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Pharmacognostical, Physicochemical studies and TLC fingerprinting of *Euphorbia tithymaloides*

Neetu Soni and Rajani Srivastava

Abstract

Background: *Euphorbia tithymaloides* (L.) Poit (Euphorbiaceae) leaves are extensively used in traditional medicine to cure asthma, mouth ulcers, persistent coughing, venereal troubles & ringworms. It is also known to possess antiprotozoal, anti-inflammatory, anti-plasmodial, anti-mycobacterial, anthelmintic and antimicrobial activities.

Aim: The aim of present study is an attempt to provide detailed information on Pharmacognostical & physicochemical features and TLC fingerprinting of *E. tithymaloides*.

Material and Methods: Fresh leaves from the plant were selected for macroscopical and microscopical studies while air-dried powdered sample of leaves were used for physicochemical evaluation. The leaf extract of petroleum ether and chloroform were used for TLC fingerprinting of selected plant. Pharmacognostical and physicochemical standardization performed as per WHO Quality control methods for medicinal plant materials.

Result: The macroscopical study of leaf showed that leaves are dark-green, alternate, ovate in shape having entire margin, acute apex and attenuate base while microscopy of leaves revealed that it consists of single layered epidermis with cuticle and trichomes, 2-3 layered collenchymas near the upper epidermis followed by 6-8 layered spongy parenchyma and a centrally placed C-shaped bi-collateral vascular bundles. Ash values, extractive values, loss on drying, foaming index etc. physicochemical parameters were also found out. TLC fingerprinting, revealed separation of appreciable numbers of Phyto-constituents.

Conclusion: The results of the study can be used as markers in the identification and standardization of this plant and also towards monograph development on the plant. TLC fingerprinting could be useful for qualitative and quantitative analysis of separated Phytoconstituents.

Keywords: *Euphorbia tithymaloides*, Pharmacognostical study, physicochemical study, Microscopy, Macroscopy

1. Introduction

Euphorbia tithymaloides (L.) Poit (Family: *Euphorbiaceae*), commonly known as devil's backbone, is widely distributed throughout tropical and subtropical North America and Central America and some areas of South Asia (India: Assam, Bihar, Gujarat, Madhya Pradesh, Maharashtra, Odisha, UP). This plant, which is often known by its old scientific name *Pedilanthus tithymaloides*, is relatively intolerant of high soil salinity levels.^[1, 2] As it has got a very impressive variegated foliage, it is mostly used as an ornamental plant and for making fencing in garden. The plant is 0.4 to 3m tall & 40-6cm wide whose fleshy tubular, zigzag stems produce thick, dark-green, ovate leaves and peculiar beak shaped flowers. Leaves are sessile, glabrous and acuminate in shape having length of about 1.4-3 inches while flowers whose color vary from bright white to pink are arranged in dichotomous congested cyme fashion. Fruits are sulcate, deeply 3-lobed having sub globose grey-brown seeds. Although flowering in flushes year-round in warm tropical regions, it blooms most heavily in summer. When flowering or chilly winter temperatures occur, the leaves may blush pink. An extended drought or winter cold spell may cause the leaves to completely drop off. It is widely used in traditional medicine to treat asthma, persistent coughing and mouth ulcers. The plant is also reported to possess various antioxidant principles like Kaempferol 3-O-b-D-glucopyranoside-6'--(3-hydroxy-3-methylglutarate), Quercitrin, Isoquercitrin, Scopoletin and other phytochemicals viz. steroids, tannins, triterpenes, coumarins and saponins which have been shown to possess anti-diabetic, anti-viral, hemostatic, anti-microbial, anti-helminthic activity. Its sap has been traditionally used to treat callouses, ear ache, insect stings, ringworm, skin cancer, toothache, umbilical hernias, and warts^[3].

2. Materials and Methods

2.1 Chemicals and instruments

Microscope, Camera Lucida, stage and eye piece micrometer, black chart paper, blade, watch glass, slide, pipette, HCl, Phloroglucinol, Chloral hydrate, Ethanol, Chloroform & Sulfuric acid. All the solvents, chemicals and reagents used were analytical grade and procured from department of pharmacognosy, SIHAS, SHUATS.

2.2 Plant collection

The fresh plant *E. tithymaloides* for quantitative microscopy and physicochemical studies was procured from herbal garden SIHAS, Allahabad with the help of field botanist and identified by department of pharmacognosy, SIHAS, SHUATS. The healthy leaves of the plant were first collected and stored properly. They were then washed with water and dried in sunlight for one hour and thereafter dried in shade. The dried leaves along with the dried plant were powdered with the help of grinder and were passed through the sieve no 60. The Pharmacognostical evaluation and physicochemical studies performed by using dried fine powder and fresh healthy leaves used for plant sectioning.

2.3 Macroscopical studies of leaf, stem & root

The morphological characters like condition, type, size, shape, apex, margin, venation, base, petiole, surface, phyllotaxy, color, odor, taste of leaves, length, branching, color, shape, internodal length of stem and type, shape, rootlets, odor, taste, presence or absence of bark of root of *E. tithymaloides* were studied and performed as per WHO guidelines for herbal drug evaluation [4].

2.4 Microscopical study

Sectioning: The fresh leaf collected from the plant was washed with clean water. A small part of leaf from the midrib was then cut off with the help of sharp blade. The cut off sections were put in a watch glass containing mixture of Phloroglucinol & concentrated HCl in equal ratio, the best one was chosen for microscopical study. The nature of stomata determined by performing microscopy of surface preparation of both leaf surfaces as per procedure mentioned in K.R. Khandelwal [5].

Photomicrographs of the Transverse Section of *E. tithymaloides* leaf at different magnifications were taken with help of microscope.

2.5 Quantitative microscopy

2.5.1 Stomatal Number

It is the average no of stomata per square mm of the epidermis of the leaf surface. First of all, a fresh leaf collected from the plant was washed with clean water. The epidermis was separated from the leaf by breaking the leaf into pieces and placed carefully on a slide with the help of a brush along with 1-2 drops of chloral hydrate. A square was then drawn on a drawing sheet with the help of camera lucida, the stomata & epidermal cells were traced in the square. The epidermal cells and the stomata outside the square were traced to completion on two adjacent sides of the square, for counting purpose. The no of epidermal cells and the stomata within the square and the cells which cover more than half on two adjacent sides were counted and stomatal no calculated [6].

2.5.2 Stomatal index

Same procedure followed as for determination of stomatal number and stomatal index calculated. It is calculated by

using this formula:

$$\text{Stomatal Index: } I = S/E + S * 100$$

I = Stomatal Index, S=No of stomata per unit area, E= No of epidermal cells in the same area.

2.6 Powder microscopy

The healthy plants were first collected and then stored properly. It was then dried under sunlight for 4 hours and then at a shade for one whole day. The dried plant was dried in a grinder and passed through sieve no 60. The fine powder was then stained with Phloroglucinol and HCl in equal ratio and observed under microscope at suitable magnification and photomicrograph of identified tissue fragments were captured.

2.7 Physicochemical studies

The physicochemical parameters such as Total ash, Water Soluble Ash, Acid insoluble Ash, Sulphated Ash, Solvent extractive value, Loss on drying, Foaming Index performed as per standard method [4].

2.7.1 Determination of ash values

2.7.2 Total ash

2g of air-dried powdered drug was accurately weighed and put into the tared silica crucible. The crucible was supported on a pipe-clay triangle placed on a ring of retort stand. Then, it was heated with a burner, using a flame about 2cm high and supporting the crucible about 7 cm above the flame heat till vapors almost ceased to be evolved; then it was lowered and heated more strongly until all carbon was burnt off. It was then cooled in a desiccator. The ash thus produced was weighed and the percentage of total ash with reference to the air-dried sample of the crude drug was calculated [4].

2.7.3 Water soluble ash

The ash obtained while calculating total Ash value was washed using 25 ml of distilled water into 100ml beaker. It was boiled for 5 minutes and then filtered through an 'ashless' filter paper. The residue thus obtained was washed twice with hot water. The crucible was ignited, cooled and weighed. The filter paper containing the residue was put into the crucible which was then heated gently until vapors ceased to be evolved and then more strongly until all carbons has been removed. After the ignition, it was cooled in a desiccator. The residue was weighed and water-soluble ash was calculated by subtracting the weight of residue from weight of total ash. The % of water-soluble ash was then calculated with reference to air-dried powdered drug [4].

2.7.4 Acid insoluble ash

First of all, the total ash was washed using 25ml of 2N HCl into 100ml beaker. It was then boiled for 5 minutes and then filtered through an 'ashless' filter paper. The residue thus obtained was washed twice with hot water. The crucible was ignited, cooled and weighed. The filter paper containing the residue was put into the crucible which was then heated gently until vapors ceased to be evolved and then more strongly until all carbons have been removed. After the ignition, it was cooled in a desiccator. The residue was weighed and acid- insoluble ash was calculated by subtracting the weight of residue from weight of total ash. The % of acid insoluble ash was then calculated with reference to air-dried powdered drug [4].

2.8 Solvent extractive values

5gm of drug was macerated with 100ml of different solvents (90% alcohol- alcohol soluble extractive, 90% chloroform water- water soluble extractive) in a dry 250 ml conical flask for 24hrs. It was shaken frequently during first 6 hrs. and allowed standing for 18hrs. Thereafter it was filtered. 25ml out of filtrate was evaporated to dryness on a water-bath. The drying was then completed in an oven at 100°C. The weight was then measured and the percentage w/w of extractive was subsequently calculated with reference to the air-dried drug [4].

2.9 Loss on drying

Firstly 3.0 gm of powdered drug was weighed accurately and put in a tarred porcelain dish which was earlier dried at 105°C using hot air ovens at constant weight. Using the difference in weight, the percentage loss of drying with reference to the air-dried substance was calculated [4].

2.10 Foaming Index

About 1 gm of coarsely powdered drug was weighed accurately and transferred to 500ml conical flask containing 100ml of water maintained at moderate boiling at 80-90C for about 30 min. After 30 min of boiling, it was cooled, filtered into a volumetric flask and sufficient water through the filter to make the volume up to 100ml (V₁). Clean 10 stopper test tubes were then taken and marked as 1, 2,....., 10. The successive portions of 1ml, 2ml,....., 10ml drug was taken in separate tubes and then remaining volume was adjusted with the liquid up to 10ml in each. After closing the tubes with stoppers, they were shaken for 15 seconds and allowed to stand for 15 min. Then the height was measured. If the height of the foam in each tube is less than 1cm, the foaming index is less than 100(not significant). While, if the foam is more than 1cm height after the dilution of plant material in the sixth tube, then corresponding number of the test tube was the index sought. If the height of the foam in every tube is more than 1cm, the foaming index is more than 1000. In this case, 10ml of the first decoction of the plant material needs to be measured and transferred to a 100ml volumetric flask (V₂) and volume is to be maintained up to 100ml and follow the same procedure. Foaming Index was calculated by using this formula:

Foaming Index = 1000/a in case of V₁;

Foaming Index = 1000 * 10/a in case of V₂

Where, a= volume (ml) of decoction used for preparing the dilution in the tube where exactly 1cm or more foam was observed [3].

2.11 Extraction of crude dried plant material

Solvent used of extraction were Petroleum ether and chloroform. The method of extraction was hot continuous extraction (Soxhletation).

2.11.1 Extraction procedure

The solvent (500 ml of Petroleum ether, chloroform) added to a round bottom flask, which was attached to a Soxhlet extractor and condenser on heating mantle. The Blended plant material loaded into the thimble, which was placed inside the Soxhlet extractor. The solvent heated by use of heating mantle when it evaporated, moved through the apparatus to the condenser. The condensate dripped into the reservoir containing the thimble. Once the level of solvent reached the

siphon, it poured back into the flask and the cycle repeated several times. The process carried out for 16 hours. After the completion of 16 hour the round bottom flask was removed for collection of extracted product by distillation process. The collected extracts (Petroleum ether and chloroform) product packed in suitable container for further study.

2.12 Thin layer chromatography (TLC) fingerprinting for Separation of Phytochemical constituents

2.12.1 Preparation of TLC plate

The glass slides were cleaned and dried in hot air oven. Slurry (2%) of silica gel was prepared by mixing silica gel with double the volume of distilled water in a clean beaker. Plates were prepared by pouring method. The slides were kept as such for few minutes for drying. Then the plates were activated by heating in hot air oven at 110 C for 30 min.

2.12.2 Preparation of solvent system: Toluene: ethyl acetate: formic acid in following ratios 7:3:1, 7:2:1, 7:1:1 were selected for best separation of Phyto constituents present in petroleum ether and chloroform extract.

2.13 Sample preparation

Petroleum ether and chloroform extracts treated with similar solvents for making required concentration of sample, for spotting of TLC plate.

2.14 Sample Loading

The prepared TLC slides were allowed to cool at room temperature and marked about 1 cm from the bottom as the origin. The samples were loaded at the center of each slide above from the edge and then slides were kept in the developing tank without touching baseline by solvent. Plates were developed with solvent front in linear ascending direction. The final solvent front was marked and the slides were dried.

2.15 Detection of separated Phyto constituents corresponding to spots

The developed plates were best detected by visual inspection, all the separated spots inspected for qualitative and quantitative characterization of Phyto constituents.

3. Results and Discussion

3.1 Macroscopical features

3.1.1 Leaf

The information obtained from the macroscopical studies of the leaf (like type, base, margin, apex, color, odor, etc.) summarized in the following table 1.

Table 1: Macroscopical features of *E. tithymaloides* leaf

Parameter	Observation
Condition	Fresh
Type	Simple
Size	Length: 4.4- 7.4cm Width: 2.6-3.9cm
Shape	Ovate to cordate
Margin	Entire
Apex	Acute
Base	Attenuate
Venation	Unicostate reticulate venation
Phyllotaxy	Alternate
Surface	Glabrous
Color	Upper surface: Dark green, Lower surface: Light green
Odor	Slightly aromatic
Taste	Mucilaginous
Extra feature	Very thick leaf with cuticle present in it

3.1.2 Stem

The observed size of stem were 28.3cm long and 1.6-3.1cm thick with branches different in both size/vigor and angle from the main axis (Anisotomous branching). It was non-woody, greenish in color and cylindrical in shape which sprouts from woody crown of the root. It has got distinct nodes and internodes (internodal length varies from 1.8cm-3.9cm). The stem when broken exudes a caustic milky sap (latex) which gives mucilaginous taste. It has slightly aromatic odor.

3.1.3 Root

The type of root observed as tap root (having length of about 13.1cm) with few branches and numerous small lateral roots that show horizontal downward growth. Roots were light brown in color. The woody crown present in the top has durable in structure. The root was externally smooth with thin bark (easily gets peeled off exposing pale yellow inner part) which featured with few cracks and fissures on its surface. It was slightly aromatic in odor but tasteless in taste.



Fig 1: Leaf (Upper surface-Adaxial) of *E. tithymaloides*



Fig 2: Leaf (Lower surface-Abaxial) of *E. tithymaloides*



Fig 3: Root of *E. tithymaloides*



Fig 4: Stem with leaves of *E. tithymaloides*

3.2 Microscopy

Transverse section of leaf revealed microscopical features of the leaf. The transverse section of leaf passing through mid rib consists of single layered upper and lower epidermis, made up of closely packed parenchyma cell, without intercellular spaces and minute stomatal pores. Upper surface of leaf covered with thick cuticle which consists of wax like substance to prevent excessive evaporation of water, this characteristic of leaf makes plant drought resistant. The

Presence of trichomes (both glandular and covering) were observed. The presence of 2-3 layers of collenchyma was seen below upper epidermis and above lower epidermis. The cells of collenchymas closely packed, without intercellular spaces and contain deposition of suberin which makes leaves rigid and strong. Anomocytic stomata were observed in the epidermis of the leaf (Lower surface has comparatively higher no of stomata as compare to upper surface of epidermis). The epidermal cells surrounding the subsidiary cells were thick

walled and irregular with walls straight at some part of the surface. Presence of C-shaped, centrally situated, bi-collateral vascular bundle (phloem-xylem-phloem) observed. Metaxylem observed towards the lower epidermis side while Protoxylem can be seen towards the upper epidermis side.

The mesophyll portion of leaf consists of 6-8 layers of loosely arranged spongy parenchyma. The mesophyll cell behaves as ground tissue substance and help in transportation of water and mineral through involvement of conducting leaf veins and veinlets.

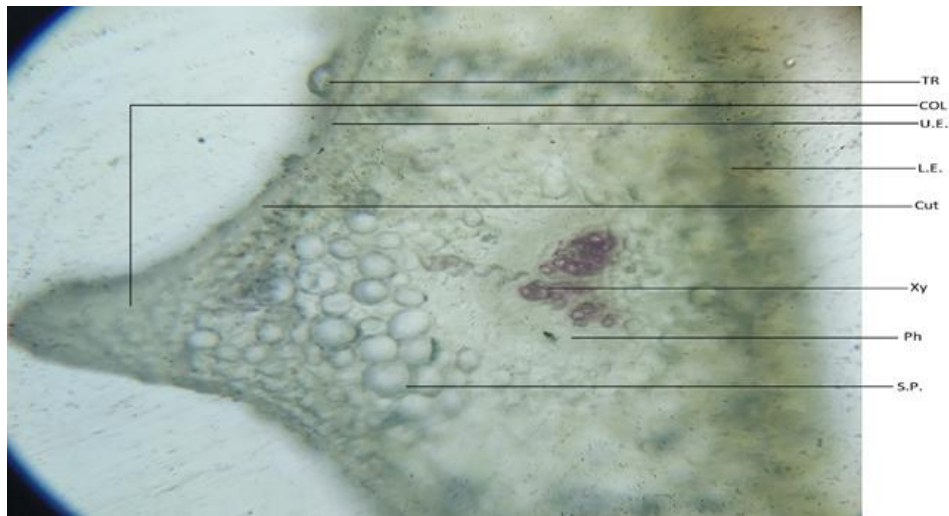


Fig 5: Transverse Section of leaf of *E. tithymaloides*. (TR- Trichome, Col- collenchyma, U.E.- Upper Epidermis, L.E.- Lower Epidermis, Cut- Cuticle, Xy- Xylem, Ph- Phloem, S.P. -Spongy Parenchyma)

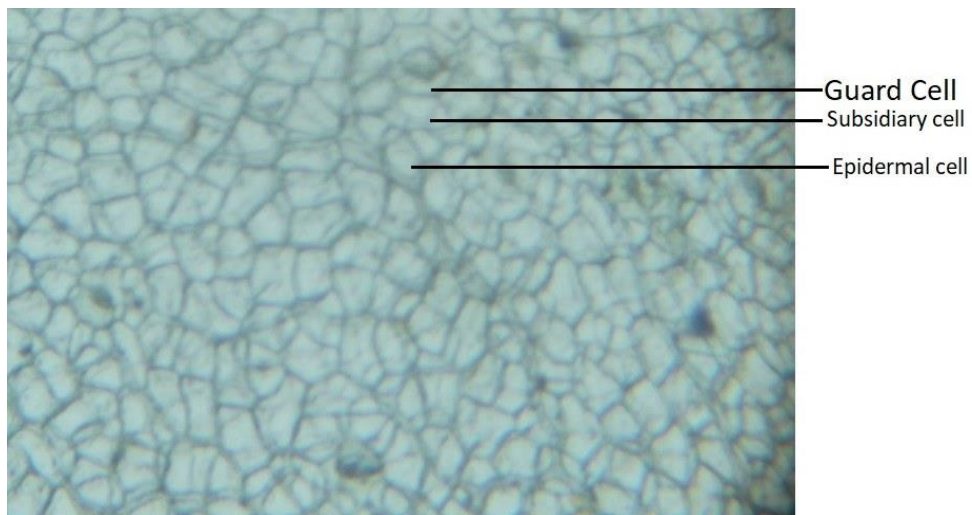


Fig 6: Leaf upper surface of *E. tithymaloides*.

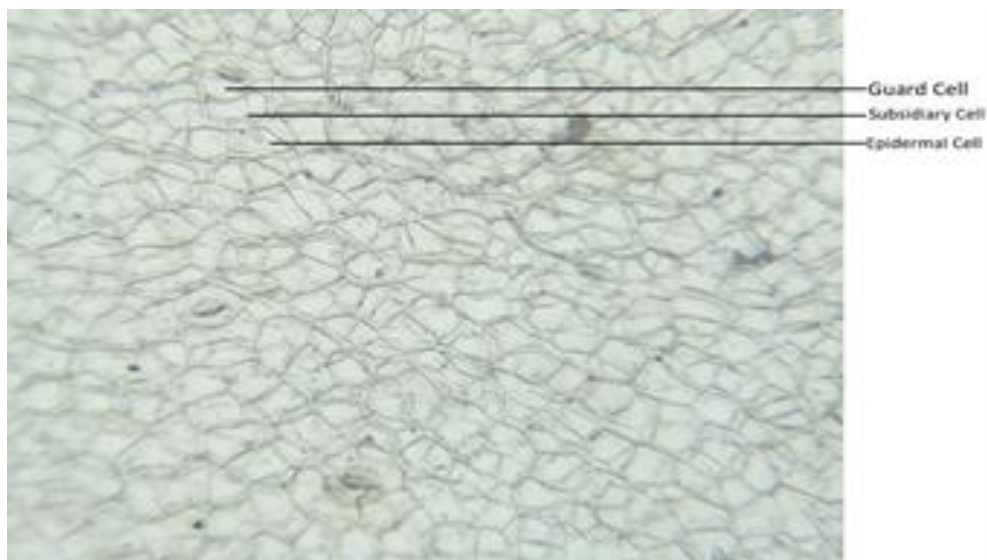


Fig 7: Leaf lower surface of *E. tithymaloides*.

3.3 Quantitative microscopy of leaf

The quantitative microscopy of the leaf of *E. tithymaloides* performed for determination of stomatal number and stomatal index. These standard parameters could be helpful for establishing identity and purity of herbal drug as well for establishing quality parameters. The results represented in table number 2.

Table 2: Leaf constants for *E. tithymaloides* leaf

Leaf Constants	Values	
	(Upper surface)	(Lower surface)
Stomatal No	11.88	22.5
Stomatal Index	4.17	7.88

3.4 Powder microscopy of whole plant of *E. tithymaloides*

Powder of the plant light brown colored, faint in odor and has slightly mucilaginous taste. The powder microscopy revealed diagnostic features. It consist of

1. Epidermal cells with numerous spongy parenchymatous cells.
2. Numerous prismatic crystals of calcium oxalate (crystals variable in size with smaller ones often forming small aggregates).
3. Very occasional covering trichomes (they were unicellular, thick-walled and conical in shape)
4. Long, non-lignified fibres with slit-shaped pit. Some of them occur as associated with vessels. Numerous lignified xylem tracheids and vessels.

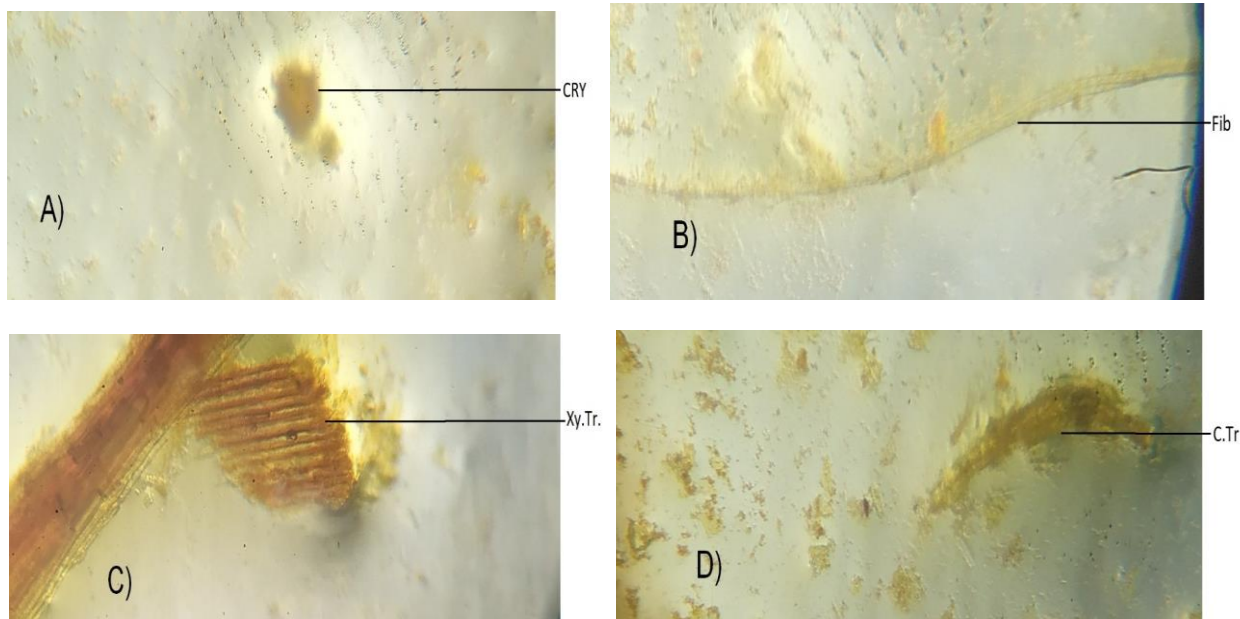


Fig 8: Powder microscopy of whole plant of *E. tithymaloides* (A-E: CRY – Crystals of calcium oxalate, Fib- Non-lignified fibre, Xy.Tr- Xylem tracheid along with vessel, C. Tr- Covering Trichome)

3.5 Physicochemical analysis

Estimation of ash value is a significant parameter for the detection of nature of plant material and any admixture which is added to the authentic drug for the purpose of adulteration, impurities and maintenance of quality & purity of herbal drug test sample. The higher Ash value of this plant indicates possibility of presence of carbonates, phosphates, silicates & silica.

The water-soluble extractive value (22.867%) indicates the presence of sugar, acids & inorganic compounds & the alcohol-soluble extractive value (11.216%) indicates the presence of polar constituents like phenols, steroids, glycosides & flavonoids. The determination of these values is primarily useful for the identification of exhausted drug. Similarly, % moisture content (4.883%) indicates the percentage loss of volatile substances along with water. Less moisture content is desirable for prevention of chemical decomposition & microbial contamination in the natural products.

The results of various physicochemical parameters of powder of whole plant of *E. tithymaloides* represented in Table 3.

Table 3: Physicochemical constants of powder of whole plant of *E. tithymaloides*

Analytical Parameter	Values obtained on dry wt. basis (%w/w)
Total Ash	11.715%
Acid insoluble ash	4.12%
Water soluble ash	5.02%
Alcohol soluble extractive value	11.216%
Aqueous extractive value	22.867%
% Moisture content	4.883%
Foaming Index	Non-significant

3.6 TLC fingerprinting of petroleum ether extract

The result of TLC fingerprinting of Petroleum ether extract represented in table 4 and best explained by figure 9.

Table 4: TLC fingerprinting of Petroleum ether extract with R_f values corresponding colour of spot.

Ratios	R _f (green)	R _f (yellow)	R _f (dark green)	R _f (greenish yellow)	R _f (orange)
7:3:1	0.56	0.78	0.85	0.92	0.96
7:2:1	0.51	0.57	0.67	0.93	0.97
7:1:1	0.51	0.55	0.61	0.78	0.96



Fig 9: TLC fingerprinting of Petroleum ether extract.

3.7 TLC fingerprinting of chloroform extract

The result of TLC fingerprinting of chloroform extract represented in table 5 and best explained by figure 10.

Table 5: TLC fingerprinting of chloroform extract with R_f values corresponding colour of spot.

Ratios	R_f (green)	R_f (yellow)	R_f (dark green)	R_f (greenish yellow)	R_f (orange)
7:3:1	0.54	0.64	0.73	0.82	0.94
7:2:1	0.52	0.66	0.82	0.88	0.92
7:1:1	0.58	0.64	0.78	0.86	0.98



Fig 10: TLC fingerprinting of chloroform extract

The comparative study on separated Phyto constituents best explained through graphical representation of different ratios

of solvent system for both extracts (petroleum ether and chloroform extract) and expressed as figure 11, 12, 13.

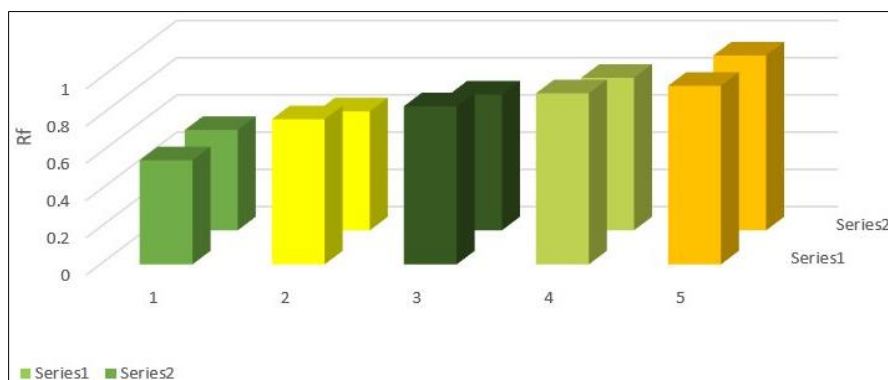


Fig 11: Elaborated chart showing no and RF values of petroleum ether and chloroform extract of *E. thymaloides* when solvent ratio = 7:3:1

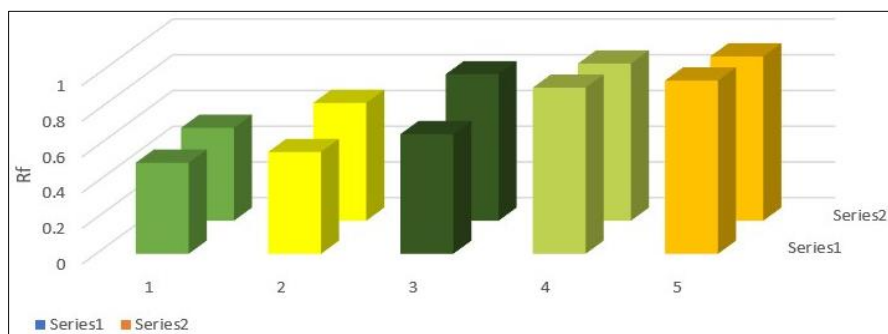


Fig 12: Elaborated chart showing no and RF values of petroleum ether and chloroform extract of *E. thymaloides* when solvent ratio = 7:2:1

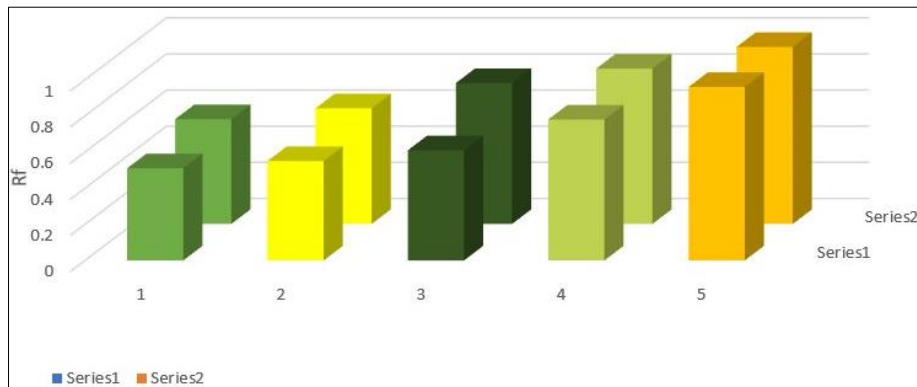


Fig 13: Elaborated chart showing no and Rf values of petroleum ether and chloroform extract of *E. tithymaloides* when solvent ratio = 7:1:1

4. Conclusion

The plant and its powder subjected for microscopic, Pharmacognostical & physicochemical evaluation provides relevant information which may be helpful in authentication of the crude drug and checking adulteration for quality control of raw material. The Pharmacognostical and physicochemical parameters observed in present study adds to the existing knowledge of *E. tithymaloides*. It could be quite useful for identification, standardization and preparation of crude drug formulation. The current observation could be helpful in differentiating the leaves of this species from closely related species of the same genus and family.

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