ISOLATION, MOLECULAR IDENTIFICATION AND BIOACTIVE POTENTIAL OF ENDOPHYTIC FUNGI ISOLATED FROM *Prunus armeniaca.*

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2023

ISOLATION, MOLECULAR IDENTIFICATION AND BIOACTIVE POTENTIAL OF ENDOPHYTIC FUNGI ISOLATED FROM *Prunus armeniaca.*

A dissertation submitted in the partial fulfillment of the requirements for the degree of Masters of Philosophy in Plant Sciences

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بسم الله الرحمن الرحيم

APPROVAL CERTIFICATE

This is certified that the dissertation entitled **"Isolation, Molecular Identification and Bioactive Potential of Endophytic Fungi Isolated from *Prunus armeniaca*."** submitted by **Hania Tariq** D/O **Waliullah Tariq** Registration No **02042111024** is accepted in its present form by the Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad as satisfying the dissertation requirement for the degree of **Master of Philosophy in Plant Sciences.**

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DECLARATION

By submitting this dissertation, I hereby declare that this thesis is my own novel work and effort that has not been submitted anywhere for any reward. Where any source of information has been used, they have been acknowledged.

Hania Tariq

DEDICATION

I dedicated this work to my family especially my father

Walíullah Tariq Awan

For his endless encouragement, support, guidance, trust, prayers and love that strengthened me to go through all the difficulties in pursuance of my goals.

AKNOWLEDGMENTS

First of all, I thank to Almighty Allah, the greatest and the most merciful, for giving me the strength and courage to carry out this work and all salutation and praises to be his beloved prophet and our true leader **Hazrat Muhammad (SAW**), who born for enlightening and guided us in every aspect of life. For me it is a great pleasure to express my deepest gratitude to my most respected supervisor prof. **Dr. Malka Saba**, Department of Plant Sciences, Quaid-i-Azam University Islamabad, for her brilliant supervision, guidance, cooperation, care, suggestions and constructive criticism throughout my research work and I express my sincerest gratefulness to her keen interest, support, encouragement, kind behavior and help especially during last days, that make me able to complete my research work in time.

I offer my special thanks to Chairman Department of Plant Sciences Dr. Mushtaq Ahmed and I also offer my deepest sense of gratitude to **Dr. Awais Rasheed, Dr. Muhammad Farooq Hussain Munis, Dr. Umar Masood Quraishi** and **Dr. Hassan Javed Chaudhary**, teachers of Plant Sciences Department Quaid-i-Azam University Islamabad, for their kind cooperation and providing all research facilities and friendly environment in their labs.

I offer my special thanks to my lab fellows Iqra Rauf, Ayesha Nawaz and Jassia Ikram for their support and cooperation and heartiest thanks to Bakhtawar Zafar and Momal Batool for their support, encouragements whenever I am disappointed, discouraged during my research work.

I can't express my feelings whenever I count amenities of my family especially my mother, she always prays for me and guided me to handle all the troubles in my life. I also offer my deepest sense of gratitude to my brother Safiullah Tariq for his guidance and helping in my research work. At the end I would like to say thanks to my all well-wishers who support me guided me either directly or in directly during my research work. May Allah Almighty bless them with prosperous and healthy life and be a source of prayers for me (Ameen).

Hania Tariq

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LIST OF ABBREVIATIONS

Abbreviation	Complete form of abbreviation
bp	Base pair
BLAST	Basic Local Alignment Search Tool
CTAB	Cetyl trimethylammonium bromide
cm	Centimeter
DNA	Deoxy ribonucleic acid
dNTPs	Deoxy ribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EB	Elution Buffer
ITS	Internal Transcribe Spacer
ITS-rDNA	Internal Transcribe Spacer-ribosomal deoxy ribonucleic acid
Km/h	Kilometer per hour
KOH	Potassium Hydroxide
M	Meter
µm	Micrometer
Mgcl2	Magnesium Chloride
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NARC	National Agriculture Research Center
nrLSU	Nuclear Large Subunit
ng/µl	Nano gram per microliter
PCR	Polymerase Chain Reaction
PB	Phosphate buffer
PE	Phosphate Ethanol buffer
Pm	Pico mole
rRNA	Ribosomal Ribonucleic Acid
RNA	Ribonucleic Acid

rDNA	Ribosomal Deoxyribonucleic Acid
Rpm	Revolution per mints
RFLP	Restriction Fragments Length Polymorphism
SSU	Small subunit ribosomal subunit
TE	Tris Ethylenediaminetetraacetic acid
TAE	Tris-acetate Ethylenediaminetetraacetic acid
µl	Microliter

ABSTRACT

Main goal of this research project is to isolate endophytic fungi of *Prunus armeniaca*, Quaid-i-Azam University, Islamabad, and evaluate the biological potential of isolated endophytes by using their crude extract, one of the least-studied areas that has received virtually no attention in terms of endophytic fungi. Plant specimens were collected for the study from Quaid-i-Azam University, Islamabad during the monsoon season (July to September 2020).

The characterization of fungi has used a variety of identification tools. Macro-morphological descriptions of the species were based on the appearance of fungal colonies i.e. colony color, colony shape, colony diameter, colony margins, texture, opacity of colony and topography. Microscopic characters of fungi i.e., conidiogenous cells, conidia were analyzed by mounting material from colonies growing on PDA on glass slides using various chemicals and molecular characterization involved amplifying the rDNA-ITS region with a couple of primers, namely ITS1F and ITS4, and creating a phylogenetic tree to show their closest relationships. To evaluate the biological potential of isolated endophytic fungi, crude extract was prepared in the organic solvent ethyl acetate. Total phenolic content, total flavonoid content and antioxidant assay were done to evaluate the biological potential of isolated endophytic fungi.

From combined morphological and molecular characterization, our research results into the identification of the four species belonging to three order, three families and four genera. The species belongs to genus Talaromyces, Pseudofusicoccum, Aalternaria and Aspergillus. From phylogenetic analysis 2 species *Talaromyces dimorphus* and *Pseudofusicoccum adansoniae* are new records for Pakistan.

Our research showed that *Prunus armeniaca* contains a wide variety of endophytic fungi, but no work has been done to document these endophytes and the plant need to be discovered for more worthy species of diverse fungi group. This is the ever first research project of the plant regarding endophytic fungi exploration.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 Study Area

The current study is carried out in the capital city of Pakistan, Islamabad. The city is situated south of the Margalla Hills. The research covers the area of Quaid-i-Azam University Islamabad to explore the endophytic fungi of *Prunus armeniaca.* The Quaid-i-Azam University is located at an elevation of 619.18 meters (2031.43 feet) above sea level. The climate here is humid subtropical with dry winters. The annual temperature of district is 27.09ºC (80.76ºF). It receives approximately 94.76mm (3.73 inches) of precipitation and 125.05 rainy days (34.26% of the time) annually.

1.2 Prunus armeniaca L

Prunus armeniaca was collected from Quaid-i-Azam University Islamabad for the research purpose. *Prunus armeniaca*, is a fruit tree belongs to family Rosaceae that is widely cultivated throughout temperate regions of the world, particularly in the Mediterranean (Gatti et al., 2009). According to its botanical name, the plant is a hardy tree with stone fruits that grows 2–10 m tall. Depending on the variety, the fruit normally ripens between the end of the summer and the mid of August. and has the shape of a drupe, resembling a plum, with a fine, downy outer skin covering mesocarp, woody inner layers forming the large, smooth, compressed stone, and the ovule maturing into the kernel, or seed.

1.2.1 Medicinal importance

Prunus armeniaca is the better source of effective medicine. Because of its antipyretic, anti-inflammatory and anticancer properties, *Prunus armeniaca* Lam., also known as the apricot tree is used medicinally to treat a variety of illnesses, including respiratory, gynecological, and digestive issues (Alajil et al., 2021). The pharmacological effects of the various apricot components are varied. Many components of the apricot plant are used as complementary and alternative (CAM) cancer treatments around the world, and some preliminary clinical studies have already shown a promising future for apricot-based medicines in this field (Sagbo and Mbeng, 2021).

The dried, ripe apricot seeds are said to have unique therapeutic benefit, used to treat arthritic pain, migraines, and gynecological conditions while seed oil is used to treat skin and ear infections (Kitic et al., 2022). The roots of apricot trees are used to treat coughs, bronchitis, asthma, constipation, and to calm swollen or irritated skin. Apricot flowers are said to increase female fertility (Lim, 2012). The seeds and oil they produce are used to treat a variety of conditions, including anorexia, vaginal infections, and insomnia. When combined with peach and walnut seeds, they are used to treat bronchitis, asthma, and respiratory infections. Additionally, they are utilized in cosmetics (Rai et al. 2016). Turkey, Pakistan, China, India, and the Western Caucasus all used the apricot's fruit, seed, stem, and gum as an anticancer treatment (Kitic et al., 2022). In vitro tests on Gram-(+)/Gram-() bacteria and fungus revealed antibacterial activity of *Prunus armeniaca* fruit, seed, leaf, root, and stem extracts (Yiğit et al., 2009), (Kitic et al., 2022).

1.3 Endophytes

Endophytes are the microbes which live inside host plant and form a symbiotic relationship with host plant in such a way that they both get benefit (Deshmukh et al., 2006). In 1886 De Bary first discovered the word endophyte for the microorganisms (Vega, 2008). There are one or more than one endophyte is present on every plant (Strobel & Daisy, 2003). which spend their lives asymptomatically in the host plant tissues (Debbab et al., 2012). Endophytes are an abundant source of naturally occurring bioactive compounds that could be useful to the pharmaceutical industries (Kogel et al., 2006). From the last two decades, endophytes gained so much attention as they are considered as a reliable source of bioactive compounds useful for medicines, agriculture and pharmaceutical industries (Stone et al., 2000, Strobel & Daisy, 2003).

Endophytes boost agricultural yields, hinder diseases, remove impurities, and also manufacture fixed nitrogen or new chemicals (Rosenblueth & Martínez-Romero, 2006). Endophytes produce diverse range of active secondary metabolites like alkaloids, flavonoids, phenolic acids, Xanthones, terpenoids, tetralones, benzopyranones and steroids (Kaul et al. 2012., Tan & Zou, 2001). The majority of bioactive natural antimicrobial metabolites, which are low-molecular weight organic chemicals that protect plants from microbes and are active against other microbes at low concentrations, are also produced by endophytes (Guo et al., 2008). Pathogens

cannot enter in plants because endophytes produce secondary metabolites that create a defense mechanism (Tan & Zou, 2001).

Human diseases have long been treated and prevented with the help of plants. During 1981 to 2002, about 61% of drugs from natural resources are discovered against cancer and other infectious diseases (Cragg & Newman, 2005). Due to development of resistance of pathogens towards synthetic drugs, production of bioactive metabolites from plants has drawn more attention recently (Joseph & Priya, 2011). More than 90% of natural compounds are derived from plants. Natural compounds are more diverse in structures and contain metabolites that are very promising and powerful (Bérdy, 2012). To discover new novel bioactive compounds, there is a need to search the unexplored habitats for new sources. Endophytes are one of the major sources of new bioactive chemicals that have grabbed the interest of researchers (Tran et al., 2010). Metabolites produced by endophytes have an antagonistic effect on the host's other microorganisms.

The search for novel drugs from endophytes was began after the discovery of penicillin from the culture of fungi *Penicillin notatum* as an antibiotic against Gram-positive bacteria by Alexander Fleming in 1928 (Oxford et al., 1939). Endophytes contain a variety of bioactive substances with powerful biological activity, which is why they seem like an interesting solution.

1.4 Endophytic Fungi

Endophytic fungi spend their whole life cycle colonizing within cells of healthy plant tissues. Endophytic fungi comprise around 1 million different species. Endophytic fungi have been connected to plants for about 400 million years (Krings et al., 2007). They also appear in a broad variety of plant orders, families, genera, and species, in ecosystem, as diverse as ferns (Swatzell et al., 1996), mosses (Davey & Currah, 2006), shrubs (Petrini et al., 1982), lichens (Li et al.,2007) and grasses (Muller & Krauss., 2005). Mainly Endophytic fungi are the members of Ascomycota but some species are connected to Oomycota, Basidiomycota and Zygomycota (Sinclair & Cerkauskas, 1996).

1.4.1 Diversity of Fungal Endophytes

Endophytic fungi have an impact on the ecology, fitness, and evolution of plants. There are many different types of organisms, each of which is capable of

producing a different bioactive substance (Jalgaonwala et al., 2011). According to the fossil record endophytic fungi have been connected with plants for more than 400 million years. Class 1 endophytes are Clavicipitaceous that represents the Clavicipitaceous species to some cool and warm season grasses (Stone et al., 2004). Class 1 endophytes transmitted to their off-spring via seed infection of the plants. Plants get benefits from those fungi depends upon species, host genotype and environmental conditions (Faeth et al., 2006). Endophytes of class 2 are confined to Ascomycota and Basidiomycota. They enhance the plant's ability to withstand stress. Vascular, non-vascular plants, woody and herbaceous angiosperms are included in Class 3 Endophytes (Davis et al., 2003). Class 3 endophytes are very diverse within certain host tissues, populations, and plants. On a single plants hundreds of endophytes are present. Endophytes belong to Class 4 are only found on roots. They are generally Ascomycota and are found in host plants like non-mycorrhizal from arctic, alpine, sub-alpine, temperate zone and non-temperate zone (Jumpponen, 2001).

1.5 Identification of fungal endophytes

There are two techniques to identify fungi which are morphological and molecular techniques.

1.5.1 Morphological Method

Numerous phenotypic traits of endophytic fungal isolates are observed using morphological techniques, including colony structure, color, elevation, and spore features (Promputtha et al.,2005). There are numerous online databases [Fungal Databases, Bibliography of Systematic Mycology, Index Fungorum, Fungal Planet, Tree of Life Web Project and Mycology] are useful for identifying fungi species. Many isolates are classified as "sterile fungus" or "sterile mycelia" because they are unable to produce spores during incubation (Kumaresan and Suryanarayanan, 2001). At the genus or species level, endophytic fungi, particularly "sterile fungi," are difficult to identify (Gamboa and Bayman, 2001).

1.5.2 Molecular Method

When compared to morphological identification, molecular approaches are far more trustworthy because phenotypic characteristics are unstable and can change depending on the environment (Schulz and Boyle, 2005). Purified endophytic fungal

isolates are tested to DNA isolation using the cetyl trimethyl ammonium bromide (CTAB) method (Arnold et al., 2007) or a DNA isolation kit (Tejesvi et al., 2011). The most often used DNA barcode for the molecular identification of fungi is the internal transcribed spacer (ITS) (U'ren et al., 2009; Sun et al., 2011). Endophytic fungal isolates are subjected to genomic DNA separation and PCR utilizing the ITS 1/4 primers to target the neighboring sequences of the 18S ribosomal RNA gene, ITS 1, 5.8S ribosomal RNA gene, and 28S ribosomal RNA gene (White et al., 1990). Amplified PCR products in agarose gel are evaluated by electrophoresis, stained with ethidium bromide or SYBR Green, and seen under UV (ultraviolet) light using a Gel Documentation (gel imaging) system. Basic Local Alignment Search Tool (BLAST) matches with the National Center for Biotechnology Information (NCBI) GenBank database are widely used in large-scale examinations of micro fungi to identify the species. To ensure the authenticity of identification based on BLAST results, comparison of taxonomic results at the genus and family levels made for the similar isolates based on BLAST searches. For species identification, an identity level of ≤ 98% ITS rDNA sequence similarity and between 95% and 98% for genus identification are required (Arnold et al., 2007). However, in addition to morphology of the isolate, several molecular markers, such as cytochrome c oxidase subunit I (CO1), -tubulin (TUB), and translation elongation factor (TEF), can be employed to confirm the fungal identity at the species level (Seifert et al., 2007; Roe et al., 2010). Consensus trees are often created using sequences found through BLASTN searches that show the highest identity and most query coverage. Fungal isolates amplified ITS sequences are then uploaded to the NCBI GenBank database.

1.6 Bioactive metabolites of Endophytic fungi

Different types of secondary metabolites are produced by endophytic fungi (Ilyas et al., 2009) which show important biological activities such as antioxidant, anticancer, immunomodulatory, antivirus, anti-tuberculosis, anti-parasite and insecticides (Hussain et al., 2014). A plant's ability to withstand biotic and abiotic challenges, its resistance to insects and pests, and their ability to absorb nutrients are all improved by endophytes that live inside the plant. Actually, they generate chemicals within plants in the form of bioactive secondary metabolites. In reality, these

endophytes act as a storehouse for phytohormones, enzymes, and pharmaceuticals (Gouda et al.,2016).

Since the 1928 discovery of penicillin, scientists have mostly focused on fungi to find novel secondary metabolites (Konakovsky, 2012). Harold Raistrick worked on the metabolites of fungi in 1949 and contributed a lot of important contributions. A billion-dollar anticancer medicine called Taxol was first identified in an endophytic fungus called *Taxomyces andreanae* (Moustafa, 2011).

1.7 Advantages of endophytic fungi as a source of secondary metabolite production

In addition to promoting plant growth, endophytic fungi also make plants more resistant to diseases. The secondary metabolites produced by endophytic fungi like Taxol, Camptothecin, Compound L-783 etc. are consider as a useful resource for drug synthesis.

1.7.1 Anti-microbial compounds

One of the main causes of illness and mortality worldwide is infectious disease. Microorganisms with multiple resistances are the cause of many infectious diseases. Research on natural products helps in the identification of novel molecules having antibacterial properties (Ramage et al., 2012). Low molecular weight natural compounds with antibacterial properties produced by endophytes are active against other pathogens at low concentrations (Guo et al., 2008). In addition, endophytes provide novel antibacterial metabolites that can be used to combat human and plant pathogens' rising drug resistance (Yu et al., 2010). Numerous bioactive substances also have antifungal properties, such as ‘sordaricin’, which was discovered from the *Xylaria* genus and has antifungal properties against *Candida albicans* (Pongcharoen et al., 2008).

1.7.2 Antioxidant compounds

Anti-oxidants are substances that prevent oxidation because oxidation results in the production of free radicals, and if this process persists, chain reaction occurs and destroys the cells. The only sources of naturally occurring antioxidants are medicinal plants, fruits, and vegetables. Endophytic fungi produce the majority of

antioxidant metabolites. Due to the fact that they are our body's first line of defense against free radical damage, antioxidants aid in the maintenance of health issues. Antioxidant-active substances have a strong inhibitory effect on substances with a range of pathogenic effects including cellular degeneration, DNA damage, and carcinogenesis like oxygen-derived free radicals and reactive oxygen species (ROS) (Huang et al., 2007). The prevention and treatment of cardiovascular disease, hypertension, diabetes mellitus, atherosclerosis, and cancer all depend on antioxidants (Münzel et al., 2010). The compounds with flavonoids and phenols generated by the endophytic *Xylaria* sp. were tested for their antioxidant potential by Liu and coworkers (Liu et al., 2007).

1.8 Scope of study

Endophytes that develop symbiotic relationships with plants increase plant productivity by providing them with vital nutrients and shielding them from plant infections (Thrall et al., 2007). Endophytes are an abundant source of important natural substances with pharmacological and biological activities (Kogel et al., 2006). The host plant is not only protected by the endophytes throughout their symbiotic association with plants, but they also give them the necessary conditions for survival. In addition, the endophytes generate a vast number of secondary metabolites that are pharmacologically active and that can be separated and characterized. These secondary metabolites have the potential to be used in both industry and medicine (Staniek et al., 2008). In the pharmaceutical industry, endophytes are important for producing drugs at a reasonable price. Endophytes are used in the pharmaceutical industry to produce medications more effectively and as a backup source of drugs (Campos et al., 2010). The problem of drug-resistant human diseases can be solved with the isolation of antimicrobial metabolites, which also aid in the creation of powerful antibiotics to combat bacterial pathogens (Ge et al., 2008).

Endophytic secondary metabolites play a significant role in human life. Many of these metabolites are used as active pharmacological components in medications such as antipyretic like aspirin, anticancer agents, antibiotics, and antivirals like lovastatin (Cholesterol lowering drugs) (Khazir et al., 2013, Mousa and Raizada, 2013) that are used as herbicides in agriculture.

1.9 Aims and objectives

The primary objective of the current study was

1. To isolate endophytic fungi from twigs and leaves of medicinally important edible plant *Prunus armeniaca.*
2. Molecular identification of endophytic fungi using universal barcode marker i.e., ITS1 &ITS4
3. Screening of isolated endophytic fungi for bioactive metabolites.
4. Evaluation of biological potential of isolated endophytes by using their crude extract.

CHAPTER 2

MATERIAL AND METHOD

2 Materials and Methods

Current research was carried out in Mycology Lab, Department of Plant Sciences, Quaid-i-Azam University, Islamabad. Several investigations were conducted in both natural and controlled environments. The experimental work was divided into three parts namely; Isolation of endophytic fungi from *Prunus armeniaca*, Identification of the fungal isolates and screening of endophytic fungi for bioactive metabolites and evaluation of their biological potential.

Part-A: Isolation of endophytic fungi from *Prunus armeniaca*

2.1 Sampling Area

Prunus armeniaca plant reported in this study was collected from Quaid-i-Azam University Islamabad, Pakistan. Herbarium specimen (Voucher no. 132104) (Shown in Fig 2.1) was deposited in the herbarium of Pakistan department of Plant Sciences; Quaid-i-Azam University Islamabad. The Quaid-i-Azam University is located at an elevation of 619.18 meters (2031.43 feet) above sea level. The climate here is humid subtropical with dry winters. The annual temperature of district is 27.09ºC (80.76ºF). It receives Approximately 94.76mm (3.73 inches) of precipitation and 125.05 rainy days (34.26% of the time) annually.

Fig 2.1: (A-B) *Prunus armeniaca*, **(B)** Research site

2.2 Isolation of endophytic fungi

The healthy plant leaves and twigs (*Prunus armeniaca)* were collected from the Quaid-i-Azam University, Islamabad. The plant leaves and twigs were surface sterilized by washing in running water for 1 minute to remove any apparent debris, followed by rinsing with deionized water, cleaning with 70% ethanol for 30 seconds, followed by cleaning with 1% sodium hypochlorite for 1 minute (Gond, et al., 2012). The samples were then thoroughly cleaned with deionized water, rewashed with 70% ethanol for 30 seconds, and left to dry by air. Smaller sections (0.5× 0.5 cm2) of the surface-sterilized samples were cut. Each plant's eight leaf and twig pieces were inoculated on PDA (Potato Dextrose Agar) (Shown in Fig 2.3) and incubated for 4–7 days at 32 °C. The fungal isolates were then sub-cultured and purified for molecular identification after the incubation period of 7 days.

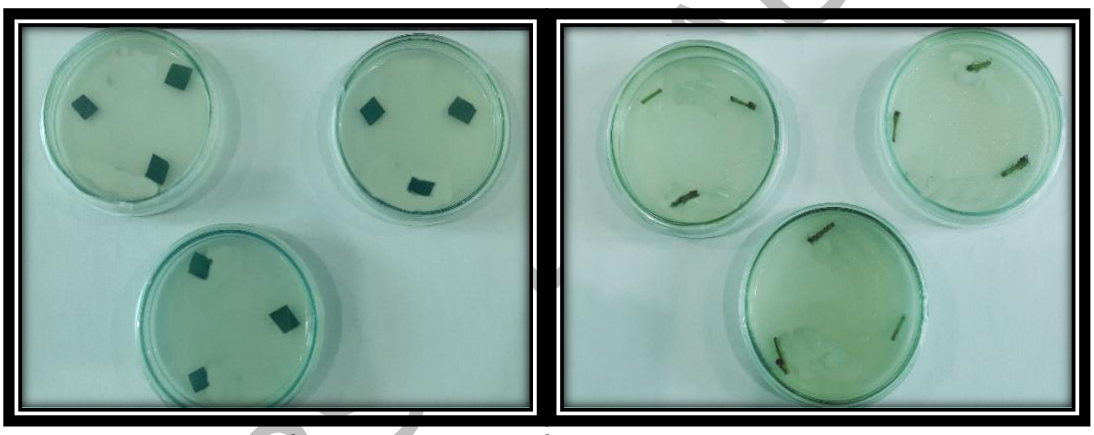

Fig 2.2: Inoculation of leaves and twigs of *Prunus armeniaca* on PDA

2.3 Morphological Characterization

Macro-morphological characterization includes following characters i.e., colony color, reverse colony color, colony shape, colony diameter, colony margins, texture, opacity of colony and topography. Color names were followed from Ridgway. Microscopic characters of fungi i.e., conidiogenous cells, conidia were analyzed by mounting material from colonies growing on PDA on glass slides using various chemicals such as 5% KOH (5g KOH in 100ml of distal water), Congo red, Lactic acid. Microscopic features were observed at magnification of 40x and 100x under Olympus (CX41) light microscope for images and measurement of sizes of various structures.

Part-B: Molecular Identification of Endophytic Fungi

2.4 Molecular characterizations

Molecular analyses of the isolated endophytic fungi were accomplished using the following methods and protocol.

2.4.1 DNA isolation

Molecular characterizations were performed by extracting the genomic DNA from fungal pure colony using 2% CTAB following the protocol of (Gardes & Bruns, 1993).

1. Small amount of pure fungal colony, picked up from the margins by sterile micropipette tip, was placed into 1.5 ml of centrifuge tube containing 300 µl of 2% CTAB buffer. Colonies immersed in were frozen and thawed (three times).
2. Inside a laminar flow hood, tissues were crushed using micro-pestles.
3. The samples were incubated in water bath at 65˚C for 30-35 min and after every five minutes, they were gently inverted.
4. 300 µl of isoamyl alcohol and chloroform was added in the ratio of 24:1. To thoroughly combine the materials with the solution a gentle vortex was used until it gives milky appearance. The samples were gently vortex to get them mixed with the solution until it gives milky appearance.
5. The sample were centrifuged for 15 minutes at 1350 rpm. It gives two prominent layers i.e., pellet and supernatant.
6. Supernatant was shifted carefully into another tube (approx. 200 µl) without touching the pellet during pipetting.
7. Ice-cold isopropanol in the ratio of 2/3 (2/3×200) was added to the supernatant for DNA precipitation and placed at 4 °C at room temperature for few minutes or in the freezer for overnight.
8. On the next day, the precipitated samples were centrifuged for 15 minutes at 1350 rpm and the solutions were discarded from tubes, the pellets were then washed by adding 200 µl of 70% ethanol, the same step was repeated twice i.e., centrifugation and washing.
9. The final pellets were dried by keeping them inside laminar flow hood for 30 minutes and then dissolved in 50 µl TE buffer and stored at 4°C in freezer.

2.4.2 PCR amplification of ITS region

DNA extracted from the fungal colonies was used directly or dilutions were prepared with double distil water where needed, depending upon DNA concentration.

2.4.2.1 Primers*:*

PCR (Polymerase Chain Reaction) was done following Gardes & Bruns (1993) to amplify the nuclear ribosomal internal transcribed spacer region (rDNA-ITS) using primer ITS1F.

Table 1: Sequences of ITS1F and ITS4 Primers

Sr. no	**Primer**	**Sequence**	**Reference**
1	ITS1F	5′-CTTGGTCATTTAGAGAAGTAA-3′	Grades &Bruns (1993)
2	ITS4 R	5′-TCCTCCGCTTATTGATATGC-3′	White *et al.* (1990)

2.4.2.2 Reaction mixture:

ITS region was amplified in 50µl reaction mixture. Reagents and their concentrations are listed below:

Table 2: Reaction mixture setup of PCR for amplification

Reagents	**Concentration (µl)**
10X Taq buffer	5
2 mM dNTP	5
5 U/ml Taq polymerase	1.25
25 mM $Mgcl_2$	10
10 pM ITS1F (forward)	1
10 pM ITS4 (reverse),	1
Nuclease free water or double distal water	21.75
Template DNA	5

2.4.2.3 Temperature cycling:

Control without DNA was run with each amplification series to check reagents contamination. The thermal cycler thermocycler profile (SimpliAmp Thermal Cycler) was used to perform the PCR reaction.

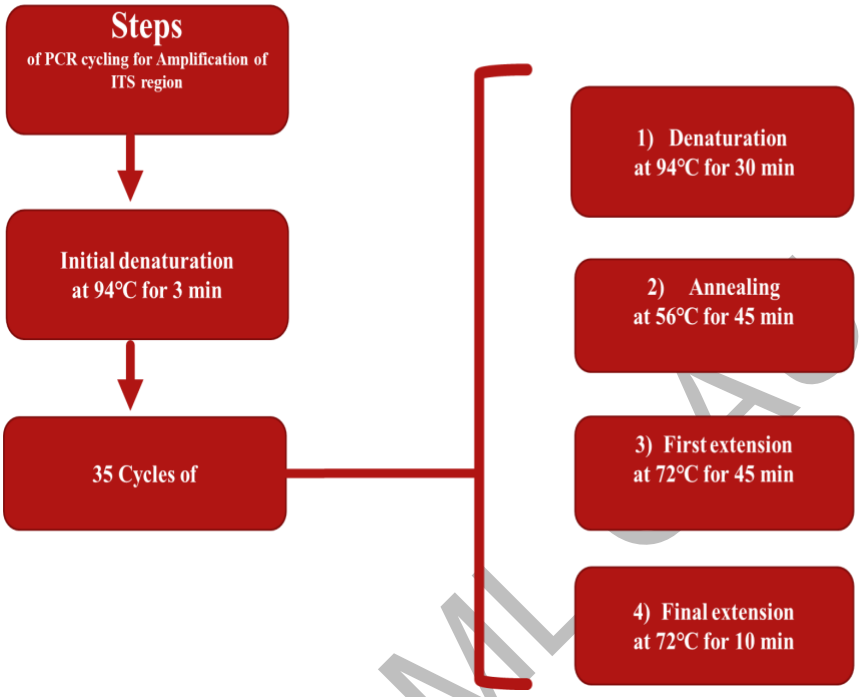

Fig 2.3: Conditions of PCR cycling for Amplification of ITS region

2.4.3 Gel electrophoreses

To visualize the PCR product, agarose gel electrophoresis was performed with 1% agarose gel followed by visualization of amplified fragments of DNA in a gel documentation system.

1. Gel was made by dissolving 0.75 g agarose powder in 75 ml of 1X TAE or TBE buffer. It was placed in microwave oven and heated until it gave transparent look.
2. Then the solution was left to cool at room temperature and 3 µl of ethidium bromide was added and agitated well.
3. Gel solution was then transferred into gel tray with inserted combs and allowed for solidification. 1X TAE or TBE buffer was added to the gel tray so that the gel was completely dipped into it (shown in Fig 2.4).
4. The comb was then removed and 2 µl of PCR product (DNA) and 2 µl of loading dye was mixed and dispensed into the wells. 2 µl of 1 kb ladder was also loaded to

assure the PCR product size. The gel was run at the voltage of 80 V for 40 minutes and DNA bands were visualized on gel after examination in gel documentation system. Images of DNA bands were saved.

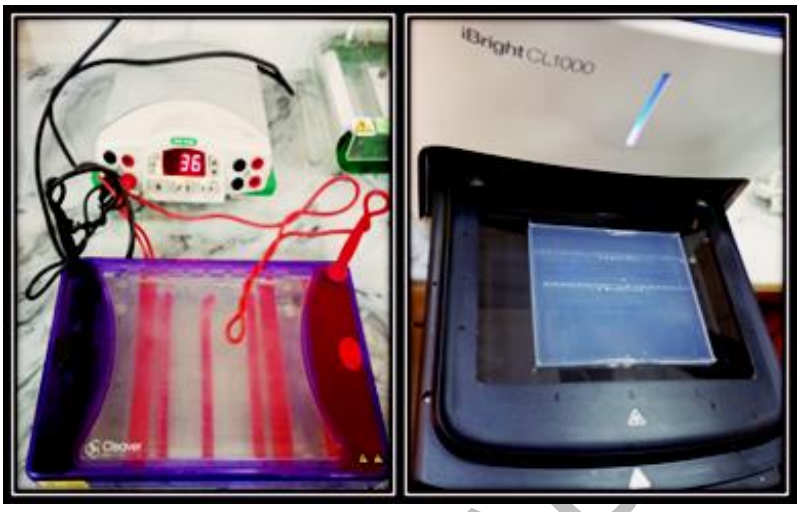

Fig 2.4: Gel Documentation system

2.4.4 Purification, Sequencing and BLAST analyses

DNAs were sent to MacroGen company (South Korea) for the purpose of purification and sequencing. The same primers used for PCR were utilized for sequencing. After sequencing, the sequences were assembled in Bio edit ver. 7.2.5. (Hall, 1999) after manual editing and trimmed up to the conserved motifs 5'-CATTA- and -GACCT-3' given by Dentinger *et al.*, (2011), and then BLAST searched in NCBI (http://www.ncbi.nlm.nih.gov/) to see the foremost similarities with other related ITS sequences deposited in the database. Sequences showing the highest percentage similarity, query cover and E value were noted for further phylogenetic analysis with our sequences. The newly generated sequences were deposited in GenBank, and accession numbers are still awaiting.

Part-C: Screening of endophytes for biological metabolites and evaluation of biological potential of their secondary metabolites.

2.5 Bioactive secondary metabolites extraction from endophytic fungi

For the extraction of fungal secondary metabolites, liquid media fermentation was performed. PDB Broth was prepared and Flasks were autoclaved at 121℃ for 20 minutes. Then fresh mycelium of fungal samples was inoculated from the DSA plates

with a loop in the broth aseptically. Flasks were then put on the shaking incubator for 20 to 30 days at 32°C with 120 rpm. (Bhardwaj A et al., 2015).

2.5.1 . Extraction of crude extracts from liquid media through filtration

The flasks were routinely examined for contamination of any kind. When the fungi had grown to their maximum potential, Vacuum filtration assembly was used to separate mycelium from broth. After 7-10 days, through filtration mycelia was separated from ethyl acetate. Mycelia was removed and ethyl acetate was evaporated at room temperature to obtain the crude extract dissolved in Dimethyl sulfoxide (DMSO) (as shown in Fig 2.5) (Bhardwaj A et al., 2015).

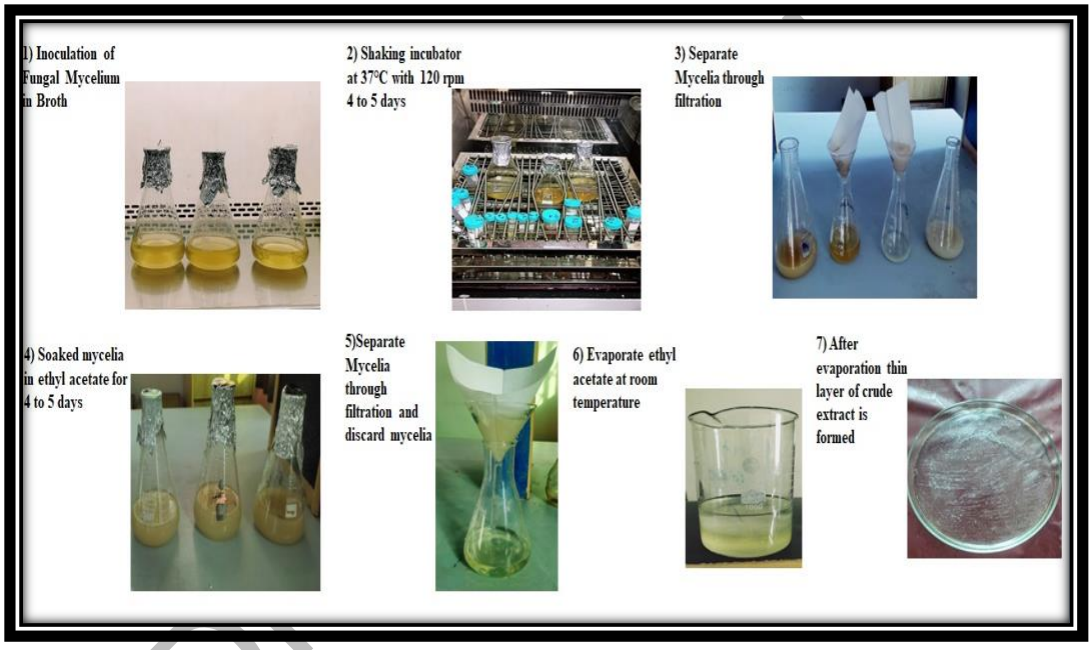

Fig 2.5: Steps of Fungi bioactive secondary metabolite extraction

2.5.2 Biological assays of crude extract

By applying the following formula, the percent extraction efficiency of each extract was determined:

% Extraction efficiency = [(We2- We1)/We3] × 100

Where W2 is weight of crude extract plus weight of sample container, W1 is the weight of sample extracts and We3 is weight of bulk powder.

Preparation of test samples for assays

Using the formula

m1v1= m2v2

40*? =? *1000µl

The crude extracts 40mg were dissolved in 1ml DMSO (Dimethyl sulfoxide).

Table 3: Different dilution of crude extracts in DMSO

mg/ml DMSO	**Equal to µg/µl**
40mg/ml	40000µg/1000µl
20mg/ml	20000µg/ml
4mg/ml	4000µg/ml

2.5.2.1 Determination of total flavonoid content

To determine total flavonoid content, Aluminum trichloride (AlCl3) method was used (Quettier-Deleu et al., 2000). In 96 well plate 20µl of activated fungal metabolites samples were taken. 10µl of 10% Aluminum chloride was added. The wells were then filled with 10µl of 1M potassium acetate. To obtained the final concentration of 200µl for each well, 160µl of distilled water was added. DMSO 20µl was taken as a negative control and quercetine (4mg/ml) in methanol was used as a positive control. For 30 min the plate was incubated at 37°C. Readings were taken at 405nm (415nm) wavelength on microplate reader.

2.5.2.2 Determination of total phenolic content

TPC in sample extracts was determined using the Folin-Ciocalteu technique (Jagadish, et al., 2009; Slinkard & Singleton., 1977). In 96 well late 20µl (4mg/ml) of activated samples metabolites were taken.90µl of 10-time diluted Folin-ciocalteu reagent (1ml Folin-ciocalteu reagent and 9ml distilled water) was added to the samples in the well plate. For 5 min the plate was incubated. Then 90µl of 6% stock solution of sodium chloride (6g/ml) was added to all samples wells and mixed properly. 20µl of DMSO was used as negative control. Positive control was 20µl (4mg/ml of Methanol) of Gallic acid. For 90 minutes the plate was incubated at room temperature. Reading was taken at 630nm (750nm) wavelength on micro plate reader.

2.5.2.3 Antioxidative activity assay (DPPH Assay)

2-diphenyl-1-picrylhdrazyl (DPPH) was used as substrate in the free radical scavenging assay (Tai, et al., 2011). In 96 well plate activated test metabolites sample in 20µl, 15µ, 10 µl, and 5 µl concentrations were taken (Fig 2.6). For DPPH stock reagent DPPH was dissolved in methanol (9.6mg/100ml) and was prepared by dissolving the DPPH in Methanol while keeping the concentration 9.6mg/100ml. 180, 185, 190 and 195µl ratio of DPPH was added to entire well to get the final concentration of 200µl in each well. 20,15,10 and 5 µl DMSO was taken as negative control instead of sample and 20, 15, 10 and 5µl 1mg/ml (Methanol) Ascorbic acid as positive control. Plate was incubated for 1 hour at room temperature. Reading was taken at 517nm wavelength on micro plate reader. The percent IC50 values of extracts were calculated as Ascorbic Acid Equivalent (AAE) µg/mg of extracts.

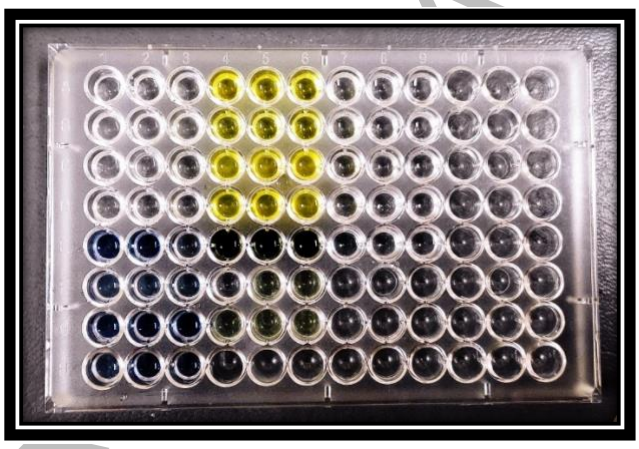

Fig 2.6: 96-well plate loaded with samples

2.5.2.4 Anti-bacterial activity

By agar well diffusion method (Balouiri, et al., 2016) antibacterial activity of crude extract was tested against five bacterial strains

- **Gram positive bacteria:** *Bacillus subtilis, Staphylococcus epidermis*
- **Gram negative bacteria:** *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa*

Assay Procedure

Nutrient agar plates were prepared by dissolving 30g per liter. After being autoclaved and put into sterile petri dishes, the media was let to solidify. The plates

were exposed to UV light for a short period of time before being incubated overnight to assure sterility. A portion of the inoculum from suspension was collected and applied to plates of nutrient agar. Inoculum was evenly dispersed over a plate to create lawns using a sterile swab, and then allowed to dry for 10 to 15 minutes. After lawn making, proper spaces wells were made by a sterile tip. After well making, wells were sealed with drops of agarose gel. Crude extract sample were used in three-fold dilutions of 10, 5 and 4µl, dissolved in DMSO. For positive (tetracycline) and negative (DMSO) controls two extra wells were made. All of the samples were dropped at a concentration of 100µl into each well and left for a while for proper diffusion. After 24 hours of incubation at 37℃, zone of inhibition was measured in mm that indicates the antibacterial activity of crude metabolites.

Fig 2.7: Research work conducted during this study period

CHAPTER 3 PART I

RESULTS

Macro and Microscopic Characterization

3 RESULTS

Results Part II

Macro and microscopic characterization of Endophytic Fungi

2.5.3 *Talaromyces dimorphus* Jiang, X. Z., Yu, Z. D., Ruan, Y. M., & Wang, L. (2018). *Scientific reports*, *8*(1), 1-11.

Etymology: The unique epithet is derived from the fact that the species frequently produces both biverticillate and monoverticillate penicillin.

Macro-morphologically characterization

PDA 32℃, 7d; Colonies 12-15mm, thin, plane, irregular, sulcate. Initially colonies colored Pistachio Green (R. Pl. XLI) with white margins and after 7 to 10d margins turned into Zinc Orange (XV); Mycelium white: Reverse Apple Green (XVII) at center, Zinc Orange elsewhere with white margins (Shown in fig 3.1).

Micromorphology

Conidiophores: Conidiophores biverticillate and monoverticillate, Stipe smooth-walled, (19.9) 20.7 - 34.9 (37.9) × (1.3) 1.4 - 2 (2.2) µm.

Metulae, (8) 9 - 12(13) × (2.4) 2.6 - 3.6 (3.9) µm, 4–6 per vertical

Philiades: phialides 3–4 per verticil, ampulliform, (7.2) 7.9 - 10 (10.4) × (1.7) 1.8 - 2.26 (2.3) µm.

Conidia: conidia smooth-walled, ovoid to ellipsoidal, (2.9) 3.8 - 5 (5.3) × (1.9) 2.1 - 2.8 (3.3) µm.

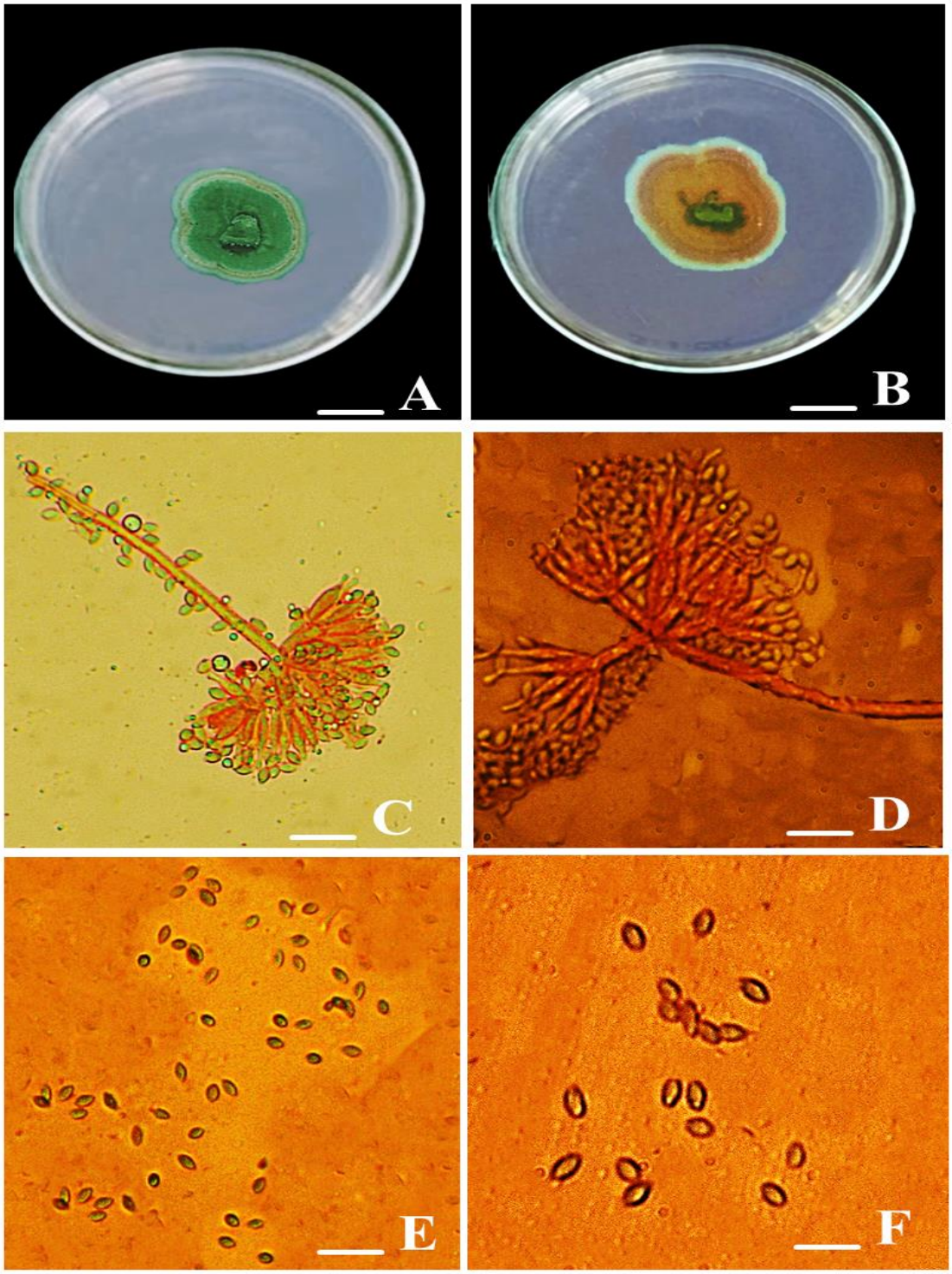

Fig 3.1: - *Talaromyces dimorphus* (A): Colony Front View; (B): Reverse colony on PDA incubated at 32°C for 7 days (C): Conidiophores 40X, (D): Conidiophores 100X, (E) Conidia 40X, (F) Conidia 100X

2.5.4 Alternaria alternata Aung, S. L. L., Liu, H. F., Pei, D. F., Lu, B. B., Oo, M. M., & Deng, J. X. (2020). Mycobiology, 48(3), 233-239.

Macro-morphologically characterization

Colonies 5o mm in diameter on PDA after 7 days at 32℃, circular, convex with entire margins, sulcate, velvety in texture. Dark Greenish olive (R. Pl. XXX) and chromium green (R. Pl. XXXII) and white on the margins displaying Dusky Green-Gray (R. Pl. LII) and white aerial mycelium: Reverse Dusky Gray-Green with white margins. After 10 to 15 days aerial mycelium become more whitish as shown in fig 3.2(A).

Micromorphology

Conidia (12) 13.7 - 27.6 (28.4) × (4.2) 4.8 - 6.8 (7.4) µm, light to dark brown in color, obclavate and in short conical flask, typically 1 to 9 transverse septa and 0 to 3 longitudinal septa, which were seen between 5 and 8 days old.

Conidiophores (20.7) 21 - 34 (37.4) × 7.2 - 9.6 (10) µm producing 4–10 units catenulate conidia.

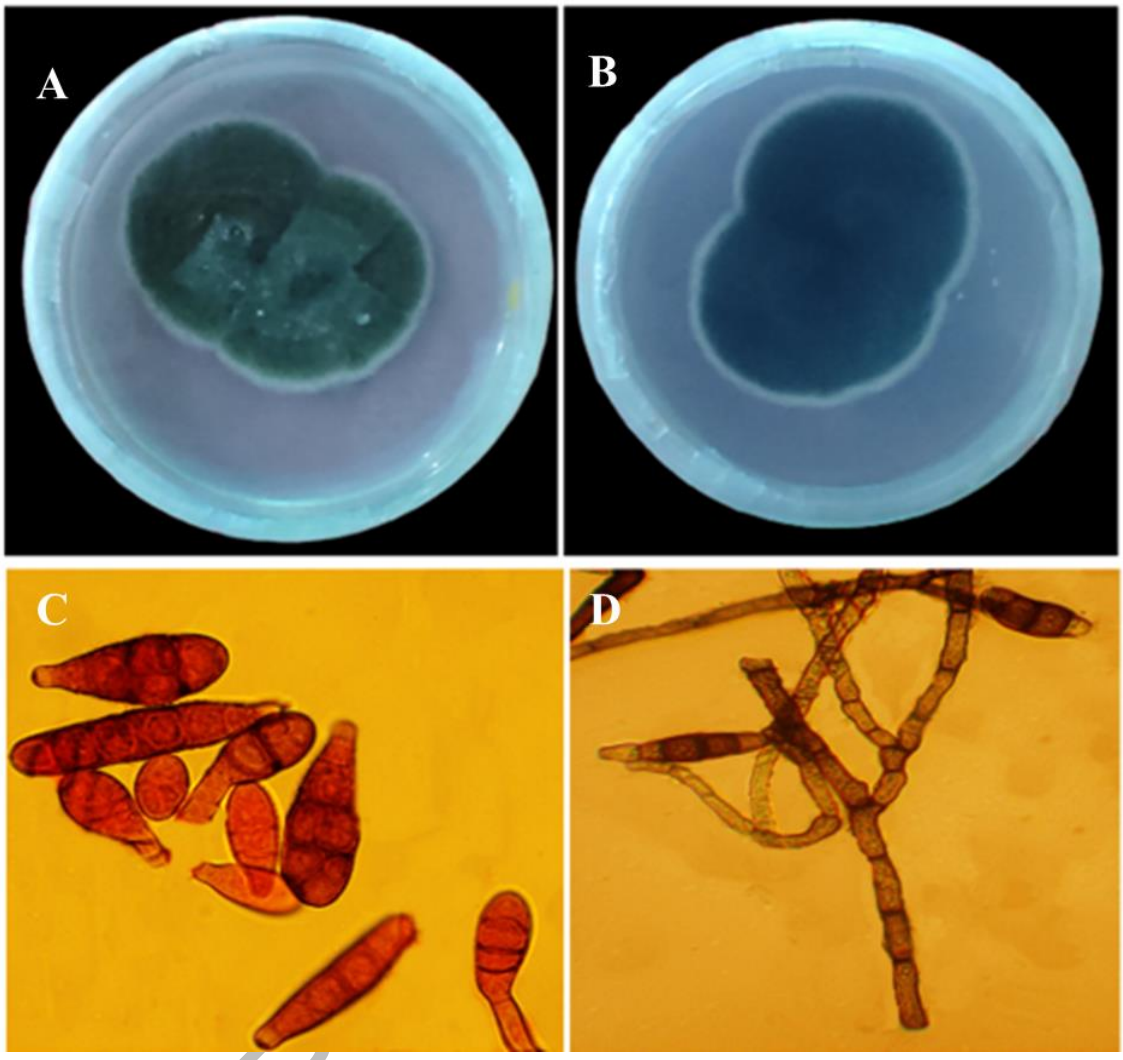

Fig 3.2: (A) Front colony, **(B)** Reverse colony, **(C)** Conidia, **(D)** Conidiophores 100

2.5.5 Aspergillus niger Zakaria, L. (2018). Malaysian Journal of Microscopy, 14(1).

Macro-morphologically characterization

Colonies 85mm in diameter on PDA at 32 ℃ after 7 days, circular, flat with entire margins, powdery in texture. Blackish white colony; Reverse Glass Green (R. Pl. XXXI) with black spotting (Fig.3.3)

Micromorphology

Conidial Head Biseriate and radiated, hyaline, Vesicles globose, 40.4 - 55.9 µm wide.

Stipe 182 - 375. × 3.83 - 20 µm, walls are thick and smooth.

Conidia (3.2) 3.3 - 3.9 (4.1) × (2.8) 2.85 - 3.59 (3.6) µm, brown, rough walled, globose to sub-globose.

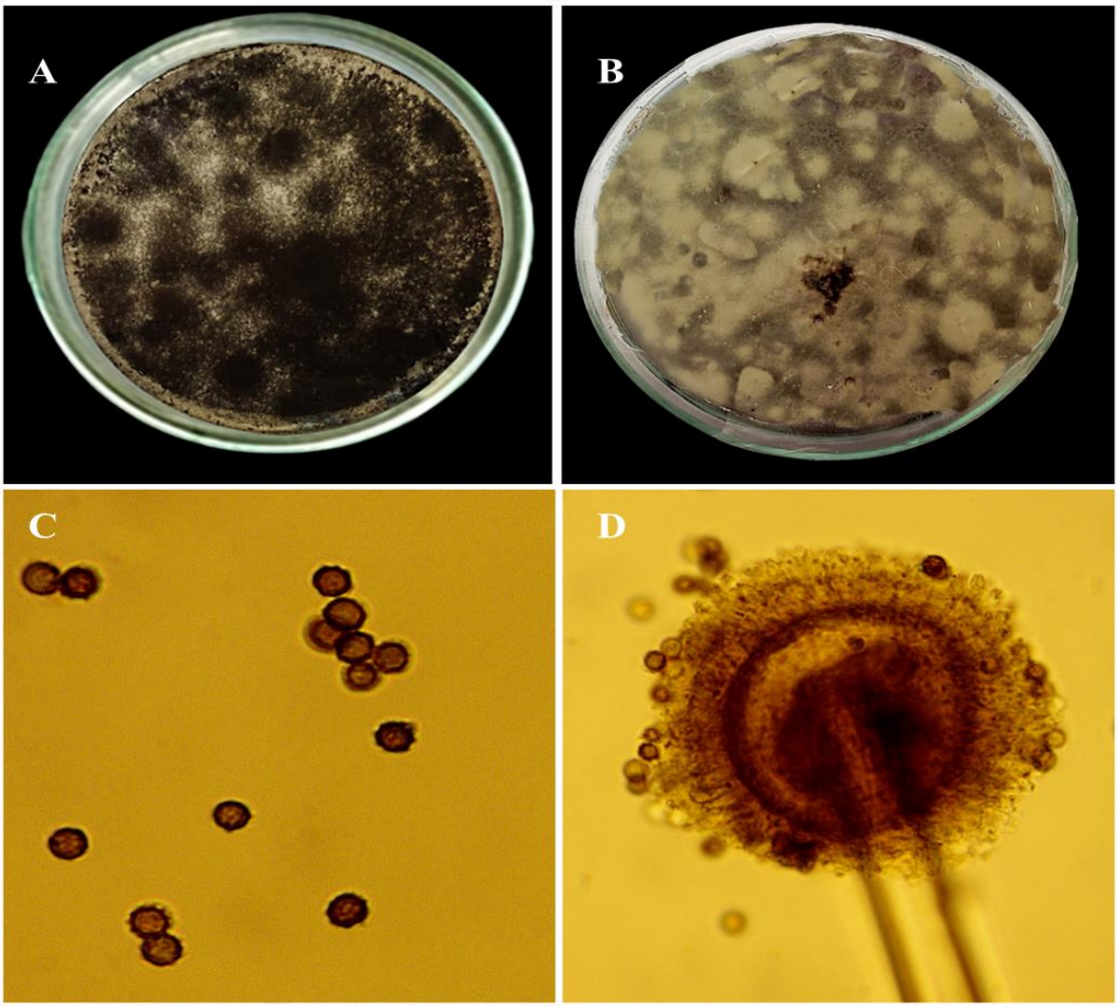

Fig 3.3: (A) Front colony, **(B)** Reverse colony, **(C)** Conidia, **(D)** Conidial head

3.1.3 *Pseudofusicoccum adansoniae* Pavlic, D., Wingfield, M. J., Barber, P., Slippers, B., Hardy, G. E. S. J., & Burgess, T. I. (2008). *Mycologia, 100*(6), 851-866.

Macro-morphologically characterization

Colonies 84mm diam on PDA at 32°C after 7 days, raised**,** irregular with lobate margins. Initially whitish colonies with a relatively dense mycelial mat; Reverse colony is white in color with Ivy Green (R. PI *XXXI*) in the center of colony. Submerged mycelium turns Cress Green (R. PI *XXXI*) to Light Grayish Olive (R. PI *XLVI*) and becoming Deep Grayish Olive (R. PI *XLVI*) within 10-15 days. Aerial mycelium are dense with cottony texture (as shown in fig 3.4). Colonies become more cottony with age. White cottony color turns into Dark grayish olive (R. PI *XLVI*) after 30 to 40 days of incubation.

Micromorphology

Conidia (7.4) 10.1 - 16.6 (23.8) × (6.3) 7.2 - 12.7 (16) µm, ellipsoidal, aseptate, hyaline, straight or slightly bent with rounded tips, smooth walls, fine granular substance, and a thin mucilaginous coating around it.

Conidiogenous Cells (7.8) 10.2 - 21.3 (25.4) × (3.7) 4.3 - 6.7 (6.9) µm, Holoblastic, cylindrical or rod shaped, hyaline, smooth walled. Conidia were formed enteroblastically after the initial conidium, which was formed holoblastically.

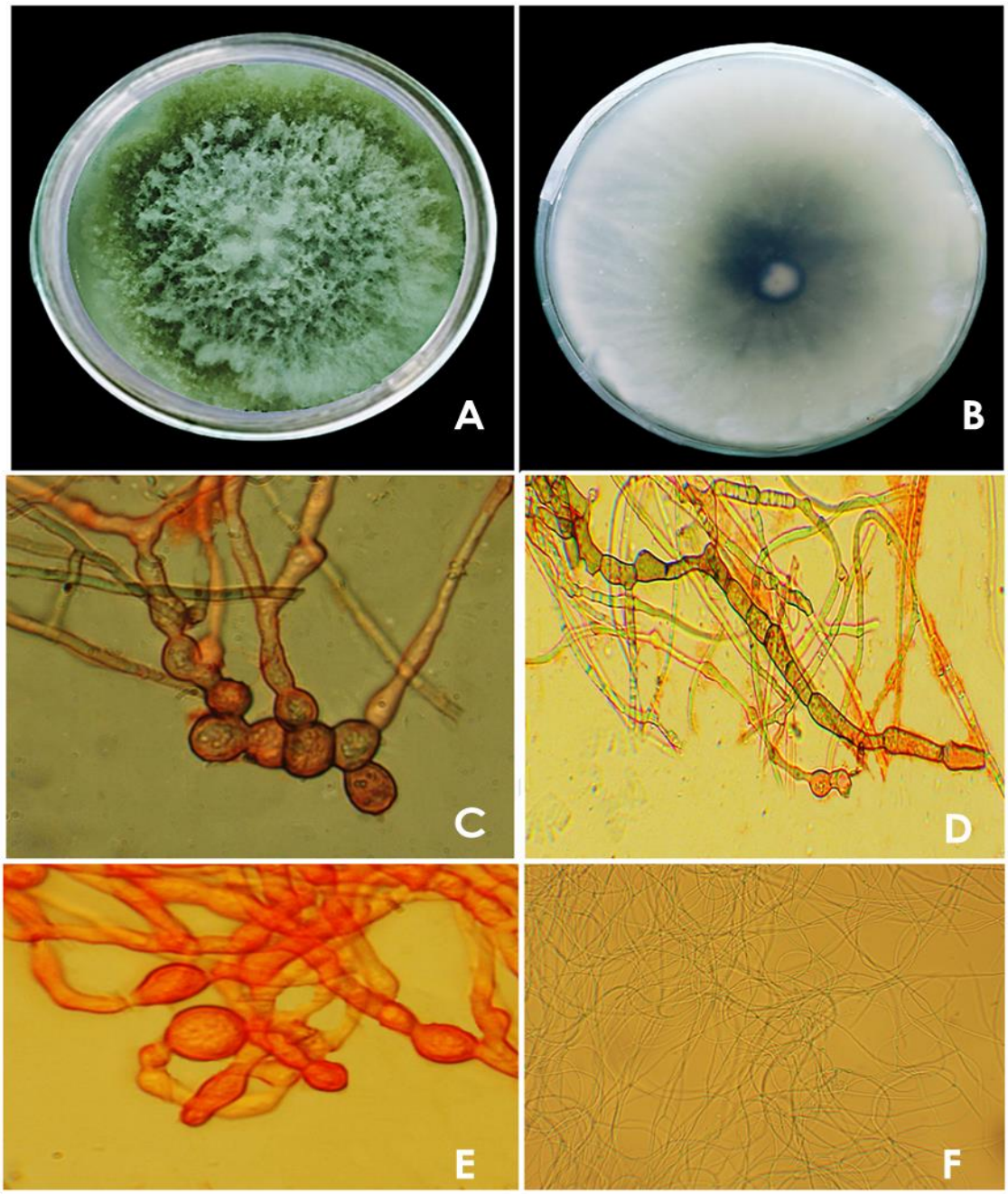

Fig 3.4: (A) Front colony, **(B)** Reverse colony, **(C, E)** Conidia, **(D)** Conidiogenous cells, **(F)** hyaline mycelium.

CHAPTER 3 PART II

RESULTS

Molecular and phylogenetic analyses of Endophytic Fungi

Results Part II

3.2 Molecular and phylogenetic analyses of Endophytic Fungi

3.2.1 Results of preliminary molecular analysis

3.2.1.1 Gel electrophoreses of genomic DNA

The following bands were produced after running genomic DNA on a 1% agarose gel.

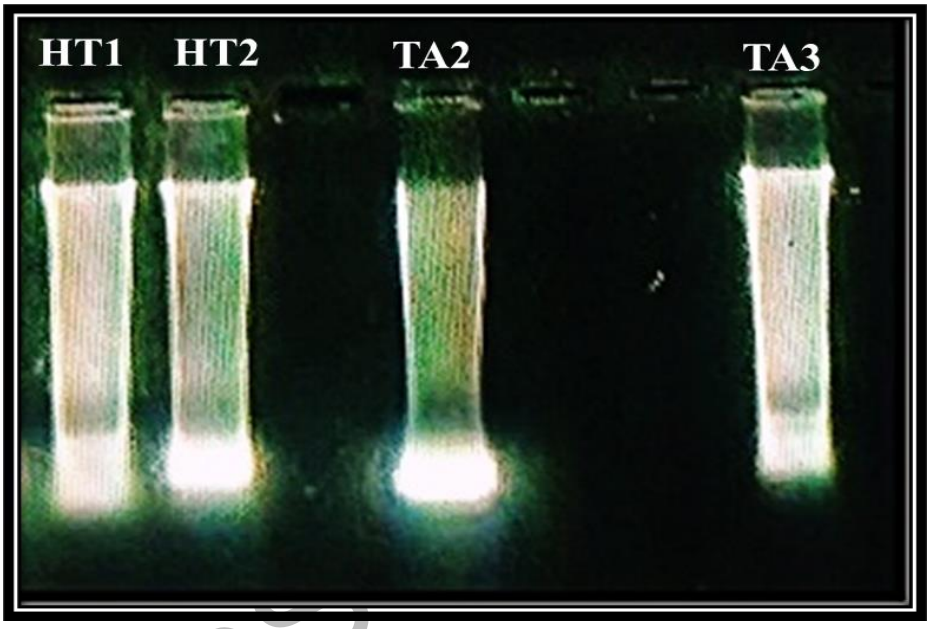

Fig 3.5: Gel electrophoresis of genomic DNA of 4 fungal specimens, HT1 (*Talaromyces dimorphus*), HT2 (*Alternaria alternata*), TA2 (*Aspergillus niger*) and TA3 (*Pseudofusicoccum adansoniae*).

3.2.1.2 Gel electrophoreses of PCR product

When the fungal specimens' rDNA-ITS regions were amplified using ITS1-F and ITS4 primers and run on a 1% agarose gel with a 1 kb ladder, the result was a 750–800 bp product as shown in fig 3.6.

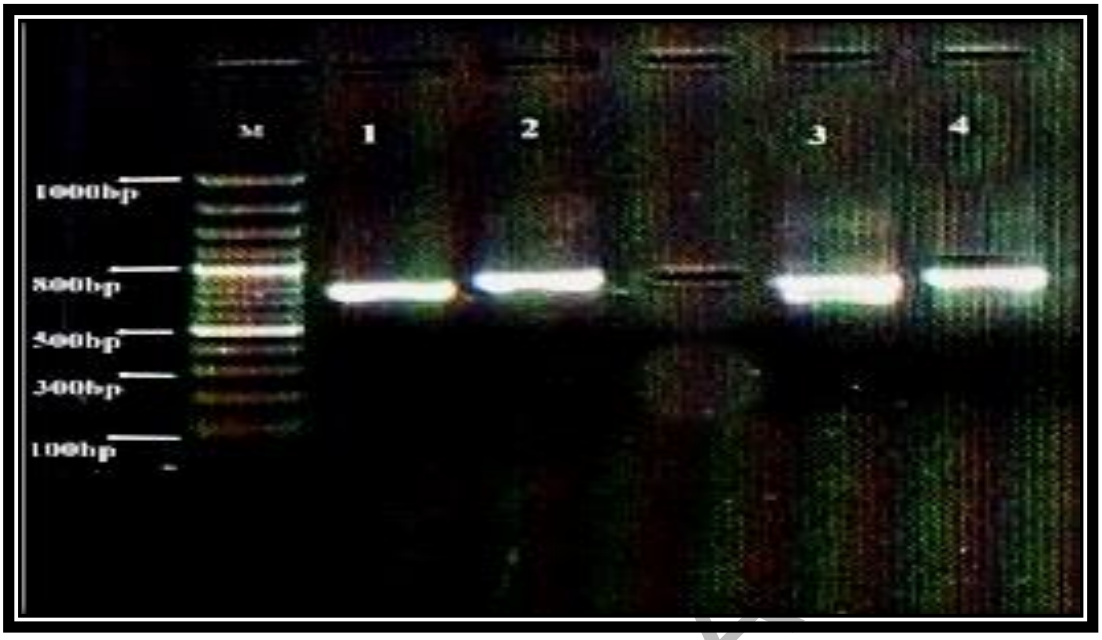

Fig 3.6: PCR amplification of rDNA-ITS region of fungal specimens, M (1kb).

3.2.2 Molecular Characterization of Endophytic Fungi

3.2.2.1 Molecular analyses of Talaromyces dimorphus

Targeted rDNA-ITS1, 5.8 and IT2 regions produced DNA fragments of 590 bp after amplification with specific primers ITS1F and ITS4. Initial Blast analysis indicated that the sequences showed maximum similarity with *Talaromyces* sp. (GeneBank accession # KY007095.1) 100.00%. Other closely related sequences exhibited percentage similarity of 100.00%, 100.00% and 100.00% with *Talaromyces* sp. (GeneBank accession # OP237472.1) *Talaromyces* sp. (GeneBank accession # OL774785.1), and *Talaromyces* sp. (GeneBank accession # JQ717338.1) respectively. Based on the initial BLAST results, phylogenetic analysis was carried out using sequences from Visagie, et al., (2018). All of the sequences were trimmed and assembled using Bioedit Software. Muscle alignment tool was used to align the sequence using MEGAX software.

All of the obtained sequences were trimmed to their conserved regions. 5"…GAT- [CATTA……GACCT]-CAAAT…3" as published by Dentinger et al.,

(2011) and the alignment portion amongst them were added in the analysis. For phylogeny construction gaps were treated as missing information.

There were 27 sequences in the final dataset. Out of these, 26 of these sequences belonged to ingroup taxa that were members of 26 taxa of genera, while *Talaromyces proteolytic* was used as outgroup following Visagie, et al., (2018).

Phylogenetic relationship was formed by using Neighbor-Joining and Maximum Likelihood method, Jukes and cantor (1969) model of ITS sequences and nearest neighbor-interchange (NNI) as ML heuristic search method using MEGAX software (Tamura et al., 2011) and tested as 1000 replicates of bootstrap value and bootstrap values >70% were shown in the phylogenetic tree.

The phylogenetic analysis used a total of 539 sites after trimming and removing the incomplete letters. Of these, 414 positions were conserved, 95 were variable positions, 49 were singleton positions, and 45 were parsimony informative sites.

Our sequences clustered with sequences of *Talaromyces dimorphus* from Genbank with strong bootstrap support (99%). Therefore, our species identified as *Talaromyces dimorphus*. In the phylogenetic tree, accession numbers for the used sequences are given. sequences assigned for this investigation were deposited in the Genbank and are pending. In the phylogenetic tree, the sequences of sample species are denoted by solid square boxes.

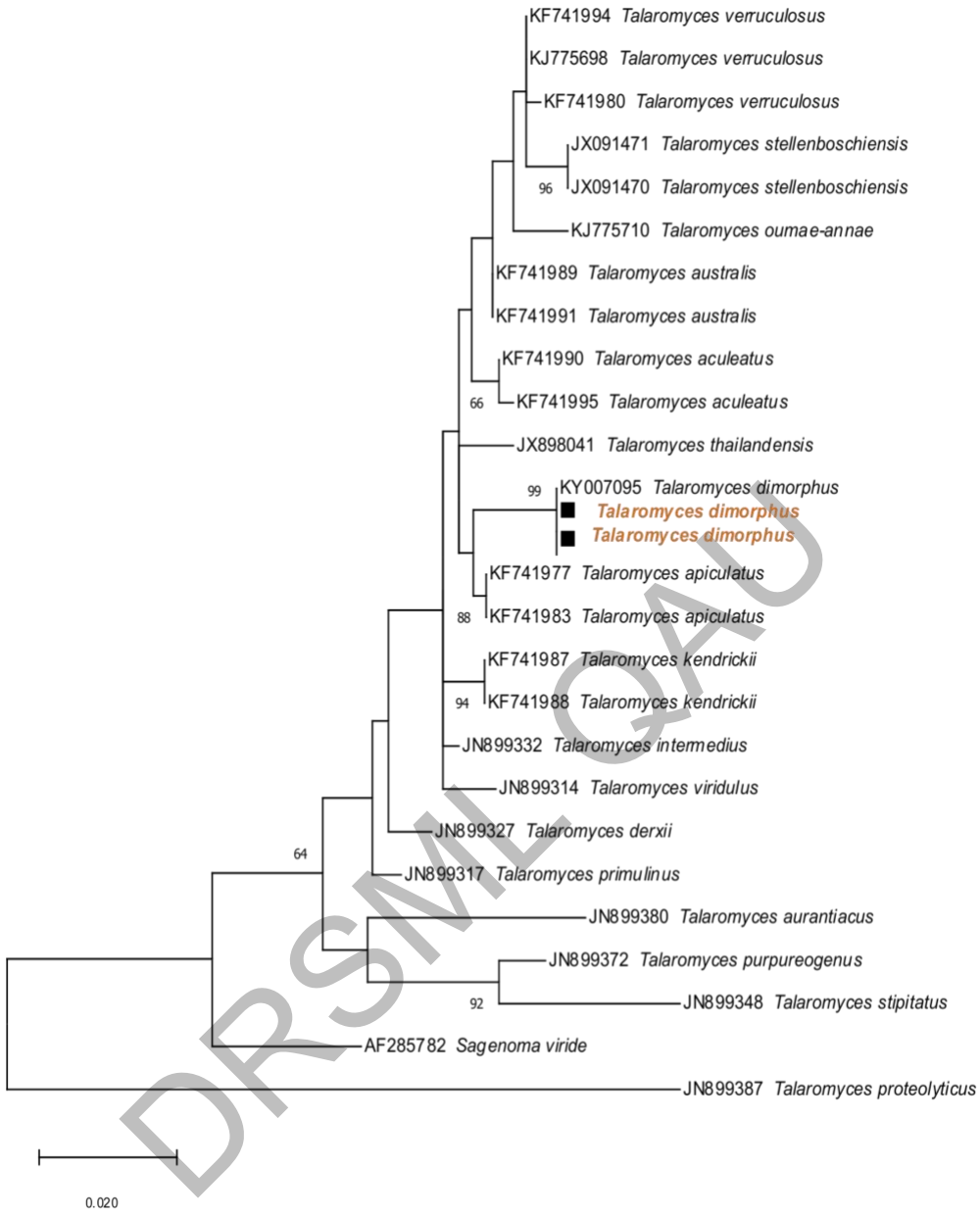

Fig 3.7: Phylogenetic analysis/relationship of *Talaromyces dimorphus* sp. was determined by Maximum Likelihood method using MEGAX software. *Talaromyces dimorphus* sp. shown in a different color in the phylogenetic tree.

3.2.2.2 Molecular analyses of Alternaria alternata

Targeted rDNA-ITS1, 5.8 and IT2 regions produced DNA fragments of 580 bp after amplification with specific primers ITS1F and ITS4. Initial Blast analysis indicated that the sequences showed maximum similarity with *Alternaria* sp. (GeneBank accession # OP850817.1) 100.00%. Other closely related sequences exhibited percentage similarity of 100.00%, 100.00% and 100.00% with *Alternaria* sp. (GeneBank accession # OP895139.1) *Alternaria* sp. (GeneBank accession # OP811259.1), and *Alternaria* sp. (GeneBank accession # OP800098.1) respectively. Based on the initial BLAST results, phylogenetic analysis was carried out using sequences from Aung, et al., (2020). All of the sequences were trimmed and assembled using Bioedit Software. Muscle alignment tool was used to align the sequence using MEGAX software.

All of the obtained sequences were trimmed to their conserved regions. 5"…GAT- [CATTA……GACCT]-CAAAT…3" as published by Dentinger et al., (2011) and the alignment portion amongst them were added in the analysis. For phylogeny construction gaps were treated as missing information.

There were 19 sequences in the final dataset. Out of these, 17 of these sequences belonged to ingroup taxa that were members of 17 taxa of genera, while *Bipolaris tetramera* and *Exserohilum pedicellatu* was used as outgroup following Aung, et al., (2020). Phylogenetic relationship was formed by using Neighbor-Joining and Maximum Likelihood method, Jukes and cantor (1969) model of ITS sequences and nearest neighbor-interchange (NNI) as ML heuristic search method using MEGAX software (Tamura et al., 2011) and tested as 1000 replicates of bootstrap value and bootstrap values >70% were shown in the phylogenetic tree.

The phylogenetic analysis used a total of 552 sites after trimming and removing the incomplete letters. Of these, 424 positions were conserved, 110 were variable positions, 12 were singleton positions, and 98 were parsimony informative sites.

Our sequences clustered with sequences of *Alternaria alternata* from Genbank with strong bootstrap support (100%). Therefore, our species identified as *Alternaria alternata*. In the phylogenetic tree, accession numbers for the used sequences are given.

sequences assigned for this investigation were deposited in the Genbank and are pending. In the phylogenetic tree, the sequences of sample species are denoted by solid square boxes.

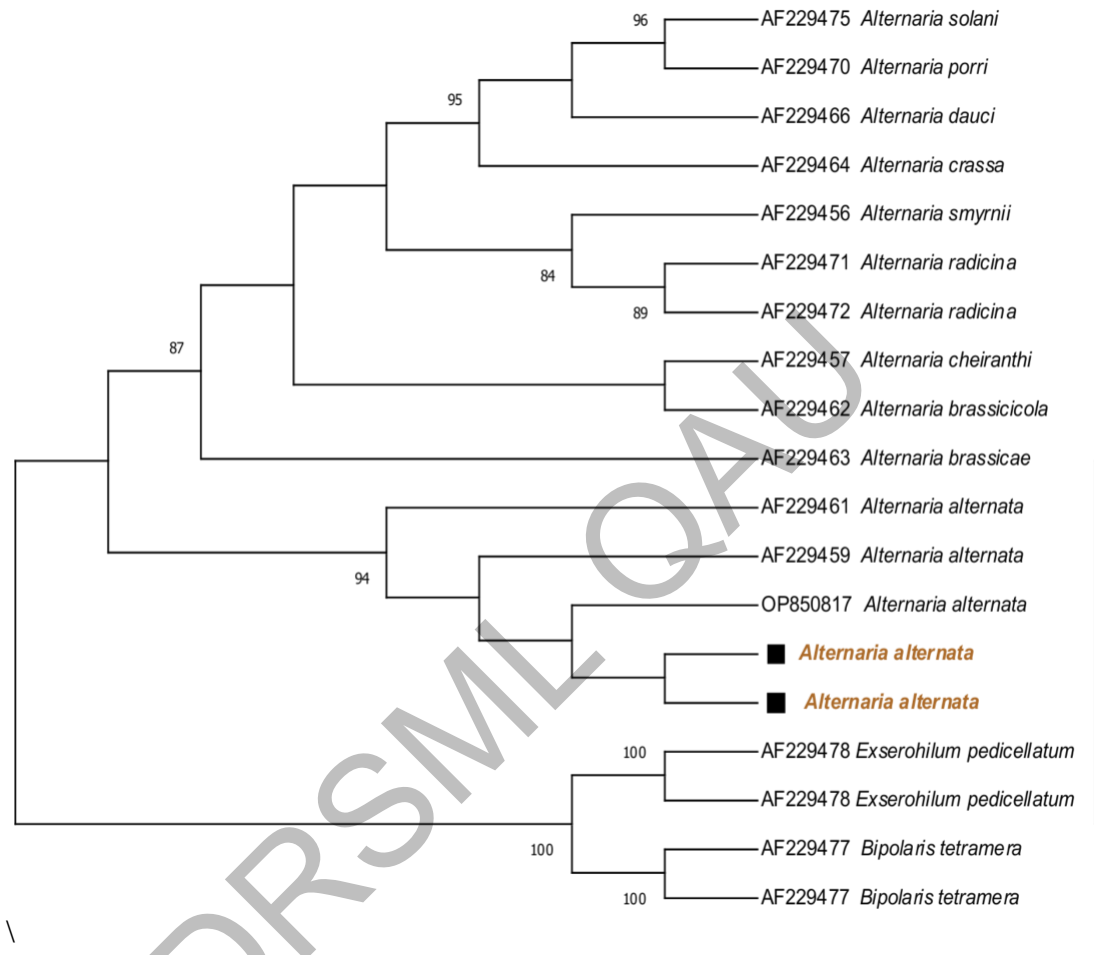

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Fig 3.7: Phylogenetic analysis/relationship of *Alternaria alternata* sp. was determined by Maximum Likelihood method using MEGAX software. *Alternaria alternata* sp. shown in a different color in the phylogenetic tree.

3.2.2.3 Molecular analyses of *Aspergillus niger*

Targeted rDNA-ITS1, 5.8 and IT2 regions produced DNA fragments of 610 bp after amplification with specific primers ITS1F and ITS4. Initial Blast analysis indicated that the sequences showed maximum similarity with *Aspergillus* sp. (GeneBank accession # OP850817.1) 100.00%. Other closely related sequences exhibited percentage similarity of 100.00%, 100.00% and 100.00% with *Aspergillus* sp. (GeneBank accession MT597434.1) *Aspergillus* sp. (GeneBank accession #MT550026.1), and *Aspergillus* sp. (GeneBank accession # MT550028.1) respectively. Based on the initial BLAST results, phylogenetic analysis was carried out using sequences from Zakaria, (2018). All of the sequences were trimmed and assembled using Bioedit Software. Muscle alignment tool was used to align the sequence using MEGAX software.

All of the obtained sequences were trimmed to their conserved regions. 5‟…GAT- [CATTA……GACCT]-CAAAT…3‟ as published by Dentinger et al., (2011) and the alignment portion amongst them were added in the analysis. For phylogeny construction gaps were treated as missing information.

There were 15 sequences in the final dataset. Out of these, 13 of these sequences related to ingroup taxa that were members of 13 taxa of genera, while *Penicillium chrysogenum* was used as outgroup following Zakaria, (2018). Phylogenetic relationship was formed by using Neighbor-Joining and Maximum Likelihood method, Jukes and cantor (1969) model of ITS sequences and nearest neighbor-interchange (NNI) as ML heuristic search method using MEGAX software (Tamura et al., 2011) and tested as 1000 replicates of bootstrap value and bootstrap values >70% were shown in the phylogenetic tree.

The phylogenetic analysis used a total of 552 sites after trimming and removing the incomplete letters. Of these, 469 positions were conserved, 61 were variable positions, singleton positions were none, and 61 were parsimony informative sites.

Our sequences clustered with sequences of *Aspergillus niger* from Genbank with strong bootstrap support (100%). Therefore, our species identified as *Aspergillus niger*. In the phylogenetic tree, accession numbers for the used sequences are given. Sequences assigned for this investigation were deposited in the Genbank and are

pending. In the phylogenetic tree, the sequences of sample species are highlighted and denoted by solid square boxes.

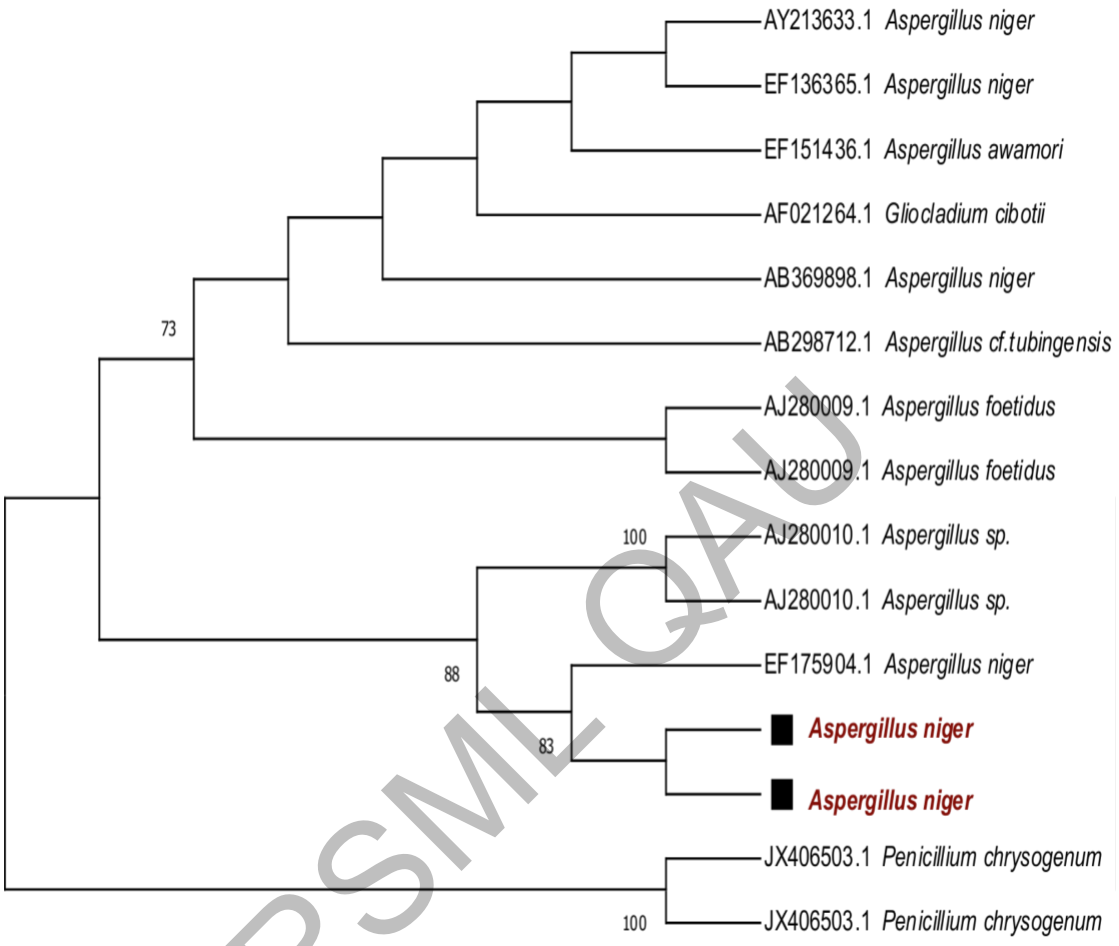


Fig 3.8: Phylogenetic analysis/relationship of *Aspergillus niger* sp. was determined by Maximum Likelihood method using MEGAX software. *Aspergillus niger* sp. shown in a different color in the phylogenetic tree.

3.2.2.4 Molecular analyses of *Pseudofusicoccum adansoniae*

Targeted rDNA-ITS1, 5.8 and IT2 regions produced DNA fragments of 610 bp after amplification with specific primers ITS1F and ITS4. Initial Blast analysis indicated that the sequences showed maximum similarity with *Pseudofusicoccum* sp. (GeneBank accession # MH863169.1) 100.00%. Other closely related sequences exhibited percentage similarity of 100.00%, 100.00% and 100.00% with *Pseudofusicoccum* sp. (GeneBank accession # MK480513.1 *Pseudofusicoccum* sp. (GeneBank accession # MK480514.1), and *Pseudofusicoccum* sp. (GeneBank accession # MK480511.1) respectively. Based on the initial BLAST results, phylogenetic analysis was carried out using sequences from Senwanna et al., (2020). All of the sequences were trimmed and assembled using Bioedit Software. Muscle alignment tool was used to align the sequence using MEGAX software.

All of the obtained sequences were trimmed to their conserved regions. 5"…GAT- [CATTA……GACCT]-CAAAT…3" as published by Dentinger et al., (2011) and the alignment portion amongst them were added in the analysis. For phylogeny construction gaps were treated as missing information.

There were 19 sequences in the final dataset. Out of these, 17 of these sequences related to ingroup taxa that were members of 17 taxa of genera, while *Botryosphaeria dothidea* was used as outgroup following Senwanna et al., (2020). Phylogenetic relationship was formed by using Neighbor-Joining and Maximum Likelihood method, Jukes and cantor (1969) model of ITS sequences and nearest neighbor-interchange (NNI) as ML heuristic search method using MEGAX software (Tamura et al., 2011) and tested as 1000 replicates of bootstrap value and bootstrap values >70% were shown in the phylogenetic tree.

The phylogenetic analysis used a total of 552 sites after trimming and removing the incomplete letters. Of these, 782 positions were conserved, 45 were variable positions, 7 were singleton positions, and 38 were parsimony informative sites.

Our sequences clustered with sequences of *Pseudofusicoccum adansoniae* from Genbank with strong bootstrap support (100%). Therefore, our species identified as *Pseudofusicoccum adansoniae*. In the phylogenetic tree, accession numbers for the

used sequences are given. sequences assigned for this investigation were deposited in the Genbank and are pending. In the phylogenetic tree, the sequences of sample species are denoted by solid square boxes.

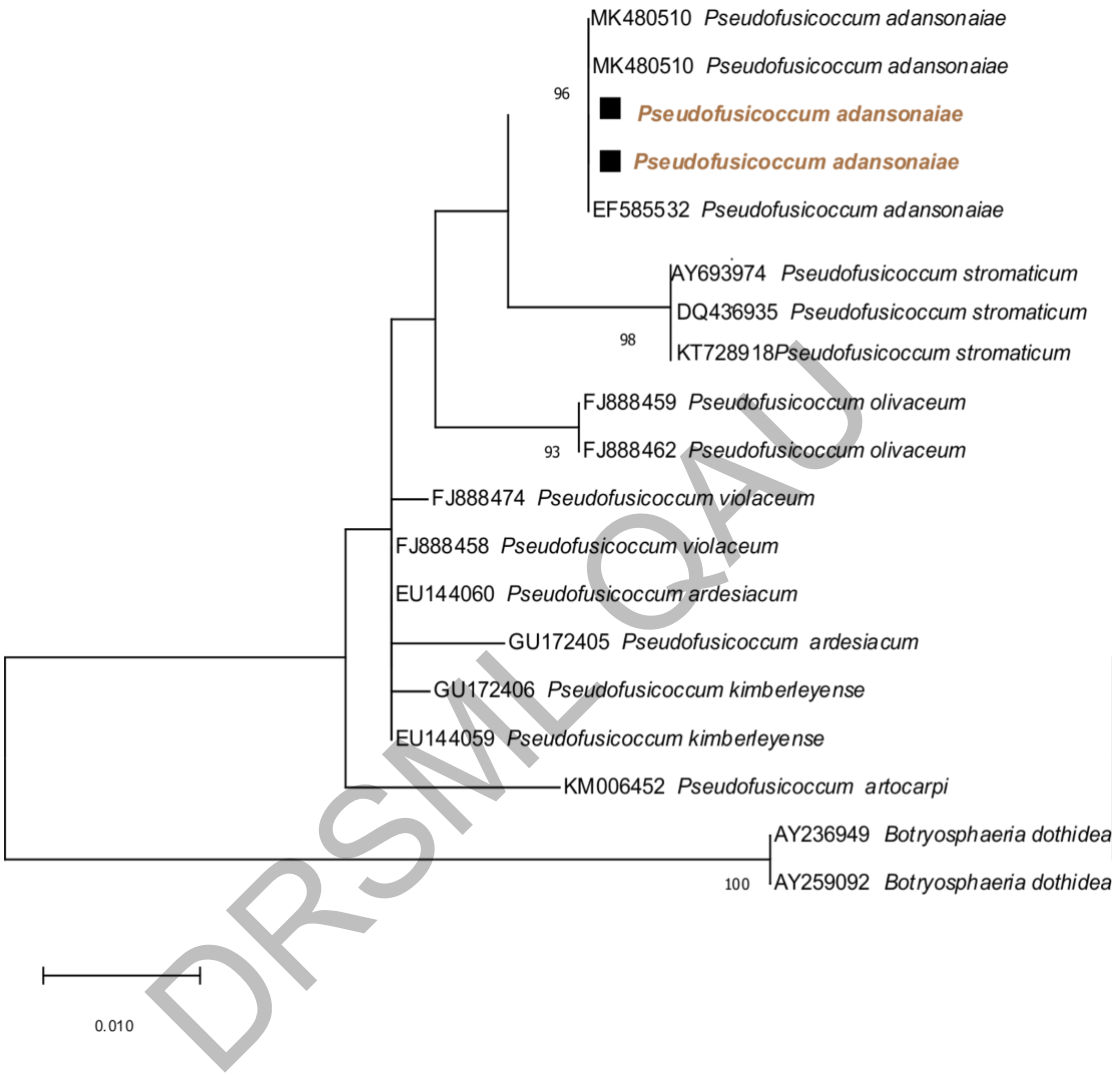


Fig 3.10: Phylogenetic analysis/relationship of *Pseudofusicoccum adansoniae* sp. was determined by Maximum Likelihood method using MEGAX software. *Pseudofusicoccum adansoniae* sp. shown in a different color in the phylogenetic tree.

CHAPTER 3 PART III

RESULTS

EVALUATION OF BIOLOGICAL POTENTIAL OF ENDOPHYTIC FUNGI

Result Part III

3.3.1 Determination of Total Flavonoid Content

To determine total flavonoid content, fungal bioactive secondary metabolites were used. The result data shows that fungal sample HT2 have high flavonoid content among all the fungal samples. i.e., 22 µg Quercetine equivalent per mg of extract which was taken as standard. The flavonoid extract average concerning quercetine was found to be 8.03, 23.2,8.03 and 7.73 (µg quercetine equivalent/ml of extract) for HT1, HT2, TA3 and TA4 respectively (as shown in fig 3.5).

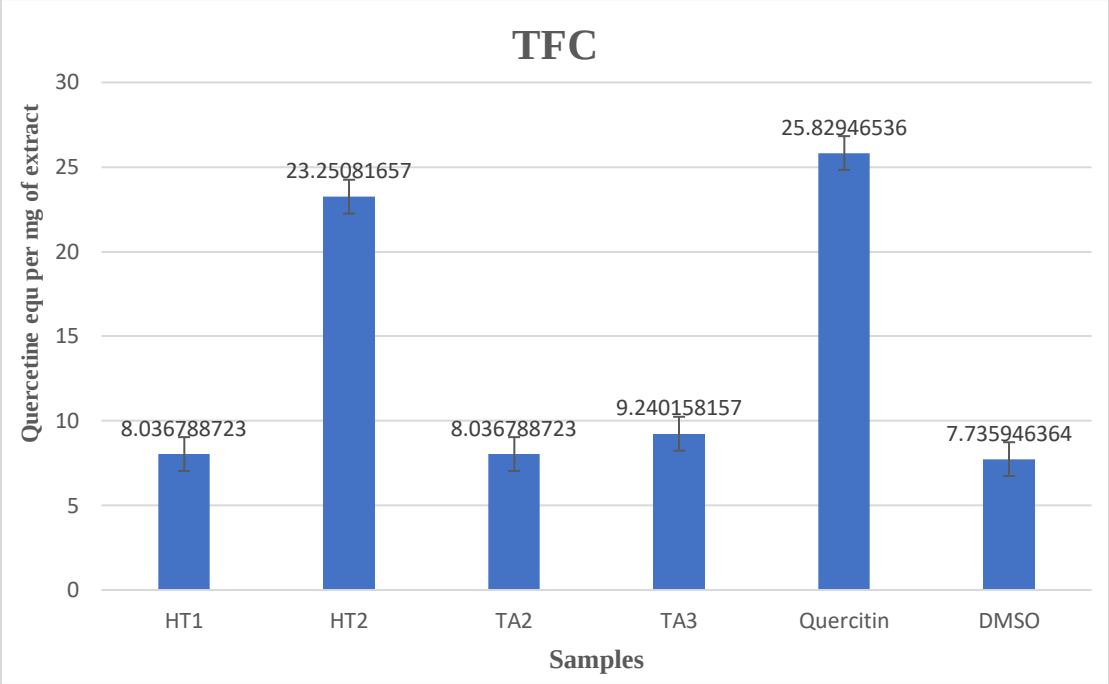


Fig 3.11: Graphical representation of total flavonoid content of isolated fungal samples

3.3.2 Determination of Total Phenolic Content

To determine total phenolic content, fungal bioactive secondary metabolites were used. The result data shows that fungal sample TA2 have high flavonoid content, as shown in figure (3.6), among all the fungal samples. i.e., 22 µg Gallic acid equivalent per mg of extract which was taken as standard. The phenolic extract average concerning gallic acid was found to be 66.9, 87.9, 138.6, and 89.1 (µg gallic acid equivalent/ml of extract) for HT1, HT2, TA3 and TA4 respectively.

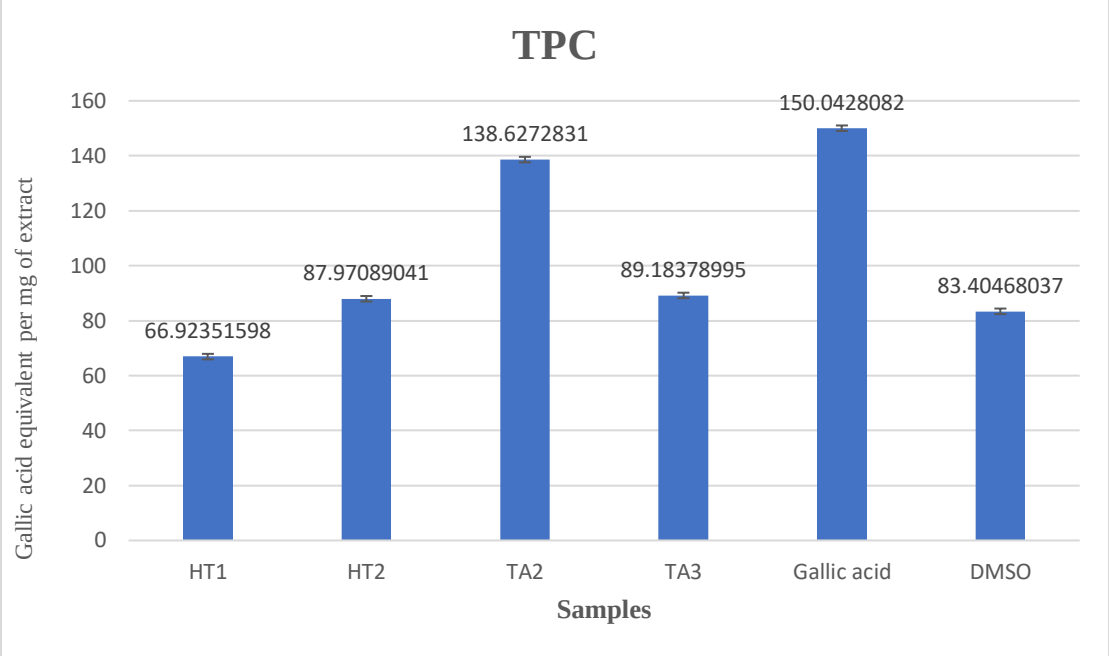


Fig 3.12: Graphical representation of total phenolic content of isolated fungal samples

3.3.3 Antioxidative activity assay (DPPH Assay)

For the purpose of determining the DPPH free radical scavenging capacities, we tested crude extract of fungal secondary metabolites extracted from the leaves and twigs of *Prunus armeniaca* extracted with organic solvent ethyl acetate. The extracts were shown in table. By calculating half maximal inhibitory concentration (IC50 value) results were evaluated. Graphical representation of IC50 value is shown in fig 3.7. As a standard, Ascorbic acid was used because of its strong antioxidant activity. Results were obtained in triplicated and were presented as mean ± Standard Deviation (SD) and for further processing Microsoft Excel was used. On the basis of DPPH absorbance IC50 value was measured. HT1 and TA3 have very strong antioxidant activity. TA2 also shows strong antioxidant activity while HT2 showed mild antioxidant activity. Results shows that HT1 and HT2 have higher free radical scavenging capacity.

Table 4: IC50 Value of samples

	Concentrations µg/ml				
Sample	**20**	**15**	**10**	**5**	**IC50**
HT1	21.63406	22.66974	26.63982	21.05869	1.2
HT2	25.51438	23.24511	22.6122	27.04258	>500
TA2	37.11162	25.83429	24.74108	22.43959	144.4
TA3	26.81243	24.91369	24.39586	25.60759	1.06

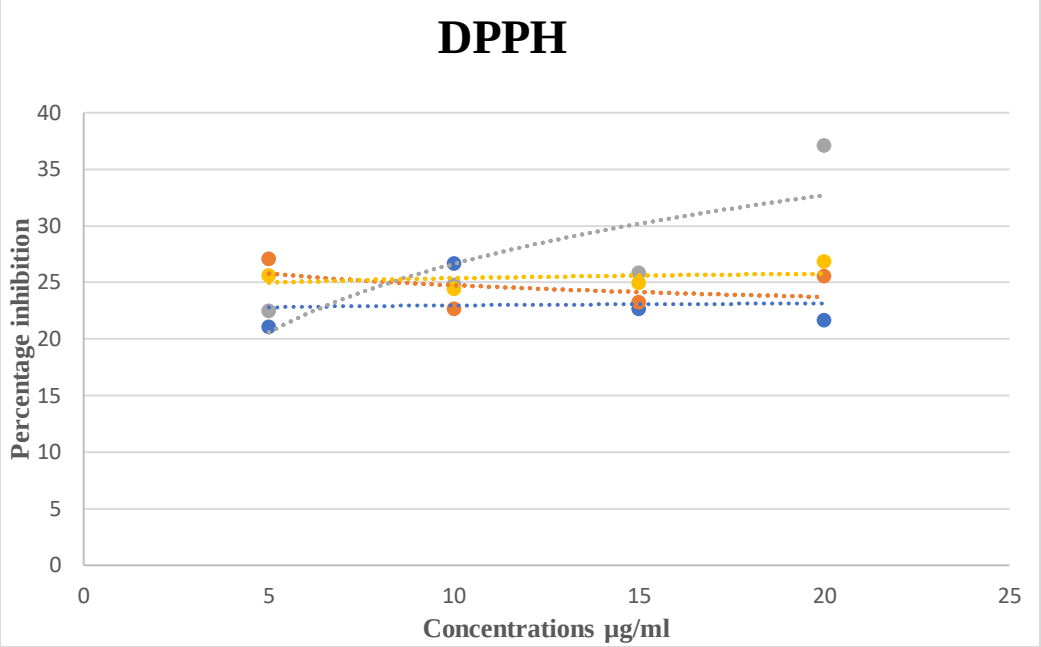


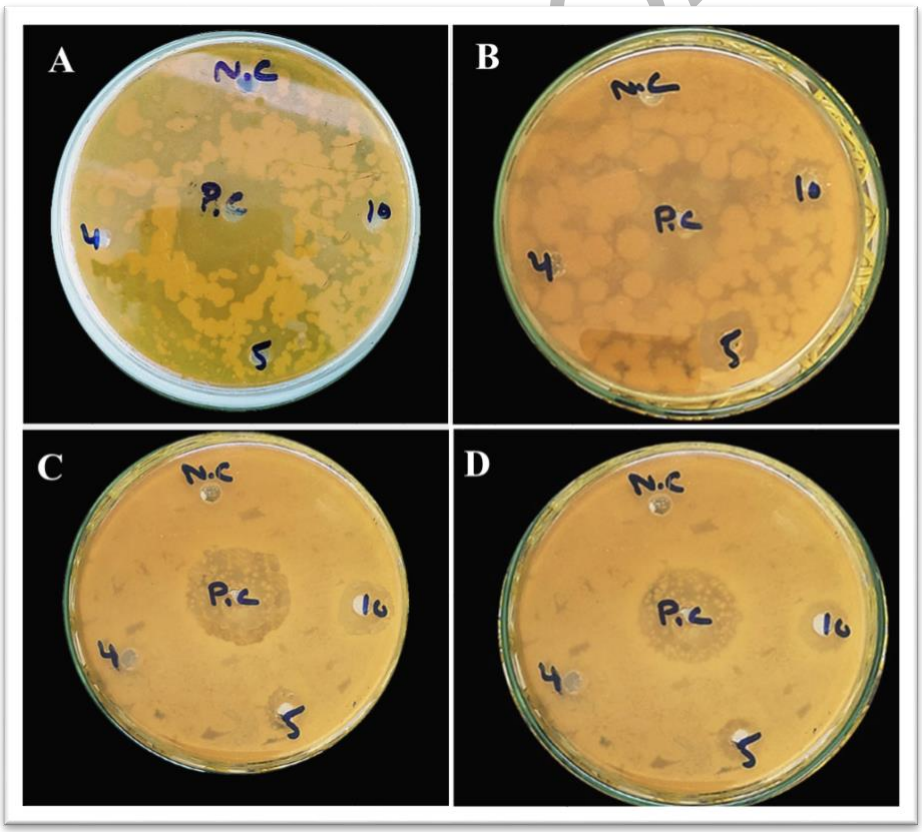
Fig 3.13: Different Logarithmic representation of IC50 value of DPPH assay

3.3.4 Anti-bacterial Activity of Crude extract

Crude extract of fungal samples was tested against five different bacteria at three different concentrations of 10,5, and 4 mg/ml by agar well diffusion method. 20µl of three concentrations was added in wells and zone of inhibition was measured after 24 hours as shown in figure 3.8. DMSO was taken as negative control which shows 0mm zone of inhibition and Tetracycline was taken as positive control. The antibacterial activity results of fungal samples are shown in below table.

Table 5: Determination of Antibacterial assay of fungal extracts

S.no.	***Bacteria***	***Fungal Extracts (5mg/ml)***				***+control***
		HT1	**HT2**	**TA2**	**TA3**	
1	*Pseudomonas aeruginosa*	15.1±0.2	13.1±0.2	-	-	25.2±0.25
2	*Escherichia coli*	15.5±0.1	13.2±0.2	-	-	25.3±0.3
3	*Klebsiella pneumonia*	-	-	20.2±0.2	20.1±0.2	40.1±0.1
4	*Bacillus subtilis*	-	20.2±0.2	-	-	40.7±1.07
5	*Staphylococcus epidermis*	20.3±0.3	-	-	5.3±0.3	20.3±0.3



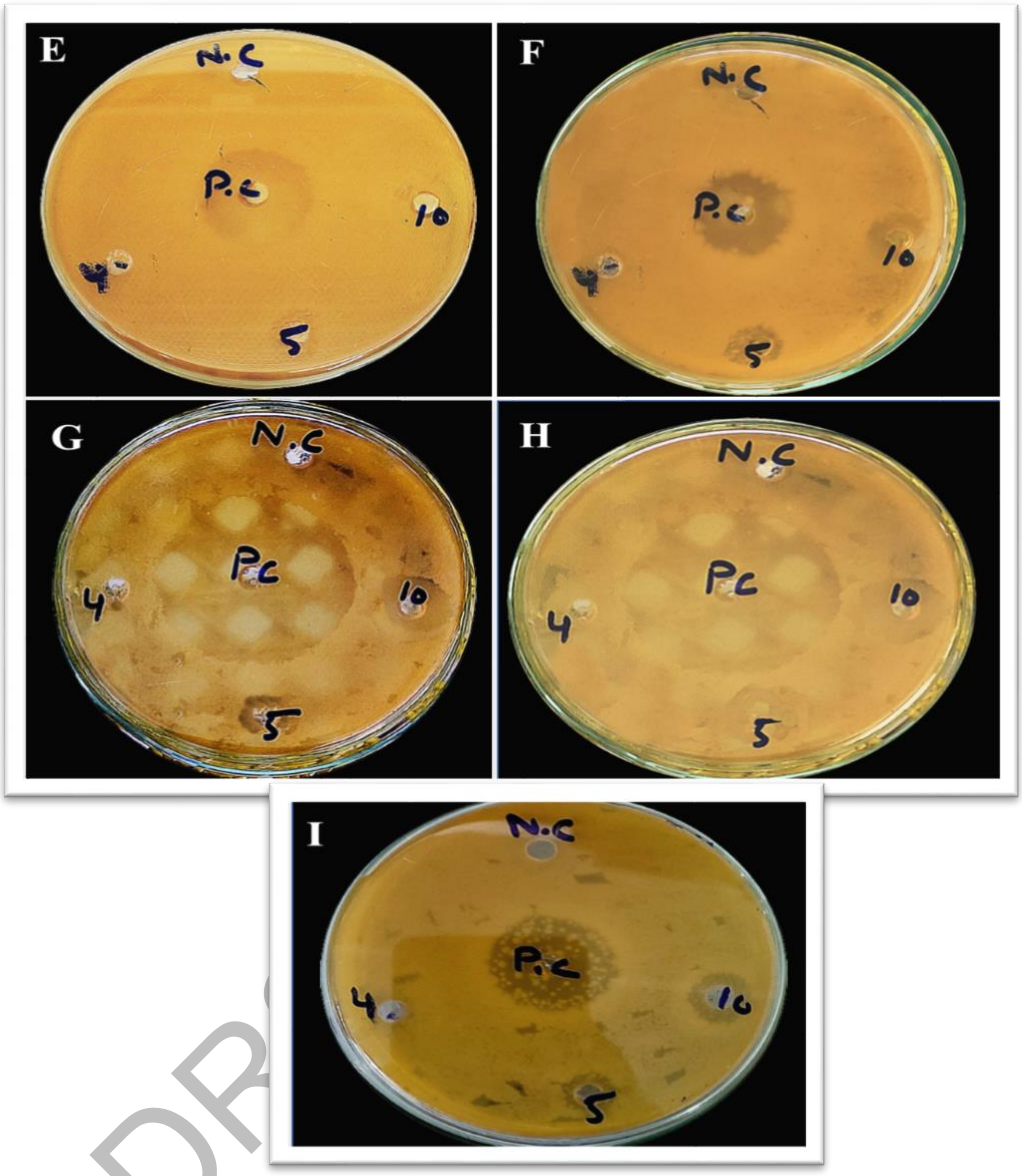


Fig 3.14: Anti-bacterial activity against: ***E. coli*** ***(A)*** *T. dimorphus,* ***(B)****A.alternata.* ***Pseudomonas aeruginosa*** **(C)** *T. dimorphus,* ***(D)*** *A.alternata*. ***Staphylococcus epidermis*** **(E)** *T. dimorphus*, **(F)** *P.adansoniae*. ***Klebsiella pneumonia*** **(G)** *A.niger,* **(H)** *P.adansoniae*. ***Bacillus subtilis*** **(I)** *A.alternata*

CHAPTER 4

DISCUSSION

4 Discussion

This research project's main goal is to isolate endophytic fungi of *Prunus armeniaca*, Quaid-i-Azam University, Islamabad, and evaluate the biological potential of isolated endophytes by using their crude extract, one of the least-studied areas that has received virtually no attention in terms of endophytic fungi. Plant specimens were collected for the study from Quaid-i-Azam University, Islamabad during the monsoon season (July to September 2020). The characterization of fungi has used a variety of identification tools (Hibbett et al., 2007; Yang, 2011; Feng et al., 2012; Khaund and Joshi, 2014) because mycologists do not solely rely on morphological characterization for accurate identification up to species level assortment. Therefore, for accurate and valid identification down to the lowest taxonomic level, both molecular and morphological characterization are required. In present study, four endophytic fungi were isolated from *Prunus armeniaca*, all endophytic fungi were processed by both tools which have been reduced to four different species.

For molecular characterization, genomic DNA of these four specimens were isolated with 2% CTAB method and then amplified rDNA-ITS conserved regions with fungal specific primers ITS1F and ITS4 (Gardes and Bruns, 1993; White et al., 1990; Zhao et al., 2011). The PCR products were amplified, then purified using a QIAquick purification kit (Khaundi and Joshi, 2014; Hussain et al., 2018) and sequenced using an ITS1F primer from Microgen in South Korea. All the sequences were then analyzed and trimmed from conserved sites i.e. 5‟ GAT[CATTA…..GACCT]CAAAT 3‟using Bioedit software and then BLAST at NCBI GeneBank database for sequence similarity and for phylogenetic tree construction using MEGAX software (Tamura et al., 2013; Saba and Khalid, 2014).

From combined morphological and molecular characterization, the four species were identified belonging to three order, three families and four genera. The species belongs to genus Talaromyces, Pseudofusicoccum, Aalternaria and Aspergillus.

From phylogenetic analysis 2 species *Talaromyces dimorphus* and *Pseudofusicoccum adansoniae* are new records for Pakistan.

Genus **Talaromyces** C.R. Benj. The species presenting symmetrical biverticillate penicilli, acerose phialides, with mycelium displaying yellow, orange, pink, or red colors and the ascocarps, when present, being gymnothecial, had been incorporated into the Penicillium section Biverticillata-Symmetrica by Raper and Thom. Pitt (1979) distinguished between the anamorphic and teleomorphic states using the dual nomenclature criteria, and it classified the species that only displayed the anamorphic condition to the Penicillium subgenus Biverticillium and the species that displayed the teleomorphic condition to the genus Talaromyces. The Melbourne nomenclatural code recently chose to use a single name for a single species instead of the previous dual naming method (McNeill., 2012). Talaromyces was finally approved as the genus name for these species.

The worldwide genus Talaromyces C.R. Benj. can be found in a variety of habitats, including soil, air, live or decaying plants, and indoors. Both its advantageous and harmful impacts on people have been extensively studied. Based on a polyphasic species idea, a description of Talaromyces was provided, divided into seven sections: Bacillispori, Helici, Islandici, Purpurei, Subinflati, Talaromyces, and Trachyspermi.There are more than 75 known species of Talaromyces, which makes up the majority of the genus.

One taxon of genus Talaromyces described during this study is *Talaromyces dimorphus*. According to phylogenetic analyses, using dataset from Visagie, et al., (2018), our sequences clustered with *Talaromyces dimorphus* with strong bootstrap value (100%). Therefore, the taxon is named as *Talaromyces dimorphus.*

Morphological characters of *Talaromyces dimorphus* are colonies thin, plane, irregular, sulcate, colored Pistachio Green (R. Pl. XLI) with white margins and reverse Apple Green (XVII) at center, Zinc Orange elsewhere with white margins (Jiang et al., 2018). Conidiophores biverticillate and monoverticillate, stipe smooth-walled, metulae 4–6 per vertical, phialides 3–4 per verticil, ampulliform, conidia smooth-walled, ovoid to ellipsoidal. The unique epithet is derived from the fact that this specie frequently produces both biverticillate and monoverticillate penicillin.

Pseudofusicoccum Mohali. Botryosphaeriales species are found on a variety of different host plants and are widely distributed around the world especially in tropical and moist regions (Phillips et al. 2013, 2019; Slippers et al. 2017). Worldwide distribution of the Dothideomycete order Botryosphaeriales, which includes the families Aplosporellaceae, Botryosphaeriaceae, Melanopsaceae, Phyllostictaceae, Planistromellaceae and Saccharataceae, has been documented (Phillips et al. 2019).

Pseudofusicoccum is currently included in Phyllostictaceae and there are eight species that have been identified. (Dissanayake et al. 2016; Jami et al. 2018). This genus is distinguished by hyaline, aseptate, cylindrical to ellipsoid conidia that are encased in a mucilaginous sheath, as well as submerged to superficial pycnidial conidiomata (Pavlic et al. 2008; Yang et al. 2017). In Botryosphaeriales, numerous sexual and asexual taxa have been introduced; however, sexual morphs of Pseudofusicoccum species have never been recorded. (Pavlic et al. 2008; Mehl et al. 2011; Doilom et al. 2015).

One taxon of genus Pseudofusicoccum described during this study is *Pseudofusicoccum adansoniae.* According to phylogenetic analyses, using dataset from Senwanna et al., (2020), our sequences clustered with *Pseudofusicoccum adansoniae* with strong bootstrap value (100%). Therefore, the taxon is named as *Pseudofusicoccum adansoniae* and it was first reported from Australia and is a new record from Pakistan.

Pseudofusicoccum adansoniae colonies on PDA at 32°C, raised**,** irregular with lobate margins. Initially whitish colonies with a relatively dense mycelial mat; Reverse colony is white in color with Ivy Green (R. PI *XXXI*) in the center of colony. Submerged mycelium turns Cress Green (R. PI *XXXI*) to Light Grayish Olive (R. PI *XLVI*). Aerial mycelium are dense with cottony texture. Colonies become more cottony with age. Conidia ellipsoidal, aseptate, hyaline, straight or slightly bent with rounded tips, smooth walls, fine granular substance, and a thin mucilaginous coating around it. Conidiogenous Cells holoblastic, cylindrical or rod shaped, hyaline, smooth walled. It was first reported from Australia (Pavlic et al., 2008) and is a new record for Pakistan.

Alternaria Nees. One of the most widespread genera of saprophytic fungi on the earth is Alternaria. It consists of a large number of species that lead necrotrophic, phytopathogenic lives. Several species have been clinically linked to allergic respiratory illnesses, despite the fact that they rarely infect people with invasive infections. Last but not least, Alternaria species are among the best-known makers of a variety of secondary metabolites produced by fungi, including toxins. More than 60 morphologically distinct or host-specific species can be categorized in this area, including the type species of the genus Alternaria, *A. alternata.* (Woudenberg et al. 2013).

One taxon of genus Alternaria described during this study is *Alternaria alternata.* According to phylogenetic analyses, using dataset from Aung, et al., (2020), our sequences clustered with *Alternaria alternata* with strong bootstrap value (100%). Therefore, the taxon is named as *Alternaria alternata.*

Morphological characters of *Alternaria alternata* are colonies circular, convex with entire margins, sulcate, velvety in texture. Dark Greenish olive (R. Pl. XXX) and chromium green (R. Pl. XXXII) and white on the margins displaying Dusky Green-Gray (R. Pl. LII) and white aerial mycelium: Reverse Dusky Gray-Green with white margins. Conidia light to dark brown in color, obclavate and in short conical flask, typically 1 to 9 transverse septa and 0 to 3 longitudinal septa, which were seen between 5 and 8 days old. Conidiophores producing 4–10 units catenulate conidia (Zhang et al.,2023).

Aspergillus P. Micheli**.** The diverse genus Aspergillus has a significant impact on society and the economy. Species are found all over the world in a variety of habitats, and they are well recognized for producing mycotoxins, spoiling food, and commonly being reported as human and animal infections. In addition, numerous species are employed in biotechnology to create a variety of secondary metabolites, including antibiotics, medications, and enzymes, as well as to act as catalysts in numerous food fermentations.

Aspergillus has traditionally been categorized and identified using phenotypic characteristics, however in recent years molecular and chemotaxonomic

characterization has had a significant impact. Aspergillus was first given the name by Micheli (1729), and the genus was established by Haller (1768). Aspergillus' infrageneric division has traditionally been based on morphology. In 1965, Raper and Fennell classified the genus into 18 groups. Gams et al. (1986) introduced names of subgenera and sections in Aspergillus because the division into groups does not have any nomenclatural status. Four subgenera—Aspergillus, Circumdati, Fumigati, and Nidulantes—and 20 sections have currently been proposed by Houbraken et al. (2014) and Hubka et al. (2014).

One taxon of genus Aspergillus described during this study is *Aspergillus niger*. According to phylogenetic analyses, using dataset from Zakaria, (2018), our sequences clustered with *Aspergillus niger* with strong bootstrap value (100%). Therefore, the taxon is named as *Aspergillus niger*.

Morphological characters of *Aspergillus niger* are colonies, circular, flat with entire margins, powdery in texture. Blackish white colony; Reverse Glass Green (R. Pl. XXXI) with black spotting. Conidial Head Biseriate and radiated, hyaline, Vesicles globose. Stipe walls are thick and smooth. Conidia brown, rough walled, globose to sub-globose.

Species belonging to the Aspergillus section Nigri occupy a wide range of habitats in both animal and plant settings (Gams et al. 1986). They are commercially significant as both harmful and advantageous microbes. At many stages, such as those before and after harvest, during processing, and during handling, they might contaminate meals and feeds.

Numerous bioactive metabolites have been extracted from the endophytes of medicinal plants and structurally described using a variety of sophisticated, and traditional techniques. Some of these substances have practical uses in medicine, agriculture, and pharmaceuticals. The majority of them have given researchers a starting point for future work on synthesizing and formulating bioactive chemicals into effective medicines with wide-ranging uses in the health care system and many other areas of human existence.

The study comprises many biological assays in which crude extracts of endophytes were screened to assess their potential as antibacterial, to determine their total phenolic content, total flavonoid content, and DPPH assay (% scavenging of free radicals).

Metabolites with antibiotic activity, known as antimicrobial compounds, are extracted from endophytes. These are naturally occurring, low molecular weight compounds that exhibit activity against other microbes even at low doses (Guo et al., 2008). The crude extracts of Isolated fungal strains were investigated for their antimicrobial activities. The crude extracts were investigated against seven human pathogenic bacterial strains in antibacterial activity assay which are as following:

Gram positive bacteria: *Bacillus subtilis, Staphylococcus epidermis*

Gram negative bacteria: *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa*

The ethyl acetate crude extract of *Talaromyces dimorphus* displayed a strong antibacterial activity against gram-positive and gram-negative bacteria except *Klebsiella pneumonia.* The minimum inhibitory activity of the EA crude extract was recorded against *P.aeruginosa* with inhibition zone of 15.1±0.2 mm followed by *S. epidermidis* 20.3±0.3mm and E. coli 15.5±0.1 mm. According to Cai et al., (2020), fungus *Talaromyces assiutensis* showed strong inhibitory activity against pathogenic bacteria *E. coli* and *S. epidermidis*.

The EA crude extract of *Alternaria alternata* displayed a strong antibacterial activity against gram-positive and gram-negative bacteria except *Klebsiella pneumonia.* The minimum inhibitory activity of the EA crude extract was recorded against *Pseudomonas aeruginosa* with inhibition zone of 13.1±0.2 mm followed by E. coli 13.2±0.2mm and B. subtilis 5.3±0.26mm. Elghaffar et al., (2022) reported fungal endophyte *Alternaria alternata*, from leaves of *Ziziphus spina-christi*, displayed strong antibacterial activity towards gram-positive and gram-negative bacteria. The ethyl acetate crude extract of *Pseudofusicoccum adansoniae* displayed mild antibacterial activity against gram-positive bacteria *S. epidermidis* with inhibition zone of 5.3±0.3.

Abba et al., (2018) recorded that *Pseudofusicoccum* sp isolated from *Annona muricata* showed no antibacterial activity against *Bacillus subtilis*. The ethyl acetate crude extract of *Aspergillus niger* only displayed antibacterial activity against gram-positive bacteria *Klebsiella pneumonia* with inhibition zone of 15.1±0.15.

Our body's first line of defense against potential damage brought on by the production of free radicals is antioxidants. At least 50 diseases have been linked to the pathophysiology of free radicals. Prior to attacking the cells, antioxidants neutralize or stabilize these free radicals. In current study, fungal isolates tested for DPPH free radical scavenging activity showed good results. *Talaromyces dimorphus* and *Pseudofusicoccum adansoniae* have very strong antioxidant activity with IC50 value of 1.2 and 1.06 respectively. *Alternaria alternata* and *Aspergillus niger* with IC50 of 144.5 and >500 respectively which shows them a weak antioxidant.

Phenols are high molecular weight substances that function as either antioxidants or substrates for oxidation processes (Tsimidou, 1998). Ruma et al. (2013) reported total phenolic content of the extracts of two species of fungi i.e., *Aspergillus fumigatus* (isolated from plant Garcinia species) with values such as 100.2 GAE/mg of extracts. In the present study, extracts of one fungal isolate *Aspergillus niger* showed the highest activity among others 138.6GAE/mg of extract, respectively. *T.dimorphus, A.alternata* and *P.adansoniae* showed total phenolic content of 66.9, 87.9 and 89GAE/mg.

With regard to antioxidant action and stabilizing lipid oxidation, flavonoid molecules play a crucial role (Maisuthisakul et al.,2007). Alternaria sp., an endophytic fungus, it was discovered that the same fungal species isolated from both the leaf and stem of Crotalaria pallida possessed 7.32 and 4.59 mg of flavonoid chemicals, respectively. In current study, endophytic fungi HT1, HT2, TA2 and TA3 isolated from *Prunus armeniaca* leaves and twigs had total flavonoid content of 23.5, 9.2. 8.03 and 8.03 µg/mg Quercetin equivalent.

CONCLUSION

Endophytes are regarded as abundant sources of organic bioactive substances. These pharmacological substances are proving to be valuable resources for use in a variety of fields, including medicine, agriculture, and industry. They offer a wide range of applications and potentials. The development of new bioactive metabolites is ongoing since secondary metabolites serve as the basis for around 40% of pharmaceuticals. In order to limit the invasion of diseases, plants may create antimicrobial substances. These substances can also be created by plant endophytes that live in harmony with their hosts. Screening of such plants for the isolation of important and novel endophytes especially from medicinal plants could lead to the discovery of possible therapeutic compounds. Our current endophytes' crude extracts were biologically examined and produced positive results; as a result, they may be regarded as a good source of undiscovered important metabolites for future research. We report that the crude extracts of our endophytes contain substances with antibacterial and antioxidant properties.

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