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Pharmacognostic Analysis of *Tinospora cordifolia* (Thunb.) Miers, with respect to Dioecy

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Abstract

Objective: *Tinospora cordifolia* (Thunb.) Miers, *Menispermaceae*, is a dioecious creeper, a plant of significant medicinal importance in the traditional systems of medicine and designated as Rasayana in Ayurveda. Vegetative parts of this plant are used as drug. This makes it important to choose right material for efficacy, especially if there could be gender-associated differences in contents (both qualitative as well as quantitative) of the medicinally active or important constituents and metabolites.

Methodology: Macro-microscopical studies, physicochemical parameters, HPTLC and *in vitro* antioxidant activity have been performed as per standard methods.

Results: Present findings have shown that such differences do exist. Male and female plants have different leaf shapes, petiole shapes and length. Quantitative anatomical features also provide basis to distinguish between male and female plants. The size of cortical region, presence of starch grains and mucilage canals were such important feature which was significantly different between the sexes. Total sugar, starch and tannin were also high in female plants. With respect to antioxidant potential and tinosporoside biomarker, female plants were better than male plants.

Conclusion: Our studies have demonstrated that there are gender based differences in morphology, anatomy, physico-phytochemical profiles and antioxidant potential. This study underscores the importance of gender in all the dioecious medicinal plants for their quality control.

Keywords: *Tinospora cordifolia*; Dioecy; Rasayana; Microscopy; HPTLC; Tinosporoside; Antioxidant potential

Introduction

Tinospora cordifolia (Thunb.) Miers, *Menispermaceae*, is one of the important dioecious plants. It is distributed throughout India as well as in China, Burma and Sri Lanka. In India its habitat ranges from Kumaon Mountains to Kanyakumari the southern tip of India [1,2]. This plant has continued to draw the attention of investigators all over the world for over 50 years. In Ayurveda it is designated as a "Rasayana", recommended to enhance general body resistance, promote longevity and as anti-stress and adaptogen [3-5]. However, its importance in use of plant material in traditional medicine seems to have escaped the attention of ancient masters as well as of the present day scientists as is evident from lack of references in global literature on traditional medicine [6]. It is notable that nearly twenty-five plant species with known dioecism are presently being used in traditional systems of medicine. This significant plant is also mentioned in important Pharmacopoeias [7,8]. Almost all the aerial vegetative parts have medicinal properties. Leaves, stem, bark, fruits and even whole plant have been variously used for treatment of a diverse range of disorders including allergies, diabetes, immune system, fever, gout, ulcer, skin inflammation, anti-viral, anti-dote to venoms, respiratory tract infections, rheumatoid arthritis and even cancer [5,9,10]. Several reports on its chemical constituents, medicinal properties and validation of therapeutic claims have already been published without considering its dioecy character [7,8,11-13].

The safety and efficacy of herbal medicines is closely correlated with the quality of the source materials used in their production. When the plant or its parts are used as drug source, it becomes essential to determine the quantitative differences that may exist inherent in the plants used on account of their genetic factors including gender. The differences at gender level there are some basic physiological differences and some metabolites which are found in one gender are absent in other [14]. The phenomenon of dioecy in plants is however, rarer and is confined to about 7% of the known taxa, widely distributed in different orders and families [15]. Thus, in nature it is possible to have a gender-associated qualitative or quantitative difference that will ultimately impact on the drug efficacy for that taxon. Though human awareness of this phenomenon is as old as from the Babylonian times (ca. 2300BC) where different sexes were known for date palms [16]. Gender based differences were first documented in *Cannabis sativa* and that remains the only dioecious plant where detailed studies were made [17]. Total phenolic concentrations were higher in dioecious *Ilex vomitoria* females than males [18]. In case of blue crabs (*Callinectes sapidus*) sex specific differences in metabolites were shown by Kleps et al. [19]. A clear understanding of the gender distinction is thus warranted in the case of medicinal plants of importance to traditional systems of medicine.

All these studies from ancient times till date have used vegetative parts without any prior distinction between male and female plants,

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thus necessitating fresh investigations on gender-based differences. In this communication we present comparison of pharmacognostical parameters of male and female plants of this important Rasayana drug for its quality control and also evaluate their antioxidant potential to validate the efficacy.

Materials and Methods

Collection of plant materials and macro-microscopic analysis

Male and female plants of *Tinospora cordifolia* were collected for studying dioecy variation during flowering season i.e. Jul-Aug. The plant materials were identified by the taxonomist Prof. M.B. Siddiqui and vouchers plant specimen of *T. cordifolia* No. A.M.U/31337 (male), A.M.U/31338 (female) were deposited in Botany Department, Aligarh Muslim University, Aligarh for future reference. Leaf with petiole, aerial roots and stem were separated out from flowering male and female plants to ensure that they represent gender. Samples for anatomical studies were processed following standard procedures. The macroscopy and microscopy of the plant parts was studied according to the well-established method [20]. Hand/cryostat sections of 20-60 µm thickness in transverse (TS) view were taken for the aerial roots, stems, leaves and petioles. The leaves from male and female plants were boiled in saturated chloral hydrate solution for surface studies and quantitative leaf constants viz. vein islet and vein termination, trichomes, epidermal cells, stomatal numbers and stomatal index were studied according to the standard methods [8,21]. All the sections and cleared leaves were examined under Nikon trinocular microscope and photographs were taken using a Lieca ATC 2000 microscope, fitted with a digital camera and were processed and saved using the software DIGI 1.

Physicochemical evaluation

The physico-chemical parameters were calculated as per standard methods given in Ayurvedic Pharmacopoeia of India [8].

Loss on drying: About 10 g of each specimen under study were accurately weighed and transferred to a petri plate which was known for its weight and kept in a hot oven at 100-105°C for an hour. Then the sample was weighed along with petri plate to deduct the actual weight of the tarred petri plate. The weight of the specimen was noted to calculate the percentage loss on drying with reference to air dried specimen.

Alcohol and water-soluble extractives: 5 g of coarsely powdered material was macerated with 100 mL of ethanol, shake frequently for six hours and allowed to stand for eighteen hours. Filtered rapidly, without any loss of solvent, evaporated 25 mL of the filtrate to dryness in a tared petri plate, and dried at 105°C in the Lyzer hot air oven (Thermotech, TIC 4000N), to constant weight and weighed. The percentage of alcohol soluble extractive was calculated with reference to the air-dried powdered material. However, the determination of water-soluble extractive was preceded in the same way, only ethanol was replaced with chloroform-water.

Ash values: About 5 g accurately weighed, powdered material incinerated in a tared silica crucible at a temperature not exceeding 450°C in the Muffle furnace (DBK/5022/3, DBK Instruments, Mumbai) until free from carbon, cooled and weighed. The acid insoluble ash was determined by added 10% hydrochloric acid was to the crucible containing total-ash collected the insoluble matter on an ash less filter paper and washed with hot water until the filtrate was neutralized. The filter paper containing the insoluble matter was transferred to the original crucible and ignited to constant weight in the Muffle furnace. The residue was allowed to cool in suitable desiccators for 30 minutes and weighed. Similarly, water soluble ash as determined by added 25 ml of distilled water and boiled for 5 min. The solution was filtered through ash less filter paper. The residue was washed twice with 5 ml distilled water. The insoluble residue left on filter paper was ignited in a silica crucible at 450-500°C until ash was formed, and the residue was allowed to cool in desiccators for 30 min, weighed. The percentage of total ash, acid insoluble ash and water soluble ash were calculated with reference to the air-dried plant material.

Total sugar and starch content: Total amount of sugar and starch present in the plant material was calculated based on the standard method of Mont Gomery [22] using a spectrophotometer (Thermo electronic, Double Beam UV-vis Spectrophotometer). 10% homogenate of the plant tissue was prepared with 80% ethanol and centrifuged at 2000 rpm for 15 minutes. The supernatant obtained used for sugar content and residue for starch content. Then the supernatant was made up to 10 mL with ethanol and 0.1 mL aliquot was taken from that and added 0.1 mL of 80% phenol and 5 mL conc. H_2SO_4. While, starch was determined by added 4 mL of distilled water to the residue and heated on a water bath for 15 minutes and macerated with the help of glass rod. The samples added 3 mL 52% perchloric acid and centrifuged at 2000 rpm for 15 minutes. The mixture was cooled and then the absorbance at 490 nm was observed to all the samples. D-Glucose was taken as positive control. Standard curve was made by plotting a graph between optical density (OD) and concentrations of different dilutions. The percentage of sugar and starch was calculated.

Tannin content: Total tannin was estimated according to the AOAC method [20]. 2 g powdered plant material was extracted with 100 mL distilled water by boiling on water bath for 6-8 hrs., filtered and made up the volume to 100 mL in the volumetric flask. 5 mL Folin and Ciocalteu"s reagent, 10 mL saturated sodium carbonate (35 g in 100 mL distilled water) were added in 1 mL aliquot of it and made the volume up to 100 mL in a volumetric flask. The instrument was calibrated through blank and took the corresponding absorbance of different samples using the Double Beam UV-vis Spectrophotometer at 760 nm. Tannic acid of different concentration was used as standard and calibration curve was plotted between concentration and optical density (OD).

High Performance Thin Layer chromatography (HPTLC)

Tinosporoside was estimated using HPTLC. The plate was performed on Higlachrosep plates coated with 0.2 mm layers of nano silica containing UV 254 fluorescent indicator (S.D. Fine Chemicals, India). The extracts were diluted to a concentration of 10 mg mL^{-1} in methanol and 20 µL of the extracts were applied on the HPTLC plates using CAMAG Linomat applicator 5. A solution (1 mg mL^{-1}) of tinosporoside was prepared by dissolving 10 mg standard in 10 mL methanol. Test solutions 10, 20 and 40 µL and various dilutions of tinosporoside reference (corresponding to 2, 4, 6, 8, 10 and 12 µL) marker were applied on the HPTLC plates with the help of applicator as described earlier. The plate was eluted to a distance of 8.0 cm at room temperature (25 ± 2°C) in a solvent system – chloroform: methanol: water (8: 2: 0.2 v/v/v) as mobile phase, in a CAMAG twin-trough chamber previously saturated with mobile phase vapour for 30 min. After run the plates were completely dried in air at room temperature and scanned at 220 nm. The linear regression graph was plotted with the help of Win Cats 3.2.1 software and used for estimation of tinosporoside in the samples. All the chemicals used in the experiments were of analytical grade. Reference standard tinosporoside was procured from Dr. Rakesh Maurya, Scientist CSIR-CDRI, Lucknow.

In vitro antioxidant activity

For antioxidant study only stem was used. 10 g of powdered material was extracted with ethanol on a boiling water bath for 25 min, consecutively three times. The combined extract was concentrated under vacuum at 45°C, dried and weighed. Four different protocols were used for evaluation of antioxidant potential.

Reducing power assay: The reducing power of the sample was determined by the method of Oyaizu [23]. An aliquot of the sample (1.0 mL) at various concentrations (20-100 µg/mL) was mixed with phosphate buffer (0.2 M, pH 6.6, 2.5 mL) and 1% potassium ferricyanide (2.5 mL). The mixture was incubated at 50°C for 20 min. After adding 10% trichloroacetic acid (2.5 mL), the mixture was centrifuged at 650 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% iron (III) chloride (0.5 mL), and the absorbance was measured at 700 nm by Spectrophotometer using an appropriate blank. Assay was carried out in triplicate.

2, 2'-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay: The capacity to scavenge the stable free radical DPPH˙ was done according to the method of Blois [24]. 1 mL of the samples mixed with 1 mL methanolic solution containing DPPH radicals (0.135 mM). The mixture was shaken vigorously and left to stand for 30 min in the dark (until stable absorption values were obtained). The reduction of the DPPH radical was determined by measuring the absorption at 517 nm by Spectrophotometer. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: %RSA=[(ADPPH-AS)/ADPPH] × 100, where AS is the absorbance of the solution when the sample extract has been added at a particular level and ADPPH is the absorbance of the DPPH solution. The assays were carried out in triplicate. The extract concentration providing 50% inhibition (IC50) was calculated from the graph of RSA percentage against extract concentration. Ascorbic acid used as standards.

Superoxide (SOD) anion radical scavenging activity: Superoxide (SOD) anion radical scavenging activity was done according to Nishikimi et al., [25]. 1 ml of NBT solution, 1 ml of NADH (468 µM) solution, 0.1 ml of plant extract (10 mg in 0.1 ml DMSO and 0.9 ml Phosphate buffer (pH 8) and 0.1 ml of phenazine methosulfate (60 µM) solution were added together and incubated at 25°C for 5 min. After 5 min the absorbance was read at 560 nm. The assays were carried out in triplicate and result given as 50% inhibition (IC50). Superoxide dismutase (SOD) is a metalloenzyme that catalyze the dismutation of superoxide radical into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) and consequently provide an important defense mechanism against superoxide radical toxicity.

Anti-lipid peroxidation assay using goat liver model: Freshly excised goat liver was processed to get 10% liver homogenate in cold phosphate buffer saline, pH 7.4 using glass Teflon homogenizer and filtered to get clear homogenate. The degree of lipid peroxidation was assayed by estimating the thiobarbituric acid reacting substance (TBARS) by using the standard method of Ohkawa et al., [26] with some modifications. Different concentration (0.1-0.5 µg/mL) of extract was added to the 3 mL of liver homogenate, lipid peroxidation was initiated by adding 100 µl of 15 mM ferrous sulphate and reaction mixture was incubated for 30 min at 37°C. To the 100 µl of this reaction mixture 1.5 mL 10% trichloro acetic acid (TCA) was added, after 10 min centrifuge the mixture at 3000 rpm for 20 min, supernatant was transfer to the fresh tube and 1.5 mL of 0.67% thiobarbituric acid (TBA) (dissolved in 50% glacial acetic acid) was added and incubated the reaction mixture in boiling water bath for half an hour the intensity of pink colour complex was read at 535 nm by Spectrophotometer. The percent inhibition was estimated using the formula (A control-A sample)/A control) × 100.

Result

Macro-microscopic characteristics are the simplest and cheapest methods to start with for establishing the correct identity of the source materials. The general description of macro-microscopy of all the parts of *T. cordifolia* has already been published [27]. However, on comparison the macro-microscopy of male and female plant parts several characteristic features were observed (Figures 1-5). Flowers light yellow in racemes or racemose panicles, arising from nodes on the old stem. The male flowers are clustered and female are solitary. Fruits are drupes, green in colour and turning red when ripe (Figure 1). Transverse section (TS) of the vegetative parts and leaf surfaces of male and female plants shows similar tissue arrangement with some quantitative variations.

TS of aerial root is circular in outline with multilayered exodermis, cells irregular in size and lignified followed by 10-12 cells broad parenchymatous cortex. Xylem vessels are arranged at radii in exarch condition with alternating 1 or 2 broad lumen vessels, surrounded by fibres and tracheids. Phloem is present in 4 groups. Ceratenchyma is observed just above the phloem towards the periferae (Figure 2).

TS of young stem have single layered epidermis, covered with thick cuticle. Hypodermis is collenchymatous, middle cortex chlorenchymatous, inner cortex parenchymatous, present in notch region. Pericycle is sclerenchymatous, broad, continuous and arranged in wavy rings with well-defined ridges and furrows at almost regular intervals. Vascular bundles, many arranged in a ring, bicollateral, closed or open. Phloem is well developed consists of sieve tubes, companion cells and parenchyma. Xylem composed of vessels, tracheids, fibres and parenchyma. Cambium is present towards the periphery. Starch grains are embedded in most of the parenchymatous cells. Starch grains are simple, varying in shapes and sizes. Mucilage canals are also present in hypodermal & cortical region. However, epidermis diminishes and lignified cork is present at maturity. Cork cambium well developed 3-5 cells broad. Hypodermis 8-10 cells broad, collenchymatous and having plenty mucilage canals in this region. Cortex is parenchymatous. Pericycle is sclerenchymatous, discontinuous in arc shaped patches followed by 3-5 cells broad layer of parenchymatous tissue. An arc of ceratenchyma is also present below this region. Phloem is well developed as in young stem, arranged in groups just above the xylem. Xylem consists of broad lumen vessels either solitary or in group of 2-3 surrounded by fibres and tracheids. Medullary rays are radiating and wedge shaped. Central portion is occupied by parenchymatous pith as in young stem. Starch grains are abundant in all the parenchymatous cells. Mucilage canals are also found scattered in cortical, phloem & pith regions. Mucilage canals and tannin containing cells are observed throughout the sections (Figure 3).

TS petiole shows single layered thick walled epidermis covered with thick cuticle followed by 10-12 cells broad hypodermis. Ground tissue is collenchymatous, a wavy line of bi-collateral vascular bundles connected with slight thickened tissue (Figure 4). TS passing through the midrib region of leaf shows slightly ridged on adaxial surface and strongly protuberated at the abaxial surface. Single layered upper and lower epidermis covered with thick cuticle. Meristele centrally located, vascular bundle bicollateral and capped with sclerenchymatous bundle sheath. Phloem and xylem are well developed. The ground tissue is collenchymatous and embedded with numerous mucilaginous canals.

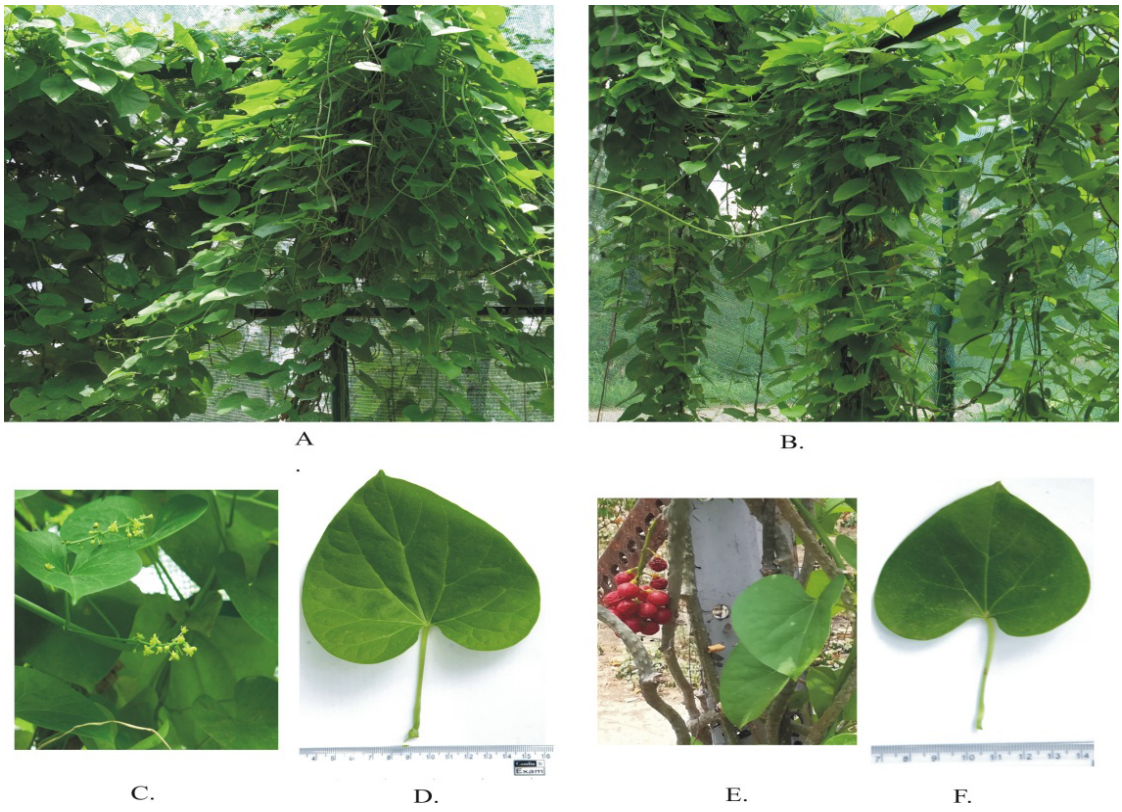

Figure 1: Macroscopy of *Tinospora cordifolia.* (A) Male plant; (B) Female plant; (C) Male flowering twig; (D) Leaf from male plant; (E) Female flowering twig; (F) Leaf from female plant.

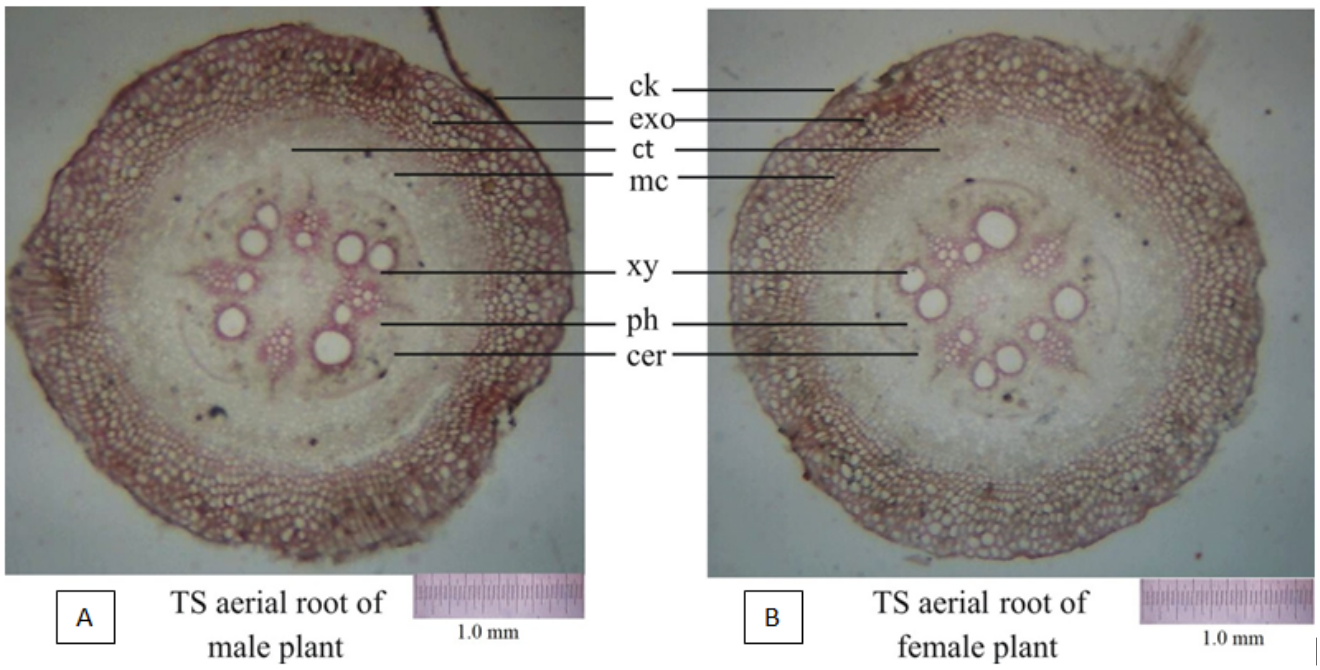

Figure 2: Microscopy of *Tinospora cordifolia*, aerial root (A) Male plant, (B) Female plant. Abbreviations: cer: ceratenchyma; ck: cork; ct: cortex; exo: exodermis; mc: mucilage canal; ph: phloem; tc: tannin containing cell; xy: xylem.

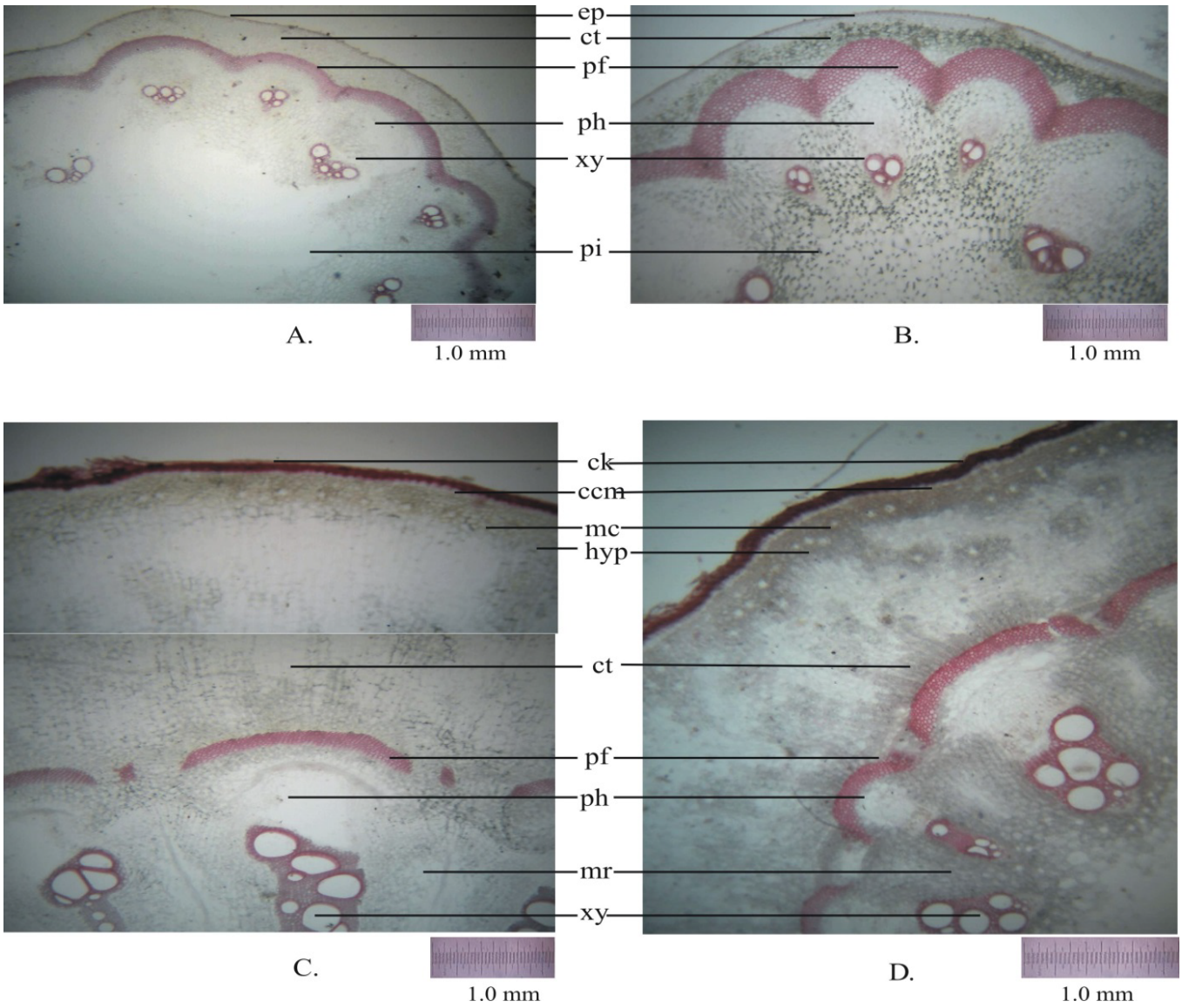

Figure 3: Microscopy of *Tinospora cordifolia*, stem (A) young stem of Male plant, (B) young stem of Female plant, (C) mature stem of male plant, (D) mature stem of female plant. Abbreviations: ccm: cork cambium; ck: cork; ct: cortex; ep: epidermis; hyp: hypodermis; mc: mucilage canal; mr: medullary rays; pf: pericyclic fibres; ph: phloem; pi: pith; tc: tannin containing cell; xy: xylem.

The lamina is dorsiventral, exhibiting a single or two rows of palisade, underneath the upper epidermis, the remaining mesophyll being of spongy parenchymatous cells. Vascular bundles traversed throughout the mesophyll tissue (Figures 5A and 5D). In surface view the upper epidermal cells are slightly thick walled, angular and devoid of stomata (Figures 5B and 5E) while the lower epidermal cells are undulated and embedded with stomata and trichomes (Figures 5C and 5F). Histological differences between male and female plant parts have been summarized in Table 1. The physicochemical parameters are presented in Table 2.

Tinosporaside in the male and female plants varied considerably in aerial root, leaf and stem. In the male plant it was found 682.77 ± 1.03, 667.7 ± 0.98 and 800.57 ± 1.57 in the aerial root, leaf and stem respectively. While in the female plant its concentration varied 897.64 ± 0.34, 778.3 ± 1.25 and 1178.73 ± 1.01 from aerial root, leaf and stem respectively. It is notable that the percent yield of extract of female plant was more than male plant. HPTLC finger print profiles are presented in Figure 6.

Quercetin was used as standard in reducing power assay and expressed as ascorbic acid equivalent. DPPH radical scavenging activity of female extract was found most powerful DPPH• scavenger as evidenced by low IC50 (136.2 µg/ml). The IC50 value of superoxide anion radical scavenging capacity for female and male were respectively, 0.44 and 0.62 mg/ml (Table 3).

Discussion

Quality control of the herbal drug is very much essential for commercial exploitation as well as the medicinal value of the raw drugs. Even authenticated plant material may not be of desired quality and strength and not conforming to the physicochemical parameters

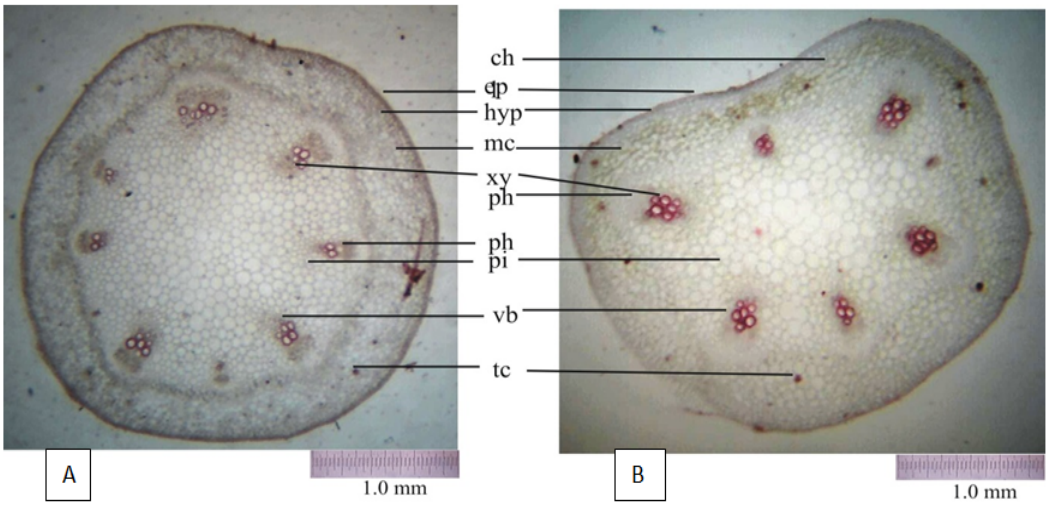

Figure 4: Microscopy of *Tinospora cordifolia*, petiole (A) Male plant, (B) Female plant. Abbreviations: ch: chlorenchyma; ep: epidermis; hyp: hypodermis; mc: mucilage canal; ph: phloem; pi: pith; tc: tannin containing cell; vb: vascular bundle; xy: xylem.

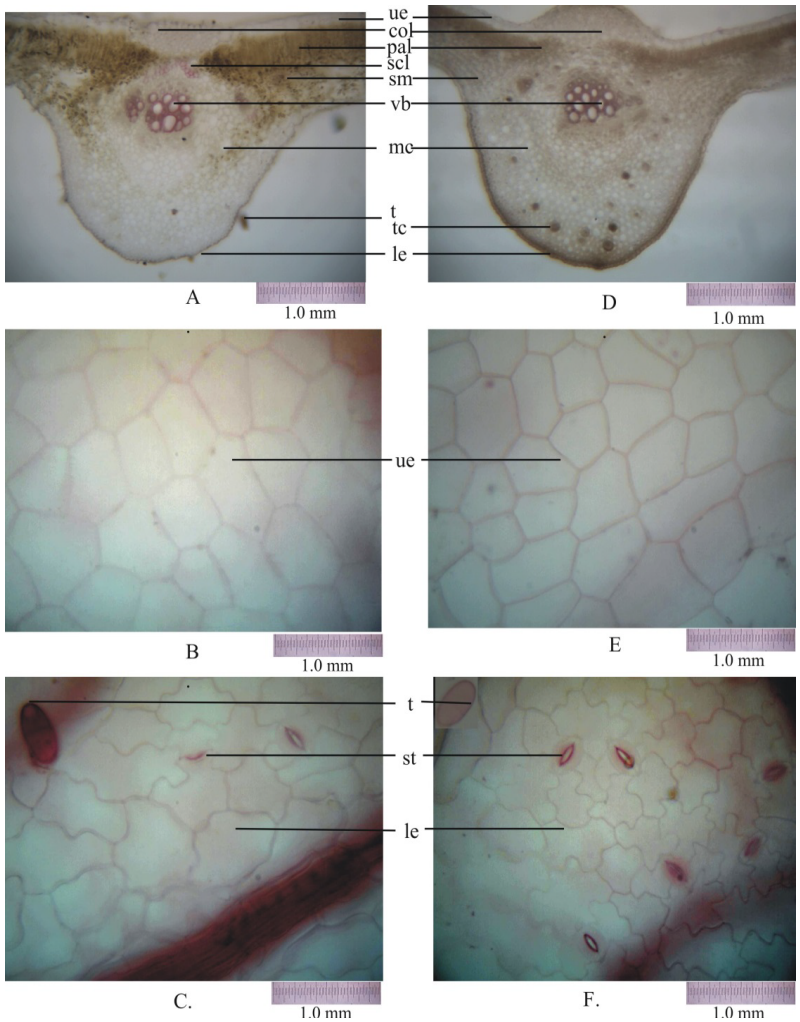

Figure 5: Microscopy of *Tinospora cordifolia*, leaf (A-C), Male plant, A. TS leaf, B. upper surface of leaf, C. lower surface of leaf; (D-F), Female plant, D. TS leaf, E. upper surface of leaf, F. lower surface of leaf. Abbreviations: col: collenchymas; le: lower epidermis; mc: mucilage canal; pal: palisade cells; scl: sclerenchyma; sm: spongy mesophyll; st: stomata; t: trichome; tc: tannin containing cell; ue: upper epidermis; vb: vascular bundle; xy: xylem.

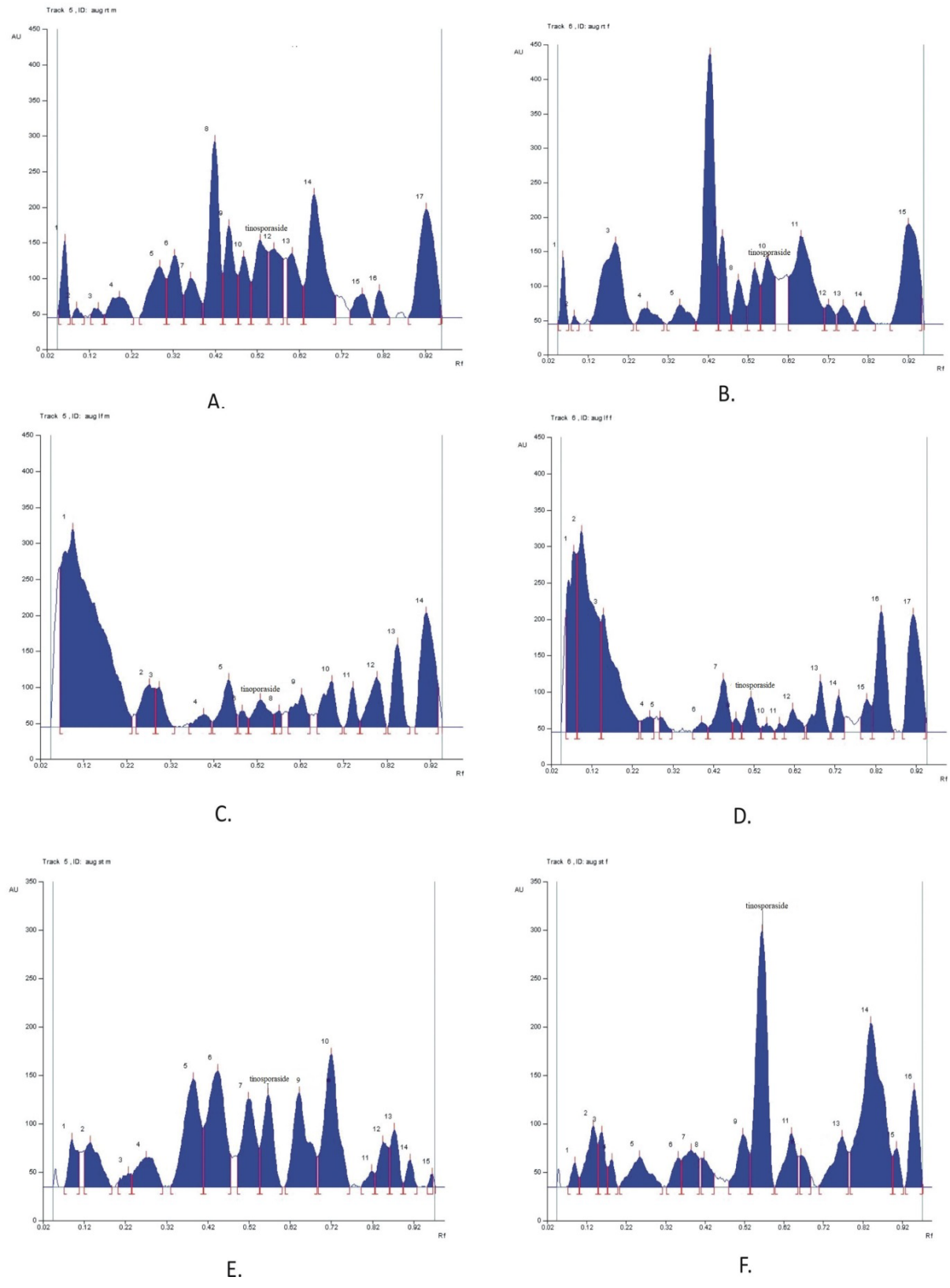

Figure 6: TLC fingerprint profile of methanolic extract of dioecious *Tinospora cordifolia* along with the Tinosporoside biomarker. (A) Aerial root of male plant; (B) Aerial root of female plant; (C) Leaf of male plant; (D) Leaf of female plant; (E) Stem of male plant; (F) Stem of female plant.

S.No.	Character	Male	Female
		Aerial root	
1.	Mucilaginous canals	Less in number (2-3/mm^2)	Comparatively more in number (4-6/mm^2)
2.	Tannin containing cells	Less in number (4-5/mm^2)	Comparatively more in number (5-8/mm^2)
		Mature Stem	
3.	Hypodermis	Collenchymatous cells with more angular thickening	Collenchymatous cells with lesser angular thickening
4.	Cortical region	Very broad (Approx. 3000-3200 µm)	Comparatively narrow (Approx. 2000-2500 µm)
5.	Tannin containing cells	Few in number (1-2/mm^2)	Numerous (6-12/mm^2), scattered in cortical, phloem and pith regions
6.	Mucilaginous canals	Less in number (2-4 /mm^2)	Comparatively much more in number (8-15/mm^2)
7.	Starch grain	Up to 10 µm in size, less in quantity	Up to 30 µm in size, more in quantity
		Petiole	
8.	shape	Somewhat circular with one side flattened	Somewhat reniform
9.	Vascular bundles	6-8	6-7
10.	Hypodermis and ground tissue	No differentiation in ground tissue i.e. only collenchymatous	Hypodermis is collenchymatous followed by 3-5 layers of chlorenchyma and remaining region collenchymatous
		Leaf	
11.	Midrib region	Some sclerenchymatous cells in mid-rib region just below the collenchymatous hypodermis	Similar to male leaf except the sclerenchymatous patches
12.	Mucilaginous canals	Few in number (1-2/mm^2)	Numerous (6-10/mm^2)
13.	Tannin containing cells	Absent	Present
		Leaf surface	
14.	Epidermal cells	Large sized epidermal cells on both the surfaces	Comparatively smaller in size and much wavy on lower surface
15.	Trichome Number	2-3/mm^2	4-6/mm^2
16.	Stomatal Number	150-200/mm^2	150-225/mm^2
17.	Stomatal Index	12-16	12-14
18.	Vein Islets Number	0-1/mm^2	2-3/mm^2
19.	Vein Termination Number	4-9/mm^2	6-17/mm^2
20.	Epidermal Number	1025-1125/mm^2	1300-1400/mm^2

Table 1: Anatomical differences between male and female plant of *Tinospora cordifolia*.

S.No.	Parameters % (w/w)	Aerial Root		Leaf		Stem	
		Male	Female	Male	Female	Male	Female
1.	Loss on Drying	3.47 ± 0.07	4.86 ± 0.10	6.01 ± 0.12	5.87 ± 0.12	5.02 ± 0.10	4.06 ± 0.08
2.	Total Ash	4.02 ± 0.08	6.81 ± 0.14	5.53 ± 0.11	8.02 ± 0.16	6.07 ± 0.12	8.61 ± 0.17
3.	Water-Soluble Ash	1.10 ± 0.02	2.00 ± 0.04	2.11 ± 0.04	3.08 ± 0.06	2.01 ± 0.04	3.52± 0.07
4.	Acid-Insoluble Ash	0.90 ± 0.02	1.13 ± 0.02	0.72 ± 0.01	0.85 ± 0.01	0.88 ± 0.02	0.91 ± 0.02
5.	Water soluble extract	13.20 ± 0.33	17.60 ± 0.37	17.60 ± 0.37	24.70 ± 0.49	15.40 ± 0.29	20.20 ± 0.40
6.	Alcohol soluble extract	12.50 ± 0.31	13.00 ± 0.32	11.10 ± 0.20	15.20 ± 0.29	13.30 ± 0.27	16.60 ± 0.33
7.	Total tannin content	4.41 ± 0.09	3.12 ± 0.10	2.41 ± 0.05	4.11 ± 0.08	3.66 ± 0.07	4.21 ± 0.08
8.	Total phenolic content	1.01 ± 0.02	1.00 ± 0.02	2.07 ± 0.04	3.22 ± 0.06	2.01 ± 0.04	2.12 ± 0.04
9.	Total sugar content	2.02 ± 0.04	1.87 ± 0.04	276 ± 0.06	2.87 ± 0.06	3.03 ± 0.06	3.23 ± 0.06
10.	Total starch content	5.98 ± 0.12	2.43 ± 0.05	7.12 ± 0.14	8.12 ± 0.16	5.03 ± 0.10	6.68 ± 0.13

Table 2: Physico-phytochemical analysis of different parts of male and female plants of *Tinospora cordifolia* (Each value is the mean of 6 replicates).

S. No.	Sample	RP (mM of ASE/ml)	DPPH IC50 (µg/ml)	SOD IC50 (mg/ml)	LPO IC50 (mg/ml)
1.	Female extract	0.68 ± 0.07	136.2 ± 8.5	0.44 ± 0.03	0.23 ± 0.02
2.	Male extract	0.52 ± 0.04	148.4 ± 7.6	0.62 ± 0.01	0.33 ± 0.01
3.	Quercetin	0.65 ± 0.04	--	0.035 ± 0.002	0.044 ± 0.003
4.	Ascorbic acid	--	60.14 ± 0.007	--	--

Table 3: Reducing power (RP) and DPPH and superoxide anion radical scavenging activities, lipid peroxidation inhibition in male and female stem extracts of *Tinospora cordifolia* (Values are mean ± SEM for n=3).

or the concentration of the phytoconstituents or active therapeutic agents as per the pharmacopoeial standards or the consumer/industry requirements. Such material is liable to be rejected or accepted at very low price causing not only economic loss to the cultivators or collectors of the medicinal plants but also entails doubtful efficacy or the potency of the raw drug in the alleviation of the human suffering. *T. cordifolia* is one of the important Rasayana drugs of Ayurveda. Though it is well known that the plant is dioecious no information on macro-microscopical features associated with gender is available in literature. Observations on plant morphology showed that leaf shape and petiole length can be used to distinguish male and female plants even in vegetative state. As far the number of flowers are concerned female flowers are few and sparse and male flowers are profuse and numerous. Anatomical investigations revealed other features to distinguish

between male and female plants. The outer margin of petiole of male plant is circular with one side flattened but reniform in female plants. Thus it is possible to differentiate between male and female plants of *T. cordifolia*, on the basis of certain morphological characters. These can also be differentiated microscopically on the basis of distribution pattern of tissues in cortical region of stem where male cell with more angular thickening than female and hypodermal regions is very broad in male (approx. 3000-3200) than female (approx. 2000-2500). Quantitative surface study of leaves also showed significant variation like in female plant there were wavy epidermal cells on lower surface but absent in male plant, however number of trichome, stomatal number, epidermal cells, vein termination and vein islets numbers are more in female plants (Table 1). Transverse section of stem also highlighted the differences between male and female plants which are also supported by quantitative anatomical data. Female plant having more mucilage canals (8-15/mm^2) than male (2-4/mm^2) and also starch grains in female (30 µm) are large in size than male (10 µm).

The difference in the level of tinosporoside in male and female was significant. It is also noteworthy that the yield of extract was also more in female plant than male (Table 2). HPTLC finger print profiles also showed some differentiating bands in methanolic extract (Figure 6). Tinosporoside is considered as biomarker for *T. cordifolia*, and used as quality parameter for this plant. It showed antihyperglycemic activity in Strptozotocin model [28]. Tinosporoside content in all the studied plant part like areal root, stem and leaf of female plants is significantly high in female plant and implying its better efficacy than male plants (Table 2).

Traditionally, Rasayanas were identified as adaptogenic agents [29]. It has been reported that the Rasayanas are rejuvenators, nutritional supplements and possess strong antioxidant activity. *T. cordifolia*, studies revealed that Female showed comparatively high in antioxidant potential which may due to higher amount of phenolics. Earlier reports suggested that extract of *T. cordifolia*, has been shown to inhibit the LPO and superoxide and hydroxyl radicals *in vitro* [30]. Similarly, higher amount of starch also found in female has significant implications. Polysaccharide isolated from *T. cordifolia*, showed good protection against iron-mediated LPO of rat brain homogenate as revealed by the TBARS and lipid hydroperoxide assays and high reactivity towards DPPH, superoxide radicals and the most damaging of radicals, the hydroxyl radical [31].

Conclusion

Based on the studies it can be concluded that while dealing with dioecious plants where only vegetative parts are used as drug it will be of significance to evaluate the differences between sexes. This will ensure better efficacy and reduce variations which are very common with plant based drugs.

Conflicts of Interest

The authors declare no conflicts of interest.

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Author's Contributions

Sayyada Khatoon created the project, analysed the HPTLC profiling, supervised and provided critical reading. Saba Irshad carried out laboratory work and formatted the manuscript. Namrta Choudhri collected the plant material and carried out the laboratory work. M. Vijayakumar carried out the antioxidant potential, Shweta Singh performed HPTLC analysis. Mumayyaza khan provided assistance in manuscript preparation. MB Siddiqui identified plant material. Nikhil Kumar edited and insightful recommendations of the manuscript. All the authors have read the final manuscript and approved the submission.

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