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Macro-microscopical and physicochemical evaluations of *Tinospora crispa* (L.) Hook. f. & Thomson stem

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Abstract

Tinospora crispa (L.) Hook. f. & Thomson stem has been employed as a traditional remedy for ailments like diabetes, hypertension, malaria, diarrhoea, etc. In the present study, macro-microscopical and physicochemical evaluation of *T. crispa* stem has been carried out to determine the standards for testing authenticity. The botanical evaluation of *T. crispa* stem revealed the presence of microscopic features like wide parenchymatous cortex with large columnar mucilage cell; inner cortical cells with plenty of ovoidal starch grains and prismatic crystals. The characteristic features like stellate arrangement of secondary xylems; semicircular strips of continuous secondary phloem, etc. Colour change upon chemical interaction of *T. crispa* stem powder with different reagents revealed characteristic colours under visible and UV light. The results of the present study will help to confirm the authenticity of *T. crispa* stem and it will aid to distinguish the plant from its allied species, even in crushed or powdered form.

Keywords: Powder study, Menispermaceae, Pharmacognosy

1. Introduction

The cordial relationship between man and medicinal plants has been well depicted from ancient documented evidence such as 5000 years old imprints of medicinal plants of Sumerian clay slabs (Sumner and Judith, 2000). In India, 65% of the population in rural areas use medicinal plants to meet their primary health care needs (Pandey *et al*., 2013). Nowadays, there is renewed interest in medicinal plants that have emerged because of their availability and less or no toxicity. But the major drawback of the therapeutic efficacy of such medicinal plants were their adulteration. To identify adulteration and confirm the purity of raw drug, standardization and authentication of natural drugs is the only way and is possible by pharmacognostical studies. Thus, most of the research in pharmacognosy have been focused on identifying controversial species of plants and authentication of commonly used medicinal plants through morphological, anatomical, physicochemical and phytochemical analysis.

Traditionally, pharmacognosy is recognized as a vital part of drug development process and pharmacy education. When the raw drug is in powdered form, the taxonomic identification of the plant species becomes more complex and is more prone to intentional adulteration. Pharmacognostical studies ensure the identity of a plant even if it is in the dried or powdered form. The standardization parameters evolved out of the pharmacognostic studies ensure the identity of a plant, thereby prevent adulterations to some extent and ensure the reproducible quality of herbal products.

The genus *Tinospora* (Menispermaceae) has been represented in India by only three species;

Tinospora cordifolia (Willd.) Miers, *Tinospora sinensis* (Lour.) Merr. and *Tinospora crispa* (L) Hook. f. & Thomson (Nidhi *et al*., 2013). All the three species are closely related to their morphological and chemical properties of stem, bark, leaves and flowers. *T. cordifolia* is mainly found in tropical regions of India and is widely used in folk and Ayurvedic systems of medicine (Nidhi *et al*., 2013). The stem of *T. crispa* has been used as an effective antidiabetic drug in the traditional/tribal system of medicine (Thomas *et al*., 2016). The plant has also been used traditionally in the treatment of jaundice, rheumatism, urinary disorders, fever, malaria, internal inflammation, fracture, scabies, hypertension, increasing appetite and cooling down the body temperature (Ahmad *et al*., 2016). The two medicinally important species of *Tinospora* namely *T. crispa* and *T. cordifolia* were very much alike, even in fresh form and the two species are difficult to distinguish from each other (Fig. 1). Therefore, the present study is aimed to establish pharmacognostical standards to distinguish *T. crispa* stem from its allied species.

2. Materials and Methods

2.1. Collection and authentication of plant material

T. crispa stem was collected from Thiruvananthapuram district (8°42'58.2"N 76°50'02.3"E) of Kerala, India and authenticated by the plant taxonomist of the department. Voucher specimens were deposited at the Jawaharlal Nehru Tropical Botanical Garden and Research Institute Herbarium (TBGT 96221).

2.2. Macroscopic and organoleptic characterization

Fresh stem of *T. crispa* was collected and analyzed for both qualitative and quantitative traits. The following macroscopic and organoleptic characters like, colour, texture, odour and taste of the fresh stem were noted (Trease and Eavans, 2002).

2.3. Microscopic characterization

2.3.1. Anatomical studies of the stem

Freehand, transverse sections of the fresh stem was taken, stained with safranin and iodine, mounted on glass slides using glycerin and observed under light microscope with camera attachment and photomicrographs were taken (Trease and Eavans, 2002).

2.4. Powder microscopy

The fresh stem was collected, thoroughly washed with fresh water, sliced, shade dried and powdered. The stem powder was boiled with chloral hydrate for 5 to 10 min, and then stained with safranin to determine the presence of lignified cells, calcium oxalate crystals and iodine solution was used to detect starch grains (Khandelwal, 2002).

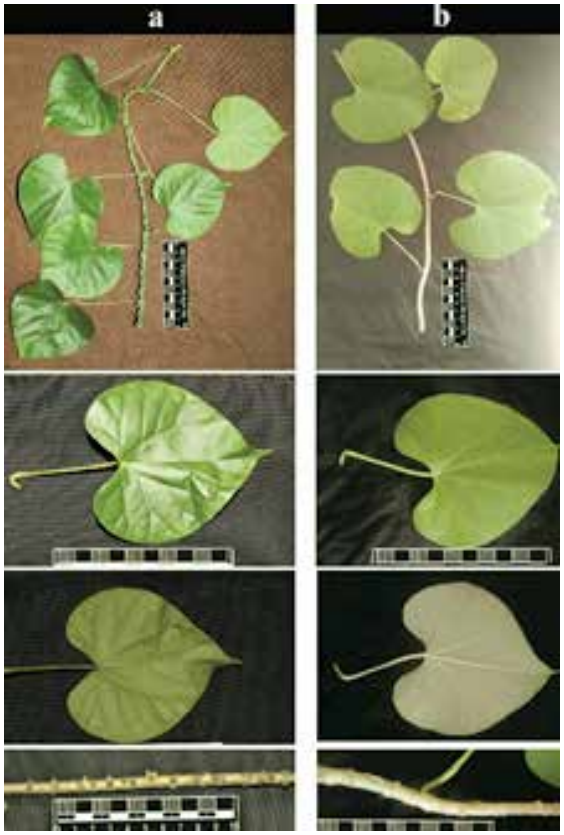

Fig. 1. Morphological similarities of leaves and stems of a. *T. crispa*; b. *T. cordifolia*

2.5. Fluorescence analysis

The fluorescence character of the plant powders (40 mesh) was studied both in daylight and UV light after treatment with different reagents like sodium hydroxide, hydrochloric acid, sulphuric acid, nitric acid, ferric chloride, potassium dichromate, Gram's iodine, toluene, methanol, ethanol, etc. (Kokoshi *et al*., 1958). The colour changes were noted using Pantone's Matching System Colour Chart.

2.6. Physicochemical analysis

Different physicochemical parameters of *T. crispa* stem powder were determined according to the quality control methods for medicinal plant materials (Anonymous, 2011).

2.6.1. Determination of pH

1% solution was prepared by dissolving 1 g of the stem powder in 100 ml of distilled water, filtered and checked pH of the filtrate with a standardized glass electrode. 10% solution was prepared by dissolving 10 g of the stem powder in 100 ml of distilled water, filtered and checked pH of the filtrate with a standardized glass electrode.

2.6.2. Loss on Drying (LOD)

About 2 to 3 g of powder was accurately weighed in a china dish and kept in a hot air oven maintained at 105° C for 5 h. After cooling in a desiccator, the loss in weight was recorded. This procedure was repeated until constant weight was obtained.

Loss on Drying (LOD)% = Loss in weight/ Weight of the drug in gms ×100

2.6.3. Determination of total ash

About 2-3 g weighed crude drug powder in a tarred silica dish was ignited and weighed. Scattered the powder drug on the bottom of the dish and incinerated by gradually increasing the heat, not exceeding dull red heat until free from carbon, cooled and weighed. The % w/w of total ash with reference to the air-dried drug was calculated.

2.6.4. Determination of acid insoluble ash

Boil the ash for 5-10 min with 25 ml of diluted hydrochloric acid, collected the insoluble matter in a Gooch crucible, washed with hot water, ignited and weighed. Percentage of acid insoluble ash is calculated with reference to air dried drug. The % w/w of acid insoluble ash with reference to the air-dried drug was calculated.

2.6.5. Determination of water-soluble ash

To the crucible containing total ash, 25 ml of water was added and boiled for 5-10 min. Collected the insoluble matter in a Gooch crucible, washed with hot water and ignite in a crucible for 15 min at a temperature not exceeding 450^0C. Subtracted the weight of insoluble matter from the weight of the ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air-dried drug. The % w/w of water soluble ash with reference to the air-dried drug was calculated. Percentage extractive and characteristic of extract yield in g/100 g leaf powder of *T. crispa* was calculated.

2.6.6. Determination of alcohol soluble extractive

Macerated 5 g of the air dried drug coarsely powdered with 100 ml of ethanol of specified strength in a closed flask for 24 h followed by shaking frequently for 6 h and allowed to stand for 18 h. The filtrate was evaporated to dryness in a tarred flat bottom shallow dish, dried at 105°C and weighed. The percentage of alcohol-soluble extractive was calculated with reference to the air dried drug.

2.6.7. Determination of water soluble extractive

Followed as directed in determination of alcohol soluble extractive using water instead of ethanol.

2.6.8. Determination of petroleum ether soluble (40-60°C) extractive

The procedure is directed to the determination of alcohol soluble extractive, using petroleum ether (40-60°C) instead of ethanol.

2.6.9. Swelling index

One gram of drug powder was taken in a measuring cylinder (25 ml) and suspended in 25 ml distilled water for 1 h by thorough mixing every 10 min. After 3 h, volume in ml was occupied by the plant material, including the sticky mucilage was measured. The experiment was repeated thrice for accuracy and the swelling index was calculated.

2.6.10. Foaming index

Finely powdered drug (1 g) was transferred into a 500 ml flask containing 100 ml of boiling water. The mixture was maintained at moderate boiling for 30 min. The mixture was cooled and filtered into a 100 ml volumetric flask and sufficient water was added to the filtrate to dilute the volume. The prepared decoction was poured into 10 stoppers test tubes, each 1 ml, 2 ml... 10 ml. The volume of the liquid each tube was adjusted (10 ml) with water. The tube was duly stoppered and shaken in a lengthwise motion for 15 sec (two shakes

per second) and allowed to stand for 15 min. The foam length in each tube was measured.

Foaming index = 1000/a

Where 'a' is the volume of the plant decoction for forming foam of height 1 cm.

2.7. Statistical analysis

All the data were expressed as mean ± standard deviation (SD).

3. Results and Discussion

3.1. Macroscopic and organoleptic characterization

The stem bark is greenish grey in colour and the stem outline surface showed plenty of warty protuberance due to circular lenticels. The dried stem is cylindrical, 1 to 2 cm diameter with a rough surface and longitudinal of cracks. Outer bark is thin and papery, brown to greenish grey in colour. The freshly transverse cut surface assumes a yellow tint when exposed to air and cut surface shows a wheel like structure. The stem is rough externally with prominent projections (Fig. 1a), internally whitish, smooth, fibrous and longitudinally striated with pleasant odour and bitter taste (Table 1).

Table 1. Macroscopic and organoleptic characters of *T. crispa* stem

Macroscopic parameters	Observation
Surface	Rough
Colour	Greenish grey
Odour	Pleasant
Taste	Bitter

3.2. Microscopic characterization of *T. crispa* stem

3.2.1. Anatomical characters

Transverse section of the mature stem showed a prominent periderm. Periderm was broken as lenticels protruded out at many places. The microscopice character of periderm showed 2 to 3 layers of phellum followed by 4 to 5 layered phellogen (Fig.2b$_2$). The cortex is wide with large columnar parenchymatous cell filled with mucilage. Cortical cells of outer rows are smaller than inner rows. Outer zone of cortex consists of 4 to 6 rows irregularly arranged chlorenchymatous cells. Inner cortical cells are polygonal in shape and filled with plenty of ovoidal starch grains and prismatic crystals (Fig. 2h). In secondary structure all the xylems united to form a stellate appearance with the semicircular strips of continuous phloem outside the secondary xylem (Fig.2b). The secondary xylem vessels with relatively smaller lumen (Fig. 2f).

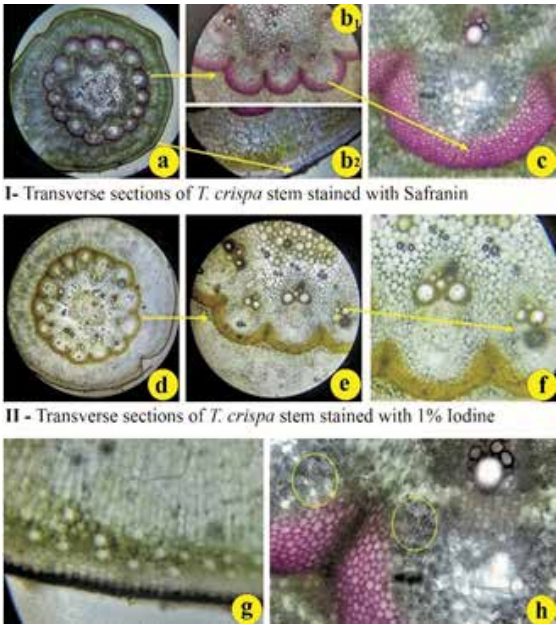

Fig. 2. Microscopic characterization of *T. crispa* stem; a. Transverse section of mature stem; b_1. Semicircular strips of continuous phloem; b_2. Outer protective periderm; c. Secondary xylem; d. Transverse section showing pith; e. Medullary cells and xylem elements; f. Secondary xylem vessels; g. Periderm enlarged; h. Ovoidal starch grains and prismatic crystals

3.2.2. Powder microscopy

Stem powder is creamish brown (Fig.3a) in colour with characteristic odour and bitter taste. It shows vessels with reticulate secondary wall thickening (Fig.3b), xylem fibers (Fig.3c). Starch grains are oval to round, mostly simple (Fig.3f).

3.2.3. Fluorescence analysis

Chemical tests of *T. crispa* stem powder with different reagents were done and observed under visible and UV light. Under UV light, the seven samples (1, 2, 3, 4, 8, 9 and 10) showed the presence of fluorescence (Fig. 4). The results were compared with their respective observations in visible light and they were represented in Table 2.

3.2.4. Physicochemical analysis

Physicochemical parameters of *T. crispa* stem powder was evaluated and observations are presented in Table 3.

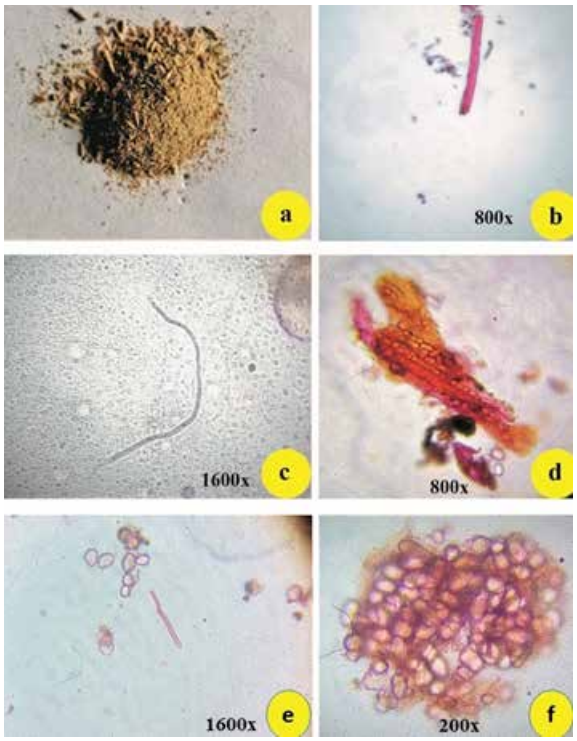

Fig. 3. Powder character of *T. crispa* stem; a. Stem powder; b. Xylem vessel; c. Xylem fibers; d. Fragmented xylem vessels with attached starch grains; e. Xylem vessel; f. Oval shaped starch grains

a. Visible light

b. UV light

Fig. 4. Fluorescence analysis of *T. crispa* stem powder: a. Colour of the stem powder observed in visible light after treatment with different reagents; b. Colour observed in UV light after treatment with different reagents

Table 2. Observations of *T. crispa* stem powder under visible and UV light after reacting with different reagents

Sl. No.	Treatment		Observation (Colour developed)
	Visible light	UV light	
1	Powder+ 5 ml NaOH (1N)	Yellow PMS 601	Yellow PMS 372*
2	Powder+ 5 ml Methanol (80%)	Green PMS 372	Green PMS 367*
3	Powder+ 5 ml HCl (0.1)	Green PMS 607	Yellow PMS 1205*
4	Powder+ 5 ml HNO_3 (40%)	Yellow PMS 1225	Yellow PMS 127*
5	Powder + 5 ml $FeCl_3$ (5%)	Orange PMS 144	Yellow PMS 119
6	Powder + 5 ml H_2SO_4 (98.08%)	Black 5 2X	Brown PMS 161
7	Powder + 5 ml Gram's Iodine	Brown 168	Black 4 2X
8	Powder + 5 ml $K_2Cr_2O_7$	Orange PMS 130 2X	Green PMS 378*
9	Powder + 5 ml Ethanol (100%)	Green PMS 587	Yellow PMS 365*
10	Powder + 5 ml Toluene (92.14%)	Green PMS 585	Yellow PMS 374*

Pantone matching system *Presence of fluorescence

The reproducibility and quality of herbal drugs are directly related to the authenticity of the plant material. Qualitative or quantitative uniqueness of different drugs with respect to the morphological, anatomical, and biochemical parameters is acknowledged as one of the reliable tools in distinguishing allied drug samples. Lack of proper documentation and the adoption of stringent quality measures continue as a major setback in the use of herbal drugs. To mitigate this quality assurance by standardization of the drug employing different pharmacognostic parameters has become the need of the hour. Hence, this might help not only to authenticate or identify the drug but also for its safe and efficacious use.

Table 3. Physicochemical parameters of *T. crispa* stem powder

Sl. No.	Test		Results
1.	pH of water solution	1% w/v	6.81±0.23
		10% w/v	6.23±0.25
2.	Alcohol -soluble extractive		9.60±0.57
3.	Petroleum ether-soluble extractive		7.20±0.85
4.	Water-soluble extractive		11.60±0.86
5.	Loss on Drying (LOD)		10.00±0.65
6.	Total ash		49.40±3.24%
7.	Acid-insoluble ash		9.25±0.74%
8.	Water-soluble ash		24.32±2.45%
9.	Swelling index		0.10±0.05
10.	Foaming index		>100

Values are expressed as mean ± SD of ten values.

T. crispa is a very scarcely studied species in pharmacognostical point of view. As far as it is known, there have been a couple of attempts made to study the pharmacognostic aspects of the species. Various pharmacognostic parameters were evaluated in the present study including macro and microscopic characteristics of the stem, powder analysis, fluorescent analysis and physicochemical parameters such as pH, swelling index, foaming index, etc.

Regarding the macroscopic characters (Table 1), the greenish rounded stem with papery outer bark is a typical character seen in other species of the genus like *T. cordifolia* (Choudhary *et al*., 2014). The characteristic large protuberances noted in the outer surface of *T. crispa* stem must be consider as a key identifying character compared to the smaller and fewer protuberance in *T. cordifolia*. The T. S. of the stem shows epidermis followed by the cortex, with wide parenchymatous zone containing large columnar type cell. The polygonal inner cortical cells with plenty of ovoidal starch grains and prismatic crystals (Fig. 2h) make it distinguishable from the allied species; whereas in the allied species like *T. cordifolia*, the starch grains may be of various shapes (Choudhary *et al*., 2014). The stellate appearance of xylem in the secondary structure together with the phloem at the radii and the sclerenchymatous regions of stem anatomy may be considered as a key identifying feature of the stem. The creamish brown (Fig. 3a) stem powder with characteristic odour will also help to check the authenticity of the plant material.

The peculiarities of microscopic characters revealed in the powder study, like the presence of vessels with reticulate secondary wall thickening (Fig. 3b), xylem fibers and ovoidal starch grains etc. will help to identify the *T. crispa* stem even in its crushed or powdered form. The specific colours as well as the fluorescence developed when the plant powder treated with specific reagents can be considered as a pharmacognostical standard for identifying the *T. crispa* stem even in powdered form. The plant powders may exhibit fluorescence phenomenon due to its chemical constituents. In different wavelengths of light, the same material in different reagents may appear similar and dissimilar. Some of the constituents may show fluorescence in the visible range in day light while some other show fluorescence only in ultraviolet light. If the plant material does not show any fluoresce phenomena, then they are made fluoresce by applying various reagents into it. The fluorescence showed in UV light by the seven samples (Fig. 4 & Table 2) in the present study strongly indicates the presence of fluorescent compounds present in the plant.

Evaluations of the physicochemical parameters aids in the formation of pharmacopoeial standards. Various physicochemical characteristics of the drug were revealed in the present study. pH 6.81 to pH 6.23 showed by the various water solutions of the drug powder indicates the almost neutral nature of crude drug. It also indicates that the plant will not cause any acidic problems while used for internal or external applications. The extractive values are one of the important parameters for determining the physico-chemical characteristics and plays an imperative part in the assessment of the crude drugs. The extraction of the drug from the plant powder with different solvents gives particular amount of yields of crude drugs. The extractive values (Table 3) of the present study; alcohol, petroleum ether and water will help to identify the presence of several types of adulteration and exhausting materials.

Swelling properties of many herbal materials may contribute to specific pharmaceuticals or therapeutic utility. The swelling of the herbal material is the indication of mucilaginous substance present in it. The relatively less swelling index (0.1) revealed in the present investigation may be due to the absence of the mucilaginous substance in the studied sample. Loss on drying is the weight of the powder obtained after the complete drying of the powder for a long period. The stem powder of *T. crispa* showed very less loss on drying value compared to other plant materials (Edwin *et al*., 2008). The foaming index is an indication of the saponin content in the plant. The foaming index (>100) of the stem *T. crispa* indicates that that the stem is less in the saponin content. Ash values are important quantitative standards (Rajesh and Latha, 2010) and criterion to analyze the identity and purity of crude drugs especially in the powder form (Patnia *et al*., 2005). The total ash value of 49.40% w/w has been noted in the plant. Total ash usually contains carbonates, phosphates, silicates and silica (Trease and Evans, 2002). The water soluble ash (24.32% w/w) is the water soluble portion of the total ash. Acid insoluble ash in *T. crispa* amounts to 9.25% w/w, which indicates that more than half of the ash is soluble in acid.

Setting up a standard for the correct identification and quality check of crude drug is a fundamental process in drug development. A great bulk of information on identity, purity and quality of plant material can be obtained from its macroscopy, microscopy, powder characters and physicochemical parameters of *T. crispa* stem. Present investigation put forward important standardization parameters for a meagerly studied medicinal herb *T. crispa* which is a new entry as an aid in the pharmaceutical industry.

4. Conclusion

Pharmacognosy isn't an issue of the past, however, it's evolved and developed over the years to adapt itself to the ever-changing surroundings, and is currently appropriate to meet the challenges of the current and also the way forward for drug discovery and development. Various pharmacognostic parameters evaluated in the present study include the macro and microscopic characterization of the stem, powder analysis, fluorescence analysis and physicochemical parameters such as pH, total ash value, extractive value, swelling index, foaming index, etc. Regarding to the macroscopic characters of the stem, the species possess key features like a greenish rounded stem with papery outer bark with large protuberances. The polygonal inner cortical cells with plenty of ovoidal starch grains and prismatic crystals. The stellate appearance of xylem with the semicircular strips of continuous phloem outside the xylem in the mature stem, *etc*. could be utilized as a reference for setting limits for the quality assurance of *T. crispa* stem.

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