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Pharmacognostical and phytochemical studies of *Viola tianschanica* Maxim. – An Uyghur ethnomedicinal plant

[Estudios farmacognóstico y fitoquímico de *Viola tianschanica* Maxim. – Una planta de la etnomedicina Uyghur]

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Abstract

Context: *Viola tianschanica* Maxim. (Violaceae) is a perennial herb widely distributed in Central Asia, especially in the Xinjiang of China. The whole herb has been used in traditional Uygur medicines as an antifebrile-detoxicate drugs.

Aims: To characterize macroscopical and microscopical features of the root, leave and rhizome of the *V. tianschanica* Maxim. Explore and establish the micro-morphology and quality control methods for this plant.

Methods: Pharmacognostic and phytochemical investigations were conducted regarding macroscopic, microscopic and preliminary phytochemical parameters.

Results: It can be identified by structural features of flowers, structural characteristics of rhizome and root, specific thickening of endothecium cells of clinandrium can be regarded as identification character. This crude drug showed the characteristic physicochemical values like total ash (12%), water soluble ash (4.0%), acid insoluble ash (3.8%) and moisture content (6.5%). The ethanolic extract mainly contained flavonoids in this herb, also contained alkaloids, tannins, saponins, coumarin and absence of fats and protein.

Conclusions: Various pharmacognostic and preliminary phytochemical characters observed in this test may help in standardization, identification and carrying out further research in *V. tianschanica* Maxim. based drugs used in Uygur traditional medicine and folk medicines. Sediment type of impurity content is higher in herbs; it should be paying attention to control quality of medicinal materials or drugs.

Keywords: Macromorphology; micromorphology; pharmacognostical identification; *Viola tianschanica* Maxim.

Resumen

Contexto: *Viola tianschanica* Maxim. (Violaceae) es una hierba perenne ampliamente distribuida en Asia Central, especialmente en Xinjiang, China. Esta hierba se ha utilizado en la medicina tradicional Uygur como antifebrífugo-desintoxicante.

Objetivos: Caracterizar las peculiaridades macroscópicas y microscópicas de la raíz, hojas y rizoma de *V. tianschanica* Maxim. Analizar y establecer los métodos de micro-morfología y control de calidad de esta planta.

Métodos: Las investigaciones farmacognósticas y fitoquímicas se llevaron a cabo en relación con los parámetros macroscópicos, microscópicos y fitoquímicos preliminares.

Resultados: La planta puede ser identificada por las características estructurales de las flores, del rizoma y la raíz, engrosamiento específico de células de endotecio del clinandrio puede ser considerado como carácter de identificación. Esta droga cruda mostró valores fisicoquímicos característicos como ceniza total (12%), ceniza soluble en agua (4,0%), ceniza insoluble en ácido (3,8%) y contenido de humedad (6,5%). El extracto etanólico contenía principalmente flavonoides, también contenía alcaloides, taninos, saponinas, cumarina y ausencia de grasas y proteínas.

Conclusiones: Varios caracteres farmacognósticos y fitoquímicos preliminares, observados en este ensayo, pueden ayudar en la normalización, la identificación y la realización de más investigaciones en preparados basados en *V. tianschanica* Maxim utilizados en la medicina tradicional Uygur y medicamentos populares. El tipo de sedimento del contenido de impurezas es mayor en las hierbas; se debe prestar atención al control de calidad de las materias medicinales o drogas vegetales.

Palabras Clave: Identificación farmacognóstica; macromorfología, micromorfología; *Viola tianschanica* Maxim.

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INTRODUCTION

Ancient traditional remedies, notably traditional Chinese medicine and Ayurveda, have been passed down and refined over their long history of clinical use (Williamson et al., 2015). The use of herbal medicinal products and supplements, principally for primary health care, has increased tremendously over the past three decades with not less than 80% of people worldwide (Ekor, 2014). *Viola* is the largest genus in the family Violaceae, with 525 - 600 species in the world, and most species distributed in the temperate Northern hemisphere (Zhou et al., 2008; Ning et al., 2012). Many *Viola* species contain anthocyanins, which have strong antioxidant activities. Most violas tested and many other plants of the family Violaceae contain cyclotides, which have a diverse range of *in vitro* biological activities, including uterotonic, anti-HIV, antimicrobial, and insecticidal activities (Tang et al., 2010; He et al., 2011; Zhang et al., 2012). *Viola canescens*, a species from India, exhibited anti-malarial activity and *in vitro* activity against *Trypanosoma cruzi* (Dua et al., 2011; Verma et al., 2011).

Viola has been evaluated for different clinical indications in human studies. Some clinical trial showed that the *Viola odorata* can improve the cough suppression in children with asthma (Qasemzadeh et al., 2015), and extract oil showed to be effective in patients with insomnia (Feyzabadi et al., 2014).

There are about 120 species of *Viola* L. genus distributed widely in China, among them *Viola tianschanica* Maxim. mainly distributed in Xinjiang, Gansu, Qinghai, Sichuan provinces and Tibet (Chen et al., 1999; Xinjiangensis, 2011). It was contained in Medicinal Flora of Uygur (Volume I) (Yongmin, 1999). The whole herb, with taste light bitter and pungent, cool in nature, is widely used in clearing away heat and eliminating toxin, reducing fever and inflammation, anti-swelling, moistening lungs to stop a cough, helping to defecate and so on (Yongmin, 1999). It was as a substitute of herba *Violae* (*V. yedoensis* Makino) in Xinjiang, mainly in Southern Xinjiang (Qin et al., 2014).

It was also documented in Chinese Pharmacopoeia Uigur Pharmacopoeia Fascicule, mainly used in the treatment fever, pyrexia, headache and influenza, acute pleurisy, pneumonia, dry pharynx, cough, and difficulty in urination, among others, but not detailed quality control standards in this Pharmacopoeia (Chinese Pharmacopoeia, 1999).

It is reported that plants of *V. tianschanica* Maxim. contains flavonols (isorhamnetin, apigenin, luteolin, kaempferol) (Yongmin, 1999; Qin et al., 2014; 2015), cyclic peptides (cycloviolacin T1, varv E) (Xiang et al., 2010), lignans (Qin et al., 2013), coumarin, alkaloids (Yu et al., 2009; Qin et al., 2014), phenolic acid (Qin et al., 2015). Modern pharmacological studies have demonstrated that the extracts of *V. tianschanica* Maxim. have anti-inflammatory (Yang et al., 2011), anti-bacterial, anti-oxidative activities (Shen and Xie, 2009), anti-complement activity (Qin et al., 2015).

In the scientific literature, there are some data of the phytochemical components and pharmacological actions while a small number of data of standards for identification and authentication about *Viola tianschanica* Maxim.

Hence, the pharmacognostic and phytochemical investigations on *V. tianschanica* Maxim. has been carried out in this research, for the development and utilization of the promising medicinal plant.

MATERIAL AND METHODS

The methodology was carried out according to with (Srikanth et al., 2013; Zhu et al., 2015; Marandi et al., 2016).

Plant material and reagents

The *Viola tianschanica* Maxim. herb bought from Uighur Medicine Limited Company of Hetian region of Xinjiang in June, 2014. The plant material was authenticated by Professor Pin Yan (College of Life Science, Shihezi University). Voucher specimens (N°. 2014050102-01) were preserved in School of Pharmacy of Shihezi University. The harvested plants were dried in shadow at room temperature (temperature 30 - 40°C), ground into powdered form and stored in airtight containers. All reagents used were of analytical grade, such as chloral hydrate, dilute glycerol, phloroglucinol.

Macroscopic and organoleptic studies

The plant was examined for morphological characters including size, shape, color, odor, taste, and extra features. The macro-morphological characteristic of the root, leaf and rhizome were observed under the magnifying lens (10x).

Microscopic studies

Dried herbs were ground to coarse powder and packed, for microscopic identification, in a suitable container. As clearing agents were used chloral hydrate and diluted. These reagents were prepared according to procedures described in the general rule of Pharmacopoeia of the People's Republic of China (Commission, 2015).

The samples were cut and immediately fixed in mixed liquid (formalin:acetic acid:ethanol 70%, 1:1:18). After fixing the samples for 24 h, 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 cut the paraffin embedded specimens. Each slice thickness was 10 - 12 µm. The dewaxed sections were stained with fast green and safranin.

Photomicrographs of the transverse section (stem, root, and leaf) and powder section were taken with the help of Biomicroscopy Primo Star (Zeiss Group, Germany) with 10x and 40 x microscope objective lens, and CX21 bio-microscopy unit (Olympus, Nikon D750 digital camera, Matrox Inspector, Matrox Electronic Systems Ltd., Japan).

Phytochemical studies

Dried herbs were ground to a coarse powder (grain size: 850 ± 29 µm) and packed in a suitable container for phytochemical identification. The powder was extracted with 70% ethanol, filtered and concentrated using vacuum distillation.

The UV spectrum 70% ethanol extracts of the herb gained with the help of ultraviolet spectrophotometer (UV-2401 spectrophotometer, Shimadzu Corporation, Japan, range from 200 to 400 nm wavelength).

The bioactive compounds such as alkaloids, flavonoids, saponins, tannins, terpenoids were screened to ascertain their presences in the 70% ethanol extract.

The total ash, acid insoluble ash, water soluble ash and moisture content were determined according to the standard procedures mentioned in the general rule of Pharmacopoeia of the People's Republic of China (Commission, 2015).

Herb powder (5 g) were placed in a conical flask and added 25 mL of 95% ethyl alcohol. Extracted it with ultrasonic (TP300-Ultrasonic extraction apparatus, frequency: 40 kHz, Tian Pong Electricity New Technology Co. Ltd, Beijing, China) for 20 min and filtrated for further use.

Conditions for thin-layer chromatography (TLC): TLCP (thin-layer chromatography plate) were activated under 100~105°C for 30 min; developing agent was chloroform-ethyl acetate-formic acid (5:4:1), distance was 8 cm, concentrated sulfuric acid was a chromogenic agent. The TLCP examined under ultraviolet (365 nm and 254 nm) and ordinary light.

RESULTS AND DISCUSSION

Original plant identification

V. tianschanica Maxim. is a perennial herb of 4 - 7 cm high common, which the whole plant is a smooth surface. The rhizomes were thick, short and vertical. The taproots were terete or inverted cone, cylindrical 2 - 5 cm long, which color was yellow-white, with few fibrous roots. There were not aerial stem and underground stem. Leaves were basal, ovate or oblong-ovate, 1 - 3 cm long, 0.5 - 0.8 cm wide, thick, apex obtuse. Base shrink to handle had the same length with leaves, hardly longer or shorter than petiole, margin entire or crenate. Stipule lanceolate or broadly lanceolate, 3/4 united with petiole, white and membranous. There were short tassels at the margin, which were sparse and glandulous. Flowers solitary in the apical portion of pedicel, pedicel was not shorter than leaves. Flowers had a diameter of 0.5 - 1 cm. Bracts were located in the middle of the scapes, opposite and linear lance shaped. There were glands along margin. Sepals 5, oblong ovate and apex acuminate. There were zonal appendages at base. Flowers were zygomorphic, and multiples of five. Petal was lavender or under which was yellow-white, or obovate petal with violet stripes. The lateral petals were not

bearded. Petals near the bottom were obcordate and longer than sepals adnexal, which in the middle was bigger than two sides, at the base of it has short calcar. Filaments were short and broad, superior ovary smooth, styles apically curved and rostellate. The capsule was ovoid and smooth. Its bloomed was at June-July, and fructified at July-August (Xinjiangensis, 2011) (Fig. 1).

Figure 1. Plant of *V. tianschanica* Maxim.

Macroscopical identification

The morphological appearance of herbs was not complete. It was usually 3 - 7 cm long and focused between 2 - 4 cm. Taproot was thick. The shape was conical, and the color was brown to grayish white. It had irregular longitudinal wrinkles. Its rhizome was 2 - 4 mm long, and the diameter was 3 mm with the ring of decorative pattern and petiole residues. The leaves were shrinking, broken clustered in a short and thick stems. Complete wet opened leaf was spoon-shaped or oval. The color was gray-green. Its top was obtusely rounded, and its basic part was cuneate and decurrent, with a long handle. The flower was pale blue with five lanceolate sepals and five obviate symmetrical petals. Capsules were usually split in a triangle and there were many light yellow seeds in it. Its quality of material was loose, and the section was light brown with sparse pores. It smelled slightly aromatic and tasted slightly pungent and bitter (Fig. 2).

Microscopic Identification

Transverse section of V. tianschanica *leaves*

Through the midrib showed the following tissue systems:

- Epidermal cells, one layer, tightly packed, round the outside wall thickening. The outside wall of lower epidermis wave-shaped bending, outside of which have cuticle thickness.
- There were two columns of main veins vascular bundles, clusters of calcium oxalate were common besides vascular bundles.
- Palisade tissue cells were almost broken, without going through the main vein, individual crystals were occasionally found in palisade tissue cells (Fig. 3).

Transverse section of the root

Oval:

- Cork layer cells 1-2 layers, rectangle or irregular, with thickened cell wall. The phelloderm was composed of 3-4 columns flat parenchyma cells, tightly packed.
- The cortex was very narrow, flat cells, tightly packed. There were few clusters of calcium oxalate in cells of the cortex. The cracks exist between cortex and phelloderm.
- The primary xylem was diarch, centrally located. Vessels were small and secondary xylem extends to both sides. Lignified tracheas two layers, in which there were xylem parenchyma cells. The diameter of outer layer vessels was big and xylogen is not lignified.
- Vascular cambium was not evident. Phloem was wide, composed of tightly packed cells.
- Clusters of calcium oxalate can be found in phloem and parenchyma cells (Fig. 4).

Figure 2. The original herb of *V. tianschanica* Maxim.

Transverse section of the rhizome

Polygon

- Cork layer cells in several layers, with irregular long polygon cells.
- Cells of cortex shrink, with big cracks, in which there were few clusters of calcium oxalate. Endodermis was not obvious and wave-shaped bending.
- Vascular bundles were scattered and the tough type of outside, forming a ring. Xylem was round like, vessels were round like, lignified, tightly packed. Inner side vessels were almost single, sparsely packed. There were single vessels scatter between vascular bundles. Phloem was separated from xylem.
- Pith was big. The cavity can be found in the center of pith. A large number of clusters of calcium oxalate existed in parenchyma cells (Figs. 5 – 6).

Powder microscopy

Yellow-green powder

- The anomocytic stoma or anisocytic stoma, subsidiary cells 3 - 4, whose long axis was about 40 μm and short axis was about 30 μm.
- Fibres were long-shuttle shape, mostly broken, of which completed ones were 400 - 900 μm in length, 65 - 110 μm in diameter, 2 - 7 μm in thickness of wall, cavity was not obvious.
- Clusters of calcium oxalate were common, whose edges and corners were broad and obtuse. Clusters of calcium oxalate, which were sporadic, have bigger diameters, up to 18 - 60 μm. In parenchyma cells, clusters of calcium oxalate were generally small.
- Single cells non-glandular hairs were conical, 200 - 400 μm in length, 15 - 20 μm in diameter, with thin walls about 2 - 4 μm.

- Spiral vessels were common, while reticulate vessels were rare, with a diameter of 9 - 22 μm, individually scattered or with parenchyma cells.
- Pollen grains were common, with near-spherical shape, canary yellow, 30 - 40 μm in diameter. The outer wall was nearly smooth, with the particulate matter on the surface and three pit canals.
- Endophragm cells of anthers were kelly, with specific thickening, of which some cells were beaded-thickened and clover shaped projections can be seen in joints of some cells. Cinclides was evident.
- Exterior epidermal cells of corollas were papillary projections.
- Epidermal cells of the stem were rectangular, whose wall was beaded-thickened.
- Cork cells were pale brown, arranged in good order, whose surface view was nearly square, the lateral view was long-shuttle shape（Fig. 7).

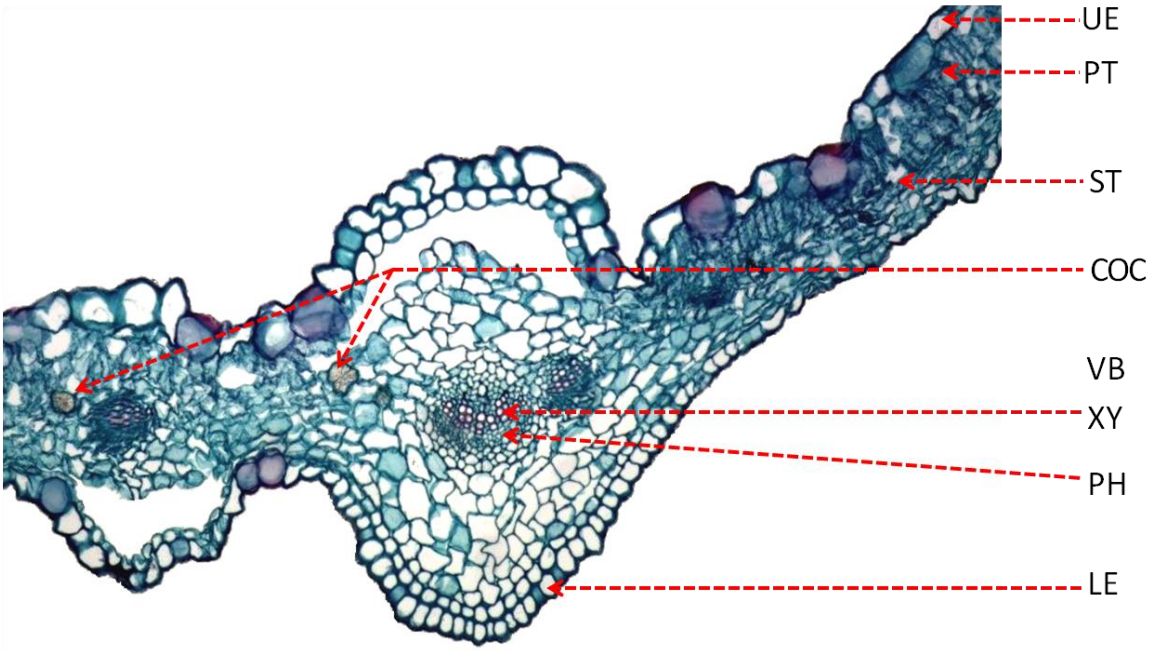

Figure 3. Transverse section of the leaf of *V. tianschanica* Maxim.

UE: Upper epidermis, **PT:** Palisade tissue, **COC:** Cluster crystal, **VB:** Vascular bundle, **ST:** Spongy tissue, **XY:** Xylem, **PH:** Phloem, **LE:** Lower epidermal.

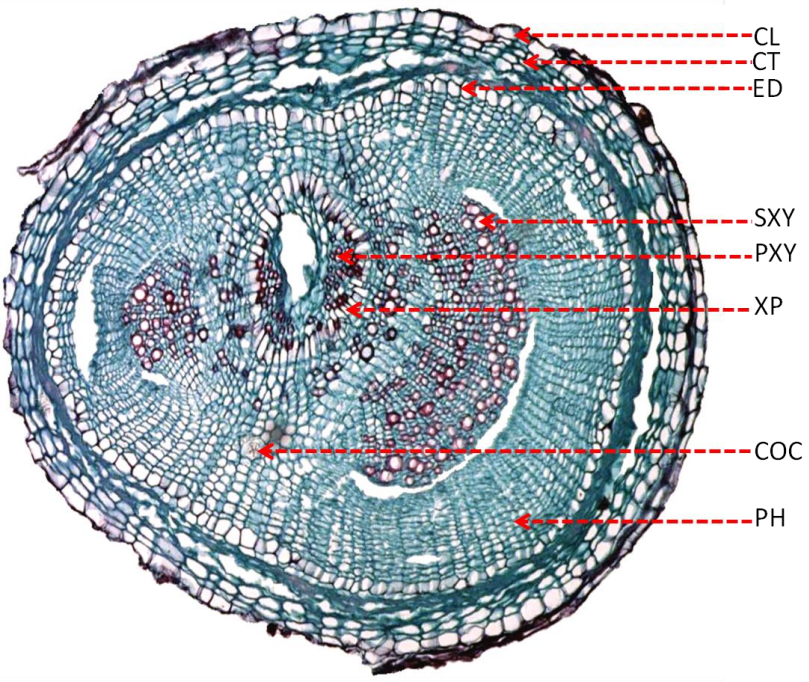

Figure 4. Transverse section of the root of *V. tianschanica* Maxim.

CL: Cork layer, **CT:** Cortex, **ED:** Endodermis, **PXY:** Primary xylem, **SXY:** Secondary xylem, **XP:** Xylem parenchyma, **COC:** Clusters of calcium oxalate, **PH:** Phloem.

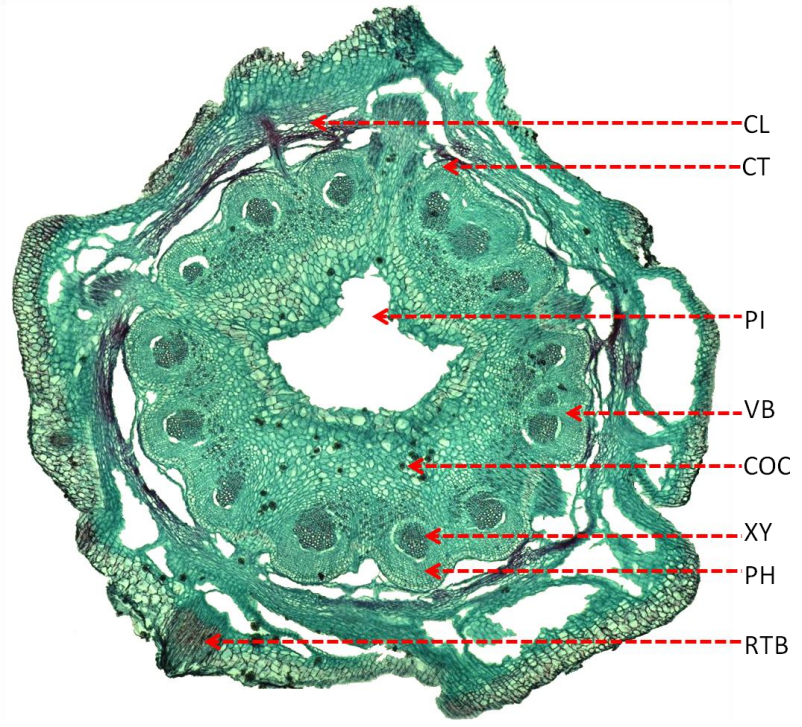

Figure 5. Transverse section of the rhizome of *V. tianschanica* Maxim.

CL: Cork layer, **CT:** Cortex, **PI:** Pith, **VB:** Vascular bundle, **COC:** Clusters of calcium oxalate, **XY:** Xylem, **PH:** Phloem, **RTB:** Root trace bundle.

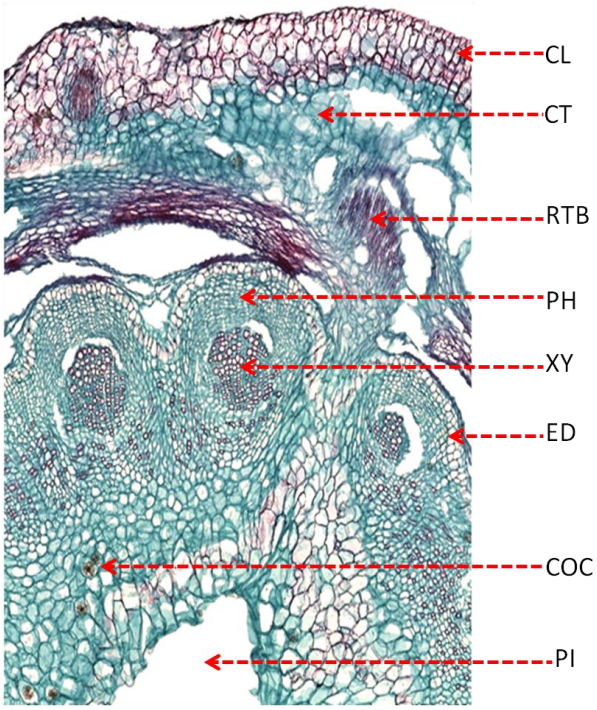

Figure 6. Rhizome transverse section of *V. tianschanica* Maxim.

CL: Cork layer, **CT:** Cortex, **RTB:** Root trace bundle, **PH:** Phloem, **XY:** Xylem, **ED:** Endodermis, **COC:** Clusters of calcium oxalate, **PI:** Pith.

Physicochemical studies

Scan of the ultraviolet spectrum

There were three absorption peaks at 204 nm, 266 nm and 351 nm. There may be the presence of flavonoids in 70% ethanol extracts (Qin et al., 2015) (Fig. 8).

Phytochemical screening

Preliminary phytochemical screening showed the presence of flavonoids, alkaloids, tannin, volatile oil, saponins and amino acids (Table 1).

Physicochemical characteristics

Several studies suggest that adulteration and misidentification of crude drugs can cause serious health problems to consumers and legal challenges for the pharmaceutical industries. The macroscopic and microscopic characters of any plant drug are considered to be the preliminary steps for establishing their quality control profile. As per the guidelines of WHO, pharmacognostical standards should be proposed as a protocol for the diagnosis and authentication of the herbal drugs (Pramanick, 2016).

Moisture content was less than the limited index, explaining dry weather in Xinjiang, it can reach the standard. Total ash and water soluble ash were all lower than the standard of Pharmacopoeia of the People's Republic of China. Acid insoluble ash was far higher than the standard for the whole herb medicine in China pharmacopoeia, the possible reason is that herbal medicine is not clean, containing soil and another inorganic impurity (Table 2).

TLC check

Fluorescence presented six obvious spots. The colorimetric detection with concentrated sulfuric acid presented eight spots (two in UV254 and six in UV365) (Fig. 9), probably due to the presence of flavonoids (Qin et al., 2015).

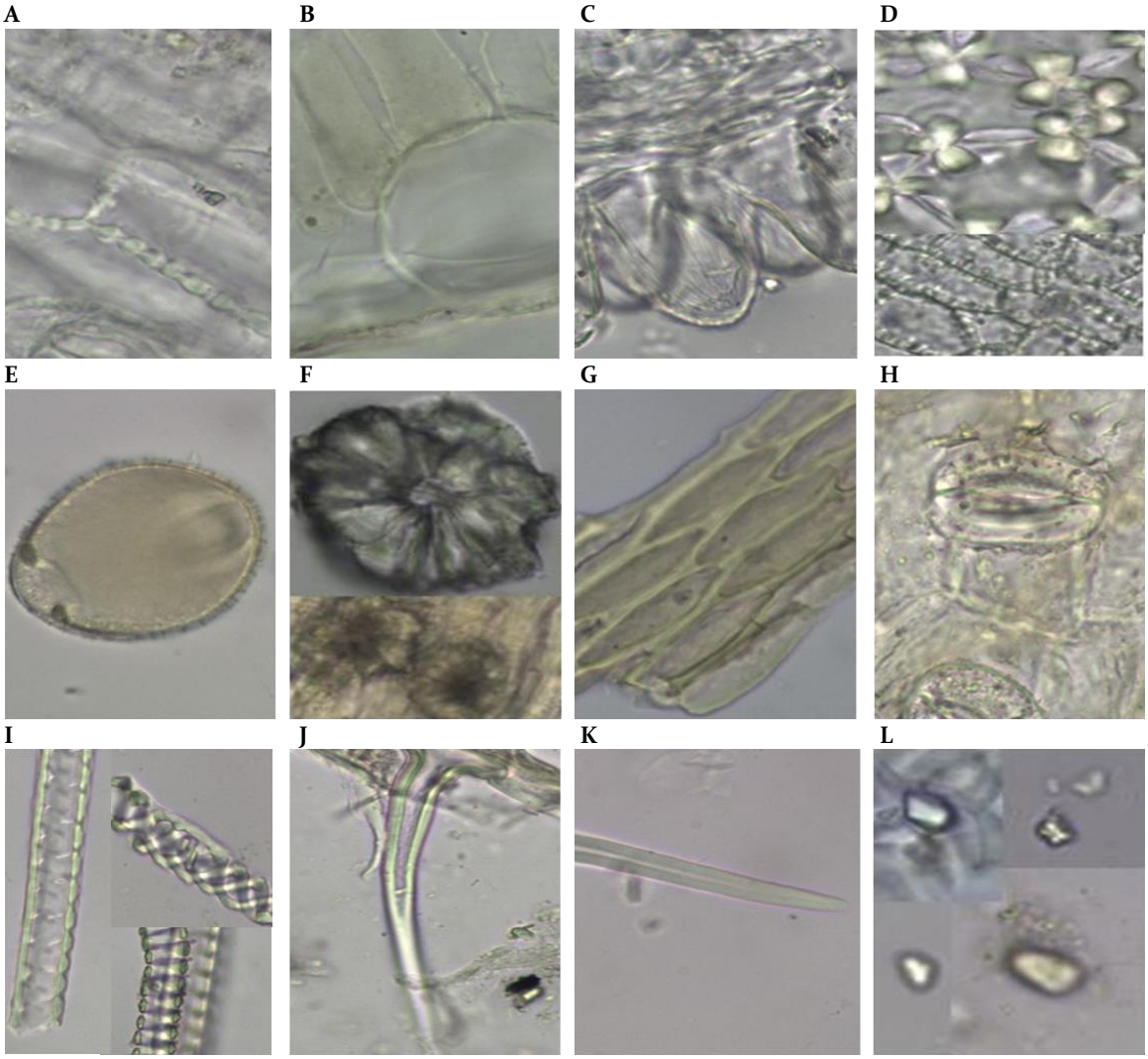

Figure 7. Powder characteristics study of *V. tianschanica* Maxim.

A: Pollen grains, **B:** Clusters of calcium oxalate, **C:** Cork cells, **D:** Stomata, **E:** Epidermis cells of stem, **F:** Palisade cells, **G:** Fragmented cells of flower epidermis, **H:** Endothecium cells of clinandrium, **I:** Vessel, **J-K:** Nonglandular hair, **L:** Solitary crystal.

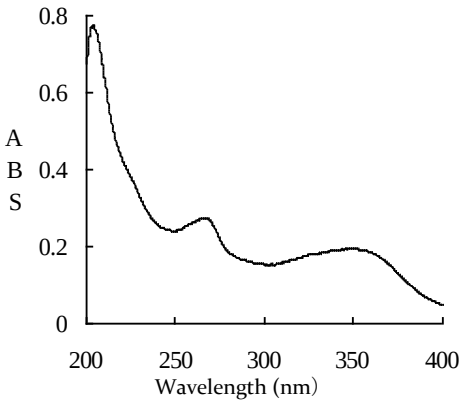

Figure 8. The UV absorption spectrum of *V. tianschanica.*

Table 1. Phytochemical analysis of *V. tianschanica*.

Test for constituent groups	Name of the test	70% Ethanol extract
Flavanoid	HCl-Mg reaction	+
	$AlCl_3$ reaction	+
Volatile oil	Microsublimation-vanillic	+
Alkaloid	Dragendorff's reagent test	+
	Wagner's reagent test	+
	Hager's reagent test	+
	Bertrand's reagent test	+
Saponin	Libermann-Burchard test	+
	Foam test	Foam 1.5 cm
Tannin	Ferric chloride Test	+
Amino acid	Ninhydrin	+

Table 2. Physicochemical characteristics of *V. tianschanica* Maxim.

Physicochemical parameter values (% w/w)		Limit value for herb(Commission 2015)
Total ash	12.0	15.0
Acid insoluble ash	3.8	1.5
Water soluble ash	4.0	8.0
Moisture content	6.5	10.0

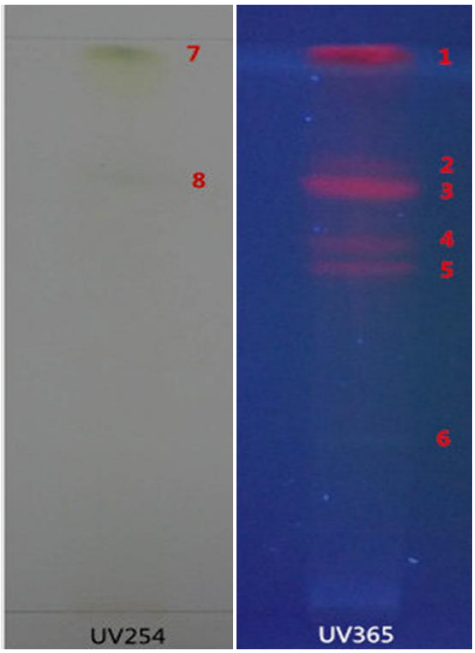

Figure 9. Thin layer chromatography of *Viola tianschanica* Maxim.

R_f1-R_f7 = 0.98,

R_f2 = 0.78,

R_f3-R_f8 = 0.74,

R_f4 = 0.66,

R_f5 = 0.60,

R_f6 = 0.42

CONCLUSIONS

Physicochemical parameters like ash values, moisture content are all indicators of quality herbal medicine, which help to determinate the physiological and non-physiological ash, possibility of microbial growth or contamination and presence of impurities respectively. The relative high acid insoluble ash value (3.8%) and a high ratio of water soluble ash content (4.0%) of *V. tianschanica* Maxim. indicates that the crude drug contains plenty of physiological ash and the non-physiological content, it will affect the clinical efficacy of drugs, so it should pay attention to the control quality of medicinal materials in herbs production.

This research showed that, *V. tianschanica* Maxim. can be identified by structural features or characteristics of flowers, rhizome and roots, specific thickening of endothecium cells of clinandrium can be regarded as distinctive identification character. In identification of ultraviolet spectrum, there are three obvious absorption peaks. There are obvious spots on thin-layer chromatography. All above have the significance of the identification in pharmacognosy.

Efforts have been made by the authors to bring out every detail on the macroscopical and microscopical characters of *V. tianschanica* Maxim. The study of pharmacognostical features had shown the standards, which will be useful for the detection of its identity and authenticity.

It provides reference basis for formulating quality standard of *V. tianschanica* Maxim., authenticity of medicinal herbs and resource utilization.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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