Plant Sci. Arc.
Plant Science Archives
An International Journal of Plant Biology

Research Article
ISSN 2518-6965
www.plantarc.com

Exploring Morpho-Anatomical Attributes, Phytochemical, and HPTLC Profile of *Enicostema axillare* (Poir. ex Lam.) A. Raynal

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Citation: P. Nampelli, S. Gangadhar Rao, P. Kamalakar (2023). Exploring Morpho-Anatomical Attributes, Phytochemical, and HPTLC Profile of *Enicostema axillare* (Poir. ex Lam.) A. Raynal. *Plant Science Archives.*
01-13. DOI: https://doi.org/10.51470/PSA.2023.8.4.01

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Received 14 September 2023 | Revised 27 November 2023 | Accepted 15 December 2023 | Available Online December 16 2023

ABSTRACT

Enicostema axillare (Lam.) A. Raynal, known as the Indian whitehead, is member of Gentiaceae family and has been used medicinally for a very long time both in India and around the world. The objective of the present work comprises collection, identification, macroscopical, microscopical, and phytochemical evaluation and HPTLC fingerprint analysis on Leaves of E. axillare. The surface studies of the leaf are amphistomatic, with anisocytic stomata and trichome complex absent in both surfaces. The T.S of Leaf is slightly flat adaxially and ribbed on abaxially at midvein, presence of one layered epidermis. Mesophyll is heterogenous, differentiated into palisade and spongy tissues, and interspersed with sphaerocrystaliferous idioblasts. The ground tissue of the mid vein consists of palisade 1-2 layered and parenchyma 1-3 layered. The midvein consists of a single oval-shaped vascular bundle, at the center. The microscopic and organoleptic characters of the powder are provided. The phytochemical analysis following hot continuous and successive extraction by Soxhlet apparatus on various extracts indicates the presence of alkaloids, phenolic glycosides, tannins, steroids and terpenoids flavonoids, saponins, phytosterols, fixed oils, gums and mucilage's, etc., It is discovered that the number of spots, and Rf values of HPTLC are distinct diagnostic features of the plant for identification. The information accumulated through the combination of chemical and botanical screening could potentially be utilized for establishing pharmacopeial standards as well as creating a standard for the identification and authentication of E. axillare.

Keywords: *Anatomical, Phytochemical, HPTLC, Enicostema axillare and Gentiaceae*

Introduction

Some traditional healers use secretly declare therapy and rare remedies. The majority of them may never tell anybody about this secret, which is why tremendous information and specific skills often disappear from our world after they pass away. Some relatively common plants are regarded as weeds until their medical usefulness is discovered by humans. Until more research is done, the potency of certain plants is unknown. Approximately 1100 medicinal plants are used in Indian medical systems today; they are mostly harvested from their natural habitats. Many common plants are not included in these records, even though tribal people sometimes employ them extensively as traditional remedies or in codified medical systems. Ayurvedic classics including the Samhitas, Nighantus, and associated text volumes have listed specific medicinal plants for therapeutic purposes and counseled doctors to discover new plant applications from shepherds, cowherds, and traditional healers to expand their knowledge.

The plants that grow meet all of humanity's requirements, including those for sustenance, clothing, and a place to live. The effectiveness of crude medications depends on using authentic samples, which necessitates plant identification. Studies in pharmacognosy, focusing on morphological and anatomical features that aid in the authenticity of crude pharmaceuticals, are a part of this field. [1]. To ensure accurate plant identification and to prevent adulterations in the original plant material, pharmacognostic studies play a crucial role in herbal technology by providing standardization parameters. With this data, plant authenticity can be assured, and the quality of herbal products can be consistently high, leading to both the safe use of these items and their continued efficacy [2,3]. Plants with medicinal properties have a long history of use, and their abundance of therapeutic substances for the treatment and prevention of illness is well-known around the globe. [4]. There are no established criteria for identifying herbs, despite their centrality to Ayurvedic therapy. Many people turn to botanical remedies as a treatment for a wide range of illnesses because of their widespread acceptance, efficacy, affordable prices, and relative safety [5], approximately 80% percent of the population in developing nations lives in rural regions and relies on plants for medicinal purposes. On a global scale, anything from 50,000 to 75,000 plants are utilized in medicinal practices, whether conventional or alternative. There are still a lot of people in more traditional societies who use herbal treatments for everyday problems. About 25 percent of today's pharmaceuticals in industrialized nations include ingredients sourced from plants traditionally used for medical purposes. [6,7,8].

The term "enicostemma" describes a wreath-like arrangement of 20 blooms at each node of the stem; the word "en" means "inside," "icos" means "twenty," and "stemma" means "wreath." [9,10,11]. *Enicostema axillare* (Lam.) A. Raynal is a perennial glabrous medicinal herb of the family Gentianaceae [12,13]. This plant is common in wastelands, along riverbanks, in grasslands, and on the coastlines of saltwater lakes; it thrives in extremely acidic environments. Tropics of Southeast Asia, Malaysia, and Africa (including the Lesser Sunda Islands) are the original habitats of this plant. Across India, you can find it.

The therapeutic effect of *E. axillare* in the treatment of humans is significant. In conventional medicine, *E. axillare* was utilized for

curing a variety of illnesses, including type 2 diabetes, leprosy, problems with the skin, the detrimental effects of malaria, and more. The low in glucose, antioxidant, hepatoprotective, and hepatomodulatory effects of the leaf contribute to its weight loss benefits. [14]. The therapeutic substances extracted from this plant were highly regarded for their efficacy because of their low toxicity, lack of adverse effects, pleasant taste, longer shelf life, and lack of environmental impact. [15].

E. axillare is traditionally used in India as a stomachic, bitter tonic, carminative to reduce fever [16], and as a tonic for appetite loss [17], antidiabetic, diastolic blood pressure and pulse rate [18], anti-inflammatory, anti-malarial, hepatoprotective, anti-hyperglycemic, hypoglycemic, antioxidant, antitumor, hypolipidemic and anthelminthic activities [19,20,21,22] and Antimicrobial [23].

Traditional medicinal uses of *Enicostema axillare*

In traditional Indian medicine, this plant has several uses, including for the prevention of liver illnesses, purification of the blood, and snake bites [24]. Traditional healers along the western coastline of India utilize a potion made out of its leaves to treat a variety of medical conditions, including diabetes, stomach ulcers, hernias, and malaria. [25]. Its leaves are used by indigenous healers in southern India to cure male infertility. [26]. Traditional herbal practitioners often employ the full plant as a means of elimination to alleviate rheumatism, skin conditions, bruising, and itching. [27,28,29].

MATERIALS AND METHODS

Chemicals

The high-quality analytical chemicals were acquired from Sigma-Aldrich, India; Thermo Fisher Scientific India Pvt. Limited; and Hyderabad Rankem laboratory reagent.

Collection and Authentication of Plant Material

In the rainy season months of July and August 2023, healthy, fresh plant materials were collected for research purposes, from many different of locations in the Eturnagaram Wildlife Sanctuary, located in the Mulugu District of Telangana, India, at latitude 18°20′28 N and longitude 80°19′48 E. The Eturnagaram Wildlife Sanctuary has year-round heat, with summers reaching very high temperatures and winters being "okay-ish." During the summer, the temperature may get as high as 44˚C. The sanctuary is known to be affected by the Northeast and Southwest monsoons from October to December. The herbarium specimen had been made and submitted to the Herbarium, Hyderabadensis Department of Botany, Osmania University, Hyderabad, Telangana, India. The submitted plant species was identified and authenticated by the Botanical Survey of India, Deccan Regional Centre, Hyderabad, Telangana, with detailed taxonomic study and given the herbarium voucher number (Voucher Number- BSI/DRC/2023-24/Identification/403).

Drying of Plant Material.

The newly picked leaves were cleaned and then cut into very small bits using sharp tools. The final measurements of these parts were 0.5 x 1.5 x1 xo.2 x 3cm. The blotting paper was spread out in the shade during the 10 days that they were exposed to air. The leaves were dried for an hour using hot air in an oven set at 40 ˚C before the anatomical studies and extraction process could start.

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Macroscopic evaluation

Observed macroscopic characteristics of a fresh *Enicostema axillare* leaf included the characteristics of the lamina, the presence of the petiole, and the leaf base. Venation, shape, texture, apex, phyllotaxis, petiole, margin, and surface distinguish laminae. Other distinguishing characteristics comprise [30].

Microscopic evaluation

Before the leaves are encased in paraffin wax, they are boiled, fixed in F.A.A. (Formaldehyde-Acetic Acid-Alcohol), and dehydrated with a sequence of xylene and alcohol. The leaves are then encapsulated with paraffin wax. To analyze sections cut at 10–12 m with an Optica rotating microtome, crystal violet, and basic fuchsin combination staining was conducted, followed by Canada balsam mounting. The microscopical examinations conducted included both quantitative and qualitative evaluations. The microphotographs were captured using a digital Sony camera and a trinocular Olympus BX-53 microscope.

Qualitative microscopy

Using a microscope, the surface and cross-section of the leaf were observed and analyzed in great detail. According to the method, the segment was stained with a 1:1 mixture of chloroglucinol and hydrochloric acid. Numerous microscopic structures were meticulously analyzed and photographed [31,32,33].

Surface view of leaf

Before creating epidermal peels, the skin was scratched and peeled with a razor blade. The peels were then stained with safranine and suspended in glycerin. After being submerged for several hours in a 4% sodium hypochlorite solution, the leaf lost all of its chemicals and color as a consequence of the treatment. The works from [34]. Various aspects of bleached leaves, including the lamina, midrib, and petiole, were examined in great detail under a microscope.

Transverse section (T.S.) of Leaf

After submerging the new leaf in water, we randomly divided it along its midrib to produce cross-sections. Fine sections were fixed on a glass plate with glycerin in the absence of a staining agent, and the resulting images were then examined under a microscope. Two components were used to produce the tissue section stain: phloroglucinol and concentrated hydrochloric acid. Trichomes, stomata, and several other distinguishing characteristics were observed [35,36].

Organoleptic parameters

Parameters like color, odor, and taste were evaluated organoleptically

Powder microscopy

There was a microscopic examination of desiccated, finely powdered leaves lignified substances that can be distinguished from other kinds of substances using a coloring solution (phloroglucinol and hydrochloric acid, mixed in a 1:1 ratio). When viewed through a microscope, a minute amount of foliage that had been reduced to a granular consistency was observed. After mounting the powder in glycerol with 1-2 drops of phloroglucinol solution (0.1% w/v) and concentrated hydrochloric acid, a coverslip was positioned on top.

Before the material was examined under a microscope, this was performed. There were indications of trichomes, stomata, epidermis cells, xylem arteries, fibers, and additional structures. As a direct result, photomicrographs of the cellular structures were able to be taken for use in subsequent research [37].

Quantitative microscopy

Estimation of the stomatal number and stomatal index

The stomatal number is the average number of stomata present in the epidermis of a leaf, measured in millimeters squared. Calculating the stomatal index of a leaf involves dividing the total number of stomata on the leaf by the total number of epidermal cells in a particular location and expressing the result as a percentage. The following algorithm will be utilized to calculate the

stomatal index: **SI = (S / S +EP) x100** Where **SI** represents the stomatal index, **S** represents the number of stomata per unit area, (stomata/ mm^2,) **EP** represents the number of epidermal cells per unit area (epidermal cells/ mm^2)

"A sample of a leaf was extracted with care to segregate the upper and lower epidermis sections separately utilizing tweezers. The piece was then immersed in glycerol on a slide. Placing the slide on a microscope stage, four identically sized sections measuring 4 mm2 were marked with black paper to outline the stomata and epidermal cells using a camera lucida. The total count of stomata and epidermal cells in each demarcated area was calculated. The average number of stomata per square millimeter was computed, and subsequently, the stomatal index was determined for both upper and lower epidermis individually following the steps outlined above." [38].

Estimation of vein termination and vein-islet number

Leaf characteristics such as the ending of veinlets and the number of vein-islets per square millimeter of leaf surface from the border to the midrib were examined. To capture this, a drawing apparatus called a camera lucida and black paper were set up. A 4 mm square was established at the center of the area. The veins situated within the square were traced, encompassing overlapping islets between the square's adjacent sides. The average counts of vein islets and veinlet endings in the four neighboring squares were documented. [38].

Estimation of palisade ratio

A portion of a leaf was utilized to examine the presence of palisade cells beneath each epidermal cell. Subsequently, the layer of palisade cells under the epidermal cells was outlined using a camera lucida on drawing paper. Four epidermal cells, each comprising five groups, were counted from various parts of the leaf, and the average count was determined as the palisade ratio. [38].

Determination of width and length of vessels and fibers

Xylem vessels were isolated from other plant structures by utilizing Schze's maceration solution. This solution was created by combining an ample amount of potassium chlorate with a nitric acid solution (50%v/v) and then heating it in a water bath. A piece of the leaf was immersed in the solution mentioned above. Potassium chlorate was periodically added to promote the breakdown and softening of leaf tissues. Subsequently, the treated leaf tissues were placed on a slide, delicately separated using a needle, and rinsed multiple times with water to eliminate any remaining acid. The width and length of the vessels were measured using a calibrated eyepiece micrometer. The measurements of 50 xylem vessels and fibers were documented. [39].

Determination of biocrystals of leaf

After treating with bleach, calcium oxate crystals were isolated and examined under a microscope. The crystals' shapes sizes were captured in photomicrographs. Various biological processes play a role in the creation of crystals in leaves, where they are primarily stored in the cytoplasm and cell vacuoles. Typically, crystals are made up of calcium salts such as calcium carbonate and calcium oxalate. As stated by Mazen in 2003, leaves contain a significant number of prismatic-shaped intracellular calcium oxalate crystals, with the conclusion that only aluminum is incorporated into these crystals. [41].

Phytochemical screening

The therapeutic effects of a drug can frequently be attributed to its secondary metabolites. Alkaloids, flavonoids, saponins, steroids & terpenoids, phenolic compounds, tannins, glycosides, coumarins, phytosterols, and resins were found in the powdered medicine extract when it was analyzed in a lab setting in ether, chloroform, ethyl acetate, and methanol [42,43].

Successive Extraction Using Soxhlet Apparatus

The Soxhlet apparatus was utilized for the successive extraction process. *E. axillare* leaf powder weighing 20 grams was placed inside a Wattmans No. 1 filter paper, inserted into the Soxhlet thimble, and 200ml (w/v) of solvent was poured into the round bottom flask (still pot). The extraction of the leaf powder occurred in a series with hexane at 70°C, chloroform at 61°C, ethyl acetate at 77°C, and Methanol at 65°C. The extraction temperatures were adjusted to the boiling points of the solvents to facilitate a quicker cycling of fresh solvents. Each solvent was allocated a five-hour duration for the hot continuous and successive extraction process. After extraction, the extracts were cooled, and filtered through Whatman No.1 filter paper, and the extraction followed the order of increasing polarity of the solvents, starting from hexane to methanol, and then proceeded for phytochemical screening [44].

Calculation of percentage yield

The dried extracts obtained with each solvent were weighed and yield was calculated concerning the air-dried weight of the plant material.

$$\text{Percentage Yield} = \frac{\text{Weight of the crude (mg/g)}}{\text{Weight of the plant material}} \text{ x100}$$

Screening of Phytochemicals

To detect secondary metabolites, a phytochemical screening was performed on the leaf samples. The well-established methods were used for conducting the phytochemical analyses. Several qualitative investigations have been carried out to ascertain the presence or absence of functional chemicals.

Detection of Alkaloids

For Mayer's Test to identify alkaloids, samples were dissolved in a diluted hydrochloric solution and subsequently sieved. Two or three drops of Mayer's reagent were found to be the sweet spot for a 2 ml filtrate.

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Test for Flavonoids
A small amount of concentrated hydrochloric acid and magnesium tube turnings were added to the test liquid, and then it was simmered for five minutes. The mixture will take on a reddish tint if it contains flavonoids.

Test for Saponins
A tiny sieve was used to eliminate contaminants after the powdered substance had been cooked with twenty milliliters of water at a low temperature for two minutes. The filtrate was after that mixed with water until it was 5 mL in volume, and then violently stirred. A clear sign of saponin presence is the presence of foaming.

Detection of Steroids and Terpenoids
A mixture of 1 mL of extract, 1 mL of chloroform, 2 mL of acetic anhydride, and 1–2 molecules of concentrated sulfuric acid was prepared for the Liebermann–Burchardt test. Steroids were visible in the liquid due to its dark green color.

Test for Phenolic compounds
The prediction that a pulverized sample will contain phenolic compounds was confirmed by the following substances. One solution of ferric chloride (5 percent): An extremely deep shade of violet-black. b. the white portion of the lead acetate solution that is still there.

Test for Tannins
A very small quantity of the medication powder was dissolved in water. The water-based extract was supplemented with a small amount of ferric chloride solution. When tannins are present, the color turns bluish-black.

Detection of Glycosides
The test solution was made by heating the extract in either alcohol or a hydroalcoholic solution.

a) Baljet's test In order to run the test, the mixture was spiked with a 2% sodium picrate solution. Glycosides were detected by the pigment's golden orange color.

b) Legal 's test
The sample was found to contain glycosides when the test solution was alkalized with pyridine and a color shift from pink to red was produced by the addition of 2% sodium nitroprusside.

c) Keller-Killiani test
Prior to mixing, 100 mg of extract they had been mixed with 1 milliliter of ferric chloride solution and 1 drop of glacial acetic acid. Next, 1 milliliter of concentrated H2 SO4 was added to the mixture to serve as a basis. When glycosides show up as a brown band at the interface, it means that the sample contains glycosides.

Detection of Coumarins
2 ml of water-based extract was diluted, and then 3 ml of a 10% NaOH solution was added. Coumarins were likely present due to the contents' golden color.

Test for Phytosterols
The solution of extract was stirred briskly before strong sulfuric acid was added and the mixture was put to storage. In the presence of phytosterols, the chloroform layer at the base of the solution will turn crimson.

Detection of Quinones
1 ml of crude extract developed a color that indicated the presence of quinones after being treated with diluted sodium hydroxide. You may get this shade of red or blue-green.

Detection of Resins
In a saucepan over medium heat, mix 2 ml of extract with 5 to 10 drops of acetic anhydrate. Add 0.5 ml of sulphuric acid. Because they formed an intense purple color, resins were likely present.

Detection of Cardiac Glycosides
For the Kellar-Kiliani test, two milliliters of clarified butter were mixed with one milliliter each of ferrous chloride, concentrated sulfuric acid, and glacial acetic acid. Presumably containing cardiac glycosides, the solution became an emerald color when illuminated.

Detection of leuco anthocyanins
The mixture consisted of an equal quantity of liquid extract and isoamyl alcohol. The presence of leucoanthocyanins has caused the top layer to become red.

Detection of anthraquinone
An ounce of finely powdered plant material was added after the mixture had been stirred in chloroform for five minutes. Quickly shaking the mixture before analysis, 5 ml of a was ammonia solution was added after sifting the contents. The presence of anthraquinone might be indicated by the vivid pink color of the sample's aqueous layer.

Detection of fixed oils
A tiny sample is compressed between two filter sheets to extract the active ingredient. The presence of fixed oils can be detected by looking for telltale signs, including oil residue on paper.

High-Performance Thin Layer Chromatography (HPTLC) analysis
An *aluminum* silica gel plate 60 F254 (5 x 10 cm with 0.25 mm thickness; Merck, Darmstadt, Germany) was coated with the concentrated methanolic extract using a 25µL syringe. Methanol was used for washing the plates prior they were used. The next thing to do was to insert the mobile phase into the TLC development chamber using filter paper. The solution samples and reference solutions were administered in bands with a width of 6 mm and a spacing of 10 mm using an automatic operation TLC Sampler 4 applicator (CAMAG, Muttenz, Switzerland, supplied by Anchrom Technologists, Mumbai) that

was fitted with a 25-µL Hamilton syringe with nitrogen flow. The flow velocity was kept constant at 15 microliters per second, while the spacing between bands remained at 20 millimeters per second. The slit size was maintained at 4 × 0.20 mm and the scanning speed was 20 mm/sec. A saturated Toluene mixture made up the mobile phase. Ethyl acetate, methanol, acetic acid, chloroform, and methanol (7:3 v/v) served as the mobile phase in the chromatography that separated the phytoconstituents. The optimal chamber saturation period was 20 minutes at room temperature, and the chromatographic run length was 8.5 cm. Following development, the TLC plates were air-dried in a vented wooden room using a hot air dryer. The CAMAG TLC Scanner III was used to conduct densitometric scanning at three different wavelengths: 254 nm and 366 nm, with a slit size of 4 × 0.20 mm and a scanning speed of 20 mm/sec. The WinCATS programme (version 1.4.3). CAMAG) ran all the instruments that used the intensity of diffusely reflected light to determine the chromatographed compounds. An illuminator, Reprostar 3, and digital camera Power Shot G2 (Canon, Tokyo, Japan) were used for photographic evidence within the Digi Store 2 system (CAMAG). The radiation source, a deuterium lamp, emitted a UV spectrum within 200-400 nm. [45,46].

Observations and results

Plant profile [47-48].

Table:1. Taxonomic classification of Enicostema axillare (Lam.) A. Raynal

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Gentianales
Family	Gentianaceae
Genus	Enicostema
Species	*Enicostema axillare*

Vernacular names: English: Indian Whitehead **Hindi:** Chota-chirayata, Chota-kirayat; **Kannad:** Karibandita, Sogade; **Malayalam:** Vellaragu, Vallari; **Marathi:** Kudavinayi; **Tamil:** Vellaragu, Vallari; **Telugu:** Nela-guli, Nela-gulimidi
Local name: Nela guridi.

Macroscopic evaluation of the leaf

Erect herb to 40 - 50 cm, stem 4 - angular; tender parts glaucous. Leaves lanceolate- oblong, 3-4.5 x 0.5-1 cm., base cuneate, apex acute; petiole to 0.5 cm. Flowers 5. merous, in axillary, sessile, ebracteate fascicles. Calyx cupular, lobes 5, oblong, unequal, herbaceous, acute, recurved with age. Corolla white, 2 mm., across, salver form; lobes 5, equal, twisted, 7 mm., obtuse. Stamens 5, all equal and fertile, included; filaments 1.2 mm., with 2-fid scales at base; anthers 1.2 mm., apiculate, dehiscence longitudinal. Ovary globose, 1.5 x 1 mm., 1- locular; bifurcate placentae, styles 1.2 mm; stigma capitate or obscurely 2-lobed. Capsule 3.5 x 3 mm., septicidally 2 - valved; seeds circular, minute, reticulate (Fig1&2A-E)
Dist: It is found throughout the greater parts of India; abundant in wastelands, follow fields, river banks, etc.

Ethnomedicinal uses in the present investigation: To prevent the formation of pus in wounds, a mixture of the leaf paste and ghee is administered. Fractures in cow bones can also be treated with this.

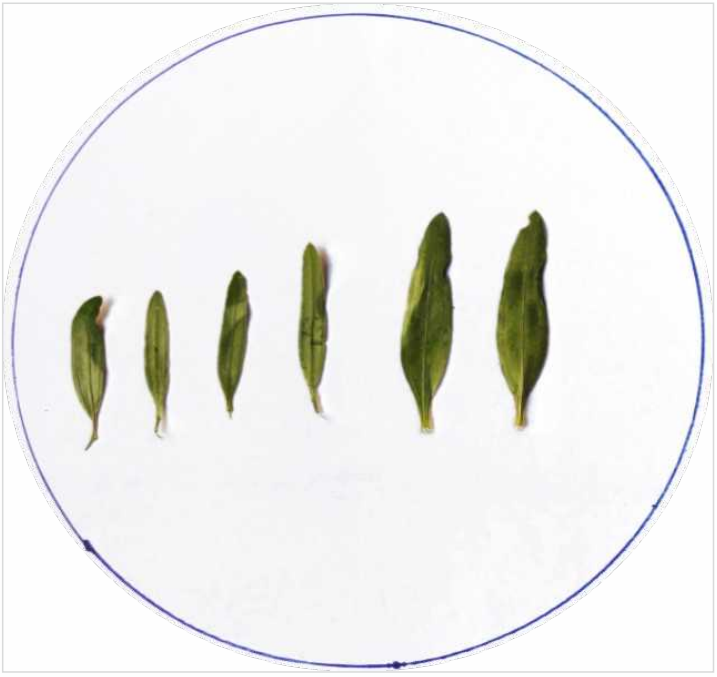


Fig:1. Macroscopic images of leaf

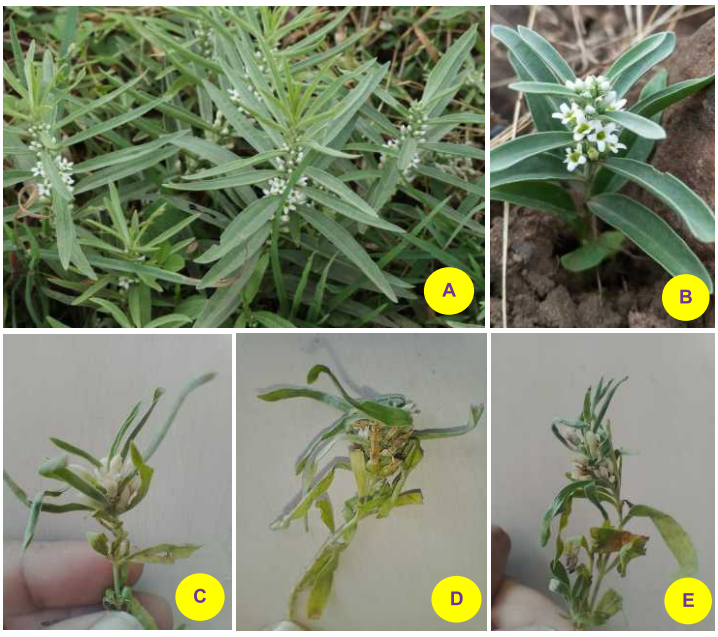


Fig: 2A. Macroscopic examination of leaves

Microscopic evaluation of the leaf (Surface View)
Leaf – Adaxial surface:
LEAF LAMINA ADAXIAL
Epidermal cell complex: The epidermal cells have 5 to 7 sides, are primarily polygonal anisodiametric with a few isodiametric, have significantly thicker sides, are striated on the surface, and have few straight to curved strands.

Dist: Common, everywhere except on veins; unevenly distributed; E.C.F. 950 per sq. mm.(Fig 3A-B).

Costal cells: 5-7 sided, polygonal anisodiametric to linear, few isodiametric, oval to spherical at margins, the sides are thick and predominantly curved to wavy, with a few straight to curve. The surface is striated, and the contents are slightly packed with starch granules.

Dist: On primary, secondary veins and at margins, irregularly arranged, parallelly oriented

Stomatal complex: Anisocytic, subsidiaries 3, monocyclic, indistinct, mostly f-type, few a-type, guard cells reniform, contents slightly dense **Dist:** Common, all over rarely on veins. The Stomatal frequency (S.F). 258 per sq.mm, stomatal index (S.I). 24. 51

Trichome complex: Trichomes absent

LEAF LAMINA ABAXIAL

Epidermal cell complex: Epidermal cells according to description on the adaxial lamina, except wavy to sinuate sides and thick crystals of different forms inside. E.C.F. 725 per sq.mm. (Fig 3C-D).

Costal cells: As stated on lamína adaxial, except crystal-rich components.

Stomatal complex: As described on lamina adaxial. The Stomatal frequency (S.F). 240 per sq.mm., stomatal index (S.I). 22.06.

Trichome complex: Trichomes absent

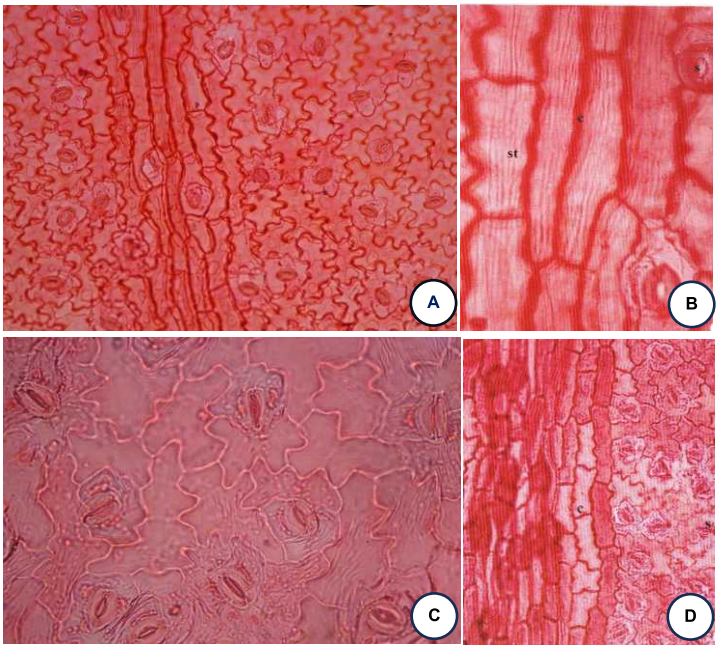


Fig: 3A. Leaf adaxial surfaceX634; 3B. Leaf adaxial surface with costal cells X450
3C. Leaf abaxial surface with striations X425; 3D. Leaf abaxial surface with costal cells X226

Transverse Section of leaf:

T.S. OF LEAF: Slightly flat adaxially and ribbed on abaxial at midvein, secondary and tertiary veins not ribbed Lamina wings 270-421 (359) µm and midvein 486-605 (543) µm in thickness

EPIDERMIS: One layered, cells mostly barrel shaped, few tabular, oval and circular elongated cells about 3568 (47) µm long. 2441 (31) µm wide and isodiametric cells 22-44 (32) µm in diameter adaxially, while smaller about 25-69 (43) µm long and 20-41 (27) µm wide and isodiametric cells 27 49 (34) µm in diameter on lamina abaxial, cells over on midvein adaxial similar as found on lamina adaxial, while abaxial cells smaller, polygonal to spherical, 1638 (26) um in diameter contents scanty margins pointed, obtuse, as those on midvein abaxial, achlorophyllous, cuticle slightly thick over the surface on adaxial.

Stomata: Amphistomatic, flushed with epidermal cells Trichomes: Absent (Fig4A-C).

MESOPHYLL: Heterogenous, differentiated into palisade and spongy tissues

Palisade: Adaxial. 1-2 layered, occasionally 3 layered at some places, throughout. extended into midvein, cells columnar, cylindrical and oblong perpendicular to the epidermis, cells about 33- 55 (45) µm long and 16 -33 (24) µm wide, often interspersed with sphaerocrystalliferous idioblasts, intercellular spaces narrow, contents slightly dense, chloroplasts abundant

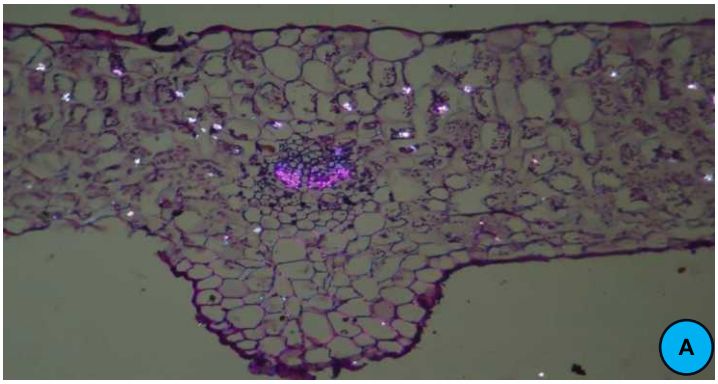
Spongy tissue: 3-5 celled thick, abaxial, cells mostly circular, oval and dumbbell shaped, about cells closely packed with few intercellular spaces, cells interspersed with sphaerocrystalliferous idioblasts, contents slightly dense with chloroplasts (Fig4A-C).

GROUND TISSUE: Of midvein heterogeneous, differentiated into palisade and parenchyma tissues.

Palisade: 1-2 layered, occasionally 3 layered on midvein adaxial cells as described on lamina mesophyll

Parenchyma: Parenchyma cells 1-3 layered, beneath the adaxial palisade and 6-8 layered on the abaxial, cells mostly polygonal, few oval to circular, about 21-44 (31) µm in diameter, walls thin, intercellular spaces narrow, few, contents scanty, dense with plastids.

VASCULAR TISSUE: The midvein is made up of a single oval-shaped vascular bundle that is about 130–205 (163) µm in diameter in the centre; several small–medium sized, oval–circular, pericyclic bundles that are enclosed by an endodermis; tracheary elements that are 30–40 in number, arranged in radial rows, polygonal, thick-walled, and about 11–22 (16) µm in diameter in the middle; secondary wall thickenings of tracheary elements that are mostly helical, bordered pitted and scalariform; helices that are double or single, rarely annular; perforation plate simple; Phloem is primarily composed of companion cells and a small number of sieve tubes. Phloem parenchyma cells are compactly arranged and lack intercellular spaces. Walls are thin and contents are scant, with a few cells having slightly dense contents. Xylem parenchyma is located between tracheary elements. (Fig 4A-C).



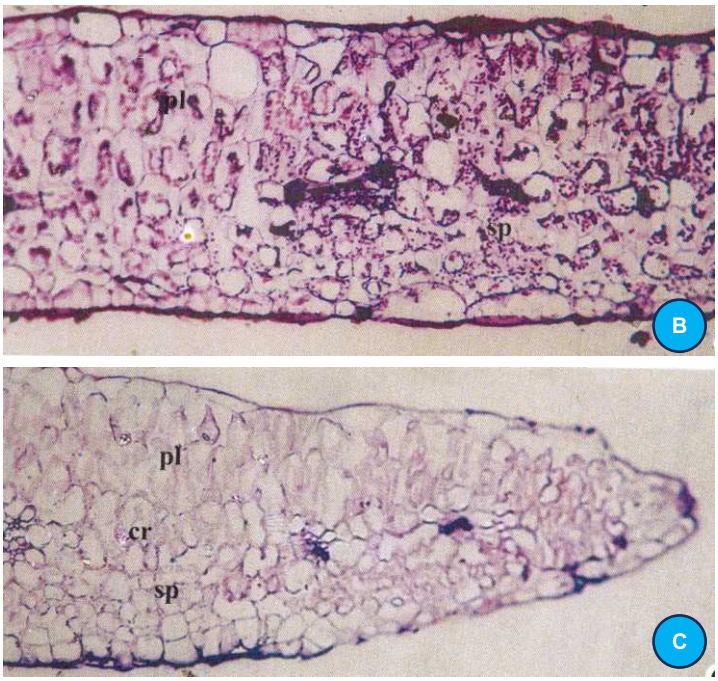


Fig:4 A.T.S. of leaf midvein X180; 4B.T.S. of leaf lamina X 158; 4C.T.S. of leaf margin X 138

Powder Microscopy of *E. axillare*

The powder is composed of up of ground tissue fragments densely packed with ca-oxalate crystals, tracheary elements with helical thickenings, costal cells with a striated surface, and epidermis fragments with underlying mesophyll. The epidermis fragments exhibit curved to wavy sides and stomata (Fig5A-F).

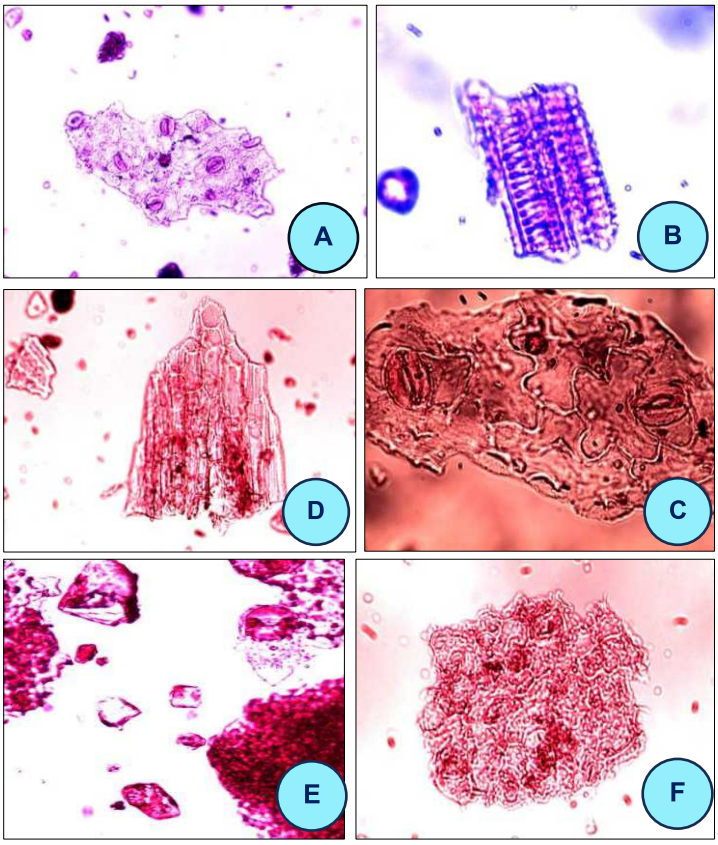


Fig: 5A-Leaf epidermis with stomata X 146; 5B- Tracheary elements with helical thickenings X 130; 5C- Stomata X 186; 5D-Costal cells X 78; 5E-Ca-oxalate crystals X60; 5F-Epidermis X 215.

Organoleptic characters

Colour – Light brown; **Touch** – Slightly coarse; **Odour** – Pungent; **Taste** – Very bitter.

Quantitative microscopy

The various parameters evaluated for leaf surface constants were observed like stomatal number (upper and lower), stomatal index (upper and lower), E.C.F, (upper and lower), Spongy parenchyma, palisade ratio, vascular bundles tracheary elements. The results are shown in Table-2

Table-2: Quantitative microscopic data

S.no	Parameters	Results
1.	Epidermal cell frequency (E.C.F adaxial)	950 per sq. mm.
2.	Stomatal index (S.I. adaxial)	24. 51
3.	Stomatal frequency (S.F. adaxial)	258 per sq.mm.,
4.	Epidermal cell frequency (E.C.F abaxial)	725 per sq.mm.
5.	Stomatal index (S.I. abaxial)	240 per sq.mm.,
6.	Stomatal frequency (S.F. abaxial)	22.06.
7.	Thickness of lamina	270-421 (359) µm.
8.	Thickness of midvein	486-605 (543) µm.
9.	Palisade cells	33- 55 (45) µm long and & 16 -33 (24) µm wide.
10.	Spongy parenchyma	16-33 (25) µm in diameter.
11.	Palisade	33- 55 (45) µm long and & 16 -33 (24) µm wide.
12.	Parenchyma (abaxial)	21-44 (31) µm in diameter.
13.	Vascular bundle	130-205 (163) µm in diameter.
14.	Tracheary elements	11-22 (16) µm in diameter.

Qualitative Phytochemical Screening

The secondary metabolites in phytochemicals are created to protect plants, but they also have medicinal properties that can help with a lot of human illnesses. Because of this, a lot of study is focused on them. A phytochemical investigation has been carried out on the *E. axillare* leaf extracts to find out the secondary metabolites. We checked extracts in the presence of hexane, ethyl acetate, chloroform, and methanol for alkaloids, flavonoids, saponins, steroids and terpenoids, tannins, glycosides, coumarins, phytosterols, quinones, anthraquinones, cardiac glycosides, leuco anthocyanins, fixed oils, and resins.

The leaf of the *E. axillare* plant was found to have several different phytochemical classifications, according to the qualitative analysis.

We can compare the solvents used in the extraction process using the findings that are given (Table 3 and Fig6).
The phytochemical analysis indicated that alkaloids were exclusively found in the methanol extract, whereas flavonoids, steroids & terpenoids were detected in all extracts except for hexane. Saponins and leucoanthocyanins were confirmed exclusively in the methanol extract, while phenols, tannins, and resins were only present in the ethyl acetate extract. Glycosides were found in both the chloroform and methanol extracts, while coumarins were identified in both the ethyl acetate and methanol extracts. Hexane and ethyl acetate extracts were found to have phytosterols, while chloroform and ethyl acetate extracts were found to contain quinones. Cardiac glycosides were discovered in all the extracts except for the methanol extract. Anthraquinones and fixed oils were seen in the hexane and chloroform extracts (Fig & table). The crude extracts were produced using several solvents, resulting in diverse percentage yields: hexane (10.6% yield), chloroform (14.16% yield), ethyl acetate (34.2% yield), and methanol (28.5%yield).

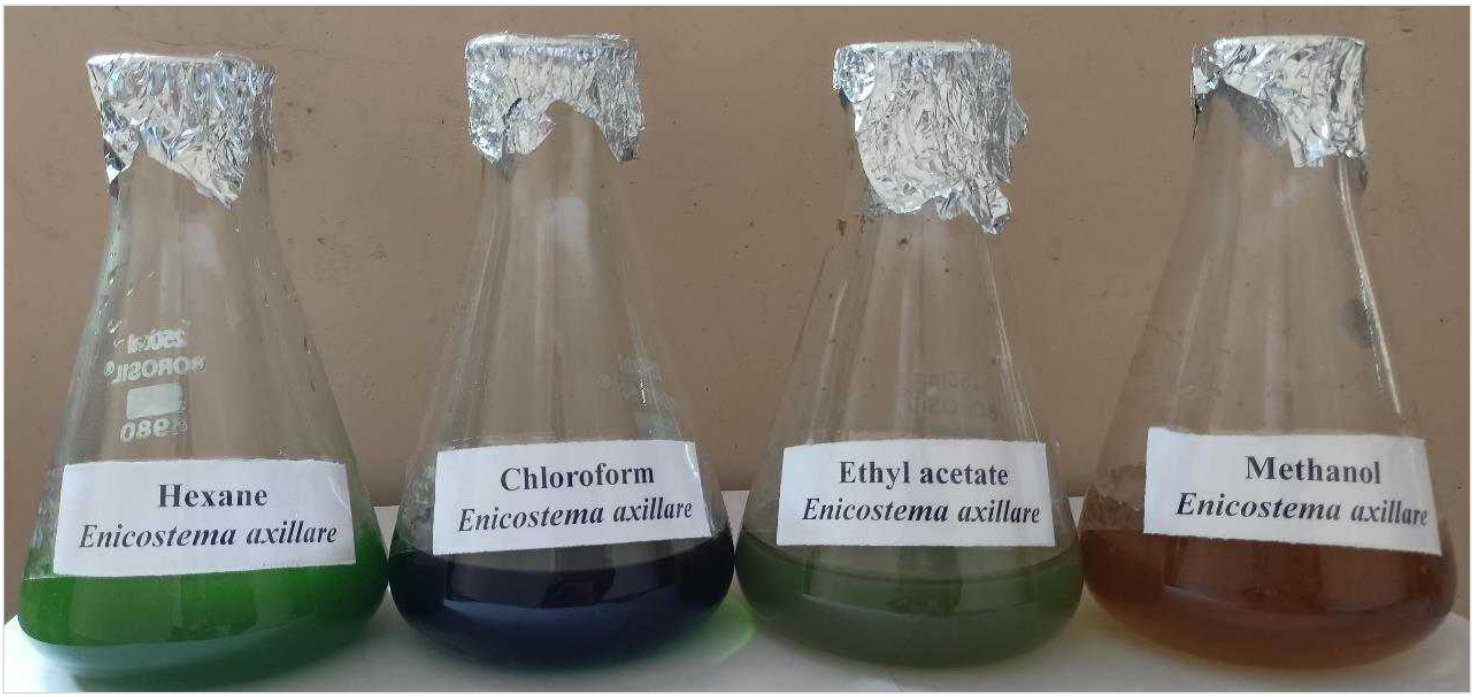


Fig.6: Phytochemical extractions with different solvents

Table-3: Phytochemical analysis of E. axillare

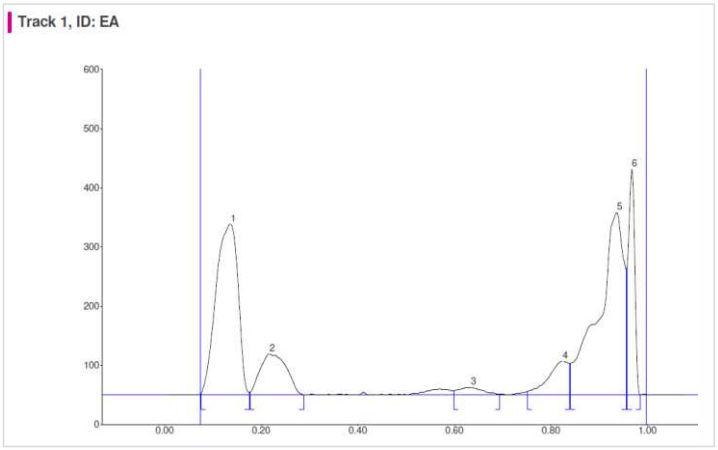
S. No	Phyto. Name	Pet. ether	Chloroform	Ethyl acetate	Methanol
1	Alkaloids	-	-	++	+++
2	Flavonoids	-	+	+++	+
3	Saponins	-	-	-	+++
4	Steroids & Terpenoids	-	++	+++	+++
5	Phenols	-	-	+++	-
6	Tannins	-	-	+++	-
7	Glycosides	-	+++	-	+++
8	Coumarins		-	++	+++
9	Phytosterols	++	-	+++	-
10	Quinones	-	++	++	-
11	Resins	-	-	+++	-
12	Cardiac Glycosides	++	++	+++	-
13	Leuco anthocyanins	-	-	-	++
14	Anthraquinones	++	++	-	-
15	Fixed oils	++	++	-	-

"+" = present; "-"= absent

The fingerprinting profile of E. axillare using HPTLC techniques.

A reliable method for rapidly assessing the botanical and plant raw material quality is of the utmost importance. Modern high-performance thin-layer chromatography (HPTLC) instruments allow for the collection of fingerprints useful for identifying and analyzing composition.
The extract was analyzed using the CAMAG HPTLC system for HPTLC fingerprint analysis. After that, it was scanned at 365 and 254 nm using the CAMAG Scanner 3 and WinCATS 4 software.
The chromatographic separation profile of the methanol extract extracted from *E. axillare* leaves, scanned at 254 nm, shows six spots (Fig. 7), of which spots 6 and 5 have a maximal composition with Rf at 0.97 and 0.94, respectively. Instead, a densitogram scanned at

366 nm, on the other hand, revealed seven spots, the seventh and second spot, of which had maximum composition at Rf 0.94 and 0.24, respectively (Fig. 9). These are characteristics of the drug under-examined, as the data abundantly shows, and they will be helpful in the leaf the field of medicine identification and authentication. These standards in pharmacopeia can be considered extremely valuable. At 254nm, six spots with varying concentrations can be seen at Rf 0.14, 0.22, 0.64, 0.83, 0.94, and 0.97 (all brown) (Fig. 8). Meanwhile, 366 nm, seven spots with varying concentrations can be seen at Rf, 0.15 (yellow), 0.24 (yellow), 0.50 (blue), 0.57 (yellow), 0.82 (blue), 0.87 (blue), and 0.94 (yellow) (Fig. 10). To guarantee the consistency and dependability of the medication, this fingerprint parameter is essential.



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.08	2.3	0.14	289.2	25.83	0.18	4.6	9506.3	31.87	unknown *
2	0.18	5.0	0.22	69.6	6.21	0.29	0.0	2753.2	9.23	unknown *
3	0.60	7.2	0.64	13.3	1.19	0.70	1.7	486.0	1.63	unknown *
4	0.75	6.5	0.83	57.3	5.12	0.84	53.4	1760.5	5.90	unknown *
5	0.84	53.5	0.94	308.5	27.55	0.96	212.4	11650.5	39.05	unknown *
6	0.96	217.8	0.97	381.6	34.09	0.99	0.0	3676.1	12.32	unknown *

Fig. 7: HPTLC densitogram of methanolic extract of E. axillare scanned at 254 nm by using chloroform: methanol (7: 3 v/v)

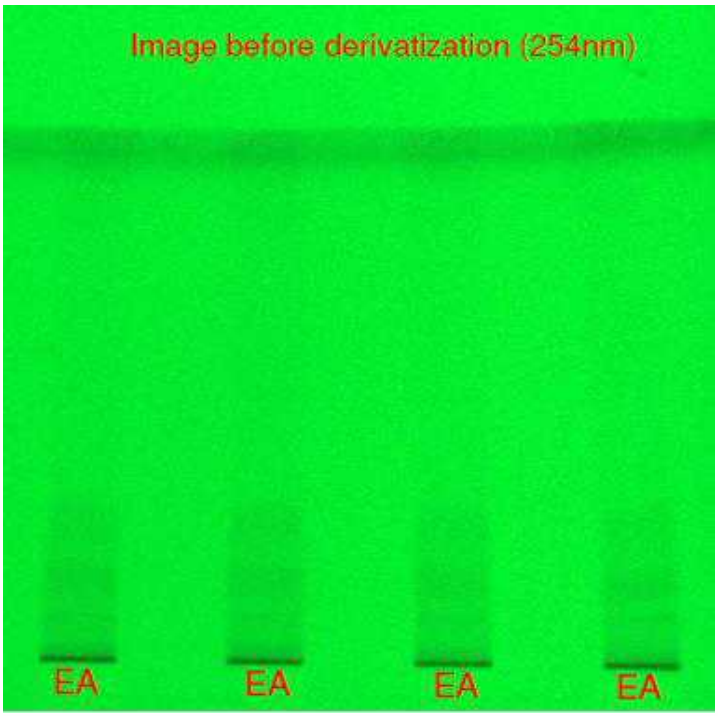
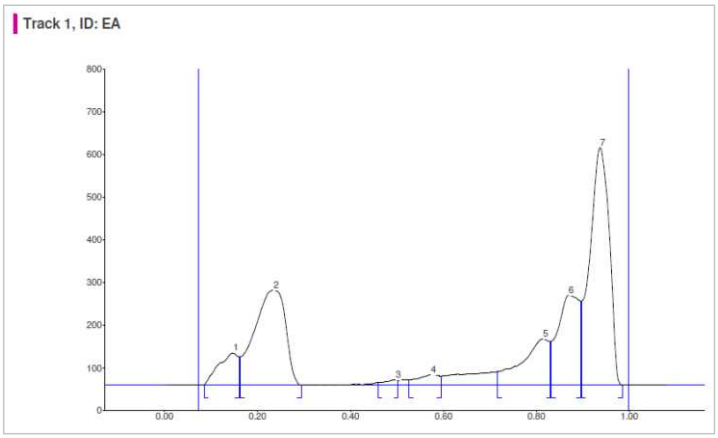


Fig.8: High performance thin layer chromatography image of E. axillare at 254 nm in chloroform: methanol (7: 3 v/v)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.09	0.8	0.15	75.7	6.25	0.16	66.6	2305.1	5.38	unknown *
2	0.16	66.7	0.24	222.8	18.39	0.30	0.2	10779.5	25.17	unknown *
3	0.46	6.1	0.50	12.8	1.06	0.50	11.8	264.8	0.62	unknown *
4	0.53	13.3	0.57	25.1	2.07	0.60	21.7	870.7	2.03	unknown *
5	0.72	31.8	0.82	108.3	8.94	0.83	102.7	4763.9	11.12	unknown *
6	0.83	103.2	0.87	210.9	17.40	0.90	197.1	6946.0	16.22	unknown *
7	0.90	197.1	0.94	556.1	45.89	0.99	0.0	16895.3	39.45	unknown *

Fig.9: HPTLC densitogram of methanolic extract of E. axillare scanned at 366 nm by using chloroform: methanol (7: 3 v/v)

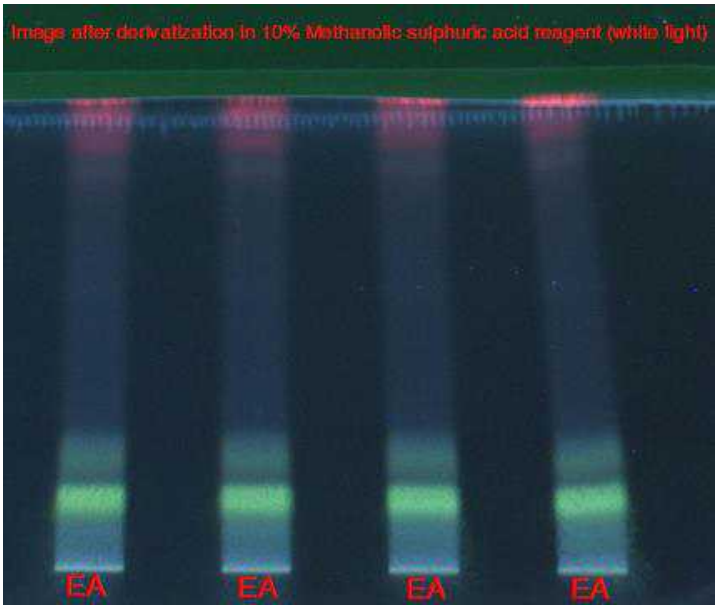


Fig.10: High performance thin layer chromatography image of E. axillare at366 nm in chloroform: methanol (7: 3 v/v)

Discussions

Enicostema axillare (Lam.) A. Raynal is a perennial glabrous medicinal herb of the family Gentianaceae [12,13 47,48] .*E. axillare* is traditionally used in India as a stomachic, bitter tonic, carminative to reduce fever [16], and as a tonic for appetite loss [49]. *E. axillare* is used in Indian ayurveda medicine in combination with other herbs, particularly for diabetes. To treat type 2 diabetes, it is given as an ayurveda pill since it dramatically improves renal function, lipid profile, systolic and diastolic blood pressure, pulse rate, and blood glucose control. It also boosts serum insulin levels [18]. *E. axillare* has demonstrated its anti-inflammatory activity, and tumour inhibition in rats [28, 50].

Leaf epidermis

Epidermal cell complex: A review of literature in the species reveals that there is no mention of the number of sides and shape of the epidermal cells. Presently the cells are 5-7 sided,

mostly polygonal anisodiametric and few isodiametric. The anticlinal walls are reported as thick [51], which is presently confirmed. Further, the sides have been reported as sinuous [52], and wavy [53, 48]. But presently they are curved to wavy and wavy to sinuate and confirming earlier observations

The epidermal cell surface was reported as papillate [48], striated [51,53,19,54], but not papillate [48]. The contents of the epidermal cells are scanty on the leaf adaxial, but they are dense with crystals of calcium oxalate towards abaxial and are reported new. The epidermal cell frequency is higher on leaf adaxial 950 per sq. mm, while lower towards abaxial 725 per sq. mm in contrast to the earlier observations [48].

The features of the costal cells were not reported in the earlier studies. Presently they are 5-7-sided, polygonal anisodiametric, linear and few isodiametric. Sides are thick, curved to wavy, and striated surface. Further, the costal cells are dense with starch grains and crystals of various shapes towards the abaxial side.

Stomatal complex: The stomata occur on either side of the leaf and confirm the earlier studies [51,55, 53, 48,]. As regards the type of stomata they have been reported as anisocytic [55, 48,19,54], anomocytic or anisocytic [52], anomocytic to anisocytic[53]. But presently they are found exclusively anisocytic with subsidiaries striated and mostly of f-type and few a-type. Further in frequency, they are 246 per sq.mm., on the adaxial and 260 per sq.mm., on abaxial, while Stomatal Index is 21 51 on the adaxial and 27.08 on the abaxial

Trichome complex: The trichomes of simple unicellular and sessile glandulam trichomes with 2- celled head have been reported in the species [53], but presently they are absent confirming the observations in earlier literature[51,53,48,54]

Leaf anatomy: The leaf has been reported isobilateral [51, 55, 48], less distinct in differentiation of the palisade and spongy [48, 54]. Presently, the leaf is found heterogenous, differentiated into palisade and spongy tissues through not markedly as also observed earlier [48,19, 54]. The palisade is 1-2 layered and occasionally 3- layered, throughout the leaf and extending into midvein. The cells are cylindrical and oblong and measure 35-55 µm long and 16-33 µm wide. The mesophyll consists of 5-6 rows of spongy parenchyma containing dense chloroplasts [53]. Mesophyll consists of horizontally elongated polygonal cells in *Enicostema* [55,60], the leaf mesophyll tissue is reported to contain druses or sphaerocrystals and mostly restricted to palisade zone [48,54]. Presently the palisade and spongy tissues are often interspersed with sphaerocrystalliferous idioblasts besides dense chloroplasts. Further, the spongy tissue is 3-5 celled thick towards abaxial with circular, dumbbell, oval shaped cells about 16-33 µm in diameter

The shape of the leaf in T.S. at midvein was reported as flat on the upper side and convexly projected on the lower side[53], prominently bulged abaxially[48], which is presently confirmed and the midvein is 486-605 µm thick. Further, the secondary & tertiary veins are not ribbed. The lamina wings are 270-421 um thick. The epidermal cells of lamina are single layered [53,54] which is also presently confirmed Further the epidermal cells were reported to wide and cylindrical with prominent cuticle [54]. Presently they are mostly barrel-shaped, a few tabular, oval, and circular, and larger towards adaxial as also reported earlier [53]. The contents of epidermal cells are scanty, but few isolated crystals occur in cells of the lower epidermis.

Ground tissue of midvein is reported to possess collenchymatous tissue [48]; cells of the parenchymatous tissue [53], homogenous and parenchymatous [54]. But presently ground tissue is heterogenous, in contrast to the earlier observation [53,48, 54], and differentiated into hypodermal palisade towards adaxial and the rest parenchymatous. A 1-2 layered and occasionally 3-layered palisade distinctly extends into the midvein and forms a hypodermal layer. The parenchymatous tissue which is predominant occupies, the rest of the ground tissue at midvein with 1-3 layered beneath the adaxial epidermis and 6-8 layered on the abaxial side enclosing the central vascular bundle. The central vascular bundle is reported as collateral with phloem surrounding the xylem [48]; Meri stele with central radiating xylem, phloem on its either side. Presently midvein vascular bundle is single, oval-shaped at the center, about 130-205 µm in diameter, conjoint, collateral, endarch, enclosed by endodermis. The xylem at the center, with tracheary elements, arranged in radial rows. The secondary wall thickenings are mostly helical, bordered pitted, scalariform, and rarely annular. Phloem is compactly arranged and encloses the xylem towards the abaxial. The wing bundles are several, oval to circular, collateral, endarch, and enclosed by an endodermis.

An analysis of the available literature indicates that the first phytochemical test used extracts from the *E. axillare* plant. Extracts from plants are shown to contain tannins, glycosides, alkaloids, and flavonoids. [56, 57]. A novel flavone C-glucoside called Verticilliside was reported along with the presence of catechins, saponins, steroids, sapogenin, triterpenoids, flavonoids, and xanthones. [58]. According to [59], A significant component of many traditional remedies sold in Japan is swertiamarin, a secoiridoid glucoside that was first identified from the leaves of *E. axillare*. The Soxhlet extraction method was utilized in the current investigation to conduct a qualitative phytochemical analysis. Alkaloids, flavonoids, saponins, terpenoids, steroids, quinones, anthraquinones, cardiac glycosides, leuco anthocyanins, fixed oils, and resins were among the phytochemicals discovered to be present. The crude extracts were produced using a variety of solvents. The hexane solvent yielded the lowest percentage yield (10.6% yield), whereas the ethyl acetate solvent provided the highest percentage yield (34.2% yield). A high-performance thin-layer chromatography (HPTLC) finger print examination was performed on the methanolic extract of *E. axillare* leaves. The results showed that there were six spots at 254 nm, spot five (Rf 0.97) with a maximum concentration of 39.04 percent, and seven spots at 366 nm, spot seven (Rf 0.94) with a maximum concentration of 39.45 percent. When it comes to the identification of the plant, these patches have the ability to act as biomarkers and fingerprint parameters.

Conclusions

The identification of this species and its reinforcement of quality control evaluations can be provided by the morphological, microanatomical, and powder microscopic characteristics. Several features might be witnessed, including glaucous portions, 5-7-sided, curved to wavy epidermal cells with striated surfaces; the lack of trichomes; the presence of calcium oxalate crystals; and the absence of collenchyma. A powerful tool for determining the identity of distinct secondary metabolites in plants is HPTLC fingerprinting. This approach is simple, cheap, renewable, and linear; it can identify plant species with ease. Additional applications include the standardization, characterization, and verification of

medicinally important plants. With high-performance thin-layer chromatography (HPTLC), different groups of secondary metabolites from the same or different plants may be profiled by using solvent systems with varying polarities. The findings show that the ethanolic extracts of *E. axillare* plant material include secondary metabolites such alkaloids, tannin, saponins, flavonoids, and more. Additionally, a unique technique for HPTLC analysis is offered. Health maintenance and chronic degenerative illnesses may both benefit from the essence of secondary metabolites. The results of HPTLC studies may be useful for writing a monograph on the *E. axillare* plant.

Acknowledgement
The facilities were provided by Prof. B. Ramadevi, Head of the Botany Department, Osmania University, Hyderabad, for which the authors are grateful. The HPTLC analytical capabilities were provided by Anchrom Enterprises, Mumbai, for which the authors are grateful.

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