Anatomical characterization, Histochemical localization and Bioactivity studies of *Gymnacranthera canarica*: a threatened species of Myristica Swamp

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PREFACE

In the vast tapestry of our natural world, there are hidden treasures waiting to be discovered, each offering valuable insights into the intricate web of life. Among these treasures are rare and threatened species that hold immense significance for the ecosystems they inhabit. *Gymnacranthera canarica*, nestled within the enigmatic Myristica swamp, is one such species deserving of our attention and study.

My research endeavour sets out to unknot the mysteries surrounding *Gymnacranthera canarica* through a comprehensive exploration of its anatomy, chemical composition, and potential bioactivity. By delving into the intricate details of this species, we aim to uncover its structural attributes, identify tissue-specific metabolites, and investigate any bioactive compounds it may harbour.

The study of *Gymnacranthera canarica* is not merely an academic pursuit; it is a quest to understand the role of this species within its ecosystem and its potential contributions to human health and well-being. Through meticulous examination and analysis, we hope to shed light on the ecological significance of *Gymnacranthera canarica* and explore its pharmacological potential.

We embarked on this journey of discovery to unravel the secrets of *Gymnacranthera canarica* and unlocking the wealth of knowledge it holds. Through our collective efforts, we aim to deepen our understanding of this remarkable species and pave the way for its conservation and sustainable utilization.

May this study serve as a beacon of knowledge, guiding us towards a greater appreciation of the natural world and the invaluable resources it provides.

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ABBREVATIONS

Entity	Abbrevations
Potassium Iodide	I_2KI
Ferric chloride	$FeCl_3$
Millilitre	mL
grams	g
Revolutions per minute	rpm
microlitre	µL
molar	M
Thin layer chromatography	TLC
nanometers	nm
Transverse section	T.S
epidermis	ep
epiblema	eb
Secretory cells	sc
Trichome	Tr

ABSTRACT

Myristica swamps, unique tropical wetland ecosystems found in the southern Western Ghats, harbour diverse and rare biodiversity. These ecosystems, located primarily in Goa, Kerala, and Karnataka, are characterized by the dominance of Myristica trees from the genus Myristicaceae. This study focuses on *Gymnacranthera canarica*, the sole species of *Gymnacrathera* in India, exclusively found in Myristica swamps. Our research encompasses anatomical characterization, histochemical analysis, and bioactivity studies of *Gymnacranthera canarica*. Thin freehand sections of various plant parts were analyzed using staining techniques to localize primary and secondary metabolites. Results revealed the presence of diverse metabolites within the plant tissues. Additionally, methanolic and distilled water extracts from the leaves and bark of *Gymnacranthera canarica* exhibited antibacterial activity against bacterial strains. Phenolic content was found to be higher in leaf extracts (211.24 ± 0.052 mg GAE/g) compared to bark extracts (164.51 ± 0.071 mg GAE/g) in methanolic extracts, also in distilled water Leaf extract showed higher phenolic content (181.83 ± 0.046 mg GAE/g) than Bark extracts (154.56 ± 0.002 mg GAE/g). Total flavonoid content was higher in methanolic leaf extracts (26.80 ±0.063 mg QE/g) and bark extracts (26.70 ± 0.048 mg QE/g), respectively. This study sheds light on the ecological significance and pharmacological potential of *Gymnacranthera canarica*, highlighting the importance of conserving Myristica swamps and their unique biodiversity.

CHAPTER 1: INTRODUCTION

1.1. MYRISTICA SWAMPS

Myristica swamps represent a distinctive ecosystem within the Western Ghats, characterized by the prevalence of tree species from the Myristicaceae family as well as a variety of rare plant and animal species (Sahib, 2017). The discovery of Myristica swamps dates back to 1960 in Travancore, establishing their presence in the Western Ghats across Kerala, Karnataka, Goa, and Maharashtra (Ranganathan, 2020). Particularly noteworthy is the physiological adaptation of plant species, notably members of the Myristicaceae, which have developed specialized aerial roots to facilitate anaerobic respiration in response to the oxygen-deficient soil of Myristica swamps. These ecosystems play a crucial role in recycling nutrients, purifying water, replenishing groundwater, regulating stream flow, and providing a diverse habitat for flora and fauna. Furthermore, they offer essential resources such as food, fodder, fuel, fiber, and a plethora of medicinal plants (Sahib, 2017).

The members of the Myristica swamps are significant in terms of their conservational status. A total of 49 species are classified under different threatened categories by the IUCN. Among these species, 12 are deemed endangered, 5 are classified as critically endangered, and 8 are considered vulnerable. Furthermore, 19 species fall under the category of least concern, while 3 species are designated as near threatened, and 2 species lack sufficient data.

1.1.1. Family: Myristicaceae

The Myristicaceae family, also recognized as the "Nutmeg family" within the order (Magnoliales), is predominantly renowned for the aromatic and pungent seeds of nutmeg. Most of its species are characterized by aromatic wood and leaves. These plants are constituents of the magnoliid order Magnoliales (APG IV 2016), comprising 21 genera and

nearly 500 species of arboreal trees, shrubs, and on occasion, climbing vines (such as *Pycnanthus sp*.).

Myristica species represent the most rudimentary among the flowering plants found on the planet as stated by Krishnamoorthy (1960). The leaves display an alternate arrangement, often appearing distichous or sometimes falsely whorled, with occasional pellucid punctation and devoid of stipules; their blades are simple and undivided. The hairs are uniseriate, branching, frequently taking on a stellate or T-shaped form. The inflorescences are either paniculate or fasciculate-racemose. The flowers are typically small, actinomorphic, and may be funnel-shaped, campanulate, or urceolate, exhibiting colors ranging from yellowish-white, yellow, pink, to red, and occasionally emitting fragrances. The perianth consists of a single row of (2-)3(-5) tepals that are basally fused, often fleshy, and valvate. Male flowers are characterized by 2-40 stamens, with their filaments partially or completely fused; the anthers are frequently united with oblong or rounded pollen sacs that often have septa, positioned outwardly, and open through longitudinal slits. Female flowers are monocarpellate, with a superior ovary that is either sessile or occasionally short-stipitate; the style may be distinct or absent, the stigma usually ± bilobed, and the ovule singular, located sub-basally to basally, either anatropous, rarely orthotropous or hemi-orthotropous, and bitegmic. The fruit is typically a fleshy, coriaceous, or woody capsule that splits open on both the ventral and dorsal sides. The seed-coat is woody, typically covered predominantly with a crustaceous to fleshy, laciniate, or whole aril. Endosperm commonly displays rumination, and the embryo is generally diminutive (Kuhn & Kubitzki, 1993).

1.1.2 *Gymnacranthera canarica* (Bedd. ex King) Warb.

It is a member of the Myristicaceae family, commonly referred to as 'Kanara' Nutmeg. This particular tree species (IUCN, 2002) is at risk and is typically found in marshy environments within the Western Ghats, which is recognized as a significant area of biodiversity in India (Myers *et al*., 2000).

a. Scientific classification

Kingdom : Plantae

Phylum : Tracheophyta

Class : Magnoliopsida

Order : Magnoliales

Family : Myristicaceae

Genus : *Gymnacranthera*

Species : *Gymnacranthera canarica* (Bedd. ex King) Warb.

b. Origin and Distribution

Fresh water swamps occur in different parts of the world such as the valleys of Mississippi and its tributaries, in Sweden, Odzala National Park, Congo, in the Malaysian region, Papua New Guinea etc. In India fresh water swamps are reported from the Siwalik, The Doon valley, Brahmaputra valley (Rao, 1994). Western ghats are one of well-known global centres of Biodiversity, endemism and it is a home to this enigmatic Myristica swamps.

(Chandran. & Mesta, 2006). This includes states such as Goa, Karnataka, Kerala and recently reported in Maharashtra. (Sreedharan & Indulkar, 2018).

c. Botanical Description

The tree is dioecious in nature and possesses knee roots. Its leaves are lanceolate, measuring 9–22 × 3.5–7.5 cm, with an acute-obtuse base, entire margins, and an acuminate-cuspidate apex. The inflorescence is axillary and paniculate, with a branched cymule bearing many flowers at different developmental stages, characterized by a puberulous nature. Male flowers are globose-ellipsoid with 3 or 4 valves that split the perianth in anthesis to the base; their lobes are involute and puberulous inside, and the synandrium is ellipsoid with an androphore around 0.5 mm long. Stamens range from 5 to 15, with linear anthers that are slightly unequal, adnate to their back up to 1 mm from the base, and incurved-spreading at anthesis, extrorse. Female inflorescences are racemose-paniculate and contracted, with ellipsoid-obovoid flower buds measuring 2–3 × 1–1.5 mm, villous inside, tomentose outside, brown, and scented. The stigma is sessile and 2-lobed, while the fruits are globose with a pointed apex, measuring 2.4–2.6 cm; the pericarp is 1.5–2 mm thick, wrinkled, glabrous-puberulous, and brown. The aril is laciniated almost up to the base with a linear pointed structure, yellow, and the seeds are globose, blunt at ends, with a persistent collar-like structure at the base. (Banik *et al.*, 2017)

d. Economic Significance

Economically, the swamps hold little significance. The primary economic resource derived from these swamps is the aril of the fruit belonging to the Myristicaceae family. The aril of *Myristica fatua var. magnifca*, *Gymnacranthera canarica*, and *Myristica malabarica* serves as a vital raw material in the paint industries. Oil obtained from the seeds of these

species is employed in varnishes and the production of veterinary medicine. Additionally, seeds are used to make wax candles.

1.1.3. Importance of Plant Anatomy and Histochemistry

Plant anatomy, a branch of botanical science, delves into the intricate structures of plant tissues and cell formations within plant organs. It involves the examination of these structures under high-powered microscopes or electron microscopes. Macroscopic evaluation and comparative anatomy reveal a vast diversity in both macro and microscopic features among different plant parts. Understanding plant anatomy is crucial in comprehending plant biology holistically. It provides the foundation for realistic interpretations of plant morphology, physiology, and phylogeny. By thoroughly understanding the structure of cells and tissues, researchers can make informed deductions about various aspects of plant life (Esau, 1953).

Moreover, plant anatomy plays a significant role in addressing practical challenges in various fields. It aids in the identification of unknown plant specimens, detection of food contaminants, and even contributes to solving forensic problems. In the context of this study focusing on *Gymnacranthera canarica*, plant anatomy takes on added significance. By examining the anatomical features of *Gymnacranthera canarica*, researchers can gain insights into its unique structural adaptations and physiological processes. This knowledge is essential for understanding the ecological niche of *Gymnacranthera canarica* within the Myristica swamp ecosystem and its potential pharmacological properties.

In this study, histochemical techniques, including the use of metachromatic stains like Toluidine blue O, are employed. These techniques allow for the visualization of various cell wall components, aiding in the identification of different cell and tissue types. Additionally, histochemical staining of compounds such as lignin, lipids, and starch will

provide further insights into the biochemical composition of *Gymnacranthera canarica* tissues.

Histochemistry combines biochemistry and histology to analyze tissue chemistry. Special stains label tissue components, aiding in the identification of various compounds within plant cells. This technique is valuable for understanding physiological functions, such as enzyme localization, and studying plant development and differentiation. Additionally, histochemistry is useful for investigating plant diseases, providing insights into pathological processes through changes in compound distribution or accumulation in diseased tissues (Kiernan, 2015).

1.1.4. Phenolic Compounds and Flavonoids: Biochemical Arsenal of Nature

Phenolic compounds are aromatic substances with one or more hydroxyl groups. There are around 8000 naturally occurring plant phenolics, with half of them being flavonoids. These compounds exhibit various biochemical activities, including antioxidant and anticarcinogenic properties, and can influence gene expression (Cheynier, 2012). Flavonoids, the largest group of phenolic compounds, are found in plants in both free and glycoside forms. They possess diverse biological activities, such as antimicrobial, antiulcer, and anticancer effects. Flavonoids consist of two benzene rings separated by a propane unit, with flavones and flavonols being the most common types. They act as antioxidants, providing protection against cardiovascular disease, cancer, and age-related cellular degeneration (Suliman & Indira, 2012)

1.1.5. Plant-derived Antibacterials

The rise of drug-resistant microbial pathogens poses a formidable challenge to combatting infectious diseases effectively. In response, plant-derived antibacterials offer promising therapeutic potential, exhibiting efficacy in treating infections while minimizing the side effects associated with synthetic antimicrobials (Kokoska, 2019). Screening plant extracts for antimicrobial activity has unveiled the capacity of higher plants to yield novel anti-infective compounds (Press, 1996). Various plant parts, including bark, leaves, flowers, and fruits, harbor natural antimicrobials that hold promise for pharmaceutical applications (Gordon and David, 2001). Even before the discovery of microbes, there was speculation regarding certain plants possessing inherent antimicrobial properties, underscoring their potential as valuable sources of antimicrobial agents.

Plants produce an extensive array of biologically active metabolites, categorized as primary and secondary metabolites (Appelzweig, 1980). Secondary metabolites, albeit present in smaller quantities compared to primary metabolites, exert potent biological effects and hold promise as therapeutic agents against drug-resistant bacteria (Dorman & Deans, 2000).

Alkaloids, tannins, flavonoids, saponins, glucosides, and other secondary metabolites exhibit broad-spectrum antibiotic properties, making them valuable resources in the fight against infectious diseases (Firas et al., 2008). Woody plants, in particular, are adept at synthesizing and accumulating a diverse range of secondary metabolites, including low molecular weight phenolics such as hydroxybenzoic and hydroxycinnamic acids, acetophenones, flavonoids, and lignans (Harborne, 1964).

Thus, the present investigation was carried out to examine its anatomical features, tissue-specific metabolites, and bioactivity potential of *Gymnacranthera canarica* plant.

1.2 AIMS AND OBJECTIVES

1. Conduct a comprehensive anatomical analysis of *Gymnacranthera canarica* plant organs to discern and document intricate structural nuances and variations.
2. Employ histochemical staining techniques to precisely localize, identify, and map tissue-specific primary and secondary metabolites across diverse tissues of *Gymnacranthera canarica*.
3. Explore the bioactivity potential of distinct plant parts of *Gymnacranthera canarica*

Based on the above objectives; the anatomical characterization, histochemical localization, and bioactivity studies of *Gymnacranthera canarica* (Bedd.ex King) Warb .plant is expected to reveal unique structural features, chemical constituents, and biological properties. These findings are expected to provide valuable insights into the species' status as an exclusively threatened species within Myristica swamps.

1.3 HYPOTHESIS

The anatomical investigation will uncover distinctive morphological features and tissue arrangements in *Gymnacranthera canarica*, while histochemical analysis will pinpoint the presence and distribution of specific metabolites within different plant tissues. Furthermore, it is hypothesized that the bioactivity studies will unveil the therapeutic potential of *Gymnacranthera canarica* extracts against microbial pathogens, contributing to its significance in traditional medicine and biodiversity conservation efforts.

1.4 SCOPE

- Investigate the bioactive compounds identified in *Gymnacranthera canarica* to elucidate their specific pharmacological properties, potentially through in vitro and in vivo studies, to uncover the plant's medicinal uses.
- Explore the biotechnological potential of *Gymnacranthera canarica*, including tissue culture techniques for mass propagation, genetic engineering for enhanced bioactive compound production, and metabolic engineering for specific compound synthesis.
- Conduct comparative anatomical studies with closely related species in Myristica swamps to gain insights into evolutionary relationships and ecological adaptations.

CHAPTER 2: REVIEW OF LITERATURE

Gymnacranthera canarica. is a unique and ecologically important habitat found in western ghats of India. It is frequently studied for its ecological significance and potential medicinal properties.

2.1. BACKGROUND

Tambat *et al*., (2006) investigated the germination of *Gymnacranthera canarica* seeds and identified that despite a high seed viability of 98%, the initial germination rate was only 40%, indicating potential dormancy issues in the species. They had applied 14 treatments among which seed coat removal alone increased germination by 20% to 60%. Also, the application of GA_3 at different concentrations revealed that 500 ppm concentration showed inhibitory effects which resulted in 10% germination.

Tambat *et al*., (2007) reported the existence of abnormal seeds in *Gymnacranthera canarica*. Although no significant differences were found between normal and abnormal seeds, the absence of germination in abnormal seeds may suggest genetic viability issues. They suggested further investigations to comprehend the causal factors behind seed abnormality.

Baby Sabulal *et al*., (2007) explored the chemical composition of leaf oils from *Myristica malabarica* and *Gymnacranthera canarica*. Their analysis revealed distinct constituents in the leaf oils of both plants. *Myristica malabarica* displayed 76 constituents, dominated by β-caryophyllene (27.3%), α-humulene (13.8%), and α-copaene (11.5%), whereas *Gymnacranthera canarica* exhibited 76 constituents, primarily β-caryophyllene (23.4%), linalool (13.4%), and α-humulene (11.3%). Sesquiterpene hydrocarbons

constituted 77.3% and 58.1% of leaf oil composition in *M. malabarica* and *G. canarica*, respectively.

Keshavachandra & Krishnakumar (2016) studied the viability, germination, and storage behavior of *Gymnacranthera canarica* seeds. They observed that these seeds germinated after two weeks with an initial moisture content. Their desiccation study revealed recalcitrant behaviour of seeds, allowing storage under lab conditions for up to two and a half months.

Salleh *et al*., (2017) focused on the diverse biological activities of Knema and its active principles, showcasing properties such as antibacterial, anti-nematodal, anti-inflammatory, cytotoxicity, and acetylcholinesterase inhibitory activities.

Anusha *et al*., (2019) conducted a pharmacological evaluation of the methanolic extract of *Gymnacranthera canarica* seeds. Their study revealed antimicrobial properties against both bacteria and fungi. The seeds displayed significant antidiabetic potential in α-amylase inhibitory assays,

Tambat *et al*., (2019) assessed the impact of disturbance on swampy species' wood specific gravity and fiber length. Through increment borer wood core samples collected from various locations in the central Western Ghats, they observed a decrease in specific gravity with increased swamp disturbance across all six locations. The frequency distribution of specific gravity values was positively skewed for less disturbed swamps but negatively skewed for highly disturbed swamps. Similarly, fibre length exhibited a similar pattern.

Anusha *et al*., (2022) evaluated the physiological and biochemical responses of *Gymnacranthera canarica* seeds to different desiccation treatments. Their findings indicated

the seeds' high sensitivity to desiccation, leading to total viability loss within 15 days. Desiccation amplified malondialdehyde and electrolyte leakage while reducing formazan formation. Seed desiccation also increased protease activity, proline, fat, sucrose, and total soluble carbohydrates while reducing the quantity of phenol starch. The early viability loss was linked to the loss of membrane integrity, associated with the formation of reactive oxygen species (ROS) and resultant lipid peroxidation products, signifying the seeds' truly recalcitrant nature.

Venkatesh *et al*., (2019) explored the comparative responses of obligate swampy and non-swampy species of the Myristicaceae family concerning wood growth in various Western Ghats locations. Their analysis revealed variations in wood growth across locations for both swampy and non-swampy species. Geographically closer locations exhibited similar growth patterns, indicating the pivotal role of climatic factors in wood growth. *Gymnacranthera canarica*, a swampy species, displayed higher growth (diameter) compared to non-swampy species, while the specific gravity was higher in the non-swampy species.

Dalvi *et al*., (2021) studied Northern Most Myristica Swamp Ecosystem in Western Ghats at Bambarde- Hewale, Maharashtra, India. Their study focused on the recently discovered Myristica swamp in Maharashtra state

2.2. Anatomical characterization & histochemical investigation

Fabio *et al*., (2013) conducted a study on seed anatomy utilizing histological techniques and microscopy. Their findings revealed a multi layered testa comprising exo-, meso-, and endotestas, exhibiting longitudinal and oblique fibres. The embryo was observed as small and straight with a moderately developed embryonic axis, rudimentary plumule, and flat, thin cotyledons showcasing the procambium.

Fernandes & Sellappan (2019) presented a comprehensive study focusing on the stem, leaf, and petiole anatomy of ten *Strobilanthes* species from the northern Western Ghats of India. Their research aimed to identify distinguishing characteristics facilitating species identification, especially when flowering material was unavailable.

Parimala & Amerjothy (2013) provided a comprehensive examination of *Myristica fragrans Houtt.* fruit microscopic details and the histochemical localization of aroma compounds. Their study also delved into identifying gaps in previous investigations.

Verenkar & Sellappan (2020) studied the effects of natural dyes *Curcuma longa* (rhizome) and *Nyctanthes arbor-tristis* (corolla tube) on various plant tissues through fluorescence microscopy. Their findings showcased autofluorescence in some tissues of unstained monocot and dicot stem sections.

Kadam *et al*., (2013) and Kadam (2019) conducted studies involving freehand sections of leaves and wood treated with specific reagents to localize components like starch, protein, tannin, saponin, fat, glucosides, and alkaloids within the tissues.

Meesawat (2019) investigated specific features, including idioblasts in leaves of varying ages in *A. muricata*. They revealed distinct histological differences among leaves of different ages, delineating the presence of subspherical idioblasts and calcium oxalate crystal-containing idioblasts. The study detected carbohydrates and fat across all leaf stages, with higher concentrations observed in the first leaf.

Ergin *et al*., (2022) studies the essential oils of the aerial parts, flower, and fruit of *Hypericum scabrum* which was characterized by the presence of monoterpene hydrocarbons, whereas roots oil include alkanes. The GC-FID and GC-MS analysis showed

that major components of roots, aerial parts, flowers, and fruits oils were undecane, α-pinene, γ-terpinene and α-thujene; α-pinene, α-thujene, and γ-terpinene, α-pinene.

2.3. Bioactivity studies

Simamora *et al*., (2018) investigated Mace extract using methanol and ethyl acetate, analyzing their chemical composition via GC–MS. They assessed antibacterial, antioxidant, and α-glucosidase inhibitory activities. Both extracts showcased four major components: sabinene, methoxyeugenol, myristicin, and elemicin. The methanol extract (ME) exhibited higher levels of methoxyeugenol, myristicin, and elemicin, while sabinene dominated in the ethyl acetate extract (EAE). Both extracts displayed notable antibacterial activity against *S. aureus* and exhibited strong antioxidant activities, with EAE demonstrating higher activity than ME.

Al-Qahtani *et al*., (2022) identified 23 phytoconstituents in *Myristica fragrans* through Gas Chromatography-mass spectrometry (GC–MS), with elemicin being the major constituent (24.44%). They conducted antioxidant studies using elemicin, revealing its antibacterial and antifungal activities against various strains, showcasing its bioactive potential.

Verma *et al*., (2021) investigated the antioxidant, antimicrobial, and anti-inflammatory activity of hydrolats and essential oil obtained through hydro-distillation of *Myristica fragrans* seeds.

Sulaiman & Balachandran (2012) focused on the total phenolics and total flavonoids content of the medicinal plants of various species based on the strong evidences of the biological activities of phenolic compounds.

Bachir & Benali (2012) examined the antimicrobial activities of the Water-distilled extracts of *E. globulus* leaves on two clinically significant microorganisms, *E. coli* and *S. aureus* by means of the agar diffusion test and dilution broth method. Various studies have highlighted the potential of plant extracts in combating microbial infections. These findings collectively support the exploration of wild plant extracts for their antimicrobial properties, underscoring their potential in developing novel antibiotics and antifungal agents.

Inampudi & Sailaja (2012) focused on evaluating the antifungal an antibacterial activities of wild plant extracts.

Ali *et al*., (2022), found that *Ilex dipyrena* was found to be good inhibitor of free radicals and lipoxygenase that could be further investigated to isolate compounds of medicinal importance.

Naik & Sellappan (2020), focused on the essential oils of various plant organs of *Annona muricata* using TLC and HPTLC they revealed the presence of Nine essential oils. Among Nine, three essential oils were uniquely identified in seeds.

Jesionek (2015) analyzed 10 phenolic compounds from extracts of five different plant and separated using optimised thin layer chromatography at normal phase mode, visualized using natural products/Polythene Glycol reagent. Antioxidant Properties of components of the extracts were assessed using directly using TLC- DPPH

CHAPTER 3: MATERIALS AND METHODS

3.1 Collection of Plant Material

Plant samples of *Gymnacranthera canarica* (Bedd. ex King) Warb. were collected from the study site located at Nirankarachi Raai, Valpoi Sattari, North Goa, India, during the study period from 2023 to 2024. Mature and healthy plant parts were carefully selected, collected in separate zip-lock polythene bags, and transported to the laboratory for further studies. GPS coordinates of the collection site were recorded as follows: Latitude 15.586434°N, Longitude 74.1904°E. These plant specimens were used for anatomical, histochemical, and bioactivity studies in this research.

Table 3.1. Collection site of the selected plant.

Sr. No.	Name of the plant	Location	Coordinates
1.	*Gymnacranthera canarica*	Nirankarachi Raai, Valpoi Sattari-North Goa	15.586434˚N &74.1904˚E

3.2. Study of Anatomical characterization

Systematic anatomical characterization of the collected plant parts, including leaves, stems, and roots, was conducted. Thin free-hand sections of fresh leaf, stem, and root were prepared using a sharp razor blade. The sections were stained with 1% safranin for 2-3 minutes and mounted on slides with glycerine. Additionally, sections were double-stained by initially staining with 1% safranin for 2 minutes, followed by washing with distilled water and de-staining with a few drops of 70% ethanol to remove excess stain. Subsequently, the sections were thoroughly washed with water and stained with Toluidine Blue-O (0.1%) for 1 minute. After washing with water, the sections were mounted on clean slides with

glycerine. Stained and unstained sections were examined under a bright-field microscope, and images were captured using a Nikon Eclipse E200 camera fitted to the microscope.

Preparation of 1% safranin stain: 1% safranin stain was prepared by dissolving 1g of safranin in 100 mL of distilled water. The solution was then filtered and stored in a reagent bottle for future use.

Preparation of 0.1% Toluidine blue O stain: 0.1% Toluidine blue O stain was prepared by dissolving 0.1g of Toluidine blue O in 100 mL of distilled water. After filtering the solution, it was stored in a reagent bottle for further use.

3.3 Study of Histochemical Localization

Histochemical tests were performed on the fresh thin hand sections of leaf, stem, root of plant *Gymnacranthera canarica.* Thin free hand sections were placed in specific stains for the histochemical localization of Primary and secondary metabolites.

3.3.1 Localization of Primary metabolites

a. Localization of Starch

Fresh free-hand sections of various plant parts of *G. canarica* were immersed in distilled water and subsequently stained with Iodine Potassium Iodide (I_2KI) solution for a duration of 15 minutes. Following staining, the sections were rinsed with distilled water and mounted in glycerine. Observation of the stained sections was conducted under a light microscope, where the presence of purple to black coloration indicated the presence of starch (Krishnan *et al*., 2001).

b. Localization of Lipids

Free hand sections were placed in 50% ethanol followed by staining in Sudan IV for 15-20 minutes. Sections were rinsed using 50% ethanol and mounted in 10% freshly prepared glycerine and observed under light microscope. Red colour indicated the presence of lipids Krishnan *et al.*, (2001).

c. Localization of Proteins

Fresh free hand sections were taken and stained in Coomassie Brilliant blue for 15-20 minutes, rinsed in Clark's solution (1 part of glacial acetic acid and 3 parts of absolute ethanol) and mounted in glycerine and observed under bright field microscope. Blue colour indicated the presence of proteins (Krishnan *et al.*, 2001).

3.3.2 Localization of secondary metabolites

a. Localization of Alkaloids

Fresh free-hand sections were obtained and subjected to staining with Dragendorff reagent, followed by rinsing with 5% sodium nitrite solution to eliminate excess stain. The stained sections were then examined under a light microscope, where the presence of a yellow to reddish-orange coloration indicated the presence of alkaloids (Kadam, 2014).

Preparation of Dragendorff's reagent

Stock solution: 5.2 g of Bismuth carbonate and 4 g of sodium iodide were boiled with 50 mL of glacial acetic acid for several minutes. After 12 hours, the resulting sodium acetate crystals were filtered using a funnel. The clear red-brown filtrate, measuring 40 mL, was then combined with 160 mL of ethyl acetate and 1 mL of water, and the mixture was stored in an amber-colored bottle.

Working solution: 10 mL of stock solution was mixed with 20 mL of acetic acid and made up to 100 mL with water.

b. Localization of phenolic compounds

Ferric chloride method

Fresh free-hand sections were taken and placed in distilled water. The sections were then transferred to 10% formalin solution containing 2% ferric chloride (in a 1:1 volume ratio). Afterward, the sections were washed with water to remove excess stain. Finally, the stained sections were mounted with dilute glycerine and observed under a bright field microscope. Presence of grey to black coloration indicated the presence of phenolic compounds.

Preparation of 10% formalin: 10 mL of Formalin was taken and 90 mL of Distilled water was added.

Preparation of 2% Ferric chloride: 2g of ferric chloride was added to 100 mL distilled water.

3.4 Bioactivity Studies

3.4.1 Preparation of Plant Extracts

Plant parts such as leaves, seeds, and bark samples of *G. canarica* were collected and washed thoroughly with distilled water to remove any impurities. Subsequently, they were shade dried and ground into a fine powder. For extraction, 10 g of each powdered sample was accurately weighed and separately mixed with 100 mL of methanol and distilled water. These mixtures were then transferred into an incubating shaker and allowed to shake for 2 days at 120 rpm and room temperature. After the incubation period, the extracts were

filtered using filter paper No. 1 to remove any solid particles, and the filtrates were stored at 4˚C until further use. To concentrate the filtered extracts, a rotary evaporator was employed at a temperature of 50˚C. The concentrated extracts were then transferred and stored in glass vials for subsequent analyses. **(Plate. 4.32)**

3.4.2 Total Phenolic Constituents studies

The total phenolic content was determined using the Folin-Ciocalteau reagent method described by Singleton *et al.,* (1965). For analysis, dry powdered plant samples and their corresponding distilled water and methanolic extracts were prepared. First, 5 mL of Folin-Ciocalteau reagent was added to each sample in the dark, and the mixture was incubated for 15 minutes. After incubation, 5 mL of sodium carbonate (Na_2CO_3) solution was added to each sample in the dark, followed by another 45-minute incubation period. Subsequently, the absorbance of each sample was measured at 760 nm and 765 nm using a UV-visible spectrophotometer at room temperature. The total phenolic contents were then calculated and expressed as milligrams of gallic acid equivalents (GAE) per gram of extract. (Ethiraj *et al.,* 2023**)**

3.4.3 Total Flavonoids determination

The total flavonoid content was measured using the aluminium chloride colorimetric assay. (Chang *et al.,* 2002) To prepare the reaction mixture, 1 mg of extract was dissolved in 4 mL of distilled water and transferred to a 10 mL volumetric flask. Subsequently, 300 µL of 5% sodium nitrite solution was added to the flask, and the mixture was allowed to stand for five minutes. Afterward, 300 µL of 10% aluminium chloride solution was added, followed by another five minutes incubation period. Next, 2 mL of 1 M sodium hydroxide was added to the flask, and the volume was made up to 10 mL with distilled water. Reference standard solutions of Quercetin (100, 200, 400, 600, and 800 µg/mL) were prepared in a

similar manner as the test samples. The absorbance of both the test and standard solutions was measured against a reagent blank at 510 nm using a UV/Visible spectrophotometer. (Anusha *et al.,* 2019)

3.4.4 Thin layer chromatography (TLC) Analysis

To assess the bioactive chemicals present in the crude plant extracts, aluminium foil-backed silica gel plates measuring 10 cm × 10 cm were utilized to analyse extracts from two different parts of *Gymnacranthera canarica* plants (i.e., bark and leaves). Plant samples extracted with methanol and distilled water solvents were spotted on a single TLC plate and dried **(Table 3.2)** lists the mobile phases, conditions, and derivatizing agents used for the detection of various secondary metabolites. The spots were examined visually before and after dipping with the derivatizing agent (Naik and Sellappan, 2020).

Table 3.2: Mobile phases and Derivatizing agents used for TLC for respective secondary metabolites.

TLC Analysis			
Class of Compounds	**Mobile phase**	**Derivatisation**	**Observation**
Alkaloids	Toluene: Ethyl acetate: Methanol: ammonia 25% (30:30:15:1) v/v/v/v	Dragendorff's reagent	Light Orange spot
Phenolic compounds	Tetra hydrofuran: Toluene; Formic acid: water (16:8:2:1) v/v/v	Alcoholic Ferric chloride	Dark blue zones
Flavonoids	Tetra hydrofuran: Toluene: Formic acid: water (16:8:2:1) v/v/v	10% Methanolic Sulphuric Acid	Florescent compounds, Red Fluorescence-plant pigment- chlorophyll.
Bioautography of antioxidant potential	Toluene: ethyl acetate: chloroform: methanol (3:2:1)	5%$KMno_4$ solution	Clear whitish spots against pink purple background

3.4.5 Antibacterial studies

Antibacterial studies were done on different plant parts of *Gymnacranthera canarica* plant.

a. Sterilization of glassware and culture vials

Sterilization of equipment's and media was carried by Dry heat sterilization. The required glasswares were washed in Teepol and kept in oven for complete drying. Petriplate, pipette, test tubes were also washed and wrapped separately in paper/aluminium foils and sterilized by autoclaving at 1 lbs for 1 hour.

b. Preparation of Media

Mueller Hinton agar (Himedia) was precisely weighed and dissolved in distilled water. The mixture was gently heated and stirred in a microwave until fully dissolved. Subsequently, it was autoclaved at 15 pounds pressure for 1 hour and then allowed to cool to room temperature.

c. Evaluation of antibacterial activity

The Bacterial strains *Escherichia coli* and *Bacillus* were collected from Microbiology Department, PES College, Farmagudi, Ponda Goa. Bacterial strain subculture was done using Nutrient agar medium. Media was poured in the pre-sterilized petriplate and kept for solidification. Loopful of the culture from the slants was taken and streaked on the plate to obtain fine isolated colonies. Streaked Bacterial strain petriplate were kept for incubation at room temperature for 24 hours.

d. Preparation of Bacterial suspension:

Bacterial suspension was prepared **i**n laminar air flow by transferring two loop full of stock culture of respective bacterial strains to 10 mL of sterile saline sodium chloride solution in conical flask. The suspension was incubated at low temperature in refrigerator for 24 hours.

Using subculture strains, antibacterial study was carried out by the following method:

1) Disc Diffusion Method

For antibacterial study, 200 µL swab of bacterial suspension was evenly spread onto petriplate containing Mueller Hinton Agar. Subsequently, sterile filter paper discs, each with a diameter of 7 mm and impregnated with plant extracts from both bark and leaf samples, were carefully positioned onto the culture plates. These prepared plates were then incubated at 37˚C for a duration of 24 hours. Following the incubation period, the antibacterial activity was assessed by observing the presence of clear inhibition zones surrounding the discs on the agar plates.

CHAPTER 3: RESULTS AND DISCUSSION

4.1 Collection and identification of Plant

Myristica swamps are critically endangered ecosystem and *Gymnacrathera canarica* is an exclusive species of this swamps which is endemic to western ghats. It is known to possess various bioactive compounds, hence to study more about its anatomy, Histochemistry and its bioactivity, plant sample was collected from Nirankarachi Raai, valpoi, sattari, North-goa. **(Table 4.2 and Plate 4.1, Plate 4.2).** Various field trips were carried out at different places to identify and locate Myristica swamps. **(Table 4.1).** It was observed that abundant of *G. canarica* plants were present at Nirankarachi Raai than other places hence we selected this site for further studies. Taxonomic Identification was done using herbarium sheets from Herbarium of Botany Discipline, SBSB, Goa University and was verified by Dr. Mandar Datar, Scientist, Agharkar Research Institute Pune. The collected specimens were preserved in formalin-Acetic acid. Further, samples were dried and stored for further use.

Table 4.1: Locations and coordinates of Places visited.

Sr. no.	Places	Coordinates
1.	Nirankarachi Raai, Valpoi, Sattari, North-Goa	15.586434˚N &74.1904˚E
2.	Netravali wildlife sanctuary	15.084152˚N & 74.2322˚E
3.	Brahmakarmali, Valpoi, Sattari, North-Goa	15.33874˚N & 74.10378˚E

Table 4.2. Name of selected plant species and location

Sr. No.	Botanical Name	Place of collection
1	*Gymnacranthera canarica*	Nirankarachi Raai, Valpoi, Sattari, North-Goa

4.2 Anatomy of different plant parts of *Gymnacranthera canarica*

The study of the transverse sections leaf, petiole, root, stem, Midrib of *Gymnacranthera canarica* plant stained with safranin and combination of safranin, Toluidine blue O revealed the following features:

4.2.1. T.S. of Leaf

In the transverse section of the leaf, the epidermal layer exhibited a highly cuticularized cell wall. Both the abaxial and adaxial sides of the leaf displayed a uniseriate palisade layer comprising rectangular epidermal cells. Additionally, the mesophyll tissue appeared well-developed, consisting of two layers: a compactly arranged cylindrical palisade parenchyma on the adaxial surface, and a loosely arranged irregular spongy parenchyma with intercellular spaces on the abaxial surface. Chloroplasts were prominently present in the mesophyll cells, with a higher concentration observed in the palisade cells compared to the spongy parenchyma cells. This anatomical arrangement likely facilitates efficient photosynthesis in *Gymnacranthera canarica* leaves. **(Plate. 4.3)**

4.2.2. T.S. of Petiole

The petiole was horse footprint shaped. The transverse section of petiole was concave at the adaxial side and convex at the abaxial side. The epidermis was covered with

thin layer of cuticle with minute multicellular trichomes. The cortex region consisted of thick collenchyma cells.. Vascular bundles were arranged in small arcs, contributing to the petiole's structural integrity and support. **(Plate. 4.4), (Plate. 4.8)**

4.2.3 T.S. of Midrib

In the transverse section of the midrib, a thin layer of cuticle was evident on both the adaxial and abaxial sides. Vascular bundles were arranged in a boomerang-shaped arc, with the elbows facing the abaxial side and the edges facing the adaxial side. Lignified cells were observed within the tissue. Additionally, compactly arranged patches of sclerenchymatous cells were found on the inner portion of the elbow region of the xylem cells. Further, 3-4 patches of sclerenchyma cells were observed cushioning the xylem cells towards the abaxial surface. **(Plate. 4.5), (Plate. 4.9)**

4.2.4 T.S. of Stem

The stem exhibited a circular outline with a thin cuticle covering its surface. The epidermis comprised a single layer of sub-rectangular cells, devoid of trichomes. Beneath the epidermis, the hypodermis was observed, consisting of collenchymatous cells. The pith cells were oval in shape and compactly arranged without any intercellular spaces. In addition to the surface features, the stem structure also revealed internal characteristics. The epidermis, being the outermost layer, serves as the primary barrier against environmental stressors. Its single-layered arrangement of sub-rectangular cells contributes to structural integrity. The absence of trichomes, which are often involved in functions like protection and moisture retention, suggests alternative mechanisms for environmental adaptation in *Gymnacranthera canarica*.

Below the epidermis, the hypodermis comprised collenchymatous cells, which provide mechanical support to the stem. This structural reinforcement likely aids in maintaining the stem's rigidity and resistance to bending forces. Furthermore, the compact arrangement of oval-shaped pith cells, without intercellular spaces, suggests efficient resource allocation and mechanical stability.

The observed structural features of the stem in *Gymnacranthera canarica* align with its habitat requirements and ecological niche. These plants are typically found in marshy swamps, and their stem anatomy reflects adaptations to this environment. The circular outline and thin cuticle of the stem indicate adaptations for efficient resource utilization and environmental resilience. The absence of trichomes may reflect a strategy to reduce water loss or prevent attachment of pathogens and pests. Additionally, the presence of collenchymatous cells in the hypodermis suggests adaptation to withstand mechanical stress, such as wind or physical contact.

Understanding the stem anatomy of *Gymnacranthera canarica* provides valuable insights into its ecological adaptations and potential physiological functions within marshy swamp ecosystems. **(Plate. 4.6), (Plate. 4.10)**

4.2.5 T.S. of Root

Transverse section of the root revealed distinct structural features essential for its function and adaptation. The epiblema, composed of thin-walled flattened cells, formed the outermost layer, providing protection and regulating nutrient uptake. The absence of root hairs suggested alternative mechanisms for water and nutrient absorption, possibly through symbiotic associations or specialized root structures.

Below the epiblema lay the cortex, contributing to structural support and storage of reserve substances. The presence of a distinct cambium ring indicated potential for secondary growth and tissue differentiation, enhancing the root's longevity and resilience. Secondary phloem, vital for nutrient transport, was evident below the cambial ring.

Notably, annual rings were distinct in the xylem region, suggesting periodic growth patterns influenced by environmental factors such as seasonality or resource availability. The radially arranged vascular bundles, situated close to the pith cells, facilitated efficient transport of water, minerals, and nutrients throughout the root system. The circular-oval arrangement of compactly packed pith cells further enhanced structural integrity and resource allocation.

The observed root anatomy of *Gymnacranthera canarica* highlights its adaptation to marshy swamp habitats. The absence of root hairs may indicate adaptation to waterlogged conditions, where conventional root structures may be less effective. Instead, the flattened epiblema cells and efficient vascular arrangement likely optimized nutrient uptake and water transport, facilitating survival in these environments.

Understanding root structure and function in *Gymnacranthera canarica* provided insights into its ecological niche and potential interactions with surrounding organisms and environmental factors. Further investigation into root adaptations and their physiological significance could elucidate strategies for plant resilience and resource acquisition in marshy swamp ecosystems. Comparative studies with related species might also reveal commonalities or unique adaptations among plants inhabiting similar habitats. **(Plate. 4.7), (Plate. 4.11)**

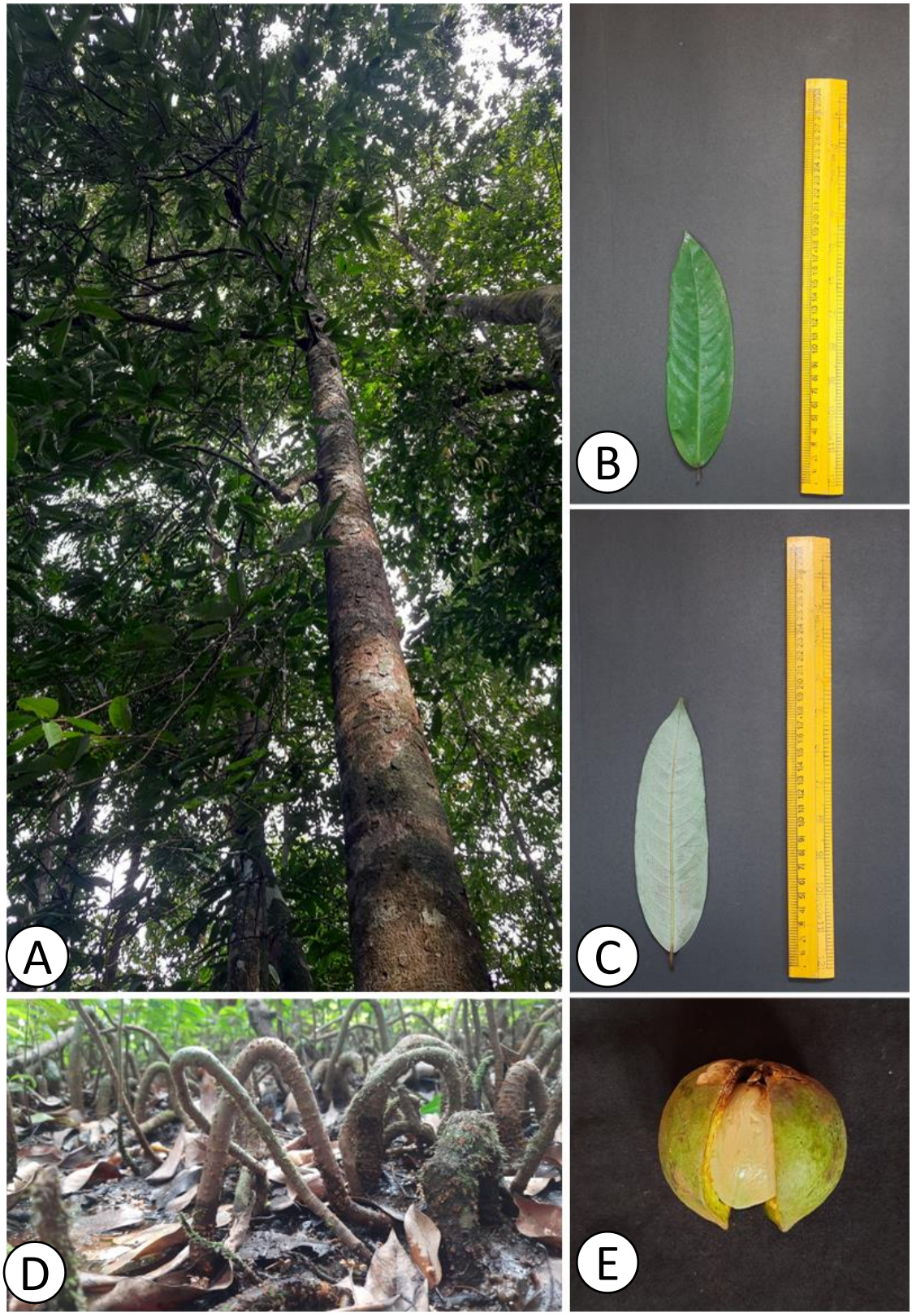

Plate 4.1: Habit of *Gymnacranthera canarica* (A) Habit, (B) Leaf-Adaxial side (C) Leaf -Abaxial side, (D) Knee Roots (E) Fruit

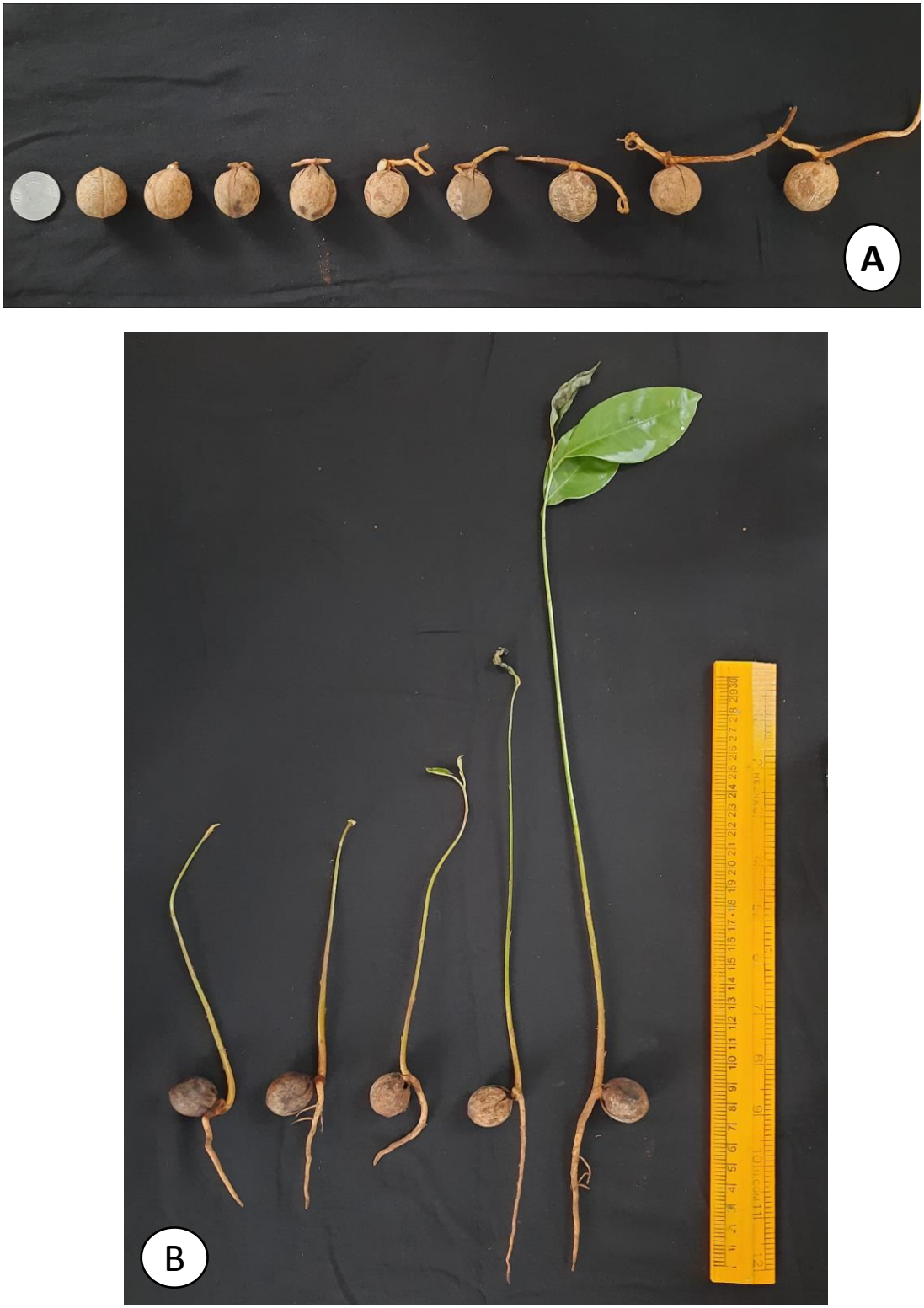

Plate 4.2: *Gymnacranthera canarica* (A) and (B) Different stages of Seed Germination

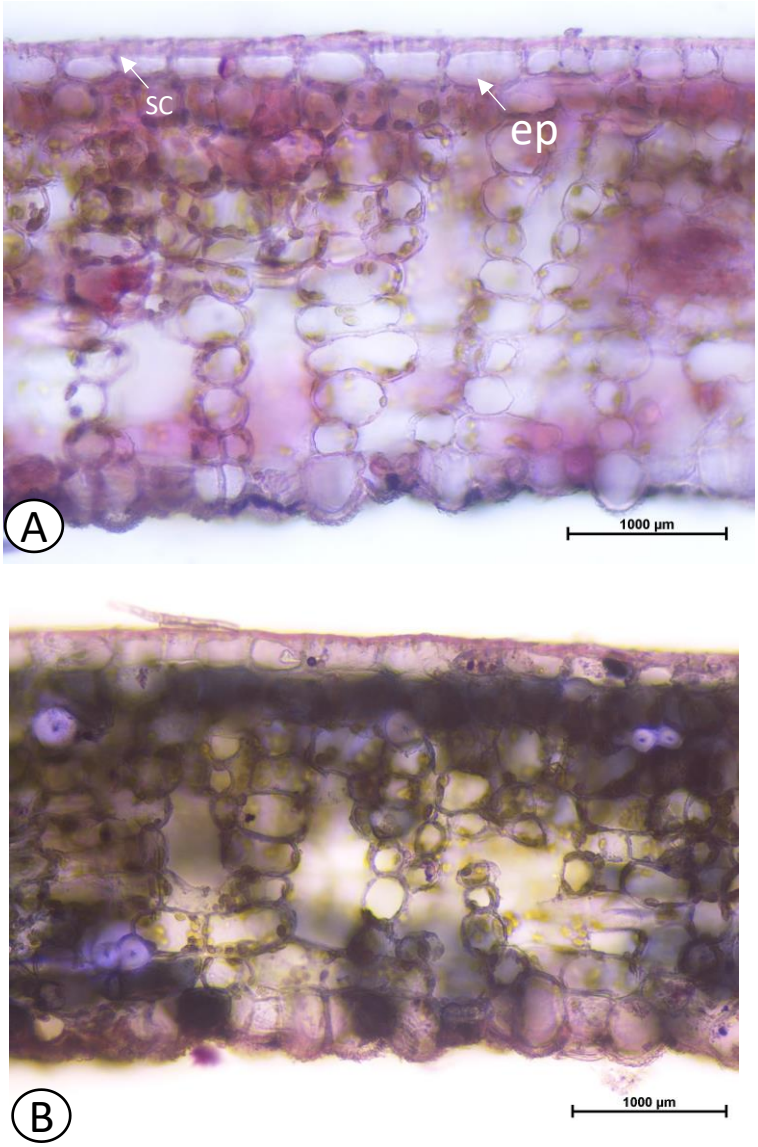

Plate 4.3: Anatomy of Leaf (A) stained with safranin stain showing epidermis (ep), secretary cells-(sc), (B) stained with combination of safranin and Toluidine Blue O.

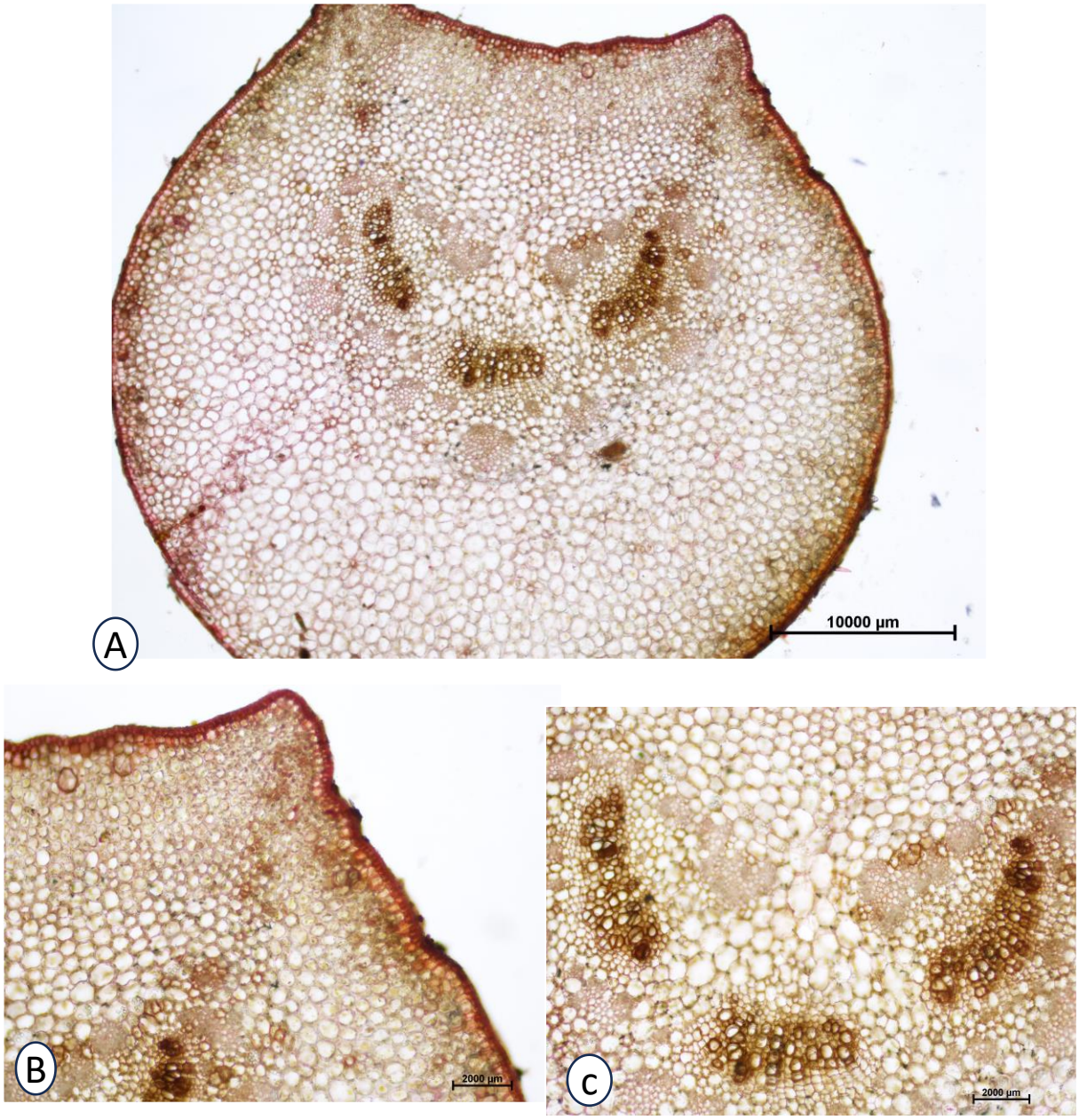

Plate 4.4: Anatomy of Petiole (A) overview of Petiole (4X), B) T.S of Petiole (10X), C) showing enlarge portion of Vascular Bundles (10x)

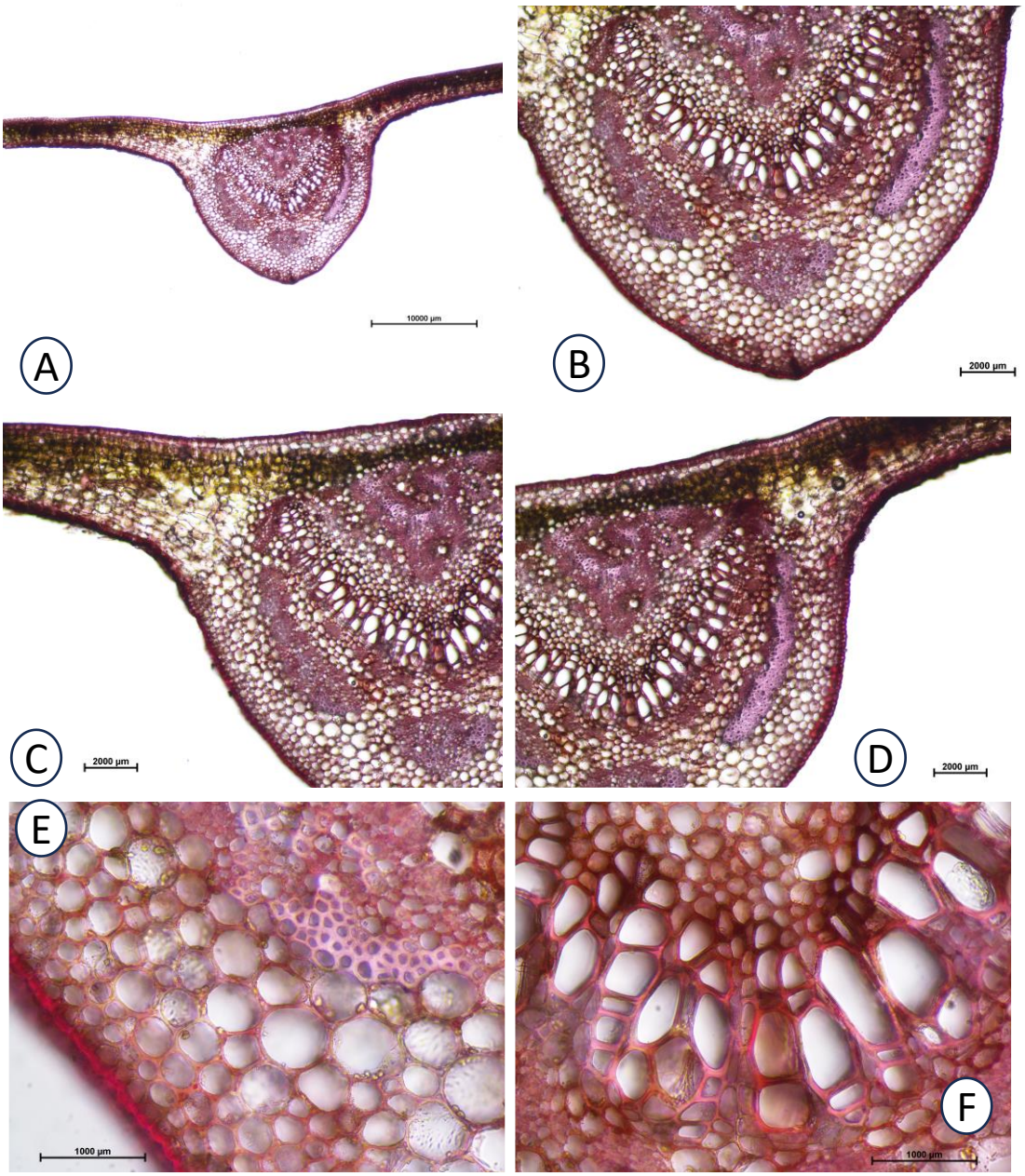

Plate 4.5: Anatomy of Midrib stained with Safranin stain (A)Overview of leaf (4X), (B) T.S of leaf showing Vascular bundles(10X), (C) and (d) T.S of leaf showing epidermal cells (10X), (E) T.S of leaf (40X) showing epidermal cells, Unstained starch granules (E) T.S of leaf (40X) showing enlarge portion of vascular bundles

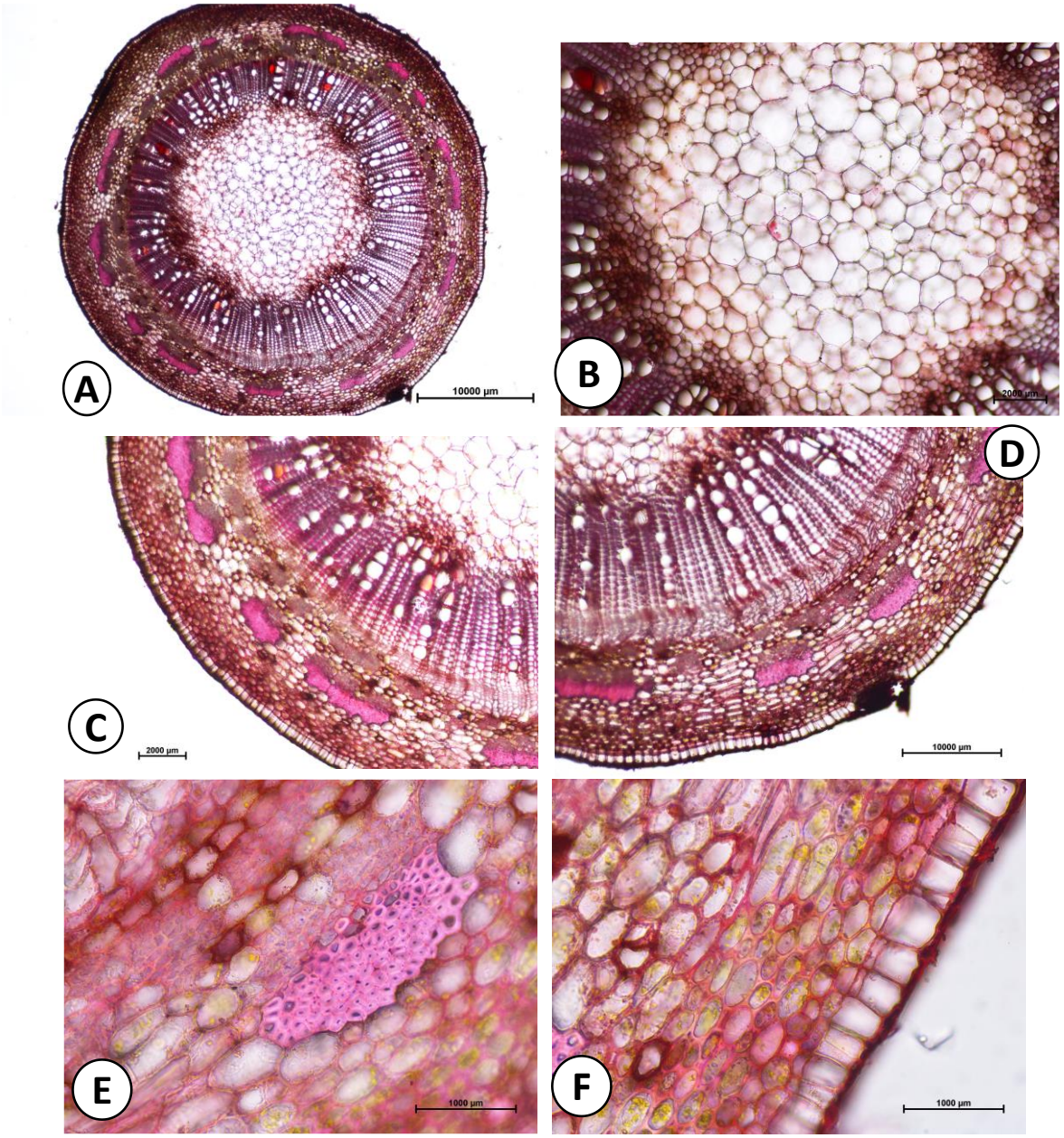

Plate 4.6: Anatomy of stem (A) overview of stem (4X), B) Enlarged view of Pith (10X), C) Enlarged view of stem (10X), D) Enlarged view of stem showing lenticels (10X), E) Enlarged view of lignified cell (40X), F) Enlarged view of epidermis (40X).

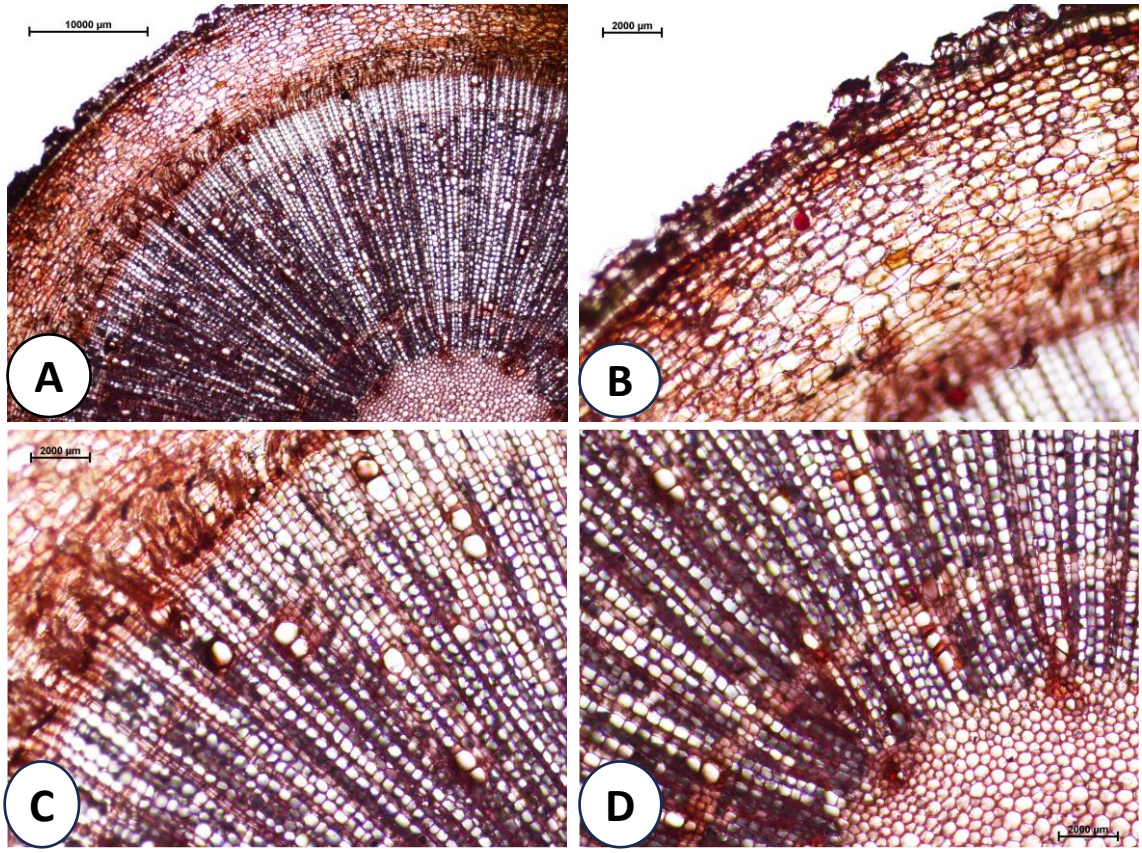

Plate 4.7: Anatomy of Root (A) overview of Root (4X), B) showing cortex (10X), C) enlarge view of annular rings (10X), D) Enlarge view of Xylem region and Pith region (10X)

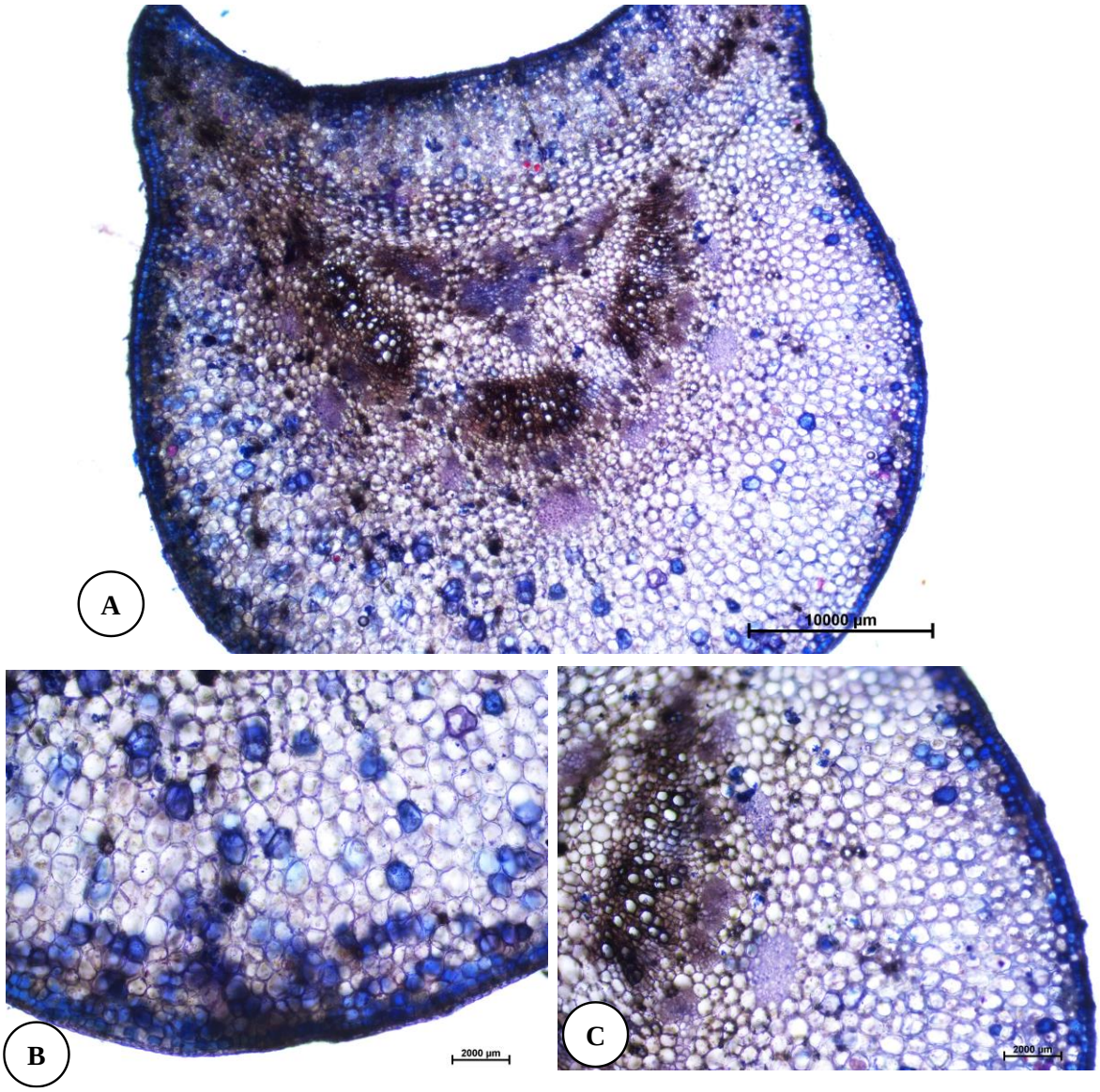

Plate 4.8: Anatomy of Petiole stained with combination of safranin and Toluidine Blue O A) Overview of Petiole (4X), B) Image showing abaxial region (4X), C) Cortex region (10X),

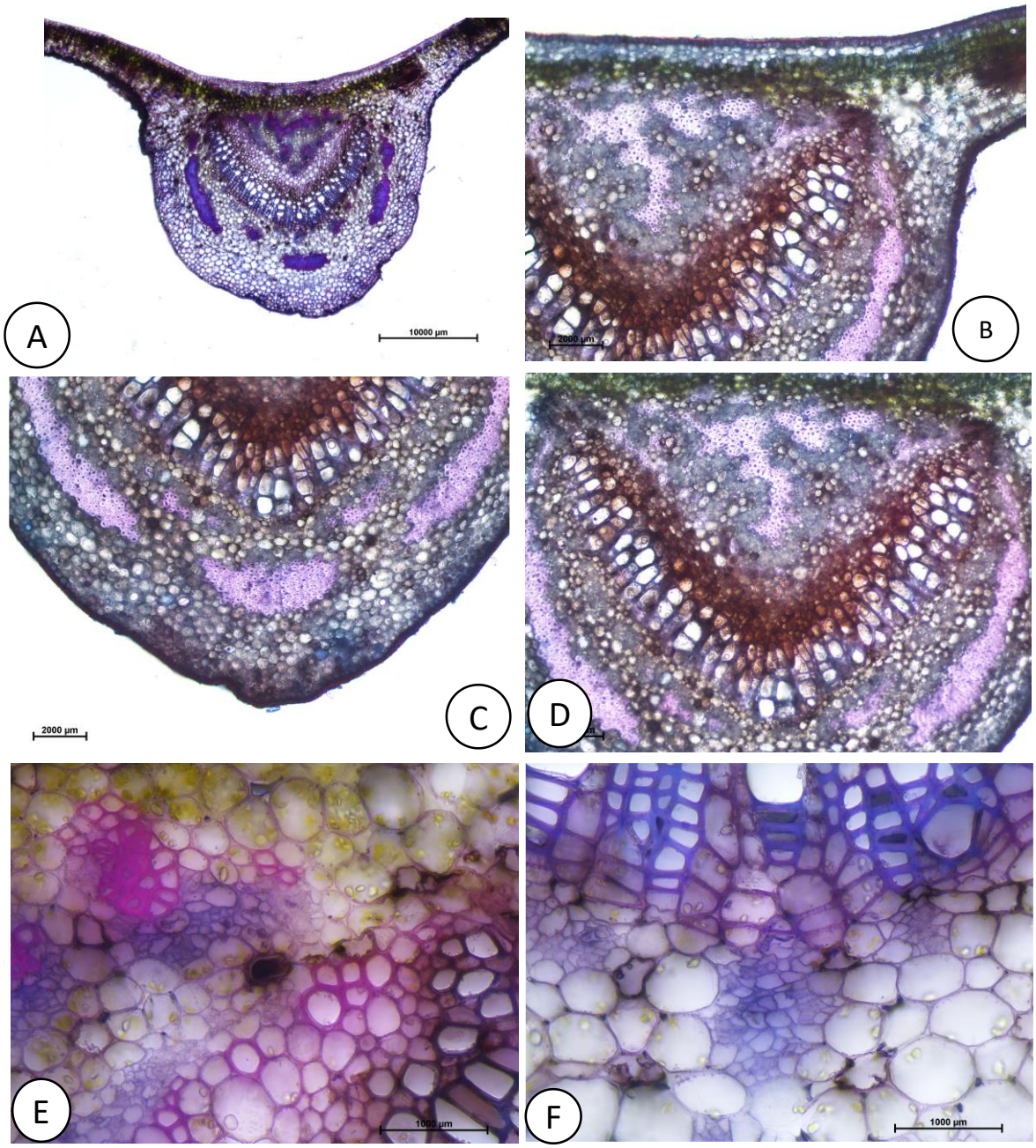

Plate 4.9: Anatomy of midrib stained with safranin and Toluidine Blue O (A) overview of Midrib, (4X), B) adaxial portion, C) Abaxial Portion, D) middle portion showing vascular Bundle (10X), E) and (F) Different colouration of Toluidine Blue o (40x)

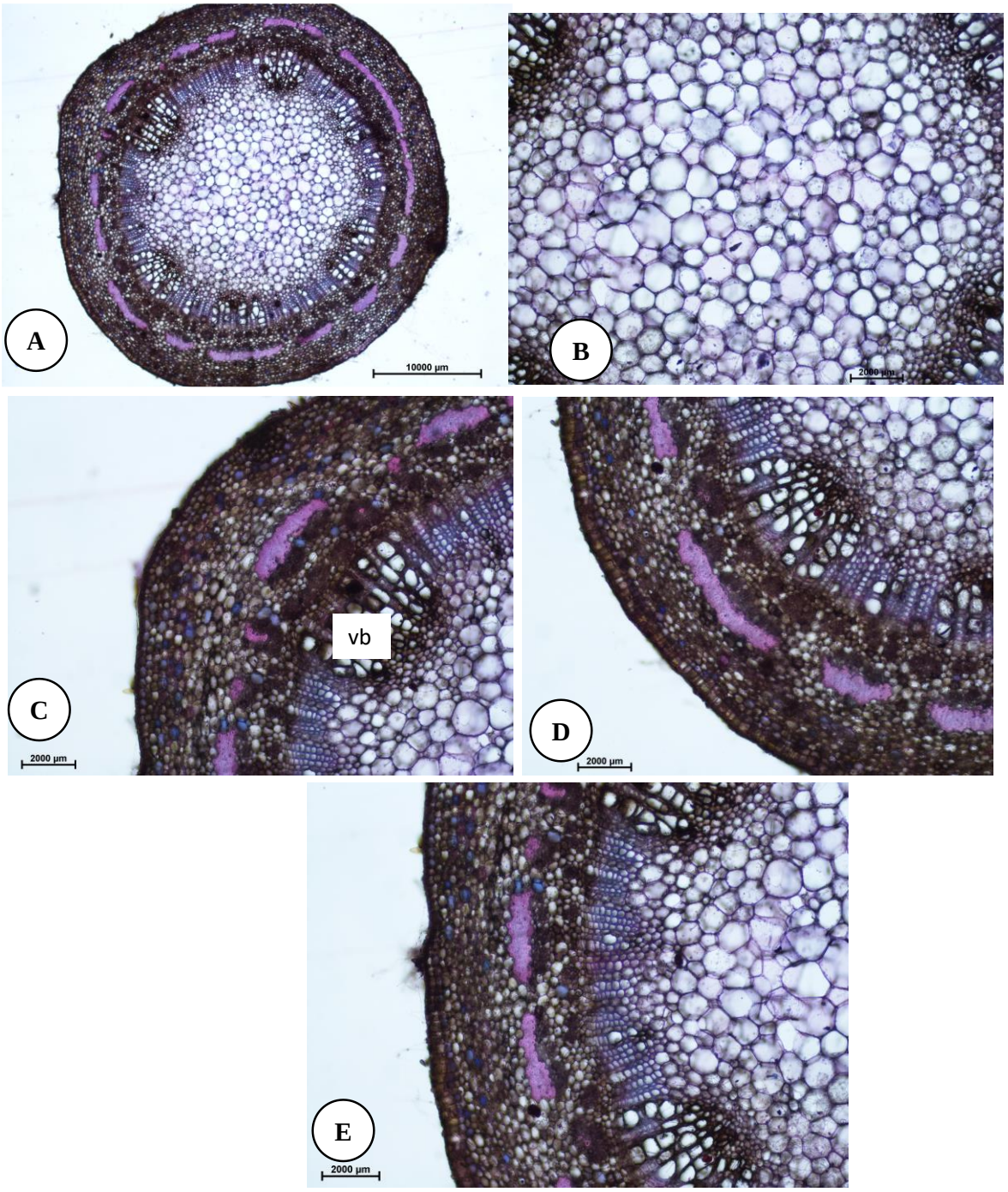

Plate 4.10: Anatomy of Stem stained with combination of safranin and Toluidine Blue O (A) Overview of stem (4X) B) Enlarge view of Pith (10X) C) showing Vascular bundle (10X) vb-Vascular bundle D), (E) enlarge view (10X)

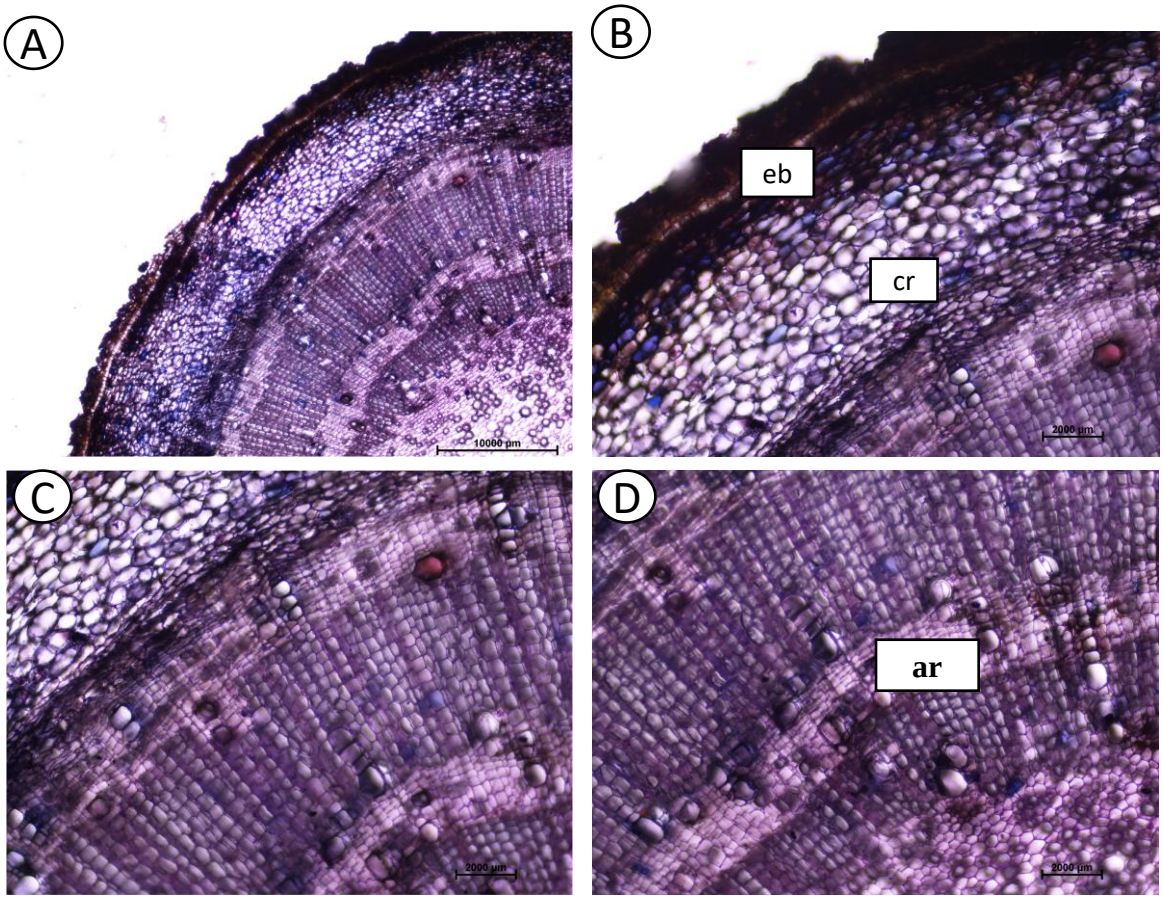

Plate 4.11: Anatomy of Root stained with combination of safranin and Toluidine Blue OA) Overview of Root (4X), B) showing outer layer epiblema and cortex region- (cr) (10X), C) showing Phloem and Cambium (10X), D) showing Annual ring (10X) ar-- annual ring

4.3 Histochemical Studies.

4.3.1 Localization of Primary Metabolites.

a. Starch

i. Localization of Starch in Midrib

Staining with I_2KI revealed the distribution of starch within the midrib of *Gymnacranthera canarica*. The highest concentration of starch was observed in the palisade tissue and mesophyll region, indicating active photosynthetic activity and carbohydrate storage in these areas. Additionally, starch was detected in lesser amounts around the vascular bundles, suggesting a potential role in energy storage and structural support. This spatial distribution of starch reflects the dynamic metabolic processes within the midrib, balancing photosynthetic productivity with storage and transport needs. The preferential accumulation of starch in the palisade tissue and mesophyll region aligns with the functional role of these regions in photosynthesis and carbohydrate synthesis. The presence of starch in the abaxial epidermal cells may indicate a protective mechanism or metabolic activity related to water and nutrient uptake. Understanding the spatial distribution of starch provides insights into the metabolic dynamics of leaf tissues and their adaptive strategies to fluctuating environmental conditions. Further investigations into the regulation of starch metabolism and its significance in plant physiology could reveal mechanisms for optimizing energy utilization and stress tolerance in *Gymnacranthera canarica*. **(Plate. 4.12)**

ii. Localization of Starch in petiole

In the petiole, staining with I_2KI revealed dark staining of both adaxial and abaxial side epidermal cells, indicating the presence of starch. Additionally, a small amount of starch was observed in the cortex region. Interestingly, young starch granules were detected around the vascular bundles, suggesting ongoing starch synthesis and storage processes in these areas. This distribution pattern of starch in the petiole highlights its role in providing energy

reserves and structural support for vascular tissues. The presence of starch in both epidermal layers may indicate a functional role in protecting underlying tissues and maintaining cellular integrity. The accumulation of starch around the vascular bundles suggests a potential role in facilitating nutrient transport and metabolism within the petiole. Further investigation into the dynamics of starch metabolism in different regions of the petiole could provide insights into its physiological significance and adaptive responses to environmental cues. **(Plate. 4.13)**

iii. Localization of Starch in stem

In the stem, staining with I_2KI revealed dark staining of epidermal cells, cortex region, and vascular bundles, indicating the presence of starch in significant amounts. Conversely, the xylem region and pith cells exhibited lighter staining, suggesting lower levels of starch accumulation. Interestingly, young starch granules were observed in higher abundance around the lignified cells.

The differential distribution of starch in various regions of the stem reflects its role in providing energy reserves and structural support for growth and development. The presence of starch in the epidermal cells and cortex region may serve as a readily accessible energy source for metabolic processes and cellular maintenance. Additionally, the accumulation of starch around the vascular bundles suggests its involvement in nutrient transport and storage within the stem. Further investigation into the regulation of starch metabolism and its functional significance in different stem tissues could provide valuable insights into plant growth and adaptation mechanisms. **(Plate. 4.14)**

iv. Localization of Starch in Root

In the root, starch content was relatively lower compared to other plant parts. Staining revealed fewer instances of starch accumulation throughout the root structure. However, young starch granules, characterized by a blue-purple coloration, were observed predominantly within the vascular cells.

The limited presence of starch in the root suggests a lesser emphasis on energy storage and metabolism in this organ. Instead, the distribution of young starch granules primarily within the vascular cells implies a role in nutrient transport and storage, facilitating the efficient uptake and distribution of essential resources within the plant. Further investigations into the regulatory mechanisms governing starch metabolism in the root could provide valuable insights into its physiological functions and adaptive responses to environmental stimuli. **(Plate. 4.15)**

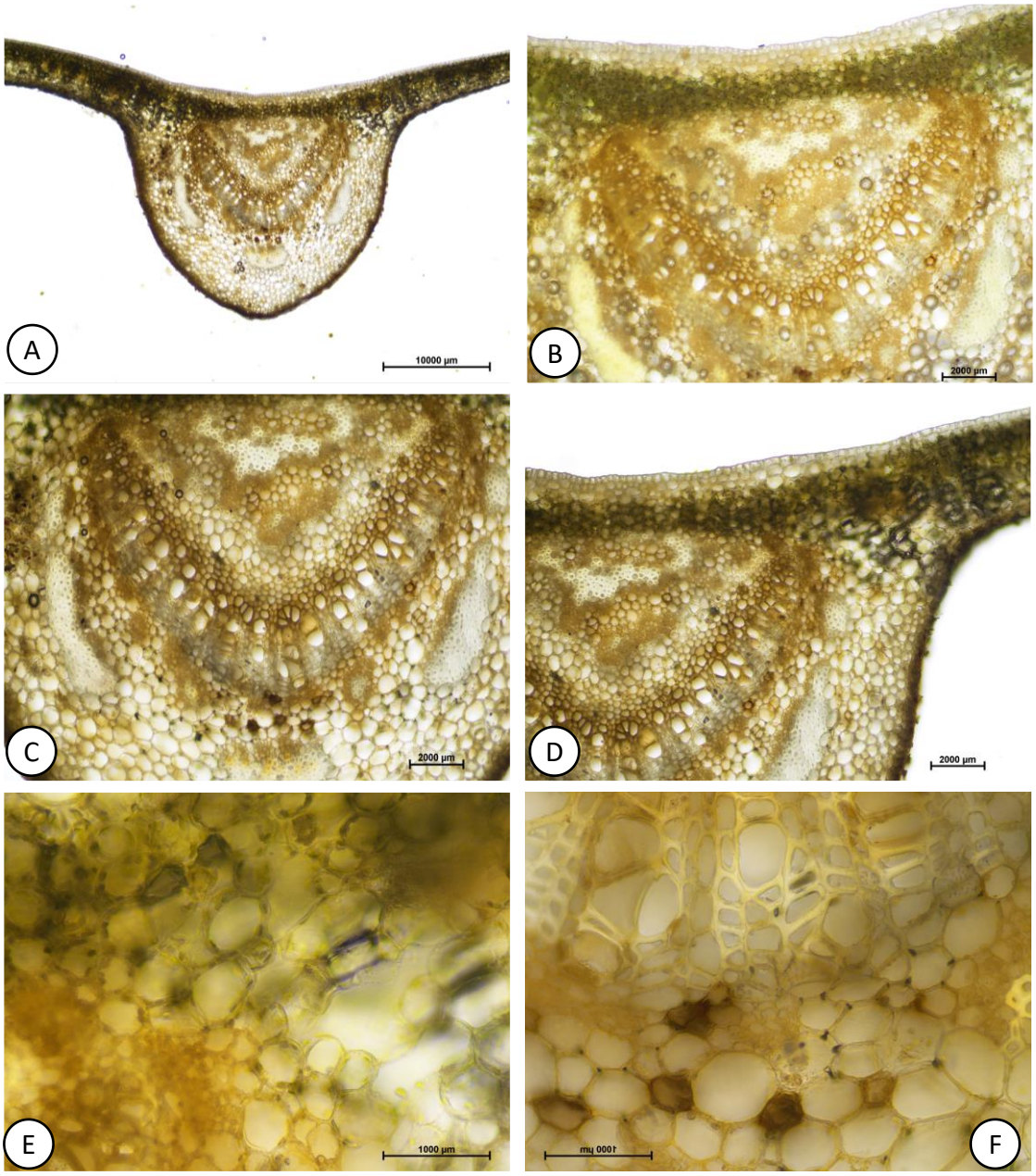

Plate 4.12: T.S of Midrib stained with I_2KI stain for localization of starch: A) overview of midrib (4X) B) and C) showing starch present in the vascular bundle and its region (10X) D) showing presence of starch in abaxial region, E) and F) enlarge view (40X)

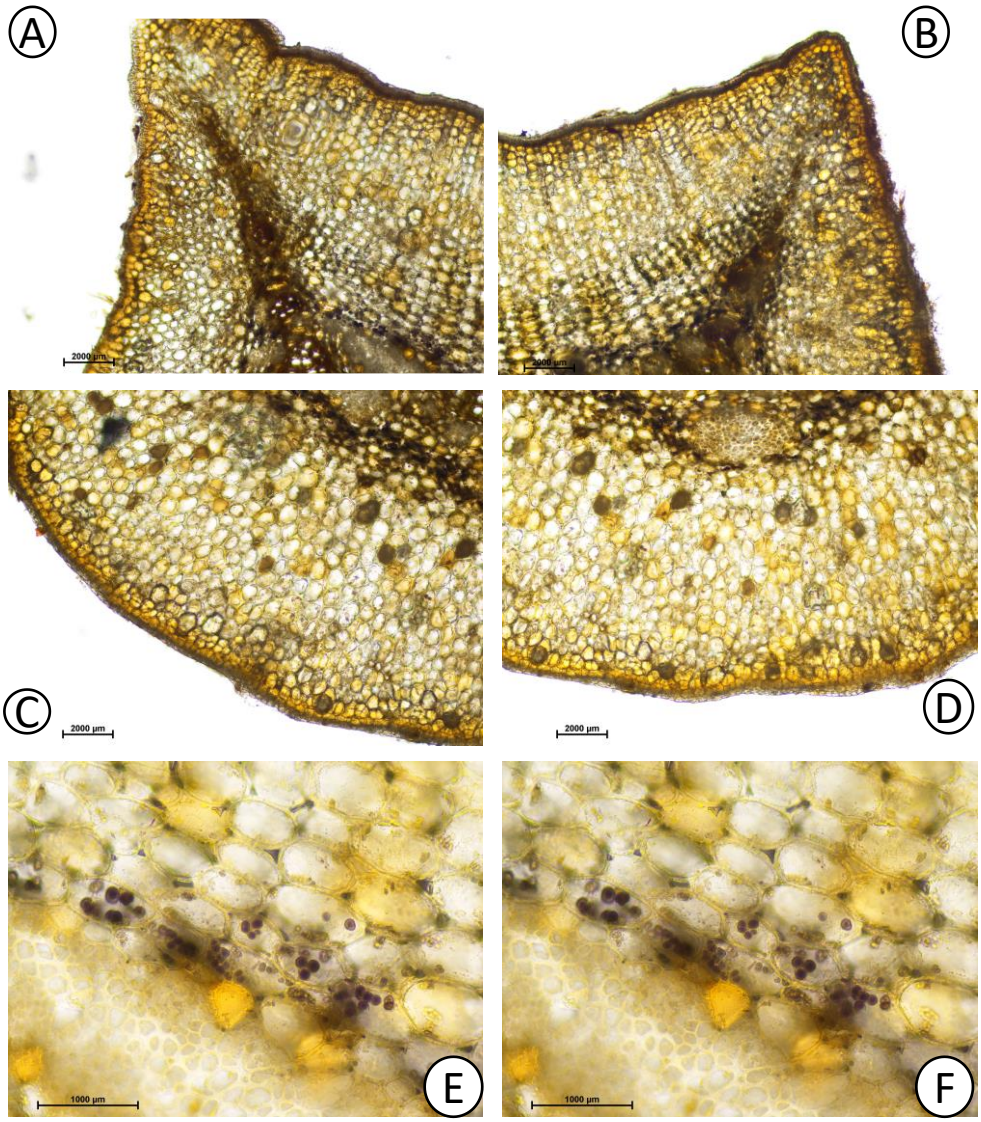

Plate 4.13: T.S Root stained with I_2KI stain for localization of starch A) and B) starch granules around vascular bundle C)and D) starch darkly stained in the epidermis and cortex region E) and F) Young starch granules around lignified cells.

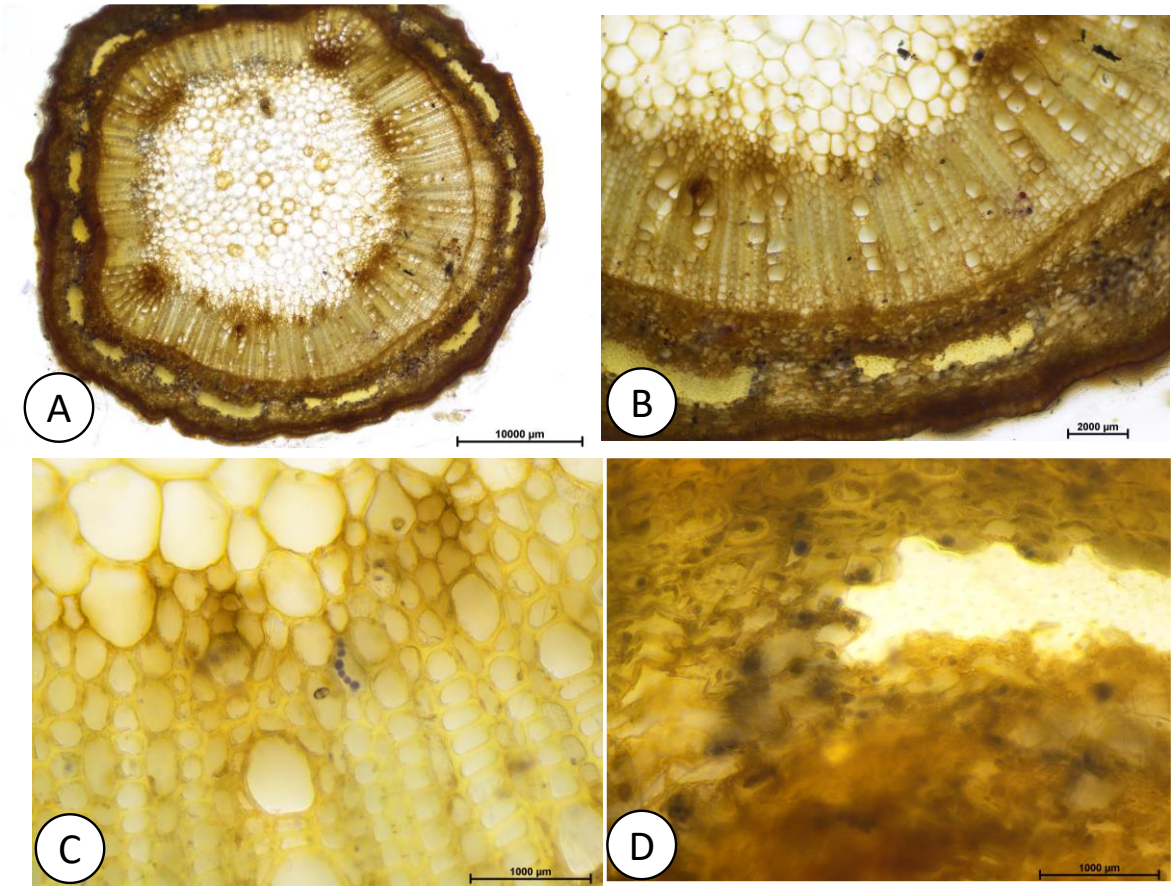

Plate 4.14: **T.S Stem stained with I_2KI stain for localization of starch** A) Overview of stem (4X) B) section under (10X), C) young starch granules near pith region, D)starch granuels surrounding lignfied cellls

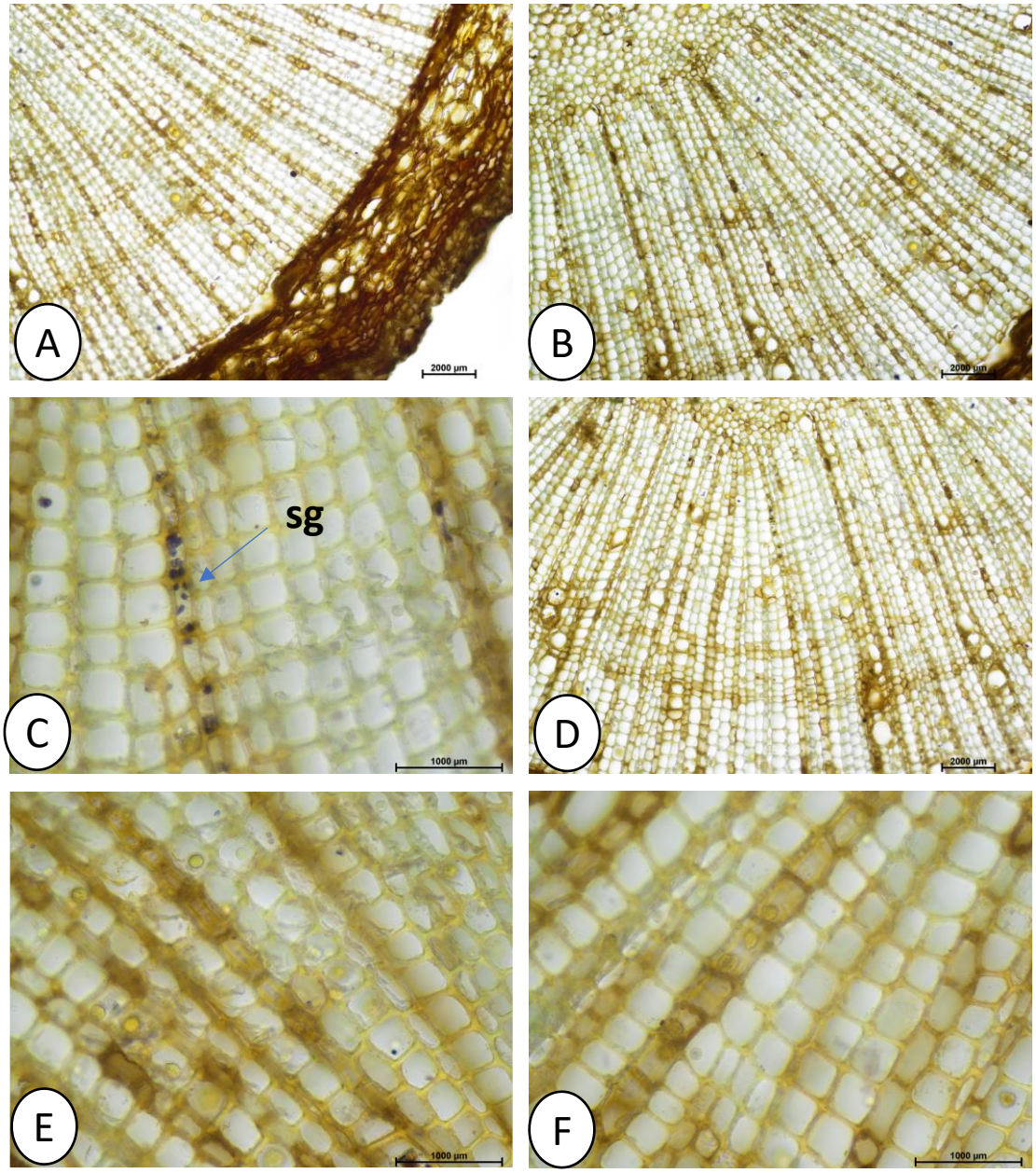

Plate 4.15: T.S Root stained with I_2KI stain for localization of starch: A) overview of Root (4X), B) showing stained regions, C) and D) showing young starch granules-(sg), E) and F) showing mature starch granules.

b. Proteins

i. Localization of Protein in Midrib

In the midrib, protein localization was evident through Coomassie brilliant blue staining, with distinct blue coloration observed in various tissue regions. Specifically, the epidermal cells, palisade layer, and mesophyll region exhibited a more intense blue staining, indicating a higher protein content in these areas. In contrast, the vascular bundles and cortex region showed lighter staining, suggesting a comparatively lower protein concentration.

The differential distribution of proteins within the midrib may reflect specific metabolic activities and functional roles associated with different tissue types. For instance, the abundance of proteins in the epidermal cells and photosynthetic mesophyll tissues likely supports cellular growth, development, and photosynthetic processes. Conversely, the relatively lower protein content in the vascular bundles and cortex region may indicate specialized functions related to nutrient transport and mechanical support, respectively.

Overall, the observed protein localization patterns highlight the spatial complexity of protein metabolism within the midrib and highlight the importance of tissue-specific protein regulation in plant physiology and development. Further investigations into the identity and function of proteins in each tissue region could provide valuable insights into their roles in plant growth, adaptation, and stress responses. **(Plate. 4.16)**

.ii. Localization of Protein in petiole

In the petiole, Coomassie brilliant blue staining revealed distinct protein localization patterns across different tissue regions. Specifically, the epidermal cells and vascular bundles exhibited dark staining, indicative of a higher protein content in these areas. In contrast, the cortex region showed lighter staining, suggesting a lower protein concentration.

The differential distribution of proteins within the petiole may reflect specific functional roles associated with different tissue types. The abundance of proteins in the epidermal cells and vascular bundles likely supports cellular functions such as growth, metabolism, and structural integrity. Conversely, the relatively lower protein content in the cortex region may indicate specialized functions related to nutrient storage or mechanical support.

Overall, the observed protein localization patterns provide insights into the spatial regulation of protein metabolism in the petiole and underscore the importance of tissue-specific protein dynamics in plant physiology. Further investigation into the identity and function of proteins in each tissue region could elucidate their roles in petiole development, function, and response to environmental cues. **(Plate. 4.17)**

iii. Localization of Protein in stem

In the stem, Coomassie brilliant blue staining revealed distinct protein distribution patterns across different tissue regions. Epidermal cells and the cortex region exhibited light staining, indicating a relatively low abundance of proteins in these areas. Conversely, the pith region displayed dark staining, suggesting a higher concentration of proteins compared to other regions of the stem. The differential protein distribution observed in the stem reflects the diverse physiological functions of different stem tissues. The epidermis and cortex are primarily involved in providing structural support and protection to the plant, while the pith region serves various roles including storage, transport, and structural support. The higher protein content in the pith region may be associated with its metabolic activities and storage functions. Proteins present in the pith region could play crucial roles in nutrient storage, metabolic regulation, and response to environmental stresses. **(Plate. 4.18)**

iv. Localization of Protein in Root

In the root, Coomassie brilliant blue staining revealed a relatively low abundance of proteins compared to other plant parts. The epidermal cells and cortex region exhibited light staining, indicating a sparse presence of proteins in these areas.

The reduced protein content in the root may reflect its specialized functions primarily related to anchorage, water, and nutrient uptake rather than active protein synthesis and metabolism. However, specific proteins localized within the root epidermis and cortex may play critical roles in root development, cell differentiation, and interactions with the soil environment. Overall, the observed protein distribution in the root highlights the tissue-specific regulation of protein metabolism and underscores the importance of understanding the functional significance of proteins in different root regions. Further research into the identity and function of proteins in the root could provide valuable insights into the molecular mechanisms underlying root physiology and adaptation to environmental conditions. **(Plate. 4.19)**

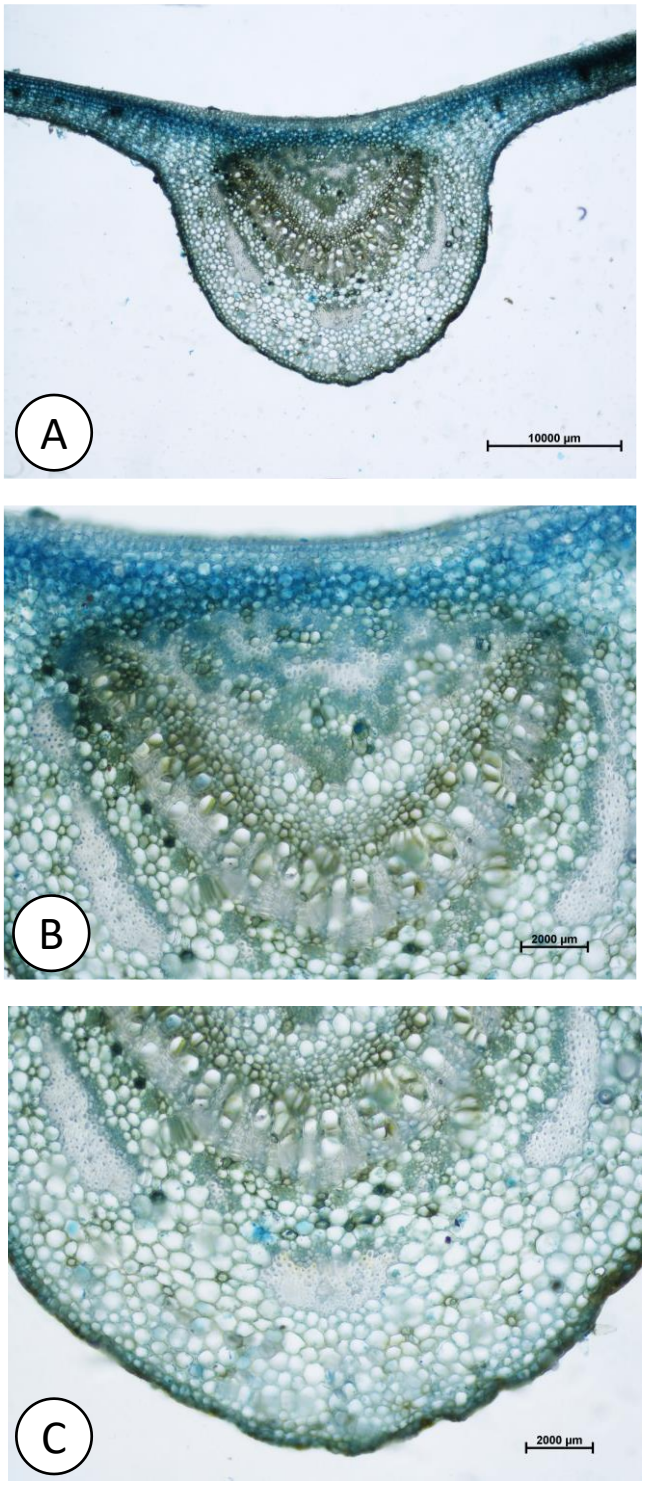

Plate 4.16: T.S. of Midrib stained with Coomassie Brilliant Blue stain for Localization of Proteins A) overview of midrib (4X), B) adaxial region with vascular bundle portion (10X), C) Abaxial region with region of vascular bundle (10X)

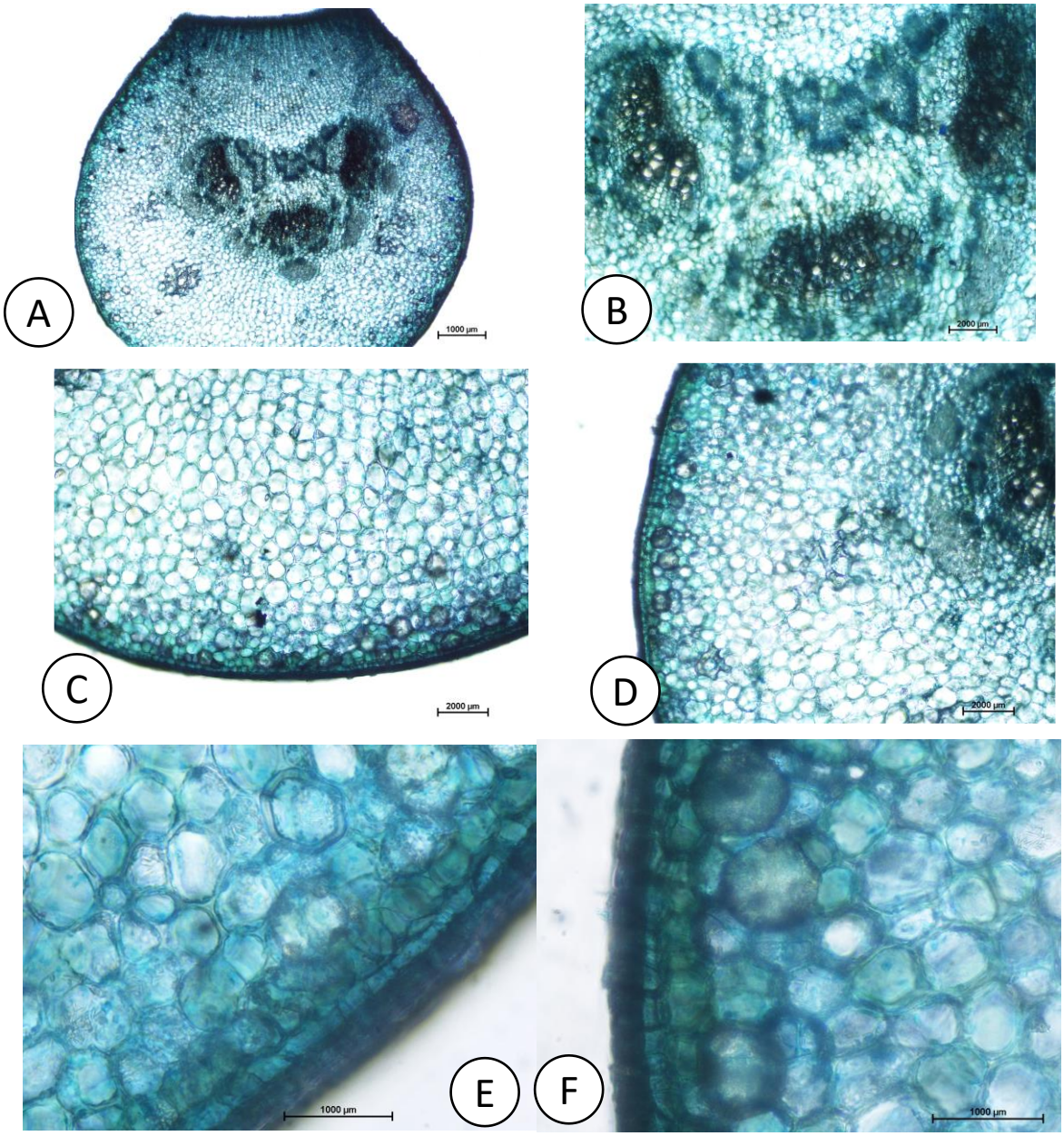

Plate 4.17: T.S. of Petiole stained with Coomassie Brilliant Blue stain for Localization of Proteins A) overview (4X), B) Vascular bundles (10X), C) and D) Abaxial region, epidermal and cortex (10X), E) and F) epidermis and cortex region (40X)

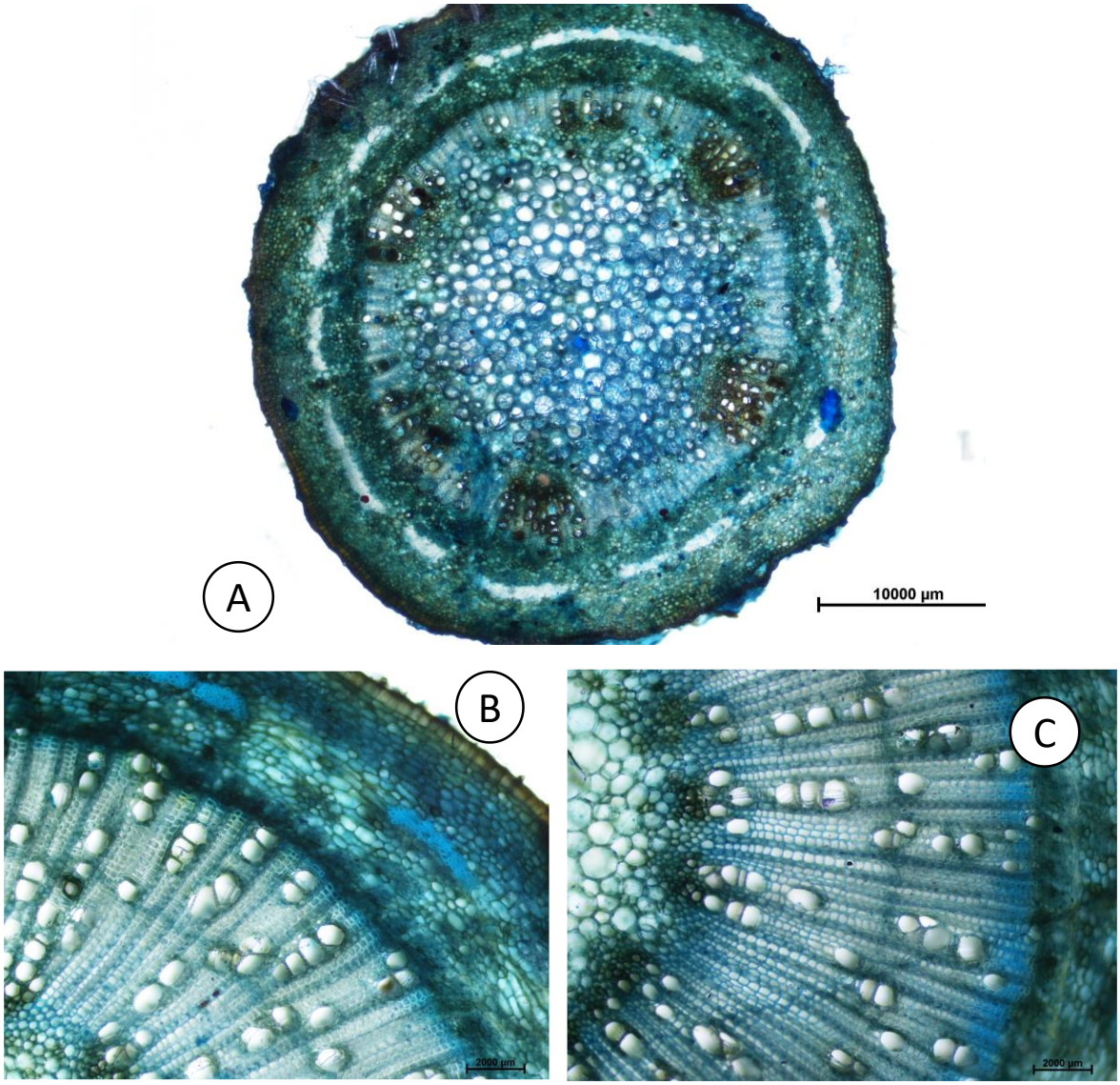

Plate 4.18: T.S. of Stem stained with Coomassie Brilliant Blue stain for Localization of Proteins A) Overview of stem (4x), B) epidermis and cortex region (10X), C) showing vascular Bundles and xylem region (10X).

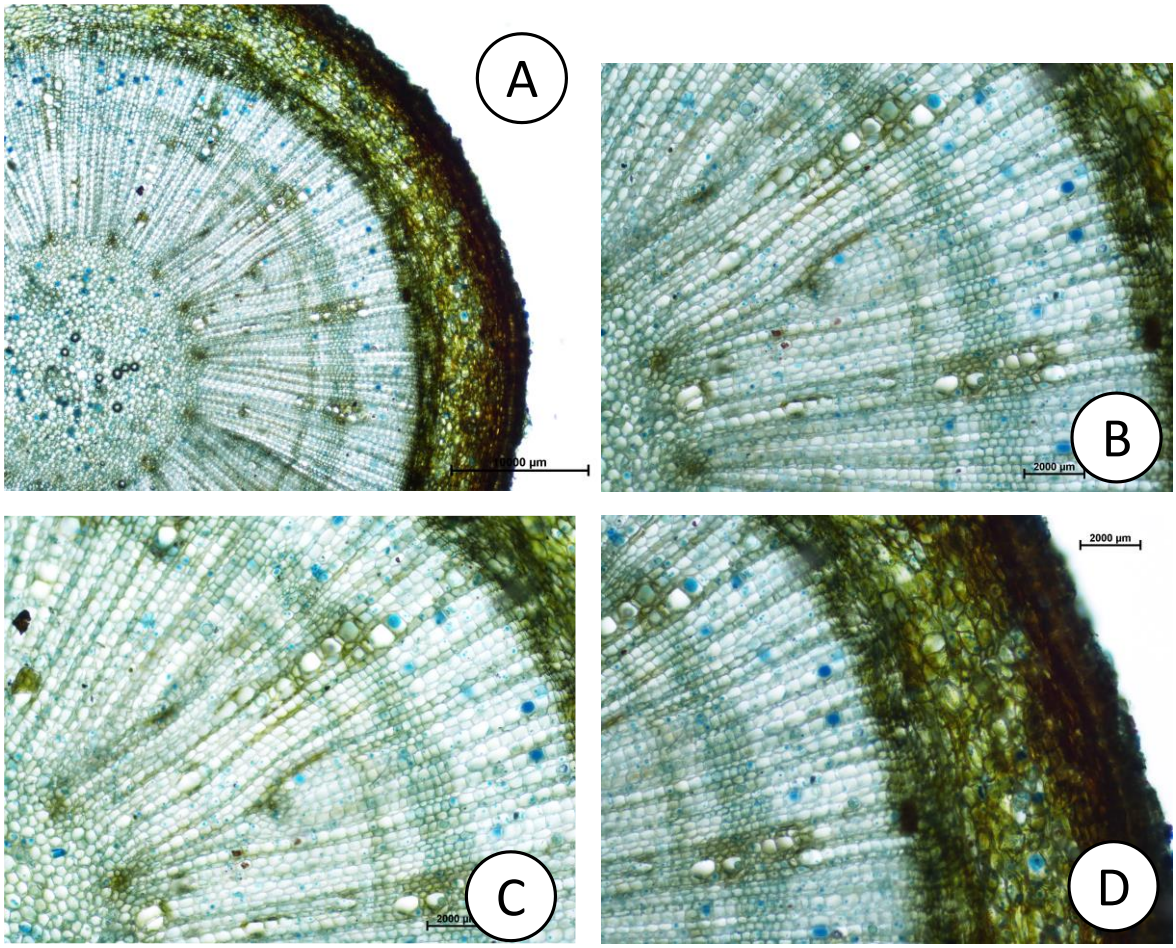

Plate 4.19: T.S. of Root stained with Coomassie Brilliant Blue stain for Localization of Proteins A) overview of Root (4X), B) and C) Cortex region and phloem region (10X), epiblema, cortex and phloem (10X)

c. Lipids

i. Localization of Lipids in Midrib

In the midrib, Sudan IV staining revealed the presence of lipids, with the epidermal cells exhibiting a deep red coloration. Abundant lipid content was observed in both the abaxial and adaxial epidermal cells, as well as around the vascular bundles and lignified cells. In contrast, the cortex region displayed a relatively lower presence of lipids.

The distribution of lipids in the midrib suggests their potential roles in various physiological processes. Lipids are essential components of cell membranes and play crucial roles in membrane integrity, signaling, and storage of energy. The higher accumulation of lipids in the epidermal cells and around the vascular bundles may be associated with their roles in providing structural support and transportation of nutrients and water. Conversely, the lower lipid content in the cortex region implies a different metabolic activity or storage function compared to other regions of the midrib. **(Plate. 4.20)**

ii. Localization of Lipids in petiole

Sudan IV staining highlighted the presence of lipids distributed across the epidermal cells, vascular bundles, and cortex region. A notable abundance of lipids was detected in the epidermal cells and around the vascular bundles, as well as surrounding the lignified cells. Conversely, the cortex region exhibited a relatively lower concentration of lipids.

The widespread distribution of lipids in various tissues of the petiole suggests their involvement in multiple physiological processes. Lipids serve as essential components of cell membranes, contributing to membrane structure and function. The high lipid content in the epidermal cells and vascular bundles may support their roles in providing structural integrity and facilitating nutrient transport. Additionally, the presence of lipids around the

lignified cells indicates their potential involvement in lignin biosynthesis or reinforcement of cell walls **(Plate. 4.21)**

iii. Localization of Lipids in stem

Sudan IV staining revealed a relatively lower presence of lipids compared to other tissues. Lipids were discernible in the epidermal cells, cortex, and vascular region, albeit in lesser amounts compared to other plant parts.

The limited lipid content in the stem suggests that lipid metabolism may not be as prominent in this tissue compared to others. However, the presence of lipids in the epidermal cells, cortex, and vascular region indicates their importance in maintaining membrane integrity and potentially facilitating various physiological processes within the stem.

The distribution of lipids across different stem tissues may reflect their diverse functional roles. While lipids in the epidermal cells may contribute to cuticle formation and protection against environmental stresses, those in the cortex and vascular region may participate in nutrient storage and transport. Overall, the modest presence of lipids in the stem suggests a potential tissue-specific regulation of lipid metabolism, highlighting the need for further investigation into the metabolic pathways and biological functions associated with lipids in stem tissues. **(Plate. 4.22)**

iv. Localization of Lipids in Root

Sudan IV staining revealed a prominent red coloration, particularly concentrated in the xylem region. Conversely, the staining intensity was notably lower in the epidermal cells and cortex region, with a comparatively lighter red coloration observed.

The significant accumulation of lipids in the xylem region suggests their potential role in various physiological processes associated with water and nutrient transport. Lipids

may contribute to the structural integrity of xylem vessels, facilitating efficient water uptake and transport from the roots to the rest of the plant.

The relatively lower presence of lipids in the epidermal cells and cortex region may indicate differences in lipid metabolism and distribution across different root tissues. While lipids in the epidermal cells may contribute to cuticle formation and protection against external stresses, those in the cortex region may be involved in nutrient storage and signalling processes. The differential distribution of lipids in the root section highlights the importance of lipid metabolism in root development and function. Further investigations into the specific lipid species and their functional roles in different root tissues are warranted to elucidate their contributions to root physiology and plant growth. **(Plate. 4.23)**

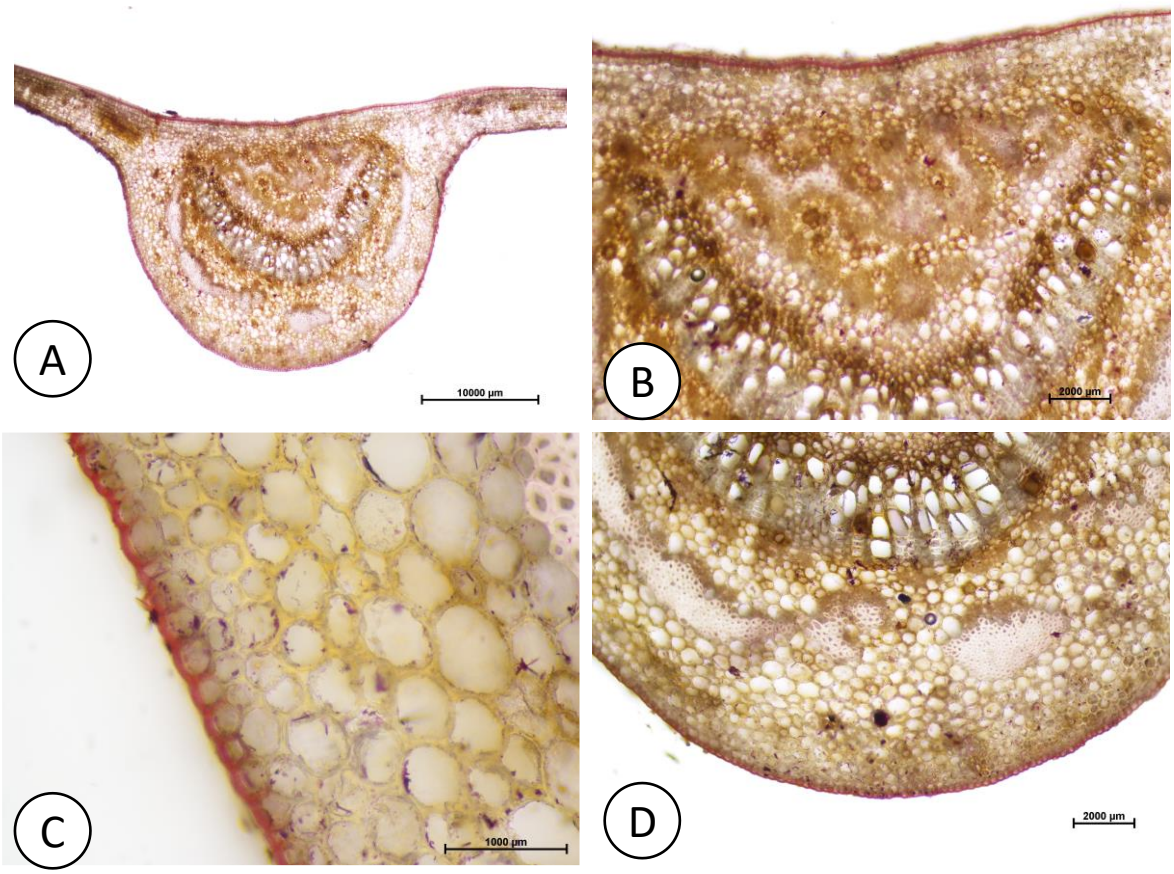

Plate 4.20: T.S. of Midrib stained with Sudan IV stain for Localization of Lipids A) Overview of midrib (4X), B) Stained adaxial region (10X) C) Stained epidermal region (40X), D) Stained Abaxial region (10X)

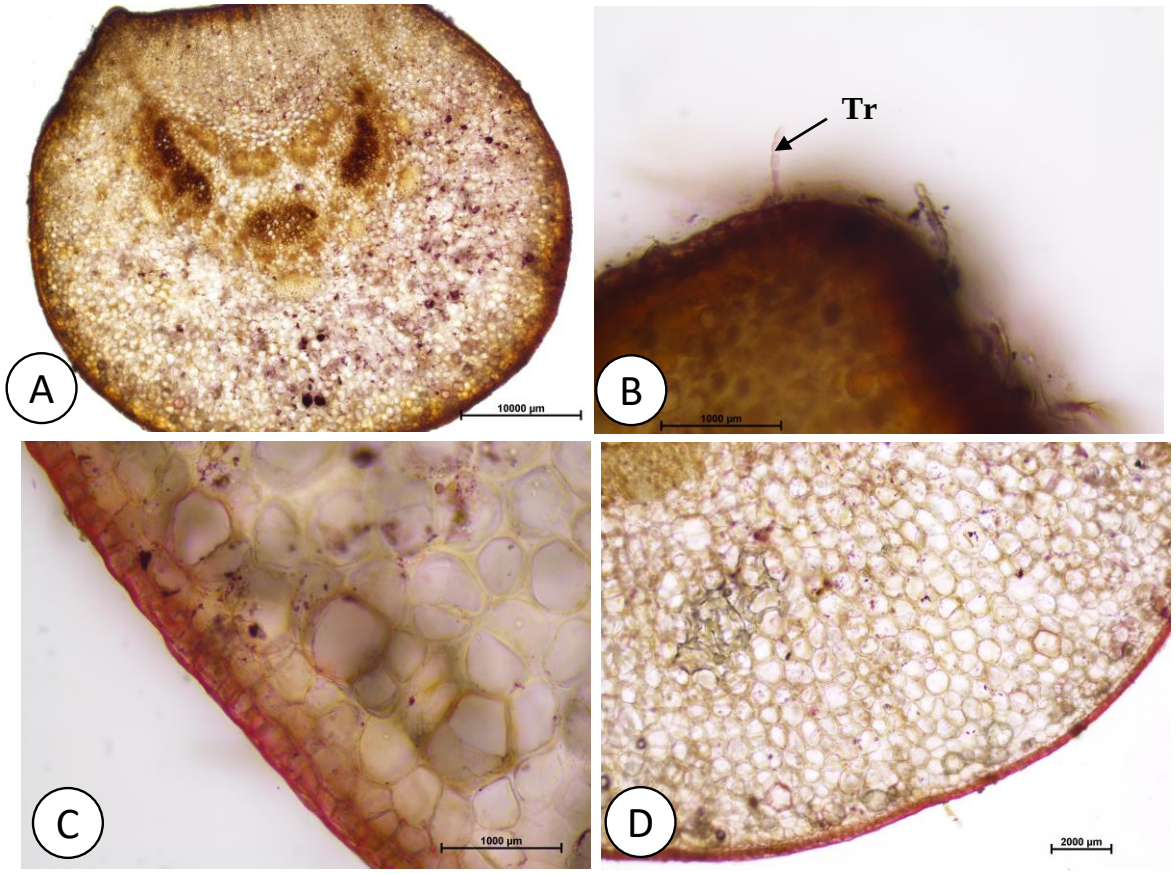

Plate 4.21: T.S. of Petiole stained with Sudan IV stain for Localization of Lipids A) overview of petiole (4X), B) Image showing Trichome-(Tr) (40X), C) Stained epidermis region (40X), D) Stained cortex region.

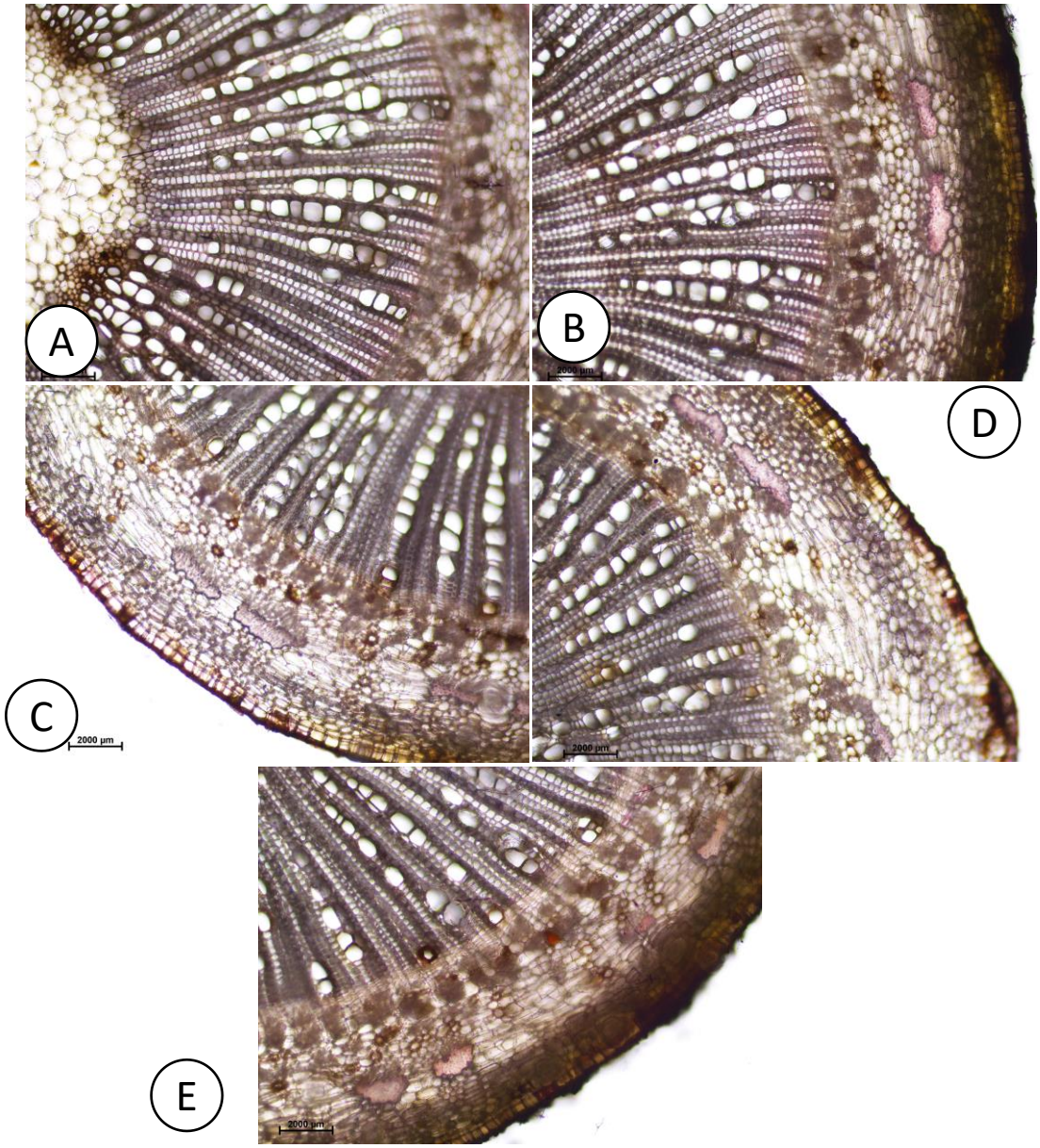

Plate 4.22: T.S. of stem stained with Sudan IV stain for Localization of Lipids: A) and B) T.S. overview (10X), C), D) and E) showing presence of starch in different regions.

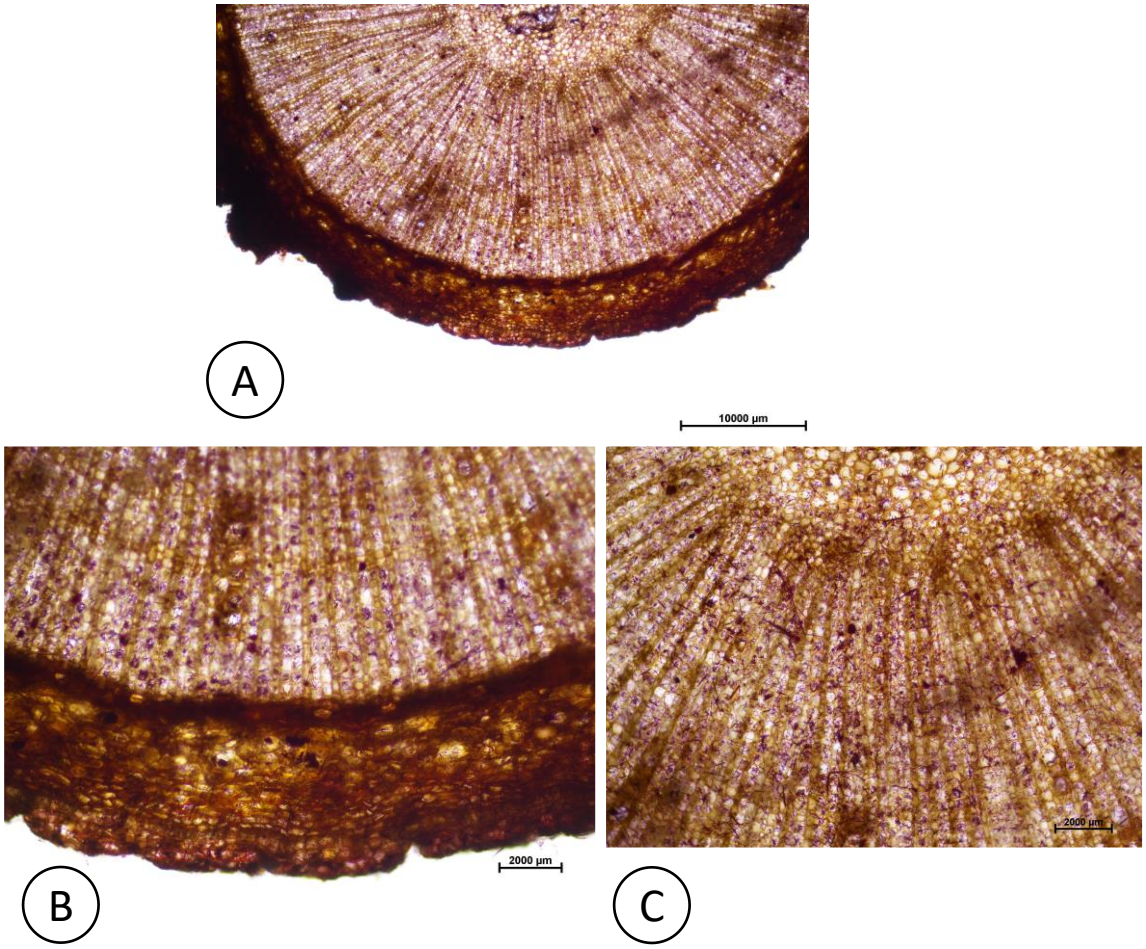

Plate 4.23: T.S. of stem stained with Sudan IV stain for Localization of Lipids: A) and B) T.S. overview (10X), C), D) and E) Showing presence of starch in different regions.

4.3.2 Localization of Secondary Metabolites.

a. Alkaloids

Sections stained with the Dragendorff's reagent showed the presence of alkaloids. Yellow, grey, greyish black, brown colour was observed in the sections

a. Localization of Alkaloid in Midrib

In the midrib section, the presence of alkaloids was evident across the entire tissue. Epidermal cells on both the adaxial and abaxial sides, as well as the mesophyll cells and palisade layer, exhibited a distinct brown staining pattern. Notably, black, or greyish spots were observed predominantly on the upper side of the vascular bundle, suggesting localized accumulation of alkaloids in this region. Additionally, lignified cells exhibited a yellowish staining coloration, indicating potential interactions between alkaloids and lignin within the tissue.

The widespread distribution of alkaloids in the midrib section suggests their involvement in various physiological processes within the leaf tissue. Alkaloids are known for their diverse biological activities and may play roles in plant defence mechanisms, as well as in regulating cellular processes such as growth and development.

The observed accumulation of alkaloids in specific regions, such as the upper side of the vascular bundle, may indicate their concentration in areas of high metabolic activity or where they are most needed for plant function **(Plate. 4.24)**

ii. Localization of Alkaloid in petiole

In the petiole section, alkaloids were observed in relatively low amounts. Epidermal cells exhibited a brown staining pattern, indicating the presence of alkaloids, albeit in small quantities. Additionally, some cortical cells displayed a yellowish or greyish staining

coloration, suggesting variable levels of alkaloid accumulation within the cortex region. The relatively low abundance of alkaloids in the petiole suggests that this plant part may not be a significant site for alkaloid biosynthesis or accumulation compared to other tissues. The variable staining patterns observed in the cortex region indicate potential differences in alkaloid distribution within the petiole structure. **(Plate. 4.25)**

iii. Localization of Alkaloid in Stem

The stem section revealed the presence of alkaloids across various tissue regions. Epidermal cells, cortex, vascular bundles, and certain pith cells exhibited a brown staining coloration, indicating the presence of alkaloids in these areas. Furthermore, lignified cells displayed a yellow staining pattern, potentially indicative of interactions between alkaloids and lignin compounds within the tissue. The widespread presence of alkaloids in the stem suggests their involvement in various physiological processes throughout this plant part. The staining patterns observed in different tissue regions highlight the heterogeneous distribution of alkaloids within the stem, possibly reflecting diverse metabolic functions or storage mechanisms. **(Plate. 4.26)**

iv. Localization of Alkaloid in Root

In the root section, the presence of alkaloids was evident, as indicated by the brownish-yellow coloration observed in the epidermal and cortex cells. This staining pattern suggests a relatively higher concentration of alkaloids in these regions compared to the xylem, which exhibited less pronounced staining. This differential distribution of alkaloids within the root tissues suggests potential functional roles or differential accumulation patterns across distinct anatomical zones. Such differential distribution of alkaloids across root tissues may reflect varied metabolic activities or localization preferences within the plant. Further investigation into the specific types and quantities of alkaloids present in each

tissue could provide insights into their physiological roles and potential pharmacological significance. **(Plate. 4.27)**

b. Phenols

Section stained with the ferric chloride solution showed Black colouration in the different tissues.

i. Localization of Phenols in Midrib

In the midrib section, phenols were observed predominantly in the epidermal layers and the region surrounding the vascular bundle. This distribution pattern suggests a potential role of phenols in providing structural support and defence against pathogens in these tissues. Further investigation into the specific types and concentrations of phenolic compounds present could elucidate their functional significance in the midrib of *Gymnacranthera canarica*. **(Plate. 4.28)**

ii. Localization of Phenols in petiole

In the petiole section, phenols were notably present in the epidermal layer and around the vascular bundles, with a higher concentration observed in the epidermal layers. This distribution suggests a potential role of phenols in protecting the petiole tissues from environmental stressors and microbial attacks. Further analysis could provide insights into the specific phenolic compounds responsible for this defensive function in petioles of *Gymnacranthera canarica* **(Plate. 4.29)**

iii. Localization of Phenols in stem

In the stem section, phenols were observed in relatively lower amounts, with distinct black coloration observed primarily in the xylem region. This distribution suggests a localized defence mechanism against potential pathogens or environmental stressors, highlighting the importance of phenolic compounds in stem physiology and protection. Further investigation could elucidate the specific phenolic profiles and their roles in stem function and adaptation. **(Plate. 4.30)**

iv. Localization of Phenols in Root

In the root section, phenols were predominantly concentrated in the xylem region, evidenced by a higher intensity of black coloration. Conversely, the epidermal and cortex regions exhibited relatively lower amounts of phenolic compounds, as indicated by less pronounced black coloration. This spatial distribution suggests a potential role of phenols in the vascular system of the root, possibly related to defense mechanisms or physiological processes. Further investigations could provide insights into the specific functions of phenolic compounds in root tissues and their implications for plant health and adaptation. **(Plate. 4.31)**

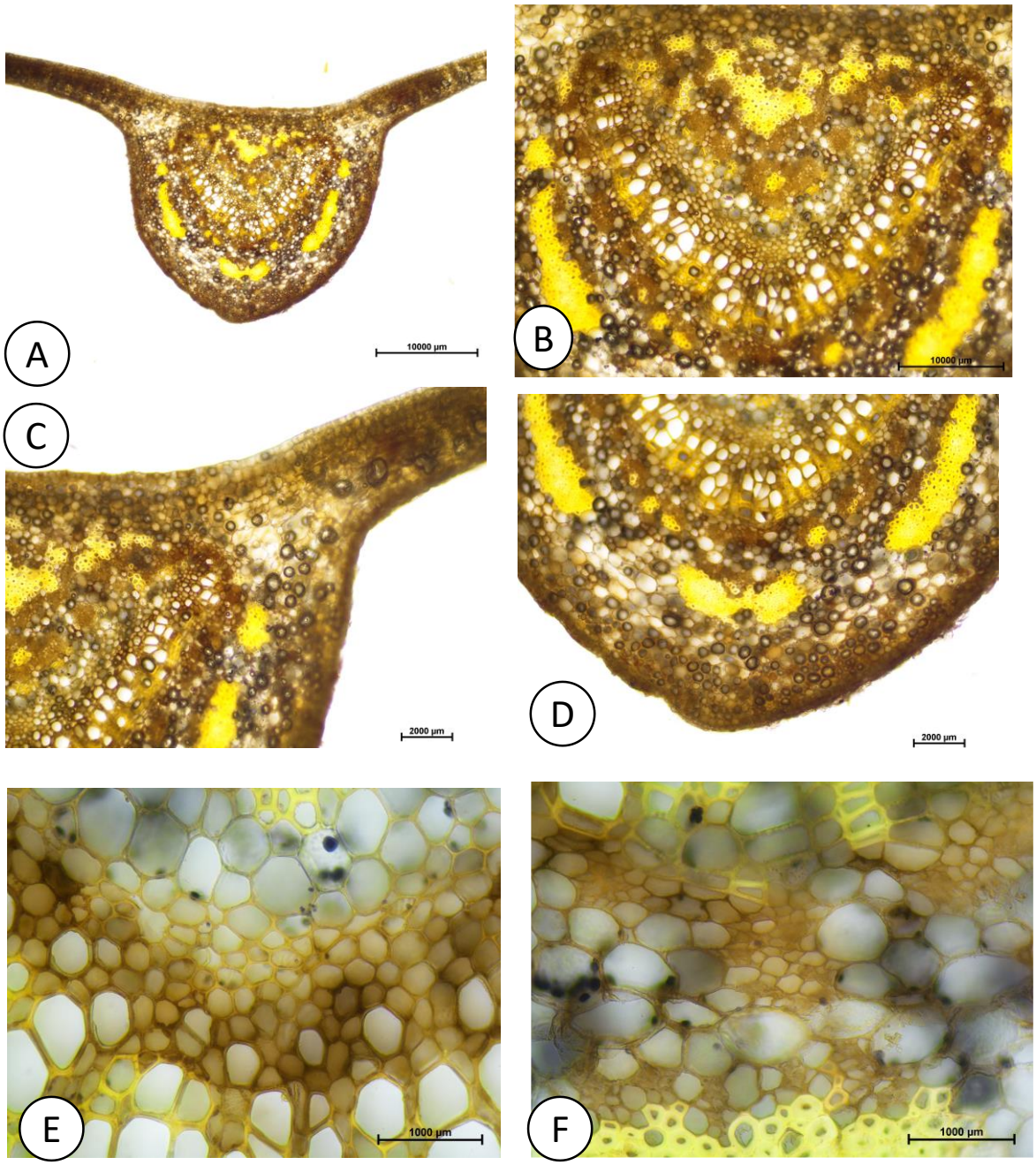

Plate 4.24: T.S. of Midrib stained with Dragendorff's reagent for localization of Alkaloids A) overview of Midrib (4X), B) Vascular bundle region (10X), C) Adaxial region (10X), D) abaxial region (10X), E) and F) colouration of alkaloids (40X)

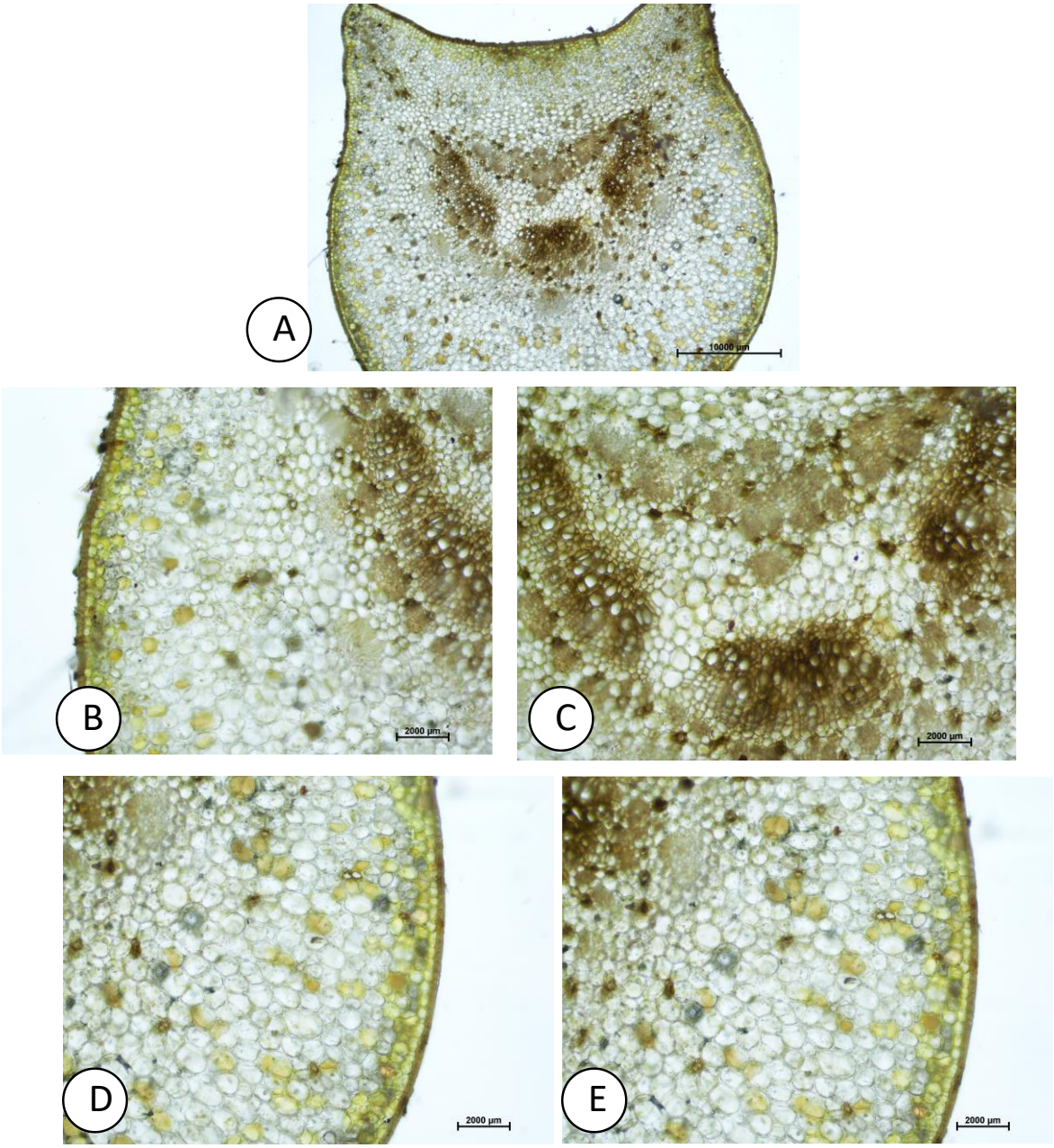

Plate 4.25: T.S. of Petiole stained with Dragendorff's reagent for localization of Alkaloids A) overview of Petiole (4X), B) Epidermal and cortex region (10X), C) Vascular bundle region (10X), D) and E) yellow brown colouration of alkaloids in cortex region. (10X).

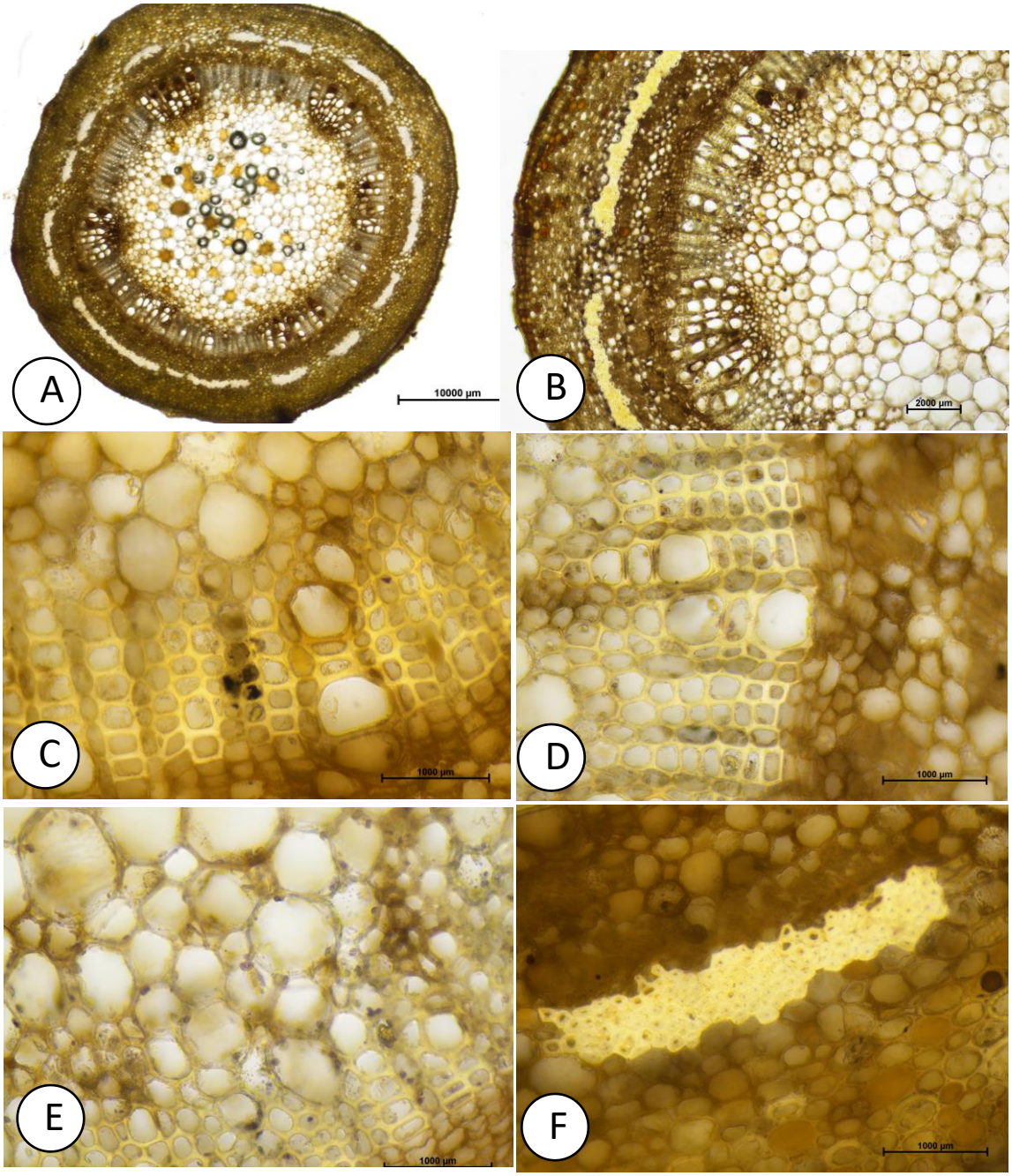

Plate 4.26: T.S. of stem stained with Dragendorff's reagent for localization of Alkaloids A) overview of stem (4X), B) Portion of stem (10X), C),D), E) and F) different colouration of alkaloids.

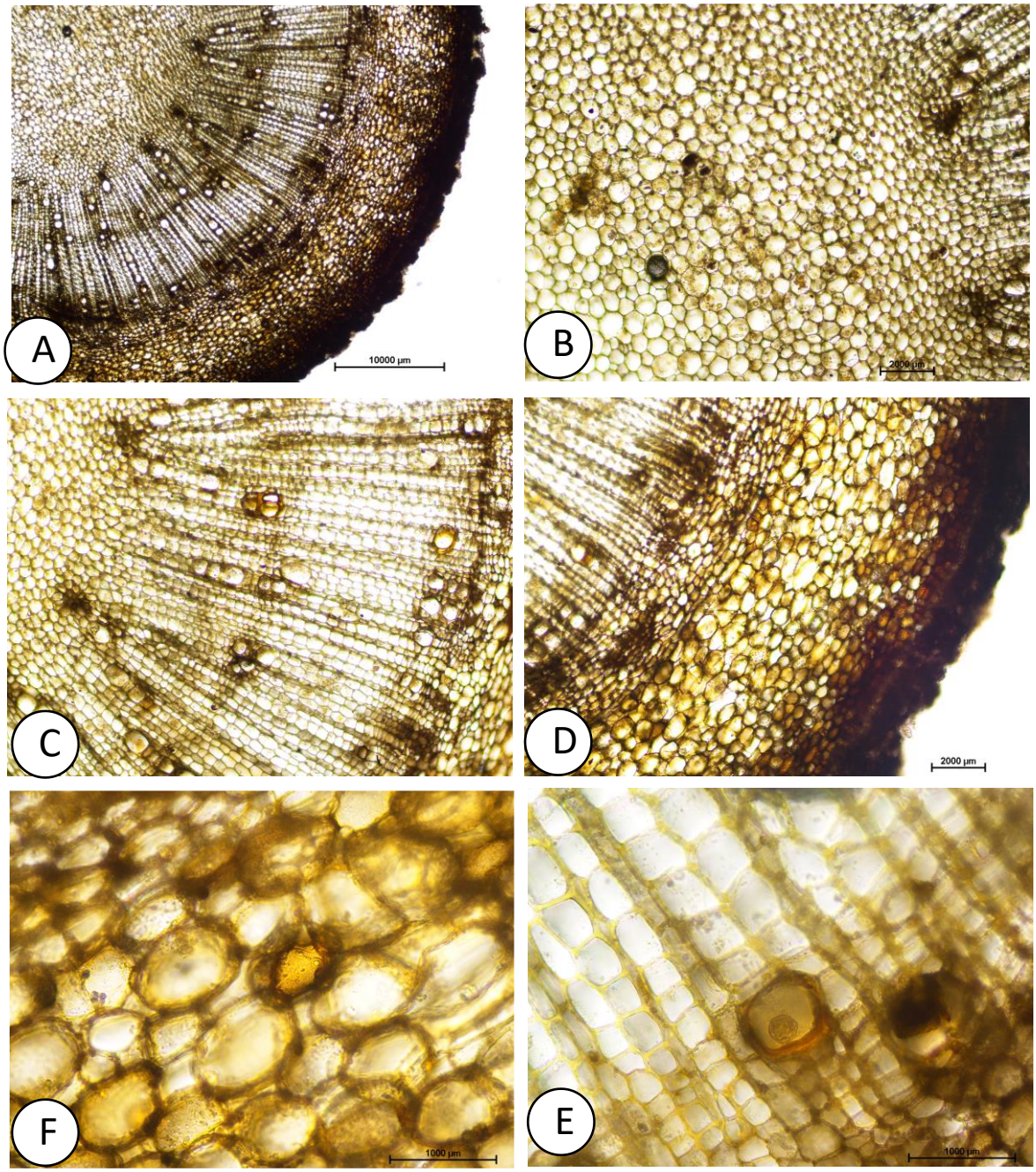

Plate 4.27: T.S. of Root stained with Dragendorff's reagent for localization of Alkaloids A) overview of root (4X), B) pith region (10X), C) pith and the xylem region (10X), D) outer and inner cortex (10X), F) and E) alkaloids I cortex region

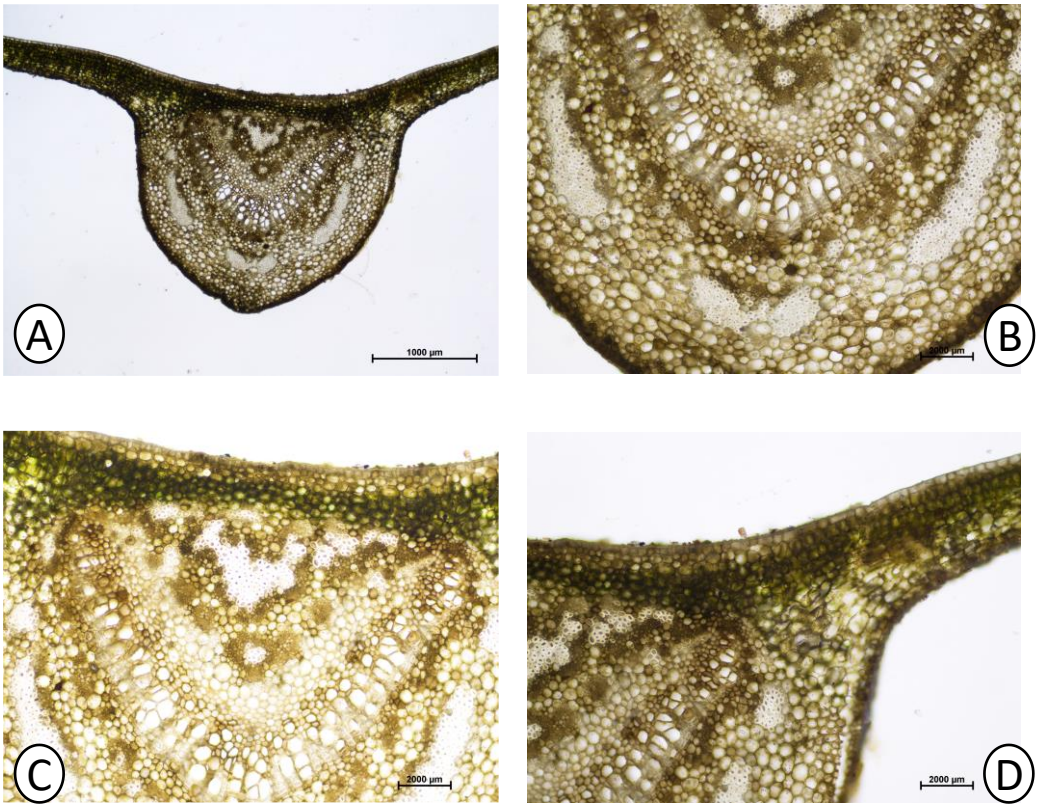

Plate 4.28: T.S. of Midrib stained with $FeCl_3$ reagent for localization of Phenols A) overview of Midrib (4X), B) Vascular bundle region (10X), C) and D) Abaxial side

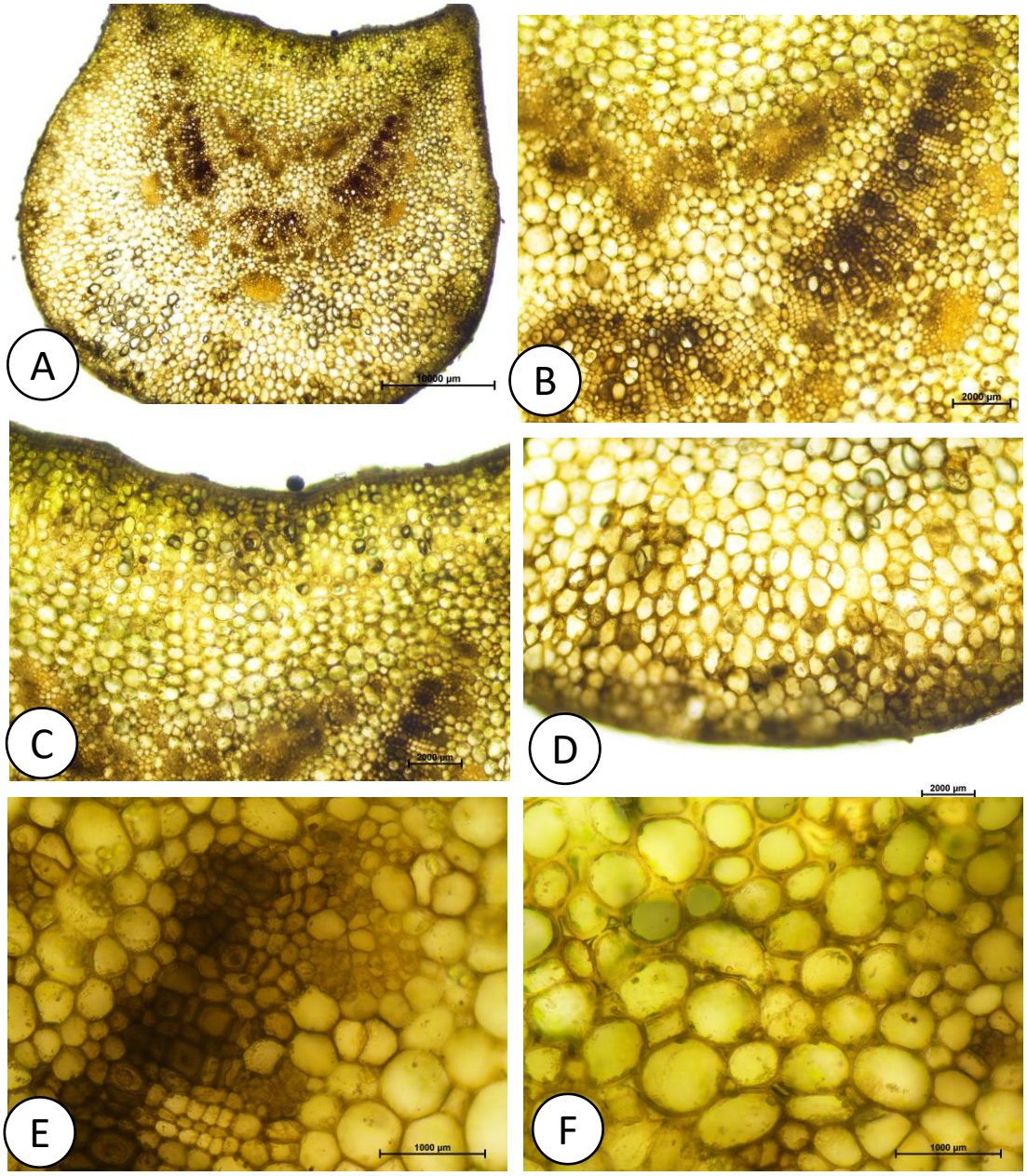

Plate 4.29: T.S. of Petiole stained with $FeCl_3$ reagent for localization of Phenols
A) overview of petiole (4X), B) Vascular bundle region (10X), C) Adaxial region, D) Abaxial region, E) Vascular region, F) Cortex region (40X)

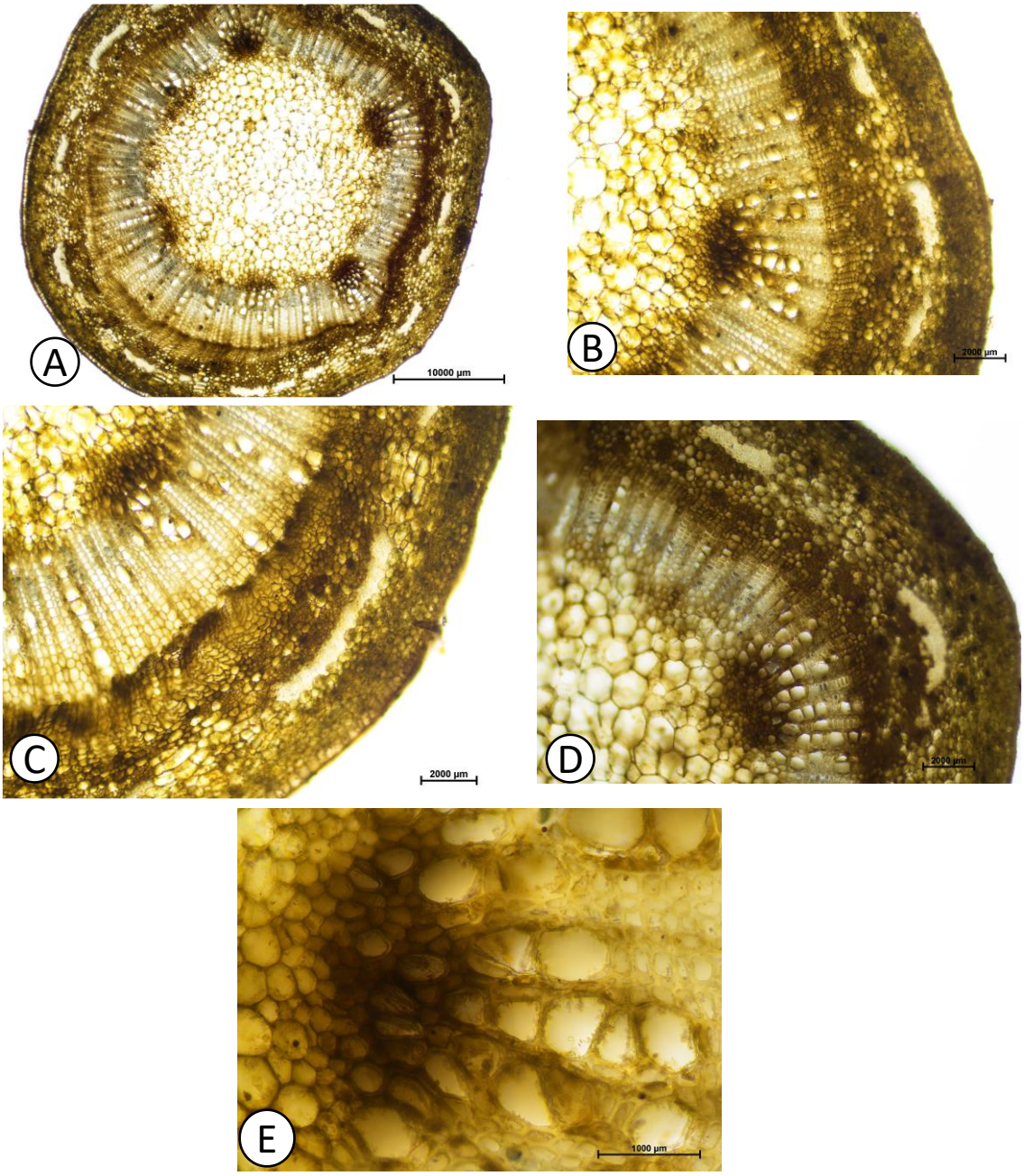

Plate 4.30: T.S. of stem stained with $FeCl_3$ reagent for localization of Phenols A) overview of stem (4X), B) Vascular bundle region(10X), C) , D) Vascular Bundle and Pith region, E) Vascular region(40X)

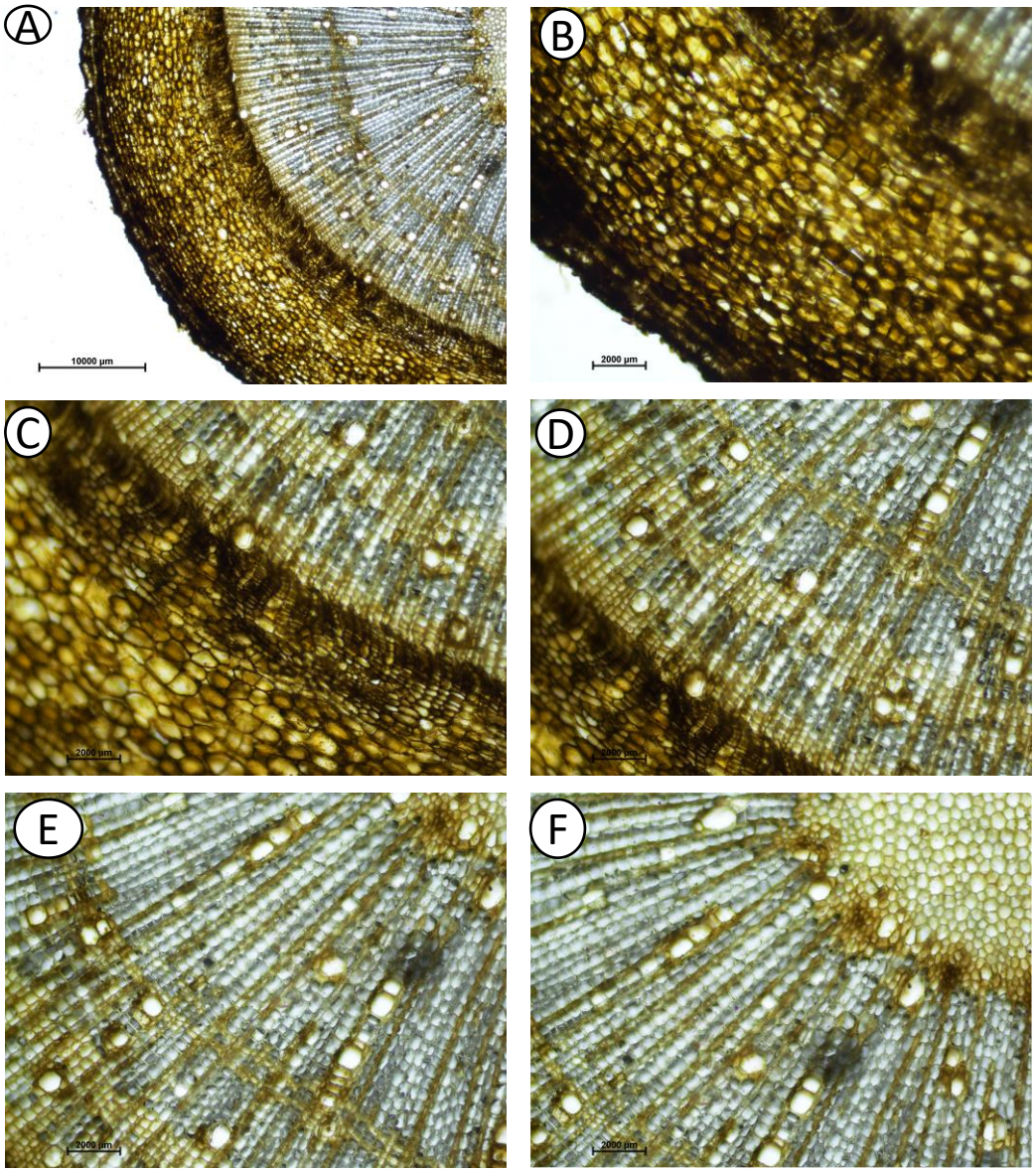

Plate 4.31: T.S. of Root stained with $FeCl_3$ reagent for localization of Phenols A) overview of Root (4X), B) Outer cortex region(10X), C) Phloem region (10X) D) Xylem region, E) annual ring region, F) pith and vascular Bundle region.(40X)

4.4 Total Phenolic Content

The Total Phenolic Content analysis of methanolic and distilled water extracts from the leaf and bark of *Gymnacrannthera canarica* revealed significant differences in phenolic concentration, as depicted in **(Table 4.3 and Fig 4.1.)** Notably, the methanolic extract exhibited higher Total Phenolic Content compared to the distilled water extract for both leaf and bark samples. This indicates that methanol was more effective in extracting phenolic compounds from the plant material than distilled water.

These findings are consistent with previous studies demonstrating the superior solvent ability of methanol for extracting phenolic compounds from plant tissues (Naidoo, 2022). The higher phenolic content in the leaf and bark extracts suggests the potential therapeutic value of *Gymnacrannthera canarica*, given the known antioxidant and medicinal properties associated with phenolic compounds (Kasmi *et al.*, 2021).

The observed differences in phenolic content between leaf and bark extracts may reflect variations in the distribution and composition of phenolic compounds within different plant parts. Further investigations could elucidate the specific phenolic profiles of *Gymnacrannthera canarica* and their pharmacological significance.

4.5 Total Flavonoid Content

The analysis of Total Flavonoid Content in methanolic and distilled water extracts from the leaf and bark of *Gymnacrannthera canarica* is presented in **(Table 4.4 and Fig 4.3)** Interestingly, the bark extracts, whether methanolic or distilled, exhibited higher Total Flavonoid content compared to the corresponding leaf extracts.

This suggests that the bark of *Gymnacrannthera canarica* may be a richer source of flavonoids compared to the leaf. Flavonoids are known for their diverse biological activities,

including antioxidant and anti-inflammatory properties, which could contribute to the medicinal potential of *Gymnacrannthera canarica*.

The observed differences in flavonoid content between leaf and bark extracts may be attributed to variations in the biosynthesis and accumulation of flavonoids in different plant parts. Further studies could focus on identifying and characterizing specific flavonoid compounds present in *Gymnacrannthera canarica* bark and their potential therapeutic applications.

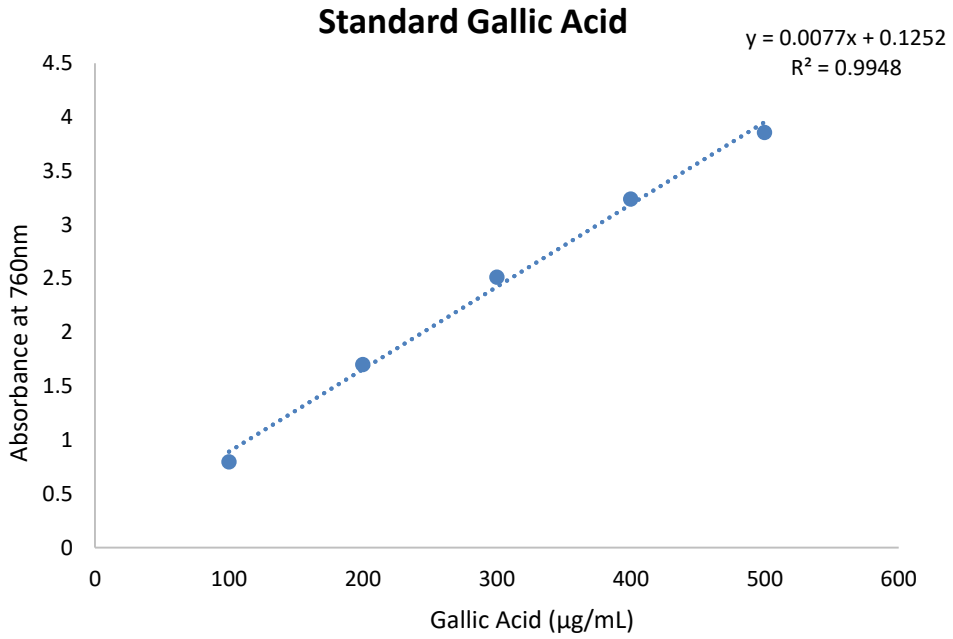

Fig4.1: Calibration curve for Gallic Acid for determination of Total Phenolic content (mg of Gallic Acid equivalent (GAE)/g of extract) at varying concentrations.

Table 4.3: The amount of total Phenolic content (TPC) in Methanolic and Distilled water extracts of *Gymnacranthera canarica* plant parts. Data represent mean values standard deviation.

Solvents	Total Phenolic content (mg of GAE/g of extract)	
	Leaf	Bark
Methanol	211.24 ± 0.052	164.51 ± 0.071
Distilled water	181.83 ± 0.046	154.56 ± 0.002

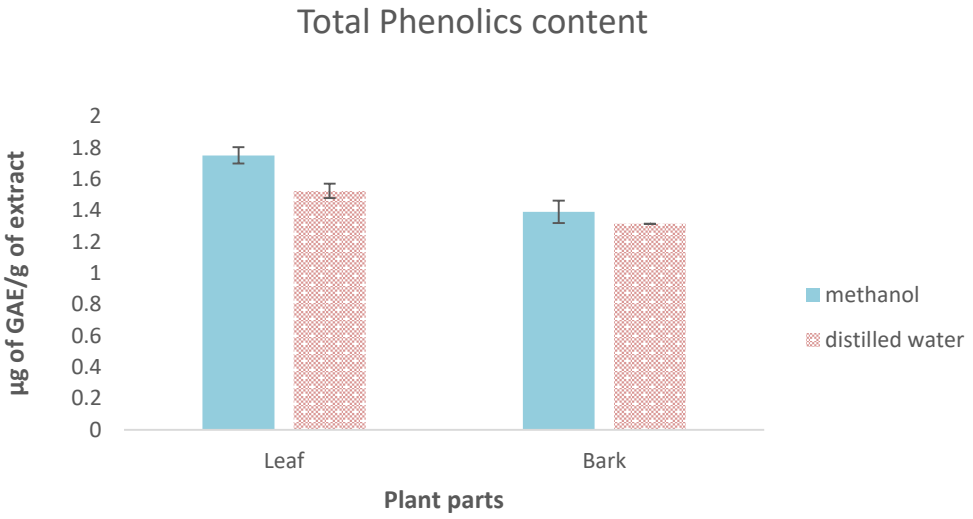

Fig 4.2: Determination of Total Phenolic content (TPC) in mg of Gallic acid equivalent (GAE)/g of Leaf and Bark; (A) Methanolic extract (B) Distilled water, Bars represent men values ± SD (n=2).

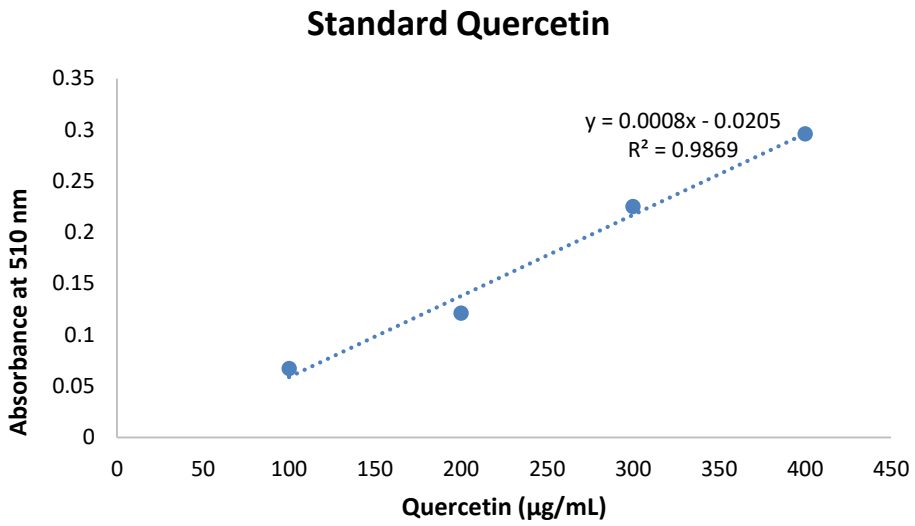

Fig 4.3: Calibration curve for Quercetin for determination of Total Flavonoid content (mg of Quercetin equivalent (QE)/g of extract) at varying concentrations.

Table 4.4: The amount of total Flavonoid content (TFC) in Methanolic and Distilled water extracts of *Gymnacranthera canarica* plant parts. Data represent mean values standard deviation.

Solvents	Total Flavonoid content (mg of QE/g of extract)	
	Leaf	Bark
Methanol	26.80 ±0.063	26.70 ± 0.048
Distilled water	25.69 ± 0.047	25.83 ± 0.052

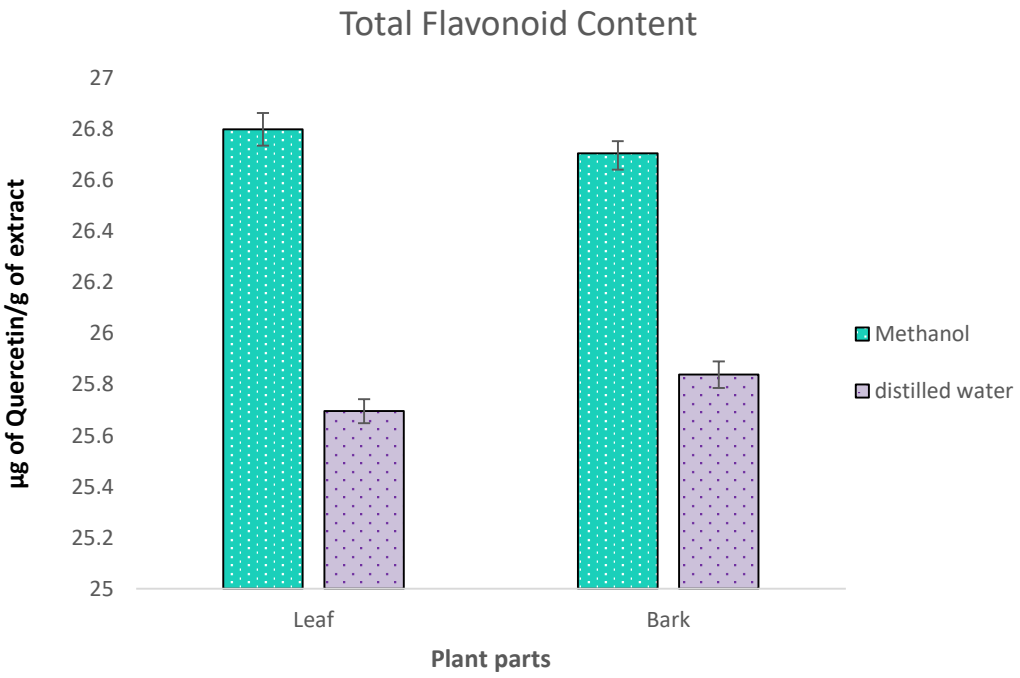

Fig 4.4: Determination of Total Flavonoid content (TFC) in mg of Quercetin equivalent (QE)/g of Leaf and Bark; (A) Methanolic extract (B) Distilled water, Bars represent mean values ± SD (n=2)

4.6 TLC profiling for Alkaloids, Phenolic compounds, Flavonoids.

The TLC profiling was done by using two different solvent system based on polarity to evaluate different classes of compounds present in plant organs. Pre-coated TLC plates were used for the same, loaded with the help of micropipette. The extract were subjected to TLC analysis with the aid of different solvents in order to separate the bioactive compounds like alkaloids, phenolic compounds, flavonoids. The study revealed the development of orange yellow spots for alkaloids after derivatizing agent indicating presence of alkaloids. Dark Blue-Black spots were observed indicating presence of Phenolic compounds and Fluorescent-Red bands were obtained for Flavonoid compounds after spraying with 10% methanolic sulphuric acid under short UV.

4.6.1 Alkaloids

Alkaloids were analyzed using TLC, resulting in the detection of seven distinct bands on the TLC plate. Among the extracts tested, the leaf methanol extract exhibited the highest number of bands, while the distilled water bark extract showed the least. Identification of these bands was accomplished using Dragendorff's reagent, which produced yellow-orange coloration, characteristic of alkaloid compounds **(Plate.4.33, Table. 4.5)**

These findings suggest variations in alkaloid composition among different plant parts and extraction solvents. Further characterization of these alkaloids could provide insights into the pharmacological potential of *Gymnacrannthera canarica*.

4.6.2 Phenolic compounds

Phenolic compounds were analysed using TLC, revealing ten distinct bands on the TLC plate. The leaf methanolic extract exhibited the highest number of bands, while the

methanolic bark extract showed the least with only two bands. Identification of these bands was achieved using 10% ferric chloride reagent, resulting in the appearance of dark zones indicative of phenolic compounds. These results emphasize the differential distribution of phenolic compounds within *Gymnacranthera canarica*, with potential implications for its medicinal properties and ecological role. Further investigation into the specific phenolic profiles of different plant parts could enhance our understanding of their biological activities. **(Plate.4.34, Table. 4.6)**

4.6.3 Flavonoids

The TLC analysis was conducted to detect flavonoids, resulting in five distinct bands observed on the TLC plate. The leaf methanolic extract exhibited the highest number of bands. Identification of these bands was achieved using 10% methanolic sulfuric acid reagent, revealing fluorescent-red bands indicative of flavonoid compounds. These findings highlight the presence of flavonoids in *Gymnacranthera canarica*, with variations in distribution among different plant parts. **(Plate.4.35, Table. 4.7)**

4.6.4 Antioxidant activity using TLC Bioautography

In the antioxidant assay, KMnO4 was employed as a substitute for DPPH due to its similar oxidative properties and cost-effectiveness. KMnO4 was utilized as a derivatizing spraying reagent after TLC plate development. The antioxidant compounds present in the plant extracts caused clear whitish-yellow bands to form against a purple or violet background, indicating potential antioxidant activity. This novel approach using TLC bioautography with KMnO4 as the spraying agent was effective in detecting antioxidant activity in all four plant extracts, namely methanolic leaf, distilled water leaf, methanolic bark, and distilled water bark extract. The methanolic leaf and methanolic bark extracts exhibited more pronounced whitish-yellow bands compared to the distilled water bark

extract. **(Plate.4.36)** These distinct banding patterns underscore the presence of antioxidant activity in the tested plant extracts, as assessed using TLC bioautography with $KMnO_4$ as the spraying agent (Naik and Sellappan, 2021).

Based on the TLC analysis results, the methanolic extract of the leaf displayed a greater number of bands compared to the other three extracts, indicating a higher diversity of phyto-constituents. Specifically, the leaf methanolic extract exhibited 7 spots of alkaloids, with 1 spot identified as alkaloids. Additionally, 3 spots of phenolic compounds were observed, of which 1 spot was identified as a phenolic compound. Furthermore, 5 spots of flavonoids were seen, with 1 spot identified as a flavonoid. In contrast, the leaf distilled water extract did not exhibit any bands in alkaloids and flavonoids but displayed 3 bands of phenols, with 1 band identified as phenols. Similarly, the bark methanolic extract only exhibited 2 bands of phenols. Notably, none of the bands were observed in the distilled water bark extract. These findings suggest that the methanolic extract of the leaf contains a more diverse range of phytochemicals compared to the other extracts, particularly in terms of alkaloids, phenolic compounds, and flavonoids. The absence of bands in certain extracts highlights the variation in phytochemical composition based on the solvent used for extraction and the plant part studied. There are no studies that have demonstrated the efficacy of TLC bioautography using $KMnO_4$ as a derivatizing agent in assessing antioxidant activity in plant extracts. This method offers a cost-effective and non-toxic alternative to traditional DPPH assays, providing valuable insights into the antioxidant potential of natural products.

Table 4.5: Retention factor (Rf) values for plant samples showing possible compounds tested for presence of Alkaloids.

Solvent system	Samples	Number of distinct spots/Bands	Rf value	Colour with spraying agent	Possible compounds
Toluene: Ethyl acetate: Methanol: ammonia 25% (30:30:15:1)	Methanol + Leaf	7	0.90	Green	Unknown
			0.83	Light green	Unknown
			0.61	Black	Unknown
			0.54	Grey	Unknown
			0.49	Light dark blue	Unknown
			0.46	Light orange brown	Alkaloid
			0.42	Grey	Unknown
	Distilled water + Leaf	-	-	-	-
	Methanol + Bark	-	-	-	-
	Distilled water + Bark	-	-	-	-

Table 4.6: Retention factor (Rf) values for plant samples showing possible compounds tested for presence of Phenolic compounds.

Solvent system	samples	Number of distinct spots/Bands	Rf value	Colour with spraying agent	Possible compounds
Toluene:Ethyl acetate: Methanol: ammonia 25% (30:30:15:1)	Methanol + **L**eaf	5	0.973	Green	Unknown
			0.907	Yellow	Unknown
			0.868	grey	Unknown
			0.842	Light brown	Unknown
			0.644	blue	Phenols
	Distilled water + Leaf	3	0.815	grey	Unknown
			0.776	Light brown	Unknown
			0.618	Blue	Phenols
	Methanol + Bark	2	0.9078	grey	Unknown
			0.842	brown	Unknown
	Distilled water + Bark		-	-	-

Table 4.7: Retention factor (Rf) values for plant samples showing possible compounds tested for presence of Flavonoids.

Solvent system	Samples	Number of distinct spots/Bands	Rf value	Colour with spraying agent	Possible compounds
Tetrahydrofuran:Toluene:formic acid:Water (16:8:2:1)	Methanol + Leaf	5	0.894	Florescent orange	Flavonoid
			0.828	White	Unknown
			0.802	Grey	Unknown
			0.75	Light pink	Unknown
			0.578	Dark blue	Unknown
	Distilled water + Leaf	-	-	-	-
	Methanol + Bark	-	-	-	-
	Distilled water + Bark	-	-	-	-

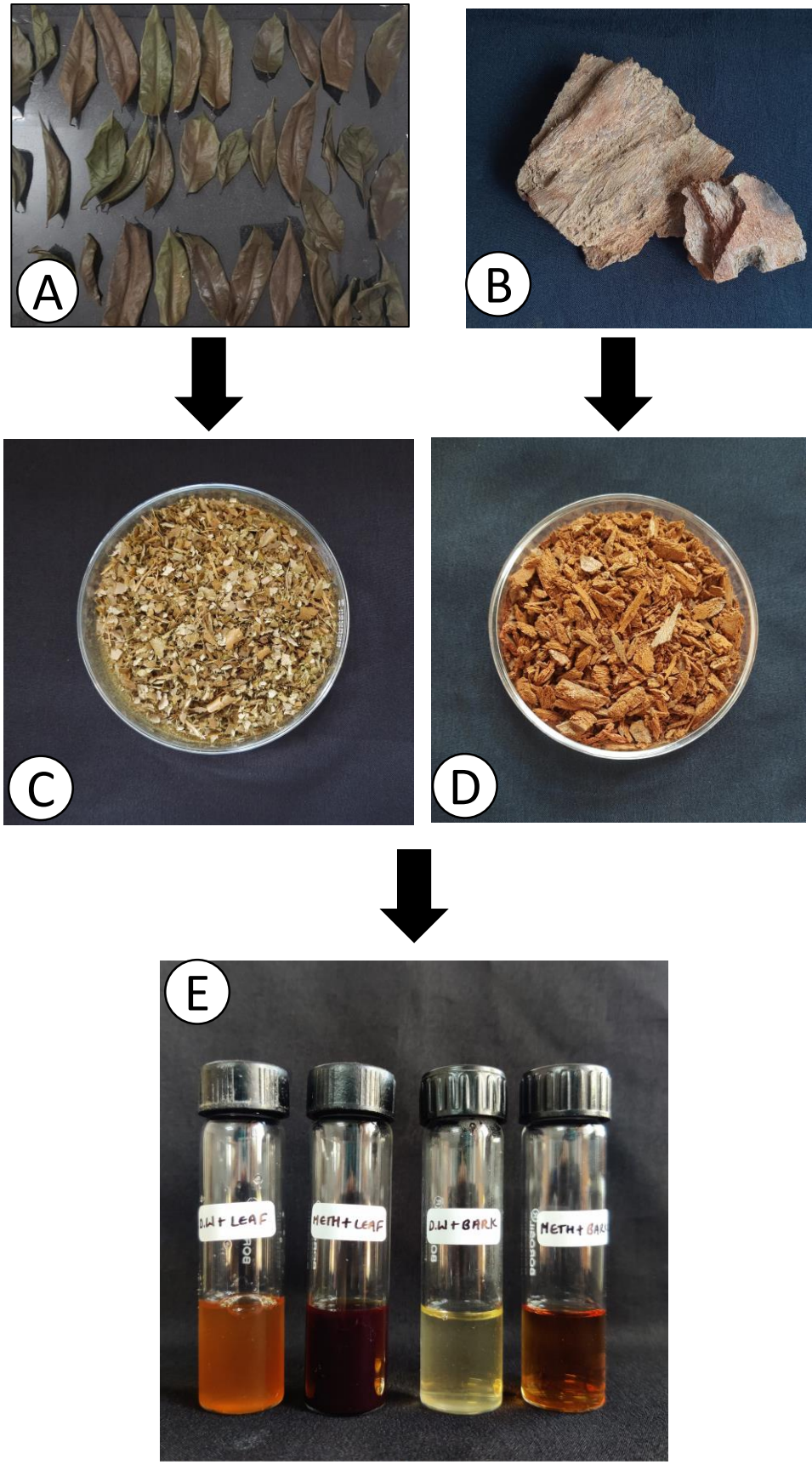

Plate 4.32: Preparation of extracts A) Shade dried leaves B) shade dried Bark C) Leaf powder D) Bark powder E) prepared extracts of methanolic leaf, Distilled water Leaf, methanolic Bark and Distilled water Bark.

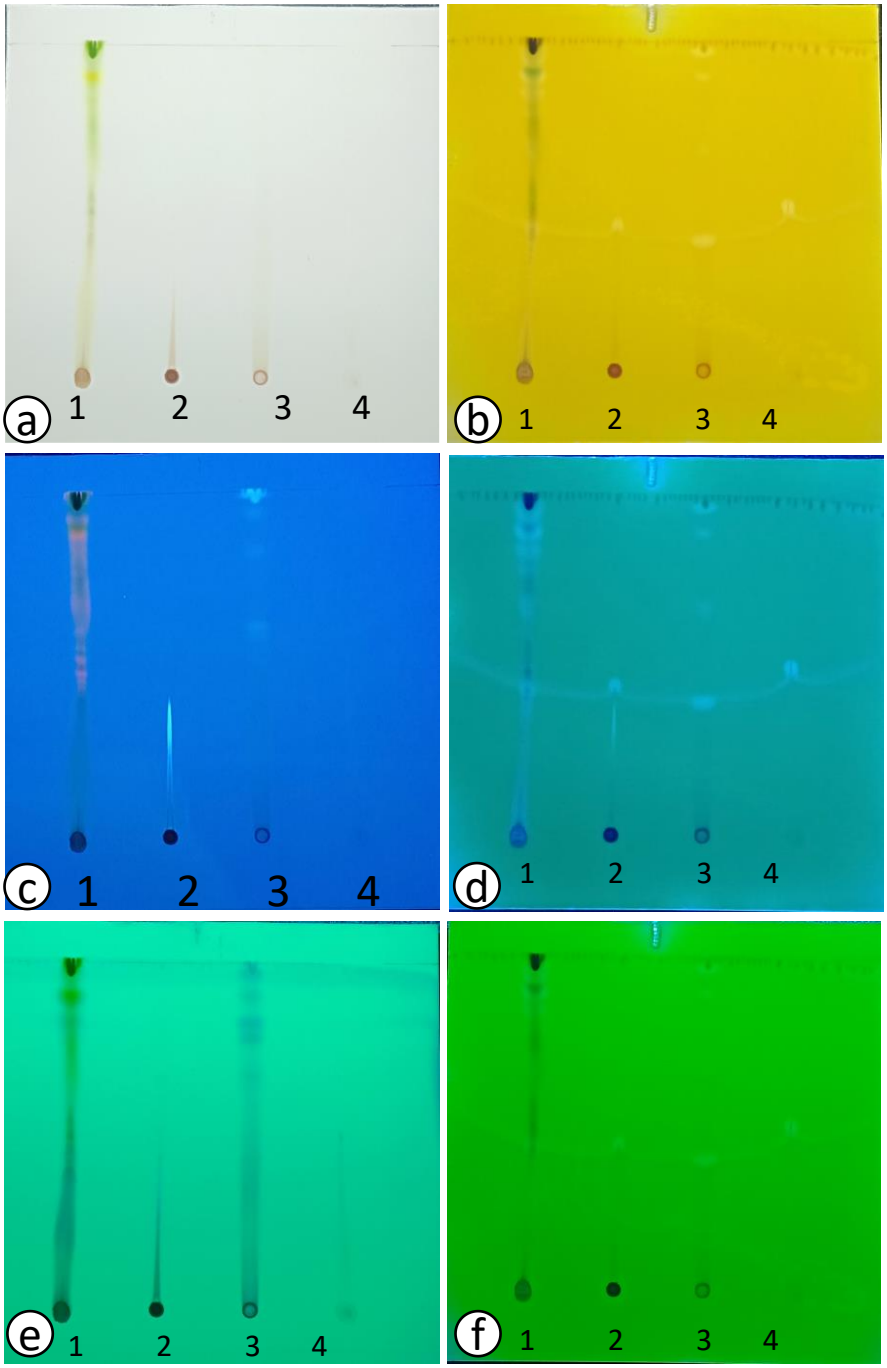

Plate 4.33: TLC isolation of Alkaloid Before And After Derivatization a)visible light before, b)visible light after, c)long UV before, d)long UV After, e)short UV before, f)short UV after. Lanes: 1-Methanollic leaf, 2- Distilled water leaf, 3- Methanolic Bark, 4- Distilled water Bark

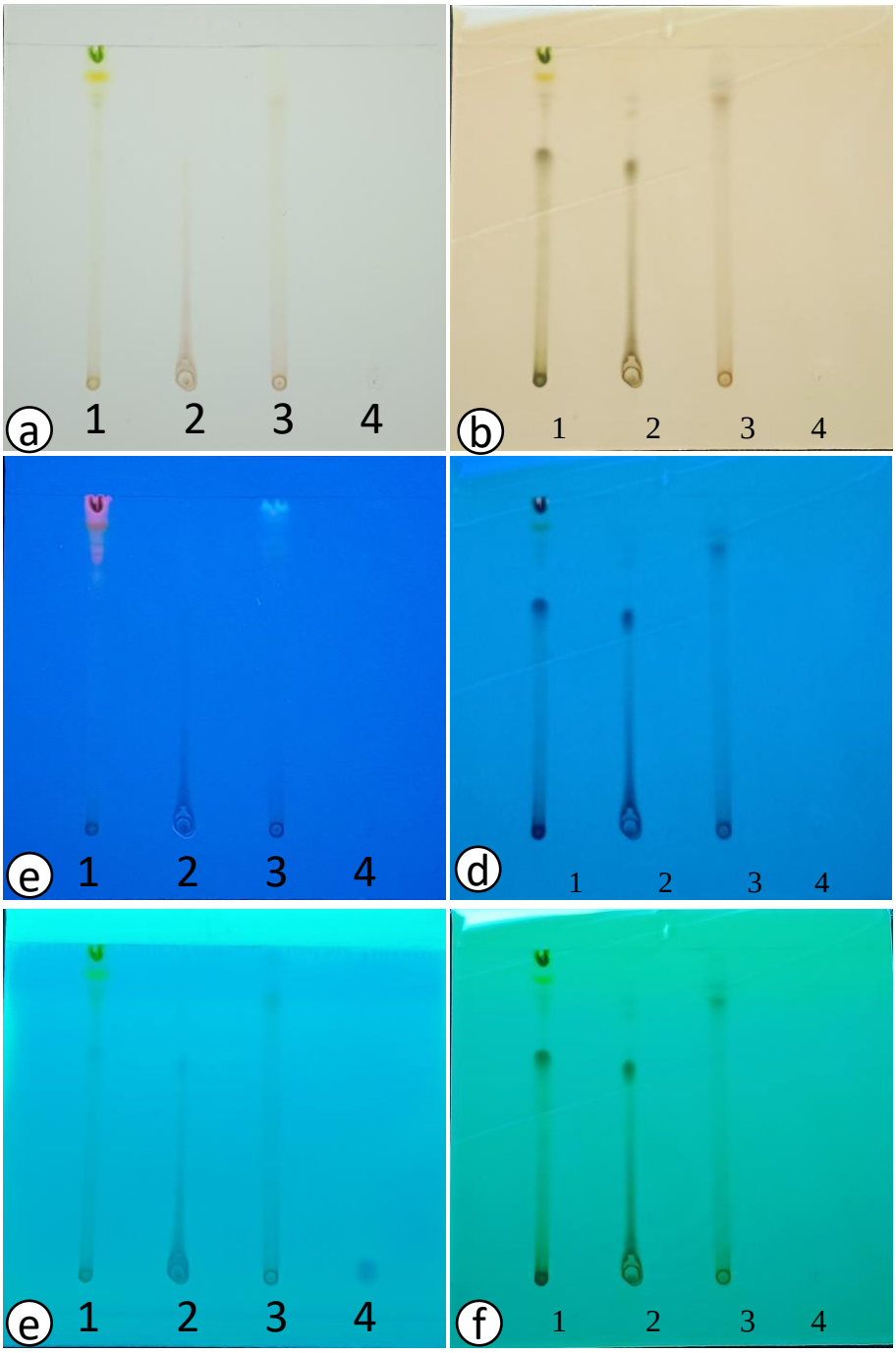

Plate 4.34: TLC isolation of Phenolic compound Before and After Derivatization a)visible light before, b)visible light after, c)long UV before, d)long UV After, e)short UV before, f)short UV after Lanes: 1-Methanolic leaf, 2- Distilled water leaf, 3- Methanolic Bark, 4- Distilled water Bark

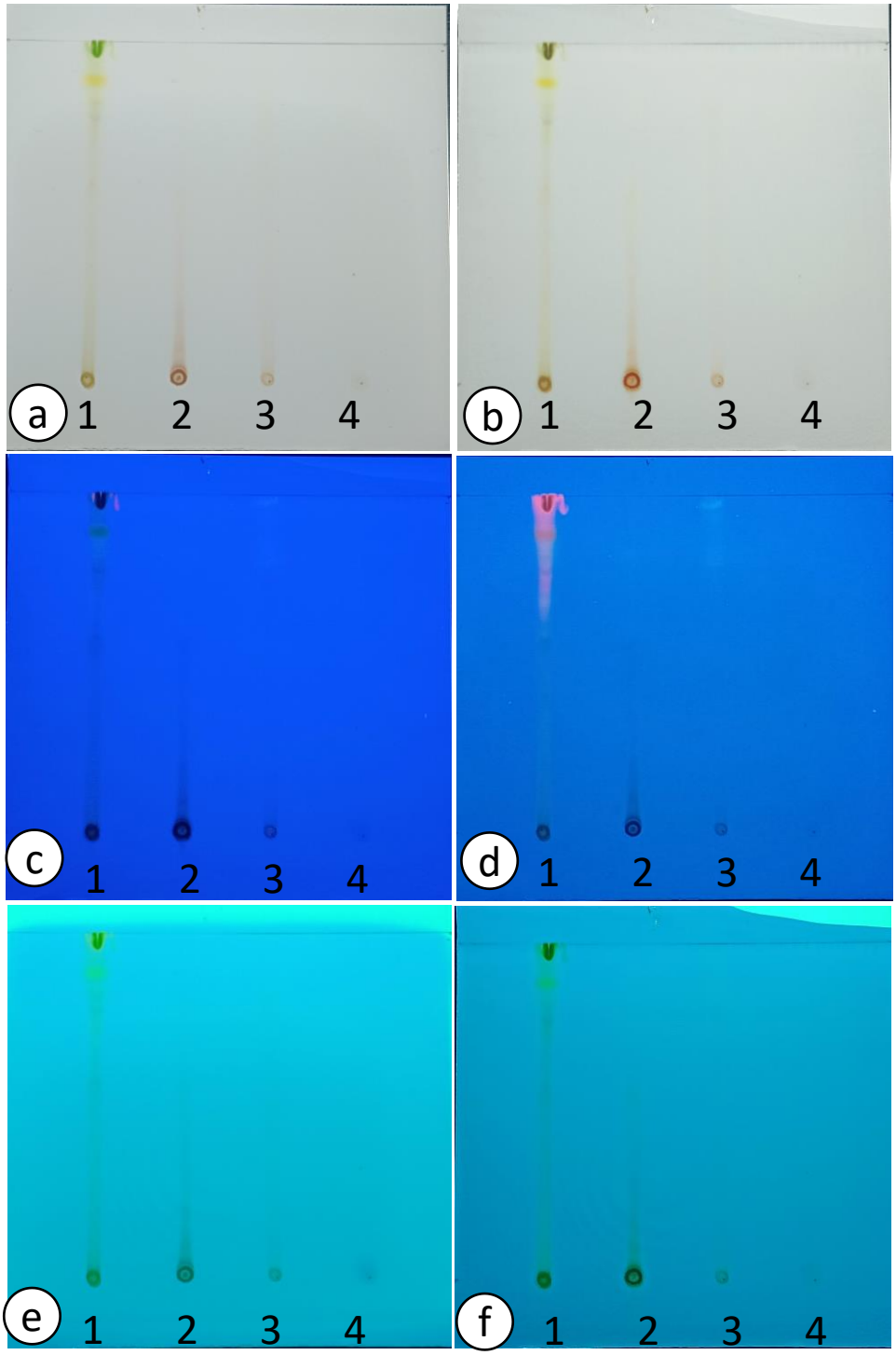

Plate 4.35: TLC isolation of Flavonoids Before And after Derivatization a)visible light before, b)visible light after, c)long UV before, d)long UV After, e)short UV before, f)short UV after Lanes: 1-Methanollic leaf, 2- Distilled water leaf, 3- Methanolic Bark, 4- Distilled water Bark

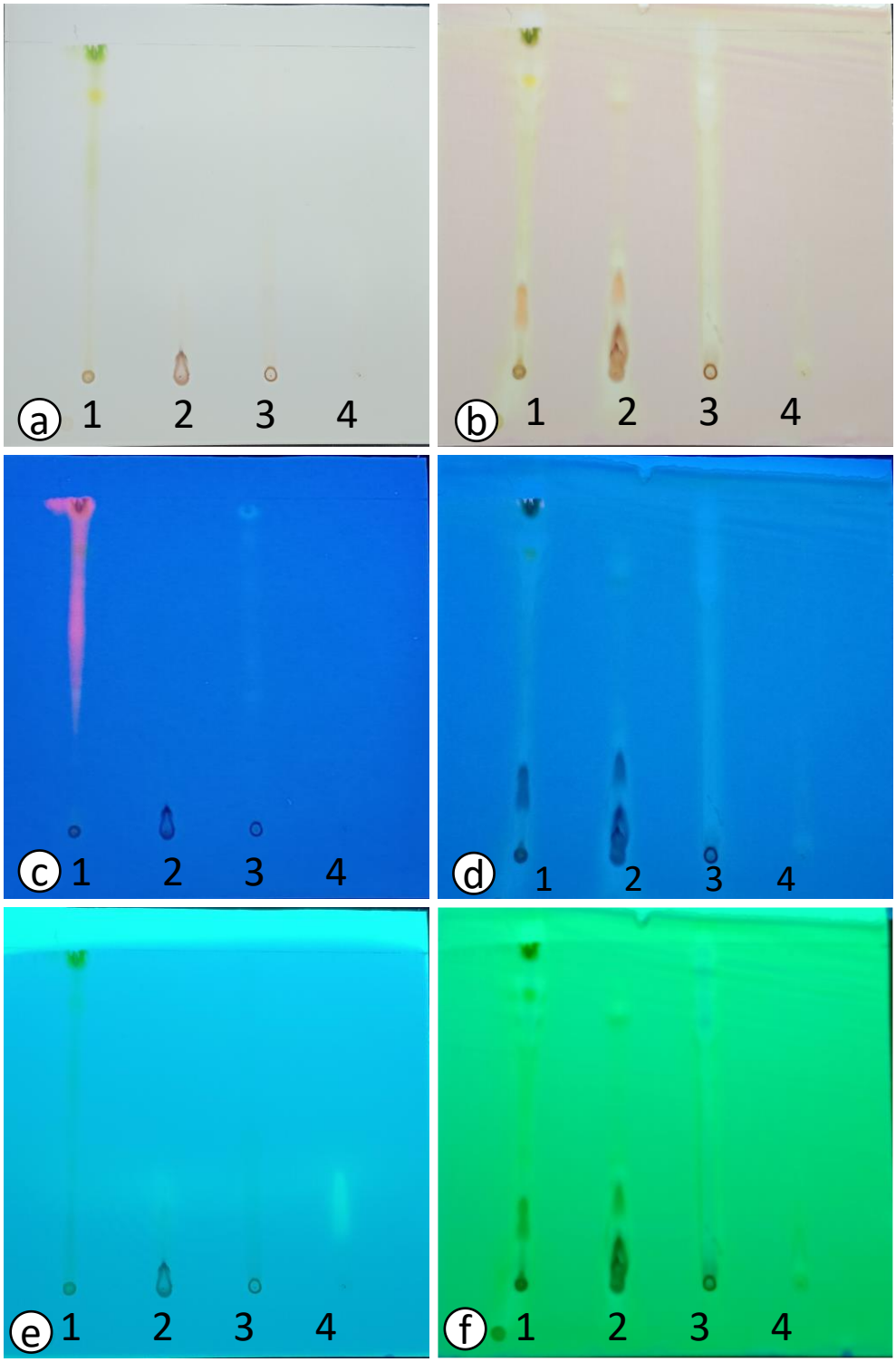

Plate 4.36: Developed TLC Bioautography Before and After Derivatization a) visible light before, b)visible light after, c)long UV before, d)long UV After, e)short UV before, f)short UV after Lanes:1-Methanollic leaf, 2- Distilled water leaf, 3-Methanolic Bark, 4- Distilled water Bark

4.7 Antibacterial studies

The antimicrobial studies of crude leaf and Bark extract of *Gymnacrathera canarica* was evaluated against two Non-Pathogenic Bacterial strains *Escherichia coli and Bacillus* using the Disc Diffusion method.

4.7.1 Disc Diffusion Method

The antimicrobial activity of crude leaf and bark extracts of *Gymnacranthera canarica* was evaluated against Escherichia coli and Bacillus sp. strains using the disc diffusion method. The results revealed minimal to no zone of inhibition on the agar plates, indicating limited antibacterial efficacy. Both methanolic and distilled water extracts of leaves and bark exhibited positive results against E. coli, while only the distilled water extracts showed activity against *Bacillus sp*.

Further comparison between the extracts demonstrated that the methanolic leaf extract displayed the highest inhibitory action against both bacterial strains, followed by the distilled water leaf extract. Conversely, the bark extracts showed lesser inhibitory effects compared to the leaf extracts, irrespective of the solvent used for extraction.

These findings suggest that *Gymnacranthera canarica* extracts possess some degree of antibacterial activity, albeit relatively modest. However, the observed variations in efficacy between different extracts underscore the importance of solvent selection and extraction method optimization in maximizing the antimicrobial potential of plant extracts.

It is worth noting that while the extracts showed some antibacterial activity, their effectiveness was limited compared to standard antibiotics. Further research employing different concentrations, extraction techniques, and purification methods may be warranted

to enhance the antibacterial properties of *Gymnacranthera canarica* extracts and explore their potential applications in combating bacterial infections.

These findings suggest that *Gymnacranthera canarica* possesses antimicrobial properties, particularly against *Escherichia coli* and *Bacillus* strains. However, the efficacy of the extracts varied depending on the solvent used for extraction and the plant part. Previous studies, such as those by Khanchandani *et al.,* (2019) and Samri et al. (2015), support the antimicrobial potential of plant extracts against multidrug-resistant bacterial strains. Additionally, Saleh et al. (2020) and Tadjine & Meeso-Moumene (2024) have demonstrated the effectiveness of plant extracts in inhibiting the growth of pathogenic bacteria.

These collective findings underscore the importance of exploring natural sources for novel antimicrobial agents, especially in the context of increasing antibiotic resistance. Further investigations are warranted to elucidate the specific bioactive compounds responsible for the observed antimicrobial activity and to assess their potential for pharmaceutical application.

Table 4.8: Antibacterial activity of Leaves and Bark showing Presence or absence of zone of inhibition using Methanol.

Sr. no.	Methanol plant extract/Negative control	Antibacterial Activity	
		E. coli	***Bacillus***
1	Leaves	+	+
2	Bark	+	+
3	Methanol	-	-

No inhibition (-), inhibition (+)

Table 4.9: Antibacterial activity of Leaves and Bark showing Presence or absence of Zone of inhibition using Distilled water

SR. NO.	Distilled water plant extract/negative control	Antibacterial Activity	
		E. coli	***Bacillus***
1	Leaves	+	+
2	Bark	+	+
3	Distilled water	-	-

No inhibition (-), inhibition (+)

Table 4.10: Antibacterial activity of Leaves and Bark using Methanol and Distilled water

SR. NO.	SOLVENT EXTRACTS	BACTERIAL STRAIN	LEAF	BARK
			Zone of inhibition (in mm)	
1	**Methanol**	***E. coli***	7.7 ± 0.963	7 ± 0.988
2		***Bacillus***	5 ± 0.865	4.33 ±0.785
1	**Distilled water**	***E. coli***	7.7 ± 0.587	6.7 ± 0.583
2		***Bacillus***	9.7 ± 0.576	7.33 ± 0.58

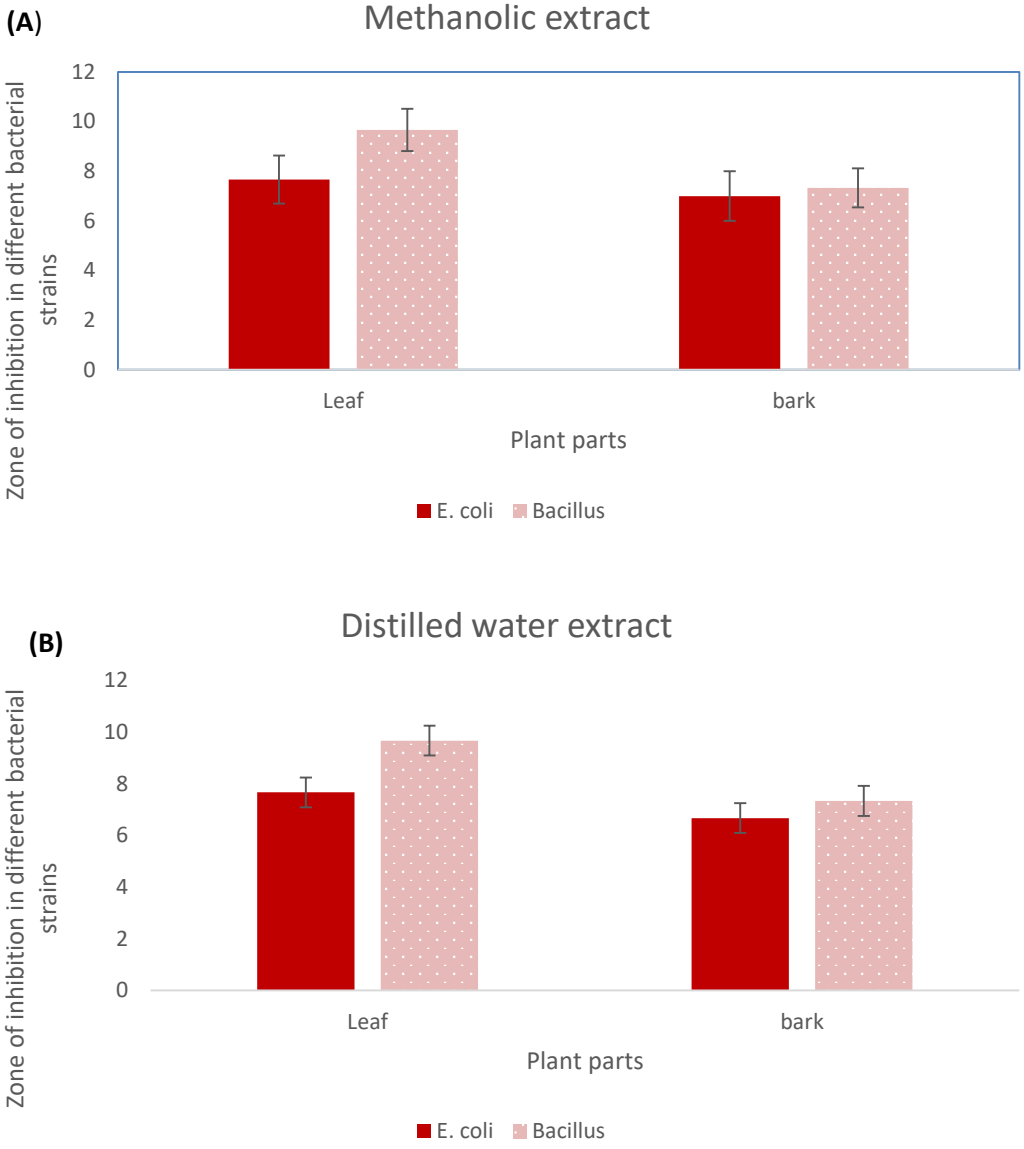

Fig 4.5: Bar graph showing *Gymacranthera canarica* Plant part leaf and Bark extract on Bacterial strains

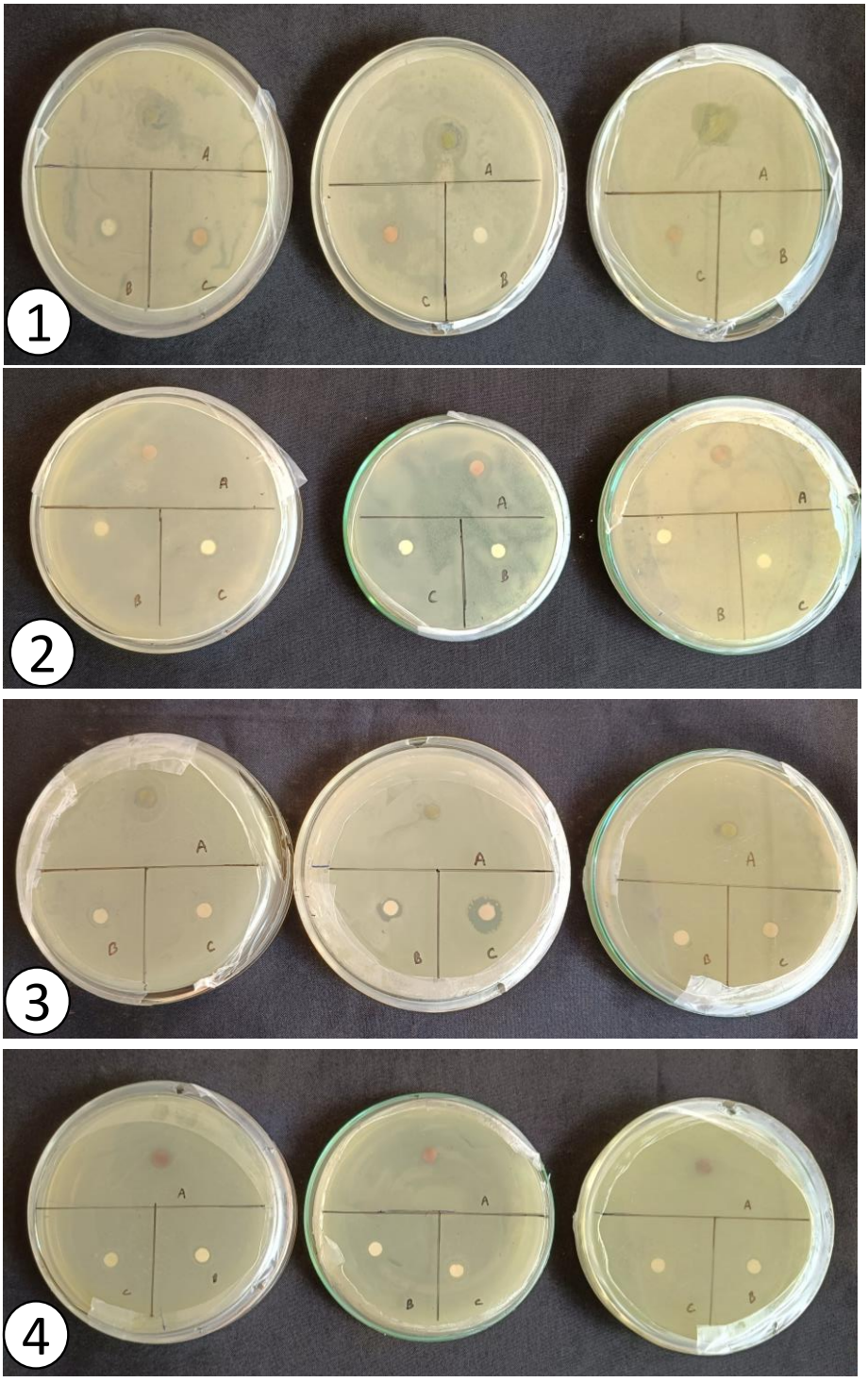

Plate 4.37: Antibacterial activity By Disc diffusion Method using leaf Extracts and Bark Extracts 1) image showing positive result Of Methanolic leaf and Methanolic Bark extract against *Bacillus subtilis* 2) image showing positive result Of D.W leaf and D.W Bark extract against *Bacillus subtilis* 3) Image showing positive results result Of Methanolic leaf and Methanolic Bark extract against *E.coli* 4) Image showing positive results result of D.W leaf and D.W Bark extract against *E.coli*

CONCLUSION

In this study on *Gymnacranthera canarica* anatomical , histochemical investigation and Bioactivity studies of its different parts various methodologies were employed to unveil and explore the structural attributes, tissue-specific metabolites, and potential bioactive compounds present also it sheds light on its ecological significance and pharmacological potential. Anatomical studies using Safranin stain and Toluidine blue O revealed features of *Gymnacranthera canarica*, such as leaf epidermal patterns, vascular tissue arrangement, and root architecture.

Histochemical studies were carried out to localize Primary and secondary metabolites which revealed the presence of Starch, Proteins, Lipids, Alkaloids & Phenols in different parts such as Petiole, Midrib, Stem and Root in different concentrations.

For the Bioactivity studies Plant parts Leaf and Bark was shed dried and extracts were prepared using maceration. Likewise, two solvents were used for extraction viz; Methanol and Distilled water. It concluded that Methanol is the best solvent for extraction than the other. Phytochemical estimation also highlighted highest Total Phenolic and Flavonoids content of methanolic extract. Thin Layer chromatography confirmed the presence of alkaloids, Phenols, Flavonoids and antioxidant potential in the extract.

Further the antibacterial studies of leaf and Bark showed positive results against two Bacterial strains i.e. *E.coli* and *Bacillus.* Leaves shows higher zone of inhibition against both the bacteria than the Bark. Hence it can be concluded that Leaves Possess more Antibacterial activity.

SUMMARY

The present study focused on the comprehensive anatomical characterization, histochemical analysis, and bioactivity assessment of *Gymnacranthera canarica*, the sole species of the genus *Gymnacranthera* found in the Western Ghats of India. *Gymnacranthera canarica* is an endangered species inhabiting the Myristica swamps, a critically endangered ecosystem primarily found in Goa, Karnataka, and Kerala.

Specimens of *Gymnacranthera canarica* were collected from Valpoi Sattari in North Goa for anatomical and histochemical analyses. Various plant parts including leaves, petioles, stems, and roots were utilized. Crude extracts of leaf and bark using methanol solvent were employed for bioactivity studies. Additionally, thin-layer chromatography (TLC) was conducted on leaf and bark extracts to analyse the phytoconstituents.

The results indicated that methanolic extracts of both leaf and bark exhibited significant resolution of phytoconstituents such as alkaloids, phenols, and flavonoids in TLC. Moreover, TLC bioautography using KMnO4 as a derivatizing agent revealed potential antioxidant activity in both leaf and bark extracts.

Furthermore, antibacterial studies were conducted using methanol and distilled water extracts of leaf and bark against *Escherichia coli* and *Bacillus* sp. strains. The methanolic extracts of both leaf and bark demonstrated notable antibacterial activity against the tested bacterial strains.

Anatomical observations revealed distinctive features such as trichomes, cuticles, palisade layers, and boomerang-shaped vascular bundles in the leaf midrib. Histochemical analysis confirmed the presence of various primary and secondary metabolites, with a higher concentration of alkaloids and phenols observed in the leaves.

Overall, this study provides valuable insights into the anatomical, chemical, and bioactivity characteristics of *Gymnacranthera canarica,* highlighting its potential pharmaceutical importance and emphasizing the need for conservation efforts due to its endangered status in the Myristica swamps ecosystem.

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