopic features, the three herbs are very similar, as can be expected since they are all members of the genus *Saussurea* DC (Table 1). However, they belong to two subgenera: *S. involucrata* in Subgen. *Amphilaena* (Stschegl.) Lipsch. while both *S. laniceps* and *S. medusa* are in Subgen. *Eriocoryne* (DC.) Hook. f.. Species from the two subgenera can be distinguished according to their capitula arrangement; this also

Fig. 4. Pollen grain from the three snow lotus herbs under scanning electron microscope (A₁, A₂: *S. involucrata*; B₁, B₂: *S. laniceps*; C₁, C₂: *S. medusa*; Scale: 5 μm).
stands for *S. involucrata*, *S. laniceps* and *S. medusa* (Table 3).

**TABLE 3. Key based on macroscopic characteristics of the three snow lotus herbs**

1. Capitula crowded at top of stem, supported or surrounded by broad, membranous, and colored involucral bracts
   
   —Subgen. *Amphilaena* (Stschegl.) Lipsch.
   
   —*S. involucrata*

1. Capitula not supported or surrounded by broad, membranous and colored involucral bracts; some capitula crowded at top of stem, usually supported or half-surrounded by involucral bracts covered with a thick layer of soft, woolly hairs

   —Subgen.*Eriocoryne* (DC.) Hook. f.

2. Capitula abundant, arranged in paniculate spikes along upper stem

   —*S. laniceps*

2. Capitula range in hemispherical racemes

   —*S. medusa*

**Differentiation of the 3 Species by Microscopic Features**

For the three types of microscopy, they contribute in different ways in authenticating herbal powders. Ordinary light microscopy shows basic microscopic features, such as cell morphological characters, sizes, and colors (Table 2; Fig. 2, 3 and 5). Polarized light microscopy reveals more information when samples are nearly identical under ordinary light, including 1) the cuticle properties of plant tissue cells, and 2) the distribution of crystals (Table 2; Fig. 2, 3 and 5). SEM can give detailed information about pollen surfaces, since pollen grains are noteworthy for the specific ornamentation of their exine layers (Table 4; Fig. 4). Therefore, with the combined use of ordinary light microscopy, polarized light microscopy and scanning electron microscopy, plenty independent characteristics of the powders can be observed, making authentication efficient and accurate.

**By Optical Microscopy**

For optical microscopy, in choosing characters by which to authenticate species, we chose characters that are least affected by preparation processes and are most stable in different populations. For example, cell color can vary with the degree of permeabilization during mounting, and cell size can vary according to plant age,
sample origin, etc. Thus, for keys to differentiate these three snow lotus herbs, we chose characteristics of non-glandular hairs, glandular hairs, cells of inner surface of microdiodange and crystal distribution underneath epidermal cells of corolla.

Non-glandular hairs are abundant in *S. laniceps* and *S. medusa*, while *S. involucrata* have less non-glandular hairs. *S. involucrata* have its unique unicellular and multicellular non-glandular hairs, while *S. laniceps* and *S. medusa* share two multicellular types: one with top cell slender, cell connections shrunk and yellow; another with cell connections smooth and colorless.

Glandular hairs, on the other hand, are rare in *S. laniceps* and *S. medusa*, while relatively more in *S. involucrata*. *S. involucrata* and *S. laniceps* share a similar type with a flat and layered head; *S. laniceps* and *S. medusa* share another close type with one-celled head and stalk, usually found on epidermal cells of corolla.

Cell walls of the inner surface of the microdiodange can also be used to further differentiate *S. laniceps* and *S. medusa*, since glandular hairs are hardly seen in the two species. The specific cell walls of *S. medusa* appear bright blue under polarized light, while the ones of *S. laniceps* do not.

The three species can also be distinguished based on the amount of crystals underneath the epidermal cells of corolla, that is, in descending order, *S. laniceps*, *S. involucrata*, and *S. medusa*. 
1. Non-glandular hairs multicellular, with top cell slender and shrunk, and cell connections yellow (A); glandular hairs on corolla epidermal cells observed, usually with one-cell head and very short stalk (B)

(A)

(B)

2. Glandular hairs on corolla epidermal cells frequently found; another kind of glandular hair with head composed of single-celled flat layers (C); cells of inner surface of microdiodange with no specific view under polarized microscope (D); epidermal cells of corolla with dense prismatic crystals, appearing bright yellow and blue under polarized microscope (E)

(C)

(D)

(E) — *S. laniceps*

2. Glandular hairs on corolla epidermal cells occasionally found; other kinds of glandular hairs rare; cells of inner surface of microdiodange appeared bright blue under polarized microscope (F); epidermal cells of corolla with few prismatic crystals, appearing bright blue under polarized microscope (G)

(F)

(G) — *S. medusa*

1. Non-glandular hairs unicellular (H), or multicellular with cell connections shrunk (I); no glandular hairs on corolla epidermal cells

(H)

(I) — *S. involucrata*

Fig. 5. Key based on powders of the three snow lotus herbs under optical microscope (Scale: 100 μm).
By Scanning Electron Microscopy

For scanning electron microscopy, interspecific differences of pollen grains, including exine ornamentation, colpus width, spinulate size, distribution density and size, were studied. As shown in Fig. 4 and Table 4, the three kinds of pollen grains can be distinguished according to spinulate density, wart density and sculptures on the outer surface.

Pollen grains from *S. medusa* can be identified from the three species by their dense spinules, lack of evident warts, and granular sculpturing on the surface. Those from *S. involucrata* and *S. laniceps*, in contrast, are with little spinules, prominent warts and reticular sculpturing on the surface. To further differentiate, pollen grains from *S. involucrata* have sparser warts and rougher reticular sculpturing than those from *S. laniceps* have.

SEM observation of exine ornamentations, shapes, and sizes of pollen grains can reflect the evolution traits of the specific species (Wang et al., 2008). As for exine ornamentation, smoothness is considered most primitive, then negative ornamentation, protrusions (e.g. granules), and spinules developed, gradually evolving into reticular, undulate or striated sculptures (Walker, 1976). According to this hypothesis, *S. medusa* (with granular sculpturing) is more primitive than *S. involucrata* and *S. laniceps* (both with reticular sculpturing). As for shape, oval is considered to be more advanced than spherical (Wodehouse, 1935; Mullaer, 1979). The pollen grains of the three species are all subrounded, indicating that they are relatively primitive in *Saussurea* genus. As for size, pollen grains are believed to have evolved from larger to smaller (Covas et Schnack, 1944). In this regard, the evolutionary sequence, from most primitive to most developed, should be *S. involucrata* (average diameter 47 μm), *S. medusa* (43 μm), and *S. laniceps* (36 μm). Therefore, the evolutionary trait above do not support the one in Flora Reipublicae popularis sinicae (Jiang et Li, 1999), which is, from most primitive to most developed, *S. laniceps*, *S. medusa*, and *S. involucrata*. As for the relationship between pollen grain characters and evolutionary sequence, further studies should be conducted in the future.
TABLE 4. Key based on pollen grains of the three snow lotus herbs under scanning electron microscope

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Spinules dense and with blunt tips; no evident warts; outer surface with granular sculptures</td>
<td>S. medusa</td>
</tr>
<tr>
<td>1</td>
<td>Spinules not evident; warts coarse and prominent; outer surface with reticular sculptures</td>
<td>S. involucrata</td>
</tr>
<tr>
<td></td>
<td>Warts widely-spaced; reticular sculptures relatively rough</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Warts densely-arranged; reticular sculptures relatively smooth</td>
<td>S. laniceps</td>
</tr>
</tbody>
</table>

CONCLUSIONS

Our study has proved that the combining characters derived from observation through macroscopic identification, ordinary light microscopy, polarized light microscopy and scanning electron microscopy can be successfully applied in the authentication of the three Sausaurea species used as the medicinal herb “snow lotus”. The macroscopic and microscopic characteristics presented in this article are proposed as a reference to establish authenticity of the drugs and may be used to differentiate the drugs from adulterants. The method has proven to be simple, convenient, and reliable. We believe that this method can be widely applied to identify other CMM.

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