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ENDOPHYTIC FUNGAL DIVERSITY AND PHYTOCHEMICAL LOCALIZATION IN THREE MEDICINAL PLANTS FROM PHANSAD WILDLIFE SANCTUARY, RAIGAD, MAHARASHTRA

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ABSTRACT:
Endophytic fungi are a prolific and underexplored source of bioactive secondary metabolites with promising pharmacological and agricultural applications. This study aimed to isolate and characterize the endophytic fungi from three ethnomedicinally important plants *Azadirachta indica* (Neem), *Centella asiatica* (Gotu kola or Indian pennywort), and *Abrus precatorius* (Rosary pea) were collected from "Phansad Wildlife Sanctuary, Murud and Roha talukas of Raigad district, Maharashtra state, India". Standard isolation protocols using surface sterilization and culture on potato dextrose agar (PDA) were employed. Isolated fungal strains were morphologically identified using light microscopy and literature keys. Total nine number of Endophytic fungi were isolated from above three plants. Crude extracts of fungal cultures and host plants were subjected to Soxhlet extraction using ethyl acetate and subsequently screened for the presence of major phytochemicals including alkaloids, flavonoids, phenols, tannins, and proteins through qualitative assays and UV-Visible spectrophotometry. Histochemical and Microtomy staining techniques were utilized to observe tissue-level localization of these metabolites. The results highlight the presence of bioactive compounds in all three plant species, with significant accumulation in treated samples compared to controls. This highlights the potential role of endophytes in enhancing the phytochemical profile of their host plants. These findings support the hypothesis that endophytic fungi may play a crucial role in the regulation and biosynthesis of secondary metabolites, which could be harnessed for industrial and therapeutic purposes.

Keywords: Endophytic fungi, Secondary metabolites, Histochemical analyses and Pharmacological properties.

INTRODUCTION:

"Endophytic fungi" refers to fungi that live in plant tissues throughout the entire or partial life cycle by establishing a mutually beneficial symbiotic relationship with its host plant without causing any adverse effect or disease (Wen J., *et al*., 2022). De Bary, 1866, first introduced the term "endophyte." (Patil R. H., *et al*.,2016). The meaning of the term endophytic has been discussed well, and different definitions have been proposed. In general, it used to be applied to any organism that lives inside (*endon*) of a plant (*phyton*), as initially postulated by De Bary (1886) (Baron N. C. and Rigobelo E. C., 2021). Many endophytes have the potential to synthesize bioactive metabolites, which may directly or indirectly be used as therapeutic agents against numerous diseases (Kusari S., 2012). Endophytic fungi produce bioactive secondary metabolites, including phytohormones (Khan *et al*., 2017b). The endophytic fungi are present in almost all plant parts, especially the leaves, where the tissue is healthy. Endophytes are an essential source of secondary metabolites (Mundu and Mehta, 2021b).

Plants are autotrophic organisms. In addition to the primary metabolism in all living beings, they have a secondary metabolism that allows them to produce and accumulate compounds of a very diverse chemical nature. The compounds derived from secondary metabolism in plants are called secondary metabolites (Mera I. F. G., *et al*., 2019). Secondary metabolites (SMs) are important indicators for evaluating the quality of medicinal materials (Li Y. *et al*., 2020). Secondary plant metabolites are numerous chemical compounds produced by the plant cell through metabolic pathways derived from the primary metabolic pathways. Albrecht Kossel, Nobel Prize winner for physiology or medicine in 1910, first defined the concept of secondary metabolite. Secondary plant metabolites are classified into several classes. Secondary plant metabolites include Phenolics, Alkaloids, Saponins, Terpenes, Lipids, and Carbohydrates (Hussein R. A. and El-Anssary A. A., 2019b).

Medicinal plants played a prominent role in ancient traditional medicine systems, like the Chinese, the Ayurvedic, and the Egyptian conventional medicines, and are still commonly used today to treat various diseases. According to the World Health Organization (WHO), over 75% of people still rely on traditional plant medicines for health care in underdeveloped or developing countries. Medicinal plants are competitive in their biological abilities according to their variation in phytochemical molecules such as vitamins, terpenoids, phenolic acids, lignins, tannins, flavonoids,

quinones, coumarins and alkaloids (Lotfy R. A., *et al*., 2015). For the large proportions of the world's population, medicinal plants continue to show a role in the healthcare system and are mainly valid in developing countries, where herbal medicine has a continuous history of long use (Dar R. A., *et al*., 2017). The histological study shows medicinal plants' localization of phytochemicals such as alkaloids, terpenoids, tannins, phenolics and flavonoids (Barman *et al*., 2023).

Azadirachta indica, known as neem, belongs to the family Meliaceae (Al-Daghari N. R., *et al*., 2020). *Azadirachta indica* derived its name from 'Azad' means free; 'Dirakht' means tree; 'i-Hind' means of Indian origin; thus, it means "the free tree of India". *A. indica* is commonly known as "Neem" or "Indian Lilac" and is mainly native to the Indian subcontinent (Kumari P., *et al*., 2021). This ancient medicinal tree is often called the "wonder tree" (Kharwar *et al*., 2020). *Azadirachta indica* is native to India and contributes to the forest cover of the northern region (Qureshi *et al*., 2019). *Azadirachta indica* is a small to medium-sized evergreen tree, up to 15 m, with a round crown up to 10 (20 max.) m in diameter; branches spreading; bark moderately thick with small, scattered tubercles. (Schmutterer H. and Simons A., 2009). Neem is an essential source of endophytes with antifungal potential (Fulzele *et al*., 2018). The phytochemical compounds of *A. indica* can be saponins, flavonoids, phenols, tannins, alkaloids, glycosides, proteins, triterpenoids, carbohydrates and alkaloids (Khanal S., 2021). Its leaves contain bioactive compounds, including steroids, phenolics, terpenoids, glycosides, alkaloids, flavonoids, and tannins (Muslihin *et al*., 2022). *Azadirachta indica* has a complex of various constituents, including nimbin, nimbidin, nimbolide, and limonoids (Alzohairy M. A., 2016). Flavonoids of *A. indica* have been reported to possess antioxidant activity (Kumaresan *et al*., 2015). Since ancient times, the plant has been used to treat several human ailments and as a household pesticide. Extracts from the bark, leaves, fruits and roots of *A. indica* have been used to control leprosy, intestinal helminthosis and respiratory disorders (Nnanna J. C., *et al*., 2018). *Azadirachta indica* is widely used to investigate endophytes and their secondary metabolites (Chutulo and Chalannavar, 2018). Neem is also used for the treatment of various diseases such as malaria, intestinal worms, piles, diabetes, respiratory disorders, constipation, treatment of rheumatism, and chronic syphilitic sores (Abubakar *et al*., 2017). Extracts of the fresh leaves are reported in folk medicine for their antimicrobial, antimalarial, anthelminthic, antiviral, and antiulcer actions (Treasure *et al*., 2020). The anatomical studies of *Azadirachta indica* leaves show the mesophyll and lamina regions. They also show the presence of a single-layered epidermis on both the upper and lower sides. A cuticle may be present on the upper epidermis. Histochemical tests of *Azardiracta indica* indicated an accumulation of compounds in epidermal cells, mesophyll, and the midrib region. (Arora *et al*., 2016).

Centella asiatica belongs to Apiaceae (Umbelliferae) also known as Gotu kola or Indian pennywort is a small, annual, slender, creeping, entwined herb that grows near swamps on damp ground (Rakotoniriana E. F., *et al*., 2007). The plant grows abundantly during the rainy season, mainly in marshy and wet areas (Harun *et al*., 2019). *Centella asiatica* are found throughout tropical and subtropical regions of India. The stem is glabrous and striated, rooting at the nodes and long-petioled leaves (Singh *et al*., 2010). Thirty secondary metabolites have been isolated from pennywort, including 18 flavonoids and 13 phenolic compounds (Truong *et al*., 2023). The leaves of *C. asiatica* form the economically most important part of the plant and are known to harbour endophytes (Gupta and Chaturvedi, 2017). The major chemical constituents found in the plant are triterpenoids, vallarine, asiaticoside, sitosterol, tannin, oxy-asiaticoside (Shastry *et al*., 2020). *C. asiatica* contains many phenolic constituents, including flavonoids, such as catechin, epicatechin, kaempferol, quercetin and related glycosides (Gray N. E., *et al*., 2017). *Centella asiatica* is one of the most significant therapeutic herbs used in Indian Ayurvedic traditions and is also known for its antibacterial, antifungal, antidiabetic, antidiuretic, and antioxidant properties (Mundu *et al*., 2024). *Centella asiatica* is used in Indian systems of medicine to enhance memory and treat skin diseases, nervous disorders and also treats various ailments, including body aches, headaches, insanity, asthma, leprosy, ulcers, eczema, and wound healing (Prakash *et al*., 2017). Histochemical tests of *Centella asiatica* indicated the presence of compounds in epidermal cells, mesophyll, and the midrib region. (Arora *et al*., 2016). The anatomical studies of leaves *Centella asiatica* show the mesophyll and lamina regions. The epidermis is compactly arranged and covered with a thick cuticle. The vascular bundle is present in the midrib region with xylem in exarch condition. (Dhivya *et al*., 2023).

Abrus precatorius, commonly known as Rosary pea, Indian liquorice, Jequirity and Crab's eye, is an important medicinal plant belonging to the family Fabaceae. It is known as *Ratti* in Hindi and *Gunja* in Sanskrit. The *Abrus precatorius* is native to India but is now found in almost all tropical and sub-tropical areas worldwide (Tabasum *et al*., 2018). *A. precatoriusis* a woody plant with red seeds with a black mark at the base. Leaves resemble tamarind leaves and stem cylindrical, wrinkled, bark smooth-textured, brown (Garaniya N. and Bapodra A., 2014). Leaves were rich in polyphenols, flavonoids, β-carotene, glutathione, α-tocopherol, and ascorbic acid (Palvai *et al*., 2014). Many phytoconstituents were reported from *A. precatorius* like Glycyrrhizin, Abrusosides A–E, triterpene glycosides, steroids, alkaloids like abrine, hypaphorine, choline and precatory, flavonoids like vitexin, toxifolin-3- glucosides (Mondal S., *et al*., 2017).This plant's roots, seeds, and leaves have been traditionally used for their purgative, emetic, tonic, aphrodisiac, and hair growth-promoting properties (Barve and Ojha, 2013). Leaves are sweet and used to treat cough, malaria, snake bites and boils (Paul *et al*., 2013). Leaves are also used for breast cancer (DharmarajSanthosam *et*

al., 2023). *A. precatorius* possesses different pharmacological activities such as antimicrobial, antifertility, antibacterial, anti-tumour, immunopotentiating and antidiarrhoeal (Taur *et al*., 2017). Leaf anatomy of *Abrus precatorius* shows the presence of a single-layered epidermis on both the upper (adaxial) and lower (abaxial) sides. A cuticle may be present on the upper epidermis. The midrib area displays a noticeable vascular bundle, with xylem on top and phloem at the bottom, enclosed by a bundle sheath (Thorat *et al*., 2025). Histochemical tests of *Abrus precatorius* show compounds in epidermal cells, mesophyll, and the midrib region (Arora *et al*., 2016).

METHODOLOGY:

Collection of plant material:

Healthy and mature plants of *Azadirachta indica* (Neem), *Centella asiatica* (Gotu kola or Indian pennywort), and *Abrus precatorius* (Rosary pea) plants were collected from "Phansad Wildlife Sanctuary, Murud and Roha talukas of Raigad district, Maharashtra state, India". Plant species were brought to the laboratory in polythene bags and processed immediately to reduce the risk of contamination. All plants were kept in sunlight and has same shade but not too much. They were watered daily with enough water to penetrate into the soil. Avoid watering the leaves because it leads to disease and damage (Kusari and Spiteller, 2012).

Isolation and Identification of Endophytic Fungi:

The leaves were washed thoroughly in sterile distilled water to remove particles and blot-dried. Leaf samples were first immersed in 70% ethanol (v/v) for one min, followed by a subsequent immersion in sodium hypochlorite (3.5%, v/v) for three min. The leaves were rinsed three times in changes of sterile distilled water and dried on sterile blotters under the aseptic condition for complete drying. Bits of 1.0 × 0.1 cm size were excised with the help of a sterile blade. The Segments of leaves were carefully placed on potato dextrose agar (PDA) media supplemented with streptomycin to inhibit bacterial growth. The plates were wrapped in clean cling film and incubated at 22 °C with 12 hrs light and dark cycles for 6 to 8 days. The effectiveness of surface sterilization of tissues was examined by setting the aliquots of sterilant on agar plates and observing fungal colonies for 2 weeks. The bits where be examined for the appearance of the fungal colony, and each fungal colony that emerged from segments was transferred to an antibiotic-free (PDA) potato dextrose agar medium to aid identification. The morphological identification of the isolates depends on the fungal colony, characteristics of the spores and reproductive structures. Identified the fungi that we isolated with the help of the Handbook of Soil Fungi by I.K. Kunwar & C. Manoharachary A. Nagamani (Author), A. Nagamani (Editor), I.K. Kunwar (Editor), C. Manoharachary (Editor) Format: Kindle Edition. All fungal mounts were produced on microscopic glass slides in lacto phenol-cotton blue and sealed with nail polish (Ramasandra Govind *et al.,* 2015).

Mat Preparation:

Fungal mycelial plugs were used to inoculate 100 ml of potato extract glucose broth in 250 ml Erlenmeyer flasks. The 250-ml Erlenmeyer flasks were incubated in the dark at 30°C for 15-20 days with rotary shaking at 105 rpm. To remove the biomass, the fungal cultures were vacuum-filtered through Whatman filter paper No.1. The Mat was dissolved in double-distilled water, homogenized, and sprayed on the incision part of the plant.

EXTRACTION OF SECONDARY METABOLITES:

Soxhlet extraction:

This process, known as continuous hot extraction, involves using a modern and efficient mechanical grinder. The leaves are sun-dried and then powdered with this grinder to obtain a coarse powder, an essential step in the Soxhlet extraction process.

Soxhlet extraction involves a series of steps that are repeated until the plant material is completely extracted. The dried, ground and finely powdered plant material is placed inside a porous bag (thimble) made of clean muslin cloth and tightly closed. (Ingle K.P *et al*., 2017; Azwanida N.N. 2015; Pandey A and Tripathi S., 2014; Doughari J.H. 2012; Majekodunmi S.O. 2015; Hossain M.A *et al*., 2014; and Harborne J.B. 1998). The bottom flask is filled with ethyl acetate, followed by the thimble into the extraction chamber. The bottom flask is heated, and the solvent starts evaporating. Then, it flows through the condenser, condenses, and down to the extraction chamber, where it is extracted by coming into contact. When the solvent level in the extraction chamber arrives at the top of the siphon, the solvent and the extracted plant material flow back to the flask. (Ingle K.P *et al*., 2017; Azwanida, N.N. 2015; Pandey A and Tripathi S., 2014; Doughari, J.H. 2012; Majekodunmi, S.O. 2015; Hossain M.A *et al*., 2014; and Harborne, J.B. 1998). This entire process continues repeatedly until the plant material is completely extracted, a point at which a solvent flowing from the extraction chamber does not leave any residue behind.

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ISOLATED ENDOPHYTIC FUNGI

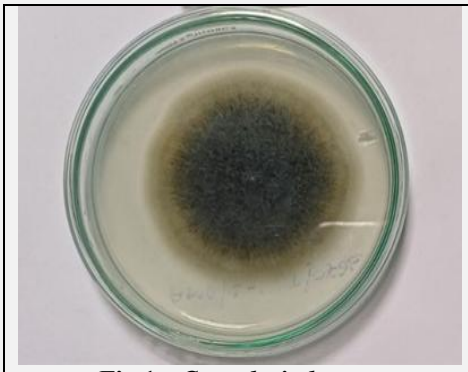

Fig 1: ***Curvularia lunata***

Fig 2: ***Aspergillus flavus***

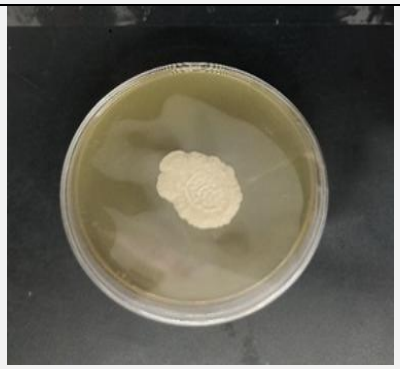

Fig 3: ***Colletotrichum gloeosporioides***

Fig 4: ***Aspergillus fumigatus***

Fig 5: ***Alternaria alternata***

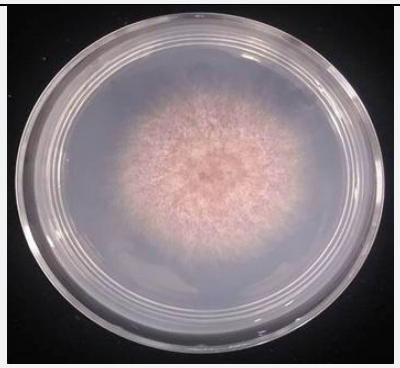

Fig 6: ***Fusarium oxysporum***

Fig 7: ***Xenodidymella humicola***

Fig 8: ***Aspergillus ochraceus***

Fig 9: ***Aspergillus niger***

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Fig 10: A - Control; A1, A2, A3 treated with *Curvularia lunata*

Azadirachta indica

Fig 11: B – Control; B1, B2, B3 - treated with *Aspergillus flavus*

Azadirachta indica

Fig 12: C – Control; C1, C2, C3 - Treated with *Colletotrichum gloeosporioides*

Azadirachta indica

Fig 13: D - Control; D1, D2, D3 - treated with *Aspergillus fumigatus*

Centella asiatica

Fig 14: E – Control; E1, E2, E3 - treated with *Alternaria alternata*

Centella asiatica

Fig 15: F – Control; F1, F2, F3 - treated with *Fusarium oxysporum*

Centella asiatica

Fig 16: G - Control; G1, G2, G3 - treated with *Xenodidymella humicola*

Abrus precatorius

Fig 17: H – Control; H1, H2, H3 - treated with *Aspergillus ochraceus*

Abrus precatorius

Fig 18: I – Control; I1, I2, I3 - Treated with *Aspergillus niger*

Abrus precatorius

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PHYTOCHEMICAL SCREENING METHODS:

Phytochemical screenings are preliminary assays that determine the presence of secondary metabolites in an extract. Several qualitative analyses described below have been used to detect the presence of alkaloids, flavonoids, tannins, phenols terpenes and protein (Abanikannda *et al.*, 2020).

Test for alkaloids

Dragendorff's test:

1 ml of extract was taken and placed into a test tube. Then, 1 ml of potassium bismuth iodide solution (Dragendorff's reagent) was added and shaken. An orange-red precipitate formed indicates the presence of alkaloids. (Pandey A. and Tripathi S., 2014; Beena P *et al*., 2016; Trease G.E. and Evan W.C., 1989; Wallis, T.E., 1989).

Test for flavonoids

Lead acetate test:

1 ml of extract was taken and placed into a test tube. Then, a few drops of lead acetate are added and shaken. Yellow precipitate shows the presence of flavonoids. (Pandey A. and Tripathi S., 2014; Beena P *et al*., 2016; Trease G.E. and Evans W.C., 1989; Wallis, T.E., 1989).

Test for phenols

Ferric chloride test:

1 ml of an extract solution was taken and placed into a test tube. Then, a 1% gelatine solution containing sodium chloride was added and shaken. Formation of a bluish-black colour indicates the presence of phenols. (Pandey A. and Tripathi S., 2014; Beena P *et al*., 2016; Trease G.E. and Evans W.C., 1989; Wallis, T.E., 1989).

Test for tannins

Gelatin's test:

1 ml of extract was taken and placed in a test tube. Then, a 1% sodium chloride gelatin solution is mixed. White precipitate indicates the presence of tannins. (Pandey A. and Tripathi S., 2014; Beena P *et al*., 2016; Trease G.E. and Evans W.C., 1989, Wallis, T.E., 1989).

Test for protein

Ninhydrin test:

1 ml of an extract was taken and placed into a test tube. Then, 0.25% of the ninhydrin reagent was added and shaken. The mixture was then boiled for a few minutes. The formation of a blue colour signifies the presence of protein.

PHYTOCHEMICAL ANALYSIS

Table 1: Phytochemical results of *Azadirachta indica* leaf extract

Sr. No.	Name Of Phytochemical	Reagent Used	*Azadirachta indica*			
			Control (C)	Test 1 A(1,2,3)	Test 2 B(1,2,3)	Test 3 C(1,2,3)
01.	Alkaloids	Dragendorff's reagent	-	+	+	+
02.	Flavonoids	Lead acetate test	-	+	+	+
03.	Phenol	Alcoholic $FeCl_3$	-	+	+	+
04.	Proteins	Ninhydrin reagent	-	+	+	+
05.	Tannins	Gelatin's test	-	+	+	+

Table 2: Phytochemical results of *Centella asiatica* leaf extract

Sr. No.	Name Of Phytochemical	Reagent Used	*Centella asiatica*			
			Control (C)	Test 1 D(1,2,3)	Test 2 E(1,2,3)	Test 3 F(1,2,3)
01.	Alkaloids	Dragendorff's reagent	-	+	+	+
02.	Flavonoids	Lead acetate test	-	+	+	+
03.	Phenol	Alcoholic $FeCl_3$	-	+	+	+
04.	Proteins	Ninhydrin reagent	-	+	+	+
05.	Tannins	Gelatin's test	-	+	+	+

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Table 3: Phytochemical results of *Abrus precatorius* leaf extract

Sr. No.	Name Of Phytochemical	Reagent Used	*Abrus precatorius*			
			Control (C)	Test 1 G(1,2,3)	Test 2 H(1,2,3)	Test 3 I(1,2,3)
01.	Alkaloids	Dragendorff's reagent	-	+	+	+
02.	Flavonoids	Lead acetate test	-	+	+	+
03.	Phenol	Alcoholic $FeCl_3$	-	+	+	+
04.	Proteins	Ninhydrin reagent	-	+	+	+
05.	Tannins	Gelatin's test	-	+	+	+

PRELIMINARY PHYTOCHEMICAL TESTS FOR SECONDARY METABOLITES IN *Azadirachta indica*

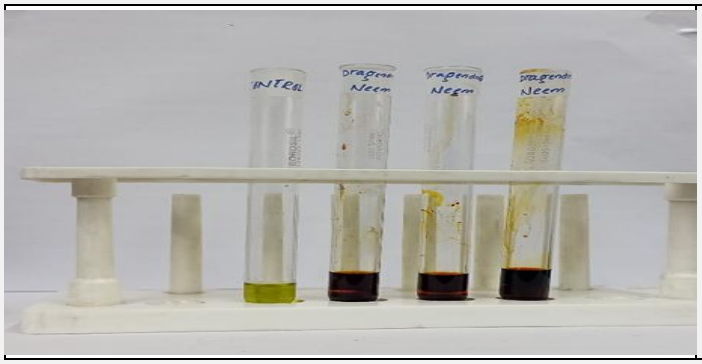

Fig 22: Preliminary phytochemical test for confirming the presence of Alkaloids

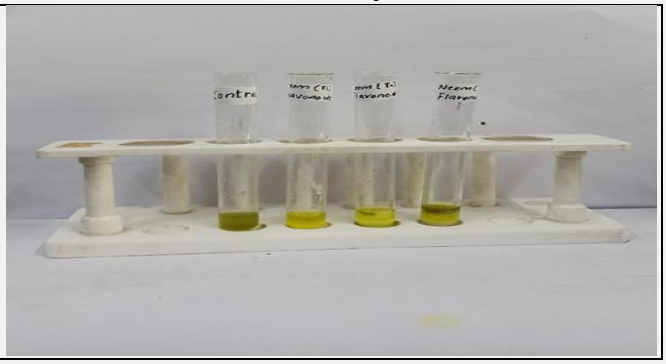

Fig 23: Preliminary phytochemical test for confirming the presence of Flavonoids

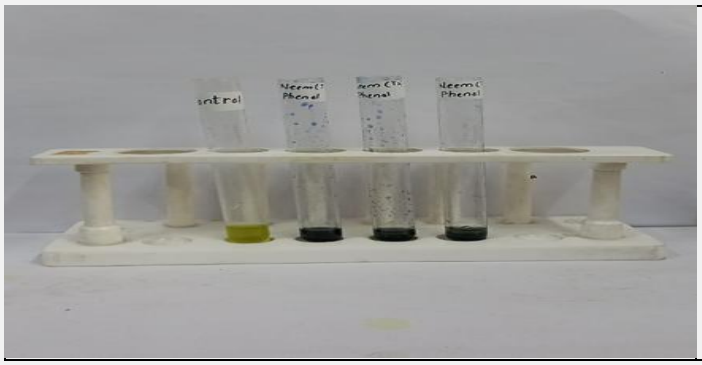

Fig 24: Preliminary phytochemical test for confirming the presence of Phenols

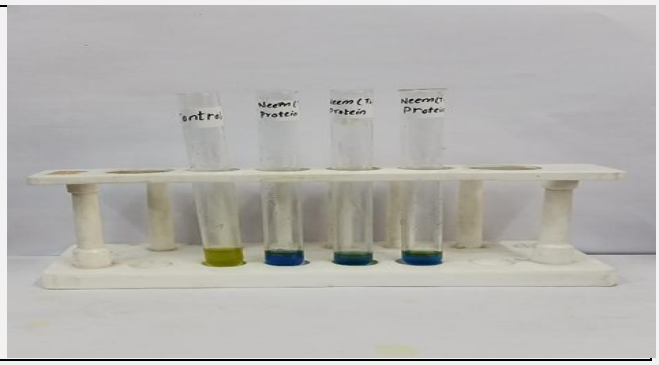

Fig 25: Preliminary phytochemical test for confirming the presence of Proteins

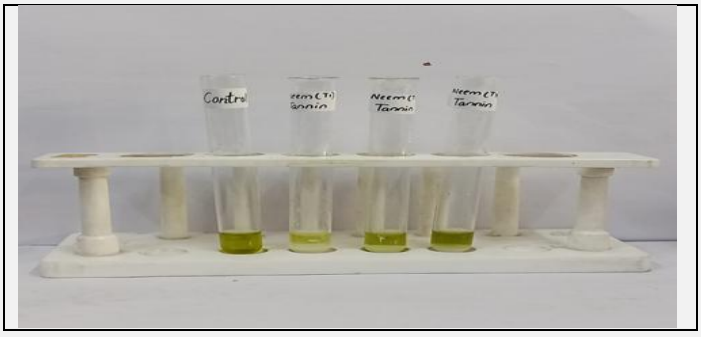

Fig 26: Preliminary phytochemical test for confirming the presence of Tannins

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PRELIMINARY PHYTOCHEMICAL TESTS FOR SECONDARY METABOLITES IN *Centella asiatica*

Fig 27: Preliminary phytochemical test for confirming the presence of Alkaloids

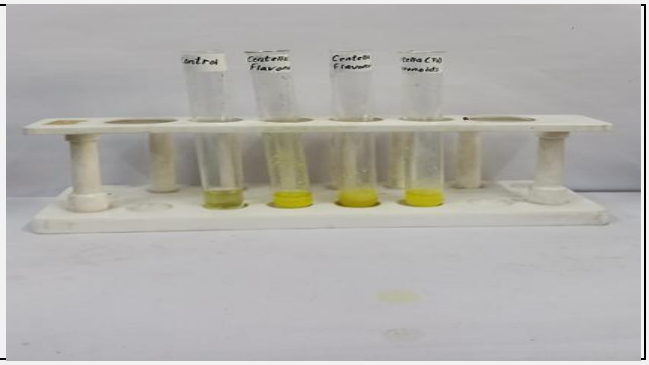

Fig 28: Preliminary phytochemical test for confirming the presence of Flavonoids

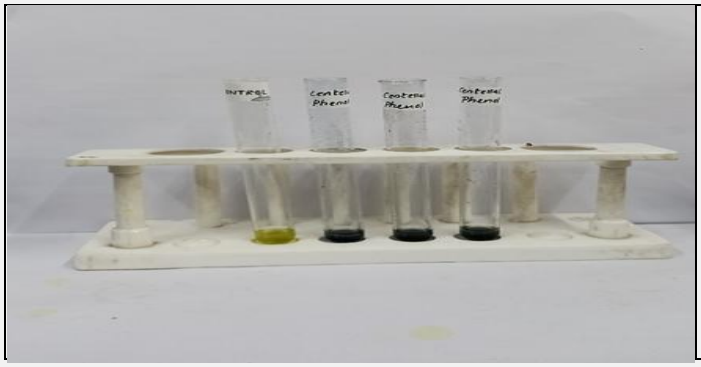

Fig 29: Preliminary phytochemical test for confirming the presence of Phenols

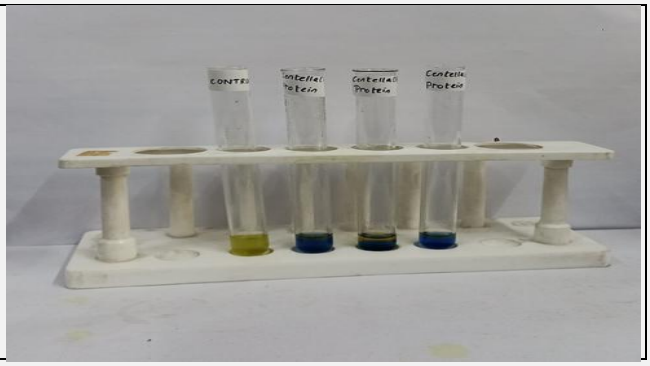

Fig 30: Preliminary phytochemical test for confirming the presence of Proteins

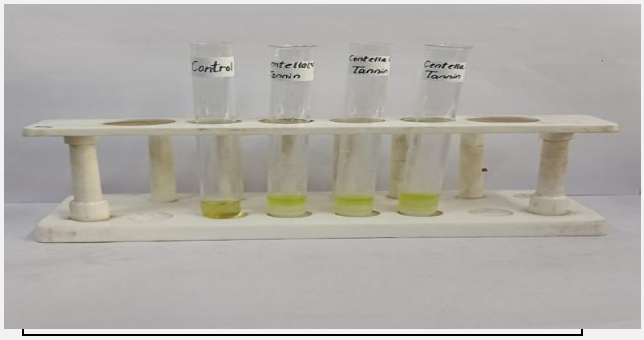

Fig 31: Preliminary phytochemical test of confirming the presence of Tannins

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PRELIMINARY PHYTOCHEMICAL TESTS FOR SECONDARY METABOLITES IN *Abrus precatorius*

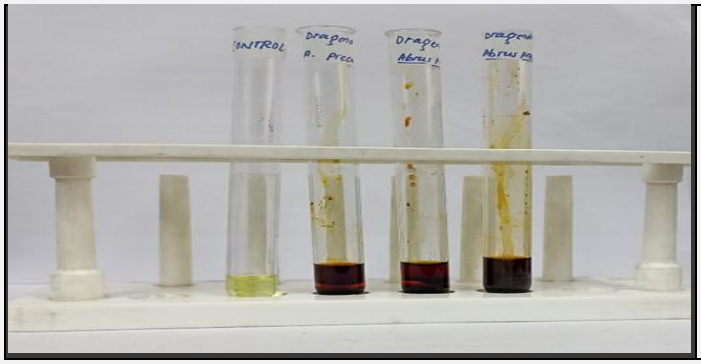

Fig 32: Preliminary phytochemical test for confirming the presence of Alkaloids

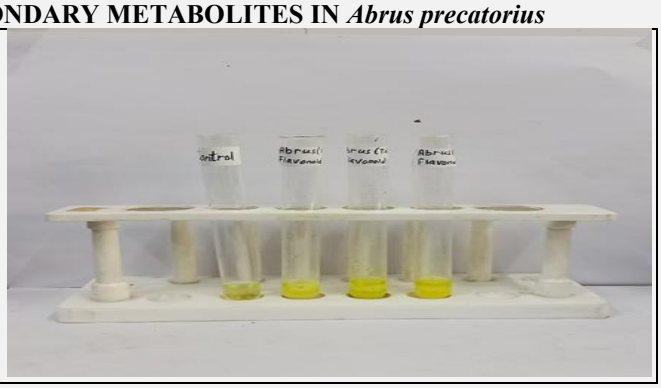

Fig 33: Preliminary phytochemical test for confirming the presence of Flavonoids

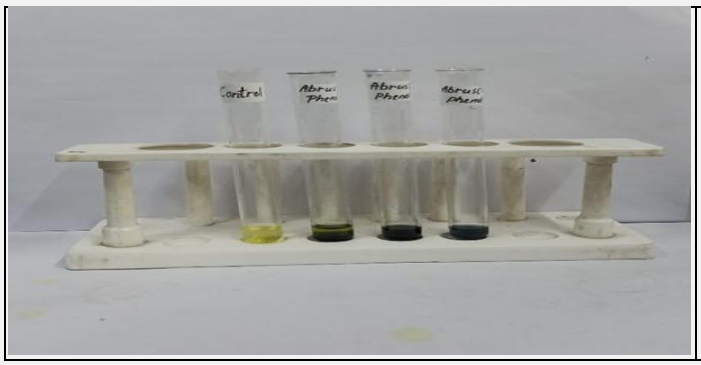

Fig 34: Preliminary phytochemical test for confirming the presence of Phenols

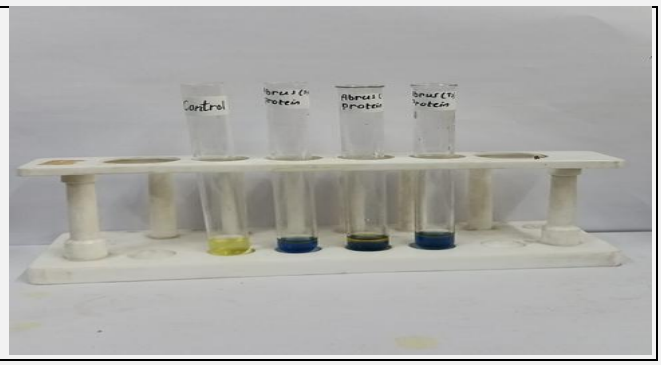

Fig 35: Preliminary phytochemical test for confirming the presence of Proteins

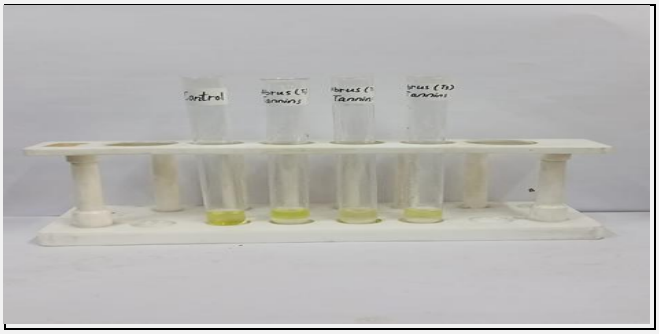

Fig 36: Preliminary phytochemical test for confirming the presence of Tannins

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SRCREENING OF PHYTOCHEMICAL CONSTITUENTS BY USING UV SPECTROPHOTOMETER:
Medicinal plants are an essential source of drugs. Secondary metabolites are chemical constituents present in plants and are crucial in determining the plant's medicinal properties. Ethyl acetate extracts of *Azadirachta indica* (Neem), *Centella asiatica* (Gotu kola or Indian pennywort), and *Abrus precatorius* (Rosary pea) were screened for Phytochemical analysis by standard procedure and subjected to analysis by UV Spectrophotometer (Pralhad and Mishra, 2015).

1. *Azadirachta indica*

Fig 37: UV – VIS Spectra of *Azadirachta indica* leaf extract

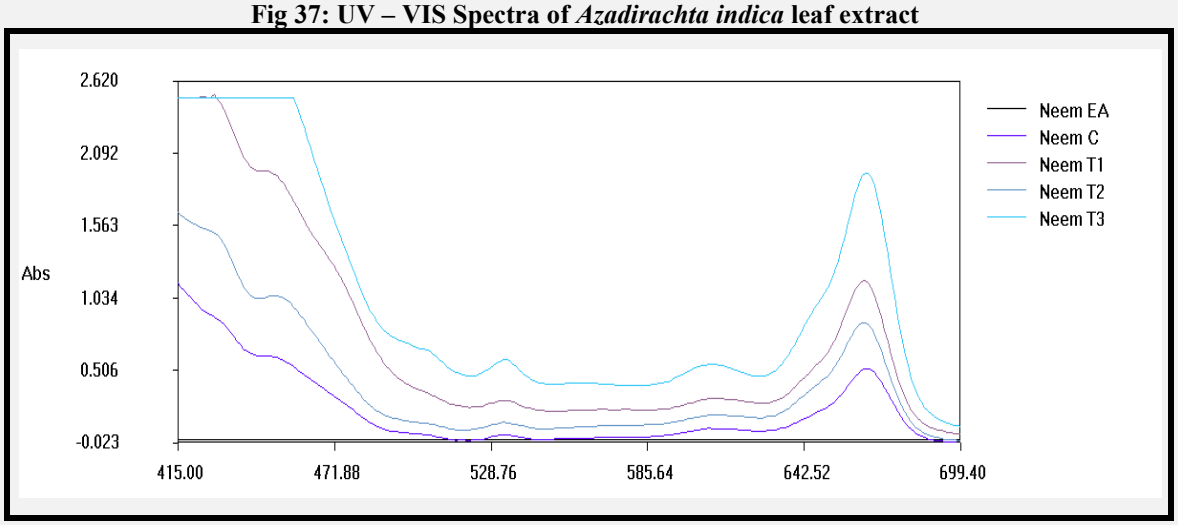

Table 4: UV – VIS peak values of *Azadirachta indica* leaf extract

Sr. No.	Samples	nm	λ
01.	Ethyl acetate (EA)	000.0	0.000
02.	Control (C)	662.2	0.515
03.	A (1,2,3)	663.4	1.159
04.	B (1,2,3)	663.4	0.854
05.	C (1,2,3)	662.2	1.917

2. *Centella asiatica*

Fig 38: UV – VIS Spectra of *Centella asiatica* leaf extract

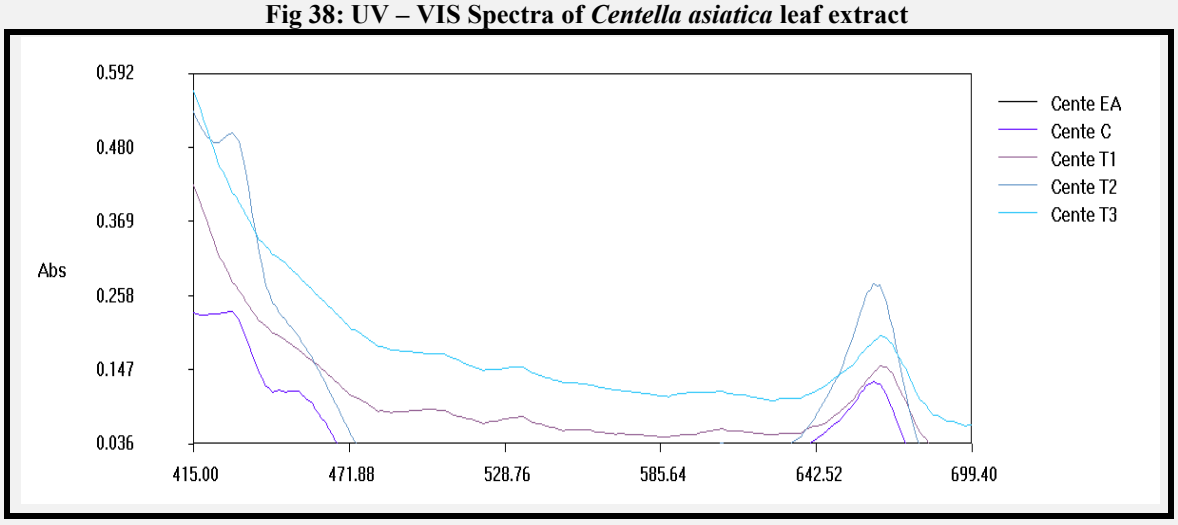

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Table 5: UV – VIS peak values of *Centella asiatica* leaf extract

Sr. No.	Samples	nm	λ
01.	Ethyl acetate (EA)	000.0	0.000
02.	Control (C)	662.2	0.130
03.	D(1,2,3)	664.6	0.153
04.	E (1,2,3)	662.2	0.276
05.	F(1,2,3)	664.6	0.198

3. *Abrus precatorius*

Fig 39: UV – VIS Spectra of *Abrus precatorius* leaf extract

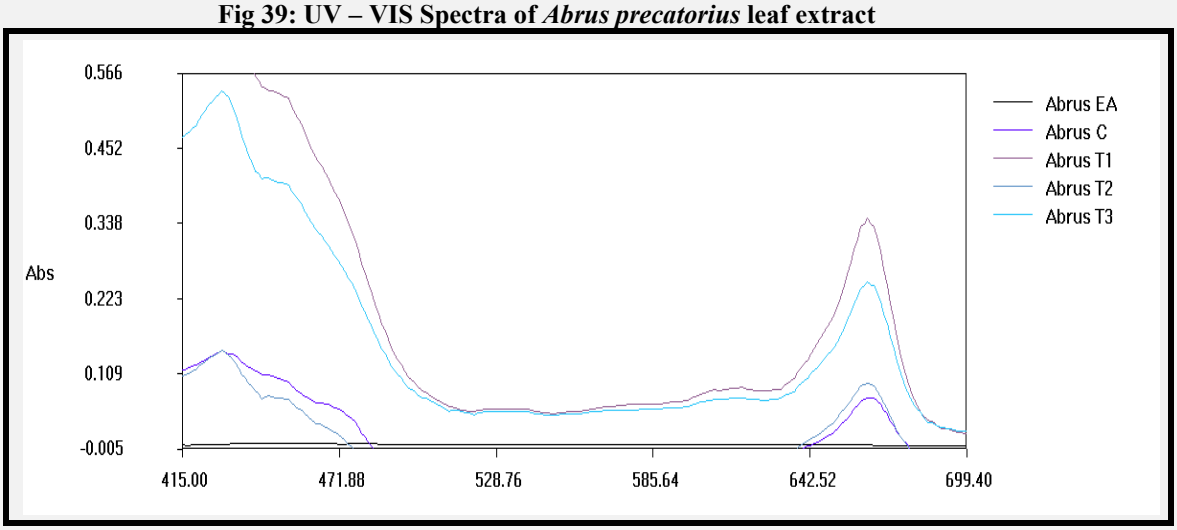

Table 6: UV – VIS peak values of *Abrus precatorius* leaf extract

Sr. No.	Samples	nm	λ
01.	Ethyl acetate (EA)	000.0	0.000
02.	Control (C)	662.2	0.073
03.	G(1,2,3)	661.0	0.337
04.	H (1,2,3)	661.0	0.092
05.	I (1,2,3)	662.2	0.249

MICROTOME:

1. Tissue Resection

Plucked the plant leaves and then cut out the midrib region of the leaves.

2. Tissue Fixation

The leaves were kept in 4% formalin for 24 hours.

3. Dehydration

Kept the leaves in different percentages of Alcohol (30%, 50%, 70%, 90%, Absolute) for 20 minutes each.

4. Clearing

- First, the leaves were kept at a 50:50 ratios of absolute alcohol and xylene for 10 minutes (The tissue colour changed from green to yellow).
- After that, kept the leaves in xylene only for 10 minutes.
- After that, four blocks were placed in an oven at 58-60°C. In the first block, xylene and wax were added in a 50:50 ratios, and the leaves was kept for 20 minutes. Wax was added to the remaining three blocks, and the leaves was transferred to the next block after every 20 minutes.

5. Wax Infiltration and Embedding

- Filtered wax was used for block making. (Wax should be filtered thrice in an oven through Whatman Filter Paper No. 1).
- Take a large petri plate and "L" pieces and apply Glycerine to it.

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- The "L" pieces were arranged in a rectangular shape on the petri plate for block making.
- The melted wax was poured in "L" pieces and allowed to solidify. To ensure that the upper surface of the block does not solidify, we keep moving the heated spatula around its surface.
- When the bottom was solidified, the leaves were inserted in the middle portion of the block, and the block was allowed to solidify completely.

6. Trimming of Block

- The block was trimmed with a clean and sharp blade and mounted on a wooden block, which was kept in a refrigerator for cooling.

7. Section Cutting

- Rotary Microtome did section cutting at 10 µm.
- The blade of the Rotary Microtome should be very sharp for thin sections.
- Cut the sections. The sections were obtained in ribbon form and were collected in a box.

8. Slide Preparation

- An albumin slide was taken, and a cut section was placed on it.
- Now, a drop of distilled water from an edge was spread in length, and the slide was put on a hot plate for a few minutes.

9. Staining Procedure

- Take the slide in a coupling jar and dewax sections in Xylene I and II for 10 minutes each.
- Rehydrate through graded Alcohols (90%, 70%, 50%, 30%) for 5-10 minutes each in distilled water. Stain sections with Safranin for 10-15 minutes.
- Rinse in tap water for 3-5 minutes. Dehydrate using 70% and 90% Alcohol for 2-4 minutes each.
- Dehydrate in 100% Alcohol for 2 minutes. Clear with Xylene for 10 minutes and mount slides with coverslip using DPX.

HISTOCHEMICAL ANALYSIS:

Histochemical studies of fresh material of in vivo, in vitro leaf and leaf-derived callus were carried out through transverse sections made with a razor blade and a microtome. Thin and transparent sections were selected and treated with different reagents/dyes to detect various classes of secondary metabolites (Table 1). The control sections (unstained) were also maintained together for comparative investigation and specific localization of secondary metabolites in various tissues and cells. Fresh sections were treated with Dragendorff's reagent (Furr and Mahlberg, 1981) for Alkaloids detection, Vanillin for Flavonoids (Mamoucha *et al*., 2016), 10% Aq. Ferric chloride for Phenol/Tannins (Mace and Howell, 1974), Bromophenol blue for Protein (Mazia *et al*., 1953). All specimens were observed under a LYNX microscope.

Sr. No	Secondary metabolites	Histochemical reagent	Observations	References
1	Alkaloids	Dragendorff's reagent	Golden color appearance	Furr & Mahlberg (1981)
2	Flavonoids	Vanillin test	Red in color	Mamoucha *et al*. (2016)
3	Phenol/ Tannins	10% Ferric chloride test (aq.)	Blue-green appearance	Mace and Howell (1974)
4	Proteins	Bromophenol blue	Appearance of blue color	Mazia *et al*., (1953)

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MICROTOME (*Azadirachta indica)*

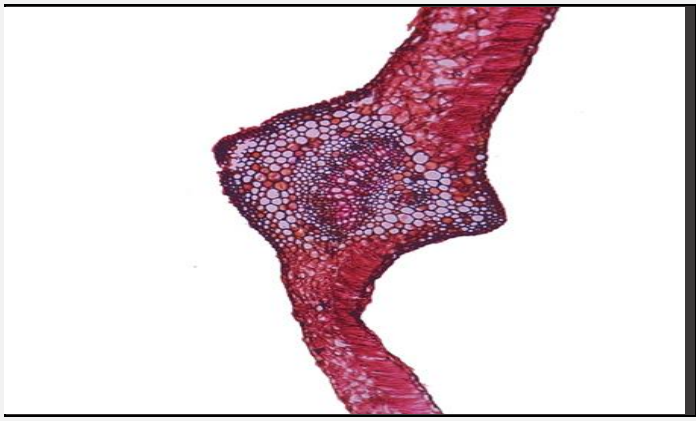

Fig 40: T.S. of leaf stain with Safranin

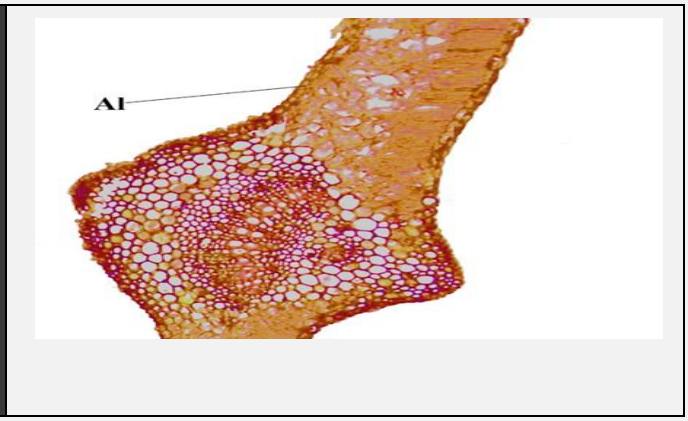

Fig 41: T.S. of leaf stain with Dragendorrf's Reagent

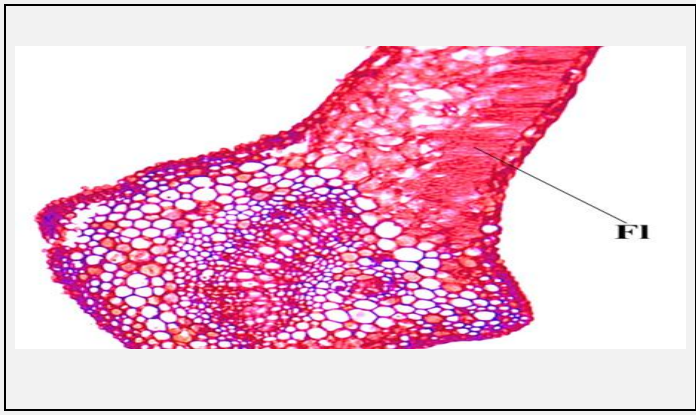

Fig 42: T.S. of leaf stain Vanillin

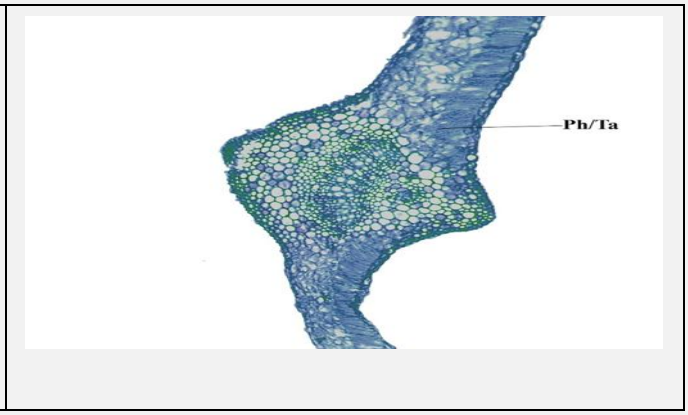

Fig 43: T.S. of leaf stain with 10% Aq. Fecl3

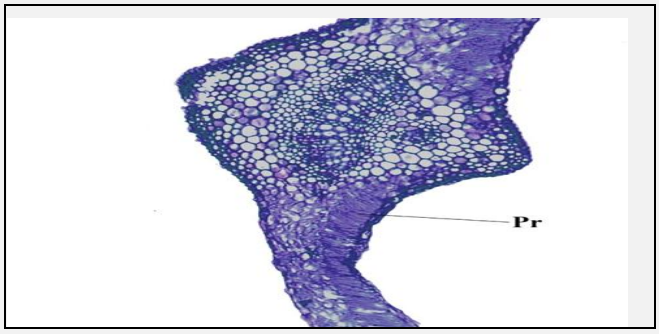

Fig 44: T.S. of leaf stain with Bromophenol blue

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MICROTOME (*Centella asiatica)*

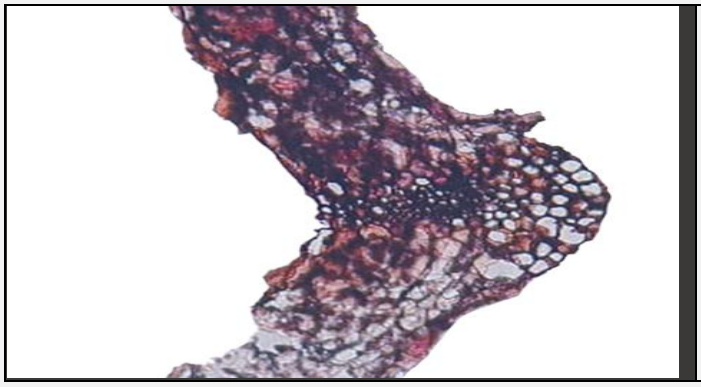

Fig 45: T.S. of leaf stain with Safranin

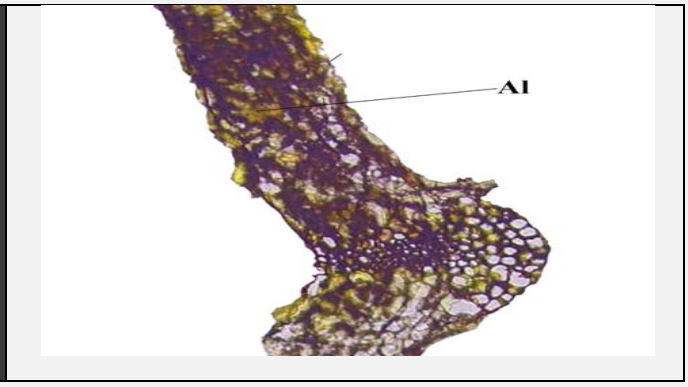

Fig 46: T.S. of leaf stain with Dragendorrf's reagent

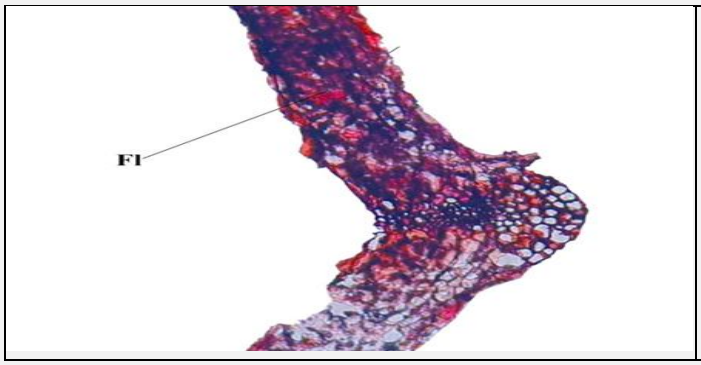

Fig 47: T.S. of leaf stain Vanillin

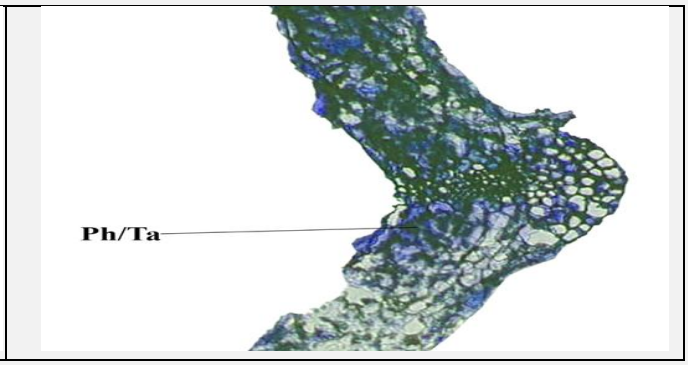

Fig 48: T.S. of leaf stain with 10% Aq. Fecl3

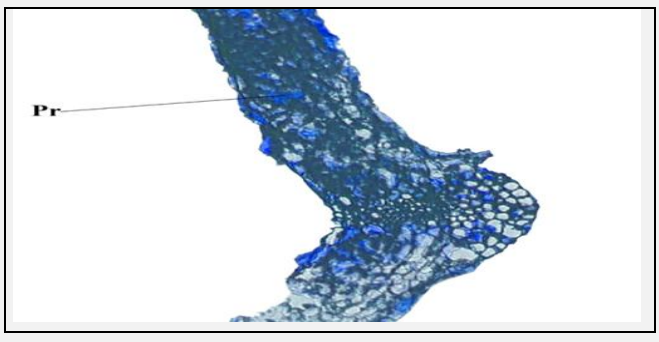

Fig 49: T.S. of leaf stain with Bromophenol blue

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MICROTOME (*Abrus precatorius)*

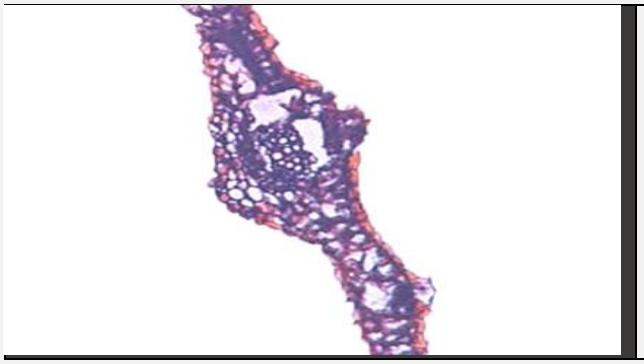

Fig 50: T.S. of leaf stain with Safranin

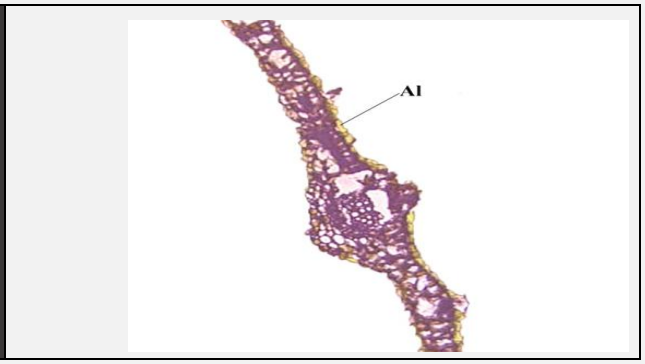

Fig 51: T.S. of leaf stain with Dragendorrf's reagent

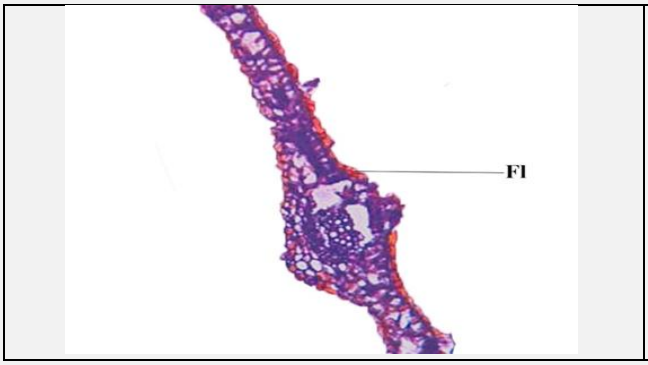

Fig 52: T.S. of leaf stain Vanillin

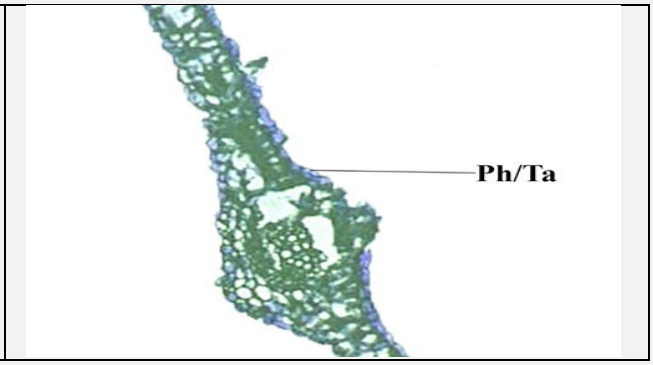

Fig 53: T.S. of leaf stain with 10% Aq.$FeCl_3$

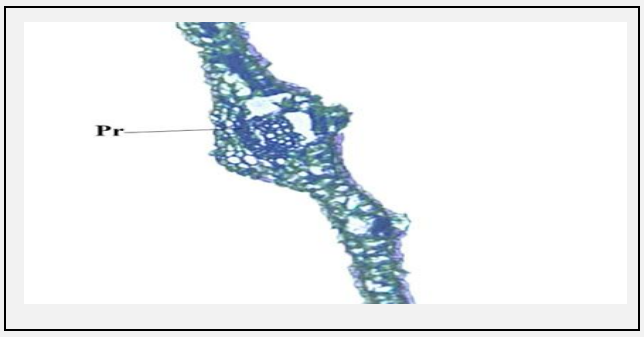

Fig 54: T.S. of leaf stain with Bromophenol blue

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RESULTS & DISCUSSIONS:

In the present study, medicinal plants *Azadirachta indica*, *Centella asiatica* and *Abrus precatorius* were selected to isolate endophytic fungi. From the leaves of *Azadirachta indica*, *Centella asiatica* and *Abrus precatorius*, endophytic fungi were isolated. A total of nine endophytic fungi were isolated from the leaves of the plants; out of which three from the leaves of *Azadirachta indica*, three from the leaves of *Centella asiatica* and three from the leaves of *Abrus precatorius*. *Curvularia lunata*, *Aspergillus flavus* and *Colletotrichum gloeosporioides* from *Azadirachta indica*, *Aspergillus fumigatus*, *Alternaria alternata* and *Fusarium oxysporum* from *Centella asiatica* & *Xenodidymella humicola*, *Aspergillus ochraceus* and *Aspergillus niger* from *Abrus precatorius*; all of these endophytic fungi have been isolated from the leaves of the plants.

After the successful isolation of endophytic fungi from *Azadirachta indica*, *Centella asiatica*, and *Abrus precatorius*, the fungal biomass (mat) of different fungal isolates, viz. *Curvularia lunata*, *Aspergillus flavus*, *Colletotrichum gloeosporioides*, *Aspergillus fumigatus*, *Alternaria alternata*, *Fusarium oxysporum*, *Xenodidymella humicola*, *Aspergillus ochraceus*, and *Aspergillus niger* were prepared separately. Each mat was dissolved in double-distilled water and homogenised to create a fungal suspension. All the suspensions were sprayed on the incision part of the plants while a control of *Azadirachta indica, Centella asiatica* and *Abrus precatorius* was left untreated for comparative analysis. In *Azadirachta indica*, A (1,2,3) treated with *Curvularia lunata*, B (1,2,3) treated with *Aspergillus flavus* and C (1,2,3) treated with *Colletotrichum gloeosporioides*. In *Centella asiatica*, D (1,2,3) treated with *Aspergillus fumigatus*, E (1,2,3) treated with *Alternaria alternata* and F (1,2,3) treated with *Fusarium oxysporum*. In *Abrus precatorius*, G (1,2,3) treated *with Xenodidymella humicola*, H (1,2,3) treated with *Aspergillus ochraceus* and I (1,2,3) treated with *Aspergillus niger*.

Plants treated with suspension cultures of endophytic fungal mat showed enhanced plant growth, exhibited healthier and greener leaves, and no visible symptoms of diseases were seen in the treated plants as compared to the control plants.

After spraying, all the treated plants of *Azadirachta indica*, *Centella asiatica* and *Abrus precatorius* were sampled and tested for secondary metabolites. Qualitative phytochemical analysis of secondary metabolites was performed using the Soxhlet extraction method with ethyl acetate. The phytochemical analysis was revealed the presence of various phytochemicals in the ethyl acetate extract of the treated plants. The presence of alkaloids was indicated by a positive result in Dragendorff's reagent, and alkaloids were present in all treated plants. The presence of Flavonoids was revealed by a lead acetate test, which gave a positive outcome, indicating that flavonoids were present in all treated plants. Phenol was detected in all treated plants, shown by the formation of a bluish-black colour. Tannins were present in all treated plants, as indicated by the formation of a white precipitate in the Gelatin test, resulting in positive results. Protein was present in all treated plants, as indicated by the formation of blue colouration in the Ninhydrin test. (Table 1, 2, 3).

Quantitative screening of phytochemicals was performed using a Spectrophotometer. Spectrophotometric scanning of the ethyl acetate extracts revealed maximum absorbance values in the range of ~661–664 nm. *Azadirachta indica* exhibited the highest absorbance in C (1,2,3), with an absorbance of 1.917λ at 662.2 nm, indicating a greater concentration of UV-absorbing secondary metabolites after treatment with *Colletotrichum gloeosporioides*. *Centella asiatica* exhibited a high absorbance value of 0.276λ at 662.2 nm in E (1,2,3), which was treated with *Alternaria alternata*, indicating the presence of phytochemicals. *Abrus precatorius* exhibited the highest absorbance with a peak of 0.337λ at 661.0 nm in G (1,2,3)**,** which was treated with *Xenodidymella humicola*.

Histochemical analysis was performed to detect the presence and localisation of various secondary metabolites in the plant tissues. The metabolites investigated include Alkaloids, Flavonoids, Tannins/Phenols, and Proteins. The detection was based on colour changes upon treatment with specific histochemical reagents. A golden colouration with Dragendorff's reagent suggested the presence of alkaloids. Histological staining using Vanillin revealed red colouration. The blue-green colour produced with 10% Aq. FeCl3 indicated the presence of phenolic compounds and tannins. Tissues treated with Bromophenol blue showed a distinct blue colouration, indicating the presence of proteins

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