Morphological and microscopic characterization of five commonly-used testacean traditional Chinese medicines

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Abstract Testacean traditional Chinese medicine (TTCM), derived from the outer shell of sea or freshwater mollusks, is a special and important category of Chinese medicinal materials. To ensure the effective use of TTCM, a comparative identification study was performed on five commonly-used testacean drugs, including Haliotidis Concha, Arcae Concha, Meretricis Concha, Ostreae Concha and Margaritifera Concha (Shijueming, Walengzi, Geqiao, Muli and Zhenzhumu in Chinese, respectively). Typical morphological photographs of the crude drugs were acquired, and the key microscopic characteristics of the derived powders under normal light microscope and polarized light microscope were summarized. The major results can be concluded as follows: (1) the original species involved in the five TTCMs could be distinguished by their respective interspecies morphological characteristics; (2) the key identification characteristics of the five powdered crude drugs were mainly crystal fragments, with the fragment features under both normal light and polarized light microscope providing powerful points for differentiating the five commonly-used testacean drugs. This study demonstrated that it is feasible to provide authentication for these five kinds of TTCMs by the combination of morphology with microscopy.

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1. Introduction

Testacean traditional Chinese medicine (TTCM) is mainly from the outer shell of sea or freshwater mollusks, with properties of both mineral and animal drugs. In China, use of this kind of medicine can be traced back to 200 BC in Shen Nong Ben Cao Jing, the first traditional Chinese medicine book. It is estimated that about 100 kinds of testacean drugs are documented in Chinese Marine Materia Medica, a contemporary monograph descriptive of marine drugs1. Recently, abundant shell resources are attracting more and more scientists to investigate their medicinal value.

Haliotidis Concha, Arcae Concha, Meretricis Concha, Ostreæ Concha and Margaritifera Concha are recorded in the Chinese Pharmacopoeia, with Chinese names of Shijueming, Walengzi, Geqiao, Muli and Zhenzhumu, respectively. The five kinds of TTCMs are used extensively as effective medicines to induce sedation, calm the nerves, improve eyesight or to soften hard masses and eliminate nodulations2–6. However, commercial shell drugs are mostly applied in coarse powder, which is prone to give rise to species confusion due to their similarity in appearance and color. What is worth noting is that authentication work for the five kinds of TTCMs has not been established.

These testacean medicinal materials are composed mainly of calcium carbonate or calcium sulfate, in hard texture. For mollusk shells, previous research was mainly on their formation, structure and organic matrix7–9. Although some methods including X-ray diffraction, infrared and atomic absorption spectrometry have been applied to identification, expensive instruments are needed10–12. It is well known that microscopic techniques are fast, accurate, economical as well as simple, and have been used widely for the authentication of herbal, animal and even mineral medicines13–15. A few microscopic studies1,16 on TTCMs have been reported, however, only cursory hand-drawing pictures on cross section were provided and none of them showed detailed color illustrations of the characteristics of powder. Herein we perform the identification of Haliotidis Concha, Arcæe Concha, Meretricis Concha, Ostreæ Concha and Margaritifera Concha by morphological and microscopic study. Morphological characteristics are depicted with color photographs and powder microscopic characteristics using normal light microscopy combined with polarized light microscopy.

2. Materials and methods

2.1. Materials

Twenty-five samples were collected from different main production areas. Among these samples, there were 7 batches of Haliotidis Concha, 5 batches of Arcæe Concha, 4 batches of Meretricis Concha, 4 batches of Ostreæ Concha and 5 batches of Margaritifera Concha. All these shells were obtained from coastal areas and used as crude experimental materials without calcination after rinsing, cracking and powdering. The details of each sample are presented in Table 1. These samples were authenticated by Prof. Huijun Li (China Pharmaceutical University, Nanjing, China) and deposited at the State Key Laboratory of Natural Medicines of China Pharmaceutical University.

2.2. Apparatus and software

A digital camera (Cannon EOS 500D, Japan) was used for taking morphological photographs of the typical shell samples. A TCM grinder (TAISITE FW135, China) was used to prepare the powdered crude drugs. An optical microscope (Nikon E200, Japan) equipped with a digital camera (Nikon Smart V550D, Japan) was used to observe the powder features and Smart V550D Capture software to record the photographs.

2.3. Reagents

Dilute glycine was prepared according to procedures described in Appendix XV B, Pharmacopoeia of the People's Republic of China, 201017.

2.4. Methods

The exterior features of each authenticated sample were examined by observing, measuring and touching. The color digital photographs of typical samples were taken with a digital camera. Each sample of these crude drugs was powdered using a grinder and passed through a 300 μm sieve, sealed with dilute glycine when observed under the microscope. The powder of each sample was observed for at least 10 slides. The distinctive representative characteristics under normal light and polarized light microscope were chosen and imaged.

3. Results

3.1. External morphology

The typical color photos and external morphological characteristics of the 5 kinds of TTCMs (15 species in total) are given in Fig. 1 and Table 2. Haliotis diversicolor Reeve, Haliotis discus hannai Ino, Haliotis ruber (Leach), Haliotis asinina Linnaeus and Haliotis laevigata (Donovan), as the original species of Haliotidis Concha; Arca subcrenata Lischke, Arca inflata Reeve, Arca granosa Linnaeus as the species of Arca Concha; Meretrix meretrix Linnaeus, Cyclina sinensis Gnelin as the species of Meretricis Concha; Ostrea taliwhanensis Crosse, Ostrea rivularis Gould, as the species of Ostreæ Concha; Hyriopsis camingii (Lea), Cristaria plicata (Leach), Pteria martensii (Dunker), as the species of Margaritifera Concha, were studied in this paper.

3.2. Powder microscopy

The key microscopic characteristics of the five powdered crude drugs under normal and polarized light microscopy are given in Fig. 2 and Fig. 3. The detailed descriptions of each drug powder composed of their respective original species are described below. The transverse section view of prismatic layer fragments can be observed in the powder of Haliotidis Concha, Ostreæ Concha and Margaritifera Concha, of which the comparison data are displayed in Table 3.

3.2.1. Microscopic characteristics of Haliotidis Concha

Nacreous layer fragments are visible everywhere, differing in size, mostly in polygonal or irregular shapes; surfaces are with slightly granular nature. These fragments are usually imbricate or in dune shape, some with a distinct wavy margin; under the polarized microscope, they show dark blue centrally and yellow at the margin. Polygonal dark brown or tan fragments can be observed, opaque under the polarized microscope. Prismatic layer fragments...
### Table 1  Collection data of five kinds of testacean drugs.

| Testacean drug | Source       | Sample number | Species               | Collection area       |
|----------------|--------------|---------------|-----------------------|-----------------------|
| Haliotidis Concha | Coastal area | SJM-1         | *H. diversicolor*     | Yulin, Guangxi        |
|                 |              | SJM-2         | *H. discus*           | Yulin, Guangxi        |
|                 |              | SJM-3         | *H. discus*           | Anguo, Hebei          |
|                 |              | SJM-4         | *H. ruber*            | Yulin, Guangxi        |
|                 |              | SJM-5         | *H. ruber*            | Anguo, Hebei          |
|                 |              | SJM-6         | *H. asinina*          | Guangzhou, Guangdong  |
|                 |              | SJM-7         | *H. laevigata*        | Guangzhou, Guangdong  |
| Arcae Concha    | Coastal area | WLZ-1         | *A. subcrenata*       | Zhuhai, Guangdong     |
|                 |              | WLZ-2         | *A. subcrenata*       | Qingdao, Shandong     |
|                 |              | WLZ-3         | *A. subcrenata*       | Nanning, Guangxi      |
|                 |              | WLZ-4         | *A. inflata*          | Putian, Fujian        |
|                 |              | WLZ-5         | *A. granosa*          | Ningbo, Zhejiang      |
| Meretricis Concha | Coastal area | GQ-1          | *M. meretrix*         | Qingdao, Shandong     |
|                 |              | GQ-2          | *M. meretrix*         | Wenchang, Hainan      |
|                 |              | GQ-3          | *M. meretrix*         | Xiamen, Fujian        |
|                 |              | GQ-4          | *C. sinensis*         | Tianjin               |
| Ostreae Concha  | Coastal area | ML-1          | *O. talienwhanensis*  | Qingdao, Shandong     |
|                 |              | ML-2          | *O. talienwhanensis*  | Lianyungang, Jiangsu  |
|                 |              | ML-3          | *O. rivularis*        | Zhanjiang, Guangdong  |
|                 |              | ML-4          | *O. rivularis*        | Wenchang, Hainan      |
| Margaritifera Concha | Coastal area | ZZM-1       | *H. cumingii*         | Nanjing, Jiangsu      |
|                 |              | ZZM-2       | *H. cumingii*         | Anqing, Anhui         |
|                 |              | ZZM-3       | *C. plicata*          | Zhui, Zhejiang        |
|                 |              | ZZM-4       | *C. plicata*          | Nanjing, Jiangsu      |
|                 |              | ZZM-5       | *P. martensii*        | Beihai, Guangxi       |

**Figure 1**  Typical color photographs of intact shells. Shells of (A) *H. diversicolor*; (B) *H. discus*; (C) *H. ruber*; (D) *H. asinina*; (E) *H. laevigata*; (F) *A. subcrenata*; (G) *A. inflata*; (H) *A. granosa*; (I) *M. meretrix*; (J) *C. sinensis*; (K) *O. talienwhanensis*; (L) *O. rivularis*; (M) *H. cumingii*; (N) *C. plicata*; (O) *P. martensii*. 1. Body whorl part; 2. spiral part; 3. protuberance with an open; 4. umbo; 5. hinge and a row of denticles; 6. radiated rib; 7. protruding long tooth; 8. dorsal margin; 9. ventral margin; 10. triangular sail-like hind wing; 11. large crest.
| Testacean drug | Original species | Morphological characteristics | Shell surface |
|---------------|-----------------|------------------------------|---------------|
| Haliotidis    | *H. diversicolor* | Elongated ovoid, inner side ear-like | The outer dark red or brown, with yellowish-brown cloudiness, spiral ribs and fine growth lines. The spiral part small, the body whorl large; dozens of protuberances arranged towards right from the second spiral whorl to the edge of the body whorl part, usually the last 6–8 open. The inner smooth, with pearly luster. |
|               | *H. discus*     | Long oval                    | The outer brown or turquoise, with many coarse and irregular wrinkles, usually with attachments such as bryozoans; the last 3–5 protuberances open. The inner showing turquoise pearly luster. |
|               | *H. ruber*      | Flat ovoid, large            | The outer usually brick-red, with attachments similar to white lime; the spiral ribs and growth lines ridged in undulation. The last 7–9 protuberances open. The inner with nacreous luster. |
|               | *H. asinina*    | Long and narrow, ear-like, slightly small and curved | The outer smooth, greyish- or yellowish-green, with triangular or irregular jade green, brown or apricot speckles. The last 5–7 protuberances, mostly ellipsoidal, open on the same level with the shell surface. |
| Arcaea        | *H. laevigata*  | Oval, somewhat large and flat | The outer ecru, growth lines relatively conspicuous. |
|               | *A. subcrenata* | Long oval and oblique fan-shaped | The shell surface bulgy, the umbo curling inwards to the ligament. The outer white, with brownish coat and minute hairs; radiated ribs distinct. The inner white, with depressions at the margin corresponding to the ribs on the outer surface, forming protruding long teeth. The hinge straight, bearing a row of denticles. |
|               | *A. inflata*    | Large                        | Similar to the shell of *A. subcrenata*. |
|               | *A. granosa*    | Somewhat small, long oval    | The outer surface with brownish coat but no minute hairs; radiated ribs distinct, with extremely obvious granular protuberances. |
| Meretricis    | *M. meretrix*   | Somewhat triangular, the ventral side arch-like | The outer smooth, with a coat like oil paint, mostly in yellowish-brown to dark brown; serrated or wavy brown stripes forming circular figured-belt, varying with individual differences. The inner white. The hinge wide, the right shell with three cardinal teeth and two front lateral teeth; the left shell with three cardinal teeth and one front lateral teeth. |
|               | *C. sinensis*   | Subrounded                   | The outer usually in pale yellow; concentric growth ring pulses above the shell surface, forming ring fine ribs. The inner white, the edge occasionally in purple, with a row of denticles. Both the right and left shells with three cardinal teeth, without lateral teeth. |
| Ostreae       | *O. talienwhanensis* | Subtriangular, the right shells somewhat flat, while the left concaved in box-shape | The outer in pale yellow or purple-brown; concentric scales arranged loosely and undulated up and down; the left shell with thick radiated ribs. The inner white, trace of adductor unobvious. |
|               | *O. rivularis*  | Oval or subtriangular        | The outer pale yellow, brown or greyish-green; concentric growth scales overlapped one another. The inner white; trace of adductor mostly in half-moon-shape, in light yellow or purple. |
| Margaritifera | *H. cumingii*   | Large, somewhat flat, in scalene triangular. Dorsal posterior margin projected upward, forming a large triangular sail-like wing; ventral margin slightly arch-like | The outer with a layer of brownish-yellow to pitchy horn, concentric growth lines distinct. The inner opalescent, with pearly luster, trace of mantle visible; both the right and left shells with two pseudo-cardinal teeth, the left with two slat lateral teeth, the right with one slat lateral tooth. |
|               | *C. plicata*    | Large, somewhat in scalene triangular. Dorsal posterior margin expanded upward, forming a large crest; ventral margin straight | The outer in tan or pitchy, margin mostly in brownish-yellow; several brownish-yellow or greenish-yellow stripes radiated from the umbo to the ventral margin visible; growth lines distinct; longitudinal ribs gradually thick arranged from the |
are rare, and single ones are usually prismatic in longitudinal section view; sometimes several arranged regularly, polygonal in the transverse section view, polychromatic and dark yellow when observed under the polarized microscope.

3.2.2. Microscopic characteristics of Arcae Concha Fragments differ in size, in strips and irregular polygons; larger ones are mostly yellowish-brown, smaller ones pale yellow. Layering of crystal fragment margins is not obvious. Smaller fragments in light yellow usually show peacock-feather-like polychromaticity under the polarized microscope. Some brown fragments with bulgy radiated ribs can be seen. Fragments with minute hair fractures are often visible. Occasionally, stripes of some brown fragments can be observed and quadrangular yellowish-brown fragments of the shell coat can be viewed.

| Testacean drug | Original species | Morphological characteristics |
|---------------|-----------------|-----------------------------|
| | | Shape and size |
| | | Shell surface |
| | | |
| P. martensi | Small, somewhat flat, in oblique square. The posterior ear large, while the anterior ear small. Dorsal margin straight, ventral margin rounded |
| | The outer with fine concentric growth lines, forming slices. The inner with strong luster. |

**Table 2 (continued)**

| Nacreous layer fragments | Haliotidis Concha | Ostrea Concha | Margaritifera Concha |
|--------------------------|-------------------|---------------|----------------------|
| Longitudinal section view of prismatic layer fragments |
| Transverse section view of prismatic layer fragments |

**Figure 2** The key microscopic characteristics of Haliotidis Concha, Ostrea Concha and Margaritifera Concha.
Figure 3  The key microscopic characteristics of Arcae Concha and Meretricis Concha.

Table 3  Comparison data of transverse section view of prismatic layer fragments.

| Testacean drug     | Species         | Transverse section view of prismatic layer fragments |
|--------------------|-----------------|------------------------------------------------------|
|                    |                 | Length (μm)   | Width (μm)   |
| Haliotidis Concha  | *H. diversicolor* | 18–52        | 6–20         |
|                    | *H. discus*     | 15–50        | 4–12         |
|                    | *H. ruber*      | 17–40        | 7–20         |
|                    | *H. asinina*    | 25–85        | 5–17         |
|                    | *H. laevigata*  | 20–70        | 6–12         |
| Margaritifera Concha | *H. cumingii*   | 40–112       | 8–23         |
|                    | *C. plicata*    | 45–200       | 17–50        |
|                    | *P. martensii*  | 24–78        | 6–30         |
| Ostreae Concha    | *O. talienwhanensis* | 33–72       | 6–22         |
|                    | *O. rivularis*  | 15–100       | 7–20         |
3.2.3. Microscopic characteristics of Meretricis Concha
Fragments differ in size, mostly in sub-squares; surface color is dark, and usually brown, opaque, or dark yellow when observed under the polarized microscope. Crystal fragments are with unobvious layer structure, some fragments in the shape of a bamboo raft. Some fragments are brown with light and dark stripes occasionally seen.

3.2.4. Microscopic characteristics of Ostreae Concha
Nacreous layer fragments are mostly irregular, larger in long strip or plate-like structures; surface is uneven and fine striations are nearly visible, pale yellow or translucent, showing yellow and blue color when observed under the polarized microscope. Prismatic layer fragments are occasionally noticeable, some appearing in rows. Longitudinal section view of the prismatic layer fragments shows prisms, while the transverse section view is polygonal under the polarized microscope, showing polychromicity or dark yellow and yellow.

3.2.5. Microscopic characteristics of Margaritifera Concha
Nacreous layer fragments are visible everywhere, dissimilar in size, mostly in polygonal or irregular shapes, with obvious granularity on the surface. These fragments are usually imbricate or in dune shape, some with serrated margins; under the polarized microscope, they show dark blue centrally and yellow at the margin. Prismatic layer fragments are rare. Longitudinal section views show obvious transverse striations, with prismatic shapes, mostly truncated at the fracture-end, polygonal in transverse section view. These fragments show polychrome and dark yellow when observed under the polarized microscope.

4. Discussion and conclusions
In this paper 25 authenticated samples gathered from different areas were studied, including 7 species of Haliotidis Concha, 5 of Arcae Concha, 4 of Meretricis Concha, 4 of Ostreae Concha, and 5 of Margaritifera Concha (Table 1).

In the morphological studies, different species of the 5 TTCMs can be distinguished from each other by their respective interspecies morphological features (Fig. 1 and Table 2). Different species of Haliotidis Concha show different shell surface colors. The outer surface of the shell of H. diversicolor is dark red or brown, with yellowish-brown or green cloudiness; H. discus has many coarse and irregular wrinkles on the shell surface, in brown or turquoise; the outer shell surface of H. ruber is brick-red, bearing attachments similar to white lime. Scattered triangular or irregular apricot or dark green cloudiness can be found on outer surface of shell of H. asinina, which is greyish-green or yellowish-green; green stripes are usually visible on pale yellow and white shell surface of H. laevigata. Three original species of Arcae Concha share similar external features. Compared to the shells of A. subcrenata and A. inflata, A. granosa is small, without minute hairs, and radiated ribs have distinct granular protuberances; A. inflata is larger than A. subcrenata. The shells of M. meretrix and C. sinensis are easily distinguished by their external morphology, for example, the shell outer surface of M. meretrix is often yellowish-brown, with serrated stripes, while C. sinensis usually pale yellow, with fine ribs. When it comes to Ostreae Concha, several distinct ribs radiated from the umbo of the left shell of O. talienwhanensis, and can be used to differentiate from O. rivularis. The key macroscopic feature of Margaritifera Concha is a hinge tooth.

### Table 4 Morphological diagnostic keys to each species of the five TTCMs.

| 1. | The original species with one shell, the outer surface of shell with three spiral whorls. |
| 2. | The outer surface usually with attachments. |
| 3. | The outer brown or turquoise, usually with attachments such as bryozoans. |
| 4. | The outer usually brick-red, with attachments similar to white lime. |
| 5. | The outer dark red or brown, with yellowish-brown cloudiness. |
| 6. | The outer ecru. |
| 7. | The outer dark red or brown, with yellowish-brown cloudiness. |
| 8. | The outer without minute hairs. |
| 9. | The shell small. |
| 10. | The shell large. |
| 11. | The right shell with one or two lateral teeth. |
| 12. | The right shell with one lateral tooth. |
| 13. | The left shell with one lateral tooth. |
| 14. | The outer with fine concentric growth lines, forming slices. |

| H. discus | H. ruber | H. diversicolor | H. laevigata | H. asinina |
| A. subcrenata | A. inflata | A. granosa | O. talienwhanensis | O. rivularis | P. martensii |
The left shell of *H. cumingii* has two pseudo-cardinal teeth and one slat lateral tooth; the left shell of *C. plicata* is the same as right shell, has one anterior lateral tooth and one posterior lateral tooth, and no pseudo-cardinal teeth; *P. martensi* has no teeth.

The morphological diagnostic keys to each species of the five TTCMs are established in Table 4. As regarding powder microscopic characteristics of the five TTCMs, the key identification points are summarized in Table 5. Due to the powder of Arcae Concha and Meretricis Concha lacking obvious structure in the nacreous and prismatic layers, Haliotidis Concha, Ostreae Concha and Margaritifera Concha will be mainly discussed in powder microscopic identification. In our normal light microscopic observation, fragments of nacreous layer showed laminated structure, while the prismatic layer showed prismatic structure, and transverse section views revealed polygonal structures. Nacreous layer fragments of Haliotidis Concha had closely arranged lamellae, somewhat thin, with the fracture margin undulating up and down, while some of *H. discus, H. asinina* margins were slight serrated; Ostreae Concha fragments appeared in long strips or were plate-like, often broom-like at one end, with a straight fracture margin, showing fine stripes; Margaritifera Concha with fine little sawteeth and lamellae arrange closely. Prismatic layer fragments of Haliotidis Concha are rare and smaller, several often in rows, and transverse section views are difficult to find, 15–85 μm in length and 4–20 μm in width; prismatic fragments of Ostreae Concha often truncate in fracture-end, some taper at the other end, with transverse section views 15–100 μm in length and 6–22 μm in width. Obvious growth lines can be observed on prismatic fragments of Margaritifera Concha, with surface views generally 24–200 μm in length and 6–50 μm in width. Colors changes due to polarization state variation after polarized light passes through mineral crystals were studied in this paper. Under the polarized light microscope, nacreous layer fragments are usually in blue at the central part, while prismatic layer fragments are often polychromatic. Nacreous layer fragments of Haliotidis Concha and Margaritifera Concha show dark blue at the center and yellow at the margin, while Ostreae Concha shows yellow at the center and blue at the margin. Longitudinal section views of prismatic layer fragments of these crude drug powders usually show bright polychromicity, sometimes in dark yellow.

Sixteen commercial crude samples of the five TTCMs bought from drug stores were examined using powder microscopy. The results indicated different TTCMs could be easily differentiated from each other under the guidance of Table 5, although the original species of each TTCM was not accurately authenticated.

In conclusion, despite the fact that the 5 commonly-used and easily confused TTCMs have similar chemical constituents, differences in powder fragment features under normal and polarized light microscope still exist. Based on our experimental results, the combination of morphological and microscopic characteristics of these drugs can be used as a simple and especially economic method to distinguish one from another. Further research on other TTCMs is needed.

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