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Standardization of wild *Krushnatulasi* (*Ocimum tenuiflorum* Linn) Leaf

Research Article

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Abstract

Background: For acceptance and globalization of Ayurveda there is needed to analyze herbal drugs according to modern techniques. Assessment of complete and accurate pharmacognostical study of herbs used in Ayurveda provides scientific basis of its quality. Objectives: To standardize the *Kushnatulasi* (*Ocimum tenuiflorum* L*inn/ Ocimum sanctum* L) collected from wild. Materials and Methods: The present study includes organoleptic, microscopic physicochemical, phytochemical and chromatographic examination of leaf of *Kushnatulasi*. Results: macro and microscopic, organoleptic, physicochemical, phytochemical and chromatographic findings are observed as per API in present study. Conclusion: Standardization of *Krushnatulasi* (*Ocimum tenuiflorum* L*inn*) is useful in authentication of genuine drug.

Keywords: *Krushnatulasi, Ocimum tenuiflorum* L*inn, Ocimum sanctum* L, Standardization, wild, Pharmacognostic.

Introduction

In ancient Ayurveda texts the concept of standardization and quality control of drugs is found. Physician used to collect the drugs himself with the help of *Shabda* (sound), *Sparsha* (texture), *Roopa* (color), *Rasa* (taste), *Gandha* (smell) and also based on habitat, morphology etc. in those days. After checking all these factors the drug would be used as a medicine. The nomenclature of many herbs denotes their physical, chemical characteristic and therapeutic uses which are considered as primitive standardization parameters (1). *Surasa*, *Sulabha*, *Surabhee*, *Shulaghnee*, *Bahumanjaree*, *Bhutaghni* these synonyms of *Krushnatulasi* depicted its useful form (juice) in many diseases, easy availability, aroma, efficacious in colic, morphological character and antimicrobial action respectively.

In current period recent advances has identified many test and parameters to evaluate quality control of drugs by pharmacognostic studies. So it is very essential to lay down pharmacognostic study of medicinal plants which are used in various formulations. It deals with authentication and standardization of natural drugs. Authentication and standardization evaluated by morphological or organoleptic tests, microscopic, chemical, and physical evaluation, chromatography, spectrophotometry etc. Pharmacognostic study includes parameters which help in identifying drug in dry powder form also. This is again necessary because once the plant is dried and made into powder form, it loses its morphological identity (2). Therefore it is necessary to provide standard parameters for the quality control of *Ocimum tenuiflorum* L*inn* (*Krushnatulasi*) leaves which can be beneficial for further quality control researches.

Krushnatulasi (*Ocimum tenuiflorum* L*inn*) or Queen of herbs has been used in Ayurveda for its varied healing properties. *Krushnatulasi* the Holy basil, the legendary 'Incomparable one' of India, is one of the holiest and most cherished of the many healing and healthy giving herbs of the orient (3).

Materials and Methods

Sample Collection

The leaves of *Ocimum tenuiflorum* L*inn* (*Krushnatulasi*) were collected as per GPS co-ordinator during the period of 21 July to 30 September 2015 from village Dabha, Wardha District (M.S.). These leaves were collected from open shrub land.

Authentication

The herbariums of *Ocimum tenuiflorum* L*inn* was prepared by standard method (4, 5) and sent for identification to Botanical Survey of India, Pune, Maharashtra.

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Preservation

After authentication of plant, the leaves of this plant collected from mature source plants. While collecting the leaves precaution was taken to avoid the insect-damaged plants. Leaves were subjected for washing under the tap water to remove adherent soil, dirt etc. for 2-3 times and finally followed by ethanol wash and then allowed to shade dry at room temperature for seven days. Finally leaves powdered to a coarse powder with mixer grinder and used for powder microscopy. For the histological profile the plant was preserved in a solution of FAA (Formalin 90: Acetic acid 7: Alcohol-3) (6).

Macroscopic and organoleptic evaluation

Macroscopic characters of the whole plant were studied for the detection of its authenticity. The characters were compared with the description given in the various floras and authenticity of the plant was confirmed. The fresh and dried leaves and their powders were evaluated separately for their macroscopic and organoleptic characters as per the standard methods described in various texts of pharmacognosy (7, 8).

Microscopic evaluation

Free hand sections of the plant material stained with Phloroglucinol and HCl and observed under the microscope for the presence of primary and secondary metabolites. the same method adopted for the powder samples (9).

Histochemical evaluation

Thick sections of plant samples subjected to Histochemical tests to find starch grains, tannin, calcium etc. by treating various reagents (10).

Physico-Chemical Study

Physicochemical analysis provides the objective parameters to set the standards for quality of raw drugs as well as finished products. With the help of analytical studies, it is possible to standardize the drug and differentiate the adulterants (11).

Phytochemical Study

A phytochemical study of a plant is necessary for understanding the significance of phyto-constituents and for its observed activities. Phyto-chemistry also helps in standardizing the herbal preparations so as to get the optimal concentrations of active constituents (12).

High performance thin layer chromatography (HPTLC) by CAMAG MUTTENZ

For obtaining the sample for HPTLC 5mg of ethanol extract of *Krushnatulasi* Leaves dissolved in 5ml of ethanol. The plate was pre-washed with Ethanol before application of spots. Sample solutions were applied to the plate as sharp bands by means of Camag Linomat-5 sample applicator. The spots were allowed to dry in a current of air. The mobile phase Toluene: Ethyl acetate: glacial acetic acid in proportion of 7:3:0.1 was poured into a twin trough glass chamber. Then whole assembly was left to equilibrate for 30min and the plate was placed in the chamber. The plate was then developed until the solvent front had travelled at a distance of 80 mm above the base of plate. The plate was then removed from chamber and dried in a current of air. Detection was done with CAMAG TLC scanner-3 at a wavelength 254 nm and 366 nm and 416nm.

Observation and Results

Dabha village situated at 20.57 N and 78.81 E (Figure 1). Intermittent presence of *Krushnatulasi* was observed while collecting from field (Figure 2). The voucher specimen number of *Ocimum tenuiflorum* L. herbarium mentioned as MSD-3 and Authentication Letter No- BSI/WRC/Tech./2014/447, Dated 29-12-2014 (Figure 3).

A) Macroscopic characters:

The leaf of *Krushnatulasi* was 6.2x2.6 cm. elliptic-oblong, obtuse or acute, entire or serrate, pubescent on both sides, minutely gland-dotted. Base is obtuse or acute; petioles 2 cm. long, slender and hairy (Fig-3).

B) Microscopic study of Leaf of *Ocimum tenuiflorum* Linn

a) Transverse Section (T.S.) of Petiole:

The diagrammatic T.S. shows Cat face shaped out line with large no. of trichomes with ground tissue consists central large vascular bundle and two meristeles. T.S. of petiole was showed the single layered barrel shaped thin cutinized epidermal cells having simple unicellular trichomes, multicellular, multiserriate, capitates sessile and glandular trichomes followed by 2-4 layered hypodermal cells. Just below the hypodermis, parenchymatous tissue is present. Some of them having chlorophyll, oil globules, The arrangement of vascular region at the centre consists large vascular bundle consists phloem towards lower side and xylem towards upper side along with two meristeles located at the edge of the main vascular bundle. Xylem consists of xylem parenchyma and its fibres and phloem consists of phloem fibres and its sieve elements (Plate 1).

b)Transverse Section through Midrib:

The diagrammatic T.S. shows boat shaped winged lamina region through mid-rib shows centrally located vascular bundle. Single layer upper epidermal cells interrupted by few multicellular trichomes epidermis covered with thin cuticle; at the region of mid rib the multicellular trichomes are bluish in colour. Lamina consists of upper 2-3 layered of palisade cells without any intra cellular spaces, with oil globules and chlorophyll pigments. 5-6 layers of spongy parenchyma cells at lower region filled with

chlorophyll with large intra cellular spaces some of the cells lead to stomatal openings (Plate-2).

Trough mid rib gives mechanical support to the centrally situated vascular bundle, below the epidermis followed by 3-4 layers of collenchymatous cells. Which is consists of xylem and phloem covered by ground tissue from both sides. Where in lower epidermal cells are smaller and often intercepted by cystoliths. There are two types of Trichomes. Epidermal Trichomes are densely distributed both on the adaxial and abaxial surfaces of the lamina.

(i) Glandular Trichomes: these are secretary structures bearing aromatic compounds. Two types of glandular Trichomes are seen (Plate-2).

(a) Peltate type of trichome: These are 'Bowl' shaped Trichomes with single short and wide stalk cell and cup shaped body. These peltate Trichomes are usually situated in shallow cavities of the Lamina. The body of the trichome is multicellular with radiating cells of darkly strained cell contents. The glands are 25 µm in height and 60 µm in diameters.

(b) Capitate type of trichome: These are multicellular Trichomes with thick, wide stalk cell and multi-cellular spherical body. The body consists of mostly 4 cells and possesses dense aroma compounds. These glands are 40 µm in height and body is 35 µm thick.

(ii) Non-Glandular Trichomes: These Trichomes are multicellular, uniserrate and un-branched. They have broad basal part and gradually tapering pointed terminal part. The trichome is 150 µm long and 20 um thick at the base (Plate-2).

C) Lamina: The lamina is bifacial with distinction into adaxial side and abaxial side. An adaxial epidermis consists of fairly wide, spindle shaped cells with prominent cuticle. The cells are 20 µm thick. The abaxial epidermis is comparatively thin, rectangular in shape and the cuticle is prominent. The mesophyll tissue consists of an adaxial band of single row of cylindrical, compact palisade cells which are 70 um in height. The lower part of the lamina includes 4 or 5 layer of lobed loosely arranged spongy mesophyll tissue (Plate-3).

Powder Microscopic Study

Diagnostic characters observed under microscope are dull green; aromatic in odour and fine corse in touch. Diagnostic characters observed under the microscope are shows groups of round to polygonal parenchymatous cells, pitted and spiral vessels, simple multicellular warty trichomes, oil globules and diacytic stomata, Pallisade parenchyma, Oil globules, Stomata, Calcium oxalate Prismatic crystals, glandular trichomes, Capitate sessile blacklist, crystal fibre, simple fibre, (Plate-4).

Organoleptic study: Orgenoleptic study of *Krushnatulasi* leaves powder was carried out and the results are depleted in the table 1.

Micrometric study of *Krushnatulasi* - leaf: The micrometric study of *Krushnatulasi* leaf 6.2x2.6 cm, Petiole 2 cm. Results are stated in table 2.

Histochemical evaluation of leaf:

Various Histo-chemical tests were conducted on the leaf powder of *Krushnatulasi.* Lignified cells, Starch grains, Ca Ox – crystals, Tannin cells, Oil globule observed where as Mucilage was absent (Table-3).

Physico-chemical parameters:

Physicochemical parameters of *Krushnatulasi* leaves powder was tested using various physico-chemical analysis such as moisture content, ash value, acid insoluble extracts and pH value was also estimated. The observed results are shown in the table 4.

Preliminary qualitative chemical test:

Leaves samples were qualitatively tested for the presence of different phytoconstituents like Carbohydrates, Protein, Reducing sugar, Steroids, Cardiac Glycosides, Saponins, Flavonoids, Alkaloids, and Tannins. The observed results are shown in the table 5.

High performance thin layer chromatography (HPTLC):

HPTLC analysis of dried leaves of *Krushnatulasi* fraction; demonstrated that the normal phase analysis carried out in silica coated plate interpreted around seven fragments resolved within 0.8 RF values. Though silica plate is not that much retentive compared with traditional C18 gel but these results proved effective for complete separation almost all fractions. This study was performed three different wavelengths; 254, 316 and 416 nm UV-vis. range. Results obtained in 254nm characterised improved peak shape compared with 316 and 416nm absorption. Importantly, after analysis most of the fractions eluted together initially. The same results were also observed in all selected wavelengths. It emphasised, the selection of silica phase in not appropriate for separation of non-polar fractions. Nevertheless, considering higher wavelengths proved much better resolution for initial retained fraction.In the middle of the HPLTC plate no any strong peak was observed. But at the end one strong component was eluted. Presumably, this fraction is a polar component that supposed to be protonated in selected mobile phase; the homogenous mixture of toluene and ethyl acetate

with small fraction of glacial acetic acid. Nonetheless, the HPTLC analysis carried out in normal phase proved effective separation of dried leaves extracts. This normal phase HPTLC technique selected to resolve better fragmentation of non-polar components from *Krushnatulasi* leaves extracts. As displayed in graph No.1; run the ethanol extract on silica gel plate with selected eluents, toluene, ethyl acetate and glacial acetic acid as modifier. These selected mobile phase optimised the complete separation of all fragments of *Krushnatulasi* leaves where the glacial acetic acid promote the protonation of acid-base strength components. Within 0.7 RF values around six components were identified. Importantly between 0.2 to 0.9 almost four components eluted together since it is presumed that they might have similar partition coefficient values. In addition, there is very partial resolution among them was occurred. Thereafter very small fractions were observed in the middle and finally one broad and blunt peak was visualised at the end of the separation (Graph No.1,2,3).

Discussion

The materials are collected from the nearest natural habitat for getting the good natural quality herbs. The Latlons are identified to make precision of the collecting area and to help the future researchers. The *Krushnatulasi* is not available as a bunch or bundled. The macroscopic features of *Krushnatulasi* leaves described in various floras are observed in the study. T.S. of *Krushnatulasi* petiole shows Cat face shaped out line in diagrammatic section with large number of trichomes with ground tissue consists of central large vascular bundle consists of phloem towards lower side and xylem towards upper side along with two meristeles located at the edge. In earlier works done by V Sharma et.al. , mentioned similar findings in pharmacognostic study of *Krushnatulasi* (13). Diacytic type of stomata was seen on both the surfaces, 4 cells of capitates trichome possess dense aroma compounds and filled with purple colour (14, 15).

The values demonstrated for *Krushnatulasi* in present study are as follows. The Foreign matter was 0%, and moisture content 0.17% w/w. The total ash-value is 0.94% w/w. and the acid insoluble ash is 0.053 % w/w. The water soluble extractive is 17.65 % w/w, and alcohol soluble extractive is 9.4 % w/w. All these values are as per standards of Ayurvedic Pharmacopeia of India and Quality Standards of Indian Medicinal Plants (Table 5.9) (16,17).

As loss on drying values (T=0.17) of *Krushnatulasi* indicating that less moisture was present in the collected material. Therefore the chances of microbial growth or contamination, and the presence of fungi or insects and plant material deteriorates quickly in presence of water was decreased. The ash values found as within normal limits (0.94) in present study, denoted the absence of an undue proportion of extraneous mineral matters introduced accidentally or mixed at the time of collection. Acid insoluble ash is the treatment of ash with hydrochloric acid leaves virtually only silica. Minimum acid insoluble ash values (0.053) found in present study indicates less quantity of silica was present in plant material. The maximum water and alcohol soluble extractive values of *Krushnatulasi* (17.65, 9.4 respectively) are denoting the more amounts of chemical constituents present and are soluble in respective solvents either water or ethanol, indicating the good potency of selected herbal drugs. pH of *Krushnatulasi* was acidic (5.09) might be because of *teekshna guna.*

In previous study by Jasmeet et. al., total ash and acid insoluble ash (15.6 & 2.5 % w/w) are more than standard and in comparison with present study. And another study by Sharma et al, express less than standard water soluble extractive and alcohol soluble extractive values (3.8 & 4% w/w) in comparison with present study. These study values of total ash and acid insoluble ash values are more than present study in comparison and less than the standards (18,19). Rf values detected for identification of alkaloids, Terpenoids, flavonoids and Saponine. 0.13, 0.25, 0.33, 0.48 Rf values reveal the presence of alkaloids. 0.04, 0.07, 0.08, 0.27, 0.72, 0.95 Rf exhibits the presence of Terpenoids. Whereas Rf 0.15, 0.17 shows presence of Saponine.

Conclusion

From the pharmacognostic study it is observed that the quality and potency of drug is good. May be because of the leaves collected are according to the Charaka said method of collection from field. During this (Varsha rutu) period the collected leaves contains highest quality of active principle, so the expected qualities in the plants offers better result in the treatments. It can be concluded that the organoleptic, pharmacognostical, physicochemical values and phytochemical study are useful in authentication and standardization of *Krushnatulasi* (*Ocimum tenuiflorum* L*inn*) while collecting the drug.

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Table 1: Organoleptic character of *Krushnatulasi* leaves powder

Characters	Colour	Taste	Odour	Nature of powder
Observations	Brown	Katu	Aromatic	Coarse

Table 2: Micrometric study of *Krushnatulasi* leaf

Characters	Measurments
Petiole	
Multicellular	3.7x0.6mm
Glandular	1.1x0.5mm
Sessile	0.5x0.4mm
Midrib	
Lamina	2mm
Through midrib	4.5mm
Sunken sessile trichome	0.7x0.5mm
Multicellular	7.5x0.5mm
Multicellular Head	1.2x1.5mm
Multicellular neck	0.8x0.7mm
Collenchyma	2-3 Layered
Palisade Cells	2 layered
Spongy Par	5-6 Layered
Palisade ratio	¼ x 2
Stomata	0.6x0.4mm
No. of Stomata	9/sq mm
No. Epidermal cells	24/sq mm
Stomatal Index	27.2

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Table 3: Histochemical tests for *Krushnatulasi* leaf

Sr. no	Reagent	Observation	Characteristics	O.t. Leaves
1.	Phloroglucinol+ Conc. HCl	Red	Lignified cells	++
2.	Iodine	Blue	Starch grains	++
3.	Phloroglucinol+ Conc. HCl	Dissolved	Ca Ox - crystals	++
4.	$Fecl_3$ solution	Dark blue	Tannin cells	++
5.	Ruthenium red	Red	Mucilage	- -
6.	Sudan III	Red	Oil globule	++
++ = Present, - - = Absent				

Table 4: Physicochemical parameters of leaves *Krushnatulasi*

***Krushnatulasi* leaves powder**		
Test	API (Stand. Values)	Found values
Foreign matter	Not >1.5	0
Loss on drying at 110^0 C (%w/w)	-	0.17
Ash value(%w/w)	Not >19	0.94
Acid insoluble ash(%w/w)	Not >0.9	0.053
Water soluble extractive value(%w/w)	Not <13.0	17.65
Ethanol soluble extractive value(%w/w)	Not <6.5	9.4
P^H 10% solution	-	5.09

Table 5: Preliminary Phytochemical investigation of leaves *Krushnatulasi*

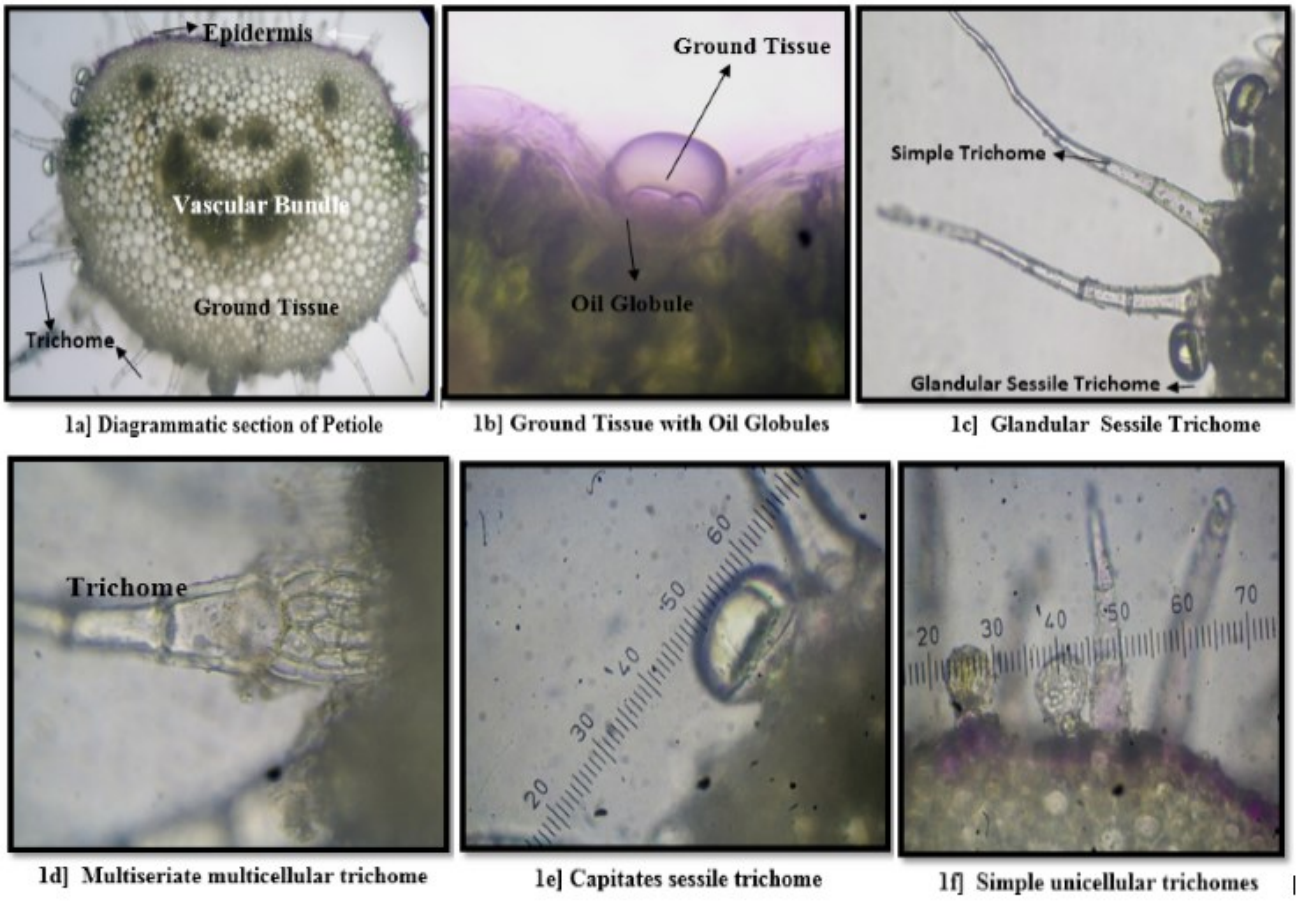
Qualitative Tests		
Sr.	**Test**	***Krushnatulasi***
1	Test for Carbohydrates	+
2	Test for Protein	-
3	Test for Reducing sugar	-
4	Tests for Steroids	+
5	Test for Cardiac Glycosides	+
6	Test for Saponin foam test	+
7	Test for Flavonoids	+
8	Test for Alkaloids	+
9	Test for Tannins and Phenolic compounds	+

Fig.1: Showing the Latitude and Longitudes of collection area (GPS)

Fig.2: Natural habitat, collection and preservation of plant material

Fig.3: Herbarium, macroscopic study of *Ocimum tenuiflorum* Linn.

Plate 1: Transverse Section (T.S.) of Petiole



1a] Diagrammatic section of Petiole

1b] Ground Tissue with Oil Globules

1c] Glandular Sessile Trichome

1d] Multiseriate multicellular trichome

1e] Capitates sessile trichome

1f] Simple unicellular trichomes

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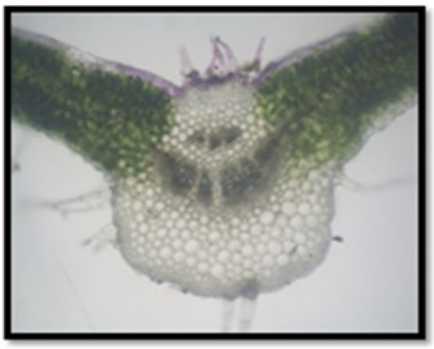
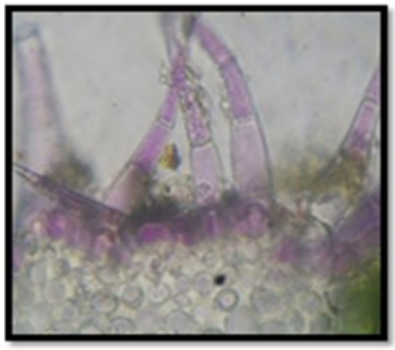
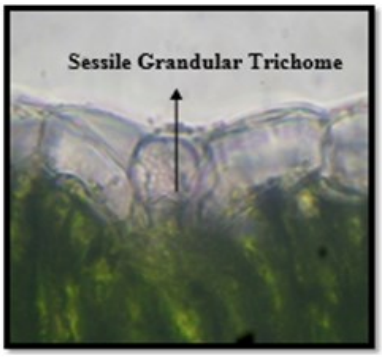
Plate 2- T.S. through midrib



2a] Diagrammatic section through Mid Rib



2b] Trichome above through mid-rib filled with purple colour(*Krishna*)



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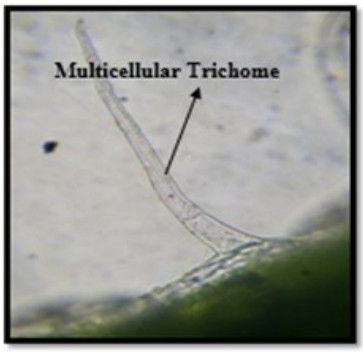

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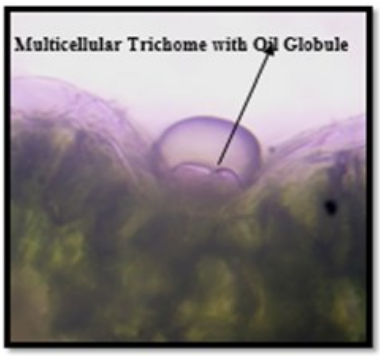

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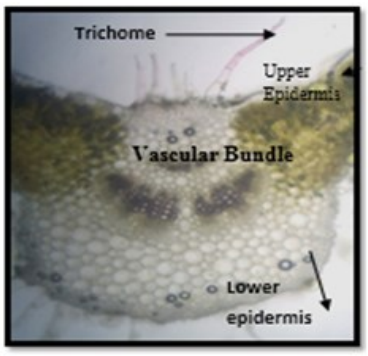
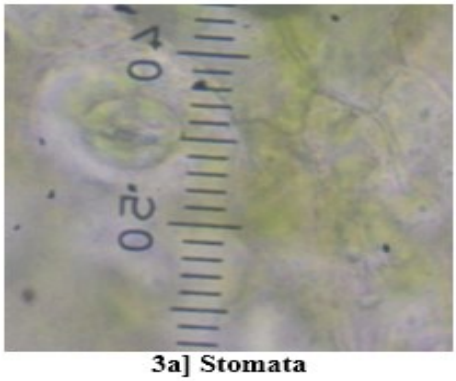


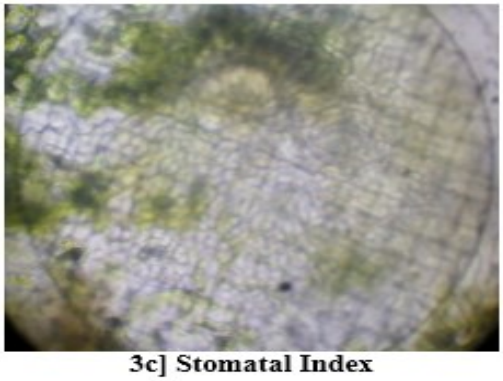
2f

Plate 3 - Micrometric surface study

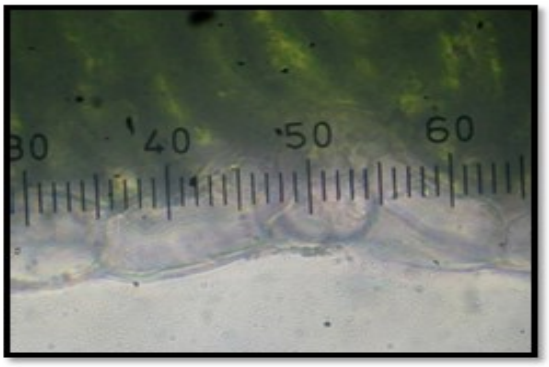


3a] Stomata

3b] Diacytic Stomata



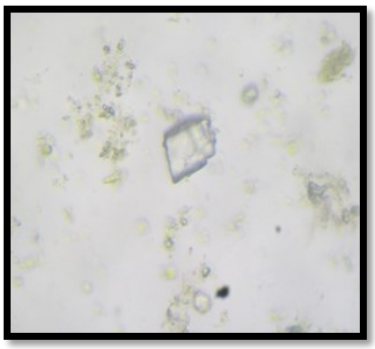
3c] Stomatal Index



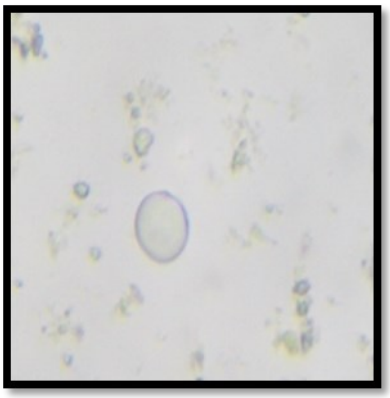
3d] Compact palisade cells

Plate 4- Powder microscopy

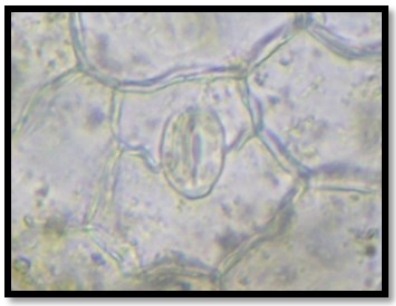
4a] Powder of Krushnatulasi



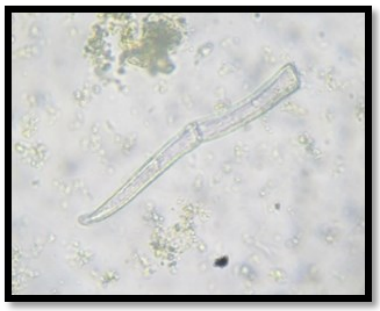
4b] Prismatic Crystal Of Calcium Oxalate



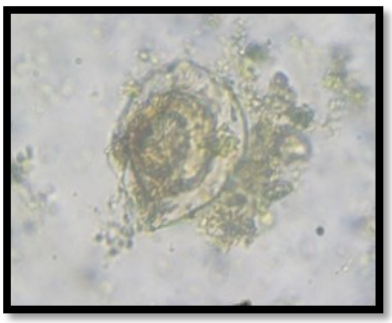
4c] Oil Globule



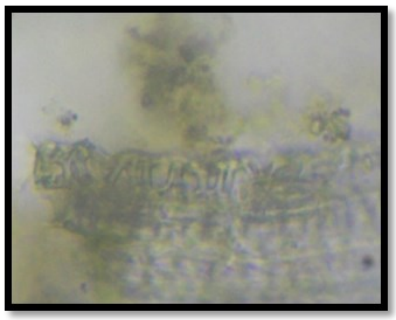
4d] Diacytic stomata



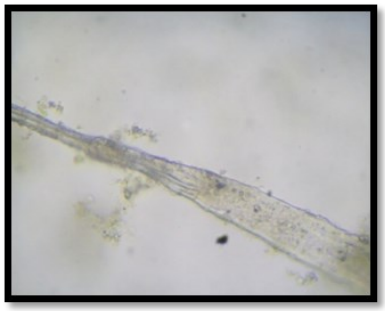
4e] Fragment of Multicellular Trichome



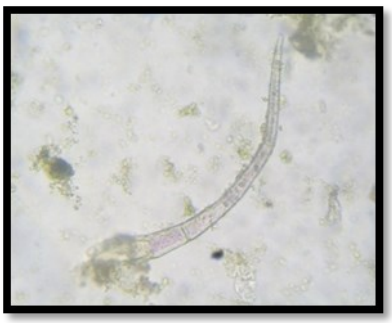
4f] Disturb Granular Trichome



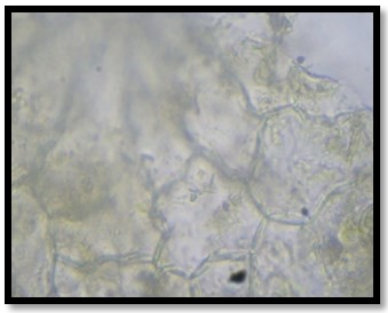
4g] Crystal Fibre



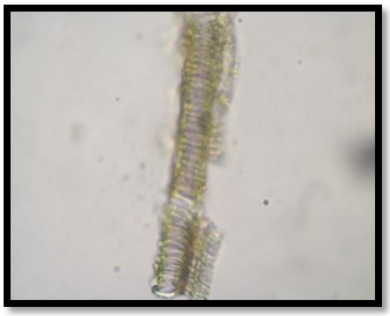
4h] Simple Fibre



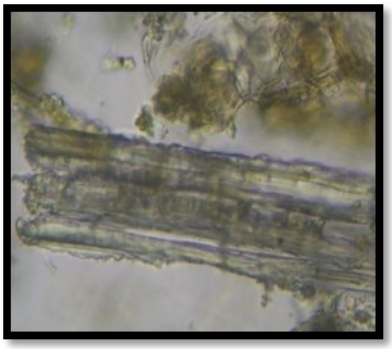
4i] Multicellular Trichome



4j] Epidermis cells

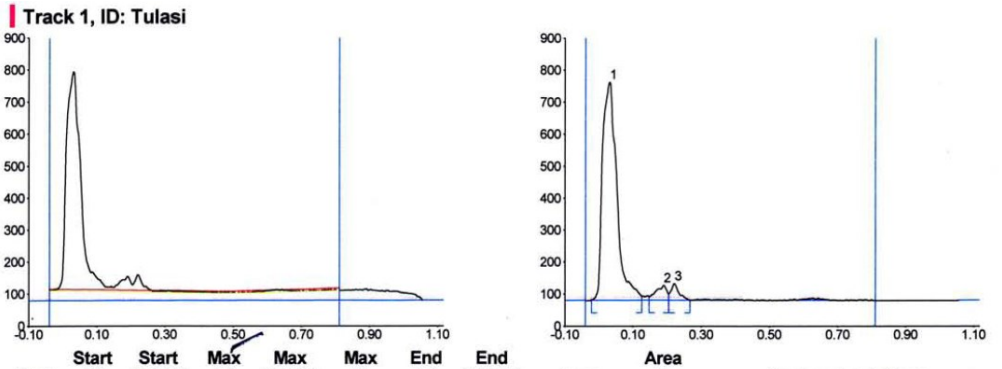


4k] Annular Vessels



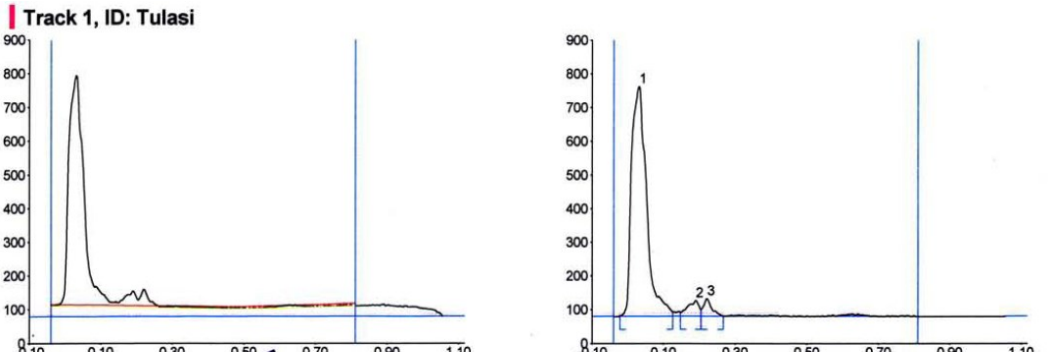
4l] Group of Fibres

Graph 1: Showing the results of HPTLC of *Krushnatulasi* at 254 wavelengths



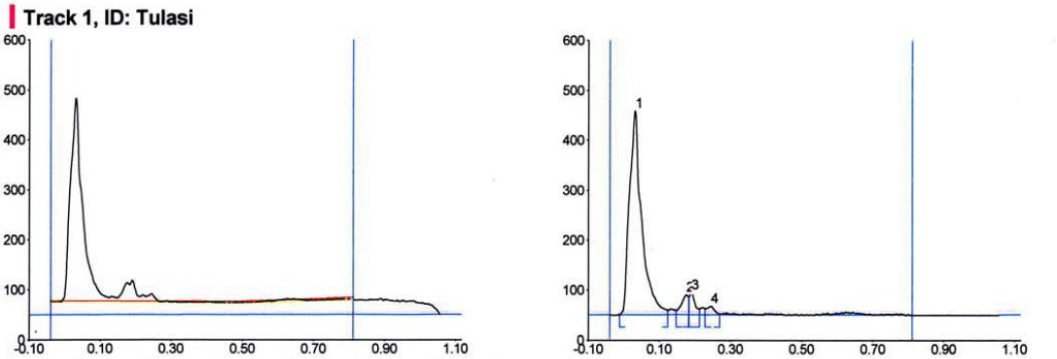
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.02	2.5	0.04	683.9	87.17	0.13	13.8	27364.0	91.07	unknown *
2	0.15	12.3	0.19	47.5	6.05	0.21	21.3	1470.1	4.89	unknown *
3	0.21	21.9	0.23	53.2	6.79	0.27	1.7	1213.0	4.04	unknown *

Graph 2: Showing the results of HPTLC of *Krushnatulasi* at 366 wavelengths



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.02	2.5	0.04	683.9	87.17	0.13	13.8	27364.0	91.07	unknown *
2	0.15	12.3	0.19	47.5	6.05	0.21	21.3	1470.1	4.89	unknown *
3	0.21	21.9	0.23	53.2	6.79	0.27	1.7	1213.0	4.04	unknown *

Graph 3: Showing the results of HPTLC of *Krushnatulasi* at 416 wavelengths



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.01	0.9	0.04	409.0	79.72	0.13	10.1	13514.3	87.81	unknown *
2	0.15	9.4	0.18	40.4	7.88	0.19	36.2	791.8	5.14	unknown *
3	0.19	36.7	0.19	45.1	8.78	0.22	12.5	690.4	4.49	unknown *
4	0.23	12.6	0.25	18.6	3.62	0.27	2.6	393.6	2.56	unknown *

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