Research Article

Pharmacognostic Studies and Preliminary Phytochemical Analysis of Cold and Hot Extracts of Leaf of *Tinospora malabarica* Miers - An Important Medicinal Plant

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ABSTRACT

Herbal medicine is one of the oldest medicines in India, which heals many diseases as mentioned in the Ayurveda and homeopathy. *Tinospora cordifolia* (Willd.) Miers. (Guduchi or Amrita) is one among the medicinal plants that has been used by Indian folk practitioners since ages to treat many diseases. In India, the genus *Tinospora* consists of four species and *Tinospora malabarica* Miers. is the major adulterant for Amrita by the local herbal healers, who are adulterating without standardization. *T. malabarica* Miers. can be confused with *T. cordifolia* (Willd.) Miers. for substitution and tampering purposes. The aim of this study was to investigate the morpho-anatomy and phytochemicals of leaf of *Tinospora malabarica* Miers. for pharmacobotanical data that may contribute to its identification and taxonomic definition from other species of *Tinospora*. The leaf powder was investigated using light microscope which shows the presence of oval or reiniform starch grains, multicellular uniseriate trichomes, annular, spiral and pitted vessels with fibers. The leaf of the plant is hypostomatic; shows anomocytic type of stomata. The preliminary phytochemical analysis of hot and cold extracts, which showed the presence of carbohydrates, proteins, amino acids, glycosides, saponins, tannins and phenols in both the extracts and gums, protein containing sulfur and flavonoids are present only in cold extracts.

Keywords: Leaf, Menispermaceae, Pharmacognosy, Phytochemical analysis, *Tinospora malabarica* Miers.

INTRODUCTION

Tinospora malabarica Miers. is native to India[1] which is commonly known as Chinese Tinospora in English, Gurch or Giloe in Hindi, Kandamrata and Padma guduchi in Sanskrit[2]. It is a large climber, its young parts covered with whitish hairs or trichomes; stem is 1.2 to 1.3 cm in diameter; smooth shining with light colored papery bark and having prominent lenticels. Membranous leaves measures 8.8 to 12.7 cm which is 7 nerved, broadly ovate, cordate, acuminate, and pubescent above whitish tomentose beneath; petioles thickened and twisted at the base reaching the length of 12-13 cm. Flowers are green and unisexual, found in racemes. Perianth 6 in number arranged in two whorls of three each, the outer whorl is small ovate to oblong, obtuse and the inner whorl is large, oblong or sub-orbicular, concave. Perianth in the male flowers are obovate, cuneate, rounded at the apex, not embracing the stamens, stamens are absent in female flowers. Drupes are 1-3 (usually 2), ovoid; endocarp marked externally with many sharp-pointed tubercles[3,4].

In India, there are four species of *Tinospora* found from north to south. In South India, only two species are found namely *T.cordifolia* (Willd.) Miers. and *T. malabarica* Miers. both the species are used to substitute one another by the local practitioners. The leaf juice of *T. malabarica* Miers. mixed with that of *Coleus amboinicus* Lour. and honey is used in the treatment of gonorrhea. In China it is reported that fresh leaves and stems of *T. malabarica* are used in the treatment of rheumatism and similar to *T. cordifolia* (Willd.) Miers. in the traditional system of Indian medicine. The stem of the *T. malabarica* Miers. is reported to treat fever, jaundice, burning sensation, diabetes, piles, skin ailments, respiratory disorders, neurological diseases and for improving intellect[2,5,6].

The various extracts of the plant is reported to show anti-inflammatory activity[7], anti-leishmenial activity[8], anti ulcer activity[9], anti-arthritic[10], anti analgesic activity[11] with different animal models and anti-cancer activity[12] with human malignant cancer cell lines.

Taking into consideration of these previous reports this work was to investigate morpho-anatomical and comparative preliminary phytochemical analysis of hot and cold extracts of leaf which is used therapeutically. The leaf and stem is being used for many medicinal preparations and it is also used as an alternative drug for *T. cordifolia* (Willd.) Miers. This work on pharmacognosy and preliminary phytochemistry on the leaf of *T. malabarica* Miers. was devoted in order to develop pharmacognostic data and identity of this medicinal plant, as well as to compare it to other species of *Tinospora*.

MATERIALS AND METHODS

Collection and Authentication

T. malabarica Miers. was collected from the Botanical Garden at the Karnatak University, Dharwad campus during the month of August, 2013. The plant was identified by one of the authors and a voucher specimen was kept in the department herbaria for the future references.

Pharmacognostic Studies

The plant material was shade dried for about 8-10 weeks in the laboratory and the dried material was coarsely powdered mechanically with the help of a grinder, passed through 20 mesh sieve and stored in an air tight container for further use. To study the anatomical features fresh leaf material was used by taking transverse hand sections and stained. Microscopical characters, physicochemical parameters and phytochemical analysis were carried out using the powder[13,14].

Organoleptic Analysis

Various sensory parameters such as color, odor, taste, size, shape, texture were studied with the help of various sensory organs[13,14].

Microscopic Analysis

Microscopic characters were studied by taking hand sections from the fresh leaf material and stained according to the standard procedures[13,14]. The powder characters were studied by staining the powder with Phloroglucinol: HCl. The microphotographs are taken with the help of Carl Zeiss Axio Imager M2 model microscope fitted with Canon powershot G2 camera.

Stomatal Type

Stomatal types were determined based on the classification of stomata on the grounds of nature and number of subsidiary cells[15].

Stomatal Number and Stomatal Index

Stomatal number is the average number of stomata per square millimeter of the leaf and stomatal index is the percentage which the numbers of stomata forms to the total number of epidermal cells, stomata are being counted on each cell and it was calculated[13] by using following equation:

$$I = \frac{S}{E+S} \times 100$$

Area of Stomatal Aperture

The length and breadth of stomatal aperture was measured using Carl Zeiss Axio Imager M2 model with inbuilt analogue camera (ProgRess® C5- JENOPTIK), measurements are noted with the help of ProgRes® Capture Pro 2.8-JENOPTIK optical system software (µm) and the area of stomatal aperture was calculated[15] using the following formula:

$A = \frac{\pi}{2} \times l \times bA = \frac{\pi}{2} \times l \times b$ μm^2 (since it is a semicircle).

Where,

A: Area.

I: Length.

b: breadth.

π/2: constant.

Palisade Cell Ratio

The Palisade cell ratio is the average number of palisade cells beneath one epidermal cell of the leaf. It is determined by counting the palisade cells beneath four continuous epidermal cells[13].

Veinlet Termination and Vein Islet Number

Veinlet termination is defined as the number of veinlet termination per square millimeter of the leaf surface, midway between the midrib of the leaf and its margin. A vein termination is the ultimate free termination of veinlet.

Vein islet is the smallest area of green tissue surrounded by the veinlets. The vein-islet number is the average number of vein-islets per square millimeter of the leaf surface. It is determined by counting the number of vein-islets in an area of 4 sq. mm of the central part of the leaf between the midrib and the margin[13].

Physicochemical Analysis

Percentage of total ash value, acid insoluble ash, water soluble ash, extractive values and total percentage of fibers were investigated[13,14].

Fluorescence Analysis

Leaf powder was subjected to freshly prepared chemical reagents tested after 24 hours to study the fluorescence behavior and were exposed to visible light and UV light (short and long wavelength)[16,17].

Extraction and Phytochemical Analysis

Powdered material was subjected to two types of extraction procedures such as hot extraction (Soxhlet extraction) and cold extraction to study the difference.

For hot extraction, 15g of powdered leaf material were taken into the Soxhlet extractor and subjected to successive extraction for 18 hours with different solvents like Petrolium ether, Chloroform, Acetone, Ethanol and Water. In case of cold extraction, 25g of powdered leaf material ware taken successively with 300ml of different solvents (Petrolium ether, Chloroform, Acetone, Ethanol and Water) extracted for 48-72 hours.

Preliminary phytochemical analysis was carried out according to the standard procedure[13,14].

RESULTS

Macroscopic Characters

Macroscopically the leaves of the *Tinospora malabarica* Miers. are cordate, acuminate, membranous, pubescent above and whitish tomentose beneath. Mid rib shows 3 – 7 nerves, nerves grows with respect to the width of the leaf lamina. Petiole of the leaf thickened at the base, cylindrical, pubescent. The leaf measures 17 cm to 20 cm in length and 15.1 to 16.2 cm in width.

(Figure- 1: Habit).

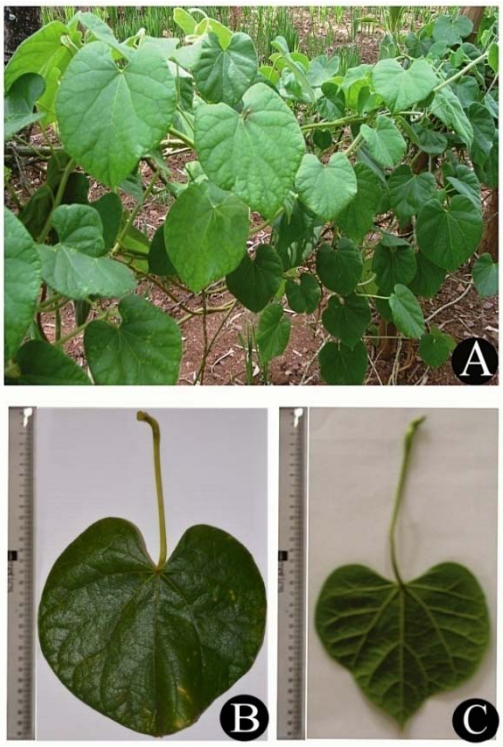

Figure 1: A- Wild plant in Habit. B- Larger leaf. C- Smaller leaf.

Organoleptic Analysis

Organoleptic analysis is one of the parameters to identify crude drug macroscopically by its color, odor, taste and texture by the sensory organs as mentioned below (Table- 1).

Table 1: Organoleptic analysis

Color	Green
Odor	Pungent
Taste	Bitter
Texture	Pubescent

Microscopic Characters

T. S of Petiole (Fig. 2, A)

Transverse section of the petiole shows circular in contour. It shows 1-2 layered tangentially elongated epidermal cells covered by multicellular uniseriate trichomes. Succeeding to the epidermis 10 – 12 layers of paranchymatous cells forming the cortex and few starch grains present throughout. Following the cortex endodermis and pericycle are present which is made of 4-5 layers of sclerenchymatous cells forming a ring around the stele. Stele is present next to the pericycle and is collateral open type consisting phloem and xylem separated by thin band of cambium. The vascular bundles are arranged to form a ring in the paranchymatous ground tissue.

T. S of Leaf (Fig. 2, B)

The transverse section passing through the midrib shows biconvex nature; however the convexity is more conspicuous on the abaxial surface. The upper and lower surface of the leaf consisting of tangentially elongated single layered paranchymatous epidermis covered with multicellular uniseriate trichomes. The epidermis is followed by collenchymatous ground tissue; the lamina is made up of Mesophyll tissues which can be divided into upper palisade and lower spongy parenchyma layers. A single median open type of vascular bundle is embedded in the paranchymatous ground tissue at the mid rib region. The xylem and phloem are arranged in the cup shaped chollenchyma layer in the ground tissue forming the vascular bundle. The transverse section passing through the lamina shows single layered palisade cells followed by several layered spongy cells. The leaf has anomocytic or Ranunculaceous type of stomata, only on the abaxial surface (hypostomatic) which is the characteristic feature of the order Ranunculales. Leaf surface also showed the presence of Vein, Vein islets, Vein terminations and palisade cells. Leaf constants such as stomatal number, stomatal ratio, vein islet number, veinlet termination number and Palisade cell ratio are presented in the table 3.

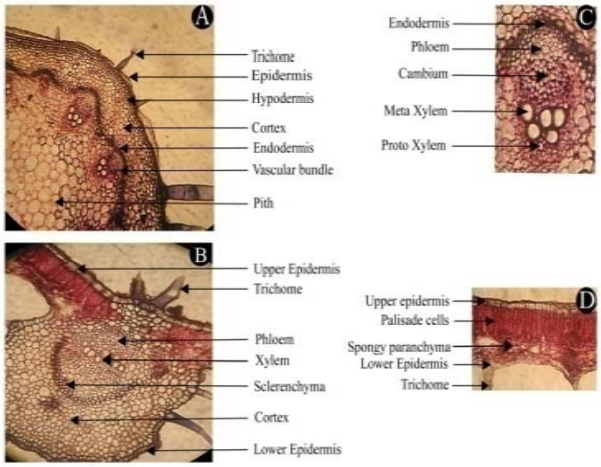

Figure 2: (A) T. S of petiole and (B) leaf of *T. malabarica* Miers.

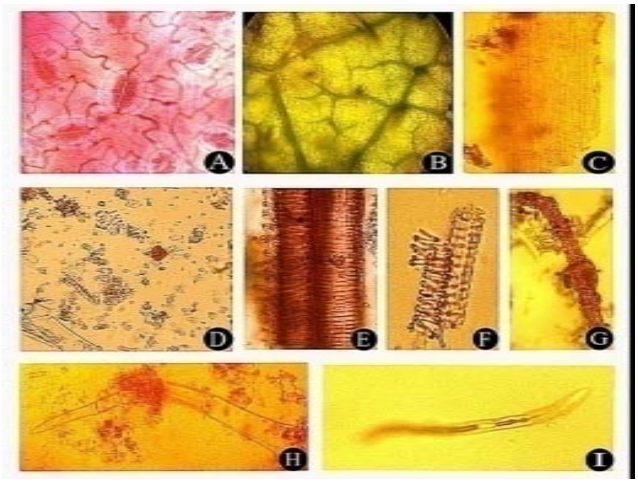

Figure 3: microscopic character of the powdered leaf material of *T. malabarica* Miers. Fig. 3, A- anomocytic stomata, Fig.3, B- vein islets and vein termination, Fig. 3, C- Epidermal cells, Fig. 3, D- oval or reiniform starch

grains, Fig. 3, E- Annular vessels, Fig. 3, F- Spiral vessel, Fig. 3, G- Pitted vessel, Fig. 3, H- multicellular uniseriate trichomes, Fig. 3, I- fiber.

Powder Microscopy

Powdered crude drug consists of multicellular uniseriate trichomes which is characteristic of this particular species as compared to the other *Tinospora* species, oval or reiniform starch grains, pitted vessels, bordered pitted vessels, annular and spiral vessels and fiber (Fig. 3, A- I).

Physicochemical Analysis

Moisture content, ash values, extractive values and determination of crude fibers by Dutch method are as mentioned below (Table- 2).

Table 2: Physicochemical analysis

Parameters		Values (w/w %)
Moisture content		11.6%
Ash values*	Total ash	8.51 ± 0.15
	Sulphated ash	21.28 ± 0.32
Extractive values*	Ether soluble	9.92 ± 0.08
	Alcohol soluble	23.27 ± 0.41
	Water soluble	30.41 ± 0.58
Crude fiber (Dutch method)		24%

* Mean of three readings ± S. D. Calculated based on dry weight of the sample.

Table 3: Leaf constants

Parameters		Values
Stomatal type		Anomocytic stomata
Stomatal number	Upper epidermis	0
	Lower epidermis	25*
stomatal index	Upper epidermis	0
	Lower epidermis	19.49
Stomatal aperture	Length	54.45µm**
	Width	12.03 µm**
	Area	10.472×10^{-2} µm^2
Vein islet number		12-14**
Vein-let termination number		9-12 **
Palisade cell ratio		4-6 (per cell)

* Mean of five readings per microscopic view (40X).
** Mean of ten readings per microscopic view (10X).

Fluorescence Analysis

Powdered material was tested with different chemical reagents and its fluorescence behavior with different wavelength of light was mentioned below (Table- 4). This study helps in identifying some particular colored compounds through its fluorescence nature.

Table 4: Fluorescence analysis

Material with chemical	Day light	UV light (254nm)	UV light (365nm)
Powder as such (P)	Green	-	-
P+ Phloroglucinol: HCl	Brown	Green	Indigo
P+ methanol	Apple green	Apple green	Ceylon red
P+ ethanol	Door country green	Apple green	Ceylon red
P+ Pet. Ether	Peach	Light green	Indigo
P+ Acetone	Apple green	Apple green	Ceylon red
P+ Chloroform	Green	Door country green	Ceylon red
P+ 50% H_2SO_4	Valencia orange	Dark green	Dark green
P+ 50% HNO_3	Valencia orange	Door country green	Dark green
P+ 50% HCl	Brown	Apple green	Ceylon red
P+ 10% NaOH	Brick red	Apple green	Brown
P+ Ammonia	Avocado green	Apple green	Dark green
P+ glacial Acetic acid	Chrome yellow	Apple green	Brown

Preliminary Phytochemical Analysis

The dried powder was successively extracted with various solvents by cold and hot extraction methods and they were tested for various phytochemicals as presented in the table 5.

Table 5: Preliminary Phytochemical analysis of cold and hot extract of leaf of *T. malabarica* Miers.

Test		Extracts									
		Pet. Ether		Chloroform		Acetone		Ethanol		Water	
		Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold	Hot
Carbohydrates	Molish's test	-	-	-	-	-	+	-	+	-	+
Reducing sugar	Fehling's test	+	-	+	+	+	+	+	+	-	+
	Benedict's test	+	+	+	+	+	+	+	+	+	+
Monosaccharides		-	-	-	-	-	+	-	+	-	+
Hexose sugars	Tollen's Phlorglucinol test	+	+	-	+	-	+	+	+	+	+
Non-reducing sugars	Iodine test	-	-	-	-	-	-	-	-	-	-

Gums	Fehling's test	-	-	-	-	+	-	-	-	-	-
	Benedict's test	-	-	-	-	-	-	-	-	+	-
Mucilage		+	+	+	+	+	+	+	+	+	+
Proteins	Biuret test	-	+	-	+	-	-	-	-	-	-
	Millon's test	-	-	-	-	-	-	-	-	-	-
Proteins containing sulfur	Precipitation test	+	-	-	-	-	-	-	-	-	-
Amino acids	Ninhydrin test	-	-	-	-	-	+	-	+	+	+
	Test for tyrosine	-	-	-	-	-	-	-	-	-	-
Steroids	Salkowski test	-	-	-	-	-	-	-	-	-	-
Glycosides	Deoxy sugars	-	+	-	+	+	-	+	-	-	-
Anthraquinone glycosides	Borntrager's test	-	-	-	-	-	-	-	-	-	-
	Modified Borntrager's test	-	-	-	-	-	-	-	-	-	-
Saponins	Foam test	-	-	-	-	-	-	-	-	+	+
Coumarine glycosides	Extract NaOH	-	+	-	+	-	-	-	-	-	-
Flavonoids	Shinodow's test	+	-	+	-	-	-	-	-	-	-
Alkaloids		-	-	-	-	-	-	-	-	-	-
Tannins and phenols	5% $FeCl_3$	-	-	-	-	-	+	-	+	+	+
	Gelatin solution	-	-	-	-	-	-	-	-	-	-
	Acetic acid solution	-	-	-	-	-	-	-	-	-	-
	Potassium di-chromate solution	-	-	-	-	-	-	-	-	-	-
	Dil. Iodine solution	-	-	-	-	-	-	-	-	-	-
	Dil. HNO_3	-	+	-	+	+	+	-	+	-	+
Organic acids	Oxalic acid	-	-	-	-	-	-	-	-	-	-
	Tartaric acid	-	-	-	-	-	-	-	-	-	-
	Citric acid	-	-	-	-	-	-	-	-	-	-
	Malic acid	-	-	-	-	-	-	-	-	-	-

DISCUSSION

Traditionally, *T. malabarica* Miers. has a greater importance in medicinal and Ayurvedic aspects in India. This plant as a reservoir of medicines used as alternate drug for *T. cordifolia* (Willd.) Meirs. by traditional practitioners without standardization[18]. Hence this study is essential for establishment of identity and purity of the drug which may help in standardization of the plant drug. The standard methods used in this study provide to prepare monographs and proper identity of the plant both in its morpho-anatomical and phytoconstituent aspects.

This study provides proper differentiation between *T. cordifolia* (Willd.) Miers. with that of *T. malabarica* Miers. both morphologically and phytochemically. The morphological differences are: (1) the leaf is densely pubescent above and whitish tomentose beneath in *T. malabarica* Miers., but in *T. cordifolia* (Willd.) Miers. it is not pubescent, (2) petioles are densely pubescent in *T. malabarica* Miers. but glabrous in *T. cordifolia* (Willd.) Miers.[19], (3) Presence of multicellular uniseriate trichome is the characteristic feature of *T. malabarica* Miers. which differentiates it from other species of *Tinospora* also.

Anatomically, the petiole show circular contour, having single layered epidermis and wide zone of cortex composed of 3-4 layer of sclerenchymatous endodermis in both species. The sclerenchymatous endodermis is continuous in *T. cordifolia* (Willd.) Miers. while in *T. malabarica* Miers. it is discontinuous or broken into areas forming a cap over the vascular bundles. The vascular bundles show poorly developed radial rows of xylem on the inner side and few rows of cambium on the outer side followed by the phloem in *T. malabarica* Miers., but in *T. cordifolia* (Willd.) Miers. the xylem is well developed, cambium and phloem are similar to *T. malabarica* Miers. The anatomical view of the leaf lamina through mid rib in *T. malabarica* Miers. shows the continuity of the palisade layer extend towards the stelar tissue but spongy is not continuous, while in *T. cordifolia* (Willd.) Miers. mesophyll is not continue towards the stellar tissue. The vascular bundles are more or less similar in both *T. malabarica* Miers. and *T. cordifolia* (Willd.) Miers. The multicellular uniseriate trichomes are present numerously on both upper and lower epidermis in *T. malabarica* Miers. but in *T. cordifolia* (Willd.) Miers. unicellular club shaped glandular hairs are present only on the lower epidermis[19]. Hence, trichomes play a major role in differentiating both species from one another. In both the species the stomata are anomocytic type and present only on the lower epidermis, but the stomatal

index of *T. malabarica* Miers. (19.49) varies with that of the *T. cordifolia* (Willd.) Miers. (10.63).

The length, breadth and area of the stomata in *T. malabarica* Miers. is 55.45µm, 12.03µm and 10.472 $\times 10^{-2}\mu m^2$ where as in *T.cordifolia* (Willd.) Miers. it is 0.561µm, 0.253µm and 0.442μm^2 respectively[20]. Presence of starch grains throughout the tissue is usually more in *T. cordifolia* (Willd.) Miers. than in *T. malabarica* Miers. As mentioned in the results, the moisture content (11.6%), total ash values (8.51 ± 0.15), sulphated ash (21.28 ± 0.32), Pet. ether soluble extractive value (9.92 ± 0.08), alcohol soluble extractive value (23.27 ± 0.41), water soluble extractive values (30.41 ± 0.58) of *T. malabarica* Miers. varies with that of the moisture content (7.4%), total ash value (6.2%), sulphated ash (0.8%), Pet. ether soluble extractive value (2.8%), alcohol soluble extractive value (7.2%) in *T. cordifolia* (Willd.) Miers.[21]

The preliminary phytochemical investigations showed the presence of carbohydrates, reducing and non reducing sugars, gums, mucilage, proteins, amino acids, glycosides, saponins, coumarine, flavonoids, phenols and tannins in different solvent extracts of *T. malabarica* Miers. but in *T. cordifolia* (Willd.) Miers. carbohydrates, proteins, amino acids, steroids, glycosides, saponins, flavonoids and alkaloids are present. Comparing the preliminary phytochemical analysis of *T. malabarica* Miers. with *T. cordifolia* (Willd.) Miers., tests for gums, mucilage, tannins, and phenols showed the presence only in *T. malabarica* Miers. but not in *T. cordifolia* (Willd.) Miers. and alkaloids, steroids showed absence only in *T. malabarica* Miers. but not in *T. cordifolia* (Willd.) Miers.[22-24]

CONCLUSION

In summary, presence of multicellular uniseriate trichomes, less starch content, higher stomatal index and area of stomata keeps *T. malabarica* Miers. differentiated from *T. cordifolia* (Willd.) Miers. morpho-anatomically. Absence of steroids, alkaloids and presence of gums, mucilage, tannins and phenols differentiates *T. malabarica* Miers. from *T.cordifolia* (Willd.) Miers. by its phytoconstituent nature of the leaves.

To conclude, this study on the pharmacognosy and preliminary phytochemical analysis on the leaf of *T. malabarica* Miers., primarily provides the useful knowledge to prepare monographs and to standardize the crude drug along with its phytochemical nature. This study serves its information that differentiates this plant from adulteration by the other plants or plant parts. Though *T. malabarica* Miers. and *T. cordifolia* (Willd.) Miers. belongs to the same genus of the family Menispermaceae, which morphologically looks alike except in one or two aspects that a common man can not differentiate. The morpho-anatomical study made them to differentiate easily from one another and the phytoconstituent nature also differs in some aspects which will add more information to it.

As the *T. malabarica* Miers. is a major adulterant for *T. cordifolia* (Willd.) Miers. by many folk practitioners, this study further helps to identify the differences in phytochemicals of both species in their structural and behavioral properties.

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