Cover Page

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PHARMACOGNOSTICAL AND HPTLC FINGERPRINTING, PHYSICO-CHEMICAL STUDIES OF SINGLE DRUG BAN AJWON OR HASHA (THYMUS SERPYLLUM L.) (WHOLE PLANT)

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Abstract

The aerial parts of Ban Ajwon or Hasha (Thymus serphyllum L.) plant has long been traditionally used in Indian System of Medicine and in other countries as antibiotic, carminative, anthelmintic, antiseptic, antispasmodic, deodorant, diaphoretic, disinfectant, expectorant, sedative and tonic. In order to ascertain the quality of drug, the present study was conducted to evaluate pharmacopoeial standards, High Performance Thin Layer Chromatography fingerprints and quality control parameters. Pharmacognostic studies (quantitative and powder microscopy) were carried out to observe stomatal number, stomatal index, veinlet number, veinlet termination number. The physico-chemical and HPTLC fingerprints studies, quality control and assurance parameters, using WHO guideline to ascertain the quality of drug. In physicochemical and HPTLC fingerprints studies like foreign matter, loss on drying, ash contained, acid insoluble ash and alcohol / water soluble extractive values, oil %, solubility at room temperature and TLC / HPTLC finger prints showed various spots at 254nm, 366nm and visible light (V-S reagent). etc. were carried out. The findings of the study reveal that the plant contains medicinally potant active phytochemical constituents such as terpenoids, terpineol and flavonoids, essential and volatile oils, some amount of silicates and considerable amount of inorganic materials. The findings also reveal that the plant contains mainly polar compounds soluble in alcohol and water. Safety parameters i.e., heavy metals, pesticide residue, microbial load and aflatoxins were also carried out to determine the safety and toxicity of Hasha.

Keywords: Ban Ajwon or Hasha, Pharmacognosy, Physico-Chemical Parameters, HPTLC Fingerprinting and Safety Parameters.

Introduction

Thymus serphyllum L. commonly called wild thyme or creeping thyme also known as Ban ajwain or Ban Ajwon in Hindi and Hasha in Unani system of medicine, is native to Europe, Western Asia and Northern Aferica. It belongs to the family Lamiaceae of which according to the World Checklist contains 7534 species (World checklist of selected plant families), including the genus Thymus L. with 220 species (Herley et al.,2004). A variable, aromatic, prostrate, evergreen shrub, 10-25 cm. high, found in the Himalayas from Kashmir to Nepal at altitude of 15000-4500 m also reported to be grown in the gardens of western India. In Himachal Pradesh, the plant is frequently found in the open, rocky slopes and ghasnis from 1800 m, onwards extending beyond in spits (Anonymous, 1998).

Hasha have been extensively used in traditional system of medicine for centuries. It is most frequently used for treating problems related to the gastrointestinal and respiratory systems. The leaves and floral tops yield a volatile oil, known as oil of Wild Thyme. The plant is considered to possess antispasmodic, antiseptic, expectorant, carminative, anthelmintic and stimulant properties due to its content of volatile oil. The literature on phyto-chemical studies of reveals the presence of Thymol, Carvacrol, p-Cymene, α-Terpinene, Limonene, γ-Terpinene, Linalool and its acetate, α-Terpineol, endo-Borneol, Terpinyl acetate, Isobutyl acetate, Caryophyllene, Geraniol and its acetate, 1,8-Cineole, Citral, Citronellal, Citronellol phenolic acids (mainly rosmarinic, caffeic and chlorogenic acid) and flavonoids (naringenin, dihydro quercetin, apigenin, eriodictyol, quercetin and rutin) etc. in the essential oil. The yield of oil from fresh plant is 0.27 per cent, and from the dried plant up to 0.60 per cent. The composition of these oils is affected by geographic region, the development stage of the plant, the harvest season, habitat, and climatic conditions. The oil is a pale-yellow liquid, with an agreeable odour, reminiscent of thyme, lemon and geranium. The herb is used in preparations of natural herbal remedies such as syrups, decoctions, tinctures, infusions, tea and oil. The shoots are employed for flavouring, and a non- alcoholic beverage is reported to be prepared from the leaves. The leaves and floral shoots are employed for treatment of various diseases, such as suppression of urine and menstruation, catarrh, and convulsive and whooping-coughs; they are also used as sedative in radiculalgia and epilepsy. An infusion of the herb is stated to be useful in the treatment of itch and eruptions on skin. The seeds are given as a vermifuge (Schery, 520; Watt & Breyer-Brandwijk, 528; Kirti & Basu III, 1988; Atlas med. Pl. U.S.S.R., 562; Dymock, Warden & Hooper III, 111), (Anonymous, 1998; Stefana et al., 2015).

The increase in multidrug resistant strains of pathogenic microorganisms has led to extensive phytochemical and pharmacological studies of T. serpyllum L. as an important source of medicinal substances with antioxidant, antimicrobial, antitumor, cytotoxic properties and their effective medicinal application, as well as use in pharmaceutical, food, and cosmetic industries. In

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addition, the increased pressure from consumers for natural products as supplements and their clinical application instead of synthetic chemicals, which are generally perceived by the public as being more toxic, has also stimulated research into many medicinal and aromatic plants of which T. serpyllum L. occupies a very important place. Gairola mention the use of wild thyme in some regions of India for treating menstrual disorders while Shinwari and Gilani state its use as an anthelmintic in Northern Pakistan. In some areas of Italy, wild thyme is used as an important herb in cookery, mainly for flavouring meat or fish. In addition, ethno-botanical studies in Catalonia and Balearic Islands have proved usage of T. serpyllum L. in ethno-veterinary particularly as anti-diarrheal. The British Herbal Pharmacopoeia classifies this species as a medicinal plant and among the indications for its use it mentions bronchitis, bronchial catarrh, whooping cough, and sore throats, (Anonymous, 1998; Stefana et al., 2015).

The present study was conducted to evaluate the pharmacognostical parameters viz., macroscopy and microscopy, HPTLC finger printing and physico-chemical parameters viz., ash contained, acid insoluble contained % values and water and alcohol extractive values %, volatile oil %, pH, Loss on drying, detection of heavy metals, aflatoxins and pesticide residue etc. (Sagar et al., 2020; Meena et al.,2017)

Material and method

Herbal drug was procured from Delhi and Ghaziabad market and identified by botanist using pharmacopoeial standards (Johnson, 1940). The physico-chemical studies of the drug were carried out according UPI and for HPTLC profile DESAGA sample applicator was used and photographs were taken with the help of DESAGA photo-documentation system.

Methods

Pharmacognostic Studies: For pharmocognostical studies microtome sections were taken for general observations. Leaf clearing, quantitative microscopy for determining stomatal number, stomatal index, palisade ratio, vein islet ratio and vein termination were carried out as per the standard procedure. (Sass ,1940).

Quantitative Microscopy: The cleared materials were washed thoroughly and stained with safranin for quantitative microscopic studies.

Maceration Study: Shade dried and coarsely powdered plant was treated with Jeffrey's reagent for a few hours. The action of the macerating fluid was stopped before the complete separation of all cells. Then the macerated tissue was carefully washed in distilled water to remove as much of the acid as possible and then transferred to 50% alcohol for study. Slides were made by placing small quantities of cells in water on a slide. The excess water was evaporated, mounted in glycerine and observed through microscope (Evans et al.,2001).

Results and Discussion

Pharmacognostical Studies, Macroscopic Features

Hasha (T. serpyllum L.) is an aromatic perennial, spreading, sub-shrub, stem up to 20 cm wide and up to 30 cm height (Fig. 1, 2); root thick, branched, variable in length, blackish-brown externally and pale brown internally; older stems up to 3 cm wide with inner hard whitish, longitudinally striated wood; young stems herbaceous, soft, cylindrical, branched, hairy, sub-quadrangular with ridges and furrows, densely tomentose, axillary and oppositely branched, externally purplish-brown to black, internally cream coloured, fracture fibrous; leaves 7–15 mm long and up to 5 mm wide; leaves sessile or a very short petiole; lamina tough, entire, lanceolate to ovate, covered on both surfaces by a grey to greenish grey pubescent hairs; the edges markedly rolled towards the lower surface; midrib depressed on the upper surface and very prominent on the lower surface ; inflorescence verticillasters ; small whorls of purple flowers up to 6 mm crowded into short terminal clusters; calyx green, tubular, 3 mm, 2 lipped of which the upper lip bent back and has 3 lobes on its end and lower longer and has 2 hairy teeth; corolla purple, twice as long as the calyx, 6 mm; odour and taste aromatic.

Microscopic Features: Root: T. S. of root circular in outline shows in (Fig. 3, 4);

Rootlets: Secondary vascular system well developed; secondary xylem exhibits distinct growth ring; primary vascular cylinder present in the centre shows in (Fig. 5).

Underground stem: Secondary vascular system well developed; secondary xylem exhibits distinct growth ring; primary vascular cylinder absent in the centre shows in (Fig. 6).

Stem: T.S. of stem shows quadrangular outline shows in (Fig. 6, 7); **Petiole:** The T. S. of petiole shows in (Fig. 8); **Leaf:** The transverse section of the leaf shows prominent midrib and thick lamina; the lamina slightly raised above the level of the midrib forming shallow concavity on the upper side and bent downwards on the lateral side (Fig. 9). **Midrib:** The T. S. of the midrib shows in (Fig. 10), **Lamina:** The T. S. of lamina shows in (Fig. 11), Epidermal surface: Epidermal cells in surface view consisting of polygonal parenchyma cells with wavy walls; the upper and lower epidermal cells show in (Fig. 12-15).

Cover Page

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Quantitative (Microscopy Study)

Quantitative microscopy such as stomatal number, stomatal index, vein islet number and veinlet termination number are given in Table 1. The mean value of stomatal number (adaxial and abaxial epidermis) and stomatal index (adaxial and abaxial epidermis) are found, as 188 and 213.5 and 16.11 and 20.5 respectively. The values show that stomatal number is comparatively more on abaxial epidermis. Vein islet number, veinlet termination number and palisade ratio were found as 26.25, 28.25 and 4.7 respectively.

Powder Microscopy (Maceration Study)

Greenish brown colour powder; epidermal cells in surface view with caryophyllaceous (diacytic stomata), numerous unicellular non-glandular covering trichomes up to 60µ; numerous glandular trichomes of two types one with a short stalk embedded in the epidermal layer and a unicellular head, the other with an 8- to12-celled head and no stalk up to 75µ wide; trichomes from the calyx uniseriate up to 7 to 8 cells with a narrow terminal pointed cell up to 400µ; spiral vessels up to 20µ; pitted vessels upto75µ with short tail at one or both the ends; fibers thin walled with broad lumen up to 25µ; cork cells in surface view; cortical parenchyma cells; two rows of palisade parenchyma cells and pollen grains small spherical to ellipsoidal up to 30µ with six furrows of six germ pores (hexacolpate), line of pits radiating from the pores (Fig. 16-29).

Analytical Studies

Physico-chemical Parameters: The parameters such as the amount of foreign matter, loss on drying at 105°C, total ash content of the sample, amount of water-soluble ash, amount of acid insoluble ash, amount of water-soluble extractive and alcohol soluble extractive of the sample are useful in establishing quality profile of Thymus serpyllum L.

High Performance: Thin Layer Chromatography Fingerprinting Analysis (HPTLC): The drug samples (2g) were soaked in chloroform and alcohol separately for 18 hours and refluxed for 10 minutes on water bath and filtered through Whatman No.1 filter paper. The filtrates were concentrates and made up to 10 ml in volumetric flask with respective solvents (Saxena and Yadav, 1983). HPTLC analysis was carried out as per the standard method. (Wagner and Bladt, 1996).

Safety Parameters: The microbial load and heavy metal parameters were carried out as per the WHO guidelines (Anonymous, 1998). Aflatoxins were estimated by Kobra cell techniques using Agilent HPLC instruments as per ASTA method (Anonymous, 1997). The heavy metals were analyzed by Atomic Absorption Spectroscopy (Anonymous, 2005) and pesticide residues were analyzed using GC-MS Agilent instruments equipped with Mass selective detector as per AOAC method (Anonymous, 2005; Sagar et al., 2020; Meena et al.,2017)

Results and Discussion

The physico-chemical standards for the dry powder of the whole plant (80 mesh) are given in Table 2. Total ash contained (14.64 %) and acid in-soluble ash contained (5.75 %) indicate the presence of inorganic materials. The alcohol soluble extractive value was (3.62 %) and water-soluble extractive value was (14.32 %) which might be due to the presence of polar organic bio-active phyto-chemical constituents and inorganic constituents respectively. The loss on drying obtained in the drug was 2.32 % which shows the amount of moisture content present in the drug. The pH of 1% &10% aq. solution was obtained (6.94 & 6.24 respectively).

High Performance Thin Layer Chromatography (HPTLC) fingerprinting was performed on 10 cm × 10 cm TLC plates pre-coated with 0.25 µm thin layers of silica gel 60 F_{254} (Merck). The chloroform extract of the sample was applied on the plates as bands 10 mm wide. Linear ascending development to a distance of 80 mm with Toluene: Ethyl acetate (8 : 2 v/v) as mobile phase was performed in a twin-trough glass chamber (20 cm × 10cm) previously saturated with vapors of mobile phase for 20 minutes. The plate was air dried and visualized under λ 254 nm. The HPTLC fingerprint of the chloroform extract of Hasha shows no spots under UV at 254 nm and Under UV at 366 nm shows seven spots at R_f 0.24, 0.30, 0.35, 0.55, 0.60, 0.67, 0.74(all red). Further, derivatized the same plate with 1 % Vanillin Sulphuric Acid Reagent followed by heating at 105^0 for about five minutes in an oven and examine under visible light shows eight spots at R_f 0.13(grayish blue), 0.26 (dark purple), 0.33 (light pinkish purple), 0.42 (pinkish purple), 0.52 (light green), 0.61 (green), 0.72 (pink), 0.76 (green) (Fig.-30, Track 1 and Table 3).

Similarly, the alcohol extract was applied on TLC plate and developed using Toluene: Ethyl acetate (8: 2 v/v) as mobile phase. After development the plate was air dried and visualized under UV light shows no spots at λ 254 nm and Under UV at 366 nm shows seven spots at R_f 0.24, 0.30, 0.35, 0.55, 0.60, 0.67, 0.74(all red). Further, the same TLC plate was derivatized with 1 % Vanillin Sulphuric Acid Reagent followed by heating at 105^0 for about five minutes in an oven and examine under visible light shows eight

Cover Page

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spots at R_f 0.13 (grayish blue), 0.26 (dark purple), 0.33 (light pinkish purple), 0.42 (pinkish purple), 0.52 (light green), 0.61 (green), 0.72 (pink), 0.76 (green) (Fig.30, Track 2 and Table 4).

Microbial Load Analysis: The microbial load and pathogens studies are shown in Table 5.
Heavy Metal Analysis: The medicinal plants materials are generally contaminated with arsenic and heavy metals due to environmental pollution. These components even in trace amounts are dangerous and can damage the important human organs such as kidney, liver and heart (Mukherjee, 2008). The amount of various heavy metals found in the plant material is given in Table 6. The heavy metal contents viz. lead, cadmium, mercury and arsenic as per WHO guidelines were found within the permissible limits viz. 10, 0.3, 1 and 3 ppm respectively. The plant is hence considered non-pollutant in the environment and it cannot cause any illness.
Analysis of Aflatoxins: The aflatoxin can be acute toxic, carcinogenic, mutagenic, teratogenic and immune suppressive to the human being if these are found in the plant above the prescribed limits (Felix and Mello, 1997). The various aflatoxins found in the plant material are given in Table 7. The aflatoxins B1, B2, G1 and G2 were found below the detecting limit so the toxic effect of the plant may be considered as nil and hence, the plant is safe for use.
Analysis of Pesticide Residues: The various pesticidal residues of the plant were tested and found nil. The results are shown in Table 8. So, the plant may be considered as pesticide resistant and plants are quite safe for humans.

Conclusion

In the present study various parameters such as pharmacognostical, physico-chemical, HPTLC finger print and WHO parameters of Ban Ajwon / Hasha (Thymus serpyllum L.) plant were carried out and can be laid down as reference standards of the drug. From the study, it can be concluded that the single drug Ban Ajwon / Hasha is safe and free from any toxic, hazardous substance.

Table 1: Quantitative Microscopy of Thymus serpyllum L.- Whole Plant

S. No.	Parameters Analysed	Observations	
		Range	Mean
1	Stomatal Number – Adaxial epidermis	184-192/sq.mm	188/ sq.mm
	Stomatal Number – Abaxial epidermis	211 to 216/sq.mm	213.5/ sq.mm
2	Stomatal Index – Adaxial epidermis	16.11/sq.mm	-
	Stomatal Index – Abaxial epidermis	20.5/sq.mm	-
3	Vein islet number	26.25/sq.mm	-
4	Veinlet termination number	28.25/sq.mm	-
5	Palisade ratio	4.7	-

Table 2: Physico-Chemical Parameters of Thymus serpyllum L.- Whole Plant

S. No.	Parameters Analysed	Batch I	Batch II	Batch III
	Foreign matter (%, w/w)	0.94	0.95	0.96
	Extractive value (%, w/w) Alcohol Soluble Water Soluble	3.59% 14.32%	3.62% 14.36%	3.62% 14.32%
	Ash contained value (%, w/w) Total ash Acid insoluble ash	14.62% 5.75%	14.64% 5.76%	14.64% 5.75%
	pH values 1% aqueous solution 10% aqueous solution	6.95 6.24	6.94 6.22	6.94 6.24
	Loss on drying at 105ºC (%, w/w)	2.32	2.34	2.32
	Volatile oil (%. w/v)	2.05	2.06	2.08

Cover Page

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Table 3: Rf Values of Chloroform Extract

Solvent system	Rf Values		
	254nm	366nm	After Derivatization
Toluene: Ethyl acetate (8.0: 2.0, v/v)	N/D	0.24 (Red)	0.13 (Grayish blue)
	N/D	0.30 (Red)	0.26 (Dark purple)
	N/D	0.35 (Red)	0.33 (Light pinkish purple)
	N/D	0.55 (Red)	0.42 (Pinkish purple)
	N/D	0. 60 (Red)	0.52 (Light green)
	N/D	0.67 (Red)	0.61 (Green)
	N/D	0.74 (Red)	0.72 (Pink)
	N/D		0.76 (Green)

Table 4: Rf Values of Alcohol Extract

Solvent system	Rf Values		
	254nm	366nm	After Derivatization
Toluene: Ethyl acetate (8.0: 2.0, v/v)	N/D	0.24 (Red)	0.13 (Grayish blue)
	N/D	0.30 (Red)	0.26 (Dark purple)
	N/D	0.36 (Red)	0.33 (Light pinkish purple)
	N/D	0.56 (Red)	0.44 (Pinkish purple)
	N/D	0. 62 (Red)	0.53 (Light green)
	N/D	0.69 (Red)	0.61 (Green)
	N/D	0.76 (Red)	0.71 (Pink)
	N/D		0.75 (Green)

Table 5: Analysis of Microbial Load of Thymus serpyllum L.- Whole Plant

S. No.	Parameter Analyzed	Results	WHO Limit
1	Total Bacterial Count	400 cfu/gm	10^5cfu/gm
2	Total Fungal Count	100 cfu/gm	10^3cfu/gm
3	Escherichia coli	Absent	Absent
4	Salmonella typhai Spp	Absent	Absent
5	Staphylococcus aurous	Absent	Absent

Table 6: Estimation of Heavy Metal of Thymus serpyllum L. - Whole Plant

S. No.	Parameter Analyzed	Results	WHO Limit
1	Lead	Not detected	10 ppm
2	Cadmium	Not detected	0.3 ppm
3	Mercury	0.0129 ppm	1 ppm
4	Arsenic	0.098 ppm	3 ppm

Where ppm: parts per million

Cover Page

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Table 7: Estimation of Aflatoxins of Thymus serpyllum L.- Whole Plant

S. No.	Parameter Analyzed	Results	WHO Limit
1	Aflatoxin, B1	Below Detectable Limit	0.5 ppb
2	Aflatoxin, B2	Below Detectable Limit	0.1 ppb
3	Aflatoxin, G1	Below Detectable Limit	0.5 ppb
4	Aflatoxin, G2	Below Detectable Limit	0.1 ppb

Where ppb: parts per billion

Table 8: Estimation of Pesticide Residues of Thymus serpyllum L.- Whole Plant

S.N0.	Parameter Analyzed	Results	WHO Limit (mg/kg)
1	DDT (all isomers, sum of ρ, ρ'-DDT, α, ρ' DDT, ρ, ρ'-DDE and ρ, ρ'-TDE (DDD expressed as DDT)	Not detected	1.0
2	HCH (sum of all isomers)	Not detected	0.3
3	Endosulphan (all isomers)	Not detected	3.0
4	Azinphos methyl	Not detected	1.0
5	Alachlor	Not detected	0.02
6	Aldrin (Aldrin and dieldrin combined expressed as dieldrin)	Not detected	0.05
7	Chlordane (cis & tans)	Not detected	0.05
8	Chlorfenvinphos	Not detected	0.5
9	Heptachlor (sum of heptachlor and heptachlor epoxide expressed as heptachlor)	Not detected	0.05
10	Endrin	Not detected	0.05
11	Ethion	Not detected	2.0
12	Chlorpyrifos	Not detected	0.2
13	Chlorpyrifos-methyl	Not detected	0.1
14	Parathion methyl	Not detected	0.2
15	Malathion	Not detected	1.0
16	Parathion	Not detected	0.5
17	Diazinon	Not detected	0.5
18	Dichlorvos	Not detected	1.0
19	Methidathion	Not detected	0.2
20	Phosalone	Not detected	0.1
21	Fenvalerate	Not detected	1.5
22	Cypermethrin (including other mixtures of constituent isomers sum of isomers)	Not detected	1.0
23	Fenitrothion	Not detected	0.5
24	Deltamethrin	Not detected	0.5
25	Permethrin (sum of isomers)	Not detected	1.0
26	Pirimiphos methyl	Not detected	4.0

Cover Page

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Fig. 1: T. serpyllum L.- Habbit

Fig. 2: Single plant

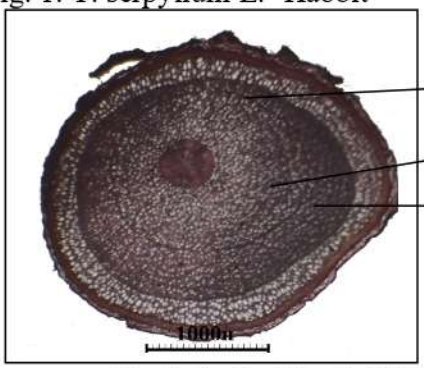

Fig. 3: T. S. of Root (1000µ)

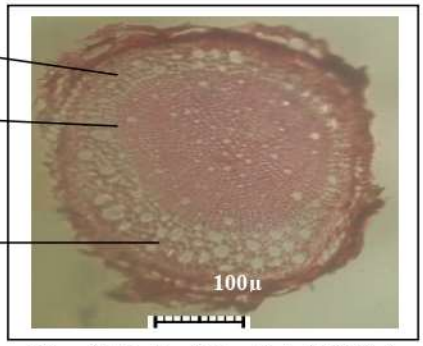

Fig. 5: T. S. of Rootlets(1000µ)

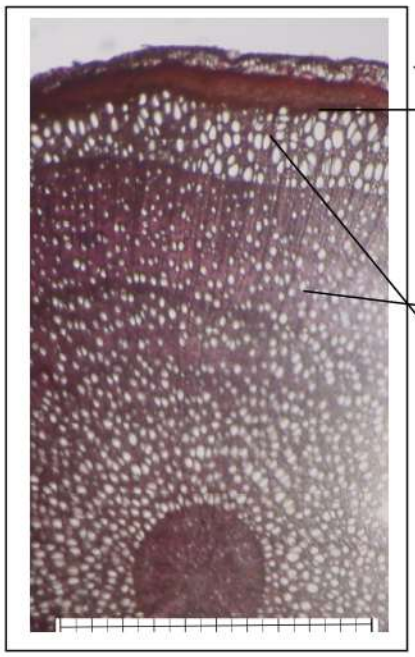

Fig. 4: T. S. of Root – Enlarged Portion (1000µ)

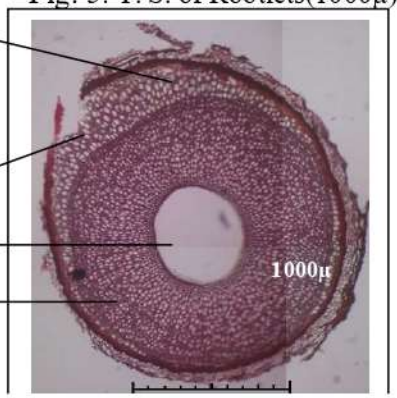

Fig. 6: T. S. of underground stem(1000µ)

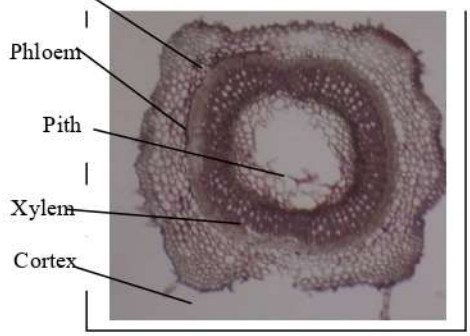

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Fig. 7: T. S. of Young Stem(1000µ)

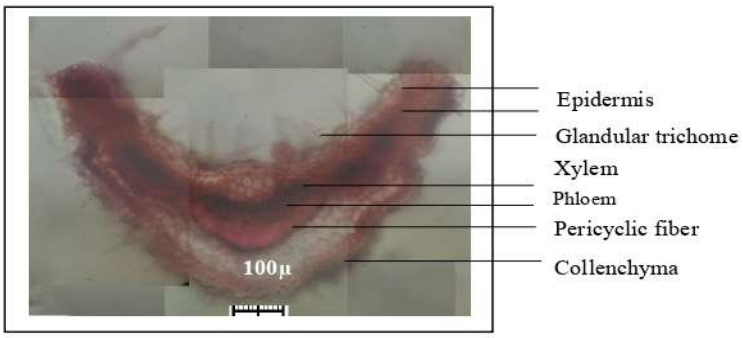

Fig. 8: T. S. of Petiole

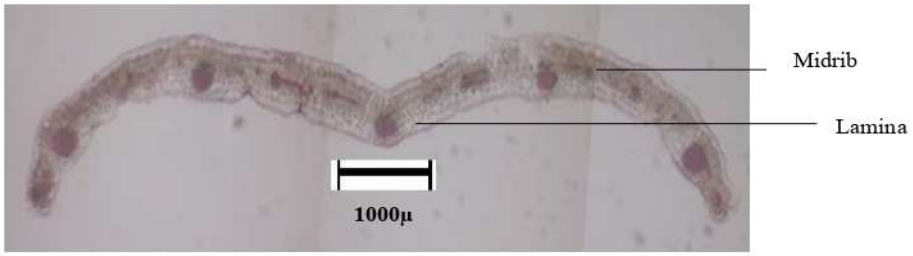

Fig. 9: T. S. of Leaf

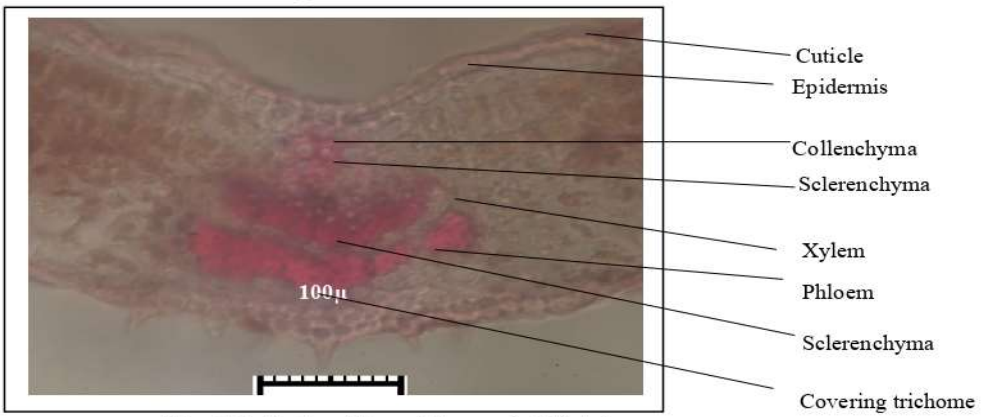

Fig. 10: T. S. of Leaf through Midrib

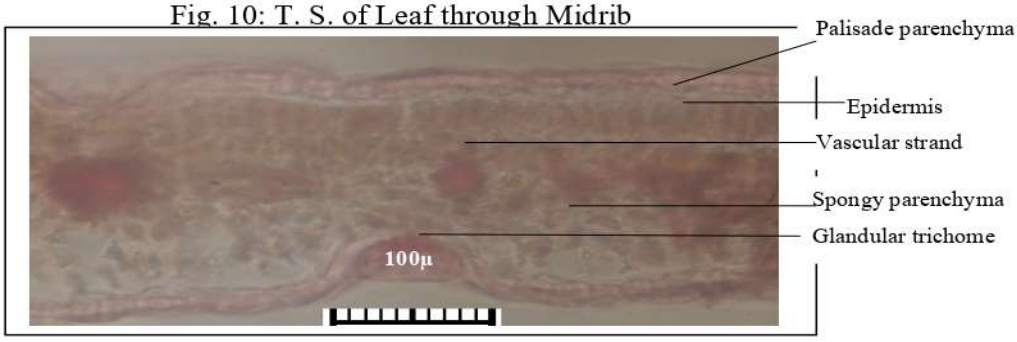

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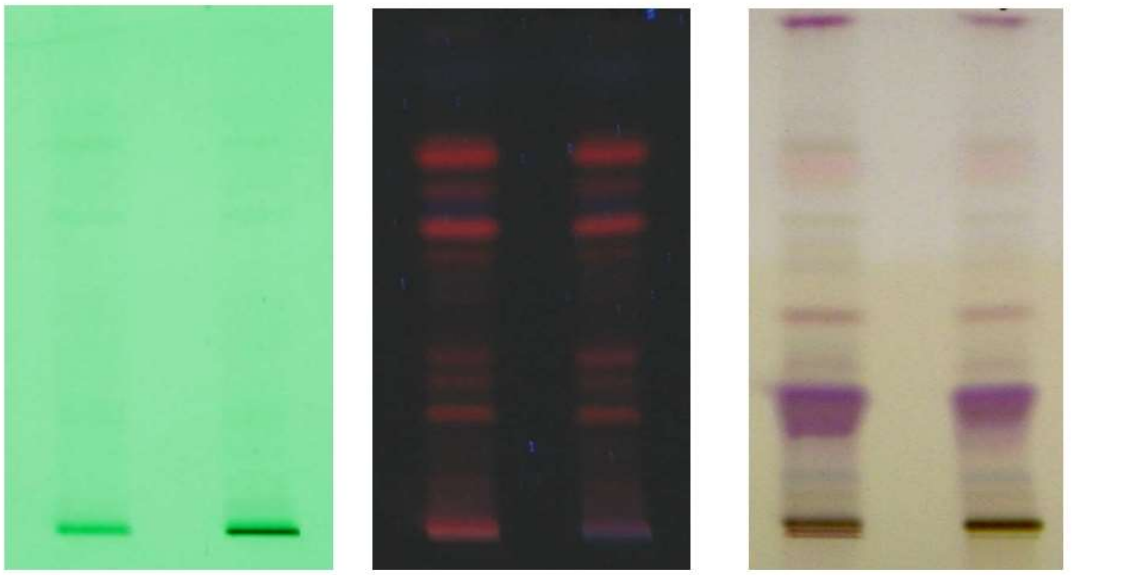
Fig. 11: T. S. of Leaf through Lamina



Solvent System: Toluene : Ethyl acetate (8 : 2 v/v)

Track 1. Chloroform extract Track 2. Alcohol extract

Fig. 30: HPTLC fingerprint of Alcohol and chloroform extracts of (*Thymus serpyllum* L).

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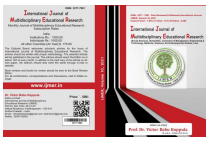
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