# COMPARATIVE MORPHO-ANATOMICAL AND HPTLC PROFILING OF *TINOSPORA* SPECIES AND DIETARY SUPPLEMENTS


## Authors

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**Abstract**

Overlapping geographical occurrence, history of traditional use, confusion in species identification and morphological resemblances among various species are some considerations that necessitate the importance of qualitative analysis for efficient quality control and safer botanical products. This paper provides detailed morpho-anatomies of the leaves and stems of *Tinospora cordifolia*, *T. crispa* and *T. sinensis*, and stems of *T. baenzigeri*. Microscopy studies of the selected *Tinospora* species revealed key diagnostic features that can help distinguish the closely related species of *Tinospora* as well as to detect any adulteration or substitution in the raw materials. HPTLC profiles of the authenticated plant materials, as well as commercial products claiming to contain *Tinospora*, were compared to distinguish *T. crispa* from other closely related species and to establish an efficient method to assess the identity and quality of the products using qualified chemical markers. HPTLC chromatograms of both plant samples and dietary supplements were compared with six reference marker compounds. The analysis revealed that borapetoside B and C were useful to identify *T. crispa* while tinosineside A was found to be characteristic to *T. sinensis*.

**Key words**

*Tinospora*, Menispermaceae, anatomy, HPTLC, microscopy, quality control

## Introduction

The genus *Tinospora* Miers (Menispermaceae) is a group of large, deciduous woody climbers of about 34 species distributed in Australia, Africa and Asia [1]. Four species, distributed in South and Southeast Asia, were selected for this study, namely *T. cordifolia* (Willd.) Hook.f. & Thomson, *T. crispa* (L.) Hook.f. & Thomson, *T. sinensis* (Lour.) Merr. and *T. baenzigeri* Forman [2, 3]. In Ayurveda, *T. cordifolia* (Sanskrit: *Guduchi*) is classified under the *Rasayana* group of plants and is used as an adaptogen, antipyretic, antiperiodic, anti-inflammatory, antirheumatic, spasmolytic, hypoglycemic and hepatoprotective [4-8]. Indigenous communities in India use the stems of *T. cordifolia* for treating various ailments such as fever, jaundice, diarrhea, bone fractures, ear pain, leucorrhea, skin diseases, poisonous bites and eye disorders [6,9-11]. Akin to *T. cordifolia*, the stems of *T. crispa* (Thai: *Boraped*) are widely used for treating fever, thirst, and loss of appetite [7,12,13]. *T. sinensis* (Chinese: *Zhong hua qing niu dan*) is used for treating bone fractures, visceral leishmaniasis, ankylosis, lumbar disc herniation and chronic rheumatism [14]. The crude extracts of T. *sinensis* leaves have high potential of immunomodulatory and anti-inflammatory activities [15-18]. *T. baenzigeri,* known as *Ching cha chali* in Thailand, is used medicinally as an antipyretic and antimalarial [19].

Overlapping geographical occurrence, historical use in traditional medicines, and morphological resemblances are important considerations that necessitate qualitative analysis for efficient quality control of *T. cordifolia*, *T. crispa, T. sinensis,* and *T. baenzigeri* as these four species are most likely to be confused with and thus adulterated or substituted with one another in global markets [20, 21]. *T. cordifolia* is often substituted with *T. sinensis* [22]. It is also reported to be adulterated with other botanicals including *Pergularia daemia* (Forssk.) Chiov. (Apocynaceae) due to the morphological similarity [23]. Several authors have in the past worked on the pharmacognostic and phytochemical profiles of *Tinospora* species [20,22-33]. However, the present study provides, for the first time, an illustrative comparative morpho-anatomy of the four closely related medicinal species of *Tinospora* and an HPTLC method to distinguish *T. crispa* from the other closely related species.

The phytochemical profile of a species may vary either qualitatively or quantitatively due to various factors thereby affecting the quality and degree of the biological effect exerted. In this context, recognizing the need to differentiate the selected species in terms of macroscopy and microscopy, the study was undertaken for characterizing and differentiating these species. An HPTLC method was developed for acquisition of fingerprints for the *Tinospora* plants with

qualified chemical markers since no such chromatographic study including different species of *Tinospora* has been initiated so far. The method was also applied for the analysis of commercially available herbal dietary supplements of *Tinospora*.

**Results and Discussion**

The morpho-anatomies of the leaves, petioles and stems of *Tinospora* species, namely *T. cordifolia*, *T. crispa* and *T. sinensis* (Figs. **1**-**5**) and stems of *T. baenzigeri* (Figs. **4**-**5**), were observed by brightfield and scanning electron microscopy (SEM). These species appear similar in their external morphologies by having broadly cordate leaves, cylindrical twining stems often with lenticels and aerial adventitious roots. However, when observed carefully, they are distinguishable by their exomorphic characteristics (Fig.**1**, Table 1).

The leaves are smooth in *T. cordifolia* (Fig. **2A**) and *T. crispa* (Fig. **2G**), but they are densely tomentose in *T. sinensis* (Figs. **2K** & **M**). The leaf surface is distinct in *T. cordifolia* with the presence of epicuticular wax coating composed of irregular crenate platelets on both sides (Figs. **2D** & **E**) as observed by SEM. Glandular trichomes with a short stalk and a small secretory head, also called the pearl glands [24, 34], are occasionally present in *T. cordifolia* and *T. crispa* leaf surfaces (Figs. **2B** & **F**). The secretory head is ellipsoid in *T. cordifolia* and ovoid-subglobose in *T. crispa*. The leaves of *T. crispa* have smooth surfaces on both sides with no epicuticular wax (Figs. **2G** & **H**). The abaxial epidermal cells are often papillate (Fig. **2J**). In *T. sinensis*, the adaxial side shows 2-3-celled uni-seriate non-glandular trichomes that are more abundant along the veins (Figs. **2K** & **L**) whereas the abaxial side is densely tomentose and matted with irregularly curved multicellular uni-seriate non-glandular trichomes (Figs. **2M**-**O**). Begum et al. [31] mentioned the presence of "uni to biseriate trichomes" in *T. sinensis* leaf but with no further details. Biseriate trichomes have not been reported previously in *Tinospora* and were also not observed during the present study. Therefore, we assume that these authors probably meant "uni to bicellular (not biseriate) trichomes". Anomocytic stomata are present in the abaxial epidermis of the leaves analyzed. Stomata are abundant in *T. cordifolia*, and the guard cells are not covered by the epicuticular wax (Figs. **2C**-**E**). In *T. crispa* the stomata are raised from the adjacent epidermal cells (Figs. **2H** & **I**) whereas in *T. sinensis*, they are slightly raised but are mostly hidden by the dense tomentose trichomes (Figs. **2O** & **P**).

Raghunathan and Mitra [20] mentioned the presence of glandular trichomes only on the lower surface of the leaf in *T. cordifolia*. However, in this study, the glandular trichomes were observed on both upper (Fig. **2B**) and lower (Fig. **2F**) epidermis of the leaves in this species.

A thin layer of cuticle was observed in the leaves of all three species, however, *T. cordifolia* has an additional waxy coating as described above. Anticlinal walls of epidermal cells are sinuous on both the sides in *T. cordifolia* (Fig. **3A**), whereas in *T. crispa* (Fig. **3B**) and *T. sinensis* (Fig. **3C**) they are straight in the adaxial side and slightly sinuous in the abaxial side. The mesophyll is dorsiventral in all studied species and consists of a single layer of palisade and 2-4 layers of spongy parenchyma cells. The average palisade ratio is 9.61 in *T. cordifolia*, 7.25 in *T. crispa* and 5.56 in *T. sinensis*.

In the transverse section, midrib anatomy shows some similarities as well as differences among the three species. The midrib is slightly convex at the adaxial side in *T. cordifolia* (Fig. **3D**), prominently convex in *T. crispa* (Fig. **3E**), and irregularly wavy with numerous trichomes in *T. sinensis* (Fig. **3F**). Vascular strands abut both above and below with 1-3 layers of sclerenchyma cells.

The petiole is pubescent in *T. sinensis* (Fig. **3I**) but glabrous in the other two species (Figs. **3G** & **H**). In transection, the petiole is more or less circular or with a wavy outline, covered externally by a layer of cuticle. The epidermis is uni-layered in all three species (Figs. **3G-I**). The primary cortex is composed of 2-4 layers of compactly arranged collenchyma cells. The pericyclic layer is continuous, 2-4 cells wide, forming a sclerenchymatous cap of lignified cells, and found to be more prominent in *T. crispa* (Fig. **3I**) than the other species. Parenchymatous cells in between the sclerenchymatous cap and the vascular strands are about 2-5 cells thick. The closed collateral vascular bundle shows the direct formation of xylem and phloem without interfascicular cambium. The number of vascular strands vary in each species of *Tinospora* which might be growth dependent. The vascular bundles observed in the study show 5-6 in *T. cordifolia* (Fig. **3G**) and 10-12 in the other two species (Figs. **3H** & **I**). The central pith consists of large and loosely arranged parenchyma cells.

The raw drug of *Tinospora* consists of pieces of stems of varying thickness ranging from 0.6-5 cm in diameter. Young stems are green with smooth surfaces swollen at nodes, the older ones show a light brown surface marked with warty protuberances due to circular lenticels. The transversely cut surface of the stem shows visible radial lines of medullary rays that run alternatively with the vascular strands capped with the sclerenchymatous arches (Figs.

**4A**, **D**, **G** & **J**) giving a wheel-like appearance, a characteristic feature of the family Menispermaceae [24,28,30]. The warty protuberances on the stem surface are more conspicuous in *T. crispa* than the other three species.

The micromorphology of the stems of *Tinospora* species shows the following characters. In *T. cordifolia*, the surface of the stem shows a few pearl glands (Fig. **2F**). In transection, the stem shows 9-10-layered cork with distinct radial files of lignified and suberized cells with two layers of secondary cork formation (Figs. **4A** & **B**). Transverse sections of the stems of *T. crispa* (Figs. **4D-F**) and *T. sinensis* (Figs. **4G** & **H**) show cork composed of 5-8 layers of regular radial files with wavy and suberized walls and 3-5 layers in *T. baenzigeri*. The outer region of the cork shows lenticels of 20-30 layers in *T. cordifolia* and *T. sinensis*, these lenticels are very common in *Tinospora* and abundant in calcium oxalate crystals. Cork cambium is 4-5-layered in *T. cordifolia* and *T. sinensis*, 5-8-layered in *T. crispa* and 1-2-layered in *T. baenzigeri*. In *T. cordifolia*, the secondary cortex is broad with 5-6 layers of chlorenchyma and 25-40 layers of parenchyma cells (Fig. **4B**). Cortex parenchymal cells are filled with abundant starch grains, which are simple, oval to elongated and sometimes irregularly shaped. A sclerenchymatous ring forms a cap encircling the vascular strands and consists of cells with thickened and densely lignified walls. Vascular strands are well-developed and arranged in an arch form (Figs. **4A** & **B**). The cortex in *T. crispa* stem is composed of 4-5 layers of tangentially arranged collenchyma and 35-45 layers of loosely packed parenchyma cells (Figs. **4D** & **E**), *T. sinensis* showed two distinct zones, the outer 1-2-layered chlorenchyma, and inner 2-12 layered parenchyma cells (Figs. **4G-H**) whereas in *T. baenzigeri*, 5-7-layered chlorenchyma and 12-17 layers of parenchymatous cells (Figs. **4J** & **K**). The cortex consists of grouped sclereid patches in *T. baenzigeri* (Fig. **4J**), the presence of sclereid fibers is not observed in the other three *Tinospora* species studied. The presence of mucilage substances was observed in cortical cells in all species.

The wood in *T. cordifolia* (Fig. **4A**), *T. crispa* (Fig. **4B**) and *T. sinensis* (Fig. **4C**) are of diffuse-porous type, but ring-porous wood is present in *T. baenzigeri* (Fig. **4J**). The vessels are wider in the earlywood and narrower in the latewood.

The vascular bundles are collateral with phloem towards peripheral and xylem at the inner side. Phloem occurs below the sclerenchyma cap and consists of phloem parenchyma, sieve and companion cells. Xylem is separated from the phloem by 4-5-layers of vascular cambium in *T. cordifolia* and *T. crispa*, 5-8-layers in *T. sinensis* and 3-4-layers in *T. baenzigeri*;

this forms an interfascicular cambium at maturity. Medullary rays run between the vascular bundles and join the sclerenchyma ring, the thickness of the medullary ray ranges from 2-8 or more cells depending on the growth and maturity of the wood [23]. Carlquist described the features of tyloses present in the xylem vessels [35]. Tyloses were observed in the stems of all species studied, often so numerous to block the entire lumen of the vessels (Figs. **4C**, **I** & **L**). They are balloon-like, oval to oblong, 1-2-celled, 20-40 µm long, often containing starch grains, as also observed by Patil and Malpathak [23]. Tyloses containing prismatic crystals were observed in the present study (Fig. **5C**). The central pith composed of loosely arranged parenchyma cells was observed in all four species of *Tinospora*. Groups of densely lignified sclereids were observed in pith as well as the cortex, only in *T. baenzigeri* (Fig. **4J**).

Histochemical studies of *Tinospora* stems showed the presence of alkaloids in the phloem and its surrounding cells in *T. cordifolia*, *T. sinensis*, and *T. baenzigeri*. Oil droplets were found in the pith as well as near the vascular strands of *T. cordifolia* and *T. sinensis*.

Ergastic substances such as calcium oxalate crystals were present in all *Tinospora* species although their shapes and occurrences varied. In *T. cordifolia*, rhomboidal prismatic crystals (Fig. **5A**)**,** styloid, cuneiforms, simple bipyramids, arrow-shaped, and oval-shaped crystals (Fig.**5B**) were observed. Tyloses showed the presence of prism-shaped crystals (Fig. **5C**). Styloids and cuneiforms crystals in *T. crispa* (Fig. **5E**), styloids (Fig. **5J-1**) and agglomerate of cuneiforms were observed in *T. sinensis* (Fig. **5J-3**) in leaf veins as well as the epidermis cells. Begum et al. [31] mentioned that calcium oxalate crystals were absent in *T. sinensis*. In contrast, we have observed several forms of calcium oxalate crystals in the leaves, petioles, and stems of *T. sinensis* (Fig. **5H-J**). Notably, druses and raphides were not observed in any of the *Tinospora* species in our studies. Patil and Malpathak [23] mentioned the presence of druses in *T. cordifolia* although this feature was not reported by any other researcher. Druses were not observed in any of the four species included in the present study. Large aleurone-like crystalline bodies that show birefringence in polarized light were observed in the leaves of *T. sinensis* (Fig. **5L-1** & **L-2**). Similar to the leaves, the petioles also showed differences in the shapes of crystals. The crystals were pyramid-shaped in *T. cordifolia*, elliptical to ovoid in *T. crispa*, elliptical as well as ovoid-shaped prisms in *T. sinensis*, (Fig. **5I-2**) and cubic or square crystals in *T. baenzigeri* (Fig. **5J-2**).

Bonde and Upaehye [25] reported that the calcium oxalate crystals were absent in *T. sinensis*. On the contrary, we observed abundant crystals of calcium oxalate in all the four

species, including *T. sinensis*, in the present study. In addition to morphological identification, the elemental compositions of these crystals were also confirmed by EDS (Energy Dispersive X-Ray Spectroscopy) analysis.

In *T. cordifolia* stems, calcium oxalate prisms of varying shapes and sizes were observed in the parenchyma cells around the vascular bundles as well as in some of the pith parenchyma cells. In *T. crispa,* the crystals were rectangular, whereas in *T. sinensis*, elliptical to ovoid prisms (Fig. **5I-1**), pyramid-shaped prisms (Fig.**5I-4**), and irregular prismatic crystals (Fig. **5J**-**4**) were present. In *T. baenzigeri* stems, tetragonal (Fig. **5M**-**1)**, hexagonal (Fig. **5M**-**2, O-2**) arrowhead-shaped (Fig.**5N**) prisms, and styloids (Fig. **5O**-1) were observed. The tyloses of *T. cordifolia* wood were often accumulated with prismatic crystals (Fig. **5C**) or starch grains. Starch grains are abundant in the leaves, petioles, and stems of *Tinospora*, and occur as simple or compound (in groups of 2-3). They are usually ovoid to round or sometimes elliptical in shape (Fig. **5 D**, **F**-**1**, **F**-**2**, **G**, **K**).

*Tinospora* products sold in the form of raw plant materials and dietary supplements as well as authenticated stem samples of the four species were analyzed by HPTLC. The profiles were compared with the reference standards (Table **2**), tinosineside A, borapetoside B, borapetoside C, N-trans feruloyl tyramine, baenzigeride A and columbin to determine authenticity of the raw materials, variations in their phytoconstituents (Fig. **6**), and quality of the *Tinospora* products sold as dietary supplements (Fig. **7**). Based on the HPTLC fingerprints, *T. cordifolia* (#20769, 5799, 5212, 8069, 10107 and 20866) showed similar band patterns with quantitative variation of compounds. The samples #17303, 20865, 20867 labeled *T. cordifolia* did not show profiles similar to the genuine stem extract of *T. cordifolia.* Tinosineside A ($R_f$ 0.17), the marker compound for *T. sinensis*, appeared among *T. sinensis* samples (# 17003, 3104, 16885, 20863). The stems of *T. crispa* (#20869 & 16849) were of similar profile except sample #17091, which showed weak bands. The major bands observed for *T. crispa* samples, borapetoside B ($R_f$ 0.24) and borapetoside C ($R_f$ 0.47), proved to be useful chemical markers to differentiate *T. crispa* from the other three species of *Tinospora*. *T. baenzigeri* showed a different profile compared to all other species of *Tinospora* studied except for the presence of columbin. The specific bands observed for the identification of *T. baenzigeri* were at $R_f$ 0.16 (yellow) and $R_f$ 0.17 (bluish-green) after derivatization with vanillin–sulfuric acid. Figure **7** shows the HPTLC fingerprint of dietary supplements in comparison with the standard reference mixture. Dietary supplements claiming to contain *T. crispa* (Tracks **2**-**7**) are easily

distinguishable from those of *T. sinensis* and *T. cordifolia*. Among *T. crispa* dietary supplements (# 20754, 20756, 20760, 20762, 20798 and 20799), sample #20798 showed the presence of all marker compounds, whereas the sample # 20754 showed weak bands probably due to low quantities of the marker compounds. *T. sinensis* product (Track **8**) showed the presence of marker compound tinosineside A. Profiles of all *T. cordifolia* products matched with the genuine *T. cordifolia* except for sample # 20864, which showed additional bands with different Rf values indicating the presence of other undisclosed ingredients in the product. The bands present in sample # 20753 (Track **9**) were not in concord with the other tracks in the group of supplements claiming *T. cordifolia* (**10**-**18**). Comparison of sample # 20753 with other samples showed a similarity of its profile with those of the supplements claiming to contain *T. crispa*. The profile for sample # 20765, a supplement purported to contain *T. cordifolia* showed a band ($R_f$ 0.13) that corresponded with tinosineside A, a chemical marker of *T. sinensis*, which disputes the quality and authenticity of the dietary supplement. These observations from HPTLC chromatograms were in agreement with an earlier report of analysis of *Tinospora* species and dietary supplements using an ultra-high performance liquid chromatography coupled with photodiode array and single quadrupole electrospray mass spectrometry detectors [37].

A pharmacognostic comparison of four species of *Tinospora*, namely *T. cordifolia, T. crispa, T. sinensis*, and *T. baenzigeri*, was made in this study by morpho-anatomy, histochemistry, and HPTLC analyses. The traditionally used part of this plant is the stem, which is widely formulated in many products and sold in the marketplace. This study provided critical information to elaborate on the differences among the often misidentified *Tinospora* species. The presence of leaf epicuticular wax is characteristic of *T. cordifolia* and a notable feature to distinguish it from the other *Tinospora* species. Of the four studied species, only *T. sinensis* presents the non-glandular covering trichomes in all studied plant parts. The morphotype and distribution of calcium oxalate crystals offer important diagnostic character to identify *Tinospora* species. HPTLC fingerprinting of these *Tinospora* species facilitated the qualitative assessment of the extracts with qualified reference standards to distinguish between different species. The HPTLC profiles of 34 *Tinospora* samples analyzed in this study showed that there exists a high chance of mix-up among them and/or usage of different *Tinospora* species in place of *T. cordifolia*, which is the species of importance in commerce. *T. baenzigeri* showed a distinct profile with two bands at $R_f$. 0.16 as yellow and $R_f$ 0.17 as bluish-green. Thus, the HPTLC fingerprinting using *T. crispa* and *T. sinensis* specific markers enabled differentiation

of these species from other closely related *Tinospora* species, providing a fast and efficient method for identification as well as quality control of the products. The present study is also expected to form a basis for future studies of *Tinospora* species involving plant materials from various geographical regions across their natural distribution at different growth levels and dioecy in order to better understand the variability of morpho-anatomical and phytochemical characteristics.

## Materials and Methods

### Botanical materials

Fresh materials of leaves, petioles, and stems of *T. cordifolia* (NCNPR # 20875), *T. crispa* (# 20877) and *T. sinensis* (# 20876) were collected from India for morpho-anatomical studies. Authentic samples of *T. baenzigeri* stems (# 20870) were obtained from Thailand. The collected plant samples were authenticated by the botanists Dr. Ganesh Babu, The University of Trans-Disciplinary Health Sciences and Technology, Bangalore, India and Dr. Vijayasankar Raman, NCNPR, University of Mississippi, USA). HPTLC analyses were conducted for 17 *Tinospora* plant samples obtained from various authentic and commercial sources. In addition, 17 dietary supplement products purported to contain *Tinospora* species were purchased from online sources. The materials (Table **3**) were assigned with unique numbers and the voucher specimens were submitted at the Botanical Repository in the National Center for Natural Products Research (NCNPR) at the University of Mississippi, USA.

### Preparation of samples for light microscopy

Fresh materials of leaves, petioles, and stems were fixed in FAA (formalin-acetic acid-alcohol) solution for seven days [38], washed in distilled water and used in microscopy analyses. Transverse sections were made using razor blades manually, double-stained with basic fuchsin and Astra blue [39]. Histochemical evaluations were performed using specific procedures for localizing starch, lignin, tannins, and total lipids and alkaloids [40-42]. Mounts were prepared on glass slides with 50% glycerol solution. Leaf specimens were cleared using bleach (2.5% sodium hypochlorite) to study epidermal features. Translucent specimens were washed with water thrice and stained in safranin. Photomicrographs were prepared using a Nikon Eclipse E600 fluorescence microscope equipped with Nikon DSFiv camera systems and Nikon Elements imaging software (Nikon Inc., Tokyo, Japan). Calcium oxalate crystals and starch

grains were observed using a Nikon Eclipse E600POL polarized microscope equipped with the Nikon DSFiv camera system and Nikon Elements imaging software.

**Micrometrics**

Various microscopic features were measured (Table **1)**. Twenty- to thirty cells/structures were measured from multiple sections from each species and the measurements were combined. The stomatal index (SI) was calculated for 100 sq. µm area as viewed under a 40x objective lens, using the formula [$SI = \frac{S}{S+E} \times 100$], where S= total number of stomata; E= total number of epidermal cells.

**Preparation of samples for scanning electron microscopy (SEM)**

Specimens fixed in FAA were washed with water and passed through 30%, 50%, 70%, 90% and 100% ethanol solutions. The samples were dried using a Leica CPD300 critical point dryer (Leica Microsystems, Wetzlar, Germany) supplied with liquid $CO_2$, mounted on aluminum stubs with double-sided adhesive carbon tapes and then coated with platinum using a Desk V HP sputter coater (Denton Vacuum, Moorestown, NJ, USA) supplied with argon gas. The samples were imaged using a JSM-7200FLV field-emission SEM (JEOL Ltd., Tokyo, Japan). Calcium oxalate crystals were analyzed and identified using an EDS (Energy-Dispersive X-ray Spectroscopy) detector (Oxford Instruments, Oxford, UK) attached to the SEM.

**Mixture of standards used for HPTLC**

The standards included tinosineside A ($R_f$ 0.17), borapetoside B ($R_f$ 0.24), borapetoside C ($R_f$ 0.47), N-trans feruloyl tyramine ($R_f$ 0.87), baenzigeride A ($R_f$ 0.92), and columbin ($R_f$ 0.95). All standards were isolated from *T. crispa* except tinosineside A which was isolated from *T. sinensis* at the NCNPR, University of Mississippi. Stock solutions of 1mg/mL were prepared for each standard and a standard mix of 0.1mg/mL was prepared as reference. The $R_f$ value of each standard in the reference mixture and color observed after derivatization is given in Table **2**.

**Preparation of plant and dietary supplement samples for HPTLC**

Dried stems were ground into fine powders using a Mixer Mill MM400 (Retsch, Haan, Germany). For HPTLC analysis, 1.0 g powder of each sample was extracted successively with 10 mL methanol. Extraction was performed by sonication in an ultrasonic bath sonicator at room temperature for 15 min, followed by centrifugation for 5 min at 3000 rpm. The

supernatant was filtered through a 0.45 μm PTFE membrane and evaporated in a speed vacuum (Savant SC250DDA SpeedVac Plus vacuum; Thermo Scientific, Waltham, MA, USA). 20 mg of dried extract was dissolved in 1 mL methanol for application.

**Instrumentation and chromatographic conditions for HPTLC**

The HPTLC system (Camag, Muttenz, Switzerland) consists of a CAMAG Automatic TLC Sampler 4, automatic developing chamber CAMAG ADC2, a TLC Visualizer, and an immersion device. VisionCATS ver. 2.1 software was used for device controlling and image processing. HPTLC plates (silica gel 60 $F_{254}$, glass-backed, 200 μm, 20 × 10 cm) (Millipore Sigma, MA, USA) were used for analysis. All samples were applied according to the following settings: 8 mm from the bottom of the plate and bandwidth 8 mm. A constant application rate of 150 nL/s was employed. The images of the chromatograms were taken using the digital camera on the Visualizer. The plates were developed using chloroform-ethyl acetate-ethanol-0.1% formic acid: 10-15-5-0.5(v/v/v/v) as mobile phase. Plates were developed in a Camag automatic developing chamber ADC2 presaturated with 25 mL of mobile phase at room temperature (23 ± 2 °C) at a relative humidity of 39 ± 5% and 10 mL of mobile phase for the development of plates at the same temperature and relative humidity. After development, the plates were derivatized by dipping into vanillin–sulfuric acid using the immersion device (dipping time 0 s, dipping speed 5 cm/s), followed by heating at 100°C for 3 min, and then images were recorded again under UV 366 nm and white light, respectively.

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**Conflict of interests**

The authors declare that there are no conflicts of interests.

**References**


[1] Ahmad SM, Hoot SB, Qazi PH, Verma V. Phylogenetic patterns and genetic diversity of Indian *Tinospora* species based on chloroplast sequence data and cytochrome P450 polymorphisms. Plant Syst Evol 2009; 281: 87− 96

[2] De Wet H, Struwig M, Van Wyk B-E. Taxonomic notes on the genera *Tiliacora* and *Tinospora* (Menispermaceae) in southern Africa. S Afr J Bot 2016: 283−294

[3] Xianrui L, Tao C, Gilbert MG. Menispermaceae. In: Wu Zy, Raven PH, Hong DY eds. Flora of China. Volume 7 Menispermaceae through Capparaceae. Beijing: Science Press China; Beijing, 2008:1−31

[4] Chi S, She G, Han D, Wang W, Liu Z, Liu B. Genus *Tinospora*: Ethnopharmacology, Phytochemistry, and Pharmacology. Evid-Based Compl Alt 2016; 2016: 9147974

[5] Nadkarni KM, Nadkarni AK. Indian Materia Medica. 3rd edition. Mumbai: Popular Prakashan Pvt. Ltd; 1976

[6] Singh J, Sinha K, Sharma A, Mishra NP, Khanuja SP. Traditional uses of *Tinospora cordifolia* (Guduchi). J Med Aromat Plant Sci 2003; 25: 748−751

[7] Khare CP. Indian Medicinal Plants: An Illustrated Dictionary. Springer. USA, 2007

[8] Tiwari P, Nayak P, Prusty SK, Sahu PK. Phytochemistry and Pharmacology of *Tinospora cordifolia*: A Review. Syst Rev Pharm 2018; 9: 70−78

[9] Singh V, Pandey RP. Ethnobotany of Rajasthan India. Jodhpur. India: Scientific publisher; 1998

[10] Lalramnghinglova H. Ethno-Medicinal Plants of Mizoram Dehradun, Dehradun: Bishen Singh Mahendra Pal Singh; 2003

[11] Upadhyay A, Kumar K, Kumar A, Mishra H. *Tinospora cordifolia* (Willd.) Hook. f. and Thoms. (Guduchi) - validation of the Ayurvedic pharmacology through experimental and clinical studies. Int J Ayurveda Res 2010; 1: 112

[12] Praman S, Mulvany MJ, Allenbach Y, Marston A, Hostettmann K, Sirirugsa P, Jansakul C. Effects of an n-butanol extract from the stem of *Tinospora crispa* on blood pressure and heart rate in anesthetized rats. J Ethnopharmacol 2011; 133: 675−686

[13] Ahmad W, Jantan I, Bukhari SNA. *Tinospora crispa* (L.) Hook. f. & Thomson: A review of its ethnobotanical, phytochemical, and pharmacological aspects. Front Pharmacol 2016; 7: 59

[14] Qin XY, Luo JY, Gao ZG. Yao Ethnic Medicinals in China. Beijing: Ethnic Publish House; 2002:50

[15] Manjrekar PN, Jolly CI, Narayanan S. Comparative studies of the immunomodulatory activity of *Tinospora cordifolia* and *Tinospora sinensis*. Fitoterapia 2000; 71: 254−257

[16] Li RW, David LG, Myers SP, Leach DN. J. Anti-inflammatory activity of Chinese medicinal vine plants. Ethnopharmacol 2003; 85: 61−67

[17] Singh N, Kumar A, Gupta P, Chand K, Samant M, Maurya R, Dube A. Evaluation of antileishmanial potential of *Tinospora sinensis* against experimental visceral leishmaniasis. Parasitol Res 2008; 102: 561–565

[18] Zhang, H.S, Zhang, l.M., and Zhang HH. The observation of curative effect of knee ankylosis after injury auxiliary through huoxue Shujin Xunxi recipe. Shaanxi J Tradit Chinese Med 2010; 31: 997–998

[19] Katib S, Ruangrungsi N, Palanuvej C, Chareonsap PP, Rungsihirunrat K. Macroscopic-microscopic characteristics and AFLP marker for identification of *Tinospora crispa* and *Tinospora baenzigeri* endemic to Thailand. J Health Res 2017; 31: 143–149

[20] Raghunathan K, Mitra R. Pharmacognosy of indigenous drugs. New Delhi, India: Central Council for Research in Ayurveda and Siddha; 1982

[21] Chandran S, Harisha CR, Pagiri BJ. Pharmacognostical variations in market samples of *Tinospora* species and their extracts. Glob J Res Med Plants Indig Med 2016; 5: 300–312

[22] Sereena K, Remashree AB. Histological, histochemical and phytochemical studies of the raw drug Amrita from different raw drug markets of Kerala. Int J Interdiscip Multidiscip Stud 2014; 1(5):182−91

[23] Patil VS, Malpathak NP. Micro-morphoanatomical approach for comparative analysis of *Tinospora cordifolia* (Willd.) Miers and its adulterant plant using SEM and Cryostat. Phcog J 2017; 9(1):39−45

[24] Solereder H. Systematic anatomy of the dicotyledons. English ed. Oxford:Clarendon Press; 1908

[25] Bonde, S.D. and Upaehye AS. Contribution to the wood anatomy of *Tinospora sinensis* (Lour.) Merrill in Relation with T. Cordifolia Miers. Anc Sci Life 1989; 9 (2): 80–85

[26] Anonymous. The Ayurvedic Pharmacopeia of India. Vol. 1 Part 1. Pharmacopoeia Commission for Indian Medicine & Homoeopathy, New Dehli: Ministry of AYUSH, Government of India; 2001

[27] Anonymous. Quality standards of Indian Medicinal Plants, Volume 1. New Delhi: Indian Council of Medical Research (ICMR); 2005

[28] Pradhan D and Pandey A. Estimation of starch extracted from different diameter thickness stem of Giloe (*Tinospora cordifolia* Willd Miers). J Pharmacogn Phytochem 2013; 2: 162–163

[29] Choudhary N, Siddiqui MB, Khatoon S. Pharmacognostic evaluation of *Tinospora cordifolia* (Willd.) Miers and identification of biomarkers. Indian J Tradit Knowl 2014; 13: 543–550

[30] Khatoon S, Irshad S, Vijayakumar M, Choudhry N, Siddiqui ZA, Kumar N. Pharmacognostic Analysis of *Tinospora cordifolia* (Thunb.) Miers, with respect to Dioecy. Single Cell Biol 2018; 7: 1-10

[31] Begum SN, Patturaj R, Ravikumar K. Comparative pharmacognostical and histochemical studies on the three different species of *Tinospora* on stem and leaf. J Pharmacogn Phytochem 2019; 8: 650–655

[32] Srinivasan GV, Unnikrishnan KP, Rema Shree AB, Balachandran I. HPLC Estimation of berberine in *Tinospora cordifolia* and *Tinospora sinensis*. Indian J Pharm Sci 2008; 70(1): 96−99

[33] Li W, Koike K, Liu L, Lin L, Fu X, Chen Y, Nikaido T. New Lignan Glucosides from the Stems of *Tinospora sinensis*. Chem Pharm Bull 2004; 52 (5): 638−640

[34] Rickson FR, Risch SJ. Anatomical and ultrastructural aspects of the ant-food cell of *Piper cenocladum* C.DC. (Piperaceae) Am J Bot 1984; 71:1268–1274.

[35] Carlquist S. Wood and Stem Anatomy of Menispermaceae. Aliso. A J Syst Evol Bot 1996; 14: 155–170

[36] Parveen A, Wang Y-H, Fantoukh O, Alhusban M, Raman V, Ali Z, Khan IA. Development of a chemical fingerprint as a tool to distinguish closely related *Tinospora* species and quantitation of marker compounds. J Pharm Biomed Anal 2019; 178: 112894

[37] Johansen DA. Plant Microtechnique. New York: Mc Graw Hill Book; 1940

[38] Roeser KR. Die Nadel der Schwarzkiefer-. Massenprodukt und Kunstw der Natur Mikrokosmos 1972; 61: 33–36

[39] Krishnamurthy KV. Plant histochemistry. Madras: S. Viswanathan Pvt. Ltd.;1988

[40] Graham DM. and Spencer M. Quantitative isolation and improved nephelometric micro determination of lupine alkaloids from plant tissues. Anal Chem 1964; 36: 210–213

[41] Yoder LR and Mahlberg PG. Reactions of alkaloid and Histochemical indicators in laticifers and Specialized Parenchyma Cells of *Catharanthus roseus* (Apocynaceae).

**Figure Legends**

Fig. **1** Leaf and stem morphology of *Tinospora*. (**A**) *T. cordifolia*, (**B**) *T. crispa*, (**C**) *T. sinensis*. Scale bar = 1 inch.

Fig. **2** Leaf micro-morphology of *Tinospora* species. **A-F** – *T. cordifolia*; **G-J** – *T. crispa*; **K-P** – *T. sinensis*. [**B**, **C** – Light microscopy; all others SEM]. **A**, **B**, **G**, **K**, **L** – Leaf adaxial surface; **C-F**, **H-J**, **M-P** – Leaf abaxial surface. Epicuticular wax is present only in *T. cordifolia* on both surfaces clearly visible in SEM images (**A**, **D**, **E**, **F**). Pearl glands (**B**, **F**, **J**) with unicellular head and a short stalk are observed in both leaf surfaces in *T. cordifolia* and *T. crispa*. Anomocytic stomata (**C-F**, **H**, **I**, **P**) are present in the abaxial leaf surface in all three species. Non-glandular covering trichomes are present only in *T. sinensis*; sparse and often with collapsed apical cells (**K**, **L**) in the adaxial surface, and densely tangled and twisted tomentose trichomes (**M-O**) in the abaxial surface. [cc- collapsed cells; ep- epidermal papillae; ew- epicuticular wax; gt- glandular trichome; st- stomata]. Scale bars: **A-D**, **H**, **L** = 50 µm; **E**, **F**, **J** = 10 µm; **G**, **O** = 100 µm; I, P = 5 µm; **K**, **M**, **N** = 500 µm.

Fig. **3** Leaf and stem anatomies of *Tinospora* species. **A**, **D**, **G** – *T. cordifolia*; **B**, **E**, **H** – *T. crispa*; **C**, **F**, **I** – *T. sinensis*. Transections of the lamina (**A-C**), midrib (**D-F**), and portions of the petioles (**G-I**). [ck- cork, co- cortex, ep- epidermis, gt- ground tissue, pa- palisade, ph- phloem, pi- pith, sc- sclerenchyma, sp- spongy tissue, tr- trichome, vb- vascular bundle, xy- xylem]. Scale bars: **A**, **C**, **D** = 100 µm; **B**, **G** = 200 µm; **E**, **F**, **H**, **I** = 500 µm.

Fig. **4** Stem anatomy of *Tinospora* species. **A-C** – *T. cordifolia*; **D-F** – *T. crispa*; **G-I** – *T. sinensis*; **J-L** – *T. baenzigeri*. Transections of stems showing arrangement of cork (ck), cortex (co), sclerenchyma (sc), vascular bundles with phloem (ph) and xylem (xy), and pith (pi). Pericyclic fibers (fi) are observed in *T. baenzigeri* (**J**) and tyloses (ty) in the xylem vessels are found in all species except *T. crispa*. Well-developed cork with multilayered phellem (pm) was found in *T. crispa* and *T. sinensis*. Scale bars: **A** = 1 mm; **B**, **L** = 100 µm; **D**, **E**, **G**, **H**, **J**, **K** = 500 µm; **C**, **F**, **I** = 50 µm.

Fig. **5**. Ergastic substances observed in *Tinospora* species. **A-D** – *T. cordifolia*; **E-G** – *T. crispa*; **H-L** – *T. sinensis*; **M-O** – *T. baenzigeri*. Various forms of calcium oxalate crystals were observed in the foliar veins (**A**, **H**), leaf epidermal cells (**B**, **E**), petioles (**I-2** and **J-2**) and stems (**C**, **I-1**,**3**,**4**, **J-4**, **M-O**) as well as stem tyloses (**C**). Starch grains (**D**, **F**, **G**, **K**) are commonly seen in the leaves, petioles, and stems. Circular, aleuron-like bodies containing crystals were observed in the leaves of *T. sinensis* (**L-1** & **L-2**). Scale bars: **A**, **H** = 100 µm; **B**, **C-1**, **E**, **F-1**, **F-2**, **O-2** = 50 µm; **D**, **G**, **I**-3, **K**, **L-1**, **L-2**, **M-2**, **N**, **O-1** = 10 µm; **C-2**, **I-2**, **J-1**, **J-2**, **J-4**, **M-1** = 5 µm; **I-1**, **I-4**, **J-3** = 1 µm.

Fig. **6** HPTLC of *Tinospora* plant materials under UV 366 nm prior to derivatization (**A**) and white light after derivatization (**B**). ***Track details***: Track **1**: Reference standards [**i**. Tinosineside A (Rf. 0.17), **ii**. Borapetoside B (Rf. 0.24), **iii**. Borapetoside C (Rf. 0.47), **iv**. N-trans feruloyl tyramine (Rf. 0.87), **v**. Baenzigeride A (Rf. 0.92), **vi**. Columbin (Rf. 0.96)]; Tracks **2-18**: Plant materials of *Tinospora* [**2-10** *T. cordifolia*, **11-13** *T. crispa*, **14-17** *T. sinensis*, **18** *T. baenzigeri*].

Fig. **7** HPTLC of *Tinospora* products under UV 366 nm prior to derivatization (**A**) and white light after derivatization (**B**). ***Track details***: Track **1**: Reference standards [**i**. Tinosineside A (Rf. 0.17), **ii**. Borapetoside B (Rf. 0.24), **iii**. Borapetoside C (Rf. 0.47), **iv**. N-trans feruloyl tyramine (Rf. 0.87), **v**. Baenzigeride A (Rf. 0.92), **vi**. Columbin (Rf. 0.96)]; Tracks **2-18**: Dietary supplements of *Tinospora* [**2-7** *T. crispa*, **8** *T. sinensis*, **9-18** *T. cordifolia*].

**Table 1 Comparative morpho-anatomy of *Tinospora* species**

| **Morpho-anatomical features** | ***T. cordifolia*** | ***T. crispa*** | ***T. sinensis*** |
|---|---|---|---|
| Leaf shape | Broadly ovate | Broadly ovate to suborbicular | Broadly ovate to subrotund-suborbicular |
| Size of leaf blade (l x b) | 8-11 x 5-9 cm | 6-13 x 6-13 cm | 7-14 x 5-13 cm |
| Leaf base and apex | Shallowly to deeply cordate and palmately 5-7-veined at base; Acute-acuminate at apex | | |
| Leaf margin | Entire, glabrous | | Entire to broadly dentate towards the apex, ciliate |
| Leaf indumentum | Glabrous on both surfaces | | Adaxially pubescent, abaxially tomentose |
| Pearl glands/glandular trichomes on leaf surfaces | Ellipsoid, about 53 x 28 µm | Ovoid-subglobose, about 38 x 39 µm | Not observed |
| Non-glandular covering trichomes | Absent | | Present in the leaf, petiole, and stem |
| Epicuticular wax | Present on both surfaces of the leaf | Absent | |
| Vein Islet number (per sq. mm) | 12.9 | 13.5 | 12.9 |
| Average size of upper epidermal cells in surface view (l x b) | 53 x 35 µm | 42 x 33 µm | 44 x 27 µm |
| Average palisade ratio | 9.61 | 7.25 | 5.56 |
| Stomatal Index | 9 | 14 | 30 |
| Average size of stomata (l x b) | 24 x 20 µm | 26 x 20 µm | 21 x 17 µm |
| Sclerenchyma cap in midrib vascular bundle | Absent | Absent | Present |
| Petiole length | 4-6 cm | 5-15 cm | 6-13 cm |
| Number of vascular bundles in the petiole | 8-9 | 12 | 13 |
| Tyloses in the vessels of the stem | Observed | Not observed | Observed |
| Alkaloids | Detected | Not detected | Detected |
| Lipids | Detected | Not detected | Detected |

**Table 2 $R_f$ value of the reference standards in the standardized mobile phase viewed before and after derivatization with vanillin-sulphuric acid at 254 nm and visible light.**

| # | Reference Standards | $R_f$ | UV active (254nm) | Color |
|---|---|---|---|---|
| i | Tinosineside A | 0.17 | - | Pink |
| ii | Borapetoside B | 0.24 | - | Pink |
| iii | Borapetoside C | 0.47 | UV active | Pink |
| iv | N-trans feruloyl tyramine | 0.87 | UV active | Pink |
| v | Baenzigeride A | 0.92 | - | Pink |
| vi | Columbin | 0.95 | - | Bluish grey |

**Table 3 *Tinospora* stem samples and dietary supplements used in this study**

| Botanical Name | NCNPR # of authentic (*) and commercial samples of *Tinospora* stems | NCNPR # of commercial products claiming to contain *Tinospora* |
|---|---|---|
| *T. cordifolia* | 5212*, 5799*, 8069, 10107, 17303, 20769*, 20865, 20866, 20867 | 20753, 20758, 20759, 20761, 20763, 20764, 20765, 20767, 20768, 20864 |
| *T. crispa* | 16849, 17091, 20869* | 20754, 20756, 20760, 20762, 20798, 20799 |
| *T. sinensis* | 3104, 16885, 17003*, 20863 | 20766 |
| *T. baenzigeri* | 20870* | |