Phytochemical Screening and Histochemical Analysis of Three *Selaginella* P. Beauv. Species

DISSERTATION

Submitted to the University of Kerala in partial fulfillment of the requirements for the degree of Bachelor of Science in Botany

by

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DECLARATION

We hereby declare that this project titled "Phytochemical Screening and Histochemical Analysis of Three *Selaginella* P. Beauv. Species" is a bonafide record of work carried out by us under the supervision and guidance of Dr. Nisha K K, Assistant Professor, Department of Botany, All Saints' College, Thiruvananthapuram, and that no part of this work has been previously formed the basis for the award of any degree or diploma.

Thiruvananthapuram,

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CERTIFICATE

This is to certify that the project entitled "Phytochemical Screening and Histochemical Analysis of Three *Selaginella* P. Beauv. Species" is an authentic work done by Aiswarya A S, Arya Aloshious U A, Renjana A R, Rudiya R, Sandra S, Varsha T M, Anjali Krishna D, Krishnapriya N, Sreelekshmi M Anil, Uthra G R & Vidhya V S for the degree of Bachelor of Science in Botany of the University of Kerala, Thiruvananthapuram, during the course of their study in this college and that this has not previously formed the basis of the award of any degree, diploma or other similar title of recognition

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Thiruvananthapuram

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INTRODUCTION AND REVIEW OF LITERATURE

Pteridophytes are unique in plant kingdom due to their peculiar location in between Bryophytes and Gymnosperms. They are an assemblage of flowerless, seedless, spore bearing vascular plants, that have successfully invaded the land. The word pteridophyte has Greek origin. 'Pteron' means a "feather" and 'Phyton' means "plants". The plants of this group have feather like fronds (ferns). The group of pteridophyte included into Cryptogams with algae, fungi and bryophytes. The algae, fungi and bryophytes are called lower cryptogams while the pteridophytes are called higher cryptogams. Pteridophytes are called vascular cryptogams, because only pteridophytes have well developed conducting system among cryptogams. Due to these reasons they are the first true land plants.

The *Selaginella* genus, commonly known as spikemoss or lesser clubmoss, is the sole genus in the family Selaginellaceae, representing a type of vascular plant. This genus is distinguished by its scale-like leaves with a ligule and its ability to produce two types of spores. They are an essential group of plants in the bryophyte lineage, exhibiting characteristics intermediate between mosses and ferns. According to Smith's classification (1955) *Selaginella* fall within the order Selaginellales of class Ligulopsida in the division Lycophyta.

Morphological Characters of *Selaginella* P. Beauv.

Selaginella plants are characterized by their unique morphological features, which include a regular anisotomous dichotomous division of the shoot apical meristem, giving rise to two new axes (branches). The leaves of *Selaginella* species are scale-like, arranged in rows along the stem, and often have a ligule. The stem of *Selaginella* plants is green, dichotomously branched, and can be either prostrate with erect branches or erect. The species often have a single apical cell at the stem's growing tip. *Selaginella* species are also known for their unique reproductive structures, which resemble a leafy, spore cone at the stem's apex. They reproduce through spores produced in specialized structures called sporangia, often

clustered into cone-like strobili. Spores germinate to form new plants (Weststrand & Korall, 2016).

The plant body is sporophyte and it is differentiated in to root, stem, leaves with ligules and rhizophore. The root of young sporophyte is of primary root while others are adventitious. The adventitious roots are at the tips of the rhizophore. The stem is green, dorsi-ventral and prostrate with short erect branches. The branches are arranged dichotomously. The shoot apex consists of a single apical cell in most cases. In some species, leaflets and colourless branches arise from the prostrate stem near point of branching. These grow downwards and have groups of adventitous roots. They are called as rhizophores. Some scientists consider them as branches and some consider them as roots and still others consider it as an organ for protection or other function. But recently they are known as adventitious roots that have dichotomous branches at tip. Leaves are small and single veined called microphylls.

Based on the nature of leaves, they are of 2 types- isophyllous and anisophyllous (Adnan et al 2021). The anisophyllous leaves are in pairs. They may be small and larger. The small leaves are inserted on the dorsal side of the stem and the larger ones are inserted on the ventral side of the stem. There is small outgrowth on adaxial side (upper side) of the leaf near base called ligule. It is a vestigial organ and provide water.

Selaginella species are found in a variety of habitats, including tropical rainforests, temperate forests, and deserts, and grow on rocks, tree trunks, or in leaf litter on the forest floor. The morphology of *Selaginella* plants is closely related to their ecological role, as they stabilize soil, provide habitat and food for various animals, and contribute to the nutrient cycle.

One of the distinctive features of *Selaginella* plants is their ability to roll into tight, brown or reddish balls during dry periods and uncurl and turn green when exposed to moisture, earning them the nickname "resurrection plants." This characteristic is not only fascinating

but also contributes to their ecological significance and ornamental value in landscaping and terrariums.

Significance of *Selaginella* P. Beauv.

Selaginella species play a significant role in both ecological systems and human practices, making them a diverse and intriguing group of plants with a range of applications and benefits. *Selaginella* species hold importance as popular plants for terrariums, hanging baskets, and landscape decoration. They are cultivated for their aesthetic appeal and are commonly used as ground cover in gardens and other landscaping settings. *Selaginella bryopteris* is a lithophyte with remarkable resurrection capabilities.

Additionally, some species of *Selaginella* have medicinal properties and are utilized in traditional medicine for their diuretic, antiseptic, and anti-inflammatory properties (Shankar and Devalla, 2012). These plants have been studied for their potential applications in treating various conditions, including arthritis, gonorrhea, hepatitis, and mastitis, highlighting their medicinal significance (Antony and Thomas, 2011). Various species are reported to provide relief from heat stroke and the burning sensation during urination (Sah et al 2005). They are used in restoring menstrual irregularities to normal and also in helping in easy delivery of pregnant women in minimizing the labor pain (Pandey et al 2017). Some species are used in the treatment of jaundice and HIV infection. They are also studied for potential cancer treatment (Gao et al, 2003; Pal et al, 2022).

Selaginella species also exhibit nutritional value (Hwang et al 2021). Several species of *Selaginella* are also used as food (raw vegetables). The aqueous extract of *S. bryopteris* possesses growth-promoting activity as well as protective action against stress (Jyothi et al, 2015). It is also reported to have induced cell death in a number of experimental cell systems including mammalian cells. *Selaginella* species have also been used in handicrafts and as ornaments since primordial times, and their use has been observed in traditional ways by people around the world for various purposes (Pandey et al 2017).

Phytochemical Studies in *Selaginella* P. Beauv.

The phytochemical studies of various *Selaginella* species have revealed the presence of a wide range of bioactive compounds, including alkaloids, flavonoids, lignans, phenols, and sesquilignans (Silva et al. 1995; Lin et al. 1994; 2000; Sun et al. 2006). These compounds have been identified in different *Selaginella* species, such as *S. sinensis, S. pulvinata, S. tamariscina,* and *S. moellendorffii,* among others. For instance, the study of *Selaginella* spp. from Java Island showed that all tested species contained alkaloid, flavonoid, saponin, and steroid compounds, while tannins were only found in some species (Chikmawati et al, 2012). The alkaloids found in *Selaginella* species have been reported to have diuretic, antipasmodic, anti-inflammatory, and analgesic effects. Flavonoids, on the other hand, have been shown to possess antioxidant, anti-inflammatory, anti-allergic, hepatoprotective, anti-thrombic, antiviral, and anti-carcinogenic activities. Saponins form strong insoluble complexes with cholesterol, making them useful in the human diet for controlling cholesterol and having antifungal and antibacterial properties that are important as components of cosmetic products.

In addition to these compounds, *Selaginella* species have also been found to contain unique alkynylphenol carbon skeleton phenols called selaginellins (Li et al, 2020). These compounds have been isolated from various *Selaginella* species, including *S. sinensis, S. tamariscina, S. pulvinata,* and *S. moellendorffii* (Xu et al, 2011; Cao et al, 2015, Křížkovská et al, 2020). Furthermore, *Selaginella* species have been found to contain selariscinins A–D, which are selaginellin derivatives via tautomerism. The phytochemical studies of *Selaginella* species have also shown the presence of flavonol glycosides, phenolic acids, and their derivatives in other species, such as *S. triloba* and *S. hastata*.

Histochemistry as a tool in Plant Science

Histochemistry is the branch of histology that deals with the identification of chemical components of cell and tissues. Histochemical methods have been developed for qualitative

and quantitative analysis of all cellular components including proteins, carbohydrates, lipids, nucleic acids and the range of ionic elements occurring in cell solutions (Gersbach et al, 2001).

Histochemistry is an essential analytical tool interfacing extensively with plant science. Plant cell structures are translucent unless they are stained. Histochemistry allows the identification and localization, at the cellular level, of biomolecules and organelles in different types of cells and tissues, based on the use of specific staining reactions and imaging. Histochemical localization of primary metabolites in plants involves the identification and mapping of essential compounds like carbohydrates, proteins, and nucleic acids within plant tissues at a cellular level. This process is crucial for understanding the metabolic activities and physiological functions of plants. By employing specific staining techniques and imaging methods, researchers can visualize the distribution of primary metabolites in different plant structures, providing valuable insights into plant growth, development, and responses to environmental stimuli.

Objectives of our Study

Studies reveal significant variations in the phytochemical profiles between different *Selaginella* species. This highlights the need for further exploration across the genus.

Hence the objectives of present study are:

- To conduct a preliminary phytochemical study in the three selected *Selaginella* species.
- To analyze the distribution of primary metabolites within the plant tissues of the selected species using histochemical techniques.

MATERIALS AND METHODS

2.1 Plant material

Three *Selaginella* species grown and maintained in the green house at All Saints' College, Thiruvananthapuram was selected for the present study (Fig.1). Plant specimens were observed and herbarium specimens were prepared and the plant material was identified with the help of Dr. Raju Antony, JNTBGRI, Palode.

1. Selaginella braunii **Baker** (Fig. 1A)

Terrestrial herbs with erect stem, 30-50 cm long, 1-2 mm thick, stout, cylindrical, pale brownish. Branches many from middle to upper part. Rhizophores towards basal portion, 2-3 cm long, stout, cylindrical, branched. Leaves heteromorphic throughout, distant towards base, compact on branches, membranous; median leaves 2.8 x 0.8 mm, oblong, cuspidate, keeled, oblique, margin entire; lateral leaves 6 x 2.1 mm, ovate-rhomboid, acute, oblique, entire; axillary leaves 3.6 x 1.8 mm, ovate acute or subacute, suboblique at base, margin enitre. Strobili 5-7 x 1-2 mm, termninal, sessile; sporophylls monomorphic, membranous, spiral, 1.5-1.7 x 0.5-0.7 mm, ovate-lanceolate, acuminate, minutely dentculate. Microspores 40 um in diameter, **o**range-red, trilete, tetrahedral, reticulate. Macrospores 450 µm in diameter, pale yellow, globose, tetrahedral, reticulate.

2. Selaginella vogelii **Spring** (Fig. 1B)

Rhizome creeping, pink, subtetragonal, leafy, with erect stems; rhizophores limited to the basal, prone part of the stem, 4-8 cm long, finely pubescent, dark streaked, ventral, most at right angles to the rhizome. *Stems* erect up to 50 cm long and 3 mm in diameter, spaced about 2 cm apart, usually pink when dry, rounded, subglabrous, sometimes rooting at the top; branches pubescent, forming an angle of 45°, triangular or deltoid outline, 2-3 pinnate, the basal pair of branches subopposed. Leaves of the main stem homomorphic, subopposite, distant, often more than 1 cm apart, symmetrical, ovate-deltoid in outline, apex pointed, base slightly auriculate, margin toothed, 3 x 2 mm; leaves of the branches heteromorphic, lateral leaves spaced, 3-4 x 1.25-2 mm, often rolled up when dry, apex

Fig. 1 *Selaginella* species selected for the study. **A.** *Selaginella braunii*; **B.** *Selaginella vogelii* & **C.** *Selaginella plana*

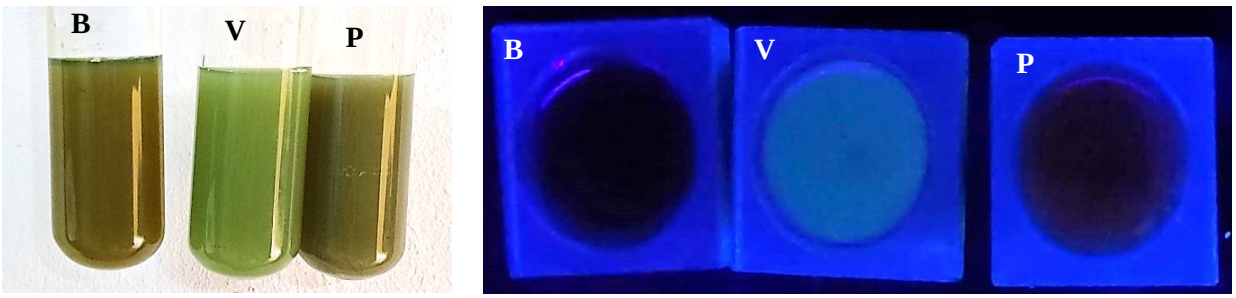


Fig. 2 Crude methanolic extract obtained from *Selaginella* species (**B**- *S. braunii*; **V**- *S. vogelii* and **P**- *S. plana*) viewed under normal visible light (**A**) and UV light (**B**)

rounded, upper half of leaf semi-oval, entire, lower half narrowly oblong, serrulate at base, midrib not reaching the top; axillary leaves as long as the lateral ones, entire, elliptic, apex rounded; median leaves spaced apart on the main stem and sometimes subopposed, regularly attenuated and imbricated on the ultimate ramifications, 1.5-2 x 0.6-0.7 mm, ovate, falcate, ciliolate on the inner face, entire on the outer face, acuminate, not awned, outer face long decurrent; axillary leaves 2 x 0.8 mm, subelliptic, apex pointed, base truncate, margin entire or rarely ciliate. *Strobili* solitary at the tips of the twigs, about 0.5-1 cm long by 1.75-2 mm in diameter, paler green than the leaves. *Sporophylls* homomorphic, 1-1.2 x 0.7-0.8 mm, ovate-deltoid, acuminate, acute, with two short quadrangular auricles at the base, loosely ciliolate, lighter green than the leaves; heterosporus.

***3. Selaginella plana* (Desv.) Hieron** (Fig. 1C)

Stems erect, ascending or climbing from a trailing base (0.4–) 2–3 m long; upper branched part frond-like, up to 60 cm long and wide; lower stems with leaves very scattered or almost absent, oblong-elliptic, ± 4 x 2 mm, rounded at apex, auriculate-cordate at base, entire. Leaves dimorphic; lateral leaves oblong-elliptic in outline, subfalcate and ± rounded to subacute at the apex, with basal auricle on acroscopic half, abruptly truncate or rounded at base of basiscopic half, entire, 3–4.8 x 1–1.8 mm; axillary leaves broadly to narrowly ovate in outline, acuminate at the apex, with 2 equal auricles at the base, entire, 2.2–4.8 x 1.6–2.5 mm; median leaves ovate-elliptic to ovate-dimidiate, ± rounded to subacute at apex with distinct auricle at base on outer side, margins entire, 2–2.3 x 0.5–1 mm. *Strobili* 6–8(–26) mm long; sporophylls equal, ovate-lanceolate to ovate-triangular, ± 2 mm long, 1 mm wide, acuminate at the apex, carinate, with pale margins, ciliolate towards base.

2.2 Dried extraction using methanol

The aerial parts of the sporophyte including stem and leaves were washed with distilled water and shade dried for three weeks. The dried samples were powdered and stored separately in polyethylene bags for further study.

2.5g of dried powder was macerated with methanol and then extracted with 25ml methanol for 3 days with intermitten shaking. The extract was filtered through gauze cloth using a Buchner funnel and poured into a pre-weighed petriplate and dried, resulted in the concentrated extract. The amount of crude extract obtained was calculated and colour of the extract was noticed under visible light and UV light.

2.3 Histochemical analysis of primary metabolites

Fresh free hand sections were made from the stem of S. *braunii, S. vogelii* and *S. plana*. Micromorphological studies were carried out using a Leica DM500 Binocular research microscope and photographs taken with Leica LCC 50 HD camera. Histochemical tests were made on the fresh sections of the stem treated with the following reagents to identify the presence or absence of primary metabolites viz., starch, proteins and lipids. Histochemical tests were performed using the following reagents: Lugol's reagent for detection of starch, Aniline blue for protein and Sudan III for total lipophilic compounds (Badria and Aboelmaaty, 2019).

A. Localisation of Starch using Lugol's Reagent (Lugol's iodine)

This reaction highlights the starch grains in dark blue to black. Almost all other structures stain yellow, but this color has no specific significance.

Preparation of Lugol's iodine

A. Dissolve 10 g of potassium iodide in 100 mL of distilled water.

B. Slowly add 5 g of iodine crystals, while shaking.

C. Filter and store in a tightly stoppered brown bottle.

Procedure

1. Submerge the sections in the Lugol's reagent for 10 min.

2. Rinse briefly with distilled water.

3. Mount the slides using distilled water or Lugol's reagent itself.

B. Localisation of Proteins using Aniline Blue Black Staining

This stain reveals proteins in blue, whether structural or acting in the primary or secondary metabolism.

Procedure

1. Dip sections into 1% aniline blue black for 1 min.

2. Wash twice in 0.5% acetic acid to remove excess stain.

3. Rinse briefly in distilled water and mount in distilled water.

C. Localization of Total Lipids using sudan III

This stain reveals total lipids as golden red globules

Procedure

1. Stained free-hand sections of fresh material in Sudan III for 15 minutes.
2. Differentiated in 50% alcohol for 1 minute.
3. Mounted in Sudan III solution.

The stained sections were observed under Leica microscope (Japan). They were photographed at different magnifications and at different views. Based on the photographs taken, localization of tested metabolites was recorded.

2.4 Preliminary phytochemical analysis of secondary metabolites

The methanol extracts were subjected to phytochemical screening using standard methods. The metabolites tested include alkaloids, phytosterol, terpenoids, glycosides, tannins and saponins using the standard protocol (Trease and Evans, 1989; Tiwari et al, 2011).

I. Test for Alkaloids (Dragendorff's Test)

The extracts were boiled for 15 minutes in 1% HCl and filtered. To the filtrate a few drops of Dragendorff's reagent were added. Formation of red precipitate indicated the presence of alkaloids.

II. Test for Phytosterols (Salkowski's Test)

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of conc. sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicated the presence of triterpenes.

III. Test for Terpenoids (Salkowski test)

0.2g extract mixed with 2ml chloroform and concentrated H_2SO_4 (3ml) was carefully added to form a layer. Reddish brown colouration at the interface indicated the presence of terpenoids.

IV. Test for Tannins

About 500ml of extract was boiled in 20ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green colouration

V. Test for saponins

0.5 mg of extract was vigorously shaken with few ml of distilled water. The formation of frothing is positive for saponins

RESULTS AND DISCUSSION

3.1 Extraction

The weight percentage yield of the crude methanol extracts of the three *Selaginella* species are shown in Table 1. Total crude extract obtained varied from 0.23g to 0.28g. The colour of the extract was also noticed under visible light and UV light. Under visible light the colour showed was olive green, dark olive green and forest green. Under UV light the appearance was brown except in *S. vogelii* which appeared milky white in colour (Table 2, Fig. 2).

Table 1. Amount of crude methanolic extract obtained from the three selected *Selaginella* species

Species	**Initial weight of the petridish (g)**	**Final weight of the petridish (g)**	**Amount of crude extract obtained (g)**
S. braunii	47.43	47.66	0.23
S. vogelii	45.34	45.62	0.28
S. plana	40.52	40.79	0.27

Table 2. The appearance of crude methanolic extract obtained from the three selected *Selaginella* species under visible light and UV light

Species	**Colour of the extract**	
	Visible light	**UV light**
S. braunii	Olive green	Dark brown
S. vogelii	Forest green	Milky white
S.plana	Dark olive green	Light brown

The colour of a plant crude extract under UV light can be attributed to the presence of various phytochemicals or secondary metabolites that fluoresce when exposed to UV light. Plant extracts often contain a complex mixture of compounds including proteins, carbohydrates, lipids, and secondary metabolites such as alkaloids, phenolics, and terpenoids. The interaction of these compounds with UV light can result in different colours or fluorescence patterns.

3.2 Anatomical features of the stem

Anatomical features of the stem were studied by taking free hand cross sections of the stem and staining by safranin. In *S. braunii*, CS of the stem is tetragonal in outline (Fig. 3A). Epidermis has many unicellular trichomes present. It is followed by a thick hypodermis made up of very thick sclerenchymatous cells and is several layered. It is followed by many layered thick walled parenchyma cells. Central stele is separated from the cortex by air space and is connected to the cortex with the help of radially elongated endodermal cells called trabeculae. Stele is monostelic and protostelic. The stelar area is elongated in outline. Xylem is exarch in condition. Metaxylem is elongated with 5 protoxylem groups.

The CS of the stem of *S. vogelii* is circular in outline (Fig. 3B). Epidermis is followed by many layered hypodermis made up of sclerenchyma. Rest of the cortex is parenchymatous. A small air space separates the stele from the cortex. The stele is connected to the cortex with the help of radially elongated trabeculae. Stele is monostelic and elongated in outline. Stele is protostelic with a central mass of metaxylem and two protoxylem groups at the periphery.

In *S. plana* the CS of the stem is elongated in outline (Fig. 3C). Epidermis is followed by hypodermis which is sclerenchymatous and 5-6 layered. It is followed by many layered thick walled parenchyma cells. A centrally located stele is connected to the cortex with the help of many long, radially elongated cells called trabeculae. The stele is separated from

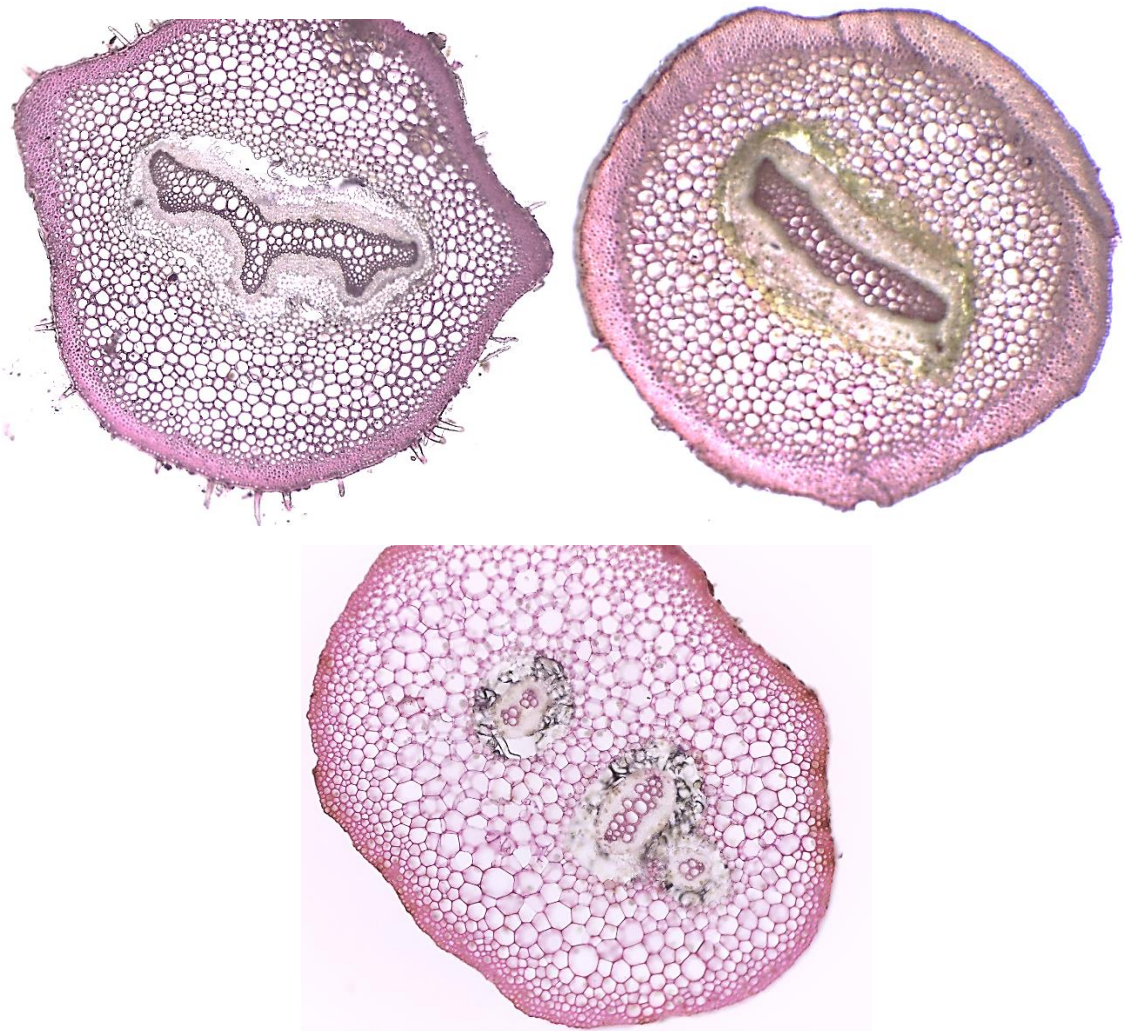


Fig 3 Anatomical features of the stem of the three selected *Selaginella* species (CS stained with safranin). **A**- *Selaginella braunii ;* **B**- *Selaginella vogelii;* **C**- *Selaginella plana*

the cortex by air space. Stelar area is elliptical in outline and is tristelic in condition with three meristeles. Each meristele is protostelic with a solid central core of the xylem that is surrounded by phloem. Xylem is exarch in condition. Xylem is linear with metaxylem centrally localised and two protoxylem groups at the periphery.

3.2 Histochemical analysis of primary metabolites

Histochemical tests were made on the cross-sections of fresh stem of the three selected *Selaginella* species for the localization of primary metabolites starch, protein and lipids. The reactions for starch were positive for Lugol's iodine solutions which presented purple blue patches in the endodermis and phloem tissues of the stem of all the three species (Fig. 4B, 5B & 6B). Starch is the most important reserve food material of the higher plants. It is usually present inside the plant cells as compact insoluble granules which may be spherical, ovoid or compact crystals and which have a distinctly layered structure. The shape and size of the starch granules varies between 2-175 microns depending upon botanical source and tissue type. Kumari et al (2018) reported that in the stem and root sections of *Jatropha curcas*, the older starch grains appeared blue black while the newly formed appeared as purple.

Aniline Blue staining revealed proteins in blue (Fig. 4C, 5C & 6C), whether structural or acting in the primary or secondary metabolism. Proteins were mainly located in the vascular tissues and cortex in all the three species. Protein accumulation in plant stems occurs in various tissues and cell types, depending on the specific function and role of the proteins. Protein accumulation in the xylem and phloem tissues is consistent with the presence of transport proteins and enzymes involved in lignin biosynthesis and cell wall formation. The parenchyma cells within the stem cortex also accumulate storage proteins. These proteins are typically found in protein storage vacuoles or in the cytosol.

Sudan III is a lipid-soluble dye commonly used to detect and localize lipids in plant tissues. When plant stem sections are stained with Sudan III, the dye preferentially

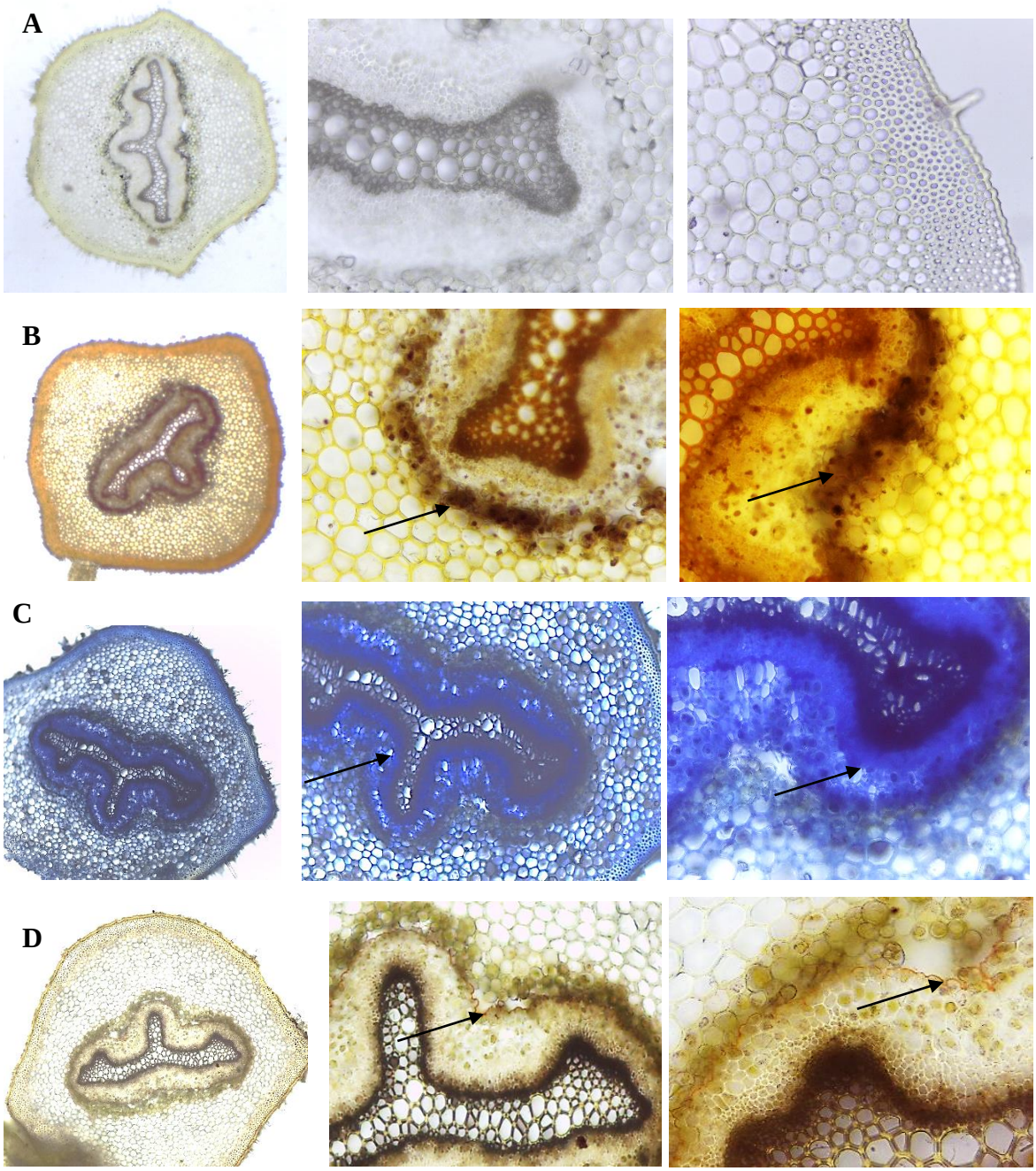


Fig 4 Histochemical studies to localize primary metabolites in *Selaginella braunii*. **A**- Unstained; **B**- Lugols; **C**- Aniline blue and **D**- Sudan III

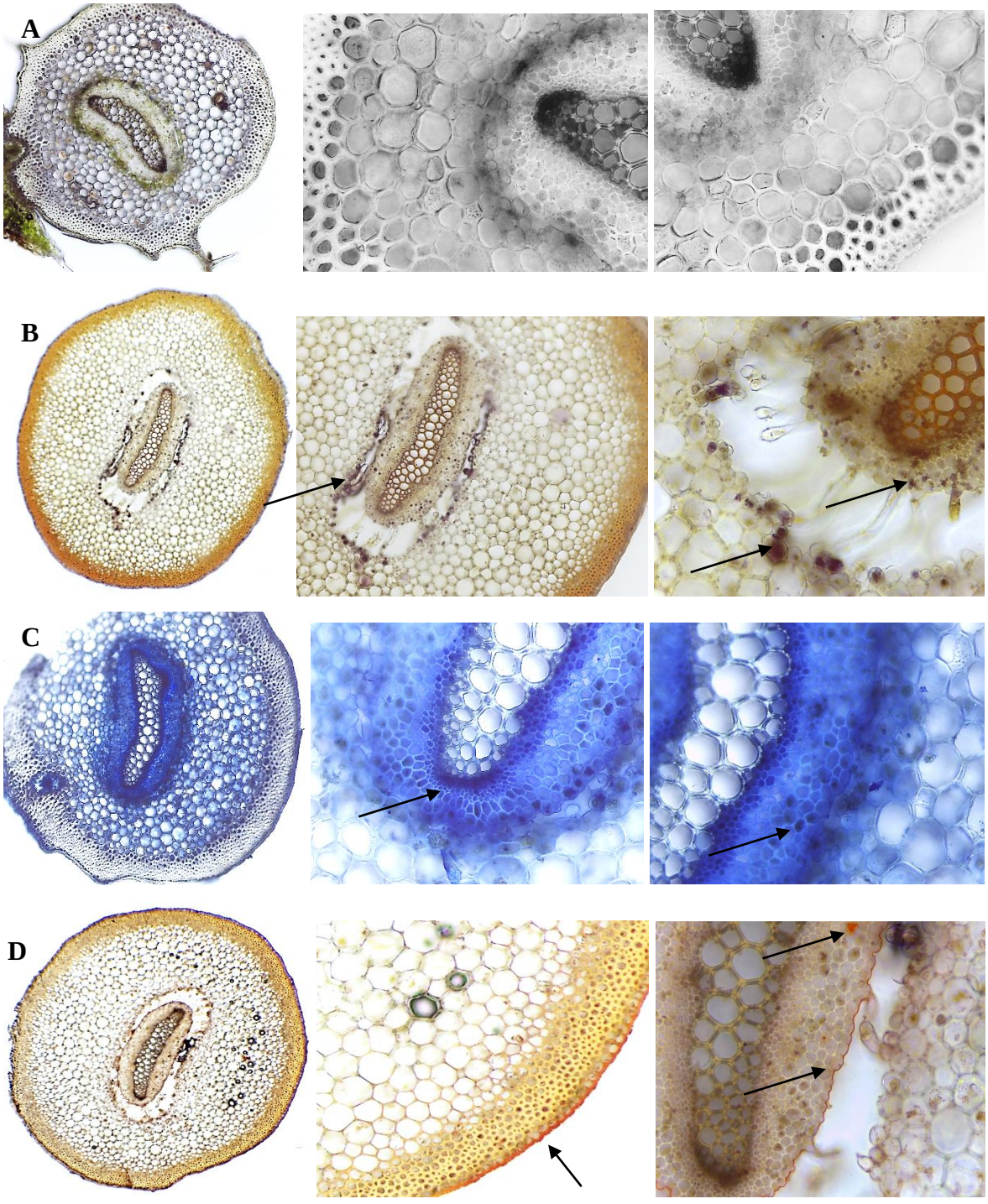


Fig 5 Histochemical studies to localize primary metabolites in *Selaginella vogelii*. **A**- Unstained; **B**- Lugols; **C**- Aniline blue and **D**- Sudan III

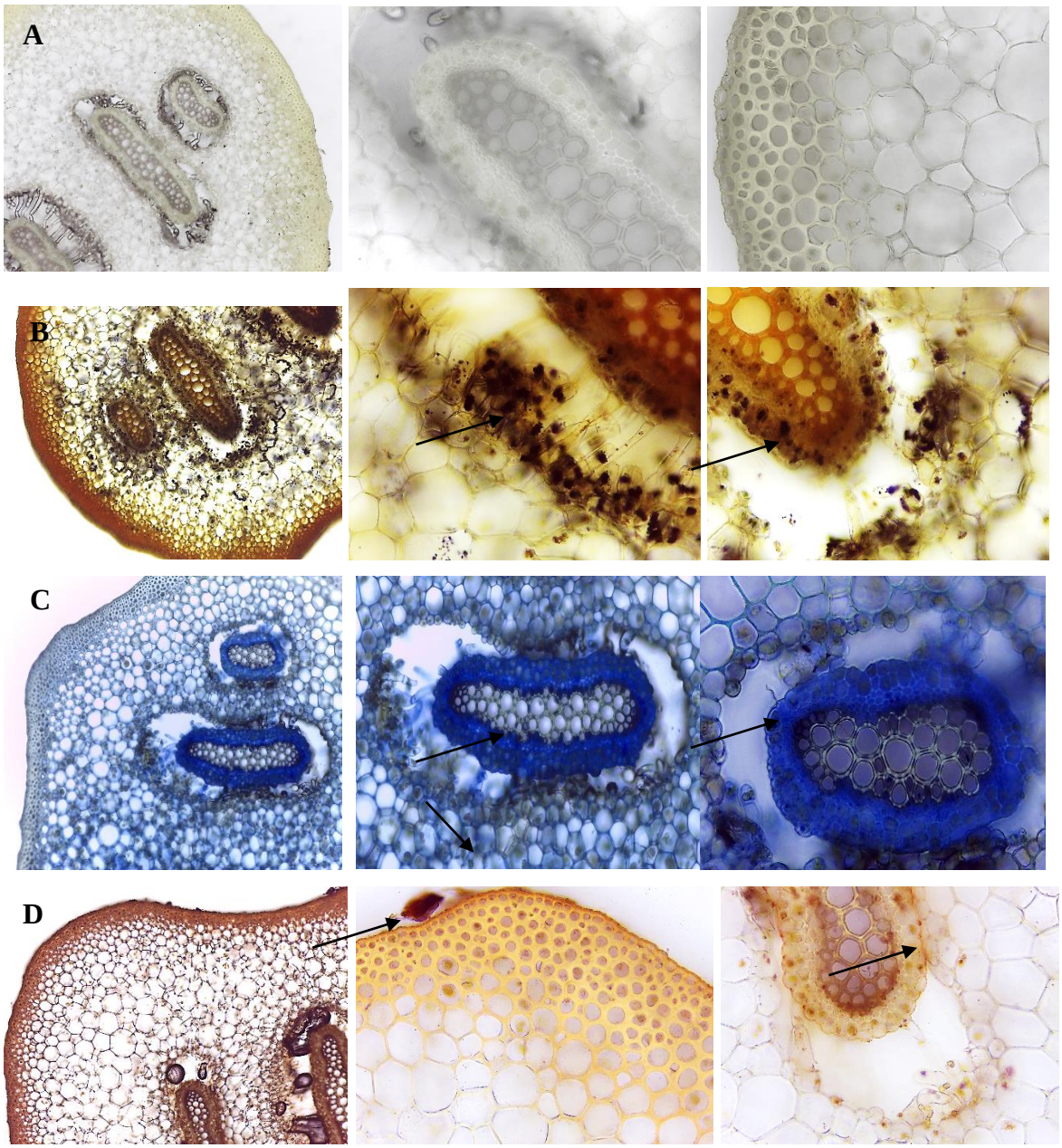


Fig 6 Histochemical studies to localize primary metabolites in *Selaginella plana*. **A**- Unstained; **B**- Lugols; **C**- Aniline blue and **D**- Sudan III

binds to lipids, producing a characteristic red or orange-red color that can be visualized under a microscope.

In the present study the localization of lipids in stems using Sudan III staining revealed the presence of lipids in the cuticle covering the epidermis of *S. vogelii* and *S. plana* (Fig 5D & 6D) while it was not very distinct in *S. braunii* (Fig 4D).

Lipid globules took a red colour when the tissue was stained with Sudan III. In all the three species lipid granules were not very distinct. Lipophilic substances were also localized in the outer phloem tissue in all the three species (Fig 4D, 5D & 6D).

Corti et al (2021) reported the presence of neutral lipids in the leaf cuticle and the cuticle covering the external epidermis of the stem of *Piper malgassicum* as revealed by Sudan III staining.

3.3 Phytochemical screening for secondary metabolites

a. Test for Alkaloids (Dragendorff's Test)

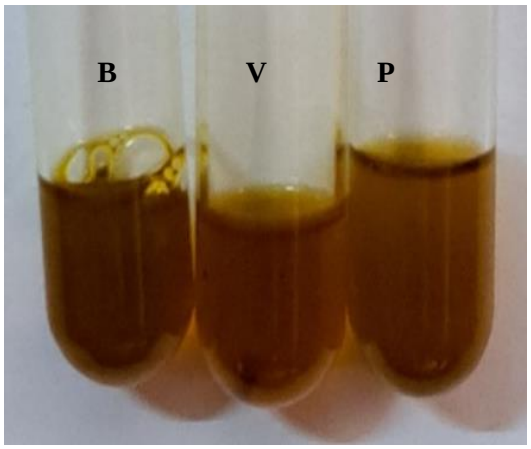
Addition of Dragendorff's reagent produced red precipitate in the methanol extracts (Fig. 7A) of all the three species indicating the presence of alkaloids. The intensity of the colour was more in *S. braunii* compared to the other two species.

b. Test for Phytosterols (Salkowski's Test)

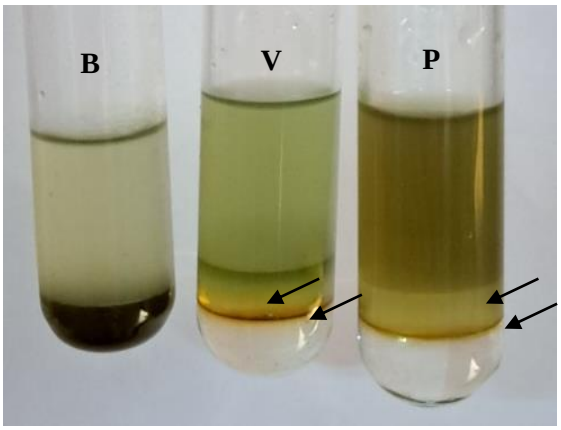
Salkowski test for phytosterols yielded golden yellow colour in the extracts of *S.vogelii* and *S.plana* which indicated the presence of phytosterols (Fig. 7B), while phytosterols were absent in *S. braunii*.

c. Test for Terpenoids (Salkowski test)

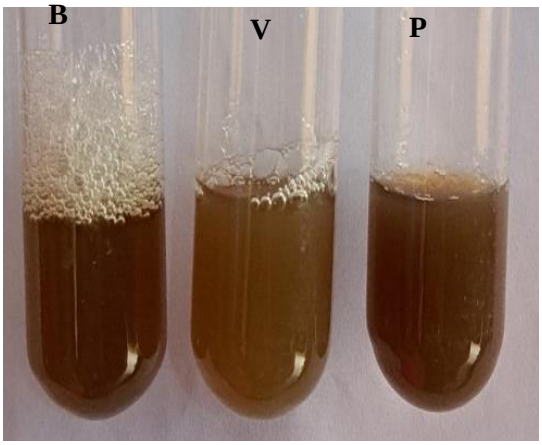
Salkowski test for terpenoids produced reddish brown colouration at the interface in the extracts which indicated the presence of terpenoids (Fig. 7B). The intensity of colour



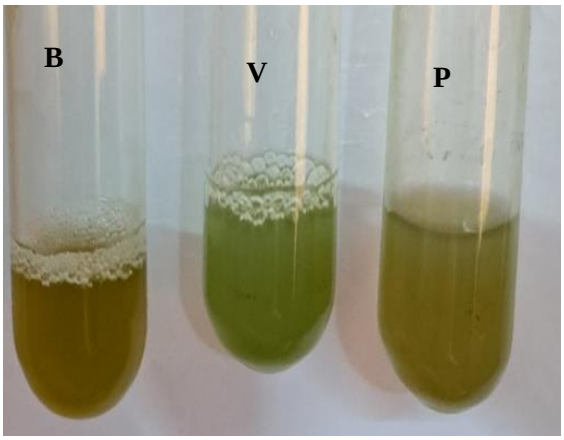
A



B



C



D

Fig. 7 Preliminary phytochemical analysis of methanolic extract of three *Selaginella* species (**B**- *S.braunii*; **V**- *S.vogelii* and **P**- *S.plana*). **A-** Dragendorff's Test; **B**- Salkowski's Test; **C**- Test for tannins & **D**- Test for saponins

was high in *S. vogelii* compared to *S. plana* while terpenoids were not detected in *S. braunii*.

d. Test for Tannins

Brownish green colouration was found in the extracts of all the there species which indicated the presence of tannins (Fig. 7C).

e. Test for saponins

The formation of frothing when the extract was vigorously shaken with few ml of distilled water showed the presence of saponins. Saponins were found in the extracts from *S. braunii* and *S. plana,* while it was absent in *S. plana.*

The qualitative phytochemical compositions of the three selected *Selaginella* species is shown in Table 3. All tested *Selaginella* species contained alkaloids, and tannins in varying levels. Phytosterols and terpenoids were absent in *S. braunii* while saponins were absent in *S. plana* (Fig. 7D).

Table 3. Phytochemical screening of the three *Selaginella* species

Experiment	***S. braunii***	***S. vogelii***	***S. plana***
1. Test for Alkaloids- Dragendorff's test	++	+	+
2.Test for Phytosterols - Salkowski test	-	++	+
3. Test for Terpenoids - Salkowski test	-	+++	++
4. Test for Tanins	++	+	+++
5. Test for Saponin	++	+	-

The secondary metabolites in plants show a lot of pharmacological properties and are the active ingredients in many medicinal plants.

In the field of medicine, alkaloids are considered as one of the leading group of phytochemicals from which powerful pain killer medications are discovered (Heinrich et al 2021). Alkaloids have diuretic, antipasmodic, anti-inflammatory, and analgesic effects. Alkaloids often have some pharmacological effects and are used for the treatment of various diseases and recreational drugs. Some alkaloids are used as the local anesthetic and stimulant as cocaine. Some alkaloids have stimulant property as caffeine and nicotine, morphine are used as the analgesic and quinine as the antimalarial drug (Khan, 2010).

Phytosterols are esters that are similar to cholesterol and found in plants. Consumption of phytosterols results in competition at the place of absorption in fat tissues between cholesterol and phytosterols. Thus having a high concentration of dietary phytosterols can efficiently reduce cholesterol blood levels. Plant sterols have been investigated as one of the safe potential alternative methods in lowering plasma cholesterol levels (Moghadasian, 2000). In addition to promoting heart health, some phytosterols have strong anticancer activity such as β-sitosterol (Kim et al., 2012). Phytosterols possess anti-inflammatory, antipyretic, antineoplastic, and immune-modulating properties as well.

Terpenoids has been reported for its noteworthy pharmacological activities such as antibacterial, anti-malarial, anti-inflammatory, anti-viral, anti-cancer activities and also shows inhibition of cholesterol synthesis. Literatures described the presence of triterpenoids in the *S. tenera* and *S. lepidophylla* which are believed to give their anticancer and cytotoxic properties to these species (Suganya et al 2011; Adnan et al 2021).

Tannins are widely distributed in plants having multiple healing properties in various health issues. They are reported as a potent antibacterial, antiviral, anti-diarrheal, antiparasitic, antihemorrhoidal agents. They are also known to be good antiviral agents (Njume, 2012). Many studies have been revealed the presence of tannins in different species of *Selaginella*

such as, S. *adoederleinii*, *S. bryopteris, S. lepidophylla, S. intermedia* and *S. inaequalifolia* (Suganya et al 2011; Adnan et al 2021A; Adnan et al 2021B).

Saponins is a group of compounds structurally related to a steroid or triterpenoidaglycone consisting one or more moieties of oligosaccharide. They are well-known for their hemolytic and foaming properties. Saponins form strong insoluble complexes with cholesterol therefore, it is believed to be useful in the human diet for controlling cholesterol. Beside that it has antifungal and antibacterial properties that are important as components of cosmetic products (Moses et al 2014; Kregiel et al 2017).

Phytochemical studies in *Selaginella* have unveiled a treasure trove of structurally diverse and potentially bioactive compounds. Continued research holds immense promise for discovering novel natural products with medicinal and agricultural significance. By unraveling the intricate relationship between *Selaginella*'s chemistry and its ecological role, we can gain a deeper understanding of plant evolution and develop sustainable strategies for utilizing these fascinating plants.

SUMMARY & CONCLUSIONS

Phytochemicals are bioactive chemical compounds occur naturally in plants. They are regarded as secondary metabolites because the plants that manufacture them may have little need for them. Phytochemicals have been the base for traditional medicine and also for modern medicine. The presence of interested phytochemical may help for further isolation, purification and characterization. Then it can be used as the base for a new pharmaceutical product.

In spite of the discovery of innumerable drugs of plant origin, the exploration of novel bioactive compounds is yet essential to enhance the range access and to search less toxic and more efficacious drugs. Medicinal and economic importance of higher plants, specifically angiosperms, has been explored thoroughly. However, lycophytes and ferns have been woefully overlooked. Though, these plants have delineated plenty of health-related benefits to humanity since ancient times. Their uses are urged in the Ayurvedic (Sushruta, Charka, Samhita), Unani, homeopathic and other systems of medicines. They exert influence on millions of human lives as conventional treatments for various diseases like, burn, cold, ascarid diseases, trauma bleeding, diarrhea and others (Benjamin and Manickam, 2007).

The cosmopolitan genus *Selaginella* also acknowledged as a "spike moss" possessing around 700–750 species distributed around the globe. The members of the *Selaginella* are well known for their uses in conventional folk medicine, food, handicrafts and as ornaments. The *Selaginella* plants are usually used by the tribal community to cure fever, jaundice, hepatic disorders, cirrhosis, diarrhea, cholecystitis, sore throat, cough of lungs, promote blood circulation, remove blood stasis and stops external bleeding after trauma and after separation of the umbilical cord (Singh & Singh 2015). A few species of *Selaginella* such as, *S. tamariscina, S. lepidophylla, S. chrysocaulos, S. bryopteris, S. labordei* and *S. moellendorffii* have been reported for their *in vitro* antimicrobial, antiviral, antidiabetic, antimutagenic, anti-inflammatory, antinociceptive, antispasmodic and anticancer potentials

due to the high content of different phytochemicals, such as flavonoids, phenylpropanoids, steroids, pigments, oxygen heterocycle, lignans, coumarins, quinoids, chromones, benzenoids, carbohydrates and alkaloids (Meng et al 1990; Lin et al 1994; De Sá et al 2012).

Preliminary phytochemical screening and quantitative estimations may be useful for the detection of bioactive principles and drug discovery. Studying the phytochemical and histochemical properties of *Selaginella* can provide valuable insights into its secondary metabolite profile, cellular organization, and evolutionary relationships. In this background the present study was conducted to perform phytochemical profiling of three *Selaginella* species grown in our campus viz. *S. braunii, S. vogelii* and *S. plana* (Fig.1).

Crude methanol extracts were obtained from the aerial parts of the sporophyte including stem and leaves of the three species using dried extraction method. 2.5g of dried powder was macerated with methanol and then extracted with 25ml methanol for 3 days with intermitten shaking. Total crude extract obtained varied from 0.23g to 0.28g. Under visible light the colour of the extract varied from olive green to dark olive green and forest green and under UV light the appearance was brown except in *S. vogelii* which appeared milky white in colour (Table 2, Fig. 2).

Histochemical tests were made on the cross-sections of fresh stem of the three selected *Selaginella* species for the localization of primary metabolites, starch, protein and lipids. The reactions for starch were positive for Lugol's iodine solutions which presented purple blue patches in the endodermis and phloem tissues of the stem of all the three species (Fig. 4B, 5B & 6B). Aniline Blue staining revealed proteins in blue (Fig. 4C, 5C & 6C). Proteins were mainly located in the vascular tissues and cortex in all the three species. The localization of lipids in stems using Sudan III staining revealed the presence of lipids in the cuticle covering the epidermis of *S. vogelii* and *S. plana* (Fig 5D & 6D) while it was not very distinct in *S. braunii* (Fig 4D). Lipophilic substances were also localized in the outer phloem tissue in all the three species (Fig 4D, 5D & 6D).

The qualitative phytochemical compositions of the three selected *Selaginella* species is shown in Table 3. All tested *Selaginella* species contained alkaloids, and tannins in varying levels. Phytosterols and terpenoids were absent in *S. braunii* while saponins were absent in *S. plana* (Fig. 7D). These secondary metabolites show a lot of pharmacological properties and are the active ingredients in many medicinal plants.

The knowledge of *Selaginella*'s phytochemical profile can be beneficial in exploring its potential for medicinal purposes. The understanding of secondary metabolite distribution can provide insights into their ecological roles, such as defense against herbivores or protection from UV radiation. Further studies are required to characterise specific biologically active compounds present in each of these species and investigate the biological activities of the identified phytochemicals. Comparative analysis of phytochemical and histochemical properties among different *Selaginella* species.

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