Pharmacogn J. 2017; 9(1): 39-45
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com | www.journalonweb.com/pj | www.phcog.net

Original Article

Micro-morphoanatomical approach for comparative analysis of *Tinospora cordifolia* (Willd.) Miers and its adulterant plant using SEM and Cryostat

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ABSTRACT

Background: *Tinospora cordifolia* (Willd.) Miers, belongs to Menispermaceae, commonly known as "Guduchi"or "Amrita" and has immense importance in Ayurvedic medicine. Several studies have been carried out on pharmacology, pharmaceutical, anticancer activity and clinical trials of *T. cordifolia*, however not much information is available on the adulterants that are added with the genuine plant. Considerable work has been done on pharmacognosy but very few studies have been performed on the anatomy of *T. cordifolia*. **Objectives:** Comparative micro-morphoanatomical analysis of *T. cordifolia and P. daemia*, is important for quality control of fresh and dried samples of the root, stem and leaves. **Material and Methods:** The plant materials of *T. cordifolia* and *P. daemia* were collected, and identified. Fixed sample subjected to cryostat microtomy and sections were observed under light microscope. Micromorphology were studied by light as well as scanning electron microscope. **Results:** Anatomical studies are cheap and important tool for identification of the correct medicinal plant. Adulterant plant does not contain active principles like Berberine and Tinosporin nor have similar morpho-anatomical characters. Because of the similar appearance and resemblance of dried adulterant plant samples, medicinally important *T. cordifolia* often get adulterated with other plants. SEM study revealed morpho-anatomical differences like the abundance of starch in cortical and ray parenchyma, different types of trichomes, etc. Cryostat techniques were used for sectioning and showed the presence of xylem plates with wide multicelluar rays. **Conclusion:** Morphological and anatomical differences were observed in *T. cordifolia* and *P. daemia*. The given results showed significant differences therefore this study aims to help in accurate identification and avoid adulteration of a medicinally important plant.

Key words: *Tinospora cordifolia, Pergularia daemia*, Morpho-anatomy, Berberine, Tinosporin, SEM study.

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History
- Submission Date: 01-09-2016;
- Review completed: 10-10-2016;
- Accepted Date: 30-10-2016.

DOI : 10.5530/pj.2017.1.8

Article Available online
http://www.phcogj.com/v9/i1

INTRODUCTION

Herbal medicine is an important branch of Ayurvedic Medical Sciences; however, there is a lack of standardised identification methods of medicinal plants and bioactive components. Medicinal plants is a subject plagued by multitudes of problems such as incorrect botanical identity, ambiguous local names due to diverse languages, similar morphological characters, active principles, pharmacognosy, and so on.

Tinospora cordifolia (Willd.) Miers belongs to Menispermaceae, has immense importance in Ayurvedic medicine and is commonly known in India as "Guduchi" or "Amrita". The drug obtained from this plant is generally prescribed for fevers, diabetes, dyspepsia, jaundice, urinary problems, skin diseases, chronic diarrhoea and dysentery.[1,2] "Guduchi-satva", the starch obtained from the stem has high nutritive value and plays an important role in cures to digestive problems and several other ailments. It is also used to treat general weakness, gonorrhoea, secondary syphilis, urinary diseases, impotency, gout, viral hepatitis, skin diseases, and anaemia. In compound formulations, "Guduchi" is used clinically to treat jaundice, rheumatoid arthritis, and diabetes.[3] The pharmaceutical significance of this plant is mainly because of various bioactive compounds found in this plant such as glucoside, alkaloidal constituents including berberine, three fatty alcohol, a bitter glucoside giloin, a nonglucosidic bitter substance gilonin.[4] Most recent studies have been focused on phytochemical, pharmacological and clinical investigations. Many interesting findings in the areas of immunomodulation, anticancer activity, liver disorders and hypoglycaemic have been reported. A micro-morphoanatomical study would play an important role in preventing adulteration. Despite the number of stud-

Cite this article : Patil VS, Malpathak N. Micro-morphoanatomical approach for comparative analysis of *Tinospora cordifolia* (Wild.) Miers and its adulterant plant using SEM and Cryostat. Pharmacog J.,2017;9(1):39-45.

ies carried out on the medicinal importance of *T. cordifolia*, not much information is available on the adulterants that are added with the genuine plant. Similarly, considerable work has been done on pharmacognosy but there have been very few studies on *T. cordifolia*. The comparative anatomical study was carried out by Bonde and Upadhye[5] and according to them *T. sinensis* is often employed in Ayurvedic preparations instead of *T. cordifolia*. Similarly, histological, histochemical and phytochemical studies carried out by Sereena and Remashree[6] also revealed the frequent substitution of *T. cordifolia* with *T. sinensis*. Use of a plant having the same morphological appearance as an adulterant in place of the medicinally important plant is common place. In the Khandesh region of Maharashtra, many tribals unintentionally use *Pergularia daemia* plant as adulterant because of the similar morphology and likeness of dried samples. Therefore this study using micro-morphoanatomical analysis is important for quality control of fresh and dried samples of the root stem and leaves of *T. cordifolia*. Additionally, the present investigation is focused on comparative study of *T. cordifolia* against its adulterant *P. daemia*, as *T. sinensis* has been studied in depth in the past.

MATERIAL AND METHODS

The plant materials of *T. cordifolia* and *P. daemia* were collected from the campus and Botanical garden of S.S. Ayurved Mahavidyalaya, Hadapsar Pune and M.J. college campus, Jalgaon respectively. Dried materials were submitted to Botanical Survey of India (BSI) Herbarium, Pune for authentication; the corresponding voucher numbers are VP-9 and VP-1. Mature leaves and stem materials were also collected and fixed in FAA for further used.[7] Suitably trimmed samples were directly embedded in Leica OCT solution. Serial transverse, tangential and radial longitudinal sections of 15-25 µm thickness were obtained with the help of a Cryostat microtome (Leica CM1520). Sections were stained with Safranin–fast green combination[7] dehydrated through ethanol-xylene series and subsequently mounted in DPX.

For Scanning Electron Microscopic analysis, samples were fixed and dehydrated in an ascending ethanol series[9] and finally in graded series of alcohol: Isoamyl alcohol. Samples were kept in pure IAA and then sputter coated with gold. The leaf surface was then observed using a Scanning Electron Microscope (JEOL JSM-6360A, Japan) at different magnifications, 100x to 10000x.

Small pieces of the stem were macerated with Jeffery's solution[8] at 55°C to 60°C for 24-38 hours and stained with 0.5% aqueous Safranin to obtain the length and width of vessels elements and fibers as well as to study morphology. One hundred readings were taken to obtain the mean and standard deviation. Important results were micro-photographed with a Leica DM 3000 LED research microscope.

RESULTS

General Morphology: General morphology of *Tinospora cordifolia* (Willd.) Miers. and adulterant plant *Pergulari adaemia* (Forssk.) Chiov. are given in Table 1, Figure 1 and Micro-morphology is given in Table 2.

Leaf Anatomy: In all studied species, transverse section of the leaf showed uniseriate epidermis covered with a thin cuticle. It consisted of stomata and trichomes on both, upper and lower surface. SEM study of *Tinospora cordifolia* leaves showed anomocytic stomata with very few trichomes on the midrib. The trichomes had an elongated globous head (Figures 1C, D). Conversely, paracytic stomata were observed in *Pergularia daemia* (Figures 1E, F). Epidermis in both was followed by mesophyll tissue with dorsiventral arrangement of one or two stratum of the palisade and three or four layers of spongy tissue (Figures 2B, D). Length and width of the palisade varied between the plant species. They had 30–40 mm length and 12-16 mm width in *Tinospora* and 40-50 mm length and 07-12 mm width in *Pergularia*. Cross section of the midrib in *Tinospora* showed broad hump on the lower side and a slight convex where-as in *Pergularia* it was convex on the lower side and slightly flat on the upper surface (Figures 2A, B). *Tinospora* had a single large collateral vascular bundle in the center surrounded by thin parenchymatous cells of the midrib whereas 6-7 in *Pergularia*. In lamina portion, minor vascular bundles were placed in both the plant species.

Stem Anatomy

The outer layer was periderm and formation was observed at a very early stage. Periderm was broken as lenticels protruded out breaking the periderm at a number of places in both the plants (Figures 3A, D). Periderm was followed by cortex and was composed of two distinct zones, outer 1 or 2 layered sclerenchymatous and inner parenchymatous 15 to 22 layered in *Tinospora*.

In *Pergularia*, starch grains were present in the parenchymatous region abundantly (Figures 3E, F). Endodermis was single layered and followed by pericycle. It was made up of thick-walled cells which formed a cap-like structure on each vascular bundle in *Tinospora*, while in *Pergularia* the endodermis and pericycle were inconspicuous (Figures 3A, D). The young stem of *Tinospora* comprised of 5 to 8 collateral vascular bundles in a ring whereas 5 to 10 in *Pergularia,* and separated by parenchyma and medullary rays in both plants. A thin layer of cambium was present between the xylem and phloem in young stems of both plants and in mature stem of *Tinospora*, a single layer of cambium was also present in ray regions which was not shown by *Pergularia*. In *Pergularia*, druses were present in phloem parenchyma and cortical parenchyma while in *Tinospora* stem was characterized by specialized starch grains (Figures 3D,C). In young *Tinospora* stem, parenchymatous pith with compact cells containing starch grains was present in the center. In both *Tinospora* and *Pergularia*, mature stem's periderm and cortex were wide and the periderm peeled off periodically (Figures 3B). Cortex was made up of both parenchymatous and sclerenchymatous tissues. In mature *Tinospora* stem, the pericyclic sclerenchyma and phloem capping the vascular bundles were fragmented forming a banded structure of secondary xylem which were not observed in *Pergularia* (Figures 3B D). In *Pergularia*, druses and secretary canals were present in the cortical region, while in *Tinospora* it was filled with starch grains and no druses were observed in the cortex.

Fiber tracheids were 600-700 µm & 400-780 µm in length and 25-30 µm & 12-25 µm in width in *Tinospora* and *Pergularia* respectively. In macerated material we observed vessel and fibre structure difference in given species (Figures 2E, F). Vessels were mostly solitary, however radial or tangential multiples of 3-5 vessels were also observed infrequently. Vessels were mostly oval too long in cross section in both the climbers. Vessel dimorphism were prominently observed in *Tinospora* and vessels were often obstructed by numerous tyloses, which sometimes resulted in complete blockage of the lumen. Such tyloses frequently showed deposition of prominent starch granules (Figures 3C). On the basis of their diameter, vessels could be categorized into wider or narrower vessel elements (Figures 2E, F). The length of the wider vessel elements was less than its diameter. In *Tinospora*, wide vessels were measured from 165-300 µm in length and 270-425 µm in diameter while the narrower vessel elements were longer than their diameter, i.e. they were 237-300 µm in length and 62-100 µm in width. On the other hand, in *Pergularia* vessels were solitary and measured 135-330 µm in length and 45-88 µm in width. Comparatively larger vessels were present and vessel dimorphism was seen prominently in *Tinospora*. Vessels possessed simple perforation plate on their slightly oblique to transverse end walls alternate bordered pits. In *Tinospora,* wide medullary rays of up to 15-25 cells with alternating

Table1: *T. cordifolia* and *P. daemia* are differentiated on the basis of following morphological characters

	***Tinospora cordifolia* (Willd.) Miers.**	***Pergularia daemia* (Forssk.) Chiov.**
Habit	Large, glabrous, perennial, deciduous, climber of weak and fleshy stem	Trellis-vine, fetid- smelling perennial climber, extensive hairy tomentose
Leaves	Simple, glabrous, alternate, exstipulate, membranous, broad ovate to round, deeply cordate at base	Opposite, reniform, ovate, acuminate, glabrous, deeply cordate at base
Flowers	Small, yellow or greenish yellow, terminal racemes/ racemose panicles, the male flowers are usually clustered and female flowers are solitary	Flowers in drooping corymbose cymes, hairy peduncle, lanceolate bracts, acute, densely pubescent
Calyx & Corolla	Sepals & Petals 6 free, smaller than sepals, obovate and membranous.	Calyx villous, sepals ovate-lanceolate and corolla glabrous outside, hairy within, dull greenish yellow or white
Stamens	Stamens 6, free, filaments clavate	Pollinia pendulus, corona double
Ovary	3-6 free carpels, uniovulate mature carpel	2 carpels, biovulate
Fruit	The drupes are ovoid, glossy, succulent, red and pea sized	Fruits (follicles) lanceolate, long-pointed, about 5 cm long, covered with soft spines and seeds are pubescent, broadly ovate

Table 2: Micro-morphology of studied plants

Micro-morphology	***Tinospora cordifolia* (Willd.) Miers.**	***Pergularia daemia* (Forssk.) Chiov.**
Stomata	Anomocytic	Paracytic
Trichome	Trichomes are few only present on vasculature with globous head	Uniseriate multicellular hair
Crystals	Starch-grains, Druses (Calcium oxalate crystals) present	Druses are present in phloem region

xylem plates were observed. Rays were measured about 1450-2300 µm in height and 300-390 µm in width in *Tinospora*. In tangential view, they were heterocellular and most of the rays were procumbent while square and upright cells were especially found on the ray periphery (Figures 3C). *Pergularia* lacked such wide rays. In both species, pith was made up of parenchymatous cells and filled with starch grains (Figures 3E, F).

Root Anatomy: In *Tinospora*, roots were of two types, aerial and underground, while in the case of *Pergularia* it was normal. The young aerial roots were thin and squarish while mature aerial root was circular. The anatomy of underground root started with a single outer epiblema layer made up of thick-walled cells. This was followed by the cortex composed of an outer layer of parenchymatous and inner sclerenchymatous layers in *Tinospora* while in *Pergularia* the outer layer was parenchymatous only. In both studied plants cortex was made up of thin walled, compactly arranged parenchymatous cells containing starch grains. In *Tinospora*, 5-8 vascular bundles were arranged in a ring while in the case of *Pergularia* the number was 5-10. Xylem was exarch with large parenchymatous pith in both studied plants.

DISCUSSION

Both *Tinospora* and *Pergularia* have enormous importance in pharmacology. Their species are widely distributed in the tropical and subtropical regions of India and China[10-12] and have multiple applications in different folk medicine, including the Indian Ayurvedic system.[10-13] The plant *T. cordifolia* has medicinal properties like anti-diabetic, antipyretic, anti-spasmodic, anti-inflammatory, anti-arthritic, antioxidant, anti-allergic, anti-stress, anti-leprotic, antimalarial, hepato-protective, immuno-modulatory and anti-neoplastic activities which have been proven by reverse pharmacological approach[11]. While *P. daemia* is used as anti-helminthic, laxative, antipyretic and expectorant, and also used to treat infantile diarrhoea and malarial intermittent fevers, the latex of this plant used as a toothache cure.[11,14,15] Compared to *Pergularia*, *Tinospora* is more commonly used in various preparations of Ayurvedic medicines as various cited references suggest.

There are various studies available on phytochemical, quantitative and qualitative analysis of both plants.[4,13,16-18] Market samples of *Tinospora* are often found to be adulterated with other species of *Tinospora* like *T. sinensis*.[6] These adulterated samples lack active principles like Berberine, Tinosporin, Tinocordiside, Tinocordifolioside, Cordioside, Cordifolioside-A, Cordifolioside-B, etc. While Sereena and Remashree, [6] mentioned that the drug obtained from *T. cordifolia* was often substituted with *T. sinensis*, no studies were done on adulteration or substitution of *T. cordifolia* with *P. daemia*. Our survey indicated that in some places of Maharashtra, *P. daemia* was frequently used as an adulterant for *T. cordifolia* because of their morphological similarities.

Comparative morphology and anatomy studies have suggested the characteristic differences between *Tinospora* and *Pergularia*, such as the presence of abundant hair on the stem of *Pergularia* which were not observed in *Tinospora*. Anatomical differences were 1) plenty of starch grains seen in *Tinospora* which was not observed in *Pergularia*, instead druses were observed in the cortex, and 2) the transverse section showed xylem plates and wide rays in *Tinospora* stem and root which were absent in *Pergularia*. However, no detailed study has been done on comparative stem and root anatomy of *Tinospora* and *Pergularia*.

Metcalfe, Chalk,[19] reported Rananculaceous (anomocytic) stomata in Menispermaceae, Rubiaceous (paracytic) in Asclapiadaceae. Similarly, present investigation showed similar results which were confirmed by SEM study in greater clarity facilitating accurate identification. Finally, our observations on stomata and trichomes were in agreement with those described by Metcalfe, Chalk.[19]

Anatomical study of *T. cordifolia* has been done by various researchers[5,6,19,20] but there was no comparative study available on *Tinospora* and its adulterant. Stem and root anatomy of *Tinospora* resembled *Aristolochia* stem which is commonly known for its atypical wood anatomical structure, i.e. lignified xylem plates embedded in parenchymatous ray cells. A similar structure was also observed in some members of Menispermaceae, for example in genera *Coscinium*, *Borismene*, *Tinomiscium* and *Penianthus*.[21] Additionally, such structure is not observed in members of Asclepiadaceae; stem cross section of *Pergularia* did not show xylem

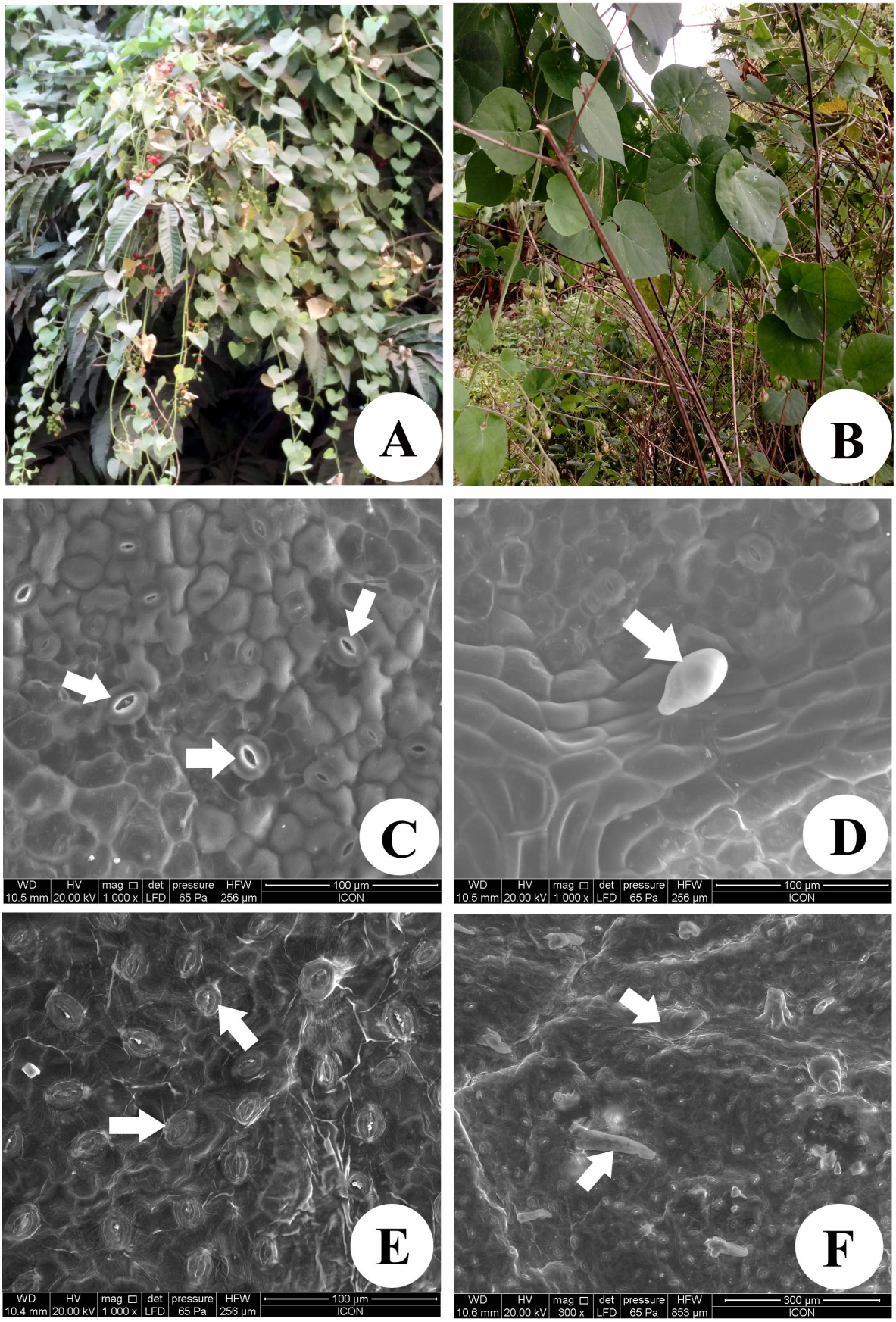

Figure 1: General morphology and scanning Electron microscopy of *T. cordifolia* and *P. daemia*

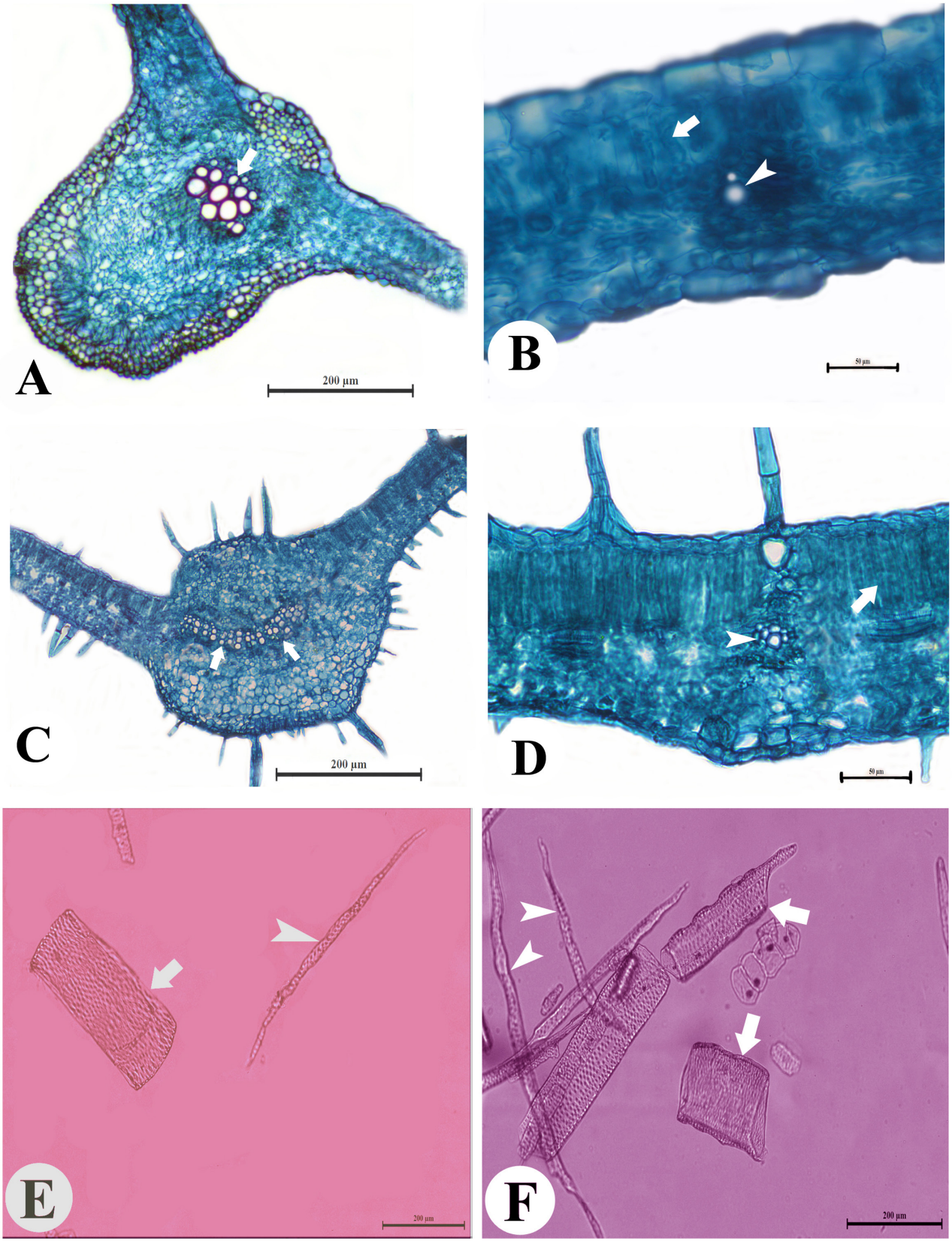

Figure 2: Transverse view of leaf A-D and macerated xylem elements of *T. cordifolia* and *P. daemia*

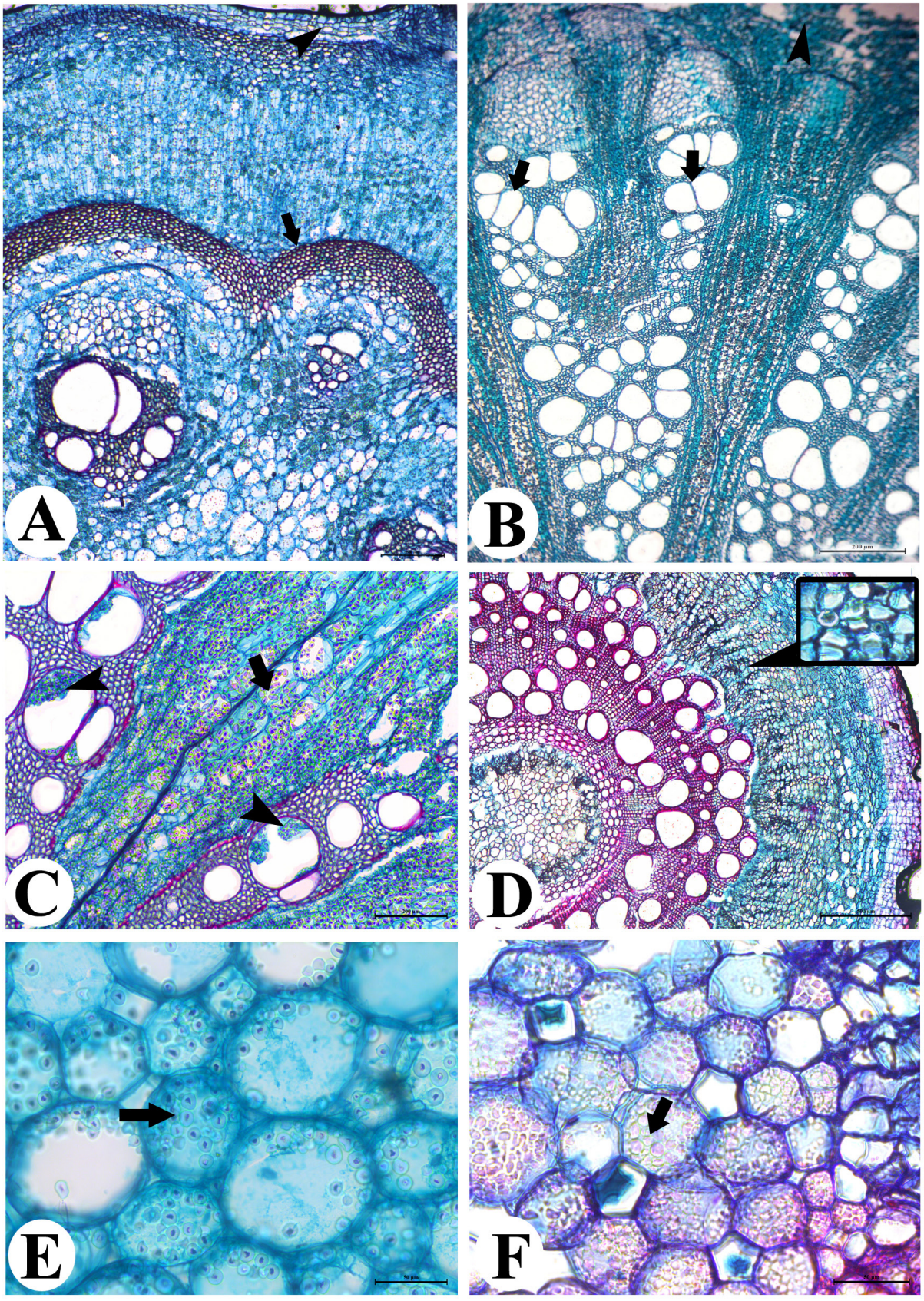

Figure 3: Transverse sections of the stem of *T. cordifolia* and *P. daemia*

plates alternate with wide parenchymatous ray. It is a distinct feature of identification to avoid adulteration. The starch grains in *Tinospora* were very specific in shape, size and structure, and were numerous when compared to the adulterant plants. Druses were present in the cortical region in *Tinospora*, while they were present in phloem region of *Pergularia*. Bonde and Upadhye,[5] reported that a group of stone cells were present in the cortical region and starch grains in all parenchymatous cells and calcium oxalate crystals were absent in cortex. In all Menispermaceae members, ray cells contained either starch grains or remnants of starch grains.[20] Our study is also supported by Bonde and Upadhye,[5] Carlquist[20] 1988 findings.

ACKNOWLEDGEMENT

The authors are thankful to the Dr. DS Kothari Fellowship program and University Grants Commission, for the financial support.

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