



Pharmacognostical and phytochemical studies on *Ziziphora clinopodioides* Lam. – A Kazakh and Uygur ethnomedicinal plant

[Estudios farmacognósticos y fitoquímicos sobre *Ziziphora clinopodioides* Lam. - Una planta etnomedicinal kazaja y uygur]

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Abstract

Context: *Ziziphora clinopodioides* Lam. (Lamiaceae) is an annual or perennial herb or subshrub widely distributed from the Mediterranean to central Asia and Afghanistan. In Xinjiang, China, the whole herb has been used in traditional Kazakh and Uygur medicines as anti-febrile and detoxicating drug.

Aims: To characterize macroscopical, microscopically of the overground part of the *Z. clinopodioides*, explore and establish the macro-morphology, micro-morphology, quality and physicochemical parameters standards for this plant.

Methods: Pharmacognostical and phytochemical investigations were conducted in terms of macroscopic, microscopic and preliminary phytochemical parameters.

Results: The vegetable material can be identified by structural features of the flowers, structural characteristics of the stem, specific pale brown hesperidin can be regarded as identification character. This crude drug showed the characteristic physicochemical values like total ash (7%), insoluble ash (1.3%), water soluble ash (3.7%), moisture (7.5%). The ethanolic extract contained flavonoids, organic acids, alkaloids, and glycosides.

Conclusions: Various pharmacognostical characters that observed in this study can be an effective supplement to further research of this ethnomedicinal plant. Meanwhile, the results of this paper deal with pharmacognostical studies on the *Ziziphora clinopodioides* in an attempt to mitigate the adulteration to the crude drug.

Keywords: micromorphology; morphology; pharmacognostical identification; *Ziziphora clinopodioides*.

Resumen

Contexto: *Ziziphora clinopodioides* Lam. (Lamiaceae) es una hierba anual o perenne o arbusto ampliamente distribuida desde el Mediterráneo a Asia central y Afganistán. En Xinjiang, China, la hierba entera se ha utilizado en las medicinas tradicionales de Kazajistán y Uygur como droga anti-febril y desintoxicante.

Objetivos: Caracterizar macroscópicamente y microscópicamente la parte aérea de *Z. clinopodioides*, explorar y establecer la macro-morfología, la micro-morfología, la calidad y los parámetros físico-químicos estándares para esta planta.

Métodos: Las investigaciones farmacognósticas y fitoquímicas se realizaron en términos de parámetros macroscópicos, microscópicos y fitoquímicos preliminares.

Resultados: El material vegetal pudo ser identificado por las características estructurales de las flores, las características estructurales del tallo, específico color marrón pálido hesperidina puede ser considerado como carácter de identificación. Esta droga cruda bruto mostró los valores físicoquímicos característicos como cenizas totales (7%), cenizas insolubles (1,3%), cenizas solubles en agua (3,7%) y humedad (7,5%). El extracto etanólico contenía flavonoides, ácidos orgánicos, alcaloides y glucósidos.

Conclusiones: Varios caracteres farmacognósticos que se observaron en este estudio pueden ser un suplemento efectivo para investigaciones posteriores de esta planta etnomedicinal. Mientras tanto, los resultados de este trabajo se refieren a estudios farmacognósticos sobre la *Ziziphora clinopodioides* en un intento por mitigar la adulteración de la droga cruda.

Palabras Clave: identificación farmacognóstica; micromorfología; morfología; *Ziziphora clinopodioides*.

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INTRODUCTION

Ziziphora, which is a genus of annual or perennial herb, less sub-shrub of family Lamiaceae, and including 25-30 species in the world, mainly distributed in the Mediterranean to central Asia and Afghanistan. *Ziziphora* are widely used as carminative, stomach tonic, expectorant and antiseptic in different areas in Iran (Shahla, 2012). In China, there are three species of *Ziziphora* distributed in gravel slope and semi-desert grass lands, such as Tianshan and Altai mountains, mountains of West Junggar, Pamir and Kunlun mountains in Xinjiang (Haiyan, 2016). The plant has been used traditionally as a natural drug for the treatment of colds and cough (Sharopov, 2011). The whole herb can relieve fever and inflammation, keep the heart pumping and eliminate dampness, so widely used in cold fever, palpitations insomnia, hypertension, acute conjunctivitis, and hemorrhoids, which recorded in Hasake Yao Zhi of Xinjiang. (Wilhan and Xin, 2009). It was also documented in Chinese Pharmacopoeia Uygur Pharmacopoeia Fascicule, and the Chinese Materia Medica also can cure swell and pain of eyes, swollen ulcers (SATCM, 1999).

Chemical research showed that the main chemical components of *Ziziphora clinopodioides* Lam are volatile oil (pulegone, limonene, menthol, pinene, menthone, and menthenone) (SATCM, 1999), vitamin, organic acids (caffeic acid, rosmarinic acid) (Zhou et al., 2011), flavonoids (acacetin, apigenin, chrysoeriol, kaempferol, hyperoside, quercetin) (Yang and Gu, 2011), alkaloids, glycosides (Shahla, 2012; Anzabi, 2016) and so on. Many documents have been conducted on the antibacterial and antifungal activities of the plants. The volatile oil has broad antibacterial activity and antioxidant effect (SATCM, 1999). For example, the research in Iran showed *in vitro* antibacterial activity of essential oil of *Z. clinopodioides* against some pathogenic bacteria. This activity could be associated to the pulegone (Shahla, 2012). In Turkey, the infusions of *Ziziphora* species, *Z. clinopodioides*, have been used as an antiseptic, carminative and sedative agent to treat cold, flu, cough, stomach ache, and diarrhea (Anzabi, 2016). Flavonoids ingredients such as acacetin have antioxidant effects. It was reported that acacetin

can prevent atrial fibrillation, and wouldn't trigger a fatal ventricular fibrillation. Thus, it is the most development potential of cardiovascular drugs. The result showed that medicinal effect of anti-myocardial ischemia might be associated with flavonoids (Yang and Gu, 2011).

There is a rich supply of data of the phytochemical components and pharmacological actions, like volatile oil and organic acids (Zhou et al., 2011), while a small number of data of standards for identification and authentication about *Z. clinopodioides*. Hence, this research focused on properties, microscopic characteristics, physical and chemical identification of *Z. clinopodioides* (above ground), aimed to set its pharmacognosy quality standards and establish the foundation of the new further application of *Z. clinopodioides* to provide a theoretical basis.

MATERIAL AND METHODS

The methodology was carried out according to (Zhu et al., 2015; Marandi, 2016; Pramanick, 2016).

Plant materials and reagents

Ziziphora clinopodioides Lam. medicinal materials collected from Xinjiang Manas forest farm. The coordinates of gathered ten batches of the sample from different elevations and harvested at different time were located at North 43°50'550" - 43°54'474" and East 86°03'279" - 86°11'118". The plant material was authenticated by Professor Pin Yan (College of Life Science, Shihezi University). Voucher specimens (No. 2016062602-03) were preserved in School of Pharmacy of Shihezi University. The harvested plants were dried in shadow at room temperature, grounded into powder and stored in airtight containers. All reagents used were an analytical grade, such as petroleum ether (60-90°C), ethyl acetate, chloroform, ferric chloride, methanol, and ethanol.

Macroscopic and organoleptic studies

The macroscopic study of a medicinal plant is helpful in rapid identification of plant material and also plays an important role in the standardization of drug. The plant was studied for morphological characters including size, shape, color, odor, taste,

and extra features. The macro-morphological feature of the root, leaf and rhizome was observed under magnifying lens (10x).

Microscopic studies

Dried herbs were grinded to coarse powder and packed in a suitable container for microscopic identification. Using chloral hydrate and diluted glycerine as clearing agents make powder section. Chloral hydrate and diluted glycerine were prepared according to procedures described in general rule of Pharmacopoeia of the People's Republic of China (Commission, 2015).

The samples were cut and immediately fixed in formalin 5 mL + acetic acid 5 mL + 70% ethyl alcohol (90 mL). After fixing the samples for 24 hours, they were dehydrated and clarified successively in graded series of ethanol and dimethylbenzene. Posteriorly, the specimens were infiltrated with paraffin wax (melting point 58-60°C) and casted into paraffin blocks. Rotary Microtome (YD-1508B, Jinhua YIDI Medical Appliance Co., Ltd., Zhejiang, China) was used to section the paraffin-embedded specimens. Each section thickness was 10-12 µm.

Photomicrographs of the transverse section (stem, root and leaf) and powder section were taken with the help of powder section and Biomicroscopy Primo Star (Zeiss Group, Germany) with 10x and 40x micro-scope objective lenses, and CX21 bio-microscopy unit (Olympus, Nikon D750 digital camera, Matrox Inspector, Matrox Electronic Systems Ltd., Japan), and BA410E biological microscope (MOTIC Electric Group Co. Ltd., P.R. China).

Phytochemical studies

The UV spectrum 70% ethanol extracts of the herb gained with the help of ultraviolet spectrophotometer (UV-2600 spectrophotometer, Shimadzu Corporation, Japan, range from 200 to 400 nm wavelength).

Dried herbs were grinded to a coarse powder (grain size: $850 \pm 29 \mu\text{m}$) and packed in a suitable container for phytochemical identification. The powder was extracted with 70% ethanol.

The bioactive compounds like alkaloids, flavonoids, and coumarin were screened ascertain

by analyzing for its coloration reactions which usually be added chemical reagents such as alkaloid precipitation agent (silicotungstic acid, iodine-potassium iodide), HCl-Mg reaction, ferric chloride test (Lin, 1977; Wu, 2002).

Physical studies

Moisture determination

The determination of moisture adopted the toluene method (Fig. 1). Three powdered sample (about 3 grams each) were prepared simply. Then, a sample was placed in the bottle A, which were added about 200 mL toluene and some dry clean zeolite. The instrument was connected and added toluene from the top of condenser pipe C until it was filled the narrow part of bottle B. Bottle A was heated slowly by the heating mantle. The temperature was adjusted when toluene began to boil, which should keep the speed of distillation of two drops per second. When the moisture was completely evaporated the inner part of the condenser tube was rinsed with new toluene. A brush imbibed of toluene or other means to brush the toluene was used which attached to the inside of condenser pipe. The distillation continued five minutes. Lastly, the samples were allowed to cool to room temperature, and the equipment was disassembled. When moisture and toluene were separated the scale (graduated tube) was read. Three sample powders were measured in parallel and was calculated the average of the results.

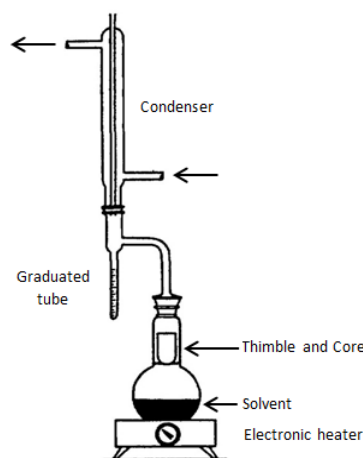


Figure 1. Instrument of toluene method.

A: Round-bottomed flask (500 mL); B: Determination tube for moisture; C: Straight condenser pipe.

Total ash measurement

The determinations of total ash and insoluble acid ash used the method of burning residue in accordance with standard procedures mentioned in general rule of Pharmacopoeia of the People's Republic of China (Commission, 2015). Dried herbs were grinded to coarse powder, which could through the No. 2 sieve (24 mesh number), then mixed evenly. Three samples, about 3 g each, were weighed and then put in the crucible, which achieved constant weight, slowly heated but not to burn, when samples were completely carbonized, gradually raise the temperature to 500-600°C. When samples were completely incinerated, then was achieved the constant weight. The total ash content of the test sample was calculated based on the weight of the residue.

Acid insoluble ash measurement

The ash (total ash measurement) was put into the crucible, in which the ash was added about 10 mL of dilute hydrochloric acid, covered with the watch-glass, and heated in a water bath for 10 minutes. Watch-glass was rinsed with hot water 5 mL, and the liquid was incorporated into the crucible and filtered with quantitative analysis filter paper. The residue in the crucible was washed with water on the filter paper and washed until the lotion did not show chloride reaction. The filtered residue was placed in the same crucible together with quantitative analysis filter paper, dried or ignited to constant weight. According to the weight of the residue, the content of acid-insoluble ash of sample was calculated.

RESULTS AND DISCUSSION

Original plant identification

Z. clinopodioides was an aromatic sub-shrub, whose root was strong and lignified, twists and turns; its stem base was woody and branched. Stems mostly from stem base, inclined to rise or nearly upright, four edges, amaranth, 12-30 cm high, not branched, erect or bow bend, usually covered

with very short, dense and downward hair, especially at the bottom of the plant. Leaves were opposite, and the axillary had any number of lobules. Leaves were wide oval, ovoid, oblong, lanceolate, or ovoid lanceolate, 0.62 cm long, 3-10 mm wide; the base was cuneate and extended into the handle, the apex was acuminate, margin entire, two sides had thin pubescence, veins on the back were clear, with yellow glandular dots. Verticillaster, which was inserted at the top of the stems and branches, was integrated as a ball. The peduncle was purple or green, 2-3 mm long, covered with dense pubescence. Bracts were small, leafy, whose margin had sparse eyelashes, calyx was tubular, 5-7 mm long, covered white hairy, inside of the throat also with white hairs, 5 calyx teeth, sub-equal, without close or open slightly in fruit. Corolla was labiates, and purple, about 10 mm long, corolla tube extended outside of the calyx, all covered with short pubescence, upper lip had two lobes, upright, apex was slightly concave, lower lip had 3 lobes, the middle lobe was long and narrow, apex emarginate, lateral lobe was circular; 4 stamens, just the prior pair developed, the back pair degenerated, extending outside of the crown, style apex with 2 shallow lobes, but they were unequal. The small nut was ovoid (Fig. 1).

Macroscopical identification

The stem was fascicular from stem base, upright, quadrangular, covered with dense pubescence, 15-25 cm long, 0.5-1.6 mm in diameter, the bottom was purple, the upper was yellow-green, the quality was hard, the transverse section was yellow-white, the pith was hollow. Leaves were opposite, ovoid, lanceolate, the apex was caudate or obtuse, the upper surface was green, the lower surface was yellow-green, but both had secretions. Verticillaster grew at the top of the stem and branches; calyx was tubular, green, peduncle was purple or green, covered with dense pubescence, corolla was purple, limb was labiate, lower lip had three lobes, covered pubescence on the outside, the handle of the bracts was long. It smelt lightly aromatic and tastes bitter with slightly sweet (Fig. 2).



Figure 1. Plant of *Z. clinopodioides*.



Figure 2. The original herb of *Z. clinopodioides*.

Microscopic identification

Surface of Z. clinopodioides leaves

Most of the epidermal cell walls were wave-shaped bending, beaded thickened, contained pale brown raphides and clustered crystal of hesperidin. Porosity was diacytic type, the surface of leaves were unicellular or multi-cellular non-glandular hairs, glandular scales had uniform distribution (Fig. 3).

Transverse section of Z. clinopodioides leaves

The upper epidermis consisted of a layer of closely arranged cells, which were elliptical or similar to the round and had non-glandular hairs and small glandular hairs, glandular scales. The outer layer of the cells had a thick horny membrane. Cells often had a pale brown crystal of hesperidin, mostly

round or fan-shaped, with radial textures. Palisade tissue were slender cylindrical, 2-3 layers, irregularly shaped spongy tissue cells, with sparse. The lower epidermis consisted of smaller round cells, with 1-2

thick layers of tissue on the inside of the epidermis near the main vein, which were arranged orderly, with non-glandular hairs outside and raphides and clustered crystal of hesperidin inside (Fig. 4).

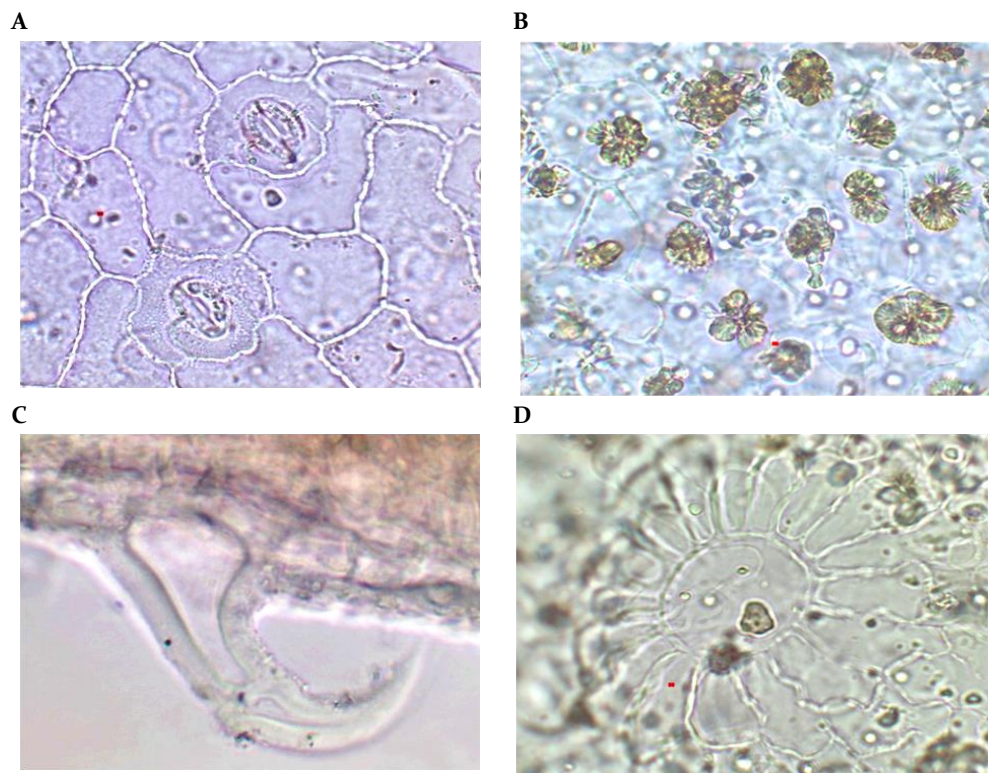


Figure 3. Surface of the leaf of *Z. clinopodioides*.

A: Epidermal cells and stomata; B: Hesperidin crystal in epidermal cell; C: Non-glandular hair; D: Glandular scale.

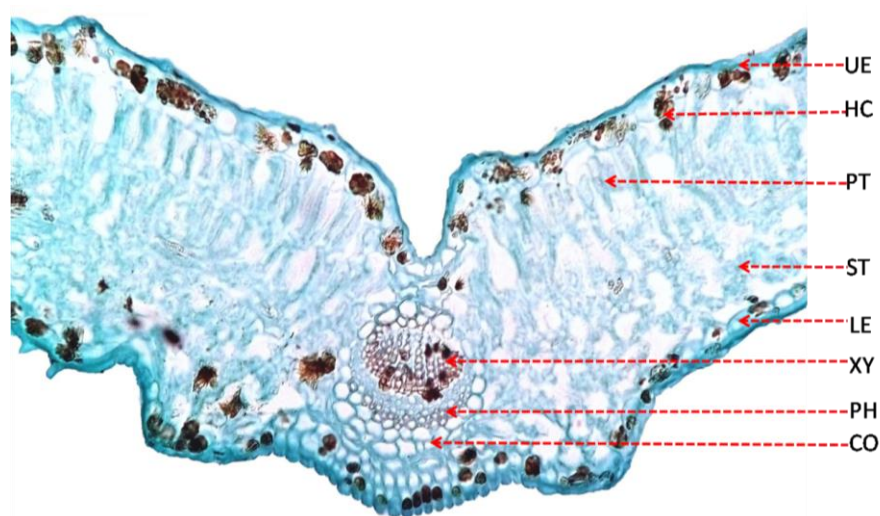


Figure 4. Transverse section of leaf of *Z. clinopodioides*.

UE: Upper epidermis; HC: Hesperidin crystal; PT: Palisade tissue; ST: Spongy tissue; LE: Lower epidermis; XY: Xylem; PH: Phloem; CO: Collenchyma.

Transverse section of stem

The epidermal cell was composed of 1-2 columns, flattened or oval, with thick horny layers outside, with non-glandular hairs, which of the base was ridge and glandular scales. Annular cortex was composed of two or one layers of parenchyma cells, and collenchyma was located at the four edges of the stem.

The medial cells of cortex were mostly flat, oblong and closely arranged. Cambium was not obvious, and phloem cells were mostly small, polygon and circular with a large number of crystals, which were closely arranged. Xylem was relatively developed whose vascular was arranged in a hash, wood rays appeared in a single line, and the cell walls of lignified wood fiber and wood parenchyma cells were thick. The pith was developed, whose big parenchyma cells were round or nearly round mostly, and the pith of old stem was hollow mostly (Figs. 5-6).

Powder microscopy

Green powder: Multi-cells non-glandular hairs were divided into three kinds. Some non-glandular hairs consisted of 3-6 cells, which distributed in calyx throat with a smooth surface, slightly bent flat, 800-1200 μm in length. Others non-glandular hairs were distributed in corolla mainly, and the surface had tiny verrucous protrusions, slightly bent and 100-400 μm in length. While others non-glandular hairs were distributed in epidermal cells, consisting of 2-3 cells mostly to expand the base, which surface had obvious verrucous protrusions, slightly bent, wall thickness with 15-40 μm in diameter.

Single-cell non-glandular hairs were expanded at base, bent mostly, smooth surface or with verrucous protrusions, which often covered on the top of calyx and leaf edge, 10-90 μm in length.

Head of glandular hairs was composed of one base dell, 10-25 μm in diameter. Moreover, there were 1 or 2 cells in petiole of glandular hairs. There were two types of small glandular hairs, which one had a single head and single handle, 10-25 μm in diameter; another had single cell, slightly large head, liking light bulb, and wall thickness were about 2-4 μm .

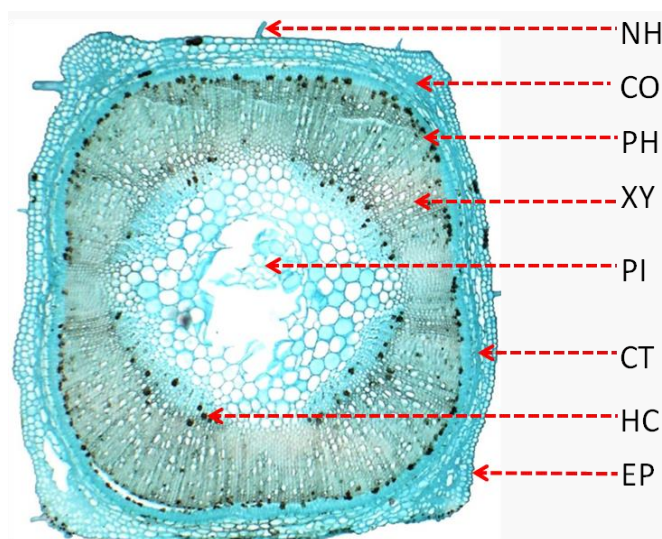


Figure 5. Transverse section of the stem of *Z. clinopodioides*.

NH: Nonglandular hair; CO: Collenchyma; PH: Phloem; XY: Xylem; PI: Pith; CT: Cortex; HC: Hesperidin crystal; EP: Epidermis.

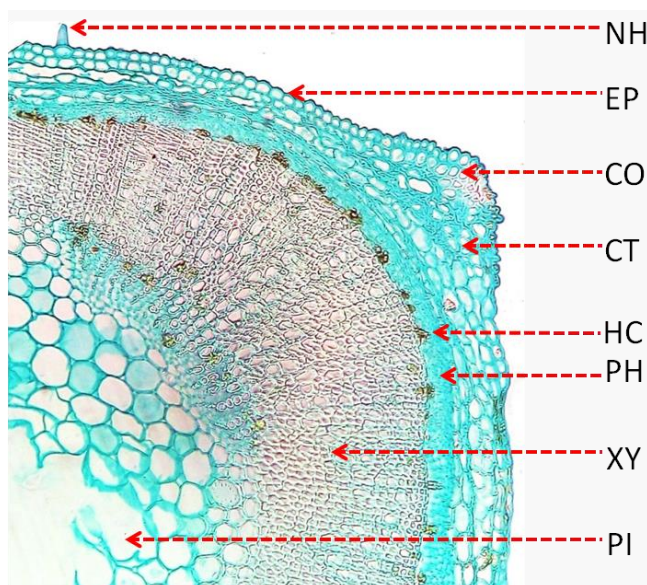


Figure 6. Transverse section of stem of *Z. clinopodioides*.

NH: Nonglandular hair; EP: Epidermis; CO: Collenchyma; CT: Cortex; HC: Hesperidin crystal; PH: Phloem; XY: Xylem; PI: Pith.

Glandular scales were light tan, whose head had 7-9 cells mostly and 11-13 cells occasionally, 45-85 μm in diameter.

Corolla epidermal cells were irregular shape and closely linked, the cell wall no obvious thickening.

Calyx tube epidermal cells were thin rectangle into spindle from the bottom to up, whose edge had water ripple and thickened wall, with transparent calcium solitary crystal and pale brown crystal of hesperidin.

Leaf epidermis stomas were diacytic type, 13-25 μm in diameter. Stem epidermis stomas were mostly anisocytic type, diacytic type occasionally, 25-40 μm in diameter.

There were two kinds of pith cells with a pit like hole grooves like texture and wall thickness. One was round like, pit intensive and small, 25-45 μm ; while another was rectangular, wall thickening obvious, 35-65 μm .

Fibers were fusiform, wall thickness and pit obvious.

Pollen grains were oblate with light yellow or colorless, which were common to observe, 15-40 μm in diameter, 6 or 3 apertures, granular carved lines on the surface.

There was oblate brown secretion beaded mostly in old stem cells, often attaching to the filamentous cells (Figs. 7-8).

Physicochemical studies

The UV spectrum of 70% ethanol extracts of *Z. clinopodioides* Lam. showed not obviously maximum absorption peaks both in number and intensity (Fig. 9). This finding could be due to little content of flavonoid, or the optimum extraction conditions should be optimized.

Preliminary phytochemical screening showed the presence of flavonoids, alkaloids, coumarin (Table 1).

Table 1. Phytochemical identification of *Z. clinopodioides*.

Metabolite	Name of the test	70% Ethanol extract
Flavonoid	HCl-Mg reaction	+
Coumarin	Ferric chloride test	+
Alkaloid	Silicotungstic acid	+
	Iodine-potassium iodide	+
	Potassium iodide mercury	+

Physicochemical characteristics

Moisture content was less than the limited index, because of dry weather in Xinjiang, which reach the standard. Total ash, acid insoluble ash, and water-soluble ash were all lower than the standard of pharmacopeia of the People's Republic of China. It showed that this herb meets the clinical medication's requirement (Table 2).

CONCLUSIONS

The standard of quality and physicochemical parameters has not been recorded in Chinese Pharmacopoeia Kazakh Pharmacopoeia Fascicule and Chinese Pharmacopoeia Uygur Pharmacopoeia Fascicule. *Schizonepeta tenuifolia* (Pharmacopoeia of the People's Republic of China, 2015), which is the same family and related genera of *Z. clinopodioides* has chemical components and efficacy very similar to this one. The standards are set initially after referenced the information of *Schizonepeta tenuifolia*, for example, macroscopical identification, physicochemical parameters (total ash 3.0%, moisture content 10.0%).

Microscopic analysis and qualitative parameters are carried out in order to establish appropriate data that can be used in identifying crude drugs. It showed that *Z. clinopodioides* could be identified by structural features of flowers, structural characteristics of rhizome and roots.

Examination of the macroscopical and microscopical features of *Z. clinopodioides* dissects a useful tool in the identification and authentication of the plant. These characters could be useful in identifying the plant earlier to its use in any pharmacological studies.

As there is no pharmacognostical work on the record, the present work could be therefore being used as one of the tools for standardization of crude drug to identify and decide the authenticity of the drug in the herbal industry. To guarantee the safety of clinical medicine, it is necessary to set the pharmacognostical standard. The data on this article can offer some references for how to ensure the stability and uniformity of quality.

Table 2. Physicochemical characteristics of *Z. clinopodioides* and *Schizonepeta tenuifolia*.

Physicochemical characteristics	Physicochemical parameter values of <i>Z. clinopodioides</i> (% w/w)	Limit value for herb (% w/w) (Commission, 2015)	Physicochemical Parameter value of <i>Schizonepeta tenuifolia</i> (Pharmacopoeia of the People's Republic of China, 2015) (% w/w)
Total ash	7.0	15.0	10.0
Acid insoluble ash	1.3	1.5	3.0
Water soluble ash	3.7	8.0	nd
Moisture content	7.5	10.0	12.0

nd: no determined

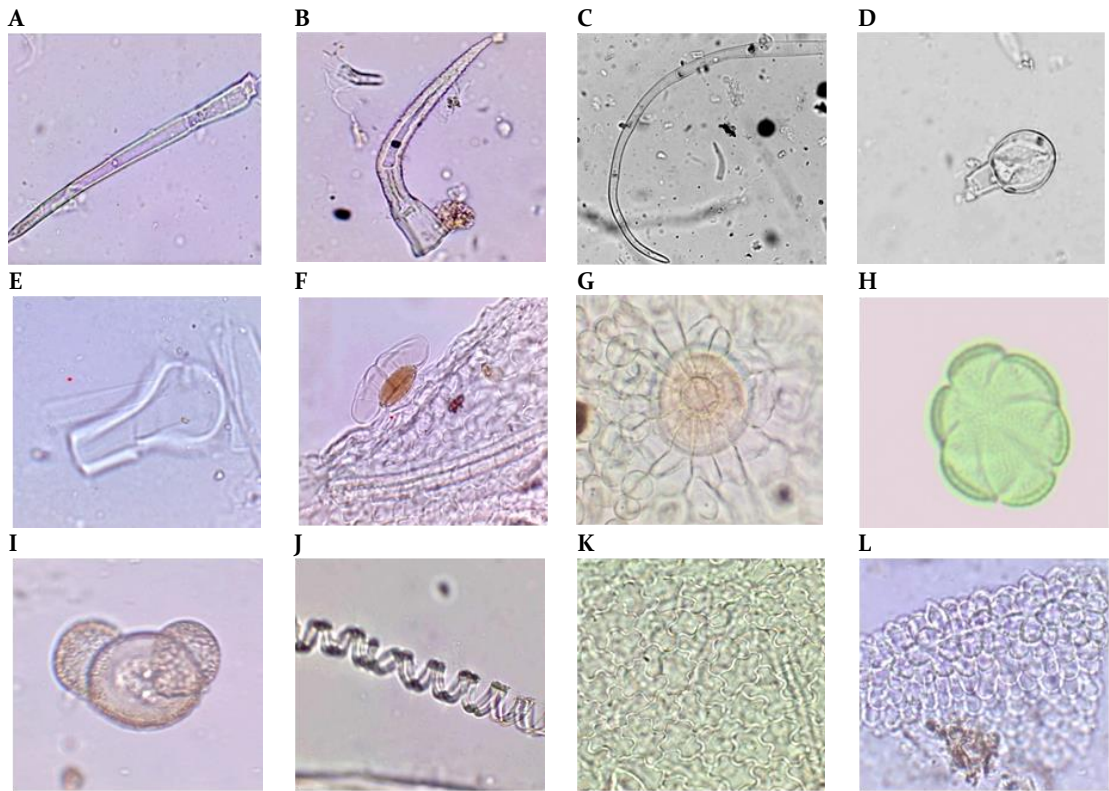


Figure 7. Powder characteristics study of *Z. clinopodioides*.

A-C: Nonglandular hair; D-E: Glandular hair; F-G: Glandular scale; H-I: Pollen grains; J: Spiral vessel; K-L: Epidermic cells of the corolla.

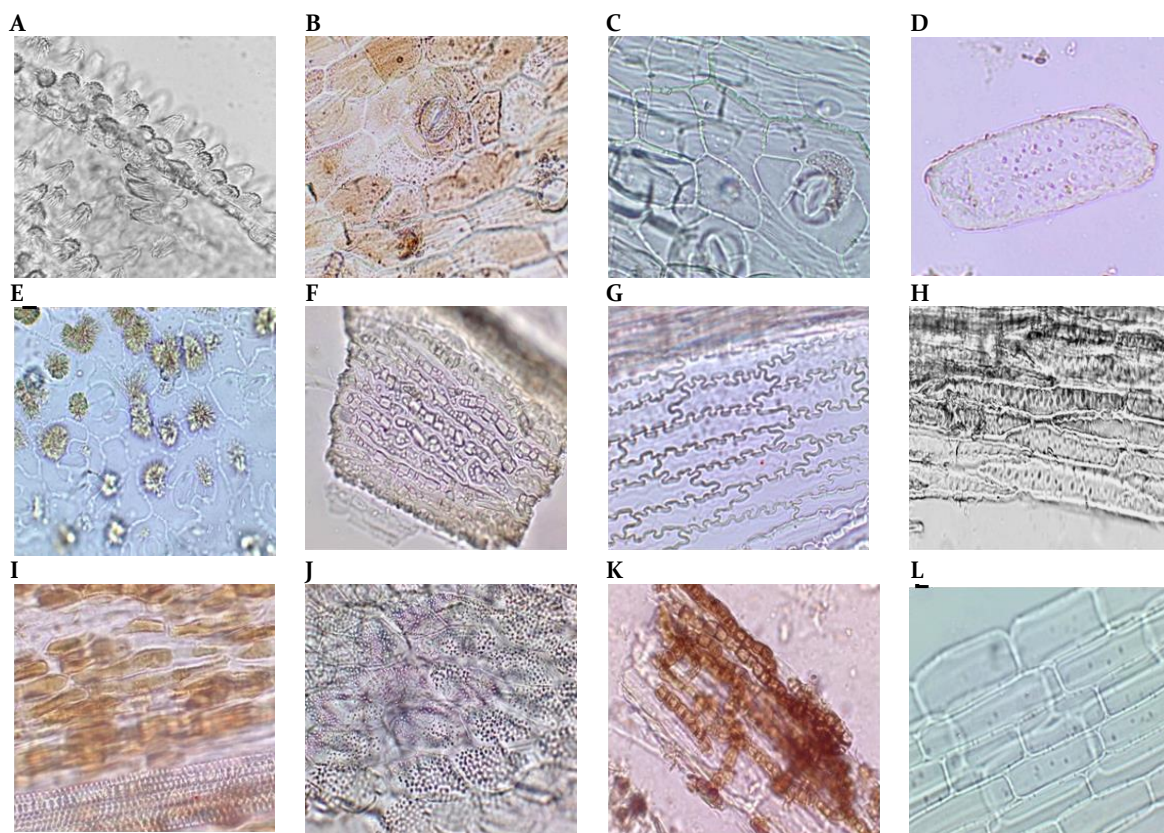


Figure 8. Powder characteristics study of *Z. clinopodioides*.

A: Endothecium cells of clinandrium; **B-C:** Epidermis cells of stem; **D:** Cells of pith; **E:** Leaf epidermis; **F-G:** Epidermis cells of Calyx; **H:** Fiber; **I:** Epidermis cells of filament; **J:** Epidermis cells of ovule; **K:** Secretory cells; **L:** Wood parenchyma cells of stem.

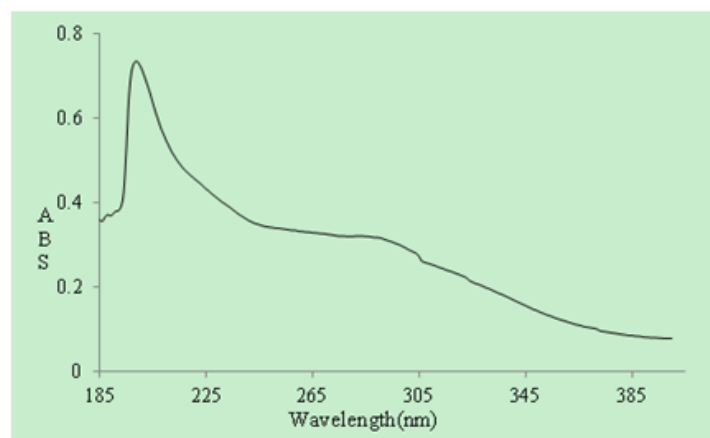


Figure 9. UV absorption spectrum of *Z. clinopodioides*.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Author contribution:

Contribution	Zhu Y	Xiong Y	Wang H	Li P
Concepts or ideas	X			X
Design	X			X
Definition of intellectual content	X			
Literature search		X	X	
Experimental studies	X	X		
Data acquisition	X	X		
Data analysis	X	X	X	
Manuscript preparation	X	X		
Manuscript editing	X	X		
Manuscript review	X	X		

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