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Comparative Study on Mineral Medicine Os Draconis (Longgu) and Counterfeit Modern Animal Skeleton

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Abstract [**Objectives**] To identify the authenticity of Longgu from the microscopic, infrared spectrum and chemical composition, and provide references for the quality control and evaluation methods of Longgu. [**Methods**] According to the mineral characteristics of Longgu, the identification research was carried out by microscope observation, near-infrared spectroscopy and X-ray fluorescence spectrometer. By comparing the single polarizing and orthogonal polarizing characteristics of genuine and fake Longgu, a qualitative identification model of genuine Longgu was established based on the near-infrared spectrum of genuine Longgu, and the detection results of elements in Longgu were analyzed. [**Results**] The genuine Longgu had apatite optical properties, and was quite different from the fake Longgu of animal bones. Compared with modern animal bones, genuine Longgus had relatively less P and Ca, but they were enriched in elements Sr and F. The correlation coefficient model with good predictive ability can be established by using the near-infrared characteristic spectrum. [**Conclusions**] Polarizing microscope, near-infrared spectroscopy and X-ray fluorescence spectroscopy can improve the identification results of Longgu.

Key words Longgu, Polarizing microscope, Near-infrared spectroscopy, X-ray fluorescence spectroscopy, Identification

1 Introduction

Os Draconis (Longgu) is derived from the bones and incisor fossils of ancient mammals such as three-toed horses, rhinoceros, cattle, elephants, *etc.*, and has the effects of calming fright, collecting perspiration and reinforcing essence, stopping bleeding and astringent intestine, generating muscle and collecting sores[1-2]. Longgu was listed as a top-grade drug in the *Sheng Nong's Herbal Classic*[3], and was widely used to cure epilepsy, blood diarrhea, ejaculation, body heat like fire, mass congealment, intestinal abscess and umbilical sore since the Tang Dynasty[4-5]. Since the Song Dynasty, more attention has been paid to its strengths of calmness, convergence, and disentangling[6-7], so its use has a long history. At present, it is commonly used in clinical treatment of depression, insomnia, anxiety, *etc.*[8-10], and is one of the most commonly used mineral drugs.

In recent years, the demand for keel in the market has been on the rise, and it is nonrenewable. As a result, the amount of its resources is decreasing day by day; the proportion of fake and shoddy products in the market is increasing sharply, and the fake products from modern animal bones are the most prominent. Among the existing standards of medicinal materials, Longgu is the mineral medicine with the highest frequency of inclusion, and is included in 10 local standards of Chinese medicinal materials and 5 standards of national medicinal materials. The main components of Longgu are apatite and a small amount of calcite, but the requirements for identification indicators of Longgu are different in various standards. The *Standard of Chinese Medicinal Materials of Shandong Province* (2002 edition) requires that the acid insoluble ash content of keel shall not exceed 8%; in the *Standard of Chinese Medicinal Materials of Guangdong Province*, it is stipulated that the calcium content of Longgu ($CaCO_3$) is not less than 65%; the *Standard of Chinese Medicinal Materials of Gansu Province* (2009 edition) stipulates that the $CaCO_3$ content of Longgu shall not be less than 20%; the *Standard of Chinese Medicinal Materials of Shanxi Province* (2014 edition) stipulates that the calcium content of Longgu ($CaCO_3$) shall not be less than 90%[11-14]. In addition, the identification of its chemical composition is too rough, and the identification criterion of Longgu and other mineral drugs such as dens draconis, calcite, *etc.* is that bubbles are produced after thin hydrochloric acid is dropped into the mineral drugs. If ore contains calcium carbonate, this reaction will appear.

Longgu is the most commonly used mineral medicine, but it is difficult to guarantee its safety and effectiveness in the clinical use of traditional Chinese medicine because of the confusion of Longgu indicators and the lack of targeted ingredients in the existing drug standards. In this paper, according to the mineral characteristics of Longgu, it was systematically studied by microscope observation, near-infrared spectrometer and X-ray fluorescence spectrometer, and was identified from the microscopic level, spectrogram and chemical composition, so as to provide reference for the quality control and evaluation methods of Longgu.

2 Materials and methods

2.1 Instruments The main instruments included DM2700P polarizing microscope (Leica, Germany), PerkinElmer Frontier near-infrared spectrometer (Perkinelmer, USA), AXS S8 TIGER wavelength dispersive X-ray Fluorescence spectrometer (Bruker, Germany), A5-40T automatic powder tablet press (Shaoxing

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Dongjing Machinery Equipment Co., Ltd.), A5-04ZT vibration mill (Shaoxing Dongjing Machinery Equipment Co., Ltd.), JA5003 electronic balance (Shanghai Liangping Instrument Co., Ltd.), and PVD-E3000 intelligent electric thermostatic air drying box (Shanghai Langgan Experimental Equipment Co., Ltd.).

2.2 Reagents and samples The main reagents were anhydrous ethanol (AR, Chengdu Kelong Chemical Reagent Factory), argon-methane gas mixture (methane content was 10% ±0.5%, Taiyu Gas Co., Ltd.). The samples of Longgu were collected from different medicinal channels.

2.3 Preparation of samples Firstly, an appropriate amount of samples were washed under running water to remove surface impurities, and divided into two parts after being died. One part was sent to Chengdu Comprehensive Rock and Mineral Testing Center of Sichuan Bureau of Geology & Mineral Resources to grind a thin slice with a thickness of 0.03 mm for polarizing microscopy identification. The other part was fully ground with a vibration mill, wrapped in a cowhide envelope, and stored for later use. Part of the powder was taken from each sample, while the remaining dried powder was divided into 3 parts and placed in a sample test cup for detection by near-infrared spectroscopy.

2.4 Identification by a polarizing microscope After being cleaned, Longgu samples were ground into thin slices with a thickness of 0.03 mm, and placed under a polarizing microscope to observe the optical properties such as the shape, color and structure of the minerals in the thin slices under a single polarizing microscope and an orthogonal polarizing microscope, respectively.

2.5 Identification by a near-infrared spectrometer The dried Longgu samples were ground with an instrument until passing through a 65-mesh sieve, and each powder was divided into 3 parts. 1.5 g of each part was placed in a sample test cup. The nea-infrared spectrometer was set to scan at 10 000 – 4 000 cm^{-1}, and the scanning resolution was 64 cm^{-1}; the data interval was 2 cm^{-1}, and the scanning frequency was 64 times. The near-infrared spectrum was obtained by scanning each sample 3 times to calculate the average spectrum. The obtained data were shown in a chart with Origin software, and the 10 000 – 4 000 cm^{-1} spectrum was selected for qualitative analysis.

2.6 Identification by X-ray fluorescence spectrometer After being fully ground by a vibration mill, the samples were sieved with a 200-mesh sieve, and dried in an oven at 60 ℃ for 2 h. 4.5 g of each sample was placed in the die of a tablet press, and kept for 20 sec at 300 kPa to make them into samples with a diameter of 34 mm. They were stored under dry conditions. Each sample was made into 3 pieces for X-ray fluorescence spectrometer detection.

After the test, Spectra plus software provided by the system was used to evaluate the measurement results, and the average of the three pieces was taken as the measurement value of the sample.

3 Results and analysis

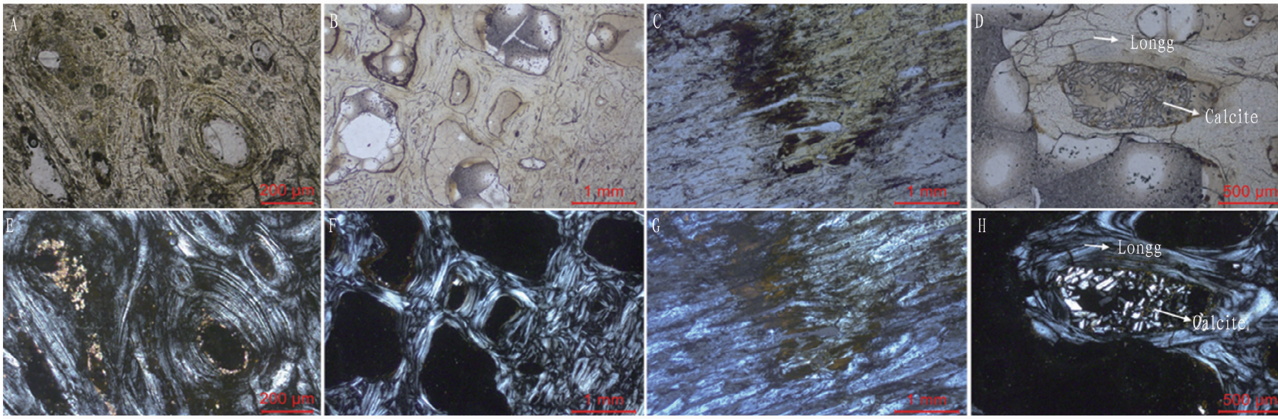
3.1 Results of identification by a polarizing microscope

Longgu slices were identified by a polarizing microscope. Under the microscope, the minerals in Longgu were produced according to the paleontological bone structure, and some of the bone spaces were filled with other materials. Under a single polarizing microscope, Longgu was brown, and had no cleavage; there was positive low-median protrusion, as well as dark dots, or annular distribution along bone pores. Under an orthogonal polarizing microscope, the highest interference color of Longgu was first-class gray, and there was parallel extinction and negative ductility. Some Longgu contained a small amount of calcite with an interference color of high-grade white and granular crystals, which filled bone spaces. Dilute hydrochloric acid was dropped into the thin flakes to produce bubbles, and alizarin red was dropped into the corresponding parts to turn red (Fig. 1).

Under a single polarizing microscope, the fake product was dark gray or gray-black, with no protrusions. Under an orthogonal polarizing microscope, there was no interference color, or it only existed in a local small area. It was non-mineralized on the whole (Fig. 2), that is, it was quite different from the genuine product.

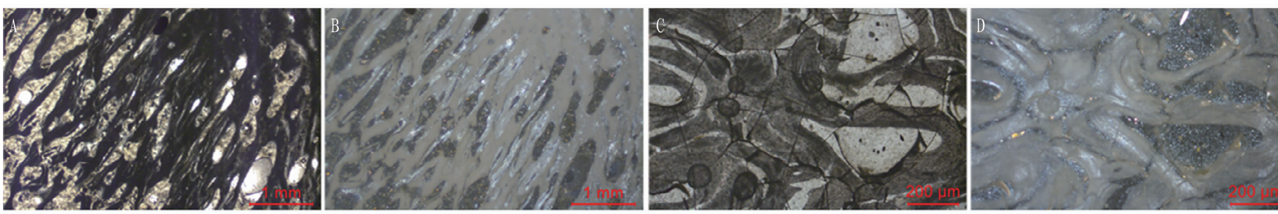
3.2 Identification by a near-infrared spectrometer There was a great difference between genuine and fake Longgu in the scanning spectrum, so the spectrum 10 000 – 4 000 cm^{-1} was used for qualitative analysis. The Longgu samples were randomly divided into a training set and a test set. The training set included 6 batches of genuine Longgu and 5 batches of fake Longgu, and the test set contained 5 batches of genuine Longgu and 5 batches of fake Longgu. The average of near-infrared spectrum of genuine Longgu samples in the training set was calculated and used as the reference spectrum. Origin software was used to preprocess the first derivative (9-point smoothing) and second derivative (9-point smoothing) of the spectral data of the samples and the average spectral data of the training set in the spectrum range of 10 000 – 4 000 cm^{-1}, so as to reduce the interference of irrelevant information or other information in the samples on the spectrum. Afterwards, Excel software was used to calculate the correlation coefficient (r) between the spectrum of each sample in the training set and the reference spectrum.

Under untreated conditions, the correlation coefficient of genuine samples in the training set ranged from 72.87% to 99.27%. However, the minimum correlation coefficient of genuine samples 72.87% was smaller than the maximum of fake samples 77.44%, which was highly likely to cause misjudgment, so this pretreatment was not applicable. Under the treatment of the second derivative (9-point smoothing), there was a big difference between the minimum correlation coefficient of genuine products (45.92%) and the maximum correlation coefficient of fake products (14.37%). However, there was also a big difference between the genuine samples in correlation coefficient, and the difference between the maximum and the minimum was up to 40.93%. Under the treatment of the first derivative (9-point smoothing), there was a big difference between the minimum correlation coefficient of genuine products (85.4%) and the maximum correlation coefficient of fake products (75.76%). The correlation coefficient of genuine



NOTE A – C. Longgu observed under a single polarizing microscope; D. Longgu and calcite observed under a single polarizing microscope; E – G. Longgu observed under an orthogonal polarizing microscope; H. Longgu and calcite observed under an orthogonal polarizing microscope.

Fig. 1 Pictures of genuine Longgu observed under a polarizing microscope



NOTE A – C. Fake product observed under a single polarizing microscope; D. Fake product observed under an orthogonal polarizing microscope.

Fig. 2 Pictures of fake Longgu observed under a polarizing microscope

samples was also relatively close, ranging from 85.40% to 98.94%, with the difference of less than 14.54%. Compared with the unprocessed data and the data treated with the second derivative, the effect was significantly better. Based on the above analysis, it is concluded that among the three pretreatment methods, the first derivative (9-point smoothing) was the best method for spectrum pretreatment.

The spectral data of samples was preprocessed by the first derivative (9-point smoothing), and then the correlation coefficient with the reference spectrum in the range of the characteristic spectrum (10 000 – 4 000 cm^{-1}) can be calculated to distinguish genuine Longgu from fake Longgu. According to the experimental results, the correlation coefficient r of genuine samples was $\geq$ 85.40%, and that of fake samples was $\leq$75.76%. The average of the two 80.58% was as the threshold for distinguishing genuine and fake products. That is, $r \geq 80.58\%$ means genuine Longgu, and $r < 80.58\%$ means fake Longgu. So far, the pre-treated spectra, characteristic spectrum segments, reference spectral thresholds and discriminant conditions of the samples in the training set constituted the qualitative identification model of genuine and fake Longgu.

3.3 Identification by X-ray fluorescence spectrometer X-ray fluorescence spectroscopy has the advantages of simple sample preparation, fast analysis, wide analysis range of elements, and can realize multi-element analysis from F to U elements in the periodic table. The main components of Longgu were detected by XRF method, and the characteristics of the elements were analyzed. The experimental results show that in 11 batches of genuine Longgu and 4 batches of fake Longgu (modern animal bones), Ca and P content were the highest, followed by Si, Fe and Al. The total content of Ca and P in modern animal bones was greater than that of genuine Longgu (Fig. 3).

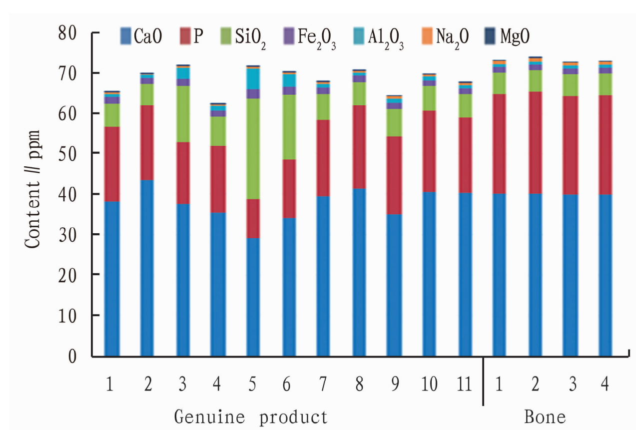


Fig. 3 Distribution of principal elements in Longgu

Longgu mainly contains inorganic components, and the original minerals include hydroxyapatite and a small amount of calcite. The molecular formula of hydroxyapatite in Longgu can be expressed as $Ca_{10}(PO_4)_6(OH)_2$, and its crystal is special, showing generally hexagonal columns with cones, in which Ca occupies two positions in the lattice. One is surrounded by nine O atoms, while the other is surrounded by six O atoms and one OH^-. Among them, Ca is easily replaced by REE, Sr, Na, K and other elements, while P is easily replaced by V, Si, As, S, and C, and OH^- is easily replaced by F^-. Longgu is formed by the fossilization of animal bones buried in the earth's crust for a long time, resulting in the enrichment of homogeneous elements. The experimental results reveal that there was no difference between genuine Longgu and modern animal bones in S, Ba, Cl, Zn, Cu, and Co, but there was a great difference in Sr and F content. In genuine Longgu, Sr content was more than 1 200 ppm, and F content was above 1 700 ppm, so the cumulative total content was over 4 000 ppm. Sr and F content in modern animal bones was between 300 and 800 ppm (Fig. 4).

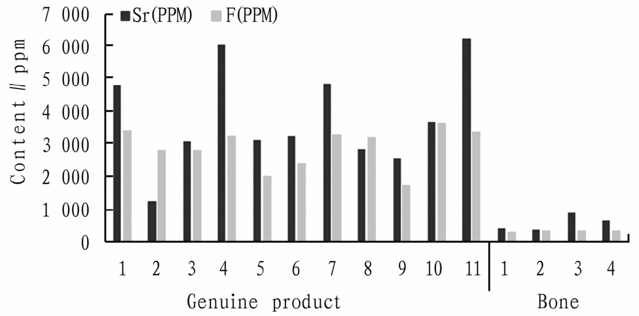


Fig. 4 Sr and F content in Longgu

4 Summary and prospects

Longgu is a precious non-renewable resource, and the state implements the principles of classified management, key protection, scientific research priority and rational utilization[15]. It has a wide range of clinical applications, and Longgu can be applied in various departments of internal medicine, external medicine, gynecology and pediatrics. Longgu is mainly used in some common chronic diseases and difficult diseases such as insomnia, depression, hidrosis and spermatospermia, and has unique medicinal value[16]. The existing stock of Longgu is decreasing year by year, and the market demand is on the rise in recent years. The demand exceeds the supply, and fake and shoddy products are on the rise. Longgu can not be accurately identified according to the existing standards.

In order to ensure the clinical safety and effectiveness of Chinese medicinal materials, it is necessary to monitor the quality of Longgu medicinal materials. In this paper, according to the mineral characteristics of Longgu, the difference between genuine Longgu and fake Longgu (modern animal bones) was observed by a polarizing microscope. It is found that genuine Longgu showed apatite characteristics, and the main component of Longgu was apatite, with only a small part of calcite. Based on the analysis of near-infrared spectroscopy, the qualitative identification model of genuine and fake Longgu was established by the pre-treated spectra, characteristic spectrum segments, reference spectral thresholds and discriminant conditions of Longgu samples. The characteristics of P, Ca, Sr and F elements in Longgu and modern animal bones were analyzed by X-ray fluorescence spectroscopy. The characteristics of Longgu identification were established through microscopic analysis, atlas and composition.

Seen from the long-term accumulation of clinical practice since the Han Dynasty, the therapeutic value of Longgu is unique, and has been recognized. As a fossil mineral medicine, its resources are endangered. So far, the alternative medicine of Longgu is not clear. In addition to the imperfect identification criteria, the material basis and activity of Longgu are still unclear. If the material basis of the biological effects of Longgu is not clarified before it disappears from the clinical practice of traditional Chinese medicine, traditional Chinese medicine will lose the opportunity to find alternative resources of Longgu to achieve sustainable utilization. It is still necessary to further study and explore the problem of Longgu resources and their substitutes.

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