# Exploring Endophytic Fungal Diversity in *Gymnacranthera canarica*: Unveiling Bioactive Compounds of The Sacred Groves of Western Ghats, Goa, India

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## DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Exploring Endophytic Fungal Diversity in *Gymnacranthera canarica*: Unveiling Bioactive compounds of the Sacred Groves of Western Ghats, Goa" is based on the results of investigations carried out by me in the Botany Discipline at the School of Biological science and Biotechnology, Goa University under the Supervision of Dr. Aditi V. Naik and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities / College will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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## COMPLETION CERTIFICATE

This is to certify that the dissertation report "Exploring Endophytic Fungal Diversity in *Gymnacranthera canarica*: Unveiling Bioactive compounds of the Sacred Groves of Western Ghats, Goa" is a bonafide work carried out by Ms. Renuka Krishnappa Malgatti under my supervision in partial fulfilment of the requirements for the award of the degree of Masters in the Discipline of Botany at the School of Biological Science and Biotechnology, Goa University.

**Dr. Aditi Venkatesh Naik**

Date: 08·04·2024

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Date: 8Th April 2024

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# DEDICATION

This Study is dedicated to my Mother Mrs Laxmava K. Malgatti and Father Late Mr. Krishnappa R. Malgatti for inculcating the importance of education and being my biggest support system throughout my journey. To my dear friend Mr. Lakshmanan G. for the constnat montivation and to Boost interest and enthusiasm for research.

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# CONTENT

# PREFACE

In the verdant swamps of Myristica, where time seems to have stood still, lies a treasure trove of biodiversity that beckons the scientific community with its untapped potential. The Sacred Groves of Myristica swamps are home to the endemic tree species of the Magnoliids, a lineage of flora with ancient origins and enigmatic attributes. These species, with their archaic features, are not just remnants of a bygone era but are living libraries waiting to divulge secrets that could revolutionize our understanding of bioactive compounds and their myriad uses for humanity.

Among these botanical wonders stands *Gymnacranthera canarica*, a species that dominates the Myristicaceae family within these relic swamps. Despite its prominence, it remains a largely unexplored reservoir of phytochemicals and bioactive compounds. The existence of endophytic fungi within this species, and their potential role in adapting to the swampy conditions, presents a fascinating avenue for research. These microscopic symbionts, through their intimate association with the host plant, may hold the key to unlocking new compounds with significant biomedical applications.

The study delves into the biogenic synthesis of silver nanoparticles using endophytic fungi, a process that not only showcases the fungi's ability to reduce and stabilize nanoparticles but also highlights their potential in creating products with substantial biomedical utility. Isolating 20 endophytic fungi from the leaves of *Gymnacranthera canarica*, the research identifies *Diaporthe sp*, *Phyllostica sp*, and *Penicillium sp* as the dominant species. These fungi, when screened for phytochemicals, exhibit a rich presence of phenols, flavonoids, alkaloids, and steroids, demonstrating antioxidant activity that surpasses that of the plant extracts. The study's findings are particularly noteworthy in the context of *Diaporthe hongkongensis*, which has been instrumental in the biogenic synthesis of silver nanoparticles. These fungal extracts, in conjunction with the synthesized nanoparticles, have shown remarkable antibacterial activity against several human pathogenic bacterial strains, including *Salmonella Typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Streptococcus pyogenes*. The efficacy of these compounds, as evidenced by the Well Diffusion Method, underscores their superiority over traditional Disc Diffusion methods.

This pioneering research lays the groundwork for further exploration of bioactive compounds with medicinal and industrial applications. It underscores the critical importance of conserving these unique species and their habitats not only for the advancement of science but also for the preservation of nature and the well-being of mankind. As we stand at the crossroads of ecological conservation and scientific discovery, the Sacred Groves of Myristica swamps emerge as a beacon of hope, guiding us towards a sustainable future where nature and human progress coexist in harmony.

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# PLATES





# TABLES

**FIGURES**

# ABBREVATIONS

| Entity | Abbrevations |
|---|---|
| Silver nanoparticles | $AgNO_3$/Ag Nps |
| Agar Well Diffuion Method | AWDM |
| Central Sophisticated Instrumentation Facility | CSIF |
| Disc Diffusion Method | DDM |
| Fungal extract 1 *Diaporthe sp* non-sprorulated stage | F1 |
| Fungal extract 2 *Diaporthe sp* sprorulated stage | F2 |
| Fungal extract 3 *Penicillium sp* | F4 |
| Fungal extract 4 *Phyllostica sp* | F3 |
| Figures | Fig |
| Gallic Acid Equivalent | GAE |
| Yeast Extract Peptone Agar | GYP |
| Potato Dextrose Agar | PDA |
| Potato Dextrose Broth | PDB |
| Plant Extract | P.E |
| Reactive Oxygen species | ROS |
| Retention Factor | $R_f$ |
| Scannig Electron Mircoscope | SEM |
| Thin Layer Chromatography | TLC |
| Total Phenolic content | TPC |
| UV-Visible Spectrophotometry | UV-VIS |



# ABSTRACT

Scared Groves of Myrsitca swamps fostering the Endemic tree species belonging to Magnoliids, having features of relatively archiac nature and are yet to explored for unvailing bioactive compunds and its potential uses for mankind. *Gymnacranthera canarica* despite being the dominant species amongst the Myristicaceae family members of the relic Myristca swamps very minimal work has been carried out in understanding the abundance of phytochemicals, bioactive compunds the existence of endophytic fungi and their potential role in adaptation to the swampy conditions. Using the endophytic fungi to reduce and stabilise silver nanoparticles during their biogenic synthesis with potential biomedical application. A total of 20 endophytic fungi were isolated from the leaves of *Gymnacranthera canarica* of which the dominat species were *Diaporthe* and *Phyllostica* and *Penicillium.* These dominant fungi were screened for phytochemicals in comparison with the plant extract using TLC method which revealed the fungal extracts were rich in phenols, flavonoids, alkaloids, steroids and exhibhited antoxidant activity in bioautography studies. The dominant endophytic fungi, *Diaporthe hongkongensis* was used for biogenic synthesis of silver nanoparticles.The Fungal extracts along with the silver nanoparticles synthesiszed showed remarkable antibacterial activity against few human pathogenic bacterial strains such as *Salmonella Typhi, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pyogene* Well Dffusion Method showed better results in comparison with Disc Diffusion method. This study contributes to the foundational work for futher exploration of bioactive compunds with medicinal and industrial application. Through this study we establish the need and importance in the conservation of these ecosystems is not just a matter of ecological importance but a step towards safeguarding a natural heritage that holds immense promise for the future of both nature and humanity.

**Key Words:** Myristica swamps, *Gymnacranthera canarica,* endophytic fungi, *Diaporthe hongkongensis*, *Phyllosticta fallopiae,* Antibacterial, Silver nanoparticles.

# CHAPTER 1: INTRODUCTION

# 1. INTRODUCTION

The Western Ghats, a UNESCO World Heritage Site, encapsulates a rich tapestry of biodiversity, hosting myriad ecosystems teeming with unique flora and fauna. Within this expanse, the sacred groves harbor a treasure trove of plant species, among them the *Gymnacranthera canarica* (Bedd. ex King) Warb., an emblematic tree species revered for its cultural significance and ecological role Chandran et al. (2010). During a study on ecological history of the Western Ghats Chandran (1997) said that Western Ghats, first came under human influence during the palaeolithic era, 12000 yr BP. The most diverse plant communities are found in the tropical rain forests Anitha (2010). The extensive variations of annual rain fall of 1000-6000 mm along with the geographical complexities and anthropogenic factors such as forest alteration for agricultural purposes has lead palaeoendemism or ‘relic’ forest formation in the Western Ghats Chandran et al., (2010).

The Endemic and Endangerd plant species show abundance of bioactive compunds which may posses potential medicinal properties Tavares *et al.,* (2012). These medicinal properties maybe induced by the endophytic fungi by production and secretion of bioactive compunds and enzymes.

Endophytic fungi, a relatively unexplored facet of the Western Ghats' ecological wealth, reside symbiotically within the tissues of host plants, often unnoticed yet holding immense potential. *Gymnacranthera canarica*, being the most dominant, having a high frequency of its occurance in most of the swamps, possessing the medicinal and cultural importance along with its remarkable adaptation to the Western Ghats' microenvironments, may harbour a diverse array of these fungi, each potentially synthesizing bioactive compounds with significant implications for various sectors, including pharmaceuticals, agriculture, and ecological conservation.

The exploration of endophytic fungi within *Gymnacranthera canarica* aligns with the urgent need to decipher the biochemical intricacies of these fungal associations. Unravelling their diversity, understanding the bioactive compounds they synthesize, and assessing their potential applications are crucial endeavours. These fungi, hidden within the plant's tissues, possess the capability to produce secondary metabolites known for their pharmacological and ecological importance. This research endeavour presents a unique opportunity to uncover these latent bioactive compounds and elucidate their potential roles in pharmaceutical development, crop protection, and environmental conservation (Bhardwaj & Agrawal 2014; Devi & Prabakaran 2014)

Furthermore, the growing human footprint in the Western Ghats, coupled with anthropogenic pressures, poses a serious threat to the fragile ecosystems, including the sacred groves housing *Gymnacranthera canarica*. Human interventions in the form of land-use changes, urbanization, and agriculture encroach upon these pristine habitats,

endangering not just the plant species but also the delicate equilibrium between the diverse organisms inhabiting these ecosystems Chandran et al., (1998). Understanding the endophytic fungi within *Gymnacranthera canarica* offers a multifaceted approach - from uncovering potential pharmaceutical leads to contributing insights into ecological conservation strategies.

### 1.1.1 MYRISTCA SWAMP ECOLOGY

Myristica swamps, fresshwater swampy forests are biodiversity hospots, harbouring a range of endemic species. Some of the plant species found in these swamps are *Gymnacranthera canarica, Syzygium travancoricum*, *Lophopetalum wightianum*, *Knema attenuata*, *Myristica malabarica*, *Myristica fatua var magnifica* and *Semecarpus kathalekanensis* which are listed on the IUCN Red List of Threatened Species (Chandran et al., 1998; Rao et al., 2014). The myristica swapms get this name as swamps are dominated by trees belonging to myristicaeae family. These swamps form an integral parts of the western Ghats' hydrological network and act as natural reservoirs, controlling floods and maintaining water quality Ranganathan *et al.,* (2022). The soil in Myristica swamps range from sandy, loamy, gravel to alluvial, rich in orgnic matter and remains waterclogged throughout the year Ragu *et al.,* (2006). These swamps provide critical ecosystem services, including carbon sequestration, supporting fisheries, and sustaining local water cycles. They also contribute to the livelihoods of indigenous communities through non-timber forest products Ranganathan *et al.,* (2022). Human activities like deforestation, conversion to agricultural land, and pollution pose a threat to the ecological significance of Myristica marshes. Since these ecosystems are currently only found in small areas, conservation activities are crucial to their preservation Venkatesh *et al.,* (2019). The environmental services provided by the wetlands, their significance as a landscape feature, and their function in watershed dynamics all require more thorough study. For these ecosystems to survive, research on how population increase and climate change affect them is equally essential Senthilkumar *et al.,* (2014).

### 1.1.2. *Gymnacranthera canarica*

Gymnacranthera canarica, commonly known as Kanara Nutmegor wild Nutmeg, is a species of plant in the family Myristicaceae. Vernacular Names: Kannada: Hedehagalu, Pindi, Pindikai; Malayalam: Pintikkaya, Undai panu; Tamil: Uudippanu. It has a loop-like pneumatophores or breathing roots, sometimes also called ' knee' with enlarged lenticels. These roots are slender and spongy when young, but turn large and woody with age. Grows along streams in wet evergreen forests, to 750 m elevation. Its Distribution in India is seen in Karnataka, Kerala, Tamil Nadu, Maharashtra and Goa.

### 1.1.3 Scientific classification (World Conservation Monitoring Centre. 1998)

Kingdom: Plantae

Clade: Tracheophytes

Clade: Angiosperms

Clade: Magnoliids

Order: Magnoliales

Family: Myristicaceae

Genus: Gymnacranthera

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

### 1.1.4 Botanical Description

Evergreen Tree, dioecious with knee roots; leaves lanceolate, 9–26 × 3.5–10 cm, acute-obtuse at base, entire at margins, acuminate-cuspidate at apex. Inflorescence axillary, paniculate, branched; cymule many-flowered, flowers at different stages of development, puberulous; male flowers globose-ellipsoid, valves 3 or 4, splitting the perianth in anthesis to the base; lobes involute at anthesis, puberulous inside; synandrium ellipsoid, androphore c. 0.5 mm long; stamens 5–15; anthers linear; slightly unequal, adnate to their back up to 1 mm from base, apical 1 mm free, incurved-spreading at anthesis, extrorse; female inflorescences racemose-paniculate, contracted; flower buds ellipsoid-obovoid, 2–3 × 1–1.5 mm, villous inside, tomentose outside, brown, scented; stigma sessile, 2-lobed; fruits globose with pointed apex, 2.4–2.6 cm; pericarp 1.5–2 mm thick, wrinkled, glabrous-puberulous, brown; aril laciniated almost up to base with linear pointed structure, yellow; seeds globose, blunt at ends with a persistent collar-like structure at base Flowering from December–April and Fruiting: June–July (Banik et al., 2017).

### 1.1.5 Importance and Uses

*Gymanacranthera canarica* showcasing genetic biodiversity dating back 500 million years is of great importance to scientific and environmental communites and needs to be conserved to preserve the ancient genetic diversity and unique ecological system and various other aspects. Various ecological factors and genetic diversity makes it hub of endophytic fungi and bacteria, bioactive compounds, medicinal properties etc Anusha *et al.*, (2019). It is potential candidate for new bioctive compund discovery and contribute towards medical feild through drug discovery Patro *et al.*, (2005). Its geneic relativity towards *Myristica malabarica,* Makes it next potential specie trees , cultivating it for its mace and nut which posses medicinal properties, this in turn will help in its conservation by increasing the population size of the plant by the locals making them the firstline caretakers of the myristica swapms willingly under EDC (Eco Development Committees). It helps in maintaing the microhabitat in the Myristica swamps, Ground water

conservation and flood control. Candles and soaps are made from seeds Banik et al., (2017). Trunk wood used in Religious rituals during Holi festival in Bambarde Village, Maharashtra Dalavi et al. (2021).

### 1.1.6 Endophytic Fungi

Endophytic fungi are fungi that live within their host plant, without causing any apparent disease to their host, but thereis a chance that, under a stressful environment for the host, they could turn pathogenic Schulz *et al.*, (1993) They are well-known for creating a large variety of bioactive chemicals, many of which have important uses in industry, agriculture, and medicine. Secondary metabolites are substances that are not directly involved in an organism's regular growth, development, or reproduction. As an alternative, they frequently participate in plant defence processes and may possess antioxidant, antiviral, antibacterial, and anticancer qualities Sunitha, S.(2016). The diversity of bioactive compounds produced by endophytic fungi is attributed to the close biological relationship between the fungi and their host plants. The compounds separated from endophytes are usually classified into various categories such as steroids, xanthones, terpenoids, isocoumarins, phenols, tetralones, benzopyranones, and enniatines Gupta *et al.*, (2023). From both terrestrial and aquatic plants, many entophytic fungal species have been identified. By enhancing plant tolerance to environmental stress and resistance to phytopathogens and herbivores, several of these endophytes may encourage development and ecological adaptability of the host (Huang et al. 2008; Sun et al. 2011).

### 1.1.7 Enzyme activity

Due to advantages including consistency, cost effectiveness, low production time and space requirements, and simplicity of process modification and optimisation, fungus enzymes have been widely used in industrial production Sunitha, S.(2016). The breakdown of organic materials, food deterioration, and host infection are all impacted by the degradative enzymes that fungus produce Hankin and Anagnostakis (1975). Enzymes produced by endophytic fungi, are important for both the fungi and the plants they inhabit. These enzymes consist of proteases, cellulases, chitinases, lipases, and amylases and they play a crucial role in the plant's defense against pathogens by hydrolyzing plant cell walls and helping the plant to absorb nutrients Bhadra *et al.*, (2022); Shankar *et al.*, (2019); Mahfooz *et al.*, (2017)

### 1.1.8 Phytochemicals

Biologically active substances that are present in plants are called phytochemicals, sometimes known as phytonutrients. Although they are not as important as vitamins and minerals, they however provide a number of health advantages and are vital to the plant's defence mechanism against parasites, bacteria, fungus, and viruses Gupta and Prakash (2014). Phytochemicals are present in all plants, including fruits, seeds, leaves, bark, and edible flowers. They are responsible for giving vibrant colors distinctive smells and medicinal properties. For example, the red, blue, and

purple hues come from polyphenol phytochemicals called anthocyanins, while the orange color is due to carotenoid pigments Wani *et al.,* (2023). The presence of phytochemicals impart various properties to the plant such as Antioxidant Properties, Anti-inflammatory Effect, Immune Support, Neuroprotection and Hormone Regulation etc.

**1.1.9 Thin Layer Chromatography**

Thin Layer Chromatography (TLC) is an analytical technique used to separate non-volatile mixtures. It's performed on a sheet of glass, plastic, or aluminum foil coated with a thin layer of adsorbent material, such as silica gel, aluminum oxide, or cellulose. The mixture to be separated is applied onto the plate, and a solvent or solvent mixture (mobile phase) moves up the plate via capillary action. Different components travel at different rates based on their affinity to the stationary phase, resulting in separation Do and Reich (2023); Sherma, J. (1992).

**1.1.10 Silver nanoparticles**

Silver nanoparticles (AgNPs) are particles of silver with a size ranging from 1 to 100 nm. They are known for their unique properties, such as high electrical conductivity, chemical stability, and antimicrobial activity, which make them suitable for various applications in medicine, electronics, and environmental science Vigneshwaran *et al.*, (2007). Biogenic synthesis refers to the production of nanoparticles using biological methods. This approach is considered eco-friendly and sustainable as it often uses plant extracts, microorganisms, and enzymes as reducing agents to convert silver ions into silver nanoparticles. The process is advantageous because it avoids the use of toxic chemicals and high energy inputs associated with physical and chemical methods of nanoparticle synthesis (Gajbhiye, *et al.,* 2009; Balouiri,. et al 2016).

**1.1.11 Antimicrobial activity**

Antibacterial Activity Against Human Pathogenic Bacteria: Research has been conducted on isolating and characterizing soil bacteria with antibacterial activity against human pathogens such as Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae. A study identified three bacterial isolates from soil samples that displayed antibacterial activity against these human pathogens. The isolates were identified as *Bacillus aryabhattai*, *Arthrobacter humicola*, and *Neomicrococcus lactis*. Khameneh *et al.,* (2019). Quorum quenching is the disruption of quorum sensing. It can prevent bacteria from communicating and coordinating certain behaviors, including virulence and biofilm formation. For example, Serratia marcescens is known for its quorum sensing abilities, which regulate the production of prodigiosin, a red pigment, and other virulence factors. Studies have investigated the quorum quenching activity of various substances against Serratia marcescens, aiming to mitigate its pathogenic effects Ravichandran *et al.,* (2018); Barcena *et al.,* (2021).

**1.2.1.Lacuna**

There exists a notable gap in the investigation of endophytic fungi within Gymnacranthera canarica, inhibiting a comprehensive understanding of their potential role in the species' adaptation and the overall ecosystem dynamics of Myristica swamps.

Additionally, the lack of studies exploring the biogenic synthesis of nanoparticles from endophytic fungi associated with Gymnacranthera canarica limits insights into potential eco-friendly nanoparticle synthesis pathways, which could be significant for various applications, including conservation strategies.

### 1.2.2 Aim

This dissertation seeks to bridge these gaps in knowledge by conducting a comprehensive investigation into the diversity of endophytic fungi within *Gymnacranthera canarica.* By assessing the bioactive compounds synthesized by these fungi, this research aims to contribute significantly to the understanding of these intricate plant-fungi associations through the comparative phytochemical analysis Between the isolated Fungi and Plant, exploring the bioactive compunds amongst the isolates and the plant, paving the way for potential applications in pharmaceutical industries, agricultural practices, and conservation strategies aimed at preserving the invaluable biodiversity of the Western Ghats.

### 1.2.3 Objectives

Explore the diversity and role of endophytic fungi within *Gymnacranthera canarica*, unraveling their significance in species adaptation and Myristica swamp ecosystem dynamics.

Conduct comparative analysis of major phytochemical classes between dominant isolated endophytic fungi and *Gymnacranthera canarica*, followed by screening bioactive compounds for antimicrobial potential, shedding light on potential medicinal applications.

Investigate the biogenic synthesis of silver nanoparticles using endophytic fungi associated with *Gymnacranthera canarica*, evaluating their bio-efficacy for various biomedical and environmental applications.

Initiate community-based awareness programs focusing on the conservation of *Gymnacranthera canarica* in Myristica swamps, emphasizing its ecological importance within fringe communities for sustainable preservation.

## 1.3. Hypothesis

The study aims to unvail the potential of the Endophytic fungi isolated from *Gymnacranthera canarica* in its contribution to the plant health and bettter survival in swampy conditions by screening them for enzyme activity and antimicrobial acivity. Also understand the similarity of distinct bioactive compounds that may contribute to the plant phytochemicals. The biogenic sythesis of silvernanoprticles using the potential dominant endophyte may have properties with potential for applications in pharmaceutical industries and agriculture. This research also focus in

conservation of these sacred grooves through cleanliness drives and awareness activities amongst the locals in collaboration with the forest department.

# CHAPTER 2: REVIEW OF LITERATURE

## 2. REVIEW OF LITERATURE

Chandran et al. (2010) stated that the Western Ghats, older than the Himalayan mountains is one of the 34 biodiversity hotspots of the world and a UNESCO World Heritage Site which shows an endemism of an execptional level and encapsulates a rich tapestry of biodiversity, hosting myriad ecosystems teeming with unique flora and fauna.The threathened habitat of myristica swamps along with mass-flowering wildflower meadows and Shola forests was one on the many reasons to declare the Western ghats as UNESCO World Heritage Site ( Natural Site) was mentioned by Senthilkumar *et al.,* (2014). Ranganathan *et al.,* (2022) highlighted that the Myristica Swamps formerly created a vast hydrological network throughout the Western Ghats which are now highly frangmented and diminishing in swampy area.

The Myristica swamps were first described by krishnamoorthy (1960) from of South Western Ghat in Travancore region, the swamps were found in Kulathupuzha, Shendurney valley and Anchal forest ranges. Ranganathan *et al.,*(2022) mentioned in thier study Champion and Seth in 1968 classified the Myristica swamps as Tropical Freshwater Swamp Forest under 4C/FSI category. Roby et al. (2018) did a phytosociological analysis of Myristica swamp forests in Kulathupuzha, Kerala and found that there was dominance of *Gymnacranthera farquhariana* and *Myristica fatua var magnifica* in those swamps but the swamps had less diveristy when compared to other forest types and Kulathupuzha Myristica swamp forests had largest population of endangered *M. Fatua var magnifica.*

Sreejith *et al.,* (2016) in their studies reavealed that a Myristica swamp in Northern Kerala displayed complete absence of species like *Gymnacranthera canarica* and *Myristica fatua var. Magnifica* which are otherwise considered the essential elements of Myristica swamps and *Myristica malabarica*'s was found to be the most dominant and due to its preference for swampy areas, their population displayed a diminishing tendency as moving away from the swamp and followed by *Knema attenuata* as the second most dominant in that swamp exhibits a reversal trend and is completely missing in the first five quadrats where the soil water content is too high.

Ragu *et al.,* (2006) concluded that the soil of Myristica swamps of Uttara Kannada were sandy loam and silty with a pH of acidic to neutral and showed lower Nitrogen, Phosphorus & Potassium contents as compare to other adjacent forest areas. High leaching losses of NPK could have resulted due to water logged condition in swamp and suggested that the soil was the dominant factor which dictated soil constitutents and the species distribution and pattern was stated by Bhat & Kaveriappa (2009) and Vijayakumar & Vasudeva (2012) in their studies.

The ecological studies conducted by Bhat & Kaveriappa (2009) on Myristica swamp forests in Uttara Kannada district of Karnataka state in India showed *G. farquhariana* appeared very frequent followed by *M. fatua var. magnifica*

the second frequent one. Other common species include, *Pinanga dicksonii*, *Hopea ponga*, *Mastixia arborea*, *Dipterocarpus indicus*, *Lophopetalum wightianum*, *Pandanus unipapillatus* and A*grostistachys meeboldii*.

Chandran *et al.,* (1998) in his study on sacred grooves shed light on major threats to the endemic and endangered tree species such as *Dipterocarpus indicus, Myristica fauta var, magnifica, Gymnacaranthera canarica, Semecarpus auriculata* belonging to the fresh water swamps by human intervention through agriculture and arecanut plantations. Verghese & Menon 1999 in thier study to better understand Myristica swamp forest dynamics, composition, structure, and diversity through phytosociological investigations revealed a total of 18 species, spanning 12 groups, were found in the 0.1 hectare in the study area. Amonsgt which *Gymnacranthera farquhariana* shows high density (1.20) followed by *Knema attenuata* (0.80) and *Myristica malabarica* (0.50). This forest is classified as Gymnacranthera farquhariana-Myristica fatua-Knema attenuata type based on dominance.

Venkatesh *et al.,* (2019) in the comparative investigation of specific gravity and wood growth in non-swampy and swampy Myristicaceae species in various Western Ghats sites, demonstrated the substantial range in wood growth and the impact of climate on growth patterns, emphasising the need for conservation methods that are specific to swamp environments. In A study conducted by Tambat *et al.,* (2019) An evaluation of the impact of disturbance on the wood specific gravity and fibre length of Swampy species was done using wood core samples were taken from specific sites in the middle Western Ghats using an increment borer. The wood specific gravity and fibre length of the samples were assessed in the laboratory where comparison was done between 6 locations, highly disturbed and less disturbed swamps. Where in two locations namely Thorme and Sampaje showed positive values of specific gravity in frequency distribution which faced least to no disturbacnce compared to the other location. Research showed that the wood specific gravity and fibre length of swamp-associated tree species were altered when there distubance to the swamps.

Chandran & Mesta (2001) stated that the Myristica swamps are named so, as the dominant species in these swamps belong to Myristicaceae family such as *Myristica fatua var magnifica* and *Gymnacranthera canarica* which have evolved several physiological and structural features enabling them to adapt to the anaerobic swampy conditions and making them entirely endemic to the fresh water swamps. The findings of Varghese and Kumar (1997) in the freshwater swamp forests of Southern Kerala obsereved that hydric soil might have led to evolution of special adaptations in Myristicaceae famliy such as knee roots hence concentration of these families in the Swamps are seen. Redox potentials of the swamps *in-situ* were greater compared to the flooded regions indicating keen roots help in oxygenation of the swamp rhizosphere, sheding light on the unique ecological dynamics of freshwater swamps in the region.

In a study by Chandran *et al.,* (2001) revealed that in a family-wise distribution of trees counted in the 37 quadrats of 400 m sq. each, Myristicaceae (37%) was found to be the most prevalent family, with *Gymnacranthera*

*canarica* accounting for 78% of the trees and also shed light on the facts that members of the Myristicaceae family are abundant in essential oils and compounds of significant scientific value, including flavonoids, lignins, quinones, phenols, etc.

Patro *et al.,* (2005) in thier studies found that the methanol extract of Myristica malabarica showed strong antioxidant activity. And isolated four known diarylnonanoids and two novel acylresorcinols, with malabaricone C showing the highest DPPH scavenging activity. Additionally, it inhibited γ-ray-induced damage to the DNA of the pBR322 plasmid in a concentration-dependent way. Sabulal *et al.,* (2007) in their studies revealed that *Gymnacranthera canarica* leaf oil contains seventy-six components (98.1% of the total) in which the main components were alpha-humulene (11.3%), linalool (13.4%), and beta-caryophyllene (23.4%) and 58.1% sesquiterpene hydrocarbons. However so far no extensive phytochemical and antioxidant analysis has been carried out on *Gymancranthera canarica*.

Ali *et al.,* (2006) in their study on Myristica swamps of Central Western Ghats, Karnataka, showed 34 species of trees were found in the Myristica Swamps of Uttara Kannada, indigenous to the Western Ghats and exhibiting high levels of endemism among trees. In these Swamps, the ground layer is characterised by uncommon plants and herbs such as *Apama siliquosa, Ochlandra scriptoria*, *Calamus spp*., *Arenga wightii,* and *Pandanus spp*.

Tambat *et al.,* (2006). Tambat *et al.,* 2006 in a study with the motto to conserve the endemic *Gymnacranthera canarica* through improved seed germination found that the *Gymnacranthera canarica* seeds exhibited excellent viability at 98% by Tetrazolium test, but only 40% initial germination was observed, suggesting presence of a dormant phase. Treatment with different GA3 concentrations led to enhanced germination and better results were obtained at doses under 500 ppm, whereas inhibitory effects was seen at concentrations over 500 ppm. The further work on seeds of *Gymnacranthera canarica* by Tambat et.al., (2007) reaveled that in the central Western Ghats, *Gymnacranthera Farquhariana* was observed to have abnormal seeds, which may have resulted from a depression caused by inbreeding. The capping on abnormal seeds lead to reduced germination indicating poor genetic viability. Keshavachandra & KrishnaKumar (2016) contributed to the conservation efforts for this swamp-obligate species by investigating the storage requirements for effective germination of the recalcitrant seeds of *Gymnacranthera canarica*. Their studies showed that under laboratory conditions (28+/- 4°C) and with critical moisture of 16.44+/-2.57%. -14.26+/-2.3%, the seeds can be kept for up to two and a half months. Anusha *et al.,* (2022) through their research showed that the early loss of viability in *Gymnacranthera canarica* seeds may be caused by a breakdown in membrane integrity, which has been connected to the production of ROS and related lipid peroxidation products, suggesting that the seeds are actually recalcitrant. Bhat & Kaveriappa (2009) in their studies stated that despite having similar stem densities and a distinct canopy with tall trees, Myristica swamp woods have less floristic variety. This reduced diversity stands out in terms of speciation within the Western Ghats.

Chandran *et al.,* 2010 stated in their studies that *Gymnacranthera canarica* inhabits 51 swamps, reaching as far north as 14° N (close to Uttara Kannada). There may be isolated swamps located even farther north in Goa's Sattari taluka. Among the noteworthy discoveries are knee-roots, or breathing roots resembling loops with expanded lenticels. promotes the discovery of additional remnant forests for their hydrological, biodiversity, and carbon sequestration properties. It also suggests integrating marginal farmers and forest tribes into carbon credit schemes to mitigate the effects of climate change.

Tambat *et al.,* 2010 in their study on swamp adapted species vulnerable to shrinkage of habitat reavealed that *Gymnacranthera canarica* shows decreased reproductive fitness with declining swamp areas, and the populations found scattered in small fragments along the Ghats region. The study disproves the theory that organisms that occur naturally in many tiny populations are less vulnerable to negative consequences from small populations.

Shenoy & Ahmad (1994) during their research on Succession of mycoflora on leaf litter in the Myristica swamps revealed Isolation of 53 genera of fungi from the leaf litter of *Gymnacranthera canarica* Warb; the main colonisers of the litter were *Beltrania rhombica*, *Ardhachandra selenoides*, and *Asterina sp.* Deuteromycetes 47 (89%), Ascomycetes 4 (7%), and Phycomycetes 1 (2%).

Goveas *et al.,* (2011) conducted a study to isolate endophytic fungi from healthy leaves and stems of the critically endangered medicinal plant *Coscinium fenestratum* in which 41 different taxa of endophytic fungi were isolated and identified. Of which the core-group fungus was found to be *Phomopsis jacquiniana*, which colonised leaves and stem segments at different rates. However, such studies have not been carried out on *Gymnacranthera canarica* being a potential host for endophytic fungi due to its medicinal properties, unique adaptation and unique ecology.

Krishnaswamy *et al.,* (2011) in their study on *Myristica malabarica* Seed Aril Extracts showed antioxidant properties and the benzene fraction showed the best effectiveness in shielding the liver from CCl4-induced damage. A study by Vinayachandra *et al.,* (2011) showed Potential larvicidal actions against *Aedes albopictus* and *Anopheles stephensi* mosquito larvae by extracts from *Knema attenuata* a species belonging to Myristicaceae family. The phytochemical tests showed the presence of phenolics, tannins, steroids, terpenes, resins, and glycolipids in the extracts. This study hints that *Gymnacranthera canarica* being rich in phytochemicals and having similar ecological condition as *Knema attenuata* may also exhibit Potential larvicidal activity.

Senthilkumar *et al.,* (2014) in their study states that, with its genetic diversity spanning 500 million years, *Gymnacranthera farquhariana* is dominant in narrow valley swamps, while *Myristica fatua var. magnifica* is dominant

in flat valley swamps and requires careful planning, manipulation, and watershed management to restore and preserve the Myristica swamp forests.

Bhardwaj & Agrawal (2014) in their studies shed light on the facts that endophytes are rich in bioactive chemicals that may be used to combat infections, including cancers in humans and animals alike. Numerous investigations have isolated and analysed various endophytic fungal species from a variety of plants, highlighting the critical role that nature plays in the discovery and development of new drugs. With this background knowledge we will screen the potential fungal isolates for various bioactive compounds.

Prabhugaonkar *et al.,* (2014) during the field visits to Myristica Swamps in Goa revealed the presence of three critically endangered tree species, *Gymnacranthera canarica*, *Syzygium travancoricum*, and *Semecarpus kathalekanensis* which are listed on the Red List of Threatened Species. Rao *et al.,* (2014) in their study highlights how important watershed-based forest management is for maintaining hydrology and protecting endangered species of the Myristica swapms. Also emphasizes mapping of the relic woods in order to stop their irreversible loss and also promotes the comprehensive preservation of riparian forests and the buffer zones that surround them, encompassing whole drainage basins.

Devi & Prabakaran (2014) evaluated *Penicillium* sp., isolated from *Centella asiatica*, for its cytotoxic and antioxidant properties. And stated that endophytic fungus *may* be a source of bioactive chemicals, as evidenced by the ethyl acetate extract's encouraging cytotoxic activity against cancer cell lines (HeLa, A431, and MCF-7) in their studies.

Uzma *et al.,* (2016) investigated the diversity of endophytic fungi and the activity of enzymes in wild medicinal plants. 112 endophytic fungi from 26 genera were isolated from two threatened plants *Hedychium flavescens* and *Hedychium coronarium*. And also identified the extracellular enzymes produce by these fungi.

Banik *et al.,* (2017) Provided a detailed review of the Gymnacranthera and other species of Myristica, with updated nomenclature, descriptions, range, blooming and fruiting times, common names, and uses; also includes photo plates, illustrations, and a key to the taxonomic class to help with identification.

A Study done Anusha *et al.,* (2019) revealed the methanolic extract of *Gymnacranthera canarica* seeds exhibits a promising antimicrobial activity against both bacteria and fungi, and is mostly sensitive against gram positive bacteria. The seeds demonstrate a good antidiabetic potential in the $\alpha$-amylase inhibitory assay (74.68%) at a concentration of 100 µg/mg of extract; however, the phenol content (8.75 µg/mg) and antioxidant activities are not significantly correlated, substantiating the direct correlation between phenol and antioxidant activities. The high flavonoid content (103.3 µg/mg) in *Gymnacranthera canarica* seeds may be the cause of the anti-inflammatory effect. Panda *et al.,* (2019) through their research explains how seed propagation is the best way to conserve since it preserves

genetic diversity. highlights issues that require vegetative proliferation, such as inadequate germination, dormancy, and reproductive constraints. He also argues that large-scale species recovery initiatives should improve propagation methods.

Dalavi et al. (2021) in their studies revealed that the locals of Bambarde-Hewale preserve the Myristca Swamps as sacred woodlands. But during the Holi festival, locals utilise the tallest Myristica tree trunk as "Ovhaliche zad" (plant growing in natural streams). One mature tree is chopped down for the celebration each year. It is necessary to run awareness campaigns among the local population, particularly the younger generation, to inform them about their scientific history, the value of the Myristica Swamps, and the necessity of protecting them and provide substitute common tree species for the same.

Neethu *et al.,* (2021) in their studies subjected *Myristica beddomei subsp Spherocarpa*, an unexplored wild nutmeg species, to chemoprofile analysis of its aerial parts. Two novel flavonoids, including an isoflavone 5,7-dihydroxy-3-(5’-hydroxybenzo[d] (7’,9’)-dioxol-1’-yl, were isolated. β-hydroxydihydrochalcone; (3(S)-hydroxy-3-phenyl-1- (2',4',6'-trihydroxyphenyl) and -4H-chromen-4-one (1) for the first time, propan-1-one (2) and eleven recognised compounds (3–13). While trimyristin (10) effectively suppressed MCF-7, partensein (8) and promalabaricone B (9) were shown to be mildly hazardous to human adenocarcinoma cell lines. Malabaricones (3, 5-7) demonstrated potential efficacy against both cell lines MCF-7 and MDA-MB-231.

Thacker & Karthick (2022) stated that during an investigation of water of Myristica swamps, a significant number of diatom taxa were found that are still unknown to science and will require formal description in the future; these findings suggest that the diatom flora of the swamp is poorly understood. The diatom assemblages in swamps were impacted by significant environmental gradients such as conductivity, pH, and dissolved oxygen. These gradients were further modified by landuse patterns within the swamps. The diatom assemblage structure in these swamps also demonstrated the potential application of diatom biodiversity as a bioindicator to comprehend the potential effects of the most significant water physicochemical parameters within the swamps.

Zhang *et al.,* (2016) during their study investigated several uses of the silver nanoparticles (AgNPs) in nanomedicine, especially in the detection and treatment of cancer. highlighted a number of AgNP bioapplications, including anti-inflammatory, anti-fungal, antiviral, anti-cancer, and anti-inflammatory properties.

Rodrigues *et al.,* (2016) assesed antiquorum sensing activity in *Chromobacterium violaceum* by measuring the inhibition of quorum sensing dependent violacein production. It was discovered that the fruit phenolic extract, at low subinhibitory concentrations, inhibited up to 96% of violacein production in *Chromobacterium violaceum*, probably because of the fruit's phenolic content

# CHAPTER 3: MATERIAL AND METHODOLOGY

# 3. MATERIAL AND METHODOLOGY

## 3.1. Study Area

The research was conducted in the scared myristica swamps of Western Ghats, Goa, India. As *Gymnacranther canarica* is confined to the myristica swamps.

### 3.1.1 Locating Myristica Swamps in Goa.

A total of 4 sites were explored during the Field visits along with few forest official from Goa Forest Department Madei Wildlife Sacnctuary. First Location was Nirankarachirai (15°34'58.7"N 74°11'18.3"E) of Bambar, Sattari, Goa. This swamp is covering an area of 0.25 ha. 2nd site was Woddyar **(**15°35'09.0"N 74°11'23.5"E), Sattari, Goa. 3rd Brahma Karmali (15 $^{0}$ 33.874'N & 74 $^{0}$ 10.378'E), Valpoi, Goa. 4th location was Netravali.

### 3.1.2 Selection of Plant

*Gymnacranthera canaric* was selected for further studies due to its unique adapation i.e. formation of knee roots and its domiance in the Nirankarachirai Swamp.

### 3.1.3 Sample selection

Leaf samples of *Gymnacranthera canaric* were selected for isolation of endophytic fungi.The three categories of leaves, young, moderatly matured and fully matured were chosen based on the colour, texture and their position on the branches. Young leaves were light green and delicate, Moderately matured leaves were medium green and little thicker, Fully matured leaves were dark green and thick with waxy coating on the surface of he leaves.

### 3.1.4 Sample collection and storage

Healthy disease free leaves were collected in a sterile ziplock bag and were dehydrated with Silica crystals to remove excess moisture. The leaves were washed throughly with tap water and with diluteTween-80 and rinsed with distilled water 2-3 times to remove any dirt particles and wiped with tissue paper and stored at -4 $^{o}$C in a sterile ziplock bag within 4-6 hr from the time of collection.

### 3.1.5 Sample Preparation and Inoculation for Isolation of Endophytic Fungi.

The leaves were cut into 5-10 cm peices and placed them in a conical flask. The leaf samples were sterilized with 75% ethanol for 30-60 seconds , decanted and washed with sterile distillled water, later the leaves were treated with 2% sodium hypochlorite for 1-2 mins. Finally the leaves were rinsed with 90% ethanol, the solution was decanted and rinsed with sterile distilled water 3 times (Petrini & Dreyfuss 1981; Schulz et al., 1993; Sawant, 2022, with slight modification) . The peices of the leaf samples were placed on sterile filter paper to remove excess water, and the leaves were cut into approximately 0.5cm to 1cm peices and placed 4 pecies on each PDA media Plate. The plates were incubate at room temprature for 2-3 days and was observed for any hyphal growth every three days for 21 day Arnold et al., (2000).

### 3.1.6 Subcultuing

To obatin pure cultures of the isolates, each isolate was subcultured on a fresh PDA media within 3-4 day of isolation.The isolated pure cultures were then stored at $4^{0}$ C for further use Bills, (1996).

### 3.1.7 Data Collection

The observation were noted down, such as the number of isloates obtained, the colony characteristics suh as colony colour, texture, Growth rate etc, for the Statistical data analysis such as Absolute frequency (f): The total number of endophytes isolated, Relative frequency (fr): The number of isolates of each species of the endophytic fungi divided by the total number of isolates and expressed in percentage. Isolation rate (IR): number of isolates obtained from tissue segments divided by total

number of tissue segments. Colonization rate (CR): Percentage of total number of isolates obtained from different tissue segments divided by total number of isolates obtained from overall tissue segments incubated Sawant, (2022) .

### 3.1.8 Identification

Identification of the endophytic fungi was done by preparing slides of the fungal cultues using Lectophenol Cotton Blue Stain and observing for spores produced by the fungi and by studing the colony characteristics such as colony colour, texture and growth patterns of the fungal hyphae etc Gaikwad et al (2013). Two dominant fungal species were identified using molecular based sequence method.

## 3.2. Extraction of Bioactive compounds from dominant endophytic fungi

### 3.2.1 Mass Production of isolated Dominant fungi

Subculturing the dominant endopytic fungi was done by using slight modified method of Uzma (2017), inoculating the fungi in 100mL of Potato Dextrose Broth in a conical flask and incubating at room temprature for 15-20 days or until sporulation was seen .

### 3.2.2 Preparation of Fungal and Plant Extracts

a) Preparation of Fungal Extracts: The fungal mycelial growth was filtered out from the liquid media using Grade 1 Filter paper and rinsed with sterile distilled water carefully to remove media composition. The Fungal mass was ground in a clean sterile Mortar and Pestle using 1:1 v/v Methanol and Ethylacetate. The extract was than centrifuged at 7000rpm for 30 mins and the supernatant was collected.

b) Preparation of Plant Extracts: The leaf samples were washed and shade dried for 10-15 days.The dried leaves were ground into coarse powder. 10g of dried leaf powder was added to 100mL ethylacetate in a 250mL conical flask, which was covered with aluminium foil. The content were soaked for 24hrs and placed in shaking incubater at 200rpm at room temperature for 24 hrs.The exctracts was filtered by passing the mixture throught glass funnel covered with Grade 1 filter paper. The extract were stored in an air tight container at -4$^{o}$C.

## 3.3. Preliminary Phytochemical Screening

The Preliminary Phytochemical Screening was done using Raman (2000).

**3.31 Test for Alkaloids**

a) Dragendroff's Test

To 500 µl Extract few drops of Dragendroff's reagent was added and observed for red precipitate. Preparation of Dragendroff's reagent:Stock solution 5.2g Bismuth carbonate and 4g sodium iodide are boiled in 50mL glacial acetic acid, for few min, After 12 hr precipitated sodium acetate crystals are filtered by sintered glass funnel 40mL clear, red-brown filtrate is mixed with 160mL of ethyl acetate and 1mL distilled water and stored it in an amber coloured bottle. Working solution: 10mL stock solution was taken to it 20mL acetic acid and distilled water was to make final volume of 100mL.

**3.3.2 Test for Flavonoids**

a) Lead acetate test

To the Extract few drops of lead acetate was added and observed for yellow precipitate

**3.3.3 Test for Tannin & Phenolic**

a) Ferric chloride Test

To 500 µl extract and distilled water 500 µl $FeCl_3$ was added & observed for Blue, Green or Violet colour.

**3.3.4 Test for Steroids**

a) Salkowski Test

To 500 µl extract, conc. $H_2SO_4$ was added and the test was confirmed by lower layer turning red.

**3.4 Determination of Total Phenolic Content (TPC)**

The Folin Ciocalteu's method was used to determine the total phenolic content. 0.001 % Gallic acid was prepared from the stock solution of 1mg/mL and used as standard. Different concentration such as 50µL, 100µL, 150µL, 200µL and 250µL and the final volume was made up to 250µL with distilled water. To each test tube 1250µLof 10% Folin-Ciocalteu's reagent was added and mixed well and incubated for 15 mins. After 15 mins incubation 1250µL of 7.5% $Na_2CO_3$ was added and the mixture was left for incubation for 45 mins. And O.D absorbance was read at 760 & 765 nm. The same procedure was repeated with the plant and fungal extracts. The presence of polyphenols is indicated by blue colouration of the mixture as Folin Ciocalteu's reagent is sensitive to polyhenols and produce a blue colour.

**3.5.1 Biogenic Synthesis of Silver Nanoparticles:**

In a conical flask 15 mL fungal extrac was added to that 1mM Silver nitrate solution was added and the volume was made up to 100mL Gaikwad et al (2013). The mixture was observed for colour change from colourless to orange-red in few hours indicating the formation silver nanoparticles Gajbhiye,et al (2009). The same procedure was repeated for the other fungal extracts. After 72hrs the samples were centrifuged at 7000 rpm for 30 mins Gherbawy, et

al, (2013). The supenatant was decanted and the precipitate was washed with distilled water, centrifuged again at 7000 rpm for 30 mins and decanted. This procedure was repeated with ethanol and the precipitate was air dried and collceted in clean container.

**3.5.2 Characterization of the silver nanoparticles:**

UV–visible spectral analysis of silver nanoparticles aws carried out as change in colour was observed in the silver nitrate solution incubated with the fungal extract. The UV–visible spectra of this solution was recorded in Spectrophotometer, from 350 nm to 500 nm Vigneshwaran *et al.*, (2007).

SEM analysis was done to study the morphology and size of the silver nanopartilces.

**3.6 Preliminary screening of endophytic fungi for enzyme activity**

The four dominant endophytic isolates were screened for amylase, cellulase, pectinase, Laccase & Lipase enzyme and phosphate solubilization activity using the methods described Hankin and Ananostakis (1975) and Sawant (2022).

**3.6.1 Amylolytic activity:**

Amylase activity was assessed by subculturing the fungi on Glucose Yeast Extract Peptone Agar (GYPA) medium supplymented with 0.2% soluble starch and the pH 6.0 was maintained. After incubating the plates for 3-4 days the plates were flooded with 1% iodine in 2% potassium iodide. The amylase activity was verified by the yellow zone surrounding the fungal colony.

**3.6.2 Cellulolytic activity:**

The fungi were grown on GYP medium containing 0.5% Carboxy-methylcellulose (CMC) for checking Cellulolytic activity of the fungi. After incubating the plates for 3-4 days. The plates wete flooded with 0.2% aqueous Congo red solution, they were destained for 15 minutes with 1M NaCl. The cellulase activity was confirmed by the appearance of yellow areas around the fungal colony in an otherwise red medium.

**3.6.3 Pectinolytic activity:**

The fungi was grown on Pectin Agar medium to determine its Pectinolytic activity.After the completion of incubation period of 3-4 days, the plates were flooded with 1% aqueous solution of hexadecyltrimethylammonoium bromide. Pectinolytic activity was indicated by the appearance of a clear zone around the fungal colony .

**3.6.4 Laccase activity:**

To asses the fungi for laccase activity, the fungal isolate was grown on Glucose Yeast Peptone Agar Medium which was amended with 0.05 g 1-naphthol L -1 at pH 6.0. The colour change of the medium from colourless to blue confirmed the laccase activity .

**3.6.5 Lipase activity:**

The fungal isolate was grown on a 1L medium supplymented with peptone (10 g) which was amended with NaCl (5 g), CaCl 2 .2H 2 0 (0.1 g), and Tween 20 (1 mL) at pH 6.0. The lipase activity was confirmed by the formation of a halo zone around the fungal colony.

**3.7 Antimicrobial activity**

Antimicrobial activity was carried out by using the methods discribed by Gajbhiye, *et al.,* (2009)**,** Bakht,. et al (2011) and Balouiri,. et al (2016) and with slight modifications. The plant and fungal extracts were prepared by refering to the methods mentioned in Raman (2001).

**3.7.1 Preparation of Extracts**

a) Preparation of Fungal Extracts: The fungal mycelial growth was filtered out from the liquid media using Grade 1 Filter paper and rinsed with sterile distilled water carefully to remove media composition. The Fungal mass was ground in a clean sterile Mortar and Pestle using 1:1 v/v Methanol and Ethylacetate. The extract was than centrifuged at 7000rpm for 30 mins and the supernatant was collected.

b) Preparation of Plant Extracts: The leaf samples were washed and shade dried for 10-15 days.The dried leaves were ground into coarse powder. 10g of dried leaf powder was added to 100mL ethylacetate in a 250mL conical flask, which was covered with aluminium foil. The content were soaked for 24hrs and placed in shaking incubater at 200rpm at room temperature for 24 hrs.The exctracts was filtered by passing the mixture throught glass funnel covered with Grade 1 filter paper. The extract were stored in an air tight container at -4$^{o}$C.

**3.7.2 Sterilization Process**

All the Glasswares were Washed with soap, dried in Hot Air Oven at 60-70$^{o}$ C for approximately 30 mins, Wrapped with news paper and packed in an autoclave bag. All the Glasswares and Media were autoclaved at 121$^{o}$C at 15 psi for 30 mins. All the working counters were swabbed with 75% ethanol. And all the work was carried out inside the laminar Air Flow under sterile conditions.

**3.7.3 Preparation of Culture Suspention**

The Bacterial cultures used for antimicrobial activity were *Klebsiella Pneumonia, Salmonella Typhi, Salmonella Typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pyogenes, Chromobacterium violaceum* and *Serratia marcescens.*The Bacterial cultures were subcultured on fresh NA media and incubated in the Incubator for 24hrs. 3-4 loop full culture was added to 20 mL of Normal Saline in sterile Testube in the LAF and mixed well by vortex method.

**3.7.4 Media and Plate Preparation**

Prepare 1L Nurtient Agar Media by dissolving 28g of readymade NA media from Himedia in 1L double distilled water. Mix well and Autoclave the media at 121ºC and 15 psi pressure for 15-30 mins.

**3.7.5 For Disc Diffusion Method**

Around 30-35mL of media was poured in the glass Petriplates under sterile conditions in LAF. The plates were left for 15-20 mins to solidify with half Lid open to avoid accumulation of water dropplets. 200 µL culture suspension was added to the NA media plates using Micro-pipette and sterile tips. The culture was spread plated using sterile L-shaped Glassrod and. The Whattman Filter papers disc of 0.7mm were soaked in the extracts for 1 min and after removing the excess extracts from the dics,they placed carefully on the media plates . The plates were than incubated for 24hrs and observed for zone of clearance and the measurements were recoded.

**3.7.6 For Agar Well Diffusion Method**

For this method pour plate technique described by Sanders (2012) was used with slightly modifications. Around 15-20mL media was poured in each plate ( Less than half the height of the Petriplate) in LAF left for 15 mins to solidify with half Lid open to avoid accumulation of water dropplets in the Petriplates. And stored for 24hrs at room temperature. In a sterile test tube 20mL of NA media was added to that 1 ml culture suspention was added and mixed it well by rotating the testubes inbetween your palms and immediately pour it in the exsiting media plates. (Note: The Media should neither be too hot while adding the culture which may kill the bacteria nor be too cold as it may solifidy before pouring the plate.). Allow the media to solidify for 10-15 mins in LAF. Bore wells in the agar media unsing a sterile cork-borer. Add 100µL extracts in the wells and incubate for 24hrs and observe for zone of clearance and note down the measurements Bakht,. et al (2011).

**3.7.7 Quorum Quenching**

Media plates with culture was prepared as Agar well diffusion method mentioned above. After adding the Extracts in the wells the plates were incubated at -4ºC to induce stress leading to quorem sensing between the bacteria which is indicate by Bright Pink and Violet colour pigment production in *Serratia marcescens* and *Chromobacterium violaceum* respectively.

The quorem quenching is indicated by the quenching of the pigments produced by the baterial colonies and formation of clear zones Rodrigues et al., (2016).

**3.8 Thin Layer Chromatography**

TLC profiling was carried out using different solvent systems to evaluate different compunds present in the plant and fungal extrats based on the polarity of different classes of compounds. The different combinations of solvent system helped in separation of avrious bioactive compounds such as phenol, flavonoids, steroids etc using 10 X 10 cm TLC Silica gel 60 $F_{254}$ Aluminium sheets.

**3.8.1. Steroids**

The TLC Plates were prepared by marking the loading points and loading the samples 30 times at the same spot. The solvent system was prepared by adding n-Butanol: Methanol: Water in the a ratio (16:4:0.2) v/v/v respectively. It was mixed well and covered with silver foil and allowed to saturate for 30 mins before placing the TLC in it for solvent run. The TLC was removed after the solvent had run for more than 3/4 length of the TLC, air dried and photographs were clicked under long UV. Sort UV and Visible light. The TLC was than derivetized by dipping in Anisealdehyde sulphuric acid and heating for 10 mins at 80$^{o}$C. And observed for Purple bands.

**3.8.2 Phenolic compounds**

The TLC Plates were prepared by marking the loading points and loading the samples 30 times at the same spot. The solvent system was prepared by adding Tetra Hydrofuran: Toluene: Formic Acid: Water in a ratio of (16:8:2:1) v/v/v/v respectively. The Air dried TLC after the solvent run was derivatized by dipping in Alcoholic Ferric Chloride and dried. The appearance of Dark blue bands indiacted the presence of phenolic compounds.

**3.8.3 Flavonoids**

The solvent system for flavonoids was Tetra Hydrofuran: Toluene: Formic Acid: Water in a ratio of ( 16:8:2:1 ) v/v/v/v respectively. And later derivetized in 10% Methanolic Sulphuric Acid. Appearance of Fluorescence under UV light indicated the presence of Flavonoids.

**3.8.4 Alkaloids**

The solvent system for Alkaloids was Toluene: Ethy acetate: Methanol: ammonia 25% in a ratio (30: 30: 15: 1) v/v/v/v and derivetized with Dragendroff reagent and observe for orange bands.

**3.8.5 Bioautography of Antioxidant Potential using TLC**

Bioautography of Antioxidant Potential using TLC was carried out by using a solvent system containing Toluene: Ethyl acetate: chloroform: methanol (3: 3: 2: 1) v/v/v/v and after the solvent was run for 3/4$^{th}$ length of the TLC, it was dried and immediately dipped in 5% $KmnO_4$ solution. Appearance pf area of clearance on the TLC indicated the presence of antioxidant compound in the plant and Fungal extracts.

# CHAPTER 4: RESULTS AND DISCUSSION

# RESULTS AND DISCUSSION

### 4.1 Locating Myristica Swamps in Goa

A total of 4 sites were explored during the field visits. First location was Nirankarachirai of Bambar village (15°34'58.7"N 74°11'18.3"E), Sattari, Goa. This swamp is covering an area of 0.25 ha. 2nd site was Woddyar **(**15°35'09.0"N 74°11'23.5"E), Sattari, Goa. 3rd Brahma Karmali ($15^0$ 33.874'N & $74^0$ 10.378'E), Valpoi, Goa. 4th location was Netravali Wildlife Scantuary (Plate 1). The Dominant species found in this these Swamps were *Gymnacranthera canarica*, *Myristic fatua Houtt. var. Magnifica*, *Lophopetalum wightianum*, *Semecarpus kathalekanensis*, and *Syzygium travancoricum* etc Prabhugaonkar et al., (2014).

### 4.2.1 Identification of Endophytic Fungi Isolated from *G. Canarica*

A Total of 20 endophytic fungi were isolated from the leaves the endecmic species *Gymnacathera canarica* belonging to different age group to understand the pattern of infection and it showed that the matured leaves exhibited more number of isolates, moderately matured leaves showed lesser number of isolates and on the other hand there were no isoltes obtanied from the young leaves (Table 4.1). This indicates that the infection and colonization of endophytic fungi increased with incraese in the age of the leaves. Of the endophytic fungi isolated in pure culture from Myristica, almost all grew well in PDA. All the isolates were grouped into four different morphotypes based on the colony characters (Table 4.2). Later the While one of the fungi remained nonsporulating, the following were recognized up to generic level. Their brief description is given under:

**1. *Diaporthe* sp.:** The fungus produced a circular, white to dull brownish colony with regular margin and cottony surface laden with glistening watery droplets. The mycelium was composed of colourless, narrow, septate, branched, smooth hyphae. In between the colony, the fungus produced many tiny, round structures and on crushing them, these were found to be fungal fruiting bodies. Conidiogenous cells developed at the tip of the branched conidiophores Alpha Conidia were hyaline, aseptate, ellipsoidal and biguttulate (Plate 7 & 8).

**2. *Fusarium* sp.:** The fungus produced a circular, white colony with regular margin. The mycelium was composed of colourless, narrow, septate, branched, smooth hyphae. The hyphae developed numerous long, septate, branched, smooth, hyaline conidiophores. Conidiogenous cells were vase-shaped, phialidic, developed at the tip of the branched conidiophores; conidiogenous cells are hyaline, phialides, and each produced many, 2-4-septate, fusiform, smooth, colourless conidia, each with a beaked tip cell and curved basal cell (Plate 7).

**3. *Phyllostica sp.*:** This fungus produced a circular, Dark Green colony with irregular marginand hard callus-like surface texture. The mycelium was composed of colourless, narrow, septate, branched, smooth hyphae.

Conidiophores were short cylindrical , and simple and conidiogenous cells were hyaline, aseptate . The conidia were ovoid to ellipsoidal and guttulate (Plate 8 & 11).

**4. *Penicillium sp.*:** The fungus produced a circular, grey green to light brownish colony with irregular margin and powdery surface. The mycelium was composed of colourless, narrow, septate, branched, smooth hyphae. The hyphae developed numerous long, septate, penicillately branched, smooth, hyaline conidiophores. Conidiogenous cells developed in a palisade manner at the tip of the branched conidiophores; the conidiogenous cells are vase-shaped, hyaline, phialides, and each one of them produced a series of one-celled, spherical, smooth, colourless conidia in linear chains (Plate 9 & 10).

**5. The Nonsporulating:** The fungus produced a circular, greyish colony with regular to irregular margin and flat surface. The mycelium was composed of colourless, narrow, septate, branched, smooth hyphae. The fungus did not produce any spores or spore producing structures (Plate 11).

### 4.2.2 Molecular Identification of Dominant Endophytic Fungi Isolated from *G. Canarica*

DNA of fungi was extracted and used for the molecular identification of the Dominant fungi. The PCR amplification of the ITS (Internal transcribed spacer) region by using the ITS region was sequenced with ITS universal primers, the sequencing and assembly was followed by NCBI Blast.

The results obtained showed Diaporthe sp showed 98.82% match with *Diaporthe hongkongensis* whereas phyllostica sp showed match with *Phyllosticta fallopiae* with a percent identity 99.82% (Fig. 4.1 & Fig. 4.2). The Phylogenetic tree of the same obtained (Fig. 4.3 & 4.4).

### 4.3. Statical Analysis

The Absolute frequency (f) i.e. the total no of isolates was 20 and the maximum no. of isolates were obtained from fully matured leaves, followed by medium matured leaves i.e. 11 and 9 respectively, however no isolation was observed in the young leaves indicating that there is an increase in infection and colonization of the leaves by fungi with increasinging age. Among the isolates *Diaporthe sp* showed highest relative frequency i.e 40% followed by *Phyllostica sp*, with R(f) 25%, *Penicillium sp* and *Fusarium sp* showed R(f) of 15% and the least was observed to be Nonsporulating (Table 4.3). Diaporthe sp. Was found to be the dominant species and also the fast growing and sporulating compared to the others, Penecillium sp was the second fastest growing and sporulating followed by Phyllostica sp and fusarium was the slowest and the last one to sporulate.

**Table 4.1: Data Of Inoculation, Isolation Of Endophytic Fungi And Its Categorization Into Diffferent Morphotypes**

| Sr.No. | Leaf Type | Plate Name | No. Of Inoculates | Morphotype | No.of Isolates |
|---|---|---|---|---|---|
| 1 | Mature Leaf | A | 4 | I | 3 |
| | | | | II | 1 |
| 2 | Mature Leaf | B | 4 | I | 2 |
| | | | | IV | 1 |
| 3 | Mature Leaf | C | 3 | I | 3 |
| | | | 1 | III | 1 |
| 4 | Medium Leaf | A | 2 | I | 2 |
| | | | 2 | III | 2 |
| 5 | Medium Leaf | B | 2 | II | 2 |
| | | | 2 | I | 1 |
| 6 | Medium Leaf | C | 4 | II | 2 |
| 7 | Young Leaf | A | 4 | - | - |
| 8 | Young Leaf | B | 4 | - | - |
| 9 | Young Leaf | C | 4 | - | - |
| | | | | Total | 20 |

**Table 4.2: Morphological characters and clasification into diffferent Morphotypes**

| **Morphotypes** | **I** | **I** | **II** | **III** | **IV** |
|---|---|---|---|---|---|
| **Colour** | White | White | Dark Green | Greyish Olive Green | Beige |
| **Texture** | Cottony | Powdery | Hard Callus like | Powdery | Matte texture |
| **Form** | Circular | Circular | Irregular | Circular | Circular |
| **Elevation** | Pulvinate | Flat | Umbonate | Flat | Flat |
| **Margin** | Filiform | Undulate | Undulate | Circular | Circular |
| **Genus Names** | *Diaporthe* Sp | *Fusarium* sp. | *Phyllostica* sp. | *Penicillium* sp. | Non-sporulating |

**Figure 4.1: NCBI Blast Results of *Diaporthe Sp***

**1. Gc W1_ITS**

**Job Title: W1**

**Program** BLASTN
**Database** nt
**Query ID** lcl|Query_99908
**Description** None
**Molecule type** dna
**Query Length** 507

| Description | Scientific Name | Max Score | Total Score | Query Cover | E value | Per. Ident | Acc. Len |
|---|---|---|---|---|---|---|---|
| *Diaporthe hongkongensis* CBS 115448 | *Diaporthe hongkongensis* | 902 | 902 | 100% | 0.0 | 98.82% | 573 |
| *Diaporthe tectonigena* MFLUCC 12-0767 | *Diaporthe tectonigena* | 885 | 885 | 100% | 0.0 | 98.22% | 548 |
| *Diaporthe aseana* MFLUCC 12-0299a | *Diaporthe aseana* | 863 | 863 | 100% | 0.0 | 97.44% | 548 |
| *Diaporthe aseana* MFLUCC 12-0299a | *Diaporthe aseana* | 863 | 863 | 100% | 0.0 | 97.44% | 548 |
| *Phomopsis lithocarpus* strain CGMCC 3.15175 | *Diaporthe lithocarpi (nom. inval.)* | 852 | 852 | 100% | 0.0 | 97.04% | 530 |
| *Diaporthe krabiensis* MFLU 17-2618 | *Diaporthe krabiensis* | 841 | 841 | 99% | 0.0 | 96.83% | 513 |

**Figure 4.2: NCBI Blast Result of *Phyllostica sp***

**2. Gc DG1_ITS5**

**Job Title: Gc DG1**

**Program** BLASTN
**Database** nt
**Molecule type** dna
**Query Length** 566

| Description | Scientific Name | Max Score | Total Score | Query Cover | E value | Per. Ident | Acc. Len |
|---|---|---|---|---|---|---|---|
| *Phyllosticta fallopiae* MUCC 0113 | *Phyllosticta fallopiae* | 1040 | 1040 | 100% | 0.0 | 99.82% | 666 |
| *Phyllosticta fallopiae* MUCC0113 | *Phyllosticta fallopiae* | 1040 | 1040 | 100% | 0.0 | 99.82% | 1208 |
| *Guignardia musicola* CBS 123405 | *Guignardia musicola* | 1040 | 1040 | 100% | 0.0 | 99.82% | 618 |
| *Guignardia alliacea* MUCC0014 | *Guignardia alliacea* | 1035 | 1035 | 100% | 0.0 | 99.65% | 1208 |
| *Phyllosticta capitalensis* CBS:128856 | *Phyllosticta capitalensis* | 1031 | 1031 | 99% | 0.0 | 99.65% | 603 |
| *Phyllosticta rhizophorae* NCY UCC 19-0352 | *Phyllosticta rhizophorae* | 1029 | 1029 | 100% | 0.0 | 99.47% | 658 |
| *Phyllosticta capitalensis* CPC 18848 | *Phyllosticta capitalensis* | 1005 | 1005 | 96% | 0.0 | 99.82% | 571 |
| *Phyllosticta paracapitalensis* CBS 141353 | *Phyllosticta paracapitalensis* | 996 | 996 | 96% | 0.0 | 99.45% | 572 |

**Figure 4.3: Phylogenetic Tree of NCBI Blast Results of *Diaporthe sp***

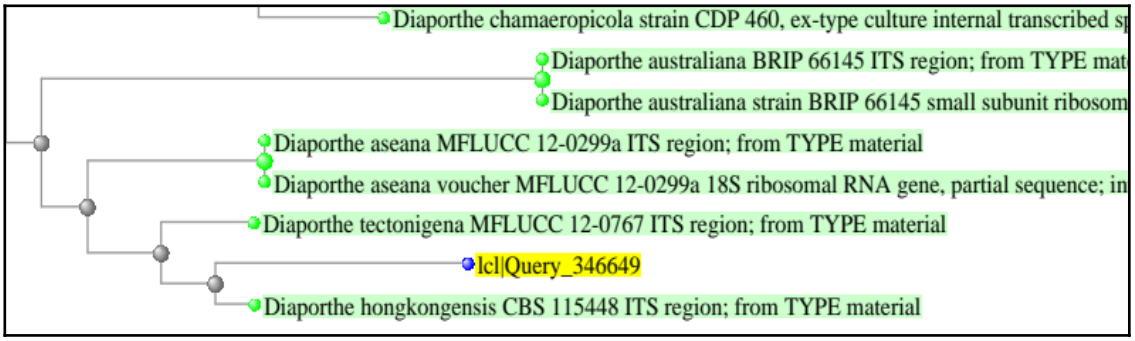


**Figure 4.4: Phylogenetic Tree of NCBI Blast Results of *Phyllostica sp***

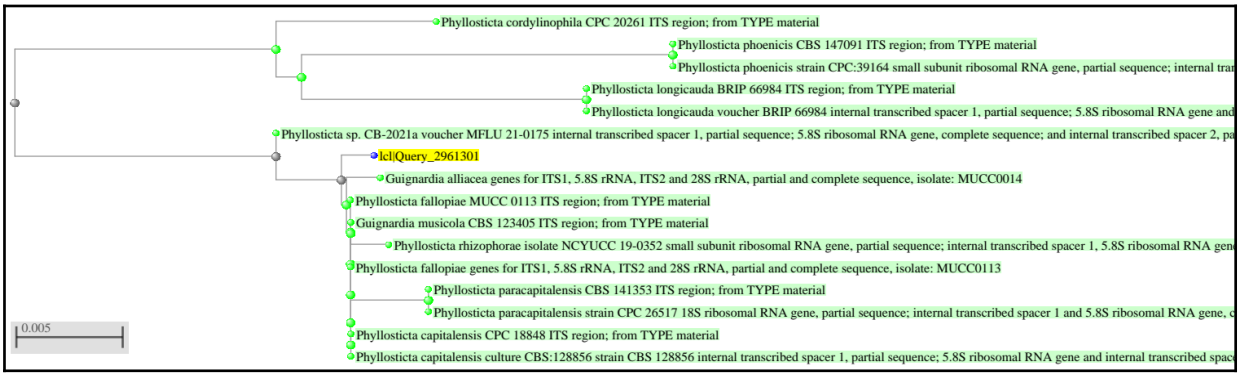



**Table 4.3: Statistical Data of Endophytic Fungal Isolates**

| Sr. No. | Morphotype | Fungal Isolates | Relative frequency (fr) |
|---|---|---|---|
| 1 | I | *Diaporthe Sp* | **40%** |
| 2 | I | *Fusarium Sp.* | **15%** |
| 3 | II | *Phyllostica Sp.* | **25%** |
| 4 | III | *Penicillium Sp.* | **15%** |
| 5 | IV | *Beige Non-Sporulating Sp* | **5%** |

### 4.4.1 Comparative analysis of the bioactive metabolites between the host plant and the isolated dominant endophytic fungi using TLC method

Comparative analysis was carried out between the host plant (P.E) , Diaporthe i non-sporulatimg stage (F1), Diaporthe in sporulatimg stage (F2), penicillium sp. (F3) and the phyllostica sp. (F4) was using Thin Layer Chromatography method (Table 4.4.1).

The dominant, diaporthe sp in the sporulating stage and the host plant exhibited Blue bands with Rf value 0.62 and 0.67 respectively indicating the presence of Phenolic compounds. However Diapothe sp in its non-sporulating phase and the other two fungi did not show any blue bands.

The screening for Alkaloids showed its presence in the fungal extracts as well as in the plant extrat which was indicated by the orange bands (Table 4.4.2).The highest Alkaloid Phyllostica sp and Diaporthe followed by Penicillium and non-sporulating Diaporthe sp, plant extract also showed presence of alkaloids. Screening the extracts for steroids revealed the prominant purple band in all three fungal extracts indicating the presence of steroids in them. However there was no presence of steroids seen in the plant extract and diaporthe sp in the non-sporulated stage. Indicating a relation between sporulation and steroid production in Fungi (Table 4.4.4).

Flavonoids were found to be present in high quatntities indicated by intense flurosent bands under long UV which was found to be equally intense in all three fungi i.e F2, F3 and F4 but little less intense in F1, and falvonoid were absent in the Host plant extract (Table 4.4.3).

**4.4.2 Bioautography**

One of the methods to check of antioxidant assay is by using DPPH method, as it is simple and quick method done using DPPH and UV spectrophotometer. But it is comparatively an expensive chemical and is also highly light sensitive. On contrary $KMnO_4$ which is relative non-toxic and inexpensive and is a similar oxidant like DPPH which in the presence of antioxidants in a sample gets reduced which is indicated by the colour change from Purple to yellow with time. Hence this commonly available oxidant was used to create antioxidant bioauotography using TLC, wherein the TLC was developed with a suitable solvent system and later derivatized using $KMnO_4$. The presence of antioxidant was indicated by whitish-Yellow clear bands were formed on the TLC against the purple background. In the plant and all the fungal extracts tested namely, *Gymnacranthera canarica*, *Diaporthe sp* sporulating and non-sporulating, *penicillium sp* and *Phyllostica sp*, whitish-Yellow bands were observed indicating their antioxidant activity. The highest clearance or White band was observed in Diaporthe sp in sporulating stage followed by *Gymnacranthera canarica*, *penicillium sp*, *Diaporthe sp* non-sporulating and the smallest abnd was observed in *Phyllostica sp.*

**Table 4.4.1: Thin Layer Chromatography for Phenols**

| **TLC – PHENOLS** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sr. No** | **Samples** | **No. of Distinct Band/Spot** | **Spot Distance (cm)** | **Solvent Front (cm)** | **Rf Value (cm)** | **Spot Colour** | **Possible compounds** |
| 1 | G.A | **1** | **6 .0** | **7.5** | **0.80** | **Dark Navy Blue** | **Phenols** |
| 2 | P.E. | **1** | **7.0** | | **0.93** | **Dark Brown** | **Unknown** |
| | | **2** | **6.5** | | **0.86** | **Mustard Yellow** | **Unknown** |
| | | **3** | **5.8** | | **0.77** | **Light Greyish** | **Unknown** |
| | | **4** | **5** | | **0.66** | **Navy Blue** | **Phenols** |
| 3 | F1 | **-** | **-** | | **-** | **-** | **-** |
| 4 | F2 | **1** | **5.8** | | **0.77** | **Faint Blue** | **Phenols** |
| | | **2** | **4.7** | | **0.62** | **Faint Blue** | **Phenols** |
| 5 | F3 | **1** | **6** | | **0.80** | **Orange** | **Unknown** |
| 6 | F4 | **1** | **6** | | **0.80** | **Light Orange** | **Unknown** |
| **G.A- Gallic Acid, P.E.- Plant Extract, F1- Diaporthe sp before sporulation, F2- Diaporthe sp.After sporulation, F3- Penicillium sp, F4- Phyllostica Sp .** | | | | | | | |

**Table 4.4.2: Thin Layer Chromatoraphy for Alkaloids**

| TLC – ALKALOIDS | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sr. No** | **Samples** | **No. of Distinct Band/Spot** | **Spot Distance (cm)** | **Solvent Front (cm)** | **Rf Value (cm)** | **Spot Colour** | **Possible compounds** |
| 1 | P.E | **1** | **7.3** | **7.5** | **0.97** | **Black** | **Unknown** |
| | | **2** | **6.7** | | **0.89** | **Dark Green** | **Unknown** |
| | | **3** | **6.2** | | **0.82** | **Light Mustard Yellow** | **Unknown** |
| | | **4** | **5.5** | | **0.75** | **Light Green** | **Unknown** |
| | | **5** | **2.8** | | **0.37** | **Dark Ornage** | **Alkaloid** |
| 2 | F1 | **1** | **2.3** | | **0.30** | **Orange** | **Alkaloid** |
| 3 | F2 | **1** | **6.1** | | **0.81** | **Orange** | **Alkaloid** |
| | | **2** | **2.3** | | **0.30** | **Orange** | **Alkaloid** |
| 4 | F3 | **1** | **2.3** | | **0.30** | **Orange** | **Alkaloid** |
| 5 | F4 | **1** | **7.3** | | **0.97** | **Orange** | **Alkaloid** |
| | | **2** | **2.3** | | **0.30** | **Orange** | **Alkaloid** |
| **P.E.- Plant Extract, F1- Diaporthe sp before sporulation, F2- Diaporthe sp.After sporulation, F3- Penicillium sp, F4- Phyllostica Sp,.** | | | | | | | |

**Table 4.4.3: Thin Layer Chromatoraphy for Flavonoids**

| TLC – FLAVONOIDS | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sr. No** | **Samples** | **No. of Distinct Band/Spot** | **Spot Distance (cm)** | **Solvent Front (cm)** | **Rf Value (cm)** | **Spot Colour** | **Possible compounds** |
| 1 | P.E | **1** | **7.5** | **7.5** | **0.96** | **Dark Blue** | **Unknown** |
| | | **2** | **6.6** | | **0.88** | **Orangish red Fluorescence** | **Chlorophyl** |
| | | **3** | **6.0** | | **0.80** | **Light Green** | **Unknown** |
| 2 | F1 | **1** | **2.3** | | **0.30** | **Light Blue Fluorescence** | **Flavonoids** |
| 3 | F2 | **1** | **6.5** | | **0.86** | **Intense Cyan Blue Fluorescence** | **Flavonoids** |
| | | **2** | **5.5** | | **0.73** | **Intense Cyan Blue Fluorescence** | **Flavonoids** |
| | | **3** | **4.3** | | **0.57** | **Faint Blue Fluorescence** | **Flavonoids or Flavonoids like compounds** |
| | | **4** | **2.3** | | **0.30** | **Very Faint Blue Fluorescence** | **Flavonoids or Flavonoids like compounds** |
| 4 | F3 | **1** | **6.8** | | **0.90** | **Light Blue Fluorescence** | **Unknown** |
| | | **2** | **5.8** | | **0.77** | **Intense Cyan Blue Fluorescence** | **Flavonoids** |
| | | **3** | **2.3** | | **0.30** | **Very Faint Blue Fluorescence** | **Flavonoids like compounds** |
| 5 | F4 | **1** | **7.3** | | **0.97** | **Light Blue** | **Unknown** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | **Fluorescence** | |
| | | **2** | **6.4** | | **0.85** | **Blue Fluorescence** | **Flavonoids like compounds** |
| | | **3** | **6.3** | | **0.84** | **Intense Cyan Blue Fluorescence** | **Flavonoids** |
| | | **4** | **2.3** | | **0.30** | **Blue Fluorescence** | **Flavonoids like compounds** |
| **P.E.- Plant Extract, F1- Diaporthe sp before sporulation, F2- Diaporthe sp.After sporulation, F3- Penicillium sp, F4- Phyllostica Sp,.** | | | | | | | |

**Table 4.4.4: Thin Layer Chromatoraphy for Steroids**

| **TLC – STEROIDS** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sr. No** | **Samples** | **No. of Distinct Band/Spot** | **Spot Distance (cm)** | **Solvent Front (cm)** | **Rf Value (cm)** | **Spot Colour** | **Possible compounds** |
| 1 | P.E | **1** | **7.7** | **7.5** | **0.96** | **Pink** | **Unknown** |
| | | **2** | **6.6** | | **0.88** | **Peach** | **Unknown** |
| | | **3** | **5.1** | | **0.68** | **Light Peach** | **Unknown** |
| | | **4** | **3.7** | | **0.49** | **Light Peach** | **Unknown** |
| | | **5** | **3** | | **0.40** | **Dark Green** | **Unknown** |
| | | **6** | **1.5** | | **0.20** | **Grey** | **Unknown** |
| | | **7** | **0.8** | | **0.10** | **Dark Grey** | **Unknown** |
| 2 | F1 | **1** | **0.8** | | **0.10** | **Light Grey** | **Unknown** |
| 3 | F2 | **1** | **7.3** | | **0.97** | **Bright Peach** | **Unknown** |
| | | **2** | **6.7** | | **0.89** | **Light Orange** | **Unknown** |
| | | **3** | **2.9** | | **0.38** | **Purple** | **Steroid** |
| | | **4** | **1.8** | | **0.24** | **Faint Grey** | **Unknown** |
| | | **5** | **1.1** | | **0.14** | **Faint Grey** | **Unknown** |
| 4 | F3 | **1** | **7.3** | | **0.97** | **Bright Peach** | **Unknown** |
| | | **2** | **6.6** | | **0.88** | **Light Orange** | **Unknown** |
| | | **3** | **2.8** | | **0.37** | **Purple** | **Steroid** |
| | | **4** | **1.5** | | **0.20** | **Grey** | **Unknown** |
| | | **5** | **0.8** | | **0.10** | **Faint Grey** | **Unknown** |
| 5 | F4 | **1** | **2.9** | | **0.38** | **Light Purple** | **Steroid** |
| | | **2** | **0.8** | | **0.10** | **Faint Grey** | **Unknown** |
| **P.E.- Plant Extract, F1- Diaporthe sp before sporulation, F2- Diaporthe sp.After sporulation, F3- Penicillium sp, F4- Phyllostica Sp,.** | | | | | | | |

**4.5 Total Phenolic Content (TPC)**

The TPC results in ethylacetate extracts of host plant *Gymnacranthera canarica*, and its endophytic fungi *Diaporthe sp* in its non-sporulating and sporulating stages , *Phyllostica sp*, and *Penicillium sp*, are shown in the table. and Fig. The highest phenolic content was seen in *Gymnacranthera canarica* i.e 408.44 µg/mL followed by *Diaporthe sp* in sporulating stage which is 276.11 µg/mL the least phenolic content was observed in *Phyllostica* sp which was 95.51 µg/mL (Table 4.5 and Fig. 4.5.1 & Fig. 4.5.2).

**4.6 Screening of the Endophytic fungi for different enzyme activity.**

The three fungi *Diaporthe sp, Phyllostica sp.* and the *Penicillium s*p were screened for production and secreation of various enzymes. All the three species showed Phosphate solubelization activity, which was confimed by the production of halo zone around the colonies on the Pikovskaya agar. Indicating their possible role in facilitating Phosphate solubilization and uptake in plants. The *Diaporthe sp* and the *penicillium sp* also showed positive result in amylolytic activity where the zone of halo was more in *penicillium sp* as compared to *Diaporthe sp*. However *phyllostica sp.* did not show any amylolytic activity. Mahfooz et al (2017) in thier studies found that amylolytic activity of a few endophytes indicates that they have the capacity to break down starch, which becomes available during plant senesces . This indicates that *Diaporthe sp* and the *penicillium sp isolated from Gymnacranthera canarica* may display saprophytic nature during plant senesces. All the three species showed negative response to Laccase, Lipase, Cellulase and Pectinolytic activity ( Table 4.6).

**4.7 Silver Nanoparticles of the Dominant Fungi : Diaporthe sp.**

Silver nanoparticles were synthesised by challenging the fungal extract of Diaporthe species with 0.001M sivler nitrate solution. The change of the colour of the mixture from colourless to brick red indicated the formation of silver nanoprticles, which was then verified by spectometric analysis. In the UV-Visible spectrum the peak was observed at 410.00 nm which is in the range as the AgNPs are knowm to exhibhit a UV-Visible absorption maximum in the range 350-500nm.

The SEM analysis shows the synthesiszed AgNPs were globular shape with particle size rangimg from 87.9 – 200 nm.

**Table 4.5: Total Phenolic content in different plnat and fungal samples**

| Sr No. | Sample ID | WL 765.0 | TPC (µg/mL) |
|---|---|---|---|
| 1 | ***Gymnacranthera canarica*** | 1.624 | 408.446115288221 |
| | Standard Deviation | 0.093 | |
| 2 | ***Diaporthe sp non-sporulated*** | 0.418 | 106.190476190476 |
| | Standard Deviation | 0.0005 | |
| 3 | ***Diaporthe sp sporulated*** | 1.096 | 276.115288220551 |
| | Standard Deviation | 0.001 | |
| 4 | ***Penicillium sp*** | 0.592 | 149.799498746867 |
| | Standard Deviation | 0.0005 | |
| 5 | ***Phyllostica sp*** | 0.377 | 95.9147869674185 |
| | Standard Deviation | 0.001 | |

**Figure : 4.5.1: Standard Gallic acid Caliberation curve for determination of Total Phenolic content 0.001mg/mL at varing concentration**

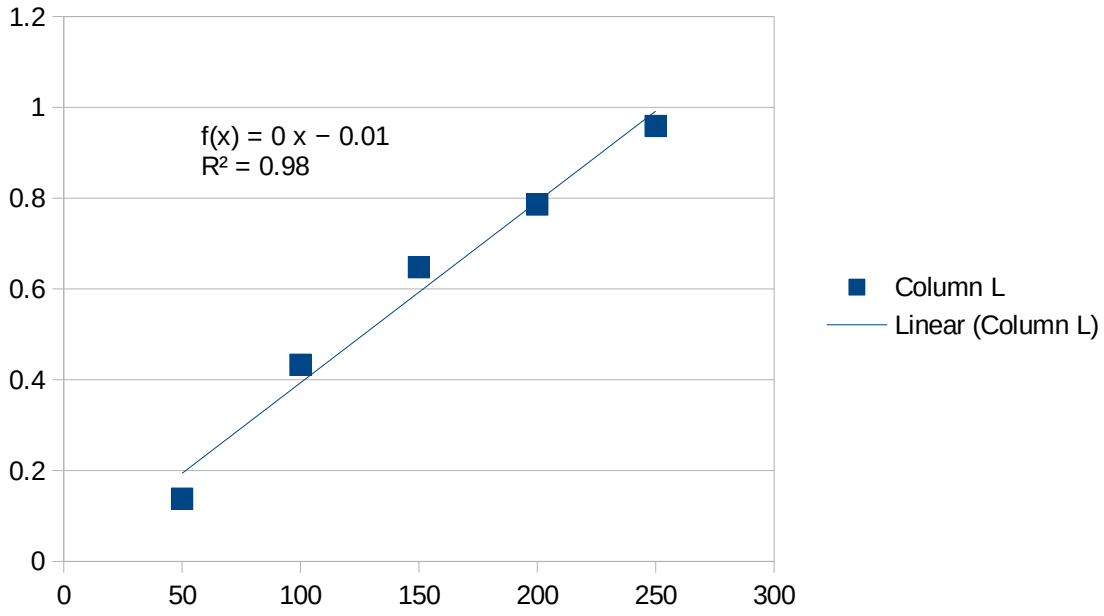



**Figure 4.5.2: Total Phenolic content of Plant and Fungal extracts**

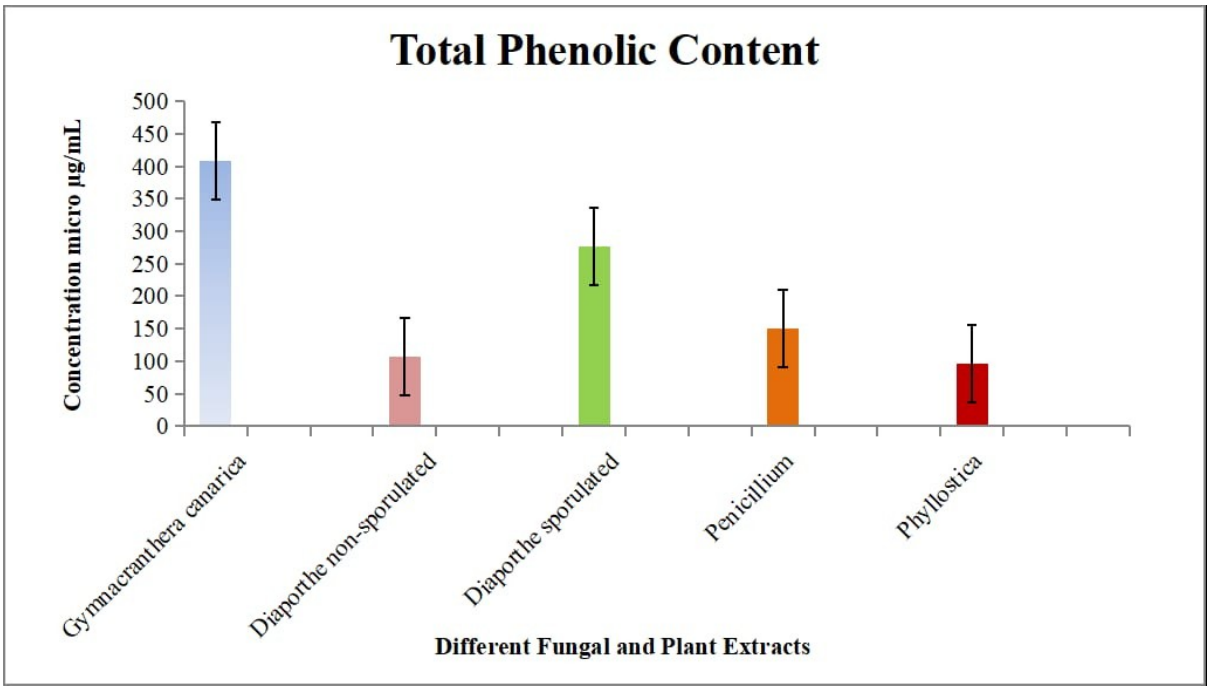



**Table 4.6: Screening the Endophytic Fungi For Different Enzyme activity**

| Sr. No. | Enzyme activity | *Diaporthe sp.* | *Penecillium sp.* | *Phyllostica sp.* |
|---|---|---|---|---|
| 1 | Phosphate solubelization activity | + | + | + |
| 2 | Amylolytic activity | + | + | - |
| 3 | Cellulolytic activity | - | - | - |
| 4 | Pectinolytic activity | - | - | - |
| 5 | Laccase activity | - | - | - |
| 6 | Lipase activity | - | - | - |

**4.8 Screening the plant and fungal extracts for Antibacterial activity against the Human pathogenic bacteria.**

The Silver nanoparticles synthesised from *Diaporthe sp*, Plant and Fungal extracts in ethy acetate were screened against Human Pathogenic Bateria such as ***Salmonella Typhi, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus pyogenes, salmonella typhimurium, Serratia marcescens*** and ***Chromobacterium violaceum*** the results are as shown in the table. The Silver Nanoparticles showed antibacterial activity against all the bacteria it was screened for. The fungal extract of *Diaporthe sp* at sporulating stage showed antibcaterial activity againts all the cultures used except for *Staphylococcus aureus* and *Serratia marcescens* where the zone of inhibhition was either equal or slightly more as compared to Silver nanoparticles and the highest as compared to all the other fungal extracts with only two exception i.e of *Pseudomonas aeruginosa* and *Chromobacterium violaceum* in Agar well Diffustion method where the silver nanoparticles showed higher zone of inhibhition than *Diaporthe sp*.

The *Diaporthe sp* in non-sporulating stage it showed antibacterial activity against *Pseudomonas aeruginosa, Streptococcus pyogenes* and *Chromobacterium violaceum.* The fungal extract of *Phyllostica s*howed growth inhibhition againt 2 strains *i.e. Pseudomonas aeruginosa* and *Chromobacterium violaceum* where the zone of inhibhition was the least as compered to all the other extracts that showed antibacterial activity. The fungal extract *of Penecillium sp* did not show any antibacterial activity againts any strains it was screened for. The Plant extract showed inhibhition of bacterial growth only againts *Streptococcus pyogenes* and *Chromobacterium violaceum* (Table 4.8, Table 4.9, Fig.4.6.1 & Fig 4.6.2).

In a comparative analysis between Disc Diffusion method and Agar Well Diffusion for ***Salmonella Typhi, Pseudomonas aeruginosa and Klebsiella pneumoniae,*** The Agar Well Diffusion Method showed more better zone of inhibhition as compared to Disc Diffusion Method.

**4.9. Quorum Quenching Activity**

The Plant and all Fungal extracts in ethy acetate along with the Silver nanoparticles synthesised from *Diaporthe sp* were screened for Quorum quenching activity against ***Serratia marcescens*** and ***Chromobacterium violaceum*** in which the quenching activity was seen in AgNPs and *Diaporthe* sp in sporulating stage against ***Serration marcescens*** which was indicated by clearance of bright red pigment by inhibhitimg its production by the bacteria. However ***Chromobacterium violaceum*** did not produce any pigmentation even after exposing it to stress ( -4$^{o}$C), hence no Quorum quenching activity was observed (Table 4.10).

**4.10. Community-Based Conservation Programs**

In our dedicated efforts to conserve the Myristica Swamps, we organized a community-driven cleanliness drive. We actively engaged with local communities to clean up the Myristica Swamps, which had been adversely affected by pollution caused by human actions. By removing plastic, glass and other pollutants, we aimed to restore the natural balance of these vital ecosystems.

Recognizing the importance of community involvement, we conducted awareness sessions for the locals. During these talks, we emphasized the significance of these Sacred Groves, areas considered sacred by local communities and their role in maintaining ecological balance. We shared existing knowledge about the swamps and supplemented it with our own research findings.

Our goal was to instill a sense of pride and moral responsibility among the locals. As the people living in close proximity to the swamps, they play a crucial role in their well-being. By understanding the ecological value of these swamps, we hoped to inspire a collective commitment to their conservation

**Table 4.7*:* Screening Plant And Fungal Extracts For Antimicrobial Activity Against Human Pathogenic Bacteria**

| **Sr. No.** | **Bacterial Strain.** | **Gram staining** | **Plant Extract** | **Diaporthe sp. non-sporulating** | **Diaporthe sp. sporulating** | ***Penecilliu m sp.*** | ***Phyllostic a sp.*** | **Silver nanoparti cles** |
|---|---|---|---|---|---|---|---|---|
| 1 | *Salmonella Typhi* | Gram negative | - | - | + | - | - | + |
| 2 | *Pseudomonas aeruginosa* | Gram negative | - | + | + | - | + | + |
| 3 | *Staphylococcus aureus* | Gram positive | - | - | - | - | - | + |
| 4 | *Klebsiella pneumoniae* | Gram negative | - | - | + | - | - | + |
| 5 | *Streptococcus pyogenes* | Gram positive | + | + | + | - | - | + |
| 6 | *Chromobacteriu m violaceum* | Gram negative | + | + | + | - | + | + |
| 7 | *Salmonella typhimurium* | Gram negative | - | - | + | - | - | + |
| 8 | *Serratia marcescens* | Gram negative | - | - | - | - | - | + |

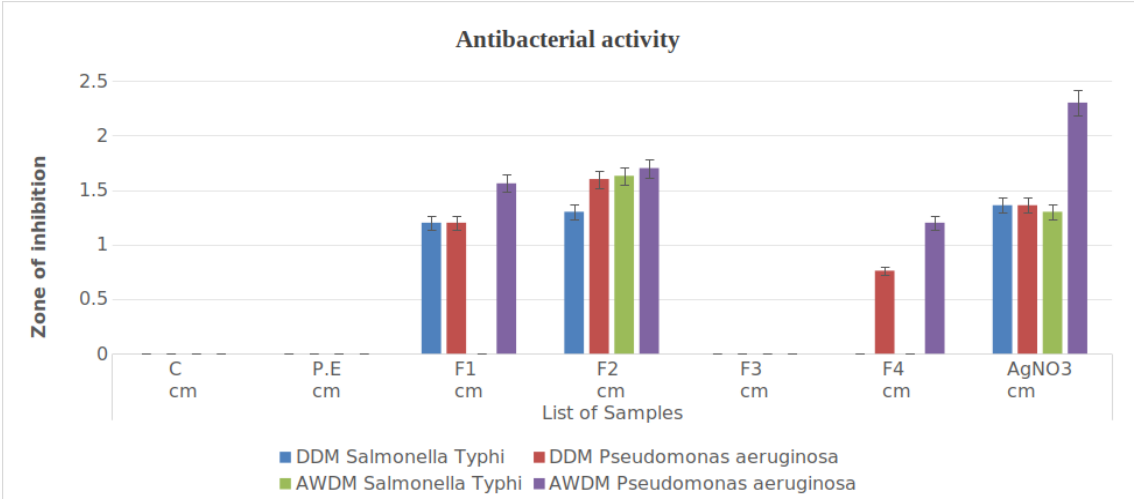
**Table 4.8: Comparative Analysis Of Antibacterial Acitivity Between Disk Diffusion Method And Agar Well Diffusion Method**

| **Sr.No.** | **Method** | **Bacteria** | **Zone of Inhibhition of different Samples** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | **C cm** | **P.E cm** | **F1 cm** | **F2 cm** | **F3 cm** | **F4 cm** | **$AgNO_3$ cm** |
| *1* | | ***Salmonella Typhi*** | - | - | + | + | - | - | + |
| | DDM | Plate 1 | 0 | 0 | 1.3 | 1.2 | 0 | 0 | 1.4 |
| | | Plate 2 | 0 | 0 | 1.1 | 1.2 | 0 | 0 | 1.3 |
| | | Plate 3 | 0 | 0 | 1.2 | 1.5 | 0 | 0 | 1.4 |
| | **Avg.** | | **0** | **0** | **1.2** | **1.3** | **0** | **0** | **1.36** |
| | AWDM | Plate 1 | 0 | 0 | 0 | 1.6 | 0 | 0 | 1.3 |
| | | Plate 2 | 0 | 0 | 0 | 1.6 | 0 | 0 | 1.3 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Plate 3 | 0 | 0 | 0 | 1.7 | 0 | 0 | 1.4 |
| | **Avg.** | | **0** | **0** | **0** | **1.63** | **0** | **0** | **1.3** |
| 2 | | ***Pseudomonas aeruginosa*** | - | - | + | + | - | + | + |
| | DDM | Plate 1 | 0 | 0 | 1.1 | 1.5 | 0 | 0.7 | 1.4 |
| | | Plate 2 | 0 | 0 | 1.2 | 1.5 | 0 | 0.8 | 1.3 |
| | | Plate 3 | 0 | 0 | 1.3 | 1.8 | 0 | 0.8 | 1.4 |
| | **Avg.** | | **0** | **0** | **1.2** | **1.6** | **0** | **0.76** | **1.36** |
| | AWDM | Plate 1 | 0 | 0 | 1.6 | 1.7 | 0 | 1.2 | 2.4 |
| | | Plate 2 | 0 | 0 | 1.5 | 1.7 | 0 | 1.2 | 2.3 |
| | | Plate 3 | 0 | 0 | 1.6 | 1.7 | 0 | 1.2 | 2.3 |
| | **Avg.** | | **0** | **0** | **1.56** | **1.7** | **0** | **1.2** | **2.3** |

**Figure 4.6.1: Antibacterial Activity- Comparative Analysis Between Disc Diffusion Method and Agar Well Diffusion Method**

**Table 4.9:**




**Screening Plant And Fungal Extracts For Antimicrobial Activity Against Human Pathogenic Bacteria Using Disk Diffusion Method**

| **Sr. No.** | **Method** | **Bacteria** | **Zone of Inhibhition of different Samples** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | **C cm** | **P.E cm** | **F1 cm** | **F2 cm** | **F3 cm** | **F4 cm** | **$AgNO_3$ cm** |
| 1 | DDM | ***Salmonella Typhi*** | - | - | + | + | - | - | + |
| | | Plate 1 | 0 | 0 | 1.3 | 1.2 | 0 | 0 | 1.4 |
| | | Plate 2 | 0 | 0 | 1.1 | 1.2 | 0 | 0 | 1.3 |
| | | Plate 3 | 0 | 0 | 1.2 | 1.5 | 0 | 0 | 1.4 |
| | **Avg.** | | **0** | **0** | **1.2** | **1.3** | **0** | **0** | **1.36** |
| 2 | DDM | ***Pseudomonas aeruginosa*** | - | - | + | + | - | + | + |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Plate 1 | 0 | 0 | 1.1 | 1.5 | 0 | 0.7 | 1.4 |
| | | Plate 2 | 0 | 0 | 1.2 | 1.5 | 0 | 0.8 | 1.3 |
| | | Plate 3 | 0 | 0 | 1.3 | 1.8 | 0 | 0.8 | 1.4 |
| | | **Avg.** | **0** | **0** | **1.2** | **1.6** | **0** | **0.76** | **1.36** |
| 3 | DDM | ***Staphylococcus aureus*** | - | - | - | - | - | - | + |
| | | Plate 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | | Plate 2 | 0 | 0 | 0 | 0 | 0 | 0 | 1.2 |
| | | Plate 3 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| | | **Avg.** | **0** | **0** | **0** | **0** | **0** | **0** | **1** |
| 4 | DDM | ***Klebsiella pneumoniae*** | - | - | - | + | - | - | + |
| | | Plate 1 | 0 | 0 | 0 | 1.2 | 0 | 0 | 1.1 |
| | | Plate 2 | 0 | 0 | 0 | 1.2 | 0 | 0 | 1.1 |
| | | Plate 3 | 0 | 0 | 0 | 1.3 | 0 | 0 | 1.2 |
| | | **Avg.** | **0** | **0** | **0** | **1.2** | **0** | **0** | **1.1** |

**Figure : 4.6.2: Antibacterial Activity Using Disc Diffusion Method**

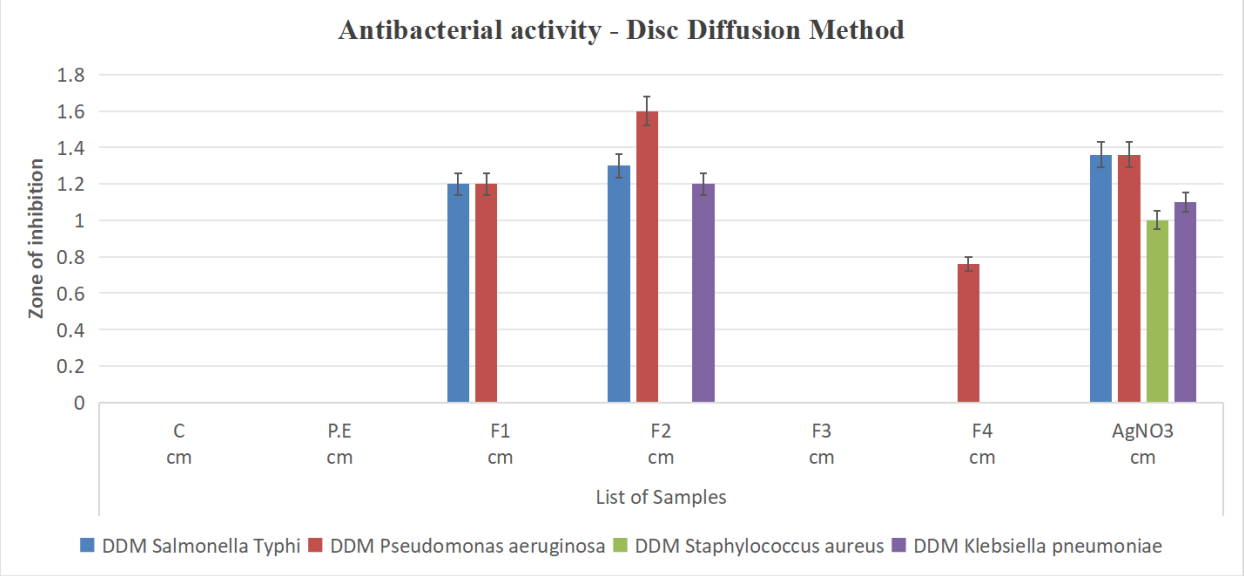



**Table 4.1: Screening Plant And Fungal Extracts For Quorum Quenching Ability**

| **Sr. No.** | **Bacteria** | **Samples** | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **C** | **P.E** | **F1** | **F2** | **F3** | **F4** | **$AgNO_3$** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1 | *Serratia marcescens* | - | - | - | + | - | - | + |
| 2. | *Chromobacterium violaceum* | - | - | - | - | - | - | - |

## DISCUSSION

The relationship between the Endophytic fungi and the host plant *Gymnacranthera canarica* shows that there is a positive impact of the endophytic funig in helping the plant in coping with various biotic and abiotic stress factors by producing secondary metabolites and overall development of the plant by facilitating various nutrient uptake (N, P, K, Fe and Zn) enzyme activity and antioxidant response as mentioned by Pereira et al., (2023) thier study expressed similar results, where the plants inoculated with Diaporthe sp plays a positive role in the modulation of tomato plant responses to drought stress by combining various processes such as improving photosynthetic capacity, nutrient uptake, enzymatic antioxidant response and osmo-protectant accumulation as compared to the uninoculated tomato plant. Tanapichatsakul et al, (2018) mentioned in their study that numerous bioactive substances with anticancer, antimicrobial, antioxidant, antidiabetic, anti-inflammatory, and immunosuppressive qualities are produced by fungal endophytes.The crude extracts of Two *Diaporthe* sp.showed significant antioxidant and antibacterial properties and their potential antioxidant activity could be attributed to phenolic compounds, which also similar results in our study wherein the diaporthe sp shows higher content of total phenols as compraed to the other isolates and exhibhiting higher antioxidant activity in bioefficacy studies. Kemkuignou et al (2023) in thier studies found that Diapothe sp displayed significant antiviral activity against three subtypes of the influenza A virus. According to Sousa, et al, (2016) A new group of chromanones and known phomosines, have been isolated from the genus Diaporthe. The outcomes of their study validate the extensive range of chemical diversity generated by endophytic fungi, particularly those belonging to the Diaporthe genus. Furthermore, phomosines A and C might be regarded as antimicrobial agents that direct the creation of novel antibiotics. A similar observation is made in our study that Diaporthe sp showed the highest range of antimicrobial activity againgsh human pathogenic bacteria and is an eligible candidate to isolate bioavtive compounds for new antibiotcs. These antimicrobial properties of isolates maybe also contributing to the protection or resistance of the plants to various bacterial and fungal infection in the swampy environment. The silver nanoparticles showing antibacterial activity against wide range of bacteria can be further screened for anticancer and cytotoxicity.Several studies have also demonstrated that endophytic fungi can produce plant-derived bioactive compounds in a way that is comparable to that of their host plants Zhao et al.(2010).Because of their capacity to promote plant growth, species in the genus Diaporthe have also been shown to be highly promising agents in the creation of biofertilizers Hilário & Gonçalves (2022). Our Current study will contribute as a foundational prilimnary work for further resaerch on *Gymancrathera canarica* and its endophytic isolates to explore the other aspects mentioned above.

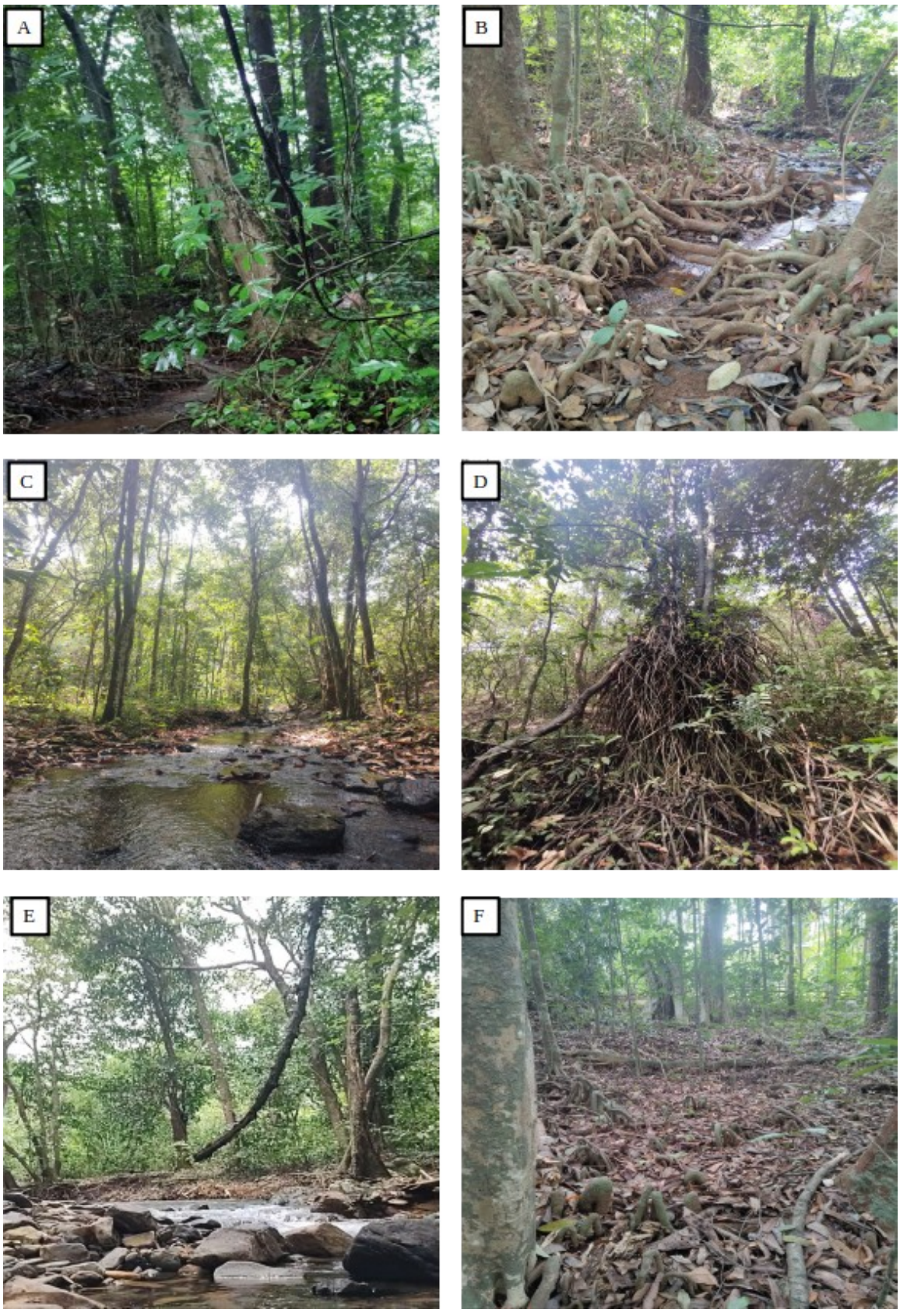



**Plate 1**: Locations of Myristica Swamps in Goa A) Nirankarachi Rai, B) *Gymnacranthera canarica* dominating Nirankarachi Rai, C) Woddyar, D) *Myristic fatua* dominating in Woddyar E) Netravali Widlife Sanctuary, F) *Gymnacranthera canarica* dominating in Netravali Widlife Sanctuary

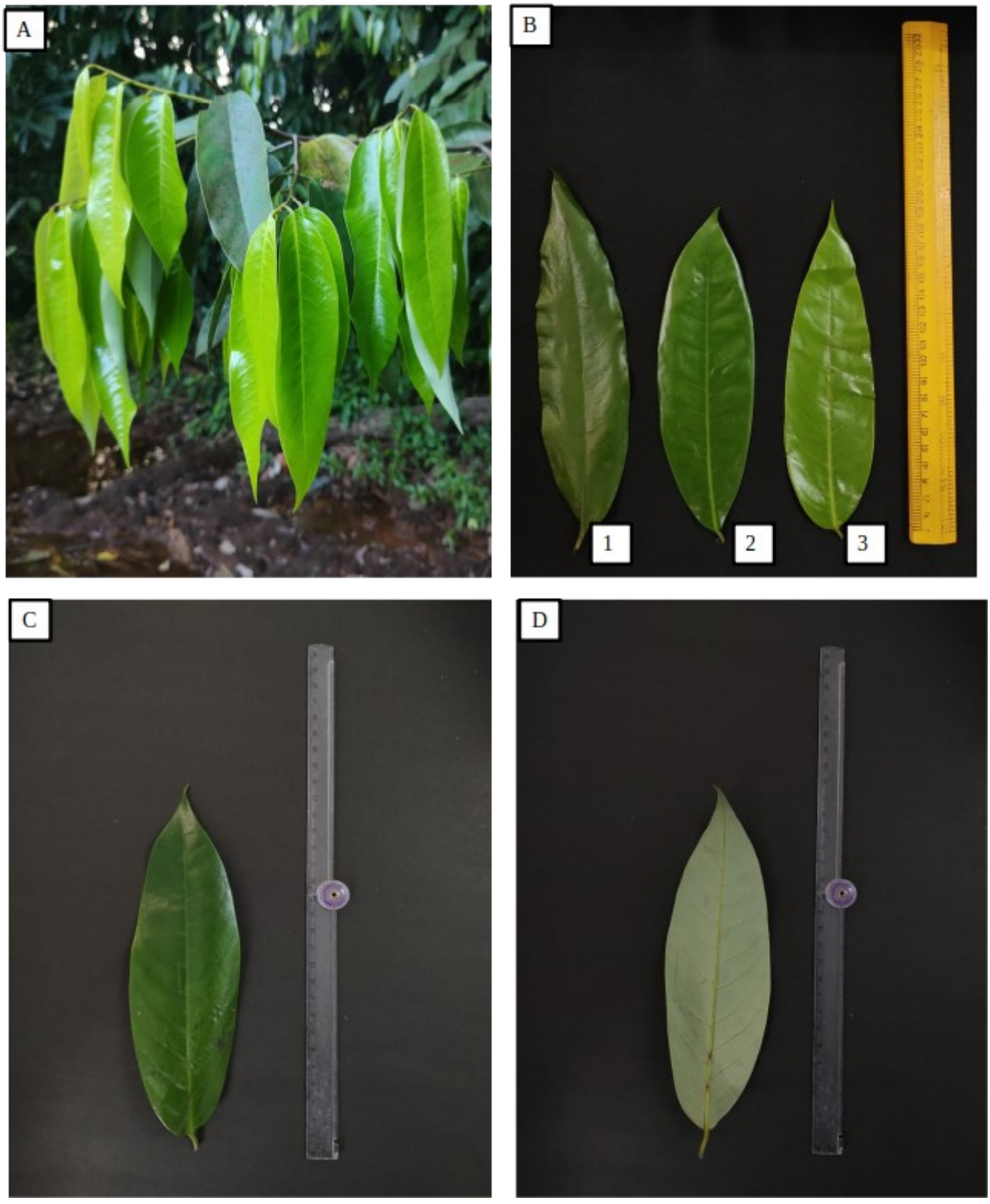



**Plate.2:** Leaf Smples Collected from the Swamps A) *Gymnacranthera canarica* in the Swamp, B) 1. Fully matured leaf, 2. Medium/ moderately matured leaf and 3. Young leaf C) Leaf characteristic- front, D. Leaf characteristic- back


**Plate 3:** Different Characteristics of Gymnacranthera canarica, A) Leaf Arrangment, B) Knee Roots, C) Height of the Plant, D) Branching pattern


**Plate.4:** Different Characteristics of *Gymnacranthera canarica* seed, A) Seed wih woddy reddish brown seedcoat, B) Seed with broken seed coat, C) Transvers Section of the seed, D) Seed with Fleshy Pericarp.


**Plate.5:** Sample Preparation, A) Washing the leaves, B) Pat drying of the leaves C) shade drying the leaves, D) Dried Leaves.


**Plate.6:** Extract preparation A ) Coaresly ground dried leaf powder B) Extraction of leaves using Soxhelet method C) Vaccum evaporation of the extracts using Rotary Vaccum Evaporator, D) Dried extract powder.

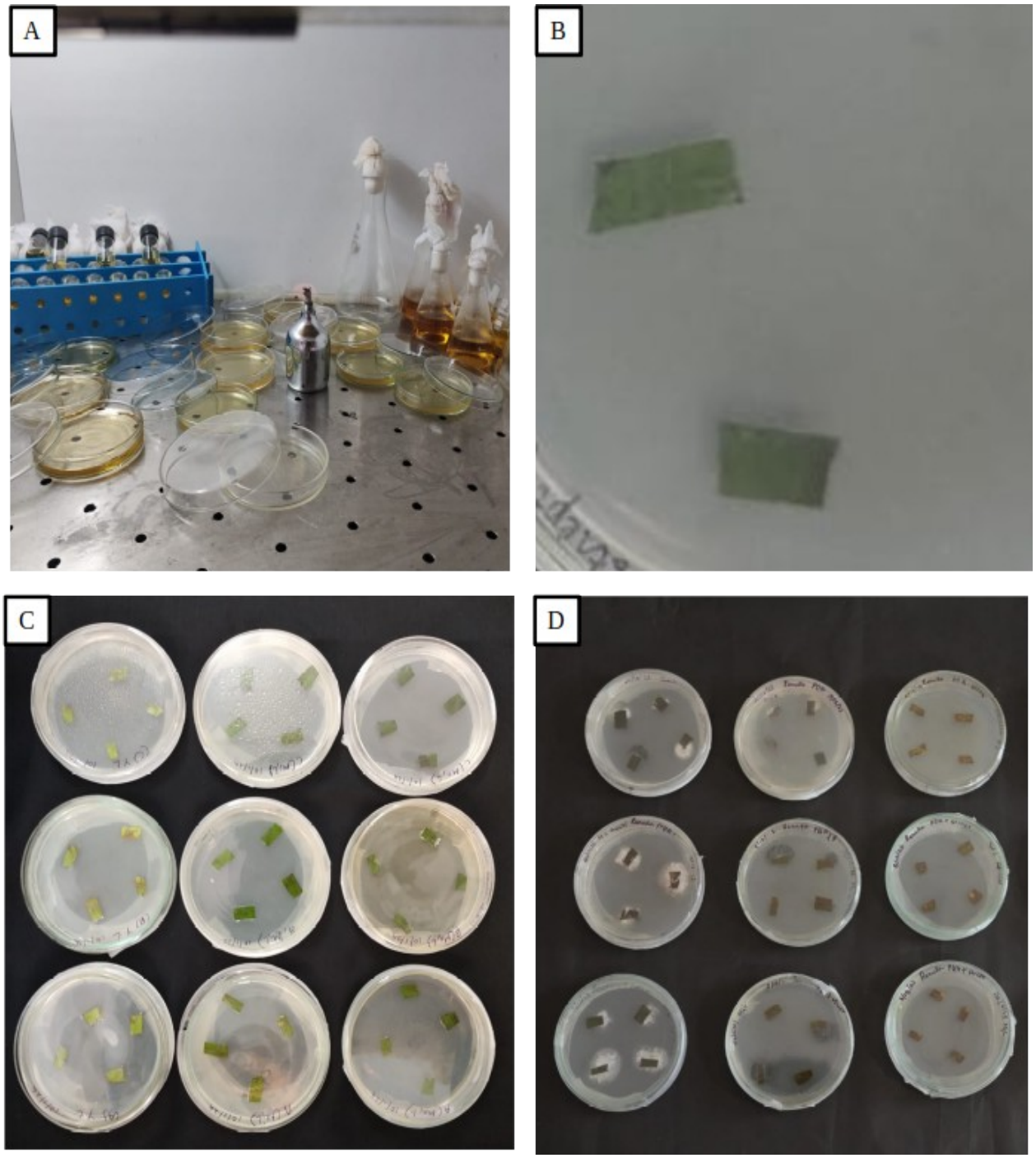



**Plate 7 :** Steps in Isolation of Endophytic fungi, A ) Media Preparation, B) Cutting and inoculating approx. 1 cm peices of leaf samples, C) No. Of plates Inoculated , D) No. Of Isolates obtained

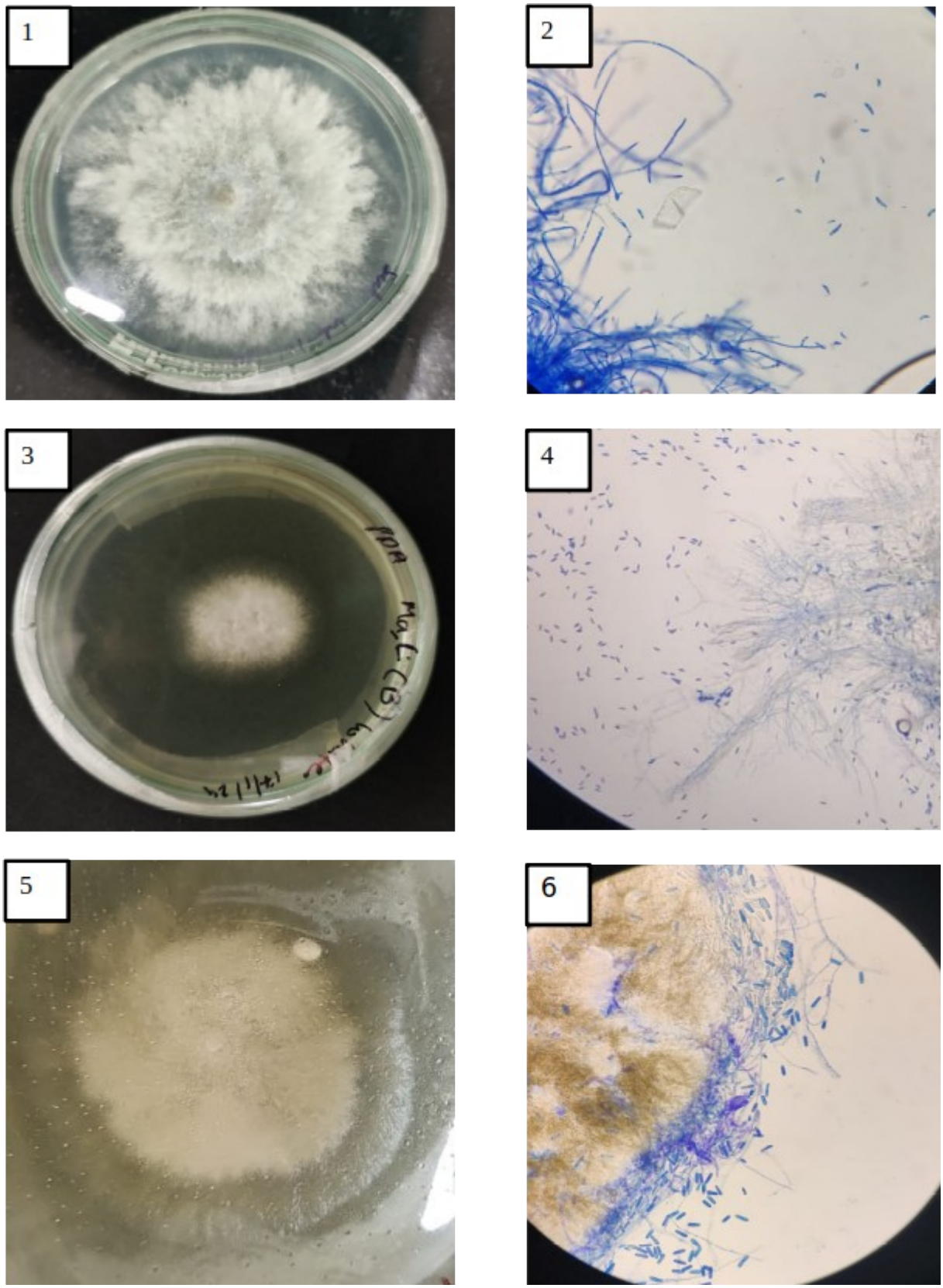



**Plate 8.1:** Endophytic Fungal isolates and their spores, 1) *Fusarium sp*, 2) Spores of *Fusarium sp* , 3) *Diaporthe sp*, 4) Spores of *Diaporthe sp*, 5) *Diaporthe sp*, 6) Spores of *Diaporthe sp*,

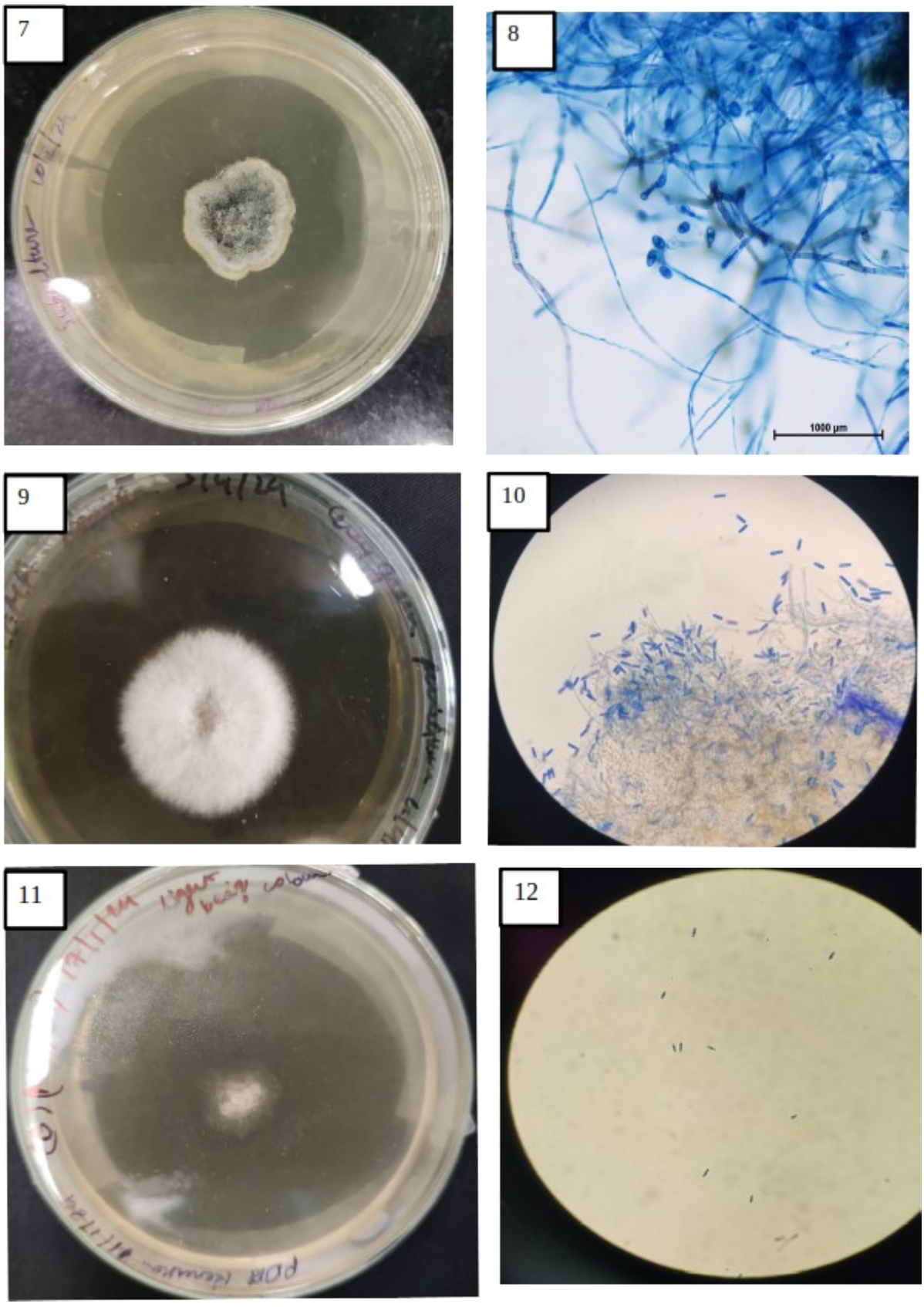



**Plate.8.2:** Endophytic Fungal isolates and their spores, 7) *Phyllostica Sp*, 8) Spores of *Phyllostica Sp*, 9) *Diaporthe sp*, 10) Spores of *Diaporthe sp*, 11) *Diaporthe sp*, 12)Spores of *Diaporthe sp*,

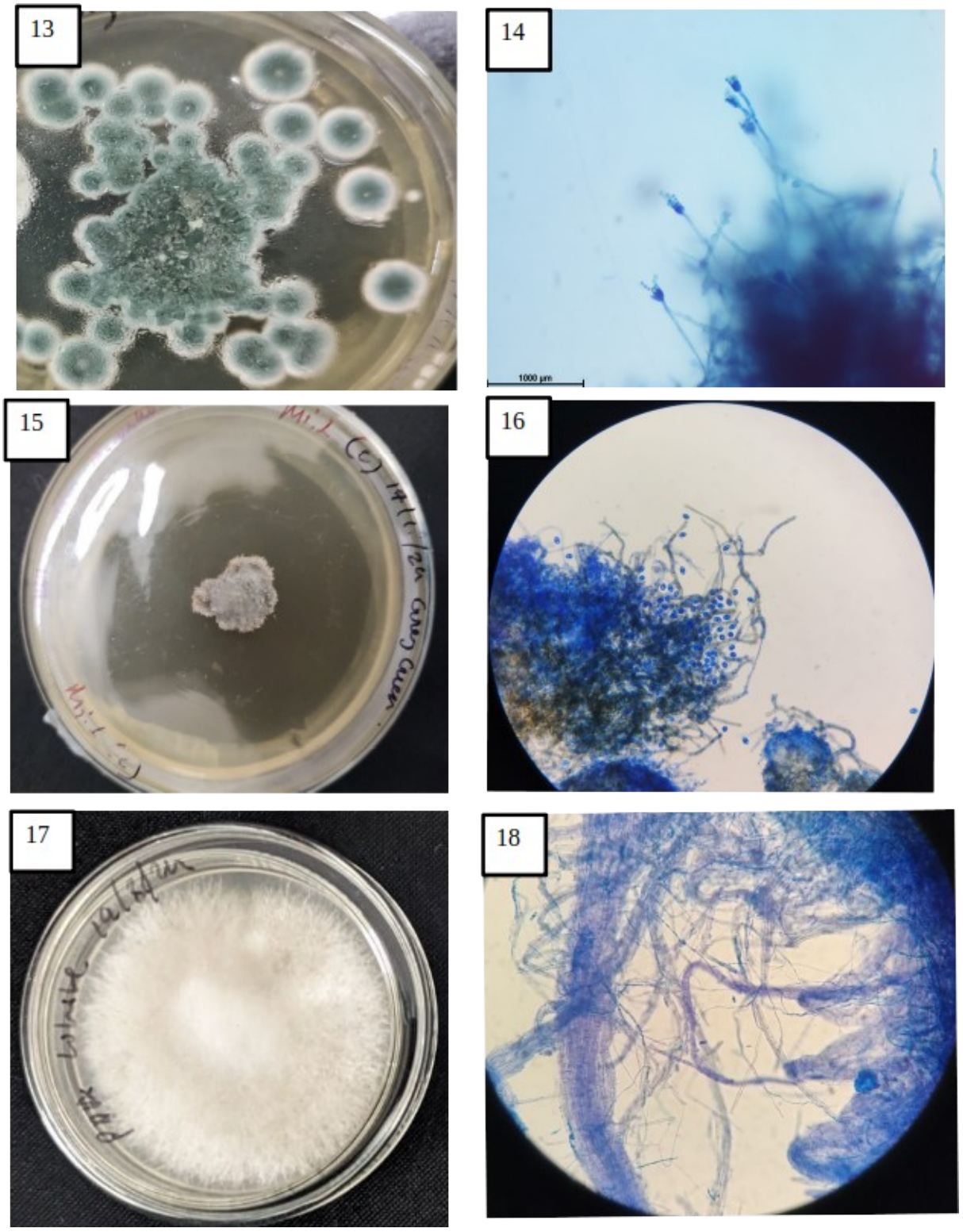



**Plate.8.3:** Endophytic Fungal isolates and their spores, 13) *Penicillium sp*, 14) Spores of *Penicillium sp*, 15) *Phyllostica Sp*, 16) Spores of *Phyllostica Sp*, 17) *Diaporthe sp*, 18) Spores of *Diaporthe sp*,

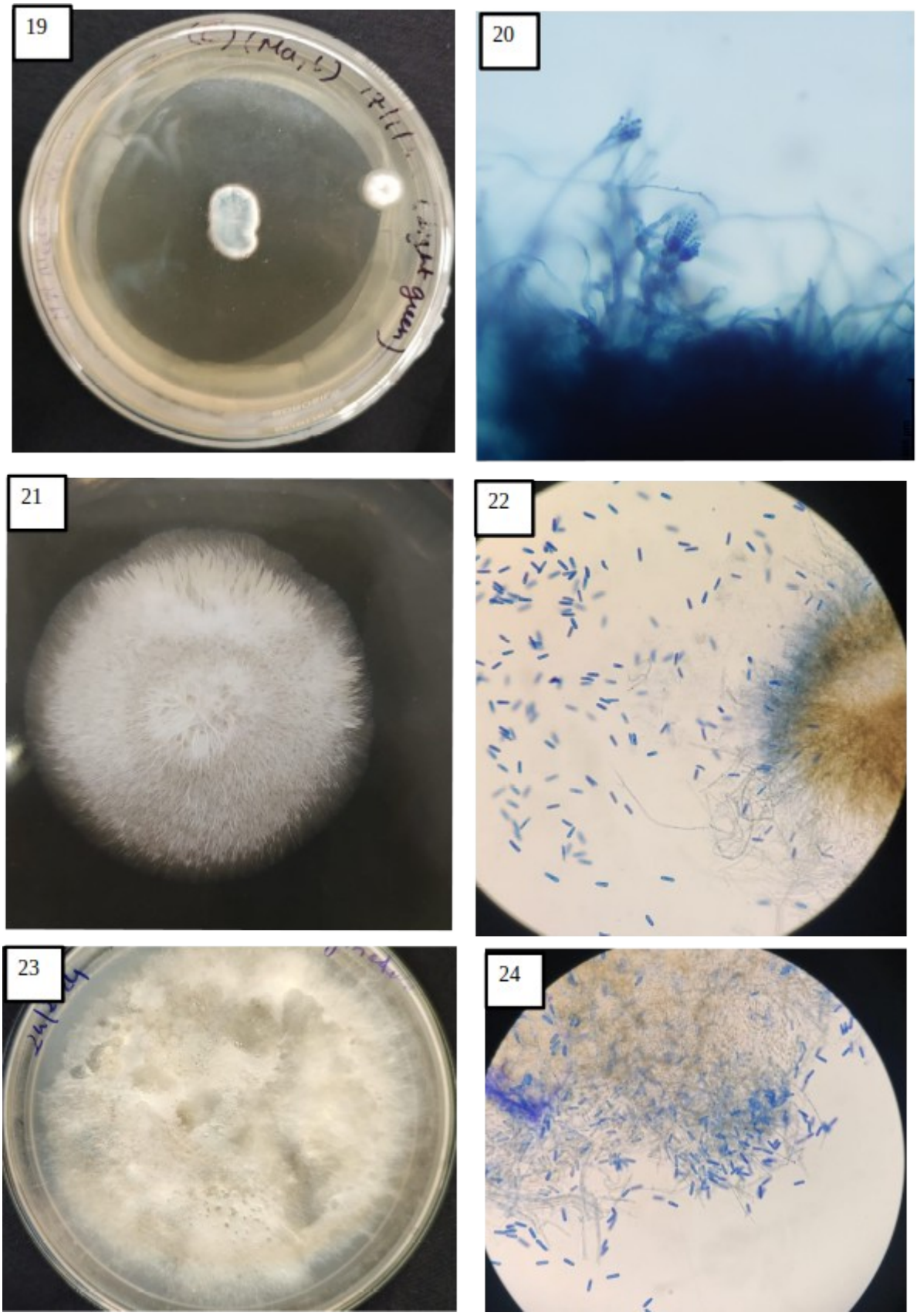



**Plate.8.4:** Endophytic Fungal isolates and their spores, 19) *Penicillium sp*, 20) Spores of *Penicillium sp*, 21) *Diaporthe sp*, 22) Spores of *Diaporthe sp*, 23) *Diaporthe sp*, 24) Spores of *Diaporthe sp*,

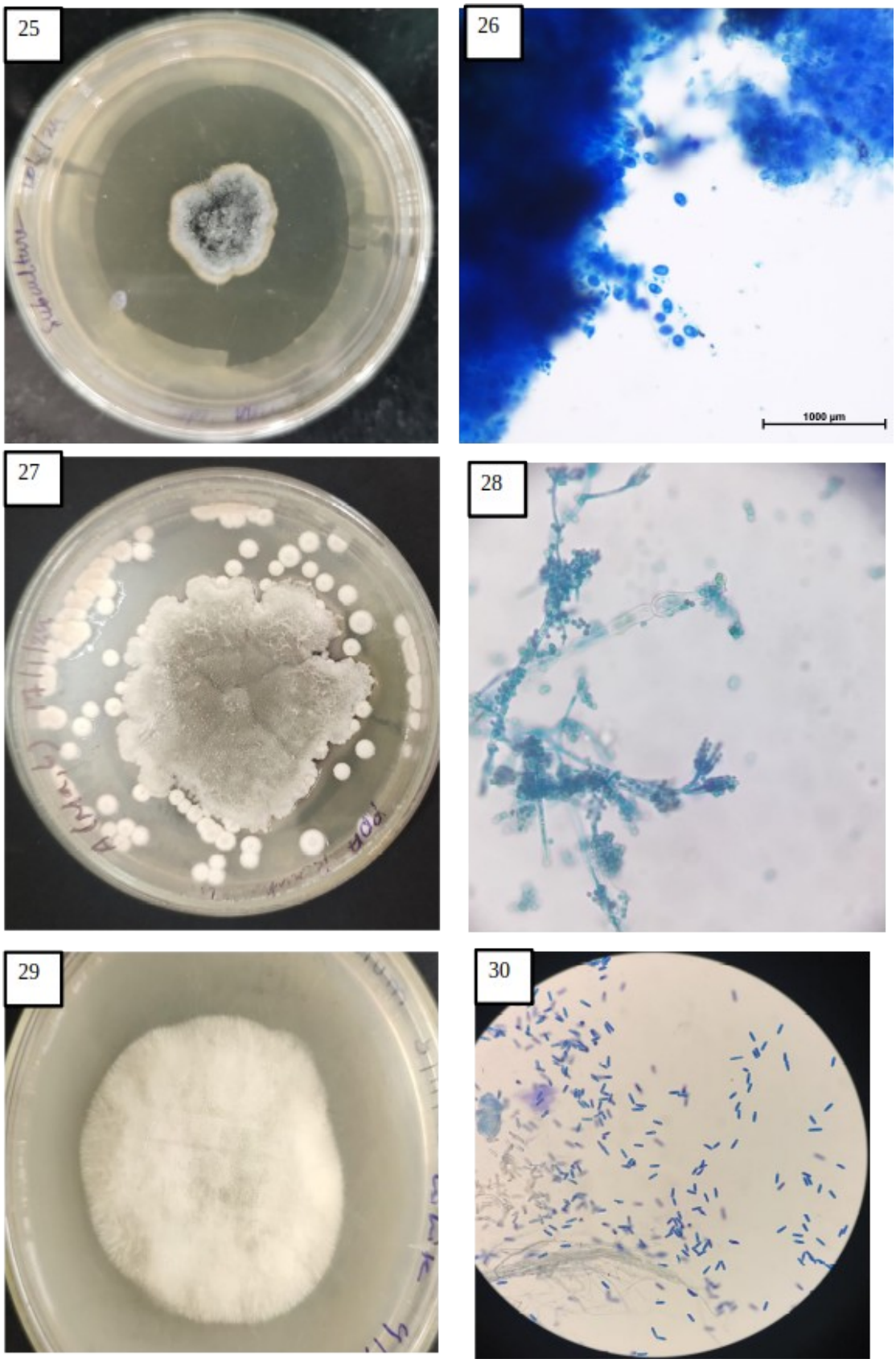



**Plate.8.5:** Endophytic Fungal isolates and their spores, 25) *Phyllostica Sp, 26*) Spores of *Phyllostica Sp,* 27) *Penicillium sp,* 28) Spores of *Penicillium sp,* 29) *Diaporthe sp,* 30) Spores of *Diaporthe sp,*

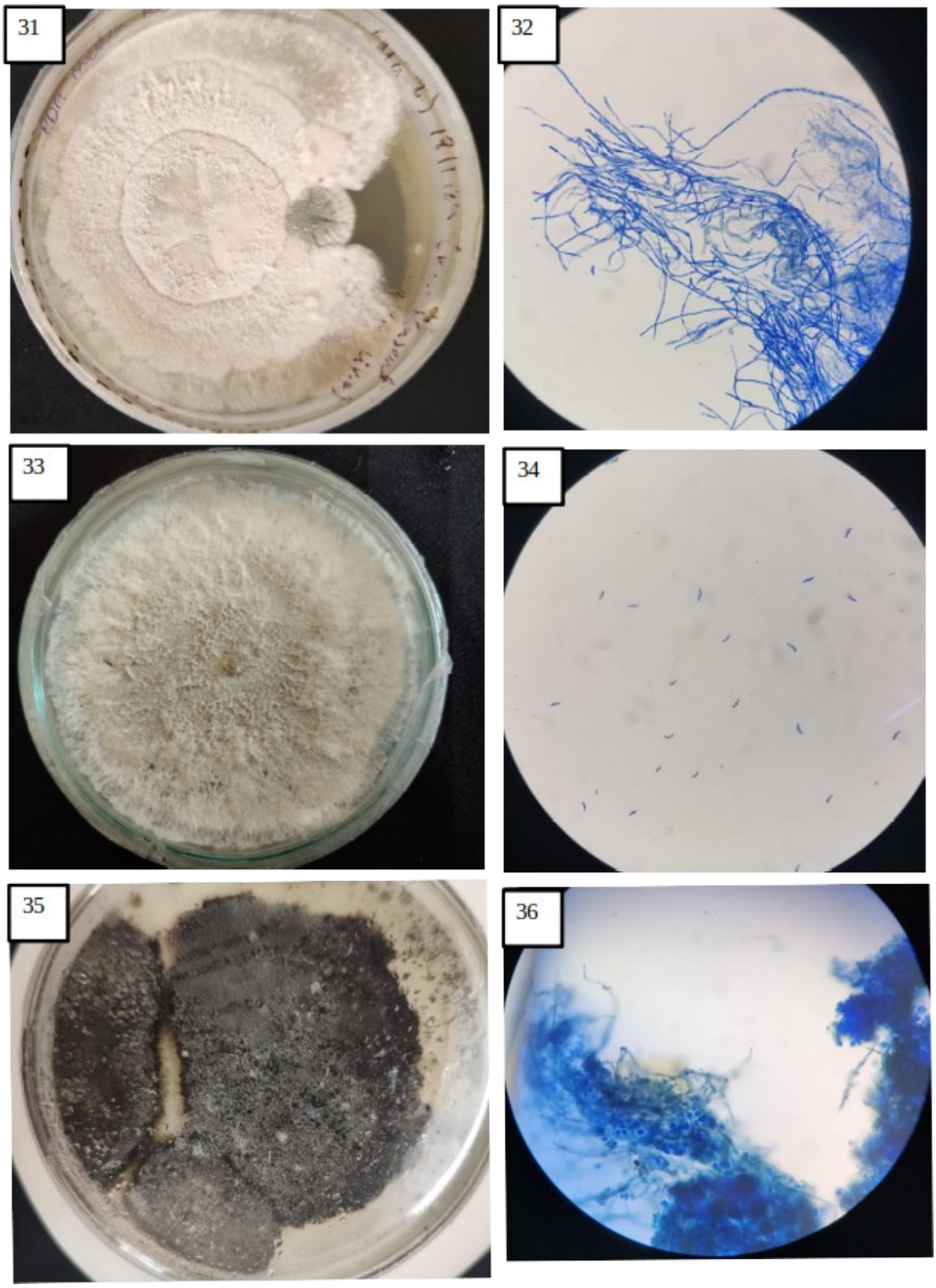



**Plate.8.6:** Endophytic Fungal isolates and their spores, 31) *Fusarium sp*, 32) Spores of *Fusarium sp* , 33)*Fusarium sp*, 34) Spores of *Fusarium sp*, *3*5) *Phyllostica Sp*, *36*) Spores of *Phyllostica Sp*,

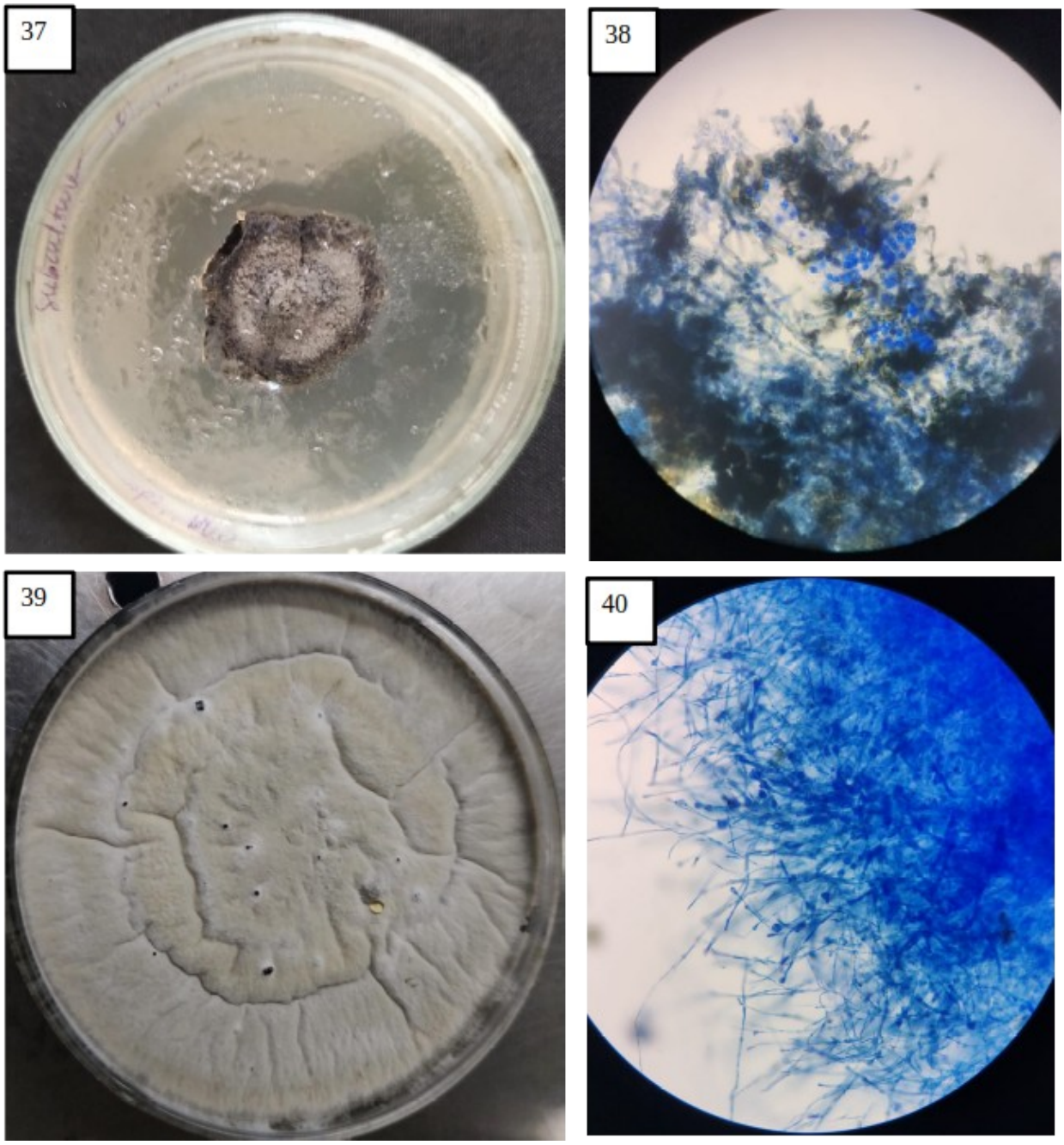



**Plate.8.7:** Endophytic Fungal isolates and their spores, *37*) *Phyllostica Sp, 38*) Spores of *Phyllostica Sp*, 39) Non-Sporulating Fungi, 40) Hyphae of Non-Sporulating Fungi.

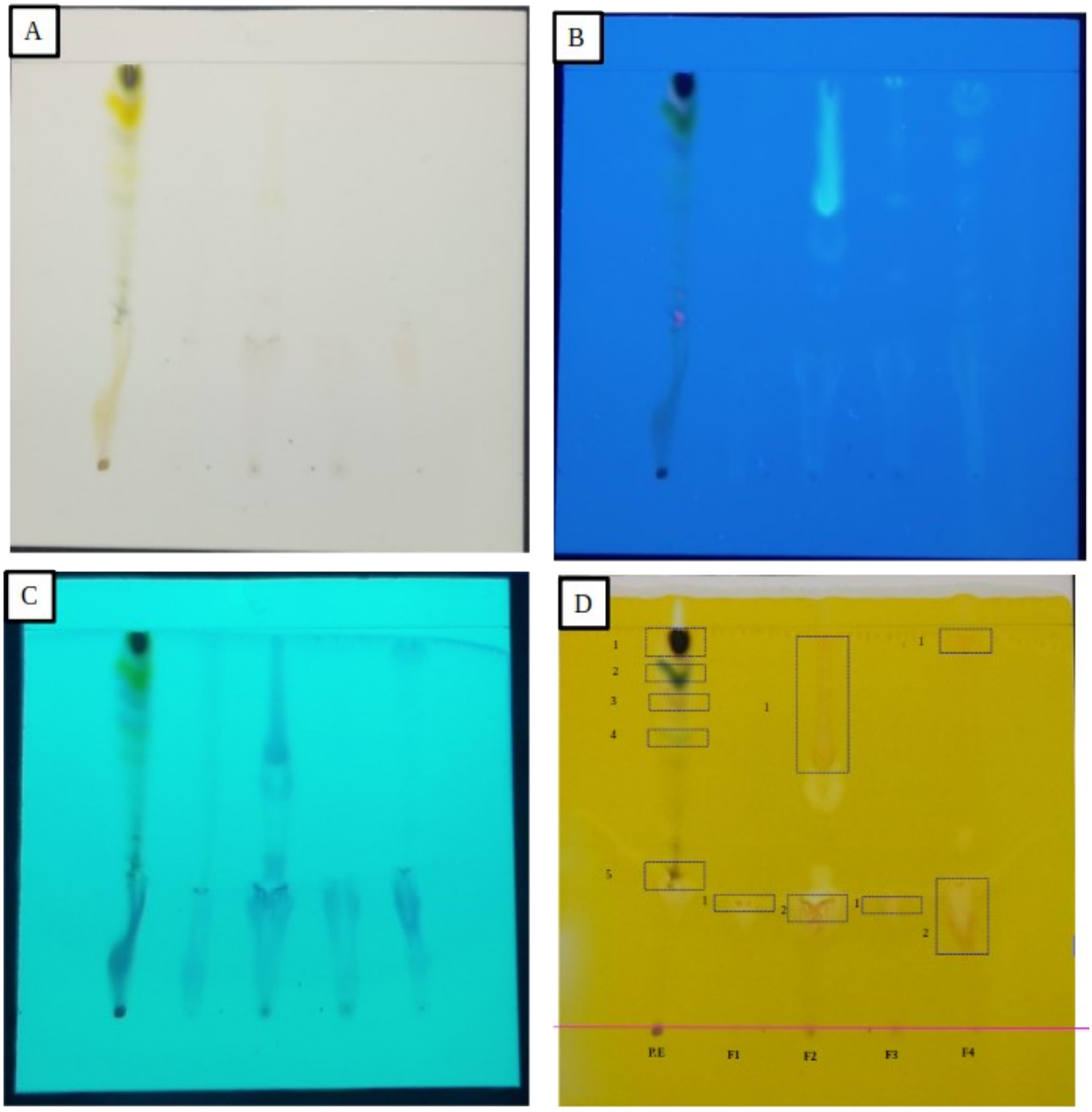



**Plate.9.1 :** TLC for Alkaloids visualisation in, A) Visible light, B) Long UV, C) Short UV, D) After Derivetaization and Band Marking.

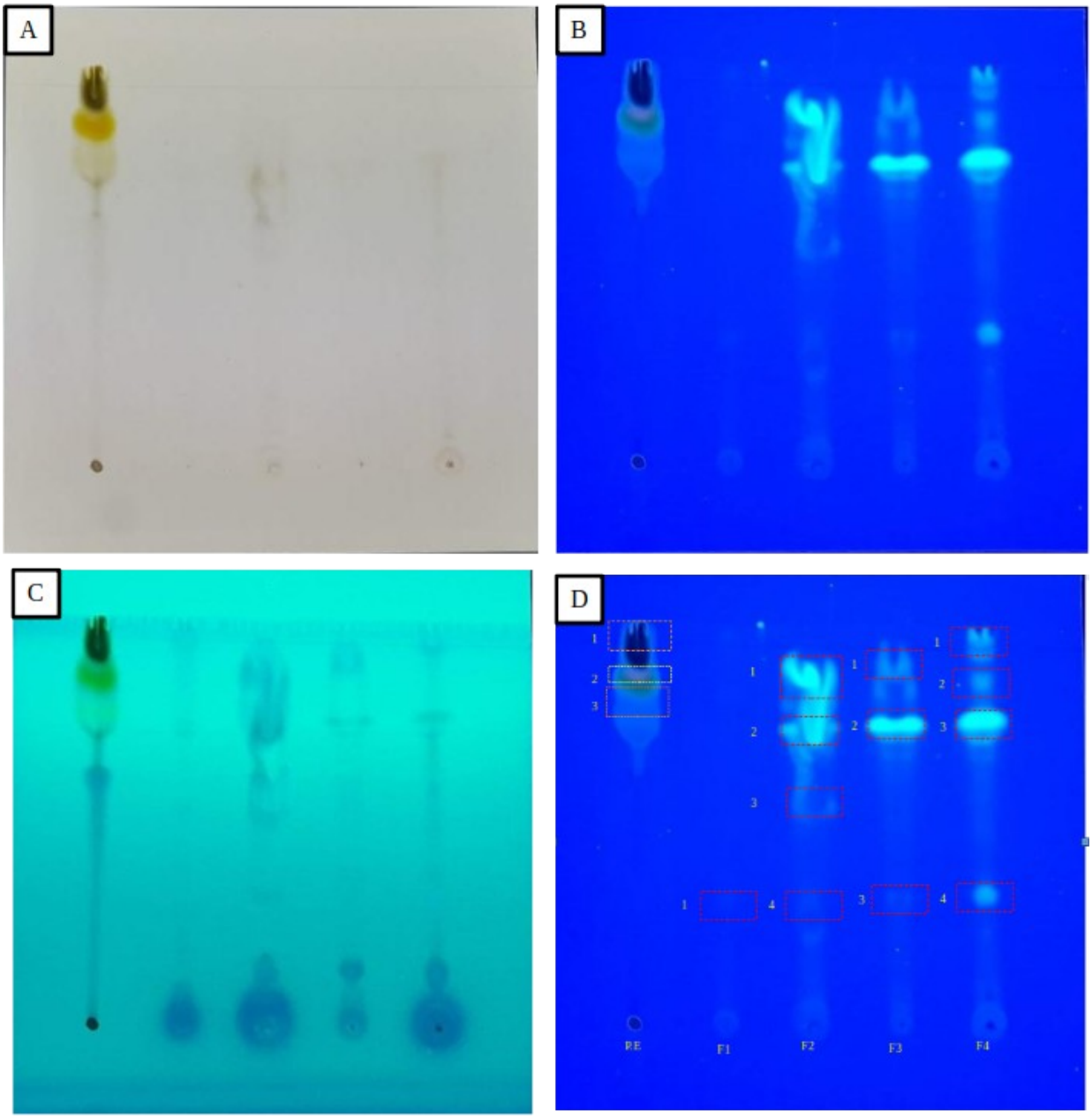



**Plate.9.2:** TLC for Flavanoids Visualization under, A) Visible light, B) Long UV, C) Short UV, D) Long UV with band marking.

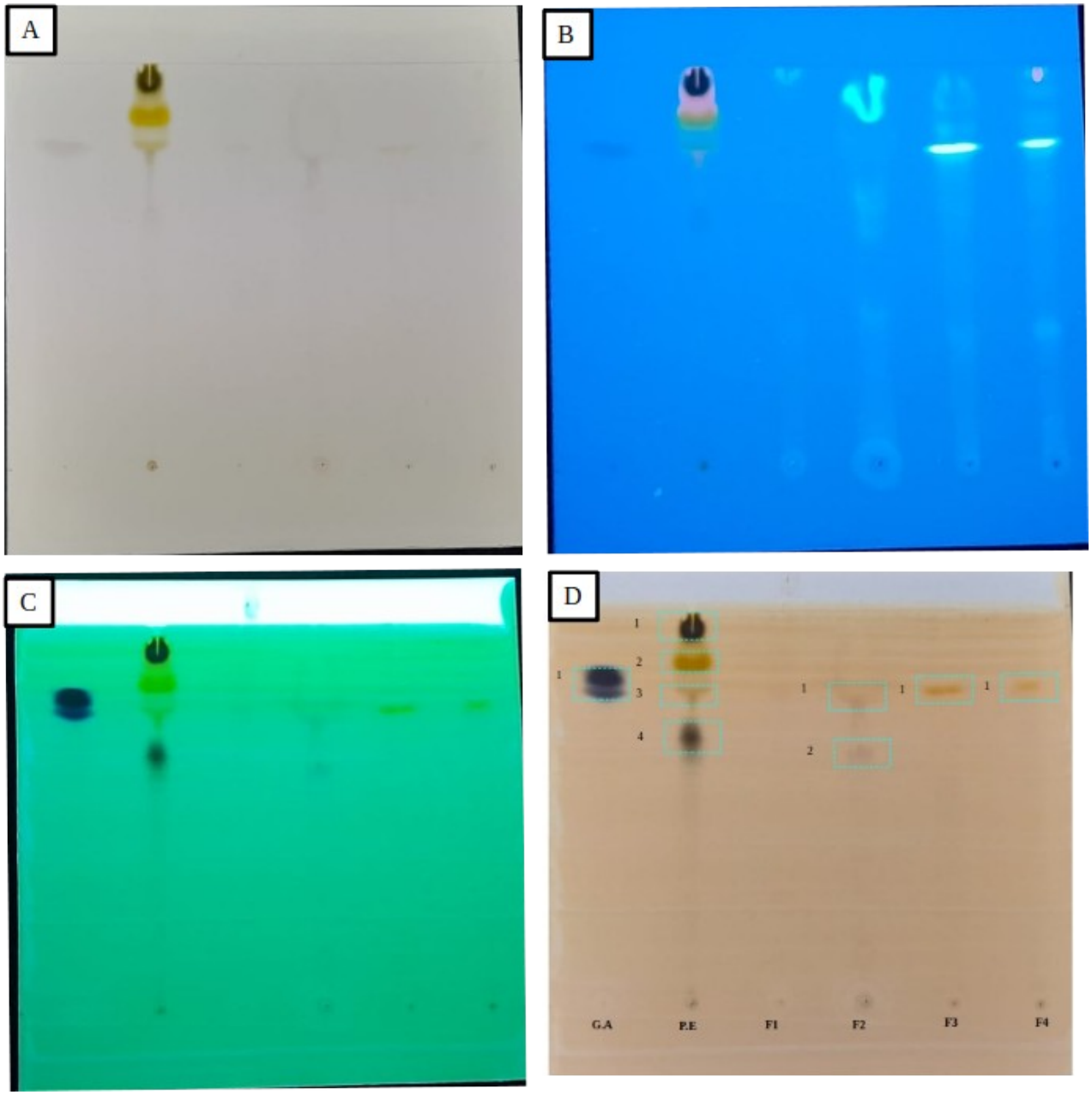



**Plate.9.3 :** TLC for Phenols visualization under, A) Visible light, B) Long UV, C) Short UV, D) After Derivetaization and Band Marking.

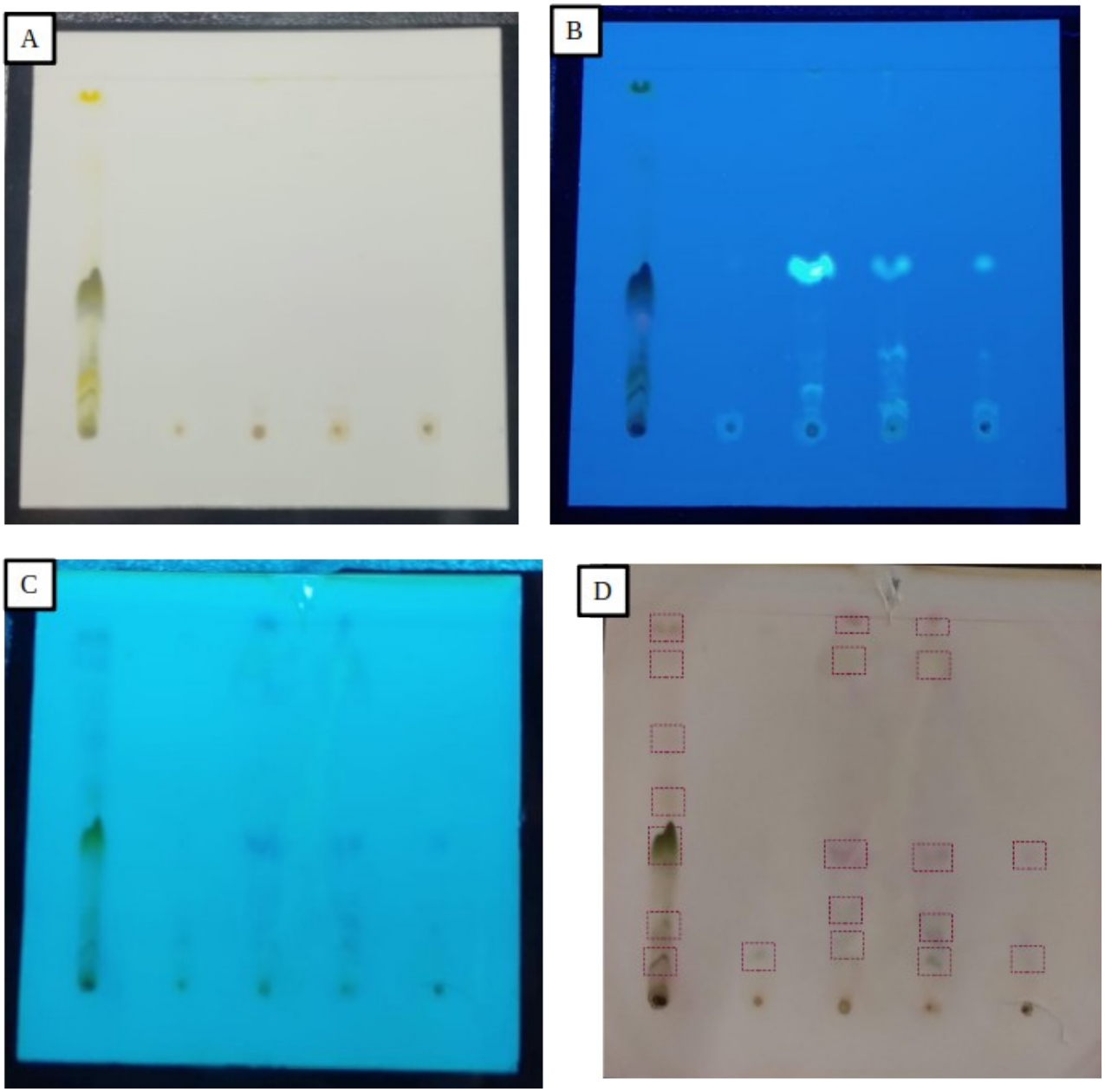



**Plate.9.4 :** TLC for Steroids vizualization under, A) Visible light, B) Long UV, C) Short UV, D) After Deivetaization and Band Marking.


**Plate.9.5 :** TLC for BioautographyVisualization under, A) Derivatized Visible light, B) Derivatized Long UV, C) Derivatized Short UV.

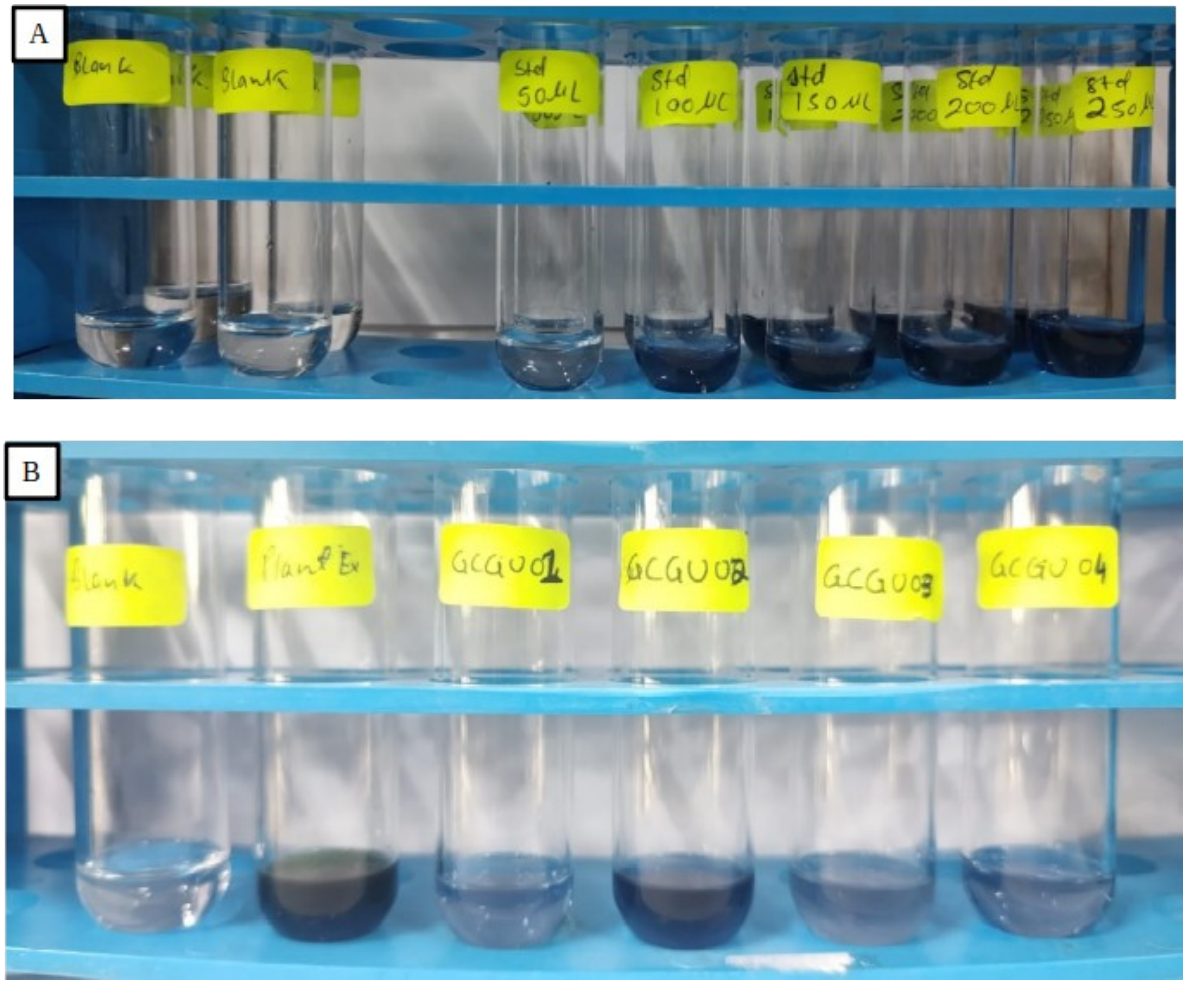



**Plate.10:** Total Phenolic Content , A) Standard Gallic Acid B) Samples ( from left to Right) , i) GCGU01: *Diaporthe* unsporulated, ii) GCGU02: *Diaporthe* sporulated, iii) GCGU03: *Penicillium* sp, iv) GCGU04: *Phyllostica* sp

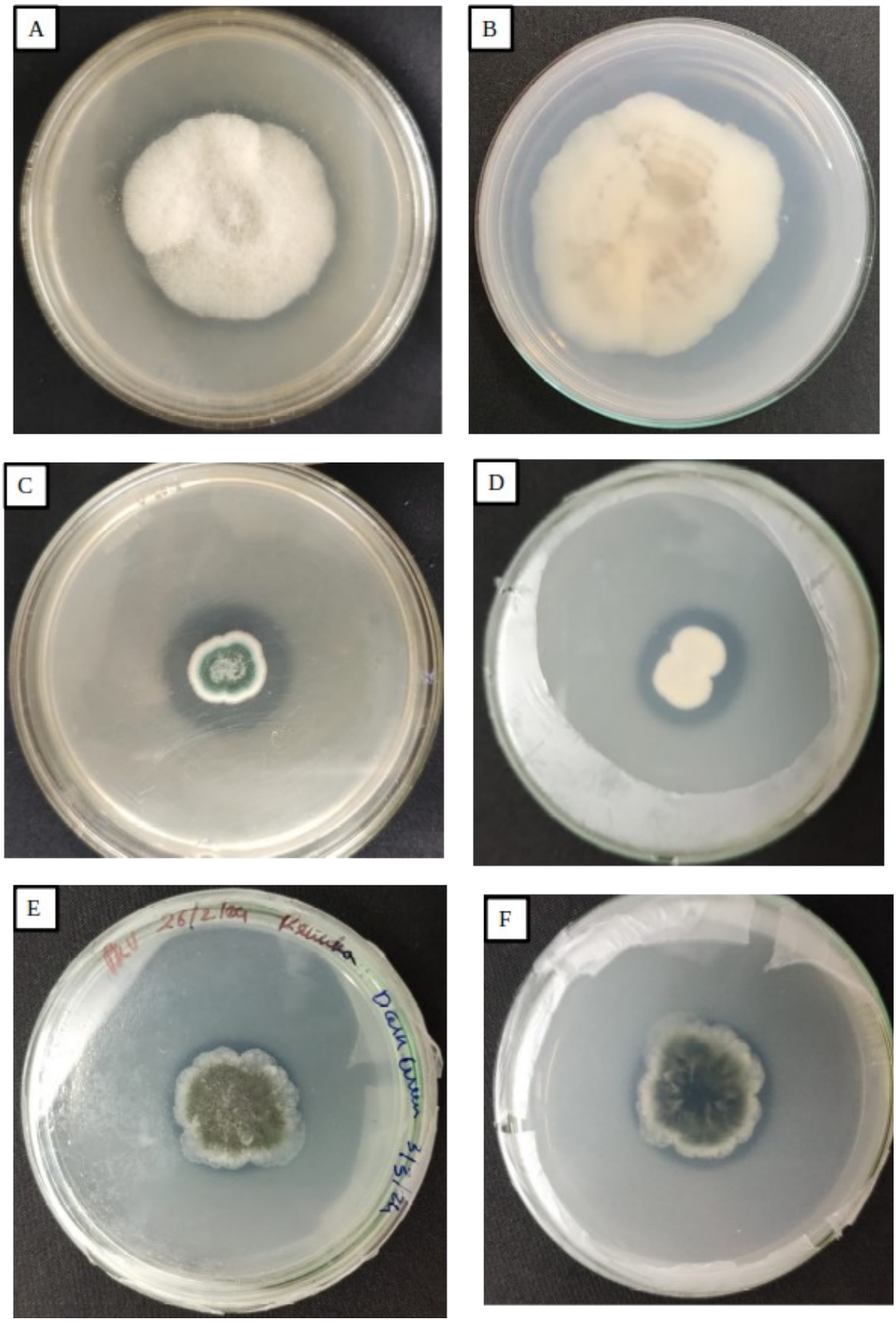



**Plate.11.1:** Phosphate solublization activity, A) *Diaporthe* sp front side, B) *Diaporthe* sp back side, C) *Penicillium* sp front side, D) *Penicillium* sp back side, E) *Phyllostica* Sp front side, F) *Phyllostica* Sp back side.

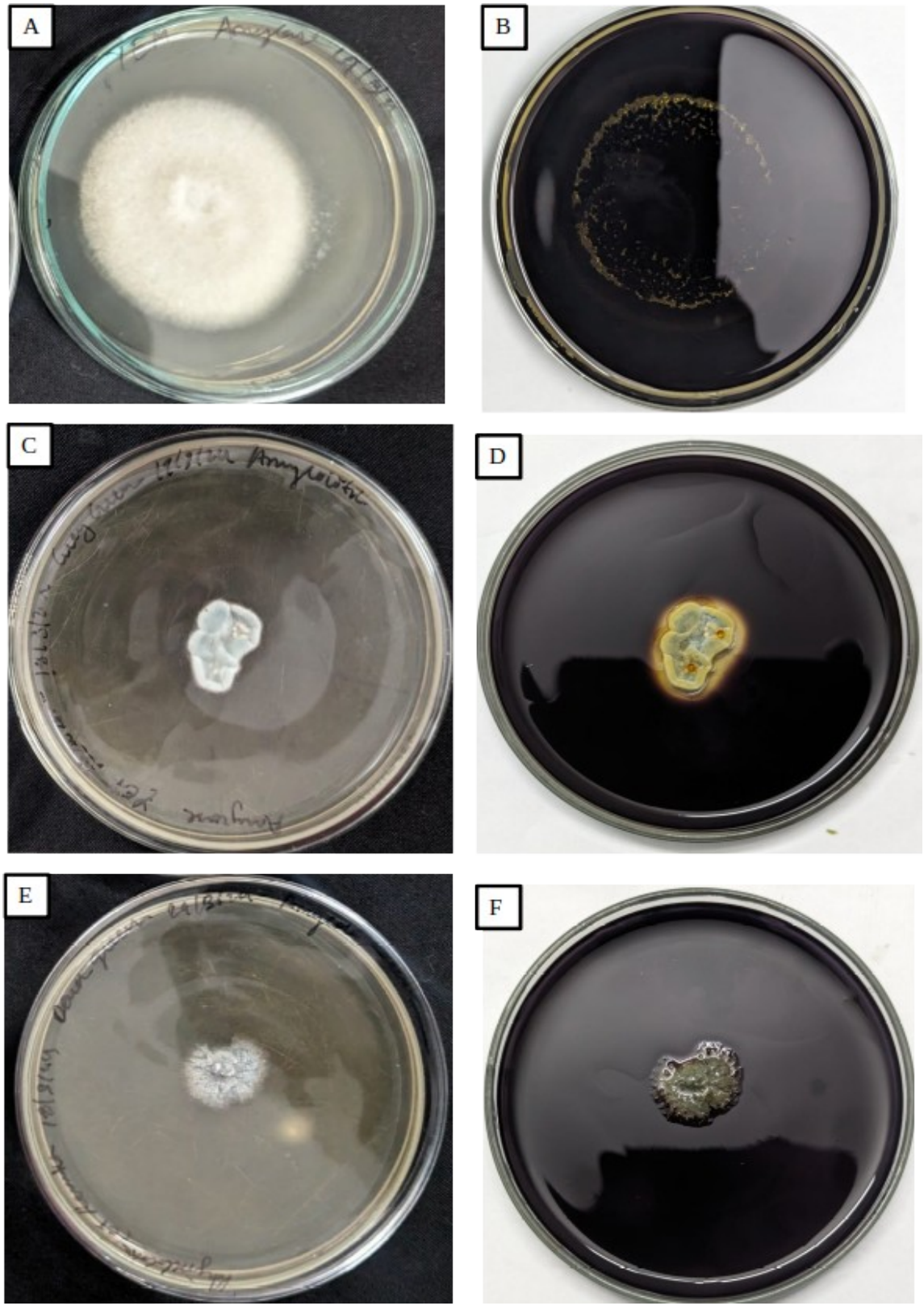



**Plate:11.2:** Amylolytic activity, A) i) *Diaporthe* sp before KI treatment ii) *Diaporthe* sp after KI treatment, B) i) *Penicillium* sp before KI treatment, ii) *Penicillium* after KI treatment t, C) i) *Phyllostica* Sp before KI treatment, ii) *Phyllostica* Sp after KI treatment.

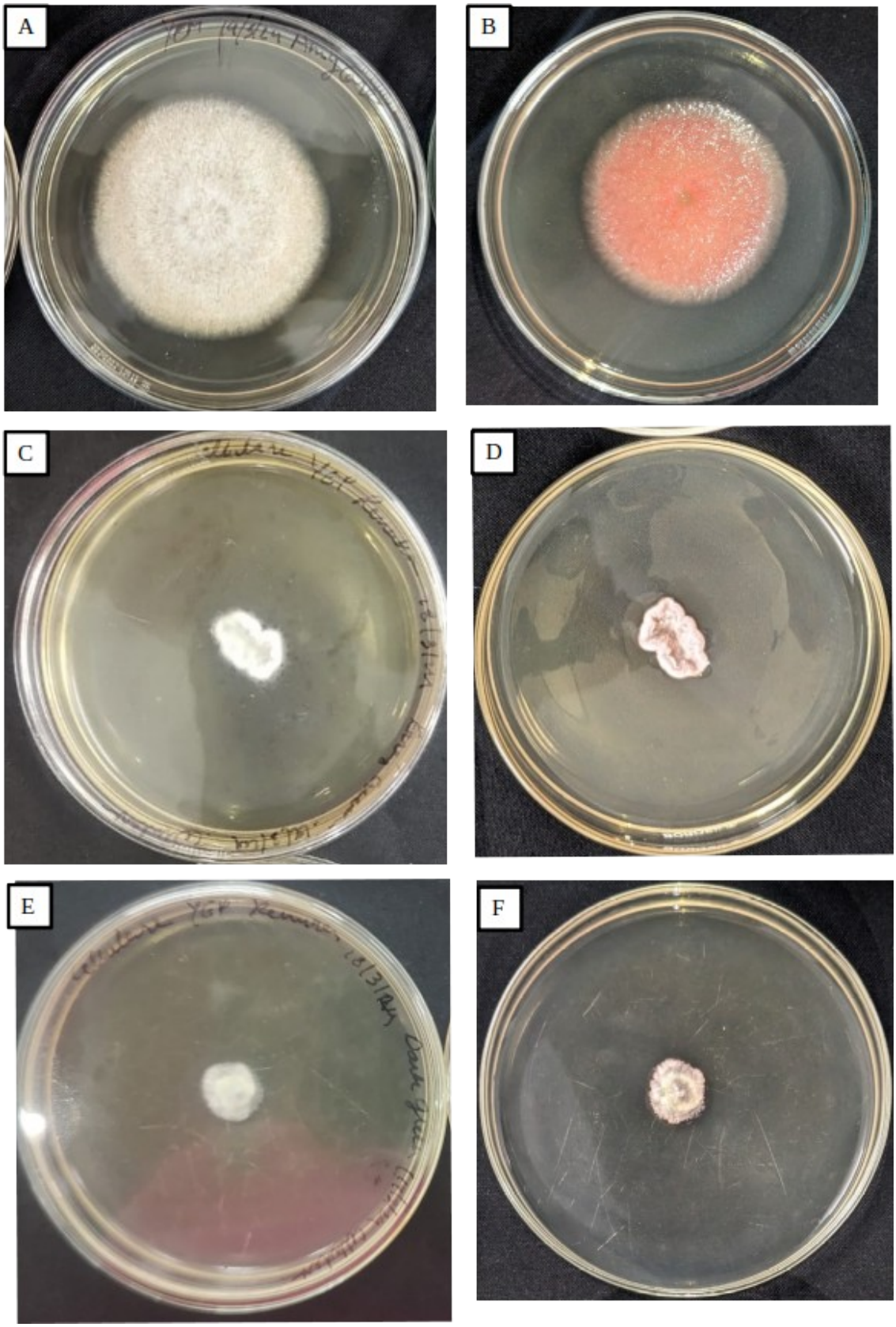



**Plate.11.3:** Cellulase activity, A) *Diaporthe* sp before Congo red treatment, B) *Diaporthe* sp after Congo red treatment, C) *Penicillium* sp before Congo red treatment, D) *Penicillium* after Congo red treatment, E) *Phyllostica Sp* before Congo red treatment, F) *Phyllostica* Sp after Congo red treatment.


**Plate.12**: Fugal Subculturing in PDB and Extract Preparation, A) *Diaporthe* sp in PDB, B) *Diaporthe* sp Fungal Mass after filteration, C) *Diaporthe* sp Fungal Extract, D) *Penicillium sp* in PDB, E) *Penicillium sp* Fungal Mass after filteration , F) *Penicillium sp* Fungal Extract, G) *Phyllostica sp* in PDB, H) *Phyllostica sp* Fungal Mass after filteration, I) *Phyllostica sp* Fungal Extract.

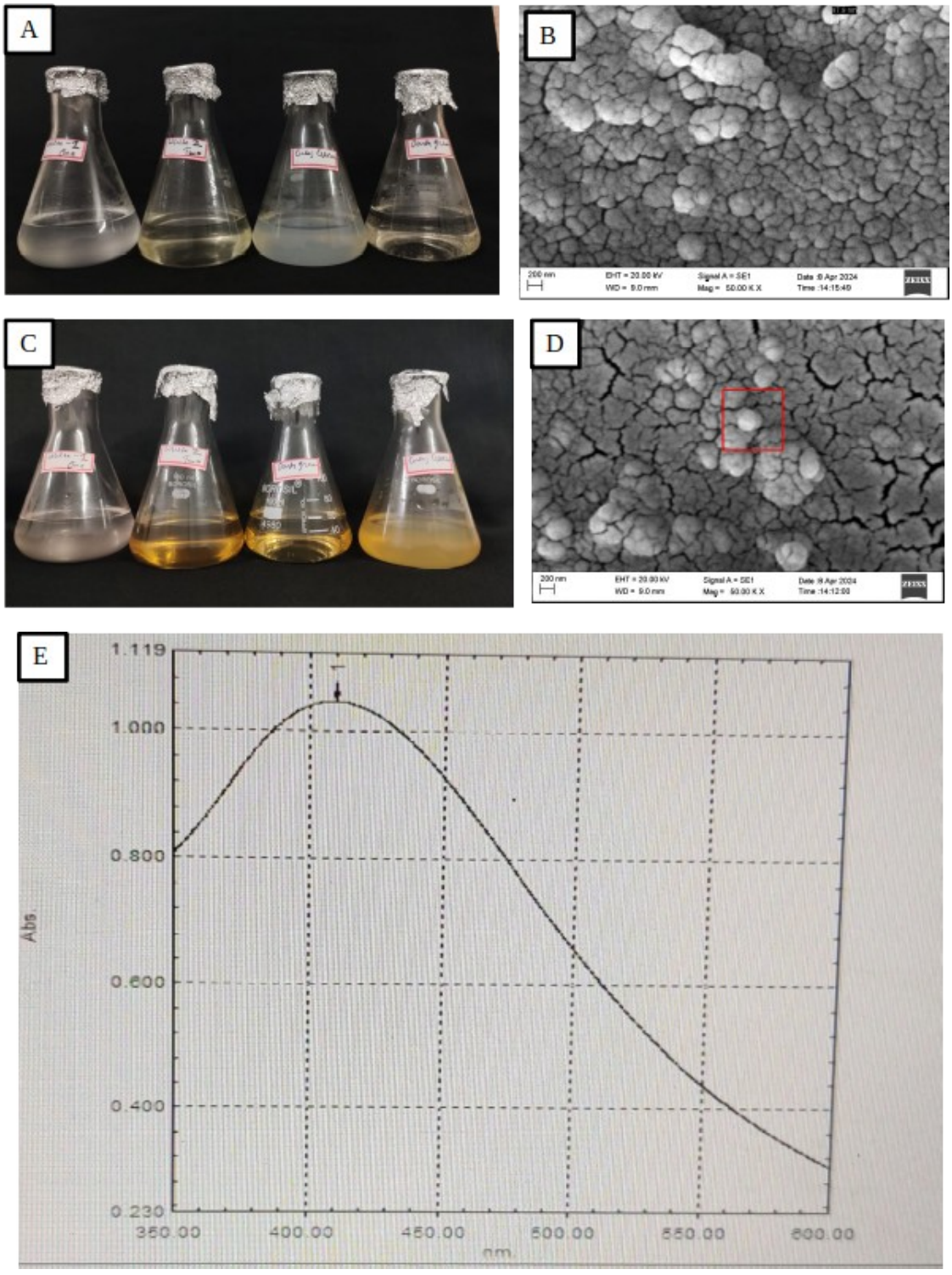



**Plate.13:** Silver Nanoparticle synthesis from Fungal Extracts, A) Before addition of Silver Nitrate solution, B) SEM Images 1 showing clusters of  of silver nanoparticles synthesised, C) After addition of Silver Nitrate solution. D) SEM Image 2 of Globular silver nanoparticles synthesised from Diaporthe sp - sporuating stage, E) Spectrometric analysis of Silver nanoparticles synthesis, absorbance peak observed at 410 nm.

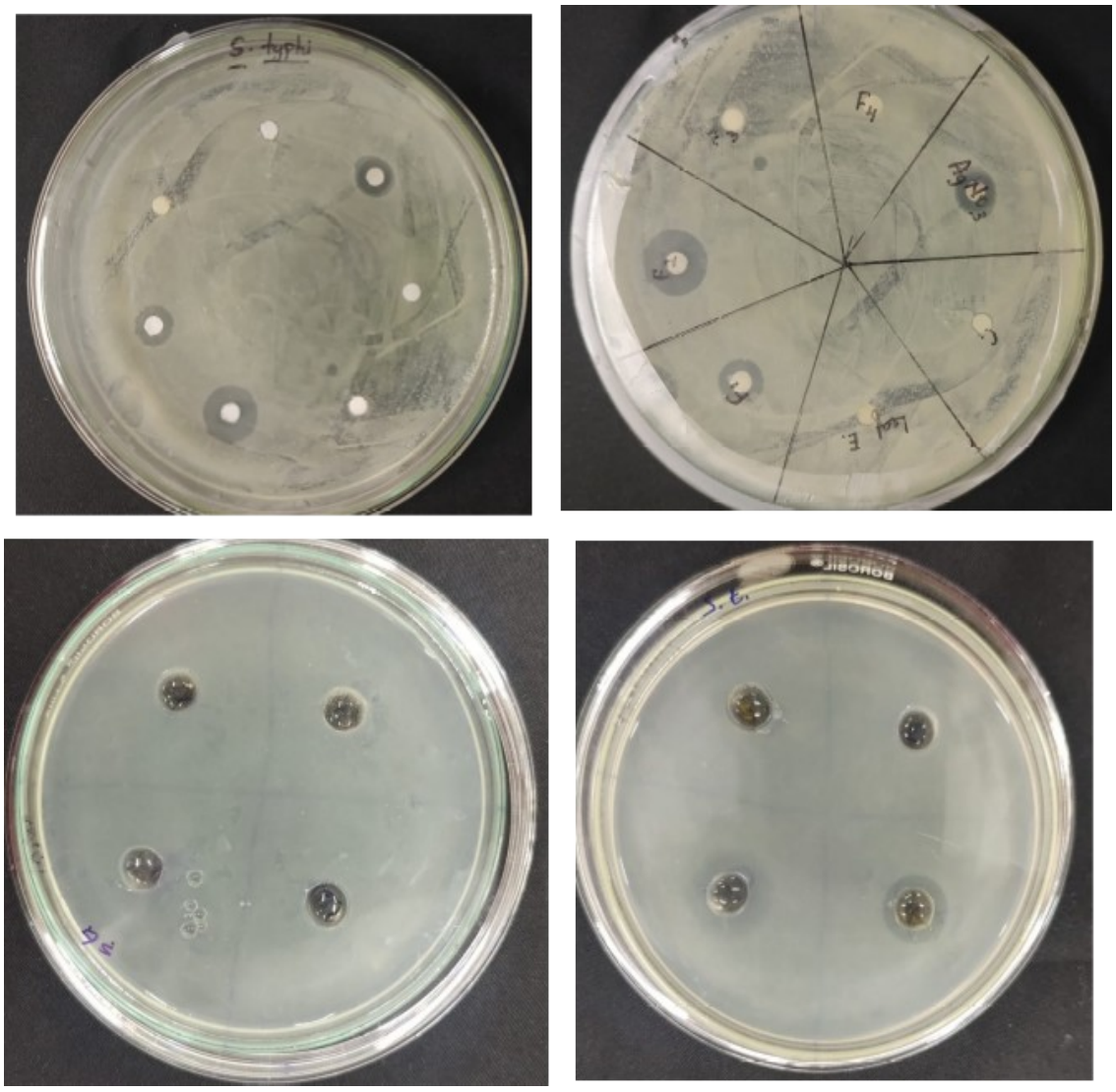



**Plate.14.1:** Antimicrobial activity against *Salmonella typhi* A) Using Disc Diffusion Metho- Front side, B) Disc Diffusion Method-Back Side, C) Agar well Diffusion Method with extracts of F1, F4, F3 and Control, D) Agar well Diffusion Method with Plant Extract, F2, Ag Nps, and Control.

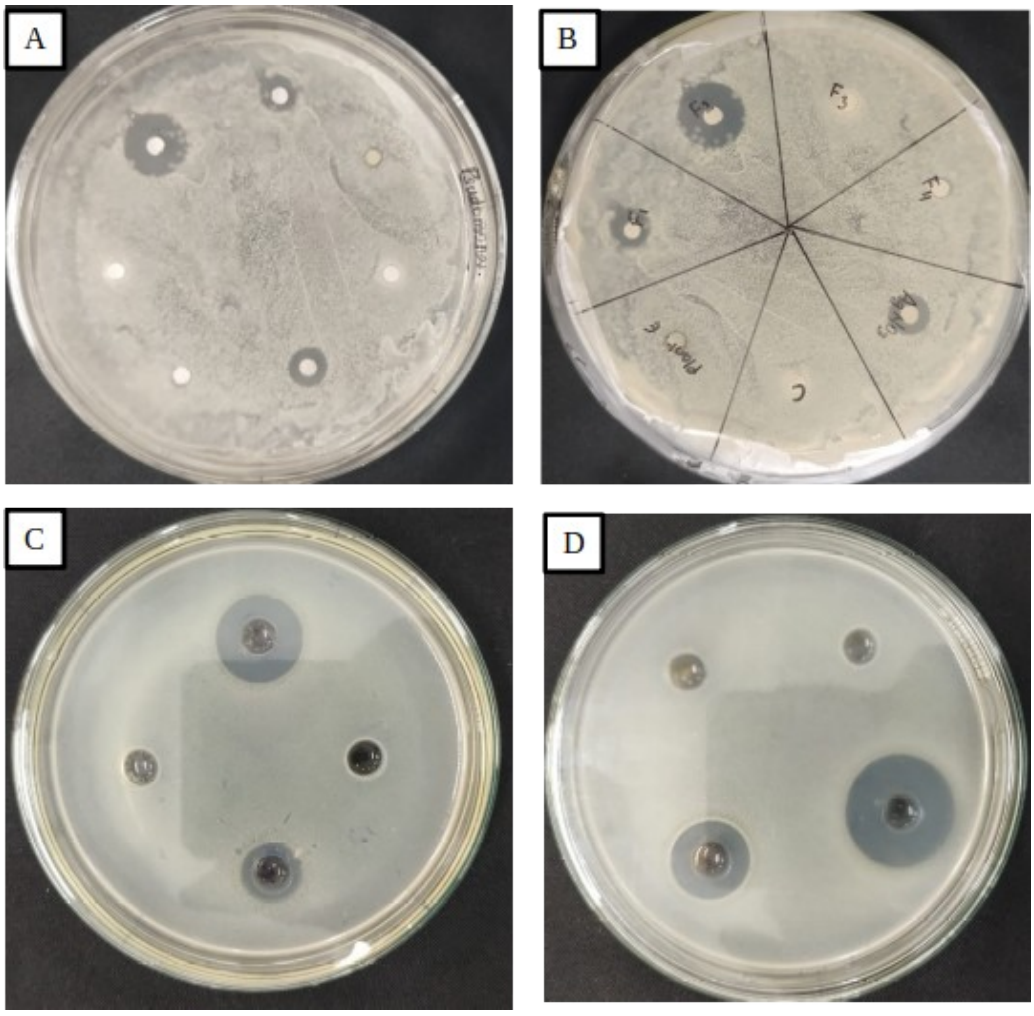



**Plate 14.2:** Antimicrobial activity against Pseudomonas areuginosa A) Using Disc Diffusion Method Front side, B) Disc Diffusion Method Back Side, C) Agar well Diffusion Method with extracts of F1, F4, F3 and Control, D) Agar well Diffusion Method with Plant Extract, F2, Ag Nps, and Control

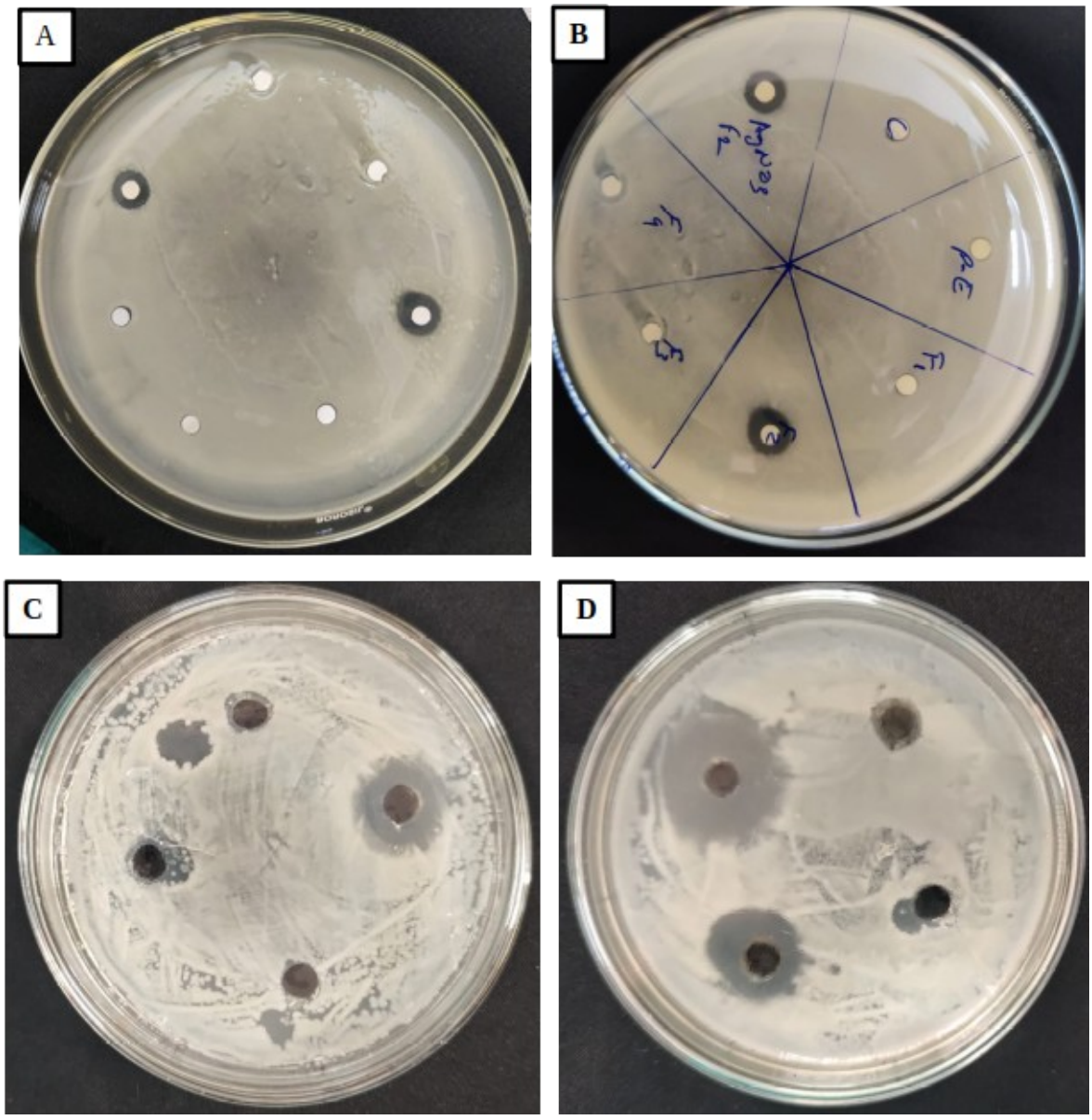



**Plate 14.3:** Antimicrobial activity against Klebsiella pneumonia A) Using Disc Diffusion Method Front side, B) Disc Diffusion Method Back Side, C) Agar well Diffusion Method with extracts of F1, F4, F3 and Control, D) Agar well Diffusion Method with Plant Extract, F2, Ag Nps, and Control

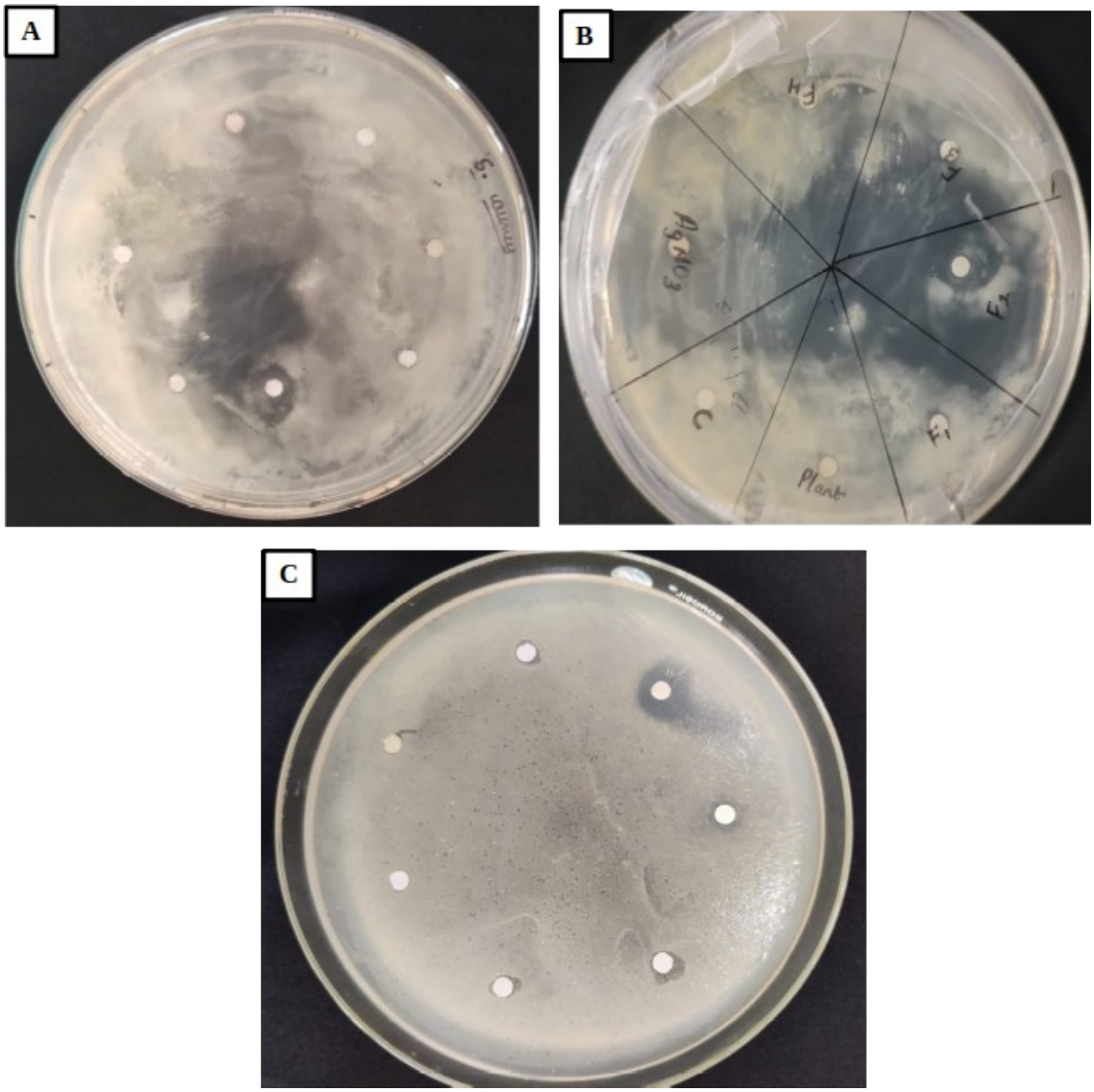



**Plate 14.4:** Antimicrobial activity against *Staphylococcus aureus* Using Disc Diffusion method, A) Spread Plate method Front side, B) Spread Plate method  Back Side, C) Pour plate Method

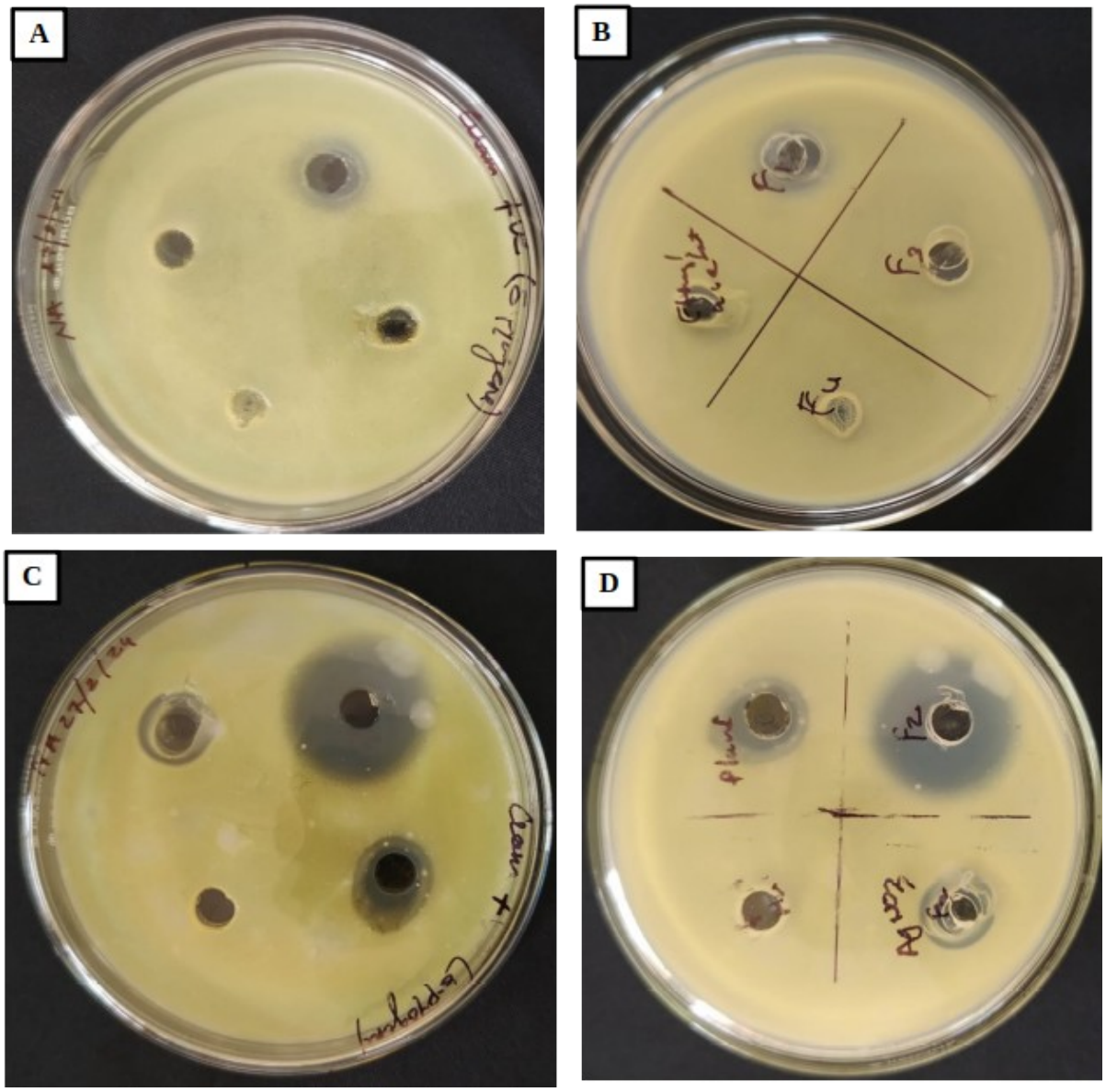



**Plate 14.5:**Antimicrobial activity against *Streptococcus pyogenes* Using Agar Well Diffusion method, A) Extracts of F1, F4, F3 and Control- front image , B) Extracts of F1, F4, F3 and Control- Back image, C) With Plant Extract, F2, Ag Nps, and Control - front image, D) With Plant Extract, F2, Ag Nps, and Controll- Back image.

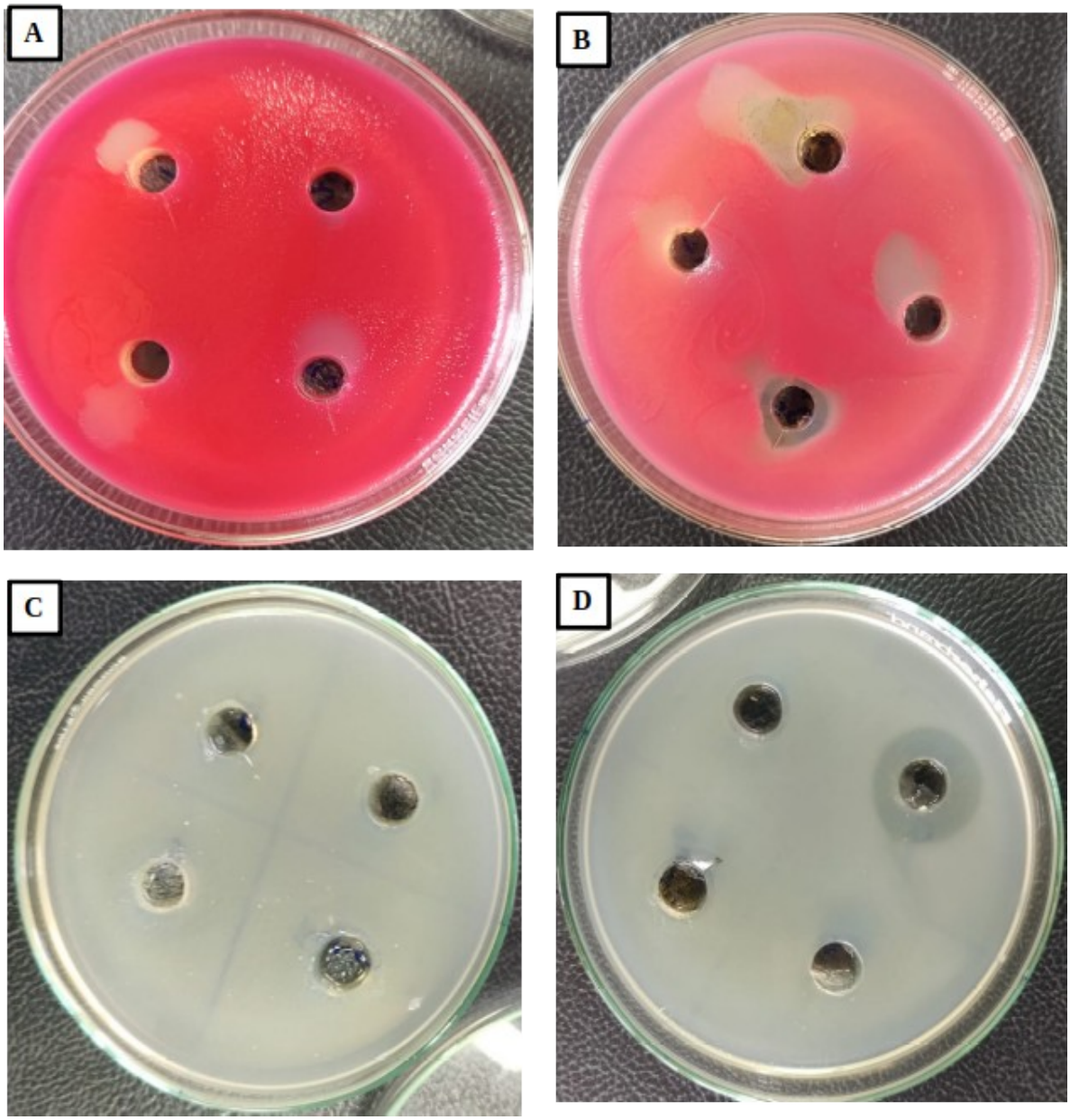



**Plate 14..6:** Antimicrobial and Quorum qurnching activity Using Agar Well Diffusion method, A) Against Serratia marcescens with fungal extrcats F1, F4, F3 and Control, B) with Plant Extract, F2, Ag Nps, and Control, C) Against Chromobacterium violaceum with fungal extrcats F1, F4, F3 and Control, D) with Plant Extract, F2, Ag Nps, and Control



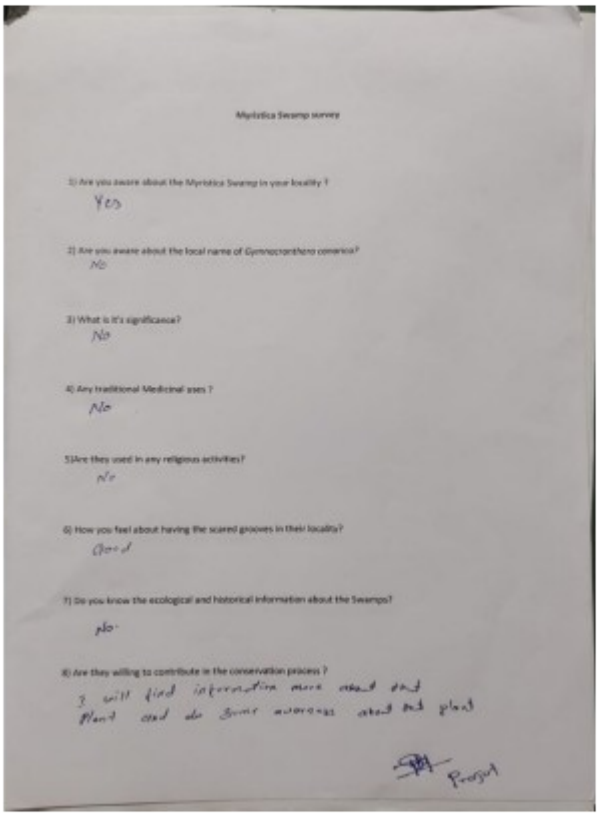






**Cleaning of the Myristica swamps was carried out involving the locals**






**Awareness created through Survey and Posters**

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