EXPLORATION OF ENDOPHYTIC FUNGI AND THEIR BIOACTIVE POTENTIAL ASSOCIATED WITH *PUNICA GRANATUM*

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A thesis submitted to the Department of Mathematics & Natural Sciences in partial fulfillment of the requirements for the degree of
Masters of Science in Biotechnology

Department of Mathematics & Natural Sciences
Brac University
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Declaration

It is hereby declared that

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Ethics Statement

No human or animal model was used in this study.

Abstract

Endophytic fungi are a group of fungi that reside in the living plant without causing any symptoms. It shows symbiotic/mutualistic characteristics with host plants. Endophytic fungi may increase the host defense system against various pathogenic microorganism and contains secondary metabolites of the respective host plant. Isolation of endophytic fungi and it's metabolites from *Punica Granatum* plant grown in Bangladesh is a recent and new approach. A total of six endophytic fungi were isolated and purified from the leaf and bark part of the plant. All of the isolated endophytic fungi namely DgB1, DgB3, DgL3, and DgL4 were identified up to genus level based on macroscopic and microscopic characteristics and up to species level based on molecular characteristics as *Diaporthe pseudomangiferae, Diaporthe pseudomangiferae*, *Phomopsis tersa, and Colletotrichum siamense* respectively. Preliminary chemical screening of the fungal extracts by thin layer chromatographic technique revealed the presence of various compounds such as flavonoids, anthraquinones, couramins, isocoumarins etc. An antimicrobial study was carried out against a wide range of both Gram-positive and Gram-negative bacteria by disc diffusion method, and testing of the antioxidant property was done by following DPPH scavenging method. Fungal strain DgL4 showed significant antioxidant activity of 21.08 µg/ml and DgB1 showed moderate antimicrobial activity. The extract DgL3 was later purified by solvent treatment with mixtures of n-Hexane, DCM, and methanol. Structural elucidation of the isolated compound was done through their spectral data (NMR). Overall, these findings indicate that the medicinal plants of Bangladesh and their associated endophytic fungi could be an ideal target for the discovery of potentially bioactive compounds or lead structures for new drugs.

Keywords: Endophytic Fungi; Pomegranate; Antioxidity; Fungal strains; Fungal extracts.

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- S.A.M. Salman Haque
  December 2022

Dedication

I dedicate this work to myself.

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Abbreviation

DMSO- Dimethyl Sulfoxide

EtOAc- Ethyl Acetate

EtOH- Ethanol

ASA- Ascorbic Acid

BHA- Butylated Hydroxy Anisole

IC50- Inhibitory Concentration 50%

PDA- Potato Dextrose Agar

NA- Nutrient Agar

DCM- Dichloromethane

TLC- Thin Layer Chromatography

PTLC- Preparative Thin Layer Chromatography

NMR- Nuclear Magnetic Resonance

Chapter 1

Introduction

1.1 Rationale of the study

Plant natural products (secondary metabolites) have been recognized as the most successful source of potential drug leads, but their recent implementation in drug discovery and development efforts has demonstrated a decline in interest (Mishra and Tiwari, 2011) The need for new compounds to provide mitigation and relief in diverse human clinical conditions is ever-growing. In addition, the challenges of bacterial drug resistance, and the emergence of highly virulent viruses and bacteria demand very drastic drug discovery approaches (Strobel *et al*., 2004). Antibiotic resistance is inevitable, but there are measures we can take to slow it down. Efforts are under way on several fronts - improving infection control, developing new antibiotics, and using drugs more appropriately.

The search for bioactive natural products with the potential of addressing these identified challenges is as old as man's existence and has always relied heavily on the study of whole plant tissues (morphological parts) such as the roots (bark and inner tissues), leaves, stem (bark and inner tissues) and animal sources (Dias *et al.*, 2012). Unfortunately, the use of whole plant or animal tissues for the isolation of bioactive natural products has some serious challenges. Firstly, the process leads to the destruction of the whole plant over time as the tissues are repeatedly collected without replenishment; most times, the existence of these plant species becomes threatened and they are even lost after some years. Closely associated with this remarkable challenge are the problems of environmental degradation, land spoilage, and the limited yield of the identified bioactive natural products. Secondly, the use of whole plant tissues in search of bioactive natural products has comparatively witnessed great wastage of research resources as a result of dereplication. These identified problems led to the loss of popularity in natural product research efforts.

At this point, there arose a dire need for a paradigm shift in the search for bioactive natural products. It appeared that the search for novel secondary metabolites should be refocused.

Endophytes were quickly recognized as veritable sources of novel bioactive natural products (Strobel and Daisy, 2003).

Endophytes are microorganisms colonizing healthy plant tissue without causing any apparent symptoms or noticeable injury to the host. Both fungi and bacteria are the most common microbes existing as endophytes and are to be found in virtually every plant on earth (Padhi et al., 2013). Endophytes have been viewed as outstanding sources of novel products because there are so many of them occupying literally millions of unique biological niches growing in so many unusual environments. In all these, endophytic fungi are exceptionally useful in the drug discovery process. Consequently, there is a need to awaken and boost research interest in the use of these endophytes for the discovery of potent bioactive molecules against the ever-increasing disease burden globally.

1.2 Natural products in drug discovery

The history of medicine dates back practically to the existence of human civilization. Historically, the majority of new drugs have been generated from natural products (secondary metabolites) and from compounds derived from natural products (Lahlou et al., 2007).

Natural products and their structural analogs have historically made a major contribution to pharmacotherapy, especially for cancer and infectious diseases (Atanasov et al., 2021). Before the 20th century, crude and semi-pure extracts of plants, animals, microbes and minerals represented the only medications available to treat human and domestic animal illnesses. The 20th century revolutionized the thinking in the use of drugs, as the receptor theory of drug action. The idea that the effect of the drug in the human body is mediated by specific interactions of the drug molecule with biological macromolecules (proteins or nucleic acids in most cases) led scientists to the conclusion that individual chemical compounds in extracts, rather than some mystical "power of life" are the factors required for the biological activity of the drug. This leads to the beginning of a totally new era in pharmacology, as pure, isolated chemicals, instead of extracts, became the standard treatments for diseases. Indeed, many bioactive compounds, responsible for the effects of crude extract drugs, and their chemical structure was elucidated. Classical examples of drug compounds discovered this way are morphine, the active agent in Opium, and digoxin, a heart stimulant originating from the flower Digitalis lanata. The evolution of synthetic chemistry also led to the chemical synthesis of many of the elucidated structures (Lahlou et al., 2013). The world's

best-known and most universally used medicinal agent is aspirin, which is related to salicin, and has its origins in the plant genera Salix sp. and Populus sp. (Strobel et al., 2004).

Even with untold centuries of human experience behind us and a movement into a modern era of chemistry and automation, it is still evident that natural product-based compounds have had an immense impact on modern medicine. For instance, about 40% of prescription drugs are based on them. Furthermore, well over 50% of the new chemical products registered by the FDA as anticancer agents, antimigraine agents, and antihypertensive agents were natural products or derivatives thereof in the time-frame of 1981-2002 (Newman et al., 2003). Excluding biologics, between 1989 and 1995, 60% of approved drugs and pre-new drug application candidates were of natural origin (Grabley et al., 1999). From 1983 to 1994, over 60% of all approved and pre-NDA stage cancer drugs were of natural origin, as were 78% of all newly approved antibacterial agents. Many other examples abound that illustrate the value and importance of natural products from plants and microorganisms in modern civilization, and Rutin which is extracted from *Punica Granatum* is an example of an important natural product that has made an enormous impact on medicine (Wani et al., 1971). The originally targeted diseases were ovarian and breast cancers, but now it is used to treat several other human tissue-proliferating diseases as well (Strobel et al., 2004).

Figure 1: Rutin

In recent times, natural products have been as widely used as chemical drugs against clinical diseases. Most chemical drugs that are widely used today were isolated from natural products, and thus natural products will continue to be important raw materials for the development of new drugs. However, since natural products are the byproducts of empirical medicine, they lack scientific

validation. Currently, various scientific experiments are being conducted to fill this gap by evaluating the efficacy of the natural product (Kim et al., 2016).

1.3 Secondary metabolites as natural products

Natural products are compounds that are produced by living systems and secondary metabolites are those which give particular species their characteristic features (Hanson et al., 2003). Secondary Metabolites are natural compounds that are not instantly concerned with the typical growth, development, or reproduction of an organism. Unlike most important metabolites, the absence of secondary metabolites does not influence on-the-spot loss of life, however alternatively in lengthy-time periods impairment of the organism's survivability, fecundity, or aesthetics, or probably in no significant alternate in any respect. Secondary metabolites are regularly restricted to a slender set of species within a phylogenetic group. Secondary metabolites most often play a principal position in plant safety towards herbivory and other interspecies defenses. People use secondary metabolites as drug treatments, flavorings, and recreational medicinal drugs. Natural products are chemical compounds or substances isolated from living organisms. Plant secondary metabolite can be found in the leaves, stem, root, or bark of the plant depending on the type of secondary metabolite that is been produced. secondary metabolite carries out a number of protective functions in the human body, it can boost the immune system, protect the body from free radicals, kill pathogenic germs and much more keep the body fit (Anulika N. P et al., 2016).

1.4 Plant as a potential source of bioactive secondary metabolites

Plant secondary metabolites are rich sources of bioactive compounds eliciting many beneficial health effects in man and animals. Plant-based foods, including vegetables, fruits, grains, seeds, nuts, and legumes, may contain hundreds of different phytochemicals. Recently, research on phytochemicals suggests their possibility as an important source of therapeutic and preventive agents against diseases (Zhao et al., 2015). 6 The four most common groups of plant-derived bioactive components are polyphenols, alkaloids, terpenes, and saponins. Some of them are found to act as defense compounds against diseases, predators, ultraviolet radiation, parasites, and oxidants, to facilitate the reproductive processes (e.g., serve as attractive smells and coloring agents), and for interspecies competition. Bioactive compounds of plant origin are those secondary metabolites possessing desired health/wellness benefit effects in man and animals (Kaur and Das

2011). Consistent evidence from epidemiological, in vitro, in vivo, and clinical studies has demonstrated that a diet rich in plant foods can reduce the risk of some degenerative diseases, such as diabetes, obesity, cardiovascular complications, and cancer. As an example, research studies have shown that about 20–50 % of all cases of cancer can be prevented by plant-based diets (Glade, 1999). Thus, dietary recommendations have always emphasized the consumption of various plant foods to reduce the risk of cancer and other chronic diseases. Many traditional plant-based medicines play an important role in health care. Phytochemicals are natural bioactive compounds found in vegetables, fruits, medicinal plants, and aromatic plants that are beneficial to treat diseases. The phytochemicals from natural products cover a diverse range of chemical entities such as polyphenols, proteins or peptides, polysaccharides, saponins, and vitamins (Zhang et al., 2018). Polyphenols are a large group of bioactive compounds that possess various activities such as antioxidant, anti-inflammatory, anticancer, anti-diabetic, and anti-microbial properties (Hung et al., 2012). Flavonoids (figure 1.3) are an important class of polyphenols isolated from natural plants that are beneficial to manage diabetics and associated complications (Arulselvan et al., 2014).

1.5 Endophyte and Endophytic Fungi

The term "endophyte" stems from the two Greek words; "endon" = within and "phyte" = plant. Thus, an endophyte is an organism surviving within a host plant without deleterious consequences. In 1809, a German botanist, Johann Heinrich Friedrich Link termed them as "Entophytae" which constituted moderately parasitic species living within plants in 1809. In 1991, Orlando Petrini re-termed these organisms as "endophytes". However, modern studies have shown that endophytes colonize plant tissues without detriment to the host and at most times, rather acts as a benefactor. An endophyte may be bacteria, fungi, algae, or oomycetes. Endophytes are an endosymbiotic group of microorganisms that colonize plants and microbes that can be readily isolated from any microbial or plant growth medium. They act as reservoirs of novel bioactive secondary metabolites, such as alkaloids, phenolic acids, quinones, steroids, saponins, tannins, and terpenoids that serve as a potential candidate for antimicrobial, anti-insect, anticancer, and many more properties (Gouda et al., 2016). Microbial endophytes have the ability to act as plant growth-promoting agents by producing phytohormones and also enable plants to grow in contaminated soils through the breakdown of hazardous compounds. Endophytes manage plant growth under

adverse conditions such as salinity, drought, temperature, heavy metal stress, and nutrient stress through different mechanisms (Eid, A.M *et al*., 2019). Endophytic fungi live inside plant tissues but don’t cause any disease symptoms. They are found in all plant species including deciduous trees, shrubs, marine algae, mosses, lichens, ferns, grasses, and palms. Endophytic fungi produce secondary metabolites that keep herbivores from eating the plant by making it poisonous or taste bad and they have other key roles in nutrient uptake, heat tolerance, plant evolution, and biodiversity. The existence of fungi inside plants has been known since the end of the 19th century. The endophytic fungus has been associated with plants for over 400 million years (Krings et al., 2007). Endophytic fungi are now considered as an important component of biodiversity (Arnold, 2005). In one survey it is estimated that over one million fungal endophytes exist in nature (Petrini, 1991). Endophytic fungus is an important source of novel, potential and active metabolites (Dreyfuss and Chapela, 1994). Bioactive compounds produced by endophytic fungus main function are to help the host plants to resist external biotic and abiotic stress, which benefits the host's survival in return. Plants lack an immune response to certain pathogens, but the endophytes that reside inside the plant tissue enhance the immune response of 9 the plants to fight against invading pathogens (Melotto et al., 2008). Plant endophytic fungus has the ability to produce the same or similar kind of compounds of from their originated host plants. Fungal endophyte communities differ in species composition, host and tissue preference worldwide distribution and differ in the polyketide and synthesis and production of bioactive compounds (Hoffman and Arnold, 2008). Endophytic fungi are considered as an important component of biodiversity.

1.6 Important Role of endophytic Fungi and its Application

There is sufficient evidence that endophytic fungi play important role in host plant physiology. They receive nutrition, protection, and propagation opportunities from their hosts (Clay and SchardI, 2002; Thrower and Lewis, 1973) and host plants also benefited from this symbiosis. Endophytes protect their host from insects, pests, and herbivores, and help their host to adapt in different stress conditions (Knop, 2007; Clay, 2005; Clay and Schardt, 2002; Malinowski and Belesky, 2006; Bonnet and Veisseire, 2000). However, endophytes also act as opportunistic under some conditions (Saikkonen, 1998; Faeth, 2004).

Plants-bearing endophytic fungi protect themselves from pathogenic microorganisms either by direct interaction with organisms or by inducing systemic resistance in the host. Some of the roles

of endophytic fungi and the application of endophytic fungi in the field of biotechnology and pharmacy are discussed below:

- **Physiological role**: Endophytic fungi protect host plants from herbivores, insects, and pathogen and act as plants' defensive mechanism by producing active secondary metabolites. It was observed that symbiotic plants activate the defense system more quickly than non-symbiotic plants after a pathogen challenge (Rodriguez, 2004). Endophytic fungus *Phomopsis oblonga* protect elm trees against the beetle *Physocnemum brevilineu* (Webber, 1981).
- **Environmental Role**: Endophytes are found to play an important role in the ecological community, to decrease the extent of environmental degradation, loss of biodiversity, and spoilage of land and water caused by excessive toxic organic insecticide, industrial sewage, and poisonous gases. Biological control using endophytes as a new efficient method is becoming widely used in environmental remediation, and in killing insects or pathogens.
- **Biotechnological Application:** Endophytes have a high ability to produce various novel and known enzymes that could be used in various biotechnological applications. These microorganisms can produce many enzymes, so they could be used as biocatalysts in the chemical transformation of natural products and drugs, due to their ability to modify chemical structures with a high degree of stereospecificity and to produce known or novel enzymes that facilitate the production of compounds of interest.
- **Pharmaceutical Application**: Endophytic fungi have been proven useful for novel drug discovery as suggested by the chemical diversity of their secondary metabolites. Many endophytic fungi have been reported to produce novel antibacterial, antifungal, antiviral, anti-inflammatory, antitumor, and other compounds belonging to the alkaloids, steroid, flavenoid, and terpenoids derivatives, and other structure types (Yu *et al*., 2010). The pharmaceutical and medical concerns of new drugs are the toxicity of these prospective drugs to human tissues. Since the plant tissue where the endophytes exist is a eukaryotic system, it would appear that the secondary metabolites produced by the endophytes may have reduced cell toxicity.

1.7 Bioactive Compounds from Endophytic Fungi

Endophytic fungi are also believed to be a potential source of novel bioactive compounds. A number of substances of pharmaceutical importance have been isolated and identified from fungi. Attempts are being made to isolate and identify bioactive metabolites from endophytic fungi (Strobel *et al.,* 2004). Endophytic fungi have been screened for antibiotics, antiviral and anticancer agents, antioxidants, insecticidal, and immunomodulatory compounds (Tan and Zou, 2001).

1.7.1 Anticancer Agents

Endophytic fungi have been studied as a source of anticancer agents since taxol was isolated from endophytic fungi *Taxomyces andreanae* (Stierle, *et al.,* 1993). Taxol is a very potent anticancer agent, first isolated from the bark of the Pacific Yew *(Taxus brevifolia).* It stabilizes microtubule formation due to its specific binding site on the microtubule polymer. Taxol is a complex molecule difficult to be synthesized chemically and its chemical synthesis is not very economical. The USA Food and Drug Administration (FDA) has approved taxol for the treatment of ovarian and breast cancer (Miller, 2002).

Strobel *et al.,* (1996; 1993) described anticancer agents from species of *Taxus* across the globe. The genus *Taxus* consists of slow-growing evergreen trees that produce taxol in a small amounts. This compound is present in the cell wall in varying amounts. There are approximately ten species in this genus and all are very slow-growing plants. The *Taxus* genus produces very little amount of taxol; therefore, other sources were searched for this compound. Fungus *Taxomyces andreanae* which is an endophyte of the plant *Taxus brevifolia* was found to produce taxol (Stierle *et al.,* 1993). Another endophytic species, *Pestalotiopsis microspora* (Strobel *et al.,* 2002, 1996) isolated from *Taxus wallichi. Pestalotiopsis guepini* isolated from *Wollemi Pine (IVollemia nobilis)* (Strobel *et al.,* 1997); *Tubercularia* sp. isolated from the Chinese yew *(Taxus mairei)* also produced taxol (Wang *et al.,* 2000). More recently, endophyte BT2 isolated from old inner bark of tree *Taxus ch'mensis* var. *mairei* was found capable of producing taxol and taxane baccatin III, an important intermediate for taxol (Guo *et al.,* 2006).

1.7.2 Antioxidant Compounds

Endophytic fungi often produce antioxidant metabolites. Pestacin and isopestacin which were isolated from *Pestalotiopsis microspora,* have the antioxidant property. This fungus is an

endophyte of the plant *Terminalia morobensis,* native of the Papua New Guinea (Strobel *et al.,* 2002). The proposed antioxidant activity of pestacin arises primarily via cleavage of an unusually reactive C-H bond, and to a lesser extent through O-H abstraction.

1.7.3 Antiviral Compounds

There are limited *compounds* reported as antiviral agents from endophytic fungi. Two novel compounds cytonic acid **A** and have been isolated from the endophytic fungus *Cytonaema* sp. These compounds are reported as inhibitors of human cytomegalovirus (hCMV) protease (Guo, *et al.,* 2000).

1.7.4 Scope of Research on Endophytic Fungi

All plant species study to date is the host of endophytic fungi (Tan and Zou, 2001). Only a tiny fraction of plant species have been investigated. Research on endophytes is in progress now. There are many aspects of endophytic organisms that could be investigated. The study of the biodiversity of endophytic fungi is important to have the primary idea about the endophytic population of particular plant species. Some scopes of research on endophytic fungi are described as follows:

- Study for novel compounds
- Identification of secondary metab
- antimicrobial activity of secondar
- Isolation of insecticidal compoun
- Isolation of pesticidal compounds
- Isolation of immunomodulating c
- Isolation of antioxidants compour
- Isolation of anticancer compound
- Isolation of plant growth stimular
- Control of plant diseases through
- Adaptation of plants in adverse er

Endophytic fungi are important components of biodiversity. More study should be conducted about them because they are elements of genetic diversity.

Chapter 2

Literature Review

The investigated plant for this research was Pomegranate *(Punica Granatum)*. A brief review of this plant is given below-

2.1 Classification of the plant *Punica Granatum*

Kingdom: Plantae

Division: Tracheophytes

Super Division: Angiosperms

Division: Tracheophyta

Class: Magnoliopsida

Order: Myrtales

Family: Lythraceae

Genus: *Punica*

Species: *P. granatum*

2.2 Habitat of *Punica Granatum*

Punica Granatum is believed to have originated long ago in the Middle East, more specifically Iran and Northern India. (Check out another plant native to this region, the Hawaiian baby wood rose.) The pomegranate has been around since ancient times; references to the pomegranate may be found throughout the Bible. The tree prefers low-humidity environments and thrives in warm areas that are protected from the wind. Although it may persist through short periods of drought or flooding, blossom formation may decrease or even halt under these circumstances. It prefers to be in full sun; shading due to overcrowding may lead to a lack of fruit production. Frost also decreases fruit production. Although mature plants may be able to tolerate frost, they often lose the ability to bear fruit after experiencing these conditions.

Pomegranate trees prefer soil with a neutral pH. However, they may be found in soil with a pH as low as 5.5 as long as an efficient drainage system is in place. They may grow in a variety of mediums - they have even been known to take root in loose gravel. The photo below shows an example of the type of environment *Punica Granatum* is known to inhabit.

2.3 Morphology

Pomegranate is a shrub that reaches 1.5 to 5 m in height, with more or less irregular and thorny branches and glossy leaves that appear as a deciduous shrub in temperate regions and as evergreen in frigid regions. *Punica Granatum* L belongs to the Punicaceae family and is the smallest plant family that includes 1 genus and 2 species, including the following*: Punica Granatum* (edible pomegranate) is indigenous to Iran and Mediterranean regions, and *Punica protopunica* (inedible) is endogenous to Socotra islands in the Pacific Ocean. The other characteristics are:

Leaves: Leaves are seen as reciprocal in newly grown branches and as integrated in spores.

Flowers: 1-5 flowers, one of them terminal and the rest marginal, short or without peduncle, their color is red and rarely yellow or white, odorless, and two-sex.

Fruit: Balausta in light red color to greenish yellow and rarely in some species dark purple. It is 5 to 20 cm in diameter and its weight varies from less than 200 g to more than 800 g.

Seed: Seeds are produced in high amounts, are triangular, albumin free, and embedded in aril. (Zamani Z, 1990)

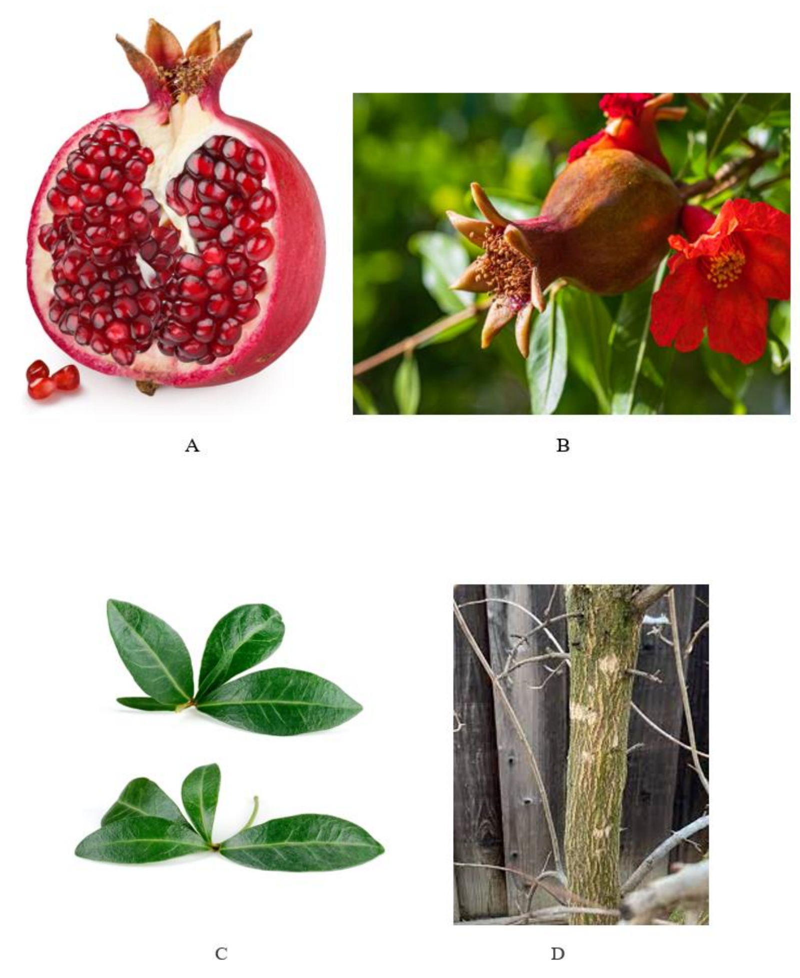


Figure 2.: Pomegranate A) fruit, B) flower, C) leaves and D) bark.

2.4 Chemical Constituents of *Punica Granatum*

A recent study demonstrated that medicinal plant shows a therapeutic role in disease management through the modulation of biological activities. (Rahmani et al., 2015) Recent findings based on in vitro and in vivo suggested that pomegranate has health-promoting effects mainly attributed due to its polyphenol content/antioxidant activity. However, its health-promoting effect has also been mentioned in traditional medicines. Fruits, seeds, peel, and leaves of pomegranate contain various types of valuable ingredients and such ingredients show therapeutics' role in the disease cure. Pomegranate peel is a rich source of tannins, flavonoids, and other phenolic compounds (Li Y et al. 2006). Its juice also contains various constituents such as polyphenols, tannins, and anthocyanins, including vitamin C, vitamin E, and lipoic acid (Van den Berg *et al*. 2011) and

punicalagin bioactive constituent responsible for more than 50% of the antioxidant activity of pomegranate juice (Seeram N *et al.* 2005).

Pomegranate fruits hold various types of constituents in different parts such as seeds, peels, and arils. Such ingredients show a therapeutic role the health management through the modulation of various biological activities. Peels of the pomegranate cover around 60% of the fruit and they hold various types of ingredients including flavonoids, ellagitannins, and panthocyanidin compounds and minerals such as calcium, magnesium, phosphorus, potassium, and sodium (Mirdehghan SH *et al.* 2007).

Pomegranate fruit arils contain huge amounts of organic acids, sugars, minerals, vitamins, and polyphenols that show antioxidant effects (Jaiswal V *et al.* 2010). Moreover, flavonoids are chief polyphenols of fruit, condensed tannins, and hydrolyzable tannins (Gil MI *et al.* 2000). Hydrolyzable tannins including ellagitannins and gallotannins consist of the common constituents present in pomegranate, and punicalagin is the major hydrolyzable tannin present in pomegranates.

2.5 Pharmalogical and Biological Activity

2.5.1 Antioxidant Activity of *Punica Granatum*

Medicinal plants make their importance in health managements through antioxidant activity and inhibition of free radical effects. Antioxidant activities of plants have been proven by earlier studies (Rahmani AH *et al,.* 2014). Pomegranate shows antioxidant activity due to the abundance of compounds such as flavonoids, flavones, anthocyanins, and catechins in different parts including fruits, seeds, and peels. The experiment was made to explore the antioxidant activity of pomegranate peels and seeds results the confirmed that extract of peels and extract of seeds showed anti-oxidant activity. Furthermore, the methanol extract of peel demonstrated the highest antioxidant activity among all of the tested extracts (Singh RP *et al.* 2002). Another study revealed that pretreatment of the extract of peel followed by treatment of CCL4 showed preservation of enzymes including catalase, peroxidase, and SOD. Additionally, lipid peroxidation was brought back by 54% as compared to the control (Kotamballi N *et al.* 2002).

The antioxidant activity of pomegranate peel powder and whey powder was evaluated and results showed that peel powder and whey powder exhibited antioxidant activity (Ashoush IS *et al,.* 2013).

Other previous reports demonstrated that leaf and peel exhibited very strong antioxidant activity (Zhang LH *et al.,* 2008) and extracts obtained from seeds exhibited various degrees of antioxidant activity and sour white peel had the highest potent antioxidant activity (Sadeghi N *et al.,* 2010).

2.5.2 Hepatoprotective Effect

Numerous plants including curcumin, green tea, date, fruits, and ginger or plant-based formulations show a very effective role in the prevention of liver damage and diseases related to the liver. Investigators demonstrated that rats fed on diets supplemented with pomegranate peel powder, whey powder, or their mixture showed potential hepatoprotective effects compared to liver injury control group (Rahmani AH *et al.* 2016) and feeding of rats with pomegranates powder show protective effect against carbon tetra chloride toxicity (Singh RP *et al.* 2002). The effect of chronic administration of pomegranate peel extract on liver fibrosis was examined and results confirmed that plasma AOC and hepatic GSH levels were considerably depressed by bile duct ligation whereas increased back to control levels in the peel extract-treated bile duct ligation group (Toklu HZ *et al.* 2007).

2.5.3 Anti-Microbial Activity

Antibiotics are effective remedies in the inhibition of bacteria growth or the growth of microorganisms. On the other side, antibiotic resistance against microorganisms is one of the major problems in the use of antibiotics against microorganisms. Plants products or ingredients of seeds, flowers, stems, bark, and leaves show a very effective role in the inhibition of the growth of pathogens. Plants are good sources of secondary metabolites including tannins, terpenoids, alkaloids, flavonoids and glycosides, which confirmed antimicrobial activities *in vivo* (Dahanukar SA *et al.* 2000). In this phenomenon, *Punica Granatum* shows a very effective role in the inhibition of microorganism growth.

2.5.3.1 Anti-Bacterial Activity

A previous study based on pomegranate peel reported that peel has an antibacterial effect (Devatakal SK *et al.* 2013). Antimicrobial activity against some food-borne pathogens via various extracts of fruit peels was examined and the finding confirmed that the methanolic extract of peels was a potent inhibitor for *Listeria monocytogenes*, *S. aureus*, *Escherichia coli,* and *Yersinia*

enterocolitica. *In vitro* antibacterial activities of different extracts of fruit peels and arils were evaluated against food-related bacteria. The finding of the study showed that all pomegranate extracts contained high levels of phenolics and exhibited antibacterial activity against all bacteria tested (Nuamsetti T *et al.* 2012). Extracts of pomegranate such as aqueous and methanolic demonstrated good antibacterial activity against *S. aureus* and *P. aeruginosa* (Sadeghian A *et al.* 2011).

2.5.3.2 Anti-Viral Activity

Earlier investigators demonstrated that tannin from the pericarp is a very effective constituent against the genital herpes virus (HSV-2) and effectively kills the virus and blocks its absorption to cells (Zhang J *et al.* 1995). Other findings reported that the acidity of juice and liquid extract solutions contributed to rapid anti-influenza activity (Sundararajan A *et al*. , 2010).

2.5.3.3 Anti-Fungal Activity

Antifungal activities of pomegranate peel extract, seed extract, juice, and whole fruit evaluated. Results confirmed that among the selected bacterial and fungal cultures, the highest antibacterial activity was noticed against *Staphylococcus aureus,* and among fungi high activity was observed against *Aspergillus niger*(Dahham SS *et al.* 2010). Data obtained in research with substances of *Punica Granatum* established the antimicrobial capacity against yeast cells of *Candida* genus (Anibal PC *et al. ,* 2013).

2.5.4 Anti-tumor activity

Cancer is a multi-factorial disease and numerous factors involve in this process. In this vista, pomegranates fruits, seeds and peels illustrate cancer preventive role seems to be due to rich source of antioxidants. The effects of an extract of pomegranate (PE) on a mouse mammary cancer cell line WA4 were investigated. It was noticed that the extract showed the inhibition of the proliferation of WA4 cells in a time- and concentration dependent manner(Dai Z *et al.,* 2010). The chemopreventive efficacy of pomegranate fruit extract and diallyl sulfide alone and in combination was investigated. Results revealed that fruit extract and diallyl sulfide alone delayed onset and tumor incidence by around 55% and around 45%, respectively, whereas their combination synergistically decreased tumor incidence more potentially at low doses (Dai Z *et al.* 2010). Earlier

investigators demonstrated that apoptotic cell numbers were significantly increased and the pro-apoptotic gene Bax expression was increased, and the anti-apoptotic gene Bcl-2 was decreased after extract treatment (Dikmen M *et al.* 2011).

2.6 Taxonomy of Endophytic Fungi

According to Petrini et al, (1986) mostly Ascomycetes, Deuteromycetes, and Bacidiomycetes class of fungi are reported as endophytic fungi. A large number of genera and species of fungi belonging to the first two classes can live endophytically in plants. The class and species of fungi depend upon the host plants. A number of coprophilous fungi belonging to the genera Ascoboius, Coprinus, Deiitschia, Geiasinospora, etc were isolated from plants as endophytes.

2.7 Physiological Role of Endophytic Fungi

There is sufficient evidence that endophytic fungi play important role in host plant physiology. They received nutrition, protection, and propagation opportunities from their hosts, and the host plant also benefited from this symbiosis. Endophytes provide protection to their host from insects, pests, and herbivores, and help their host to adapt to different stress conditions. However, endophytes also act as opportunistic under some conditions. Plants in their natural environment are continuously challenged by a variety of attacking agents. For example, herbivorous animals, insects, and microorganisms include fungi, bacteria, and viruses. The factors determining the survival of plants against these agents include a large arsenal of constitutive and inducible active defense mechanisms. The typical preformed, constitutive defenses are the structural barriers (waxes, cutin, suberis, iignin, phenolics, cellulose, callose, and cell wall proteins) which are often rapidly reinforced upon the infection process. However, plants-bearing endophytic fungi protect themselves from pathogenic microorganisms either by direct interaction with organisms or by inducing systemic resistance in the host.

2.8 Biological properties of Endophytic Fungi

The endophytic fungus is a rich source of secondary metabolites which act as a biologically active agents in the higher plants. The fungal derivatives play a vital part in human life and their compounds are the source of drugs for cancer, microbial and viral diseases. The natural compounds from the endophytes act as a growth inhibitor of plant pathogenic organisms. Endophytes are rich

sources of natural products which are used in agriculture (plant growth and insecticidal), pharmaceutical industries, and also used for phytoremediation (Sudha *et al.*, 2016).

2.8.1 Anticancer activity

Taxol is a highly functionalized diterpenoid that is widely used as an anticancer drug. It was first isolated from the bark of western yew, Taxus brevifolia. During cell division, taxol prevents the depolymerization of tubulin. Production of taxol from a different genus of endophytes by fermentation is a cheaper method. The anticancer drug, taxol has been found in many genera of endophytic fungi (*Alternaria, Fusarium, Monochaetia, Pestalotia, Pestalotiopsis, Pithomyces, and Taxomyces*). More than 15 fungi genera produced paclitaxel and its analogs.

Pandi *et al.* (2011) reported that taxol isolated from *Lasiodiplodia theobromae* showed activity against the breast cancer cell line. Alternariol 9-methyl ether is a major mycotoxin produced by fungi of the genus Alternaria. It can induce mitochondrial apoptosis in human colon carcinoma cells and induce DNA strand breaks, micronuclei, and gene mutations in various cultured mammalian cells.

Giridharan *et al*. (2012) reported that sclerotiorin isolated from *Cephalotheca faveolata* showed anti-proliferative activity against cancer cells and also it induces apoptosis in colon cancer cells.

2.8.2 Antimicrobial activity

Fungal endophytes such as *Phaeosphaeria avenaria*, *Leptosphaeria sp., Fusarium sp., P. chrysanthemicola, Cladosporium sp., Cylindrocarpon sp., Saussurea involucrata, Fusarium solani, Cordyceps memorabilis, P. longicolla and Dothideomycete sp.,* are found to have antimicrobial activity against human pathogenic bacteria and fungi such as *Micrococcus luteus, Enterococus shirae, Escherichia coli, Klebsiella pneumonia, Bacillus subtilis, Staphylococcus aureus, Shigella flexneri, S. epidermidis, Candida tropicalis, C. albicans, Cryptococcus neoformans, A. fumigatus* and *Salmonella typhi* (Lv et al. 2010; Tayung *et al.* 2011).

According to Bungihan *et al.* (2013) the extracts from *Colletotrichum gloeosporioides* and *Chaetomium globosum* exhibited antimicrobial activity against *Mycobacterium tuberculosis*, *Gordonia terrae*, *S. aureus* and *E. coli*.

Nath *et al.* (2014) reported that the extracts isolated from *C. gloeosporioides* showed antimicrobial activity against *Streptococcus pyogenes* and *Enteroccocus faecalis.*

Chapter 3

Methods and Material

3.1 Introduction

This study was designed to investigate the endophytic fungi of *Punica Granatum.* The method was divided into two separate parts-

- Biological assay
- Chemical assay

This protocol consists of the following synchronized steps on the basis of biological and chemical activity-

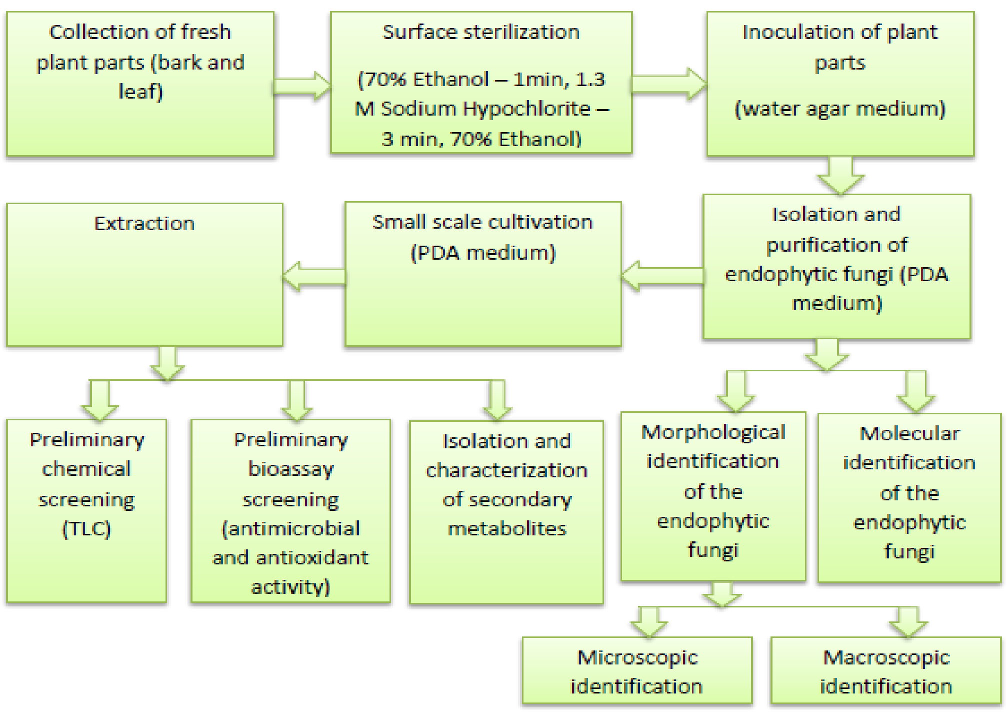
- ✓ Collection of the plant in fresh condition.
- ✓ Proper identification of the plant.
- ✓ Isolation of endophytic fungi from fresh plant material (bark and leaf).
- ✓ Purification of the isolated endophytic fungi
- ✓ Small-scale cultivation of purified endophytic fungi
- ✓ Morphological and molecular identification of endophytic fungi
- ✓ Extraction of cultured media of endophytic fungi using an organic solvent
- ✓ Preliminary bioassay and chemical profiling of the crude fungal extract
- ✓ Chromatographic screening of the fungal extracts.
- ✓ Identification of possible metabolites of fungi by different chromatographic and spectrophotometric techniques.

3.2 Collection and preparation of the plant material

In September 2020, the bark and leaf part of the plant *Punica Granatum* was collected from the grounds of BCSIR, Dhaka. The plant was identified by Dr. Farhana Afroz, Senior Scientific Officer, Pharmaceutical Sciences Research Division, BCSIR, Dhaka. A voucher specimen ought to be submitted to the herbarium for future reference.

3.3 Isolation of endophytic fungi from plant

Endophytic fungi were isolated from the plant *Punica Granatum.* The leaf and bark parts were sampled for the investigation of endophytic fungi and their bioactivity



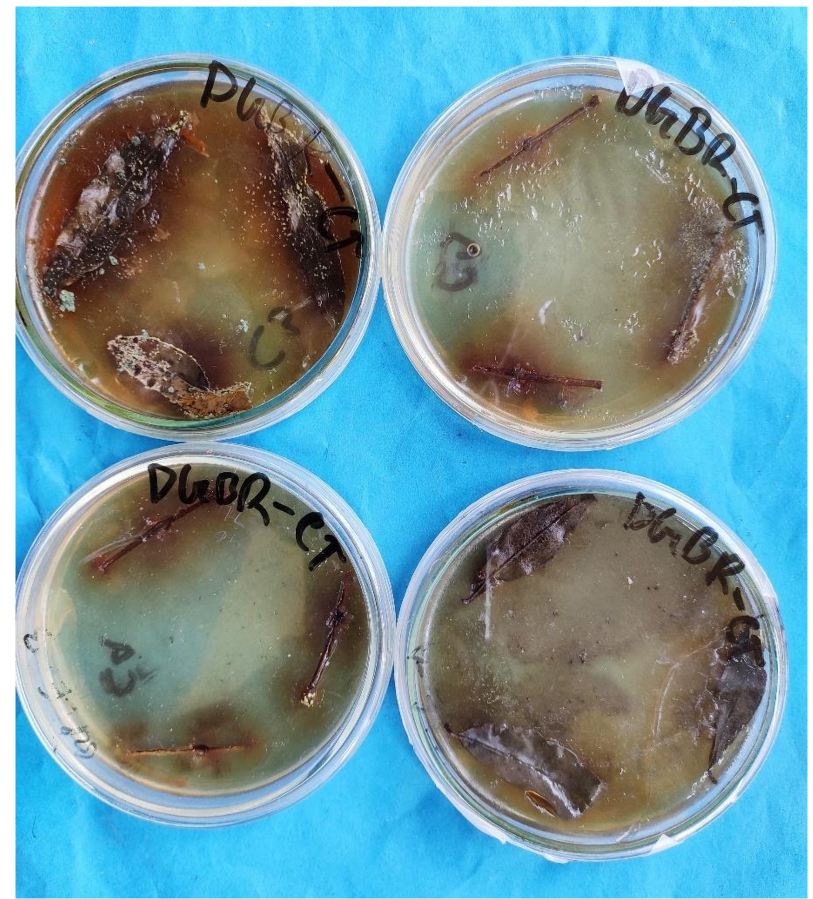
Scheme 1: Procedure of isolation, purification, identification, cultivation and extraction of endophytic fungi of *Punica Granatum.*

3.3.1 Sampling and general consideration before isolation of fungi

Healthy and mature plants were carefully chosen for sampling. The bark and leaf part of the plant were randomly collected for the study. The plant materials were brought to the laboratory in sterile bags and processed within a few hours after sampling or stored at 4 ℃ until the isolation procedure starts and excess condensation is prohibited. Otherwise, there is the risk that phylloplane fungi will colonize the plant material leading to false results during the isolation of endophytes. Fresh plant materials were used for isolation work to reduce the chance of contamination.

3.3.2 Instruments and vessel

- 100 ml beaker
- 1000 ml beaker
- Petri dish
- Large petri dish
- 250 ml conical
- Microwave oven
- Scissors
- 1 pc of small petri dish wrapped in polythene
- 1000 ml conical flask
- Forceps of various sizes
- Jar for containing forceps
- Measuring cylinder
- Scalpel
- 200 ml distilled water contained in two 250 ml conical flasks
- Large filter paper
- Cotton
- Anti-cutter
- Aluminum foil
- Razor blade
- Polythene for covering or capping conical flasks and petri dishes
- Glass plate
- Water for Injection (WFI)
- 70% Ethanol



A: **Inoculation of Bark and Leaves**

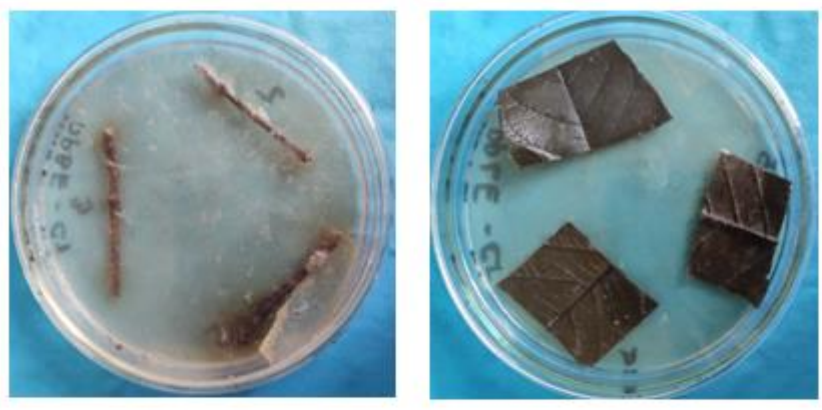


Figure B: **Inoculation of Control**

Figure 3Inoculation of plant parts; A: Leaves and Barks B: Leaves and Bark (control).

3.3.3 Medium and chemicals for isolation

Table 1: Ingredients of medium and chemicals with their amounts for isolation

Ingredients	**Amounts**
Water agar medium (HIMEDIA)	20 gm water agar in 1000 ml
Streptomycin	100 mg/ 1000 ml in WFI
1.3M NaOCl	96.772 g in 1000 ml distilled water

3.3.4 Process for isolation of endophytic fungi

- The respective plant parts were washed with tap water, followed by washing with distilled water to remove dust and debris.
- The plant was then cut with an anti-cutter, over a sterile glass plate.
- Then, the smaller plant parts were surface-sterilized by sequential immersion in 70% ethanol for 1 min, 1.3 M sodium hypochlorite (3-5% available chlorine) for 3 min, and 70% ethanol for 30s.
- Finally, these surface-sterilized leaves and bark were rinsed three times in sterile distilled water for 1 min each, to remove excess surface sterilants.
- After surface sterilization, the plant parts are dried on sterile filter paper and placed on separate petri dishes.
- Surface-sterilized leaves and bark fragments were evenly spaced in petri dishes containing water agar (WA) medium amended with streptomycin (100 mg L-1) to eliminate any bacterial growth.
- Petri dishes were sealed using parafilm and incubated at 28 ± 2° C in an incubator until fungal growth started.
- To ensure proper surface sterilization, unsterilized plant segments were prepared simultaneously and incubated under the same conditions in parallel to isolate the surface contaminating fungi as control.

- The cultures were monitored every day to check the growth of endophytic fungal colonies from the sample segments.
- The hyphal tips, which grew out from sample segments over 4-6 weeks, were isolated and subcultured onto a rich mycological medium, Potato Dextrose Agar (PDA) medium, and brought into pure culture (Kusari, 2008).

3.4 Purification of endophytic fungi

3.4.1 Materials and instrument

- Sterile slant
- Laminar Hood
- 70% ethanol
- Loop
- Sterile Potato Dextrose Agar media (MERCK)

Table 2: Composition of Potato Dextrose Agar media.

Ingredients	Amounts
Potato infusion	4 gm
D (+) Glucose anhydrous	20 gm
Agar-agar	15 gm
Distilled water	1000ml
pH	5.6 ±0.2 at 25°C

3.4.2: Process of purification

- After isolation from the petri dish, the isolated pure fungal colony was transferred to slant on to PDA media purification media.
- The transfer process was careful as no bacterial contamination or different fungal mycelia didn’t touch the loop. It was transferred to one or a few fungal mycelia to avoid fungal contamination.

- Before the transformation loop was burnt till red color to ensure sterilization and maintained by repeated subculture. Fungi were transferred from both the sample and control study.
- Then it was observed to match the test fungi with controlled fungi after 1 week. Which matched morphologically, these indicate exophytic fungi, and these were discarded.
- The unmatched fungi were isolated as endophytic fungi. By repeated transfer of these fungi, the growth was maintained and every strain was multiplied. For long time preservation, these fungi were stored at less than 4°C temperature.

3.5 Identification of endophytic fungi

Endophytic fungi were identified using morphology (macroscopic and microscopic observation) and molecular identification (DNA sequencing).

3.5.1 Morphological identification of endophytic fungi

3.5.1.1 Macroscopic identification

Macroscopic morphology deals with the study of the form and structure of organisms and their specific structural features that includes shape, structure, color, pattern, and size. Morphological identification was conducted by culturing the endophytic fungi in petri dishes using potato dextrose agar (PDA) media. The morphological features in the 3rd, 6th, 9th, and 12th days of culture were observed. Both top and bottom views of culture were observed and the features were recorded in a data sheet. Different types of fungi produced different-looking colonies, some colonies were colored, were circular, and others were irregular. Specific terminology is used to describe common colony types. These are:

- ✓ Form- the basic shape of the colony. For example, circular, filamentous etc.
- ✓ Size-the diameter of the colony. The tiny colony is referred to as punctiform.
- ✓ Elevation- this describes the side view of a colony.
- ✓ Margin/Border-the magnified shape of the edge of a colony.
- ✓ Surface-the appearance of the surface of the colony for example, smooth, rough, or dull.

- ✓ Opacity-for example, transparent (clear), opaque, translucent (like looking)
- ✓ Color-means pigmentation for example white, buff, red, purple etc.

Morphological identification was conducted by the following parameter

- Growth rate
- Type of the growth
- Diameter
- Color
- Texture of the colony surface
- Side view and
- Margin shape of the colony

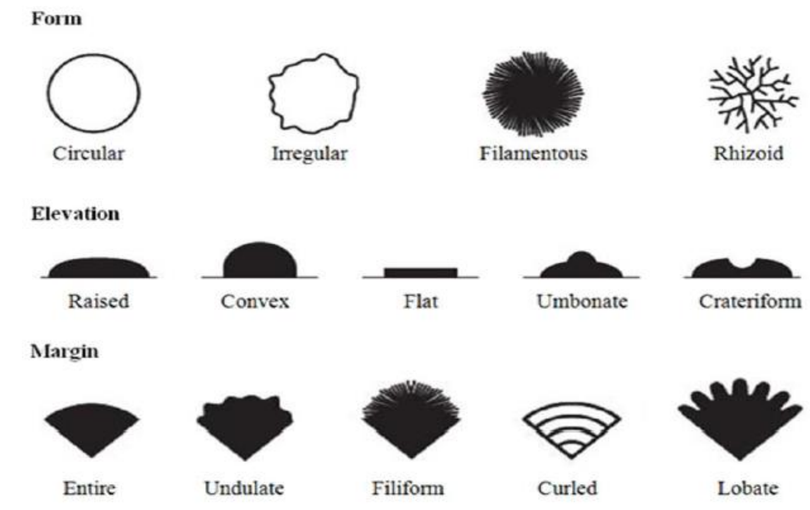


Figure 4: Morphological characteristics of fungi in petri dish.

3.5.1.2 Microscopic identification

For microscopic identification of endophytic fungal isolates, slides were prepared from cultures and stained with lactophenol cotton blue reagent and examined with a bright field and phase contrast microscope. After 3-4 days of incubation at 28°C on potato dextrose agar media, a small portion of the colony was taken into lacto-phenol cotton blue solution (0.05gram cotton blue in 100 ml lacto-phenol). A drop of the sample was poured on a glass slide and spread with the help of a sterilized needle, then covered with a cover slip. It was then examined for a characteristic

arrangement of spores under 10X, 40X, and 100X objective lenses of a compound microscope (Figure 3.3). Identification was based on morphological characteristics such as growth pattern, hyphae, the color of the colony and medium, surface texture, margin character, aerial mycelium, sporulation and production of acervuli, coloration of the medium, and the size and coloration of the conidia using standard identification manuals (Devi N *et al,* 2014). The fungi were identified using relevant keys and taxonomic notes from various standard manuals (Barnett, 1998).

3.5.2 Molecular identification of fungal strain and their taxonomy

3.5.2.1 Instruments used for molecular identification

- **DNA isolation:**

i. Automated DNA extractor, Model: Maxwell 16, Origin: Promega, USA

ii. Homogenizer, Pro Scientific.

- **DNA quantification:**

i. NanoDrop Spectrophotometer, Model: ND2000, Origin: Thermo Scientific, USA.

- **PCR:**

i. Gene Atlas, Model: G2, Origin: Astec, Japan.

- **Gel electrophoresis system:**

i. Horizontal, Model: Mini, Origin: CBS Scientific, USA.

- **Gel documentation:**

i. Alpha Imager, Model: mini, Origin: Protein Simple, USA.

- **PCR clean-up:**

i. Centrifuge, Model: Kitman24, Origin: Tomy-Japan.

3.5.2.2 Reagents used for molecular identification

- **DNA isolation:**

i. Maxwell® 16 LEV Plant DNA Kit, Model: AS1420, Origin: Promega, USA.

- **PCR:**

i. Hot Start Green Master Mix, (dNTPs, Buffer, MgCl2, Taq Pol), Cat: M7431, Origin: Promega, USA.

ii. Primer ITS4: TCC TCC GCT TAT TGA TAT GC

iii. Primer ITS5: GGA AGT AAA AGT CGT AAC AAG G

- **Gel:**

i. Agarose, Cat: V3125, Origin: Promega, USA.

ii. 100 bp DNA Ladder, Cat: G2101, Origin: Promega, USA.

iii. 1kb DNA Ladder, Cat: G5711, Origin: Promega, USA.

iv. Ethidium Bromide Solution, Cat: H5041, Origin: Promega, USA.

v. TAE Buffer: Cat: V4251, Origin: Promega, USA.

3.5.2.3 Fungal identification

Fungal strains were identified using a molecular biological protocol by DNA amplification and sequencing of the internal transcribed spacer (ITS) region and 5.8s rRNA gene.

3.5.2.4 DNA extraction

Fungal mycelium (~100 mg) was scratched with liquid nitrogen, put into an eppendorf tube, added 1.3 mL lysis buffer (100 mMTrisHCl (pH 8.0), 50 mM EDTA, 3% SDS), slightly vortexed and kept in a water bath (65º C) for 30 minutes. The mixture was then centrifuged at 13000 rpm for 13 min, collected supernatant, added an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 13000 rpm for 15 min. The supernatant was collected, added 0.5 mL isopropanol, and kept at 40º C for 30 min. The supernatant was then centrifuged at 12000 rpm for 10 min, collected pellet and added 70% ethanol (1.0 mL), and centrifuged at 13000 rpm for 5 min. The supernatant was removed, air-dried, and dissolved in water.

3.5.2.5 The amplification of DNA

The isolated DNA was then amplified by Polymerase Chain Reaction (PCR). The PCR was carried out using Hot Start Green Master Mix (Promega, USA). ITS 5 (with base sequences 5´- TCC TCC GCT TAT TGA TAT GC-3´) and ITS 4 (with base sequences 5´-TCC GTA GGT GAA CCT GCG G-3´) the universal ITS primers, were mixed with Hot Start Green Master Mix Kit and DNA template. Thus, each PCR reaction mixture contained 5-10 ng of genomic DNA, 1µM each of the primers ITS 4 and ITS 5, and 1 U of Hot Start Green-Polymerase (Promega) in a total volume of 50 µL.

Table 3: The reaction profile for PCR

Sl	Items	Volume	Reaction Number	Total Volume
1.	Master Mix	12.5 µl	X2	25
2.	T DNA (Concentration 25-65 ng/µl)	1 µl	X2	2 (1µl from each)
3.	Primer F (Concentration 10-20 pMol)	1 µl	X2	2
4.	Primer R (Concentration 10-20 pMol)	1 µl	X2	2
5.	Water	9.5 µl	X2	19
Total		25 µl	Total	50

The PCR Product Size is around 700 base. And extension time is directly related to product size. The Promega Taq Polymerase can amplify 1000 bases per min. So the extension time for PCR Work was 50 sec. The mixture was then applied to the thermal M7431 Master Mix Cycler using the programmed PCR cycle as outlined below:

- Initial activation step in 95°C for 2 minutes to activate Promega Taq DNA polymerase
- Cycling steps were repeated 32 times.
- Denaturing: 30 second at 95°C, annealing: 30 second at 54°C, extension: 50 second at 72°C
- Final extension for 5 minutes in 72°C

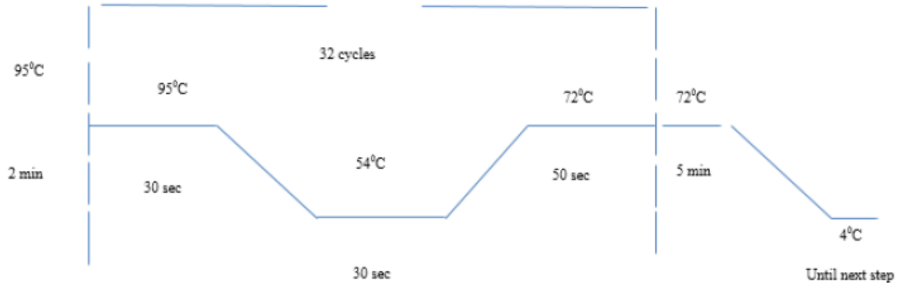


Figure 5: PCR thermal cycling condition.

3.5.2.6 Purification of PCR products

The product was purified using 2% Agarose-Gel-Electrophoresis at 75 V for 60 minutes in TBE buffer. The agarose gel was then stained using 1% ethidium bromide. A 100 bp stained DNA fragment was then excised from the agarose gel. The next step of PCR product purification was performed using Gel Cleanup Kit. The binding buffer was mixed with the PCR product and incubated at 50⁰ C for 10 minutes at 1000 rpm. The mixture was mixed with a volume of isopropanol and then centrifuged. The filtrate was discarded and the column was washed with wash buffer twice followed by centrifugation. Amplified fungal DNA (PCR product), which was incorporated into the column, was eluted by the addition of elution buffer or molecular biology grade water to the center of the column. The column was then centrifuged to collect the filtrate, which was the fungal DNA dissolved in the elution buffer.

3.5.2.7 DNA sequencing and phylogenetic analysis

The purified amplified fungal DNA was then submitted for sequencing by a commercial service and the base sequence was compared with publicly available databases such as Gene Bank with the help of Blast-Algorithms.

The purified amplified fungal DNA was sequenced using electrophoretic sequencing on an ABI 3730X 1 DNA analyzer (Applied Biosystems, USA) using Big Dye Terminator v 3.1 cycle sequencing kit. Sequences were compared with consensus sequence data from public databases Genbank (http://www.ncbi.nem.nih.gov) by using the BLASTn sequence match routines. The sequences were aligned using the CLUSTAL W program and phylogenetic and molecular

evolutionary analyses were conducted using MEGA version 6.0. The phylogenetic reconstruction was done using the neighbor-joining (NJ) algorithm, with bootstrap values calculated from 1,000 replicate runs, using the software routines included in the MEGA software.

3.6 Cultivation of endophytic fungi for small scale

The fungi were cultured in appropriate media for the production of secondary metabolites. Small-scale cultivation was carried out primarily to perform bioassays for the detection of active metabolites. Fungi were cultured on Potato Dextrose Agar (PDA).

3.6.1 Materials and instruments used for cultivation

- Petri dish (10mm)
- 250 ml Conical flask
- Microwave oven
- 1000 ml Conical flask
- Measuring cylinder
- Cotton
- Cotton plug
- 70% Ethanol
- Loop
- Para film
- PDA media
- Fresh culture of fungi

3.6.2 Small-scale cultivation

Work was focused on the culture and extraction of novel fungal endophytes. All isolated fungal strains were cultivated on small scale (25-30 Petri dishes/fungus) at room temperature for 21 days in potato dextrose agar (PDA) medium.

3.7 Extraction of secondary metabolites

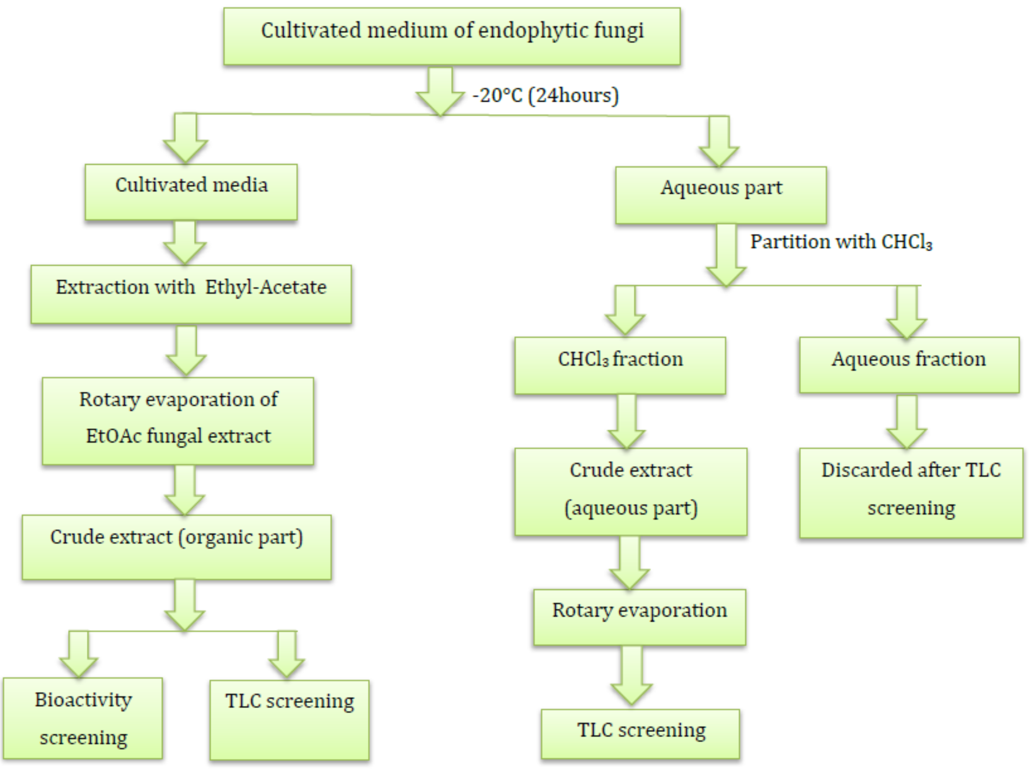
3.7.1 Materials and instruments used for extraction

- Conical flask
- Funnel
- Separating funnel
- Beaker
- Cotton
- Filter paper

- Rotary evaporator
- Solvent (chloroform)

3.7.2 Process of extraction

- After the full growth of fungi on a small scale, these were kept in deep freeze for about 24 hours
- Then these were kept in a fume hood at normal temperature.
- When it turned to normal temperature, it released water. It was checked that the freeze cultures were sufficiently released water due to sufficient rising of temperature.
- The watery portion was filtrated and solid culture was collected in another beaker.
- The watery portions were extracted by a solvent extraction process using 100ml Chloroform. Using a separating funnel, the lower chloroform portions were separated. Then these extracts were kept at normal temperature until full evaporation occurred.
- The solid cultures were ground into a blender and then these were soaked into 200ml chloroform.
- After one week these were filtrated and using a rotary evaporator at 45°C temperature the metabolites were concentrated. The extracts of the fungi were made concentrated into solid residue by evaporation under a rotary evaporator. Concentrated metabolites were transferred into a weighed vial. The recoveries were further used to soak ground fungi for the second time. After 1 week the chloroform portions were filtrated to get metabolite.



Scheme 2: Extraction process of fungal metabolites.

3.8 Determination of Bioactivity of fungal extracts

There are different test parameters to investigate bioactivity such as

- ✓ Antioxidant test
- ✓ Antimicrobial test

3.8.1 Antioxidant activity: DPPH assay

3.8.1.1 Principle

The free radical scavenging activities (antioxidant capacity) of the fungal extracts on the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) were estimated by the method of Brand –Williams et al., 1995. A stock solution of 1.6 mg of each extract in 0.4 ml methanol was prepared. The test solution was prepared at a concentration of 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, and 200 (μg/mL) by using methanol.

2.0 ml of methanol solution of the extract at different concentration were mixed with 2.0 ml of a DPPH methanol solution (20 µg/ mL). The mixture was properly mixed and kept in a dark place at room temperature for 30 min. The absorbance of the solutions was read at 517 nm against blank. The antioxidant potential was assayed from the bleaching of purple-colored methanol solution of DPPH radical by the plant extract as compared to that of Butylated hydroxyanisole (BHA) and Ascorbic acid (AA) by UV spectrophotometer.

Inhibition of free radical DPPH in percent (I%) was calculated as follows:

Scavenging ability (I%) = (A517 of control – A517 of sample / A517 of control) ×100

Where A517 of control is the absorbance of the control reaction (containing all reagents except the test material).

Extract concentration providing 50% inhibition (IC50) was calculated from the graph plotted inhibition percentage against extract concentration.

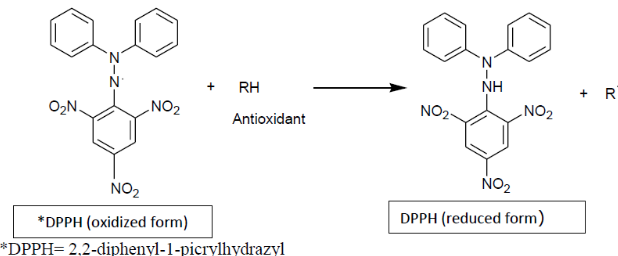


Figure 6: Redox reaction of DPPH

3.8.1.2 Materials

- Apparatus
- Test tubes
- Beakers
- Vials
- Thermometer
- Micropipette
- UV spectrophotometer
- Electronic balance

3.8.1.3 Reagents

- Methanol
- 0.02 mcg/ml methanolic solution of DPPH
- Standard: Butylated hydroxyanisole (BHA) and Ascorbic acid (AA)
- Distilled Water

3.8.2 Antimicrobial activity test

The currently available screening methods for the detection of antimicrobial activity of natural products fall into three groups, including-

- ✓ Disc diffusion method
- ✓ Bio-autographic method
- ✓ Serial dilution method

The bio-autographic and disc diffusion methods are known as qualitative techniques since these methods will give only an idea of the presence or absence of substances with antimicrobial activity. On the other hand, dilution methods are considered quantitative assays once they determine the minimal inhibitory concentration. The disc diffusion technique provides more suitable conditions for the microbial growth and is a widely accepted in vitro investigation which for preliminary screening of agents which may possess any antimicrobial activity.

3.8.2.1 Principle

Solutions of known concentration (μg/ml) of the test samples are made by dissolving measured amounts of the samples in a definite volume of solvents. Discs containing the test material are placed on a nutrient agar medium uniformly seeded with the test microorganisms. These plates are then kept at a low temperature (4°C) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved and diffused out of the media.

The plates are then incubated at 37°C (for bacteria) and 25°C (for fungi) for 24 hours to allow maximum growth of the organisms. The antibacterial activity of the test agent is determined by measuring the diameter of the zone of inhibition expressed in millimeters.

3.8.2.2 Apparatus and reagents

- Filter paper discs
- Sterile cotton
- Micropipette
- Refrigerator
- Laminar air flow hood
- Chloroform
- Sterile forceps
- Petri dishes
- Screw cap test tubes
- Autoclave
- Nutrient Agar Medium
- Spirit burner
- Inoculating loop
- Ethanol
- Nose mask and Hand gloves
- Incubator

3.8.2.3 Test organisms

The bacterial strains used for the experiment were collected as pure cultures from the Institute of Food Science & Technology (IFST), BCSIR. Both Gram positive and Gram negative organisms were taken for the test and they are listed in the Table.

Table 4: List of test microorganisms

Gram-positive Bacteria	**Gram-negative Bacteria**	**Fungi**
Staphylococcus aureus (ATCC 9144)	*Escherichia coli* (ATCC 11303)	*Aspergillus niger*
Bacillus megaterium (ATCC 9885)	*Salmonella typhi* (ATCC 13311)	*Aspergillus flavus*

3.8.2.4 Procedures

3.8.2.4.1 Culture medium selection

Nutrient agar medium (MERCK) and Potato Dextrose Agar medium (MERCK) were used most frequently for testing the sensitivity of the organisms to the test materials and to prepare fresh cultures.

3.8.2.4.2 Preparation of medium

To prepare the required volume of this medium, the calculated amount of each of the constituents was taken in a conical flask and distilled water was added. The test tubes, petri-dish and media were sterilized by autoclaving at 15-lbs. pressure at 121℃ for 20 minutes. The slants were used for making fresh cultures of bacteria that were in turn used for sensitivity study.

3.8.2.4.3 Sterilization procedures

In order to avoid any type of contamination by the test organisms the antibacterial screening was done in Laminar Hood and all types of precautions were highly maintained. UV light was switched on an hour before working in the Laminar Hood.

3.8.2.4.4 Preparation of subculture

In an aseptic condition under a laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants (for bacteria) and PDA slants (for fungi) with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37℃ (for bacteria) and 25°C (for fungi) for their optimum growth.

3.8.2.4.5 Preparation of discs

Three types of discs were used for antibacterial screening.

a) Standard discs

These were used as a positive control to ensure the activity of standard antibiotics against the test organisms as well as for comparison of the response produced by the known antimicrobial agent with that produced by the test sample. In this investigation, kanamycin (30 μg/disc) and ketoconazole (30 μg/disc) standard disc were used as the reference.

b) Blank discs

These were used as negative control which ensures that the filter paper was not active itself.

c) Discs for solvent

These were used as negative control which ensures that the residual solvents (left over the discs even after air-drying) were not active themselves.

3.8.2.4.6 Preparation of test sample discs

The extracts of endophytic fungi were tested for antimicrobial activity against a number of both gram-positive and gram-negative bacteria and fungi. The amount of sample per disc was 100 µg of the endophytic fungi extracts.

3.8.2.4.7 Preparation of the test plates

The test organisms were transferred from the subculture to the test tubes containing about 10 ml of sterilized agar medium with the help of a sterilized transfer loop in an aseptic area. The test tubes were shaken by rotation to get a uniform suspension of the organisms. The bacterial suspension was immediately transferred to the sterilized petri dishes. The petri dishes were rotated several times clockwise and anticlockwise to assure homogenous distribution of the test organisms in the media.

3.8.2.4.8 Application of the test samples

The sample discs, the standard antibiotic discs, and the control discs were placed gently on the previously marked zones in the plates pre-inoculated with test bacteria and fungi.

3.8.2.4.9 Diffusion and Incubation

The plates were then kept in a refrigerator at 4°C for about 24 hours to allow sufficient diffusion of the materials from the discs to the surrounding agar medium. The plates were then inverted and kept in an incubator at 37°C for 24 hours.

3.8.2.4.10 Determination of antibacterial activity by measuring the zone of inhibition

After incubation, the antibacterial activities of the test materials were determined by measuring the diameter of the zones of inhibition in millimeter with a transparent scale.

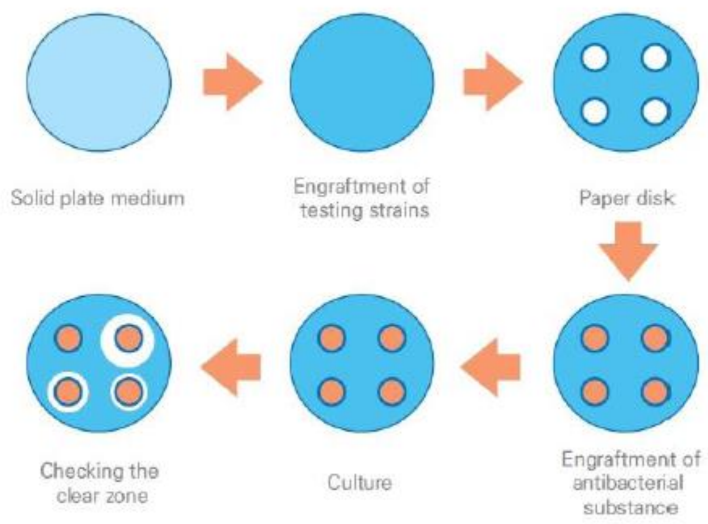


Figure 6 In vitro investigation of antimicrobial activity following the disc diffusion assay.

3.9 Chemical assay of fungal extracts

3.9.1 Detection of possible metabolites in extracts of isolated endophytic fungi

Preliminary qualitative chemical tests for the identification of secondary metabolites were carried out for the fungal extracts by the method described by Trease (1989) and Harborne (1973). This screening of the extract was performed by visual detection, UV light (in short and long wavelength and vanillin - H2SO4 spray reagent) of TLC plate.

3.9.1.1 Thin Layer Chromatography (TLC)

Ascending one-dimensional thin layer chromatographic technique is used for the initial screening of the extracts and column fractions and checking the purity of isolated compounds. For the latter purpose commercially available pre-coated silica gel plates are usually used. TLC plates are usually commercially available, with standard particle size ranges to improve reproducibility. They are prepared by mixing the adsorbent, such as silica gel, with a small amount of inert binders like

calcium sulfate (gypsum) and water. This mixture is spread as a thick slurry on an unreactive carrier sheet, usually glass, thick aluminum foil, or plastic. The resultant plate is dried and *activated* by heating in an oven for thirty minutes at 110 °C. The thickness of the absorbent layer is typically around 0.1 – 0.25 mm for analytical purposes and around 0.5 – 2.0 mm for preparative TLC.

A cylindrical glass chamber (TLC tank) with an airtight lid is used for the development of chromatoplates. The selected solvent system is poured in sufficient quantity into the tank. A smooth sheet of filter paper is introduced into the tank and allowed to soak in the solvent. The tank is then made airtight and kept for a few minutes to saturate the internal atmosphere with the solvent vapor. A small amount of dried extract is dissolved in a suitable solvent to get a solution (approximately 1%) (Harborne, 1983; Touchstone and Dobbins, 1978). A small spot of the solution is applied on the activated silica plate with a capillary tube just 1 cm above the lower edge of the plate. The spot is dried with a hot air blower and a straight line is drawn 2 cm below the upper edge of the activated plate which marks the upper limit of the solvent flow.

The spotted plate is then placed in the tank in such a way as to keep the applied spot above the surface of the solvent system and the cap/lid is placed again. The plate is left for development. When the solvent front reaches the given mark, the plate is taken out and air-dried. The properly developed plates are viewed under UV light of various wavelengths as well as treated with suitable reagents to detect the compounds.

3.9.2 Isolation of compounds

Pure compounds were isolated from the crude and fractionated extracts using Preparative Thin Layer Chromatography and other techniques.

3.9.2.1 Preparative Thin Layer Chromatography (PTLC)

The preparative thin layer chromatographic technique is routinely used in separating and for the final purification of the compounds. The principle of preparative TLC is the same as that of TLC. Here larger plates (20 cm x 20 cm) are used.

The sample to be analyzed is dissolved in a suitable solvent and applied as a narrow uniform band rather than a spot. The plates are then developed in an appropriate solvent system previously determined by TLC. In some cases, multiple development technique was adopted for improved separation. After development, the plates are allowed to dry and the bands of compounds are

visualized under UV light (254 nm and 365 nm) or with appropriate spray reagents on both edges of the plates. The required bands are scraped from the plates and the compounds are eluted from the silica gel by treating them with suitable solvent or solvent mixtures.

3.9.2.2 Solvent treatment

Solvent treatment is a process by which a compound consisting of the major portion of a mixture of compounds can be purified utilizing selective solvent washing. Initially, a solvent or a solvent mixture in which the desired compound is practically insoluble and other components are soluble is chosen. The undesired components are separated with repeated washing with this solvent or solvent mixture. If required other solvents or solvent mixtures can be used until a pure compound is obtained.

3.9.3 Visualization/detection of compounds in TLC

The following techniques are used for detecting the compounds in TLC/PTLC plates.

i. Visual detection

The developed chromatogram is viewed visually to detect the presence of colored compounds.

ii. UV light

The developed and dried plates are observed under UV light at 254 nm and 365 nm to detect the spot/band of any fluorescent or quenching compound.

iii. Spray reagents

Different types of spray reagents are used depending upon the nature of compounds expected to be present in the fractions or the crude extracts.

a) Vanillin/sulfuric acid (Stahl, 1966):

1% vanillin in concentrated sulfuric acid is used as a general spray reagent followed by heating the plates to 100° C for 10 minutes.

iv. Determination of R*f* (retardation factor) values

R*f* value is characteristic of a compound in a specific solvent system. It helps in the identification of compounds. R*f* value of a compound can be calculated by the following formula:

R*f* = Distance traveled by the compound / Distance traveled by the solvent system

3.9.4 Isolation and purification of compounds from DgB1 by solvent treatment

Thin layer chromatographic technique was used for the initial screening of the fungal extracts in different solvent systems. The developed chromatogram was viewed visually to detect the presence of colored compounds and was observed under UV light at 254 nm and 365 nm to detect the spot/band of any quenching or fluorescent compound. A spray reagent, such as 1% vanillin in concentrated sulfuric acid, was used to detect the nature of compounds expected to be present in the extracts.

Solvent treatment is a process by which a compound consisting of the major portion of a mixture of compounds can be purified utilizing selective solvent washing. Initially, a solvent or a solvent mixture in which the desired compound is practically insoluble and other components are soluble is chosen. The undesired components are separated with repeated washing with this solvent or solvent mixture. If required other solvents or solvent mixtures can be used until a pure compound is obtained.

To remove the undesired component, various mixtures of methanol and DCM was used. The primary solvent was methanol as the possible compound did not dissolve in it.

After, this was followed by TLC screening for purity. The isolated compound was denoted as DgB1.

Chapter 4

Results

4.1 Introduction

This chapter is designed to study-

- The morphological and molecular characterization of endophytic fungi isolated from *Punica Granatum.* plant,
- Bioassay of the fungal extracts,
- Identification of possible metabolites of the fungi by different chromatographic and spectrometric techniques.

Isolation, cultivation, and preparation of crude extracts from both the mycelia and cultivated medium of fungal endophytes have been done following the published method (Kusari, 2008).

4.2 Selection of endophytic fungi

A total of four endophytic fungi were isolated and purified from the plant *Punica Granatum.* Among them, two endophytic fungal strains were isolated from the bark of *Punica Granatum* – DgB1, DgB3, and two strains were isolated from the leaf of *Punica Granatum* – DgL3, DgL4. All the fungal strains were then identified by using macroscopic and microscopic morphological characteristics and molecular characterization.

Table 5: Code of test samples

No.	Test sample	Sample code
1.	Endophytic fungal strain-1 from the bark of *Punica Granatum*	DgB1
2.	Endophytic fungal strain-2 from the bark of *Punica Granatum*	DgB3
3.	Endophytic fungal strain-3 from the bark of *Punica Granatum*	DgL3
4.	Endophytic fungal strain-1 from the leaf of *Punica Granatum*	DgL4

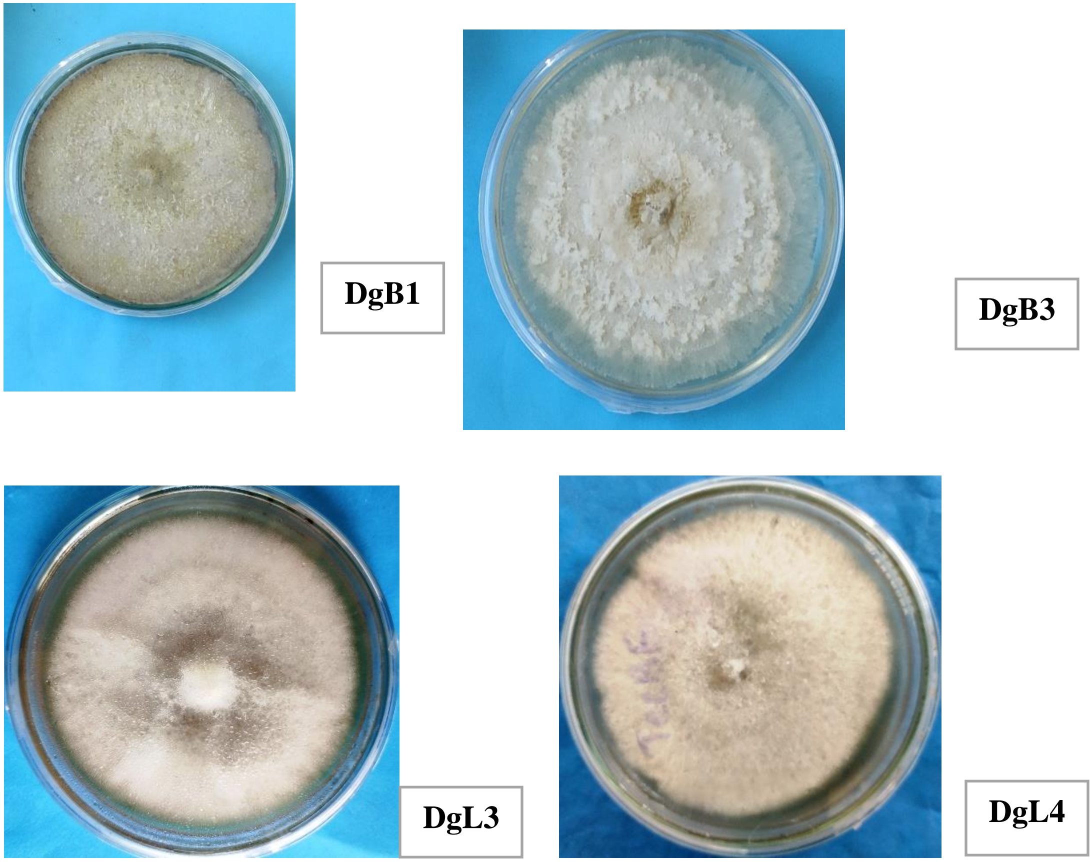


Figure 7: Four endophytic fungal strains isolated from *Punica Granatum*

4.3 Macroscopic characteristics of the isolated fungi

All the endophytic fungi exhibited characteristics of the colony and macroscopic morphology that could be used to differentiate them. The macroscopic characteristics of the fungi were documented in the following tables and figures-

Table 6: Morphological features of fungal strain DgB1

Characteristics	Observation (after 3 days)	Observation (after 6 days)	Observation (after 9 days)	Observation (after 12 days)
Type of media	PDA	PDA	PDA	PDA
Growth rate of the fungi	Moderate	Moderate	Moderate	Moderate
Nature of growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter	4.1	7.9	9	9
Surrounding temperature	28ºC	28ºC	28ºC	28ºC
Hyphae	Superficial	Superficial	Superficial	Superficial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Circular	Circular	Circular	Circular
Color of the top view	White	White with green center	White-green	White-green
Color of the bottom view	White with black center	White with black center	Black	Black
Texture of the colony surface	Wooly	Wooly	Wooly	Wooly
Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of colony	Entire	Entire	Entire	Entire
Opacity	Opaque	Opaque	Opaque	Opaque

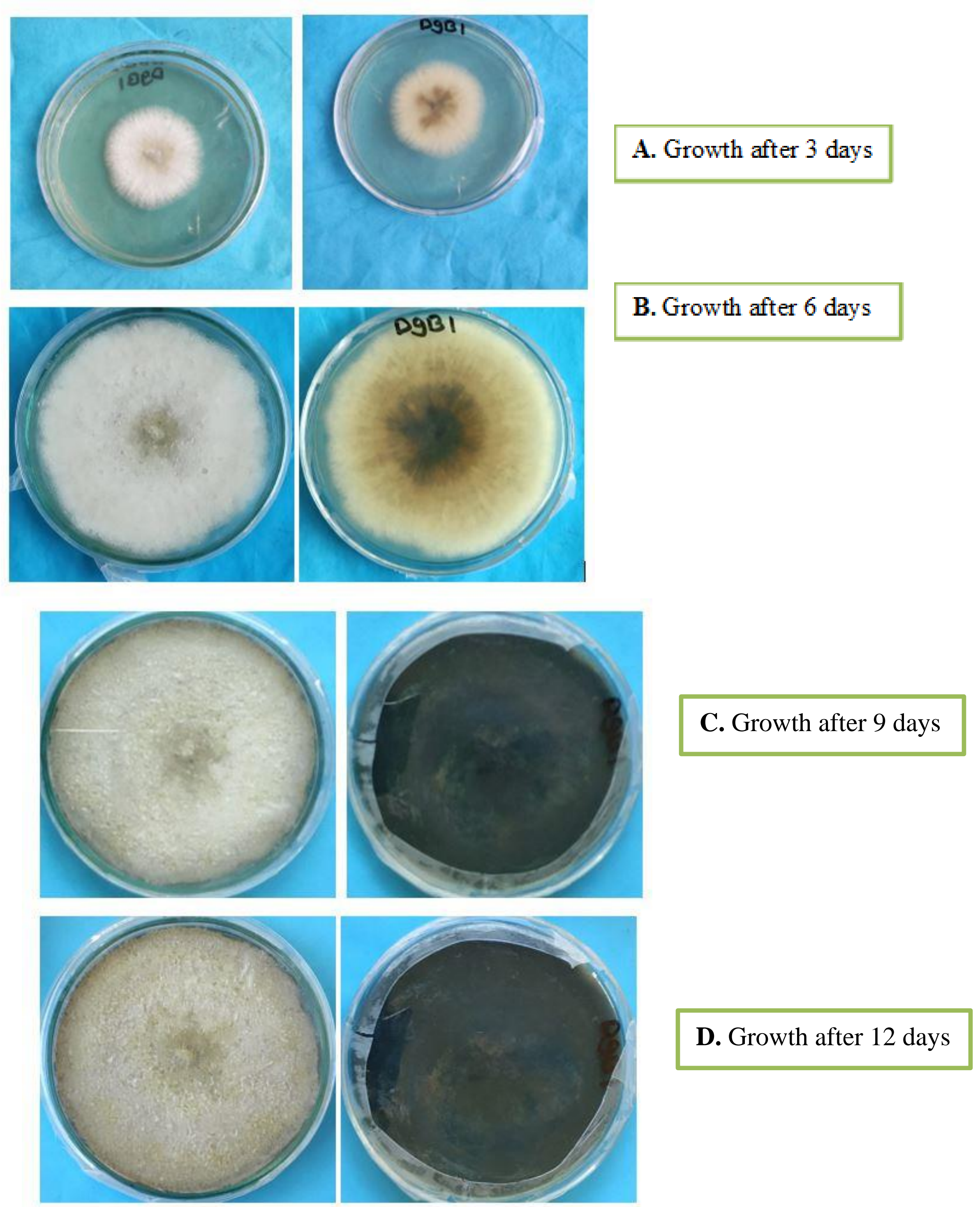


Figure 8: Top and bottom view of the fungal strain DgB1 after (A) 3 days, (B) 6 days, (C) 9 days, and (D) 12 days of small-scale cultivation

Table 7: Morphological features of fungal strain DgB1

Characteristics	**Observation (after 3 days)**	**Observation (after 6 days)**	**Observation (after 9 days)**	**Observation (after 12 days)**
Type of media	PDA	PDA	PDA	PDA
Growth rate of fungi	Moderate	Moderate	Moderate	Moderate
Nature of growth	Center to edge	Center to edge	Center to edge	Center to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter (cm)	3.5	7	9	9
Surrounding temperature	28ºC	28ºC	28ºC	28ºC
Hyphae	Surficial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Irregular	Irregular	Irregular	Irregular
Color of a top view	White	White	White with greenish-white center	White with green spots
Color of bottom view	White with an orange center	Off-white with brown center	Brown center with orange edge	Yellow with brown center
The texture of the colony surface	Wooly	Wooly	Wooly	Wooly
Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of the colony	Filiform	Filiform	Filiform	Filiform
Opacity	Opaque	Opaque	Opaque	Opaque

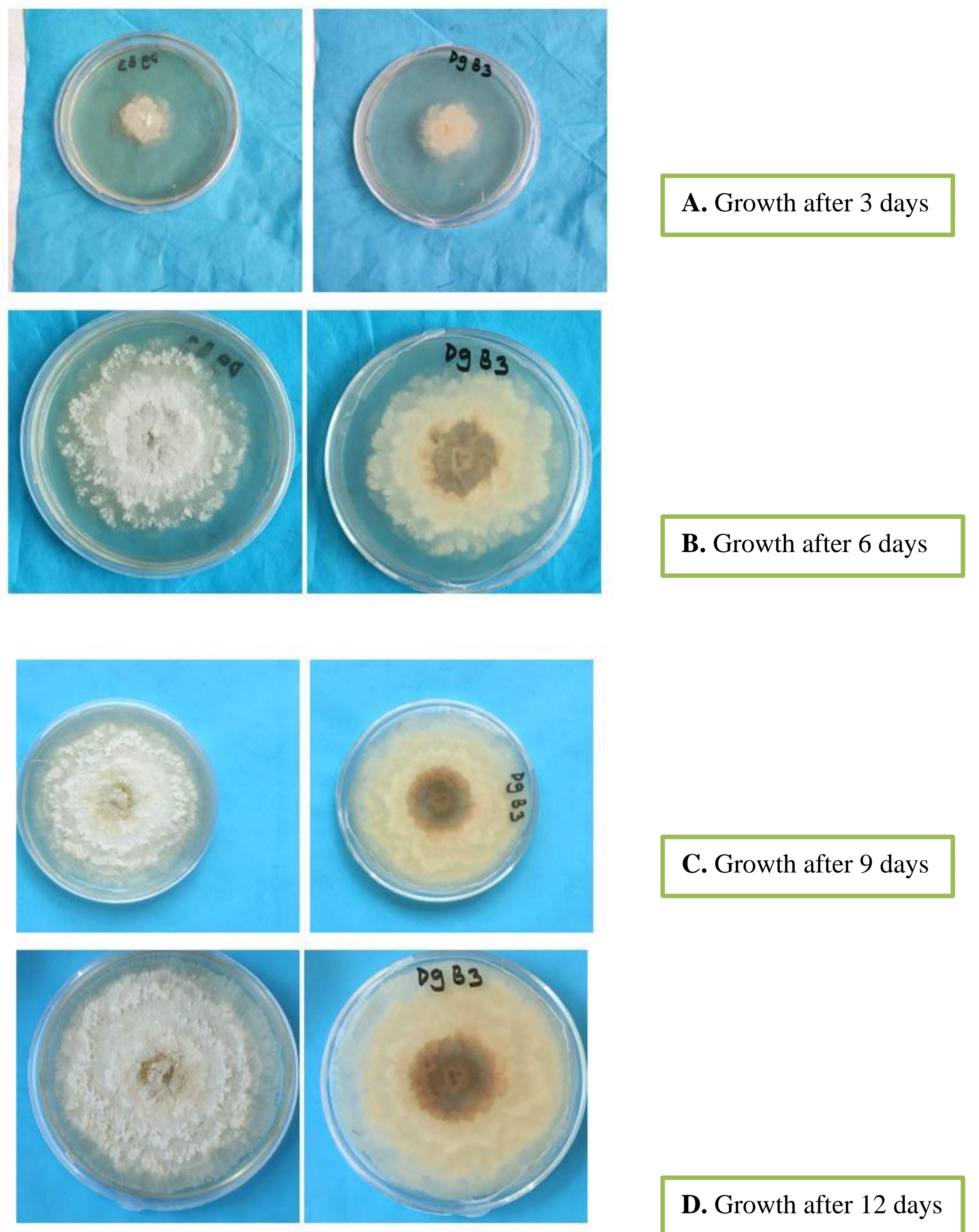


Figure 9: Top and bottom view of the fungal strain DgB1 after (A) 3 days, (B) 6 days, (C) 9 days, and (D) 12 days of small-scale cultivation

Table 8: Morphological features of fungal strain DgL3

Characteristics	**Observation (after 3 days)**	**Observation (after 6 days)**	**Observation (after 9 days)**	**Observation (after 12 days)**
Type of media	PDA	PDA	PDA	PDA
Growth rate of fungi	Moderate	Moderate	Moderate	Moderate
Nature of growth	Center to edge	Center to edge	Center to edge	Center to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter (cm)	3	6	8	9
Surrounding temperature	28ºC	28ºC	28ºC	28ºC
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Irregular	Irregular	Irregular	Irregular
Color of top view	White with orange center	White with orange center	White	White
Color of bottom view	White with orange center	White with orange center	Orange-white	Orange-white with black center
Texture of colony surface	Wooly	Wooly	Wooly	Wooly
Side view of colony/ Elevation	Raised	Raised	Raised	Raised
Margin shape of colony	Filiform	Filiform	Filiform	Filiform
Opacity	Opaque	Opaque	Opaque	Opaque

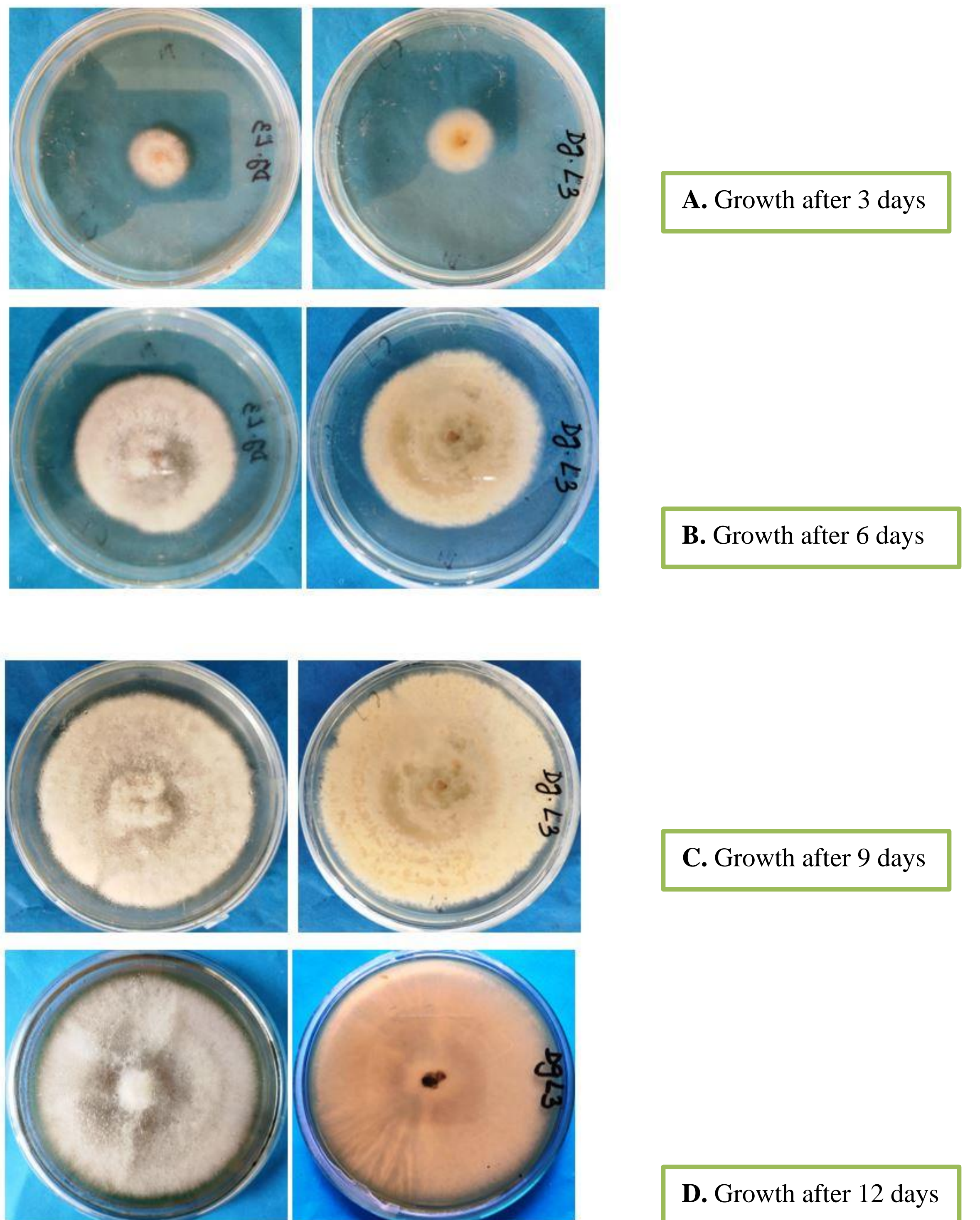


Figure 10: Top and bottom view of the fungal strain DgL3 after (A) 3 days, (B) 6 days, (C) 9 days and (D) 12 days of small scale cultivation

Table 9: Morphological features of fungal strain DgL4

Characteristics	Observation (after 3 days)	Observation (after 6 days)	Observation (after 9 days)	Observation (after 12 days)
Type of media	PDA	PDA	PDA	PDA
Growth rate of fungi	Slow	Moderate	Moderate	Moderate
Nature of growth	Center to edge	Center to edge	Center to edge	Center to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter (cm)	2	6.5	8.4	9
Surrounding temperature	28ºC	28ºC	28ºC	28ºC
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Irregular	Irregular	Irregular	Irregular
Color of top view	White with black center	White	White	White
Color of bottom view	Orange-white	Orange- white with black spots	Orange- white with black spots	Orange- white with black spots
Texture of colony surface	Wooly	Wooly	Wooly	Wooly
Side view of colony/ Elevation	Umbonate	Umbonate	Umbonate	Umbonate
Margin shape of colony	Filiform	Filiform	Filiform	Filiform
Opacity	Opaque	Opaque	Opaque	Opaque

Figure 11: Top and bottom view of the fungal strain DgL4

4.4 Microscopic Identification of the Isolated Fungi

For microscopic identification of endophytic fungal isolates, slides were prepared from cultures and stained with lactophenol cotton blue reagent and examined with a bright field and phase contrast microscope.

The fungi were identified using relevant keys and taxonomic notes from various standard manuals (Barnett, *et al.*, 1998). The microscopic observation of the characteristic mycelium and conidiophores of the fungi is given below.

Table 10:Summary of microscopic and morphologican identification

Strain	Mycelium	Conidiophores	Fungi Name	Figure
DgB1	Less abundant white mycelium. Petri dish filled out in about 7 days.	Conidia are filiform, straight or slightly curved at one end.	*Diaporthe* Sp.	
DgB3	Mycelium was less abundant. Petri dish filled out in about 8 days.	Conidia are filiform, straight or slightly curved at one end.	*Diaporthe* Sp.	
DgL3	White mycelium with long branched hyphae. Filled out the petri dish in 10 days.	Conidia were hyaline, unicellular, filiform and mostly slightly curved at end.	*Phomopsis* Sp	

DgL4	Mycellium were branched, septate and hyaline. Acervuli were subepidermal.	Conidiophore was simple, elongated, disc shaped or cushon shaped. Conidia was hyaline, one celled, ovoid to oblong. Setae were present.	*Colletotrichum* Sp	

4.5 Molecular identification of endophytic fungi

The isolated four endophytic fungi from the plant *Punica Granatum* were sub-cultured on PDA media for 7 days. The mycelium from these four fungi was scraped from the surface of the culture media and weighed. Nucleic acid was extracted and purified using a DNA isolation kit for genomic DNA using the manufacturer's protocol. For identification and differentiation of the endophytic fungi, the Internal Transcript Spacer Regions (ITS 4 and ITS 5) and the intervening 5.8S rRNA region were amplified and sequenced using electrophoretic sequencing on an ABI 3730X 1 DNA analyzer using Big Dye Terminator v 3.1 cycle sequencing kit. The ITS regions of the fungus were amplified using PCR and the universal ITS primers, ITS 4 (TCC TCC GCT TAT TGA TAT GC) and ITS 5 (GGA AGT AAA AGT CGT AAC AAG). The PCR products were purified and desalted using the Hot Start Green Master Mix. The sequences were aligned and prepared with the software Bioedit and matched against the nucleotide-nucleotide database (BLAST) of the U.S. National Center for Biotechnology Information (NCBI) for the final identification of the endophytic isolate.

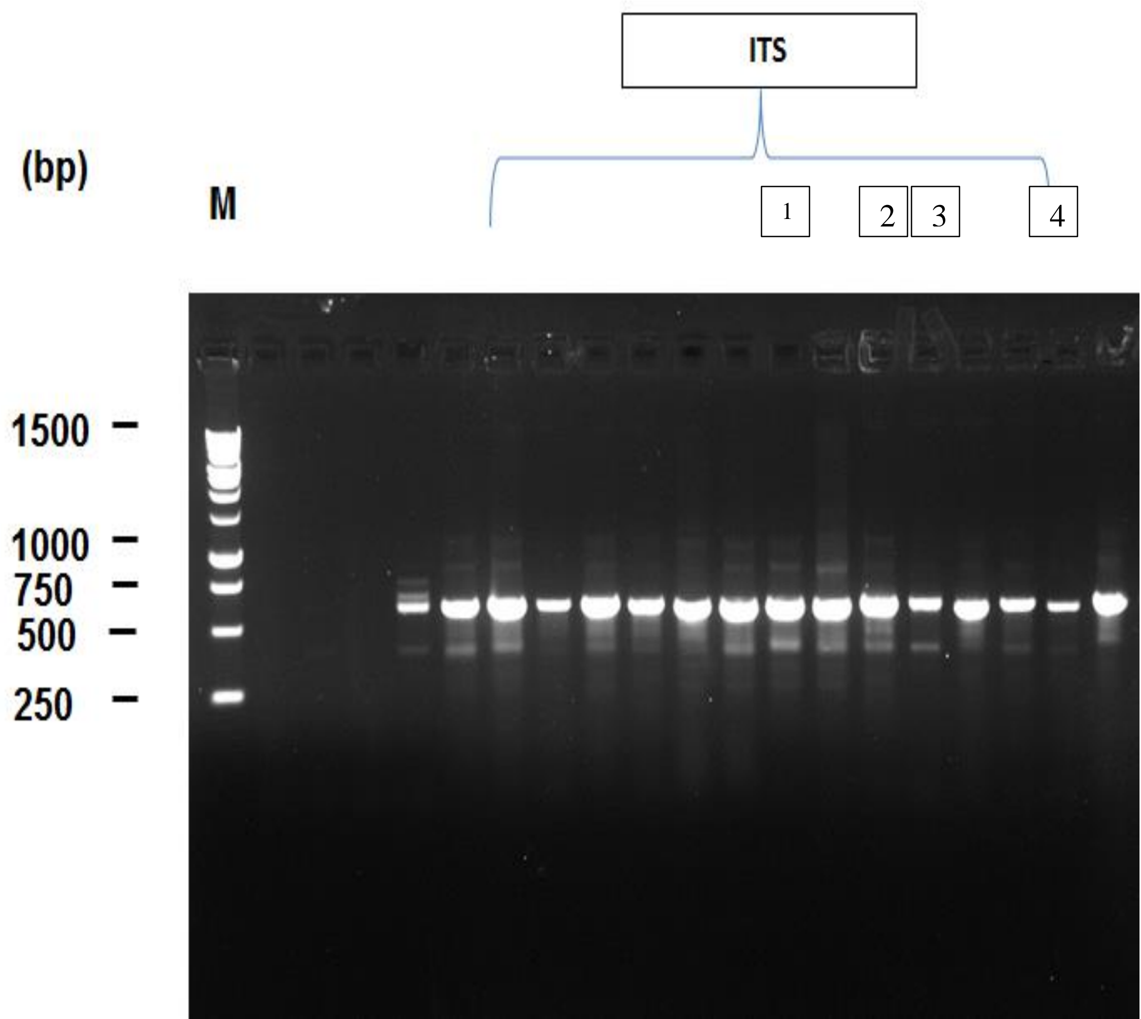


Figure 12: .: ITS profiles of ITS 4 and ITS 5 primers generated from 5 different fungi; here, 1 = DgB1, 2 = DgB2, 3 = DgL3, and 4 = DgL4, and M: denotes 1 kb DNA ladder (Marker)

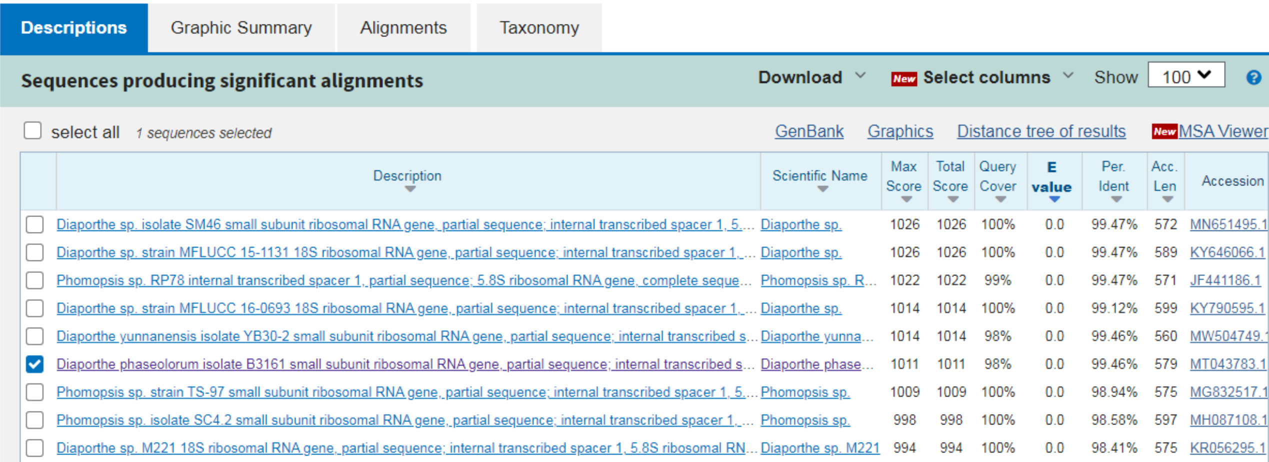
4.5.1 Molecular identification of fungal strain DgB1

4.5.1.1 The nucleotide sequence analysis of DgB1

Molecular analysis of the fungus DgB1 based on 5.8s rRNA gene revealed 99.46% similarity with another fungal isolate of *Diaporthe phaseolorum,* accession number: MT043783.1, deposited in the U.S National Center for Biotechnology Information (NCBI). Partial sequencing of the ITS region for sample DgB1 resulted in a 564 bp long sequence:

ACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCCTCGGCG
CACCCAGAAACCCTTTGTGAACTTATACCTTACTGTTGCCTCGGCGCAGGCCGGCCT
CTTAGCTGAGGCCCCCCGGAGACGGGGAGCAGCCCGCCGGCGGCCAACCAAACTCT

TGTTTCTACAGTGAATCTCTGAGTAAAAACATAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTAT
TCCGGAGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGTTG
GGGCACTGCTTCGAGAGAAGCAGGCCCTGAAATCTAGTGGCGAGCTCGCTAGGACC
CCGAGCGTAGTAGTTATATCTCGTTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAA
CCCCCAACTTCTGAAATTTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTA


Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — New Select columns — Show 100

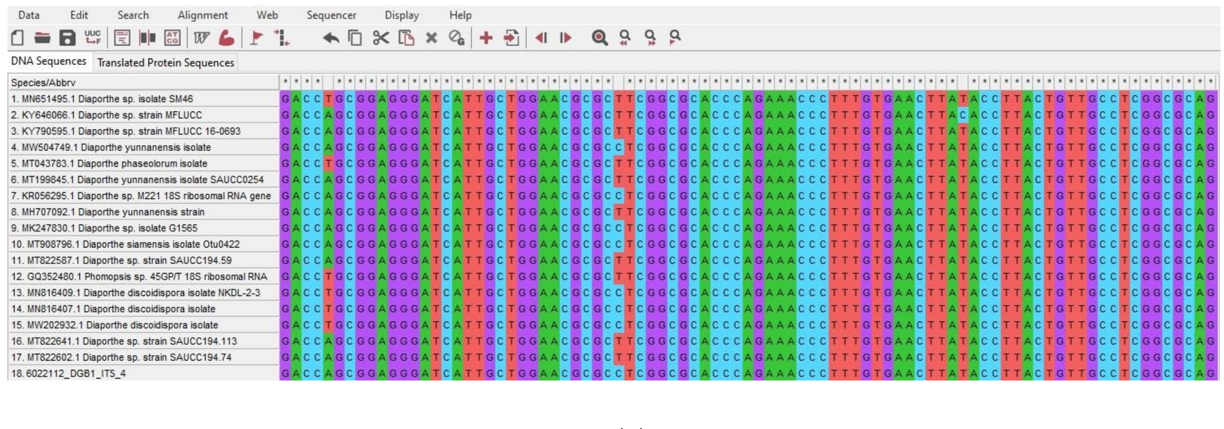
select all — 1 sequences selected — GenBank — Graphics — Distance tree of results — New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Diaporthe sp. isolate SM46 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5....	Diaporthe sp.	1026	1026	100%	0.0	99.47%	572	MN651495.1
Diaporthe sp. strain MFLUCC 15-1131 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	Diaporthe sp.	1026	1026	100%	0.0	99.47%	589	KY646066.1
Phomopsis sp. RP78 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete seque...	Phomopsis sp. R...	1022	1022	99%	0.0	99.47%	571	JF441186.1
Diaporthe sp. strain MFLUCC 16-0693 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	Diaporthe sp.	1014	1014	100%	0.0	99.12%	599	KY790595.1
Diaporthe yunnanensis isolate YB30-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Diaporthe yunna...	1014	1014	98%	0.0	99.46%	560	MW504749.1
Diaporthe phaseolorum isolate B3161 small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Diaporthe phase...	1011	1011	98%	0.0	99.46%	579	MT043783.1
Phomopsis sp. strain TS-97 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5....	Phomopsis sp.	1009	1009	100%	0.0	98.94%	575	MG832517.1
Phomopsis sp. isolate SC4.2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, ...	Phomopsis sp.	998	998	100%	0.0	98.58%	597	MH087108.1
Diaporthe sp. M221 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RN...	Diaporthe sp. M221	994	994	100%	0.0	98.41%	575	KR056295.1

Figure 13: Blast analysis of DgB1

4.5.1.2 Molecular identification of fungal strain DgB1 by DNA taxonomy method

In DNA taxonomy, after BLAST search, the most homologous sequences are downloaded from the International Nucleotide Sequence Database Collaboration (INSD) and incorporated into a multiple sequence alignment, and the phylogenetic tree-building method such as the Neighbor-joining method (NJ) is applied. Using this method, the unknown sequence is identified within an evolutionary framework.



(a)

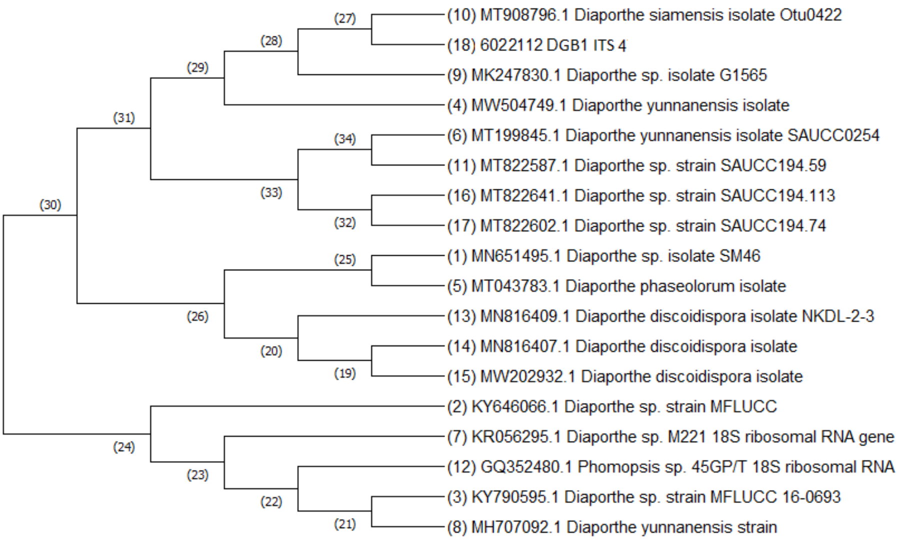


Figure 14: DNA taxonomy of fungal strain DgB1

(a) Multiple sequence alignment by ClustalW. (b) Phylogenetic tree constructed using Neighbor-joining (NJ) method with bootstrap support based on 1000 replicates and evolutionary distances computed using the Maximum Composite Likelihood method based on 5.8S rRNA-ITS sequences.

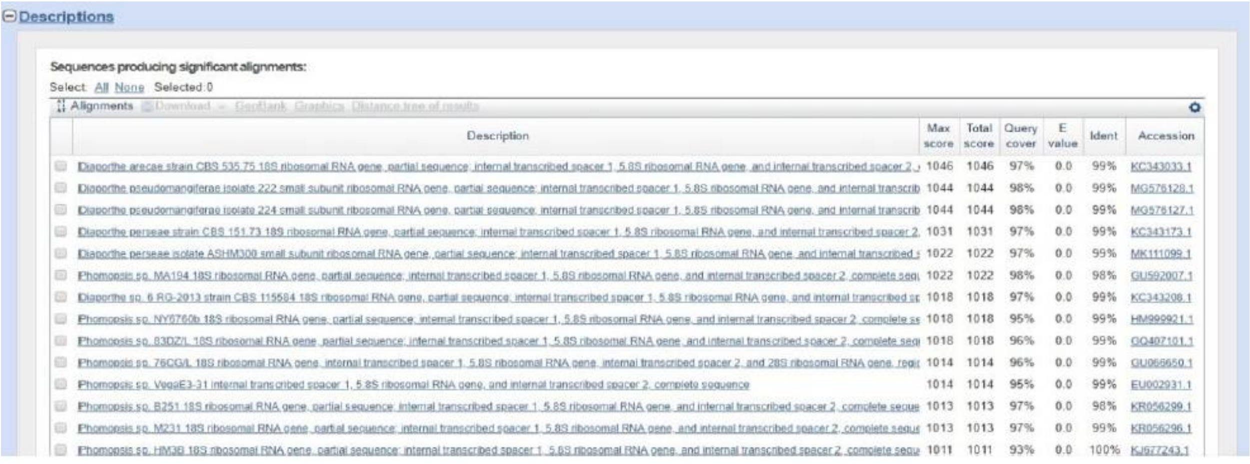
4.5.2 Molecular identification of fungal strain DgB3

4.5.2.1 The nucleotide sequence analysis of DgB3

Molecular analysis of the fungus DgB3 based on 5.8s rRNA gene revealed 98% similarity of another fungal isolate of accession number MG576128.1 that itself is identified as *Diaporthe pseudomangiferae*, similarly to other related texa, for example, *Diaporthe pseudomangiferae*

(97%, accession numbers MG576127.1, KC343173.1, MK111099.1, MK111100.1, etc.) deposited in the U.S National Center for Biotechnology Information (NCBI). The sequence of the fungus is:

ACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCCTCGGCG
CACCCAGAAACCCTTTGTGAACTTATACCTTACTGTTGCCTCGGCGCAGGCCGGCCT
CTTAGCTGAGGCCCCCCGGAGACGGGGAGCAGCCCGCCGGCGGCCAACCAAACTCT
TGTTTCTACAGTGAATCTCTGAGTAAAAAACATAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTAT
TCCGGAGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGTTG
GGGCACTGCTTCGAGAGAAGCAGGCCCTGAAATCTAGTGGCGAGCTCGCTAGGACC
CCGAGCGTAGTAGTTATATCTCGTTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAA
CCCCCAACTTCTGAAATTTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTA


Descriptions

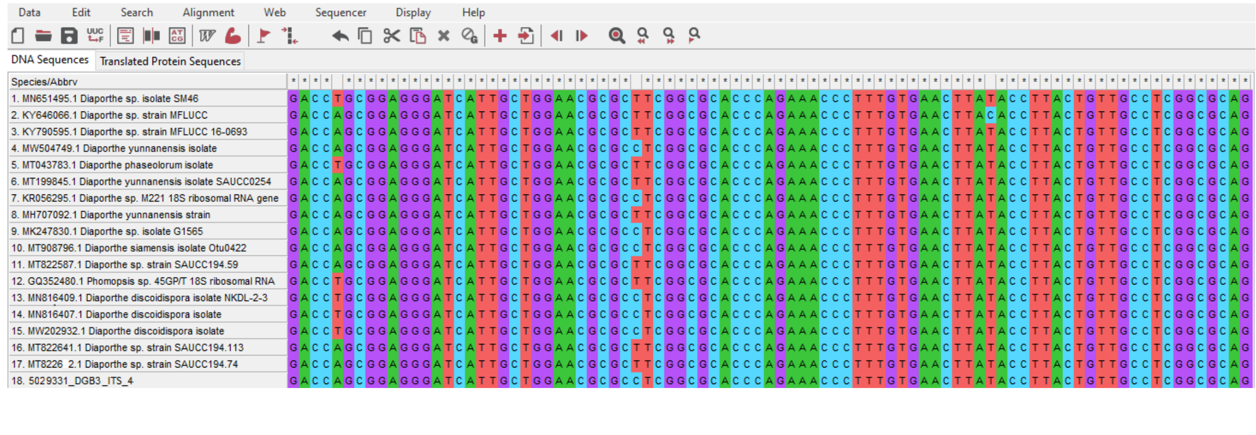
Sequences producing significant alignments:

Select: All None Selected: 0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Diaporthe arecae strain CBS 535.75 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2,	1046	1046	97%	0.0	99%	KC343033.1
Diaporthe pseudomangiferae isolate 222 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcrib	1044	1044	98%	0.0	99%	MG576128.1
Diaporthe pseudomangiferae isolate 224 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcrib	1044	1044	98%	0.0	99%	MG576127.1
Diaporthe perseae strain CBS 151.73 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2,	1031	1031	97%	0.0	99%	KC343173.1
Diaporthe perseae isolate ASHM300 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed	1022	1022	97%	0.0	99%	MK111099.1
Phomopsis sp. MA194 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seq	1022	1022	98%	0.0	98%	GU592007.1
Diaporthe sp. 6 RG-2013 strain CBS 115584 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed sp	1018	1018	97%	0.0	99%	KC343208.1
Phomopsis sp. NY6760b 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete se	1018	1018	95%	0.0	99%	HM999921.1
Phomopsis sp. 83DZ/L 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seq	1018	1018	96%	0.0	99%	GQ407101.1
Phomopsis sp. 76CG/L 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, regi	1014	1014	96%	0.0	99%	GU066650.1
Phomopsis sp. VegaE3-31 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence	1014	1014	95%	0.0	99%	EU002931.1
Phomopsis sp. B251 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seque	1013	1013	97%	0.0	98%	KR056299.1
Phomopsis sp. M231 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete seque	1013	1013	97%	0.0	99%	KR056296.1
Phomopsis sp. HM3B 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequ	1011	1011	93%	0.0	100%	KJ677243.1

Figure 15: BLAST search of DgB3



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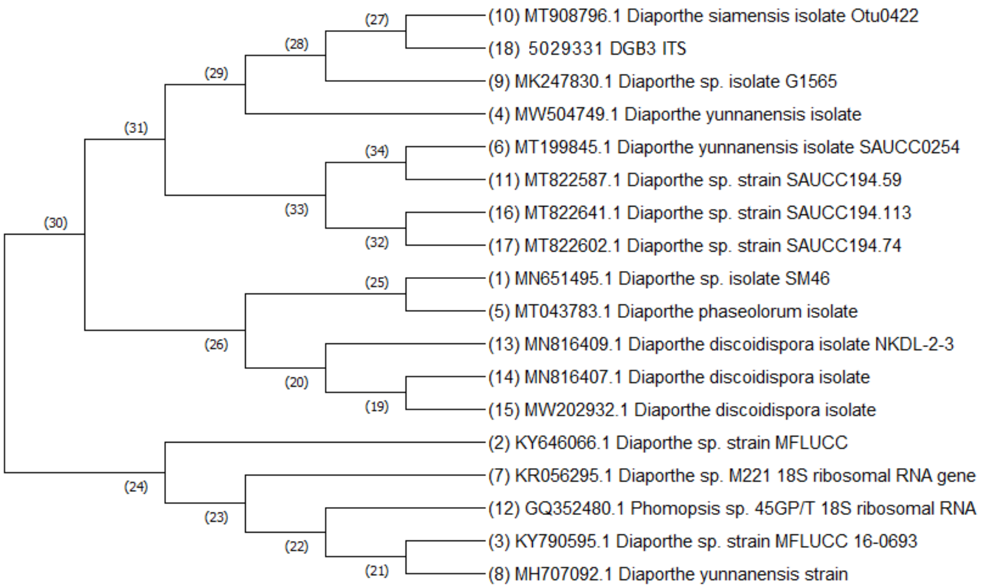


Figure 16: DNA taxonomy of fungal strain DgB3

(a) Multiple sequence alignment by ClustalW. (b) Phylogenetic tree constructed using Neighbor-joining (NJ) method with bootstrap support based on 1000 replicates and evolutionary distances computed using the Maximum Composite Likelihood method based on 5.8S rRNA-ITS sequences.

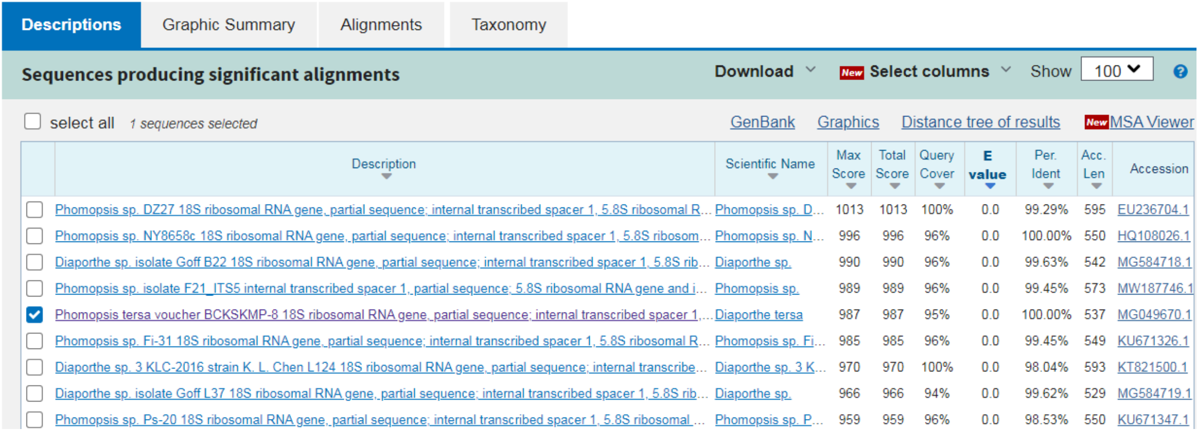
4.5.3 Molecular identification of fungal strain DgL3

4.5.3.1 The nucleotide sequence analysis of DgL3

Molecular analysis of the endophytic fungus DgL3 based on 5.8s rRNA gene revealed 100% similarity with another fungal isolate of *Phomopsis tersa,* accession number: MG049670.1,

deposited in the U.S National Center for Biotechnology Information (NCBI). Partial sequencing for the ITS region for sample DGL3 resulted in a 561 bp long sequence:

TCTCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCCCCAGGCGCACCC
AGAAACCCTTTGTGAACTTATACCTTACTGTTGCCTCGGCGTACGCTGGCCCCCAGG
GGTCCCTCTGTCTACAGAGGAGCAGGCACGCCGGCGGCCAAGTTAACTCTTGTTTTT
ACACTGAAACTCTGAGAAAAAAACACAAATGAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCCGGTATTCCGGA
GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGTTGGGGCAC
TGCTTCTCTCGCGGGAAGCAGGCCCTCAAATCTAGTGGCGAGCTCGCCAGGACCCCG
AGCGTAGTAGTTAAACCCTCGCTTTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACC
CCCAACTCTTGAAAATTGAACCTCGGATCAGGTAGGAATACCCGCTGAACT


Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments

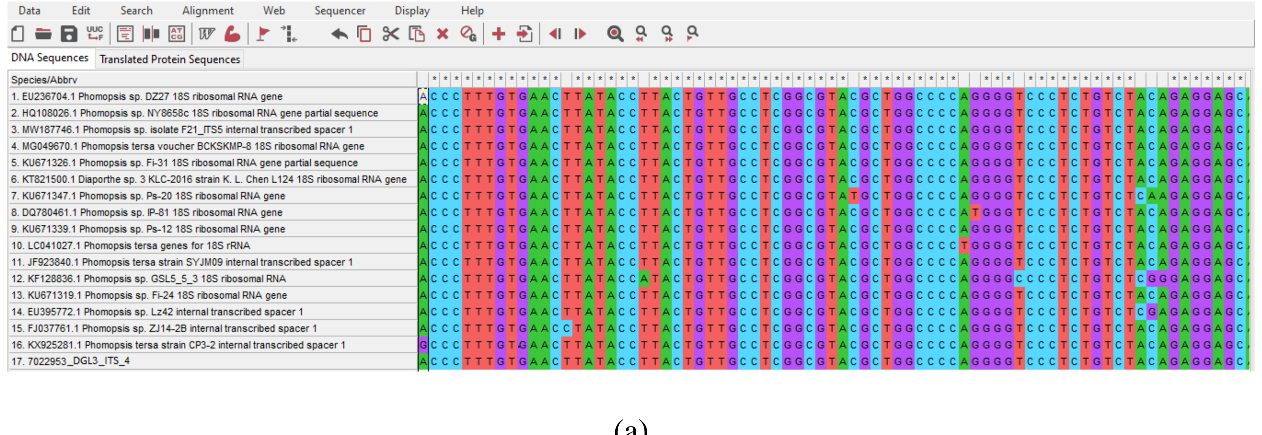
select all 1 sequences selected

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Phomopsis sp. DZ27 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal R...	Phomopsis sp. D...	1013	1013	100%	0.0	99.29%	595	EU236704.1
Phomopsis sp. NY8658c 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosom...	Phomopsis sp. N...	996	996	96%	0.0	100.00%	550	HQ108026.1
Diaporthe sp. isolate Goff B22 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S rib...	Diaporthe sp.	990	990	96%	0.0	99.63%	542	MG584718.1
Phomopsis sp. isolate F21_ITS5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and i...	Phomopsis sp.	989	989	96%	0.0	99.45%	573	MW187746.1
Phomopsis tersa voucher BCKSKMP-8 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,...	Diaporthe tersa	987	987	95%	0.0	100.00%	537	MG049670.1
Phomopsis sp. Fi-31 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal R...	Phomopsis sp. Fi...	985	985	96%	0.0	99.45%	549	KU671326.1
Diaporthe sp. 3 KLC-2016 strain K. L. Chen L124 18S ribosomal RNA gene, partial sequence; internal transcribe...	Diaporthe sp. 3 K...	970	970	100%	0.0	98.04%	593	KT821500.1
Diaporthe sp. isolate Goff L37 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S rib...	Diaporthe sp.	966	966	94%	0.0	99.62%	529	MG584719.1
Phomopsis sp. Ps-20 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal ...	Phomopsis sp. P...	959	959	96%	0.0	98.53%	550	KU671347.1

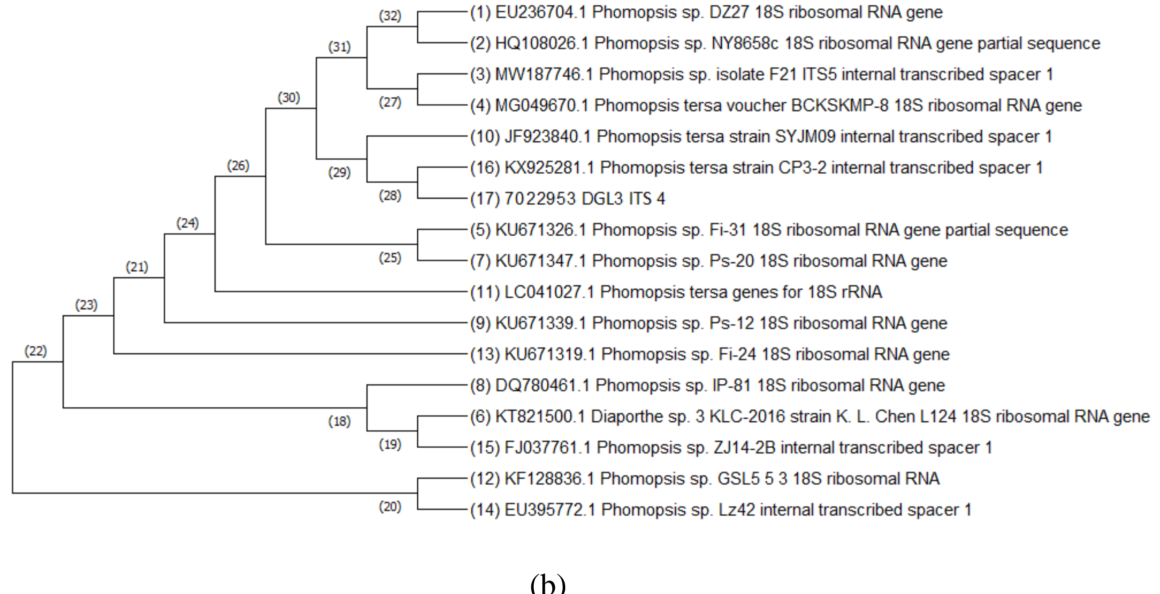
Figure 17: Blast analysis of DgL3

4.5.3.2 Molecular identification of fungal strain DgL3 by DNA taxonomy method

In DNA taxonomy, after BLAST search, the most homologous sequences are downloaded from the International Nucleotide Sequence Database Collaboration (INSD) and incorporated into a multiple sequence alignment, and the phylogenetic tree-building method such as the Neighbor-joining method (NJ) is applied. Using this method, the unknown sequence is identified within an evolutionary framework.



(a)



(b)

Figure 18: DNA taxonomy of fungal strain DgL3

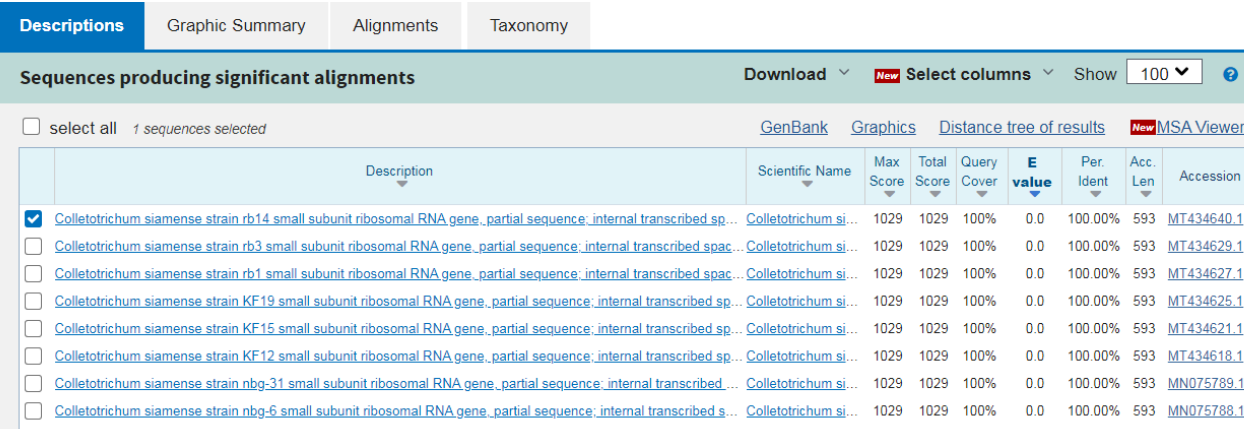
(a) Multiple sequence alignment by ClustalW. (b) Phylogenetic tree constructed using the Neighbor-joining (NJ) method with bootstrap support based on 1000 replicates and evolutionary distances computed using the Maximum Composite Likelihood method based on 5.8S rRNA-ITS sequences.

4.5.4 Molecular identification of fungal strain DgL4

4.5.4.1 The nucleotide sequence analysis of DgL4

Molecular analysis of the fungus DgL4 based on 5.8s rRNA gene revealed 100% similarity with another fungal isolate of *Colletotrichum siamense,* accession number: MT434640.1, deposited in the U.S National Center for Biotechnology Information (NCBI). Partial sequencing of the ITS region for sample DgL4 resulted in a 557 bp long sequence:

GGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTTACGCTCTACAACCCT
TTGTGAACATACCTATAACTGTTGCTTCGGCGGGTAGGGTCTCCGCGACCCTCCCGG
CCTCCCGCCTCCGGGCGGGTCGGCGCCCGCCGGAGGATAACCAAACTCTGATTTAAC
GACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTAACAACGGATCTCTT
GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGG
CATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACA
GCTGATGTAGGCCCTCAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGT
AACTTTACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCAATTTT
CCAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTA


Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

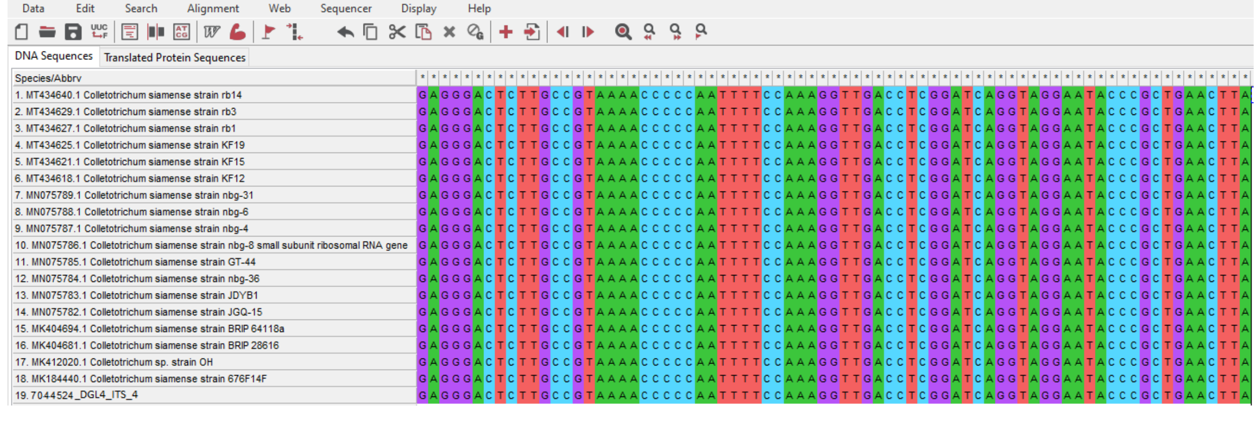
select all — 1 sequences selected — GenBank — Graphics — Distance tree of results — MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Colletotrichum siamense strain rb14 small subunit ribosomal RNA gene, partial sequence; internal transcribed sp...	Colletotrichum si...	1029	1029	100%	0.0	100.00%	593	MT434640.1
Colletotrichum siamense strain rb3 small subunit ribosomal RNA gene, partial sequence; internal transcribed spac...	Colletotrichum si...	1029	1029	100%	0.0	100.00%	593	MT434629.1
Colletotrichum siamense strain rb1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spac...	Colletotrichum si...	1029	1029	100%	0.0	100.00%	593	MT434627.1
Colletotrichum siamense strain KF19 small subunit ribosomal RNA gene, partial sequence; internal transcribed sp...	Colletotrichum si...	1029	1029	100%	0.0	100.00%	593	MT434625.1
Colletotrichum siamense strain KF15 small subunit ribosomal RNA gene, partial sequence; internal transcribed sp...	Colletotrichum si...	1029	1029	100%	0.0	100.00%	593	MT434621.1
Colletotrichum siamense strain KF12 small subunit ribosomal RNA gene, partial sequence; internal transcribed sp...	Colletotrichum si...	1029	1029	100%	0.0	100.00%	593	MT434618.1
Colletotrichum siamense strain nbg-31 small subunit ribosomal RNA gene, partial sequence; internal transcribed ...	Colletotrichum si...	1029	1029	100%	0.0	100.00%	593	MN075789.1
Colletotrichum siamense strain nbg-6 small subunit ribosomal RNA gene, partial sequence; internal transcribed s...	Colletotrichum si...	1029	1029	100%	0.0	100.00%	593	MN075788.1

Figure 19: Blast analysis of DgL4

4.5.4.2 Molecular identification of fungal strain DgL4 by DNA taxonomy method

In DNA taxonomy, after BLAST search, the most homologous sequences are downloaded from the International Nucleotide Sequence Database Collaboration (INSD) and incorporated into a multiple sequence alignment, and the phylogenetic tree-building method such as the Neighbor-joining method (NJ) is applied. Using this method, the unknown sequence is identified within an evolutionary framework.



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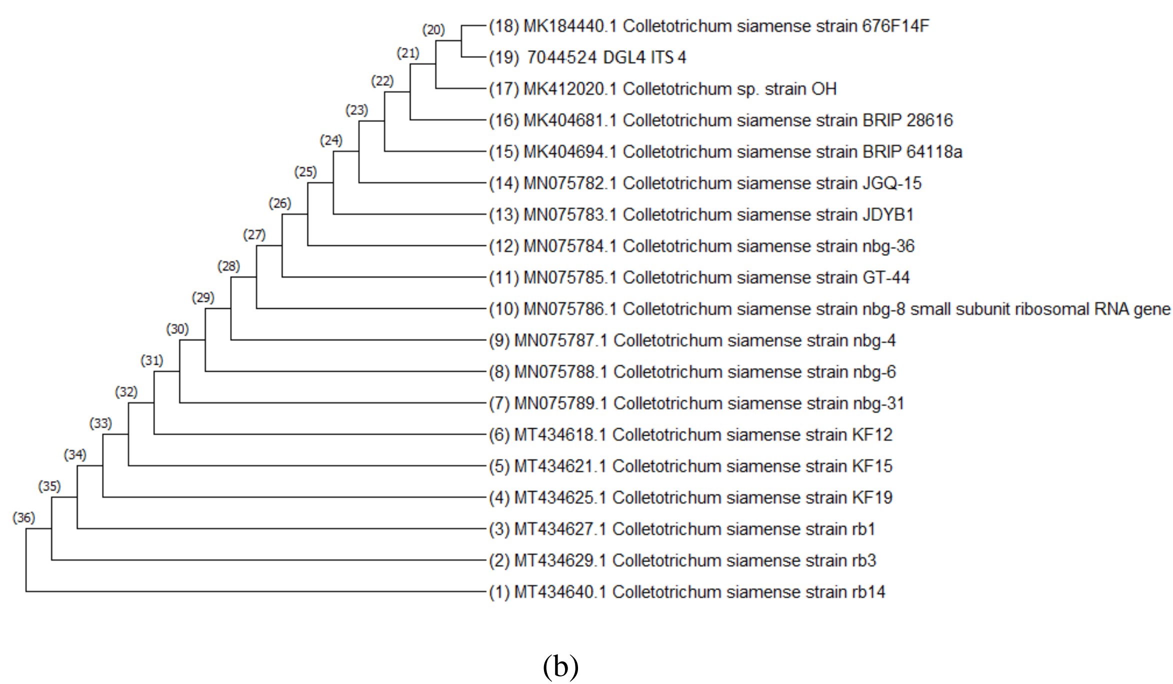


(b)

Figure 20: DNA taxonomy of fungal strain DgL4

(a) Multiple sequence alignment by ClustalW. (b) Phylogenetic tree constructed using the Neighbor-joining (NJ) method with bootstrap support based on 1000 replicates and evolutionary distances computed using the Maximum Composite Likelihood method based on 5.8S rRNA-ITS sequences.

4.6 Small-scale culture and extraction

The isolated four endophytic fungal strains from *Punica Granatum* were cultivated on small scale (25-30 petri dishes) at 27 °C for 21 days in a PDA medium. After 21 days the cultured plates were kept at -20°C overnight. The next day, the cultured medium was then extracted two times with a suitable solvent (chloroform, ethyl-acetate). On evaporation in a rotary evaporator at 45°C and 200

rpm, the extracts yielded a crude mixture of secondary metabolites. In the following table, the quantity of all the extracts is given-

Table 11: Collected Extracts

Endophytic fungi	**No of petri dish**	**Ethyl-acetate/ Organic extract (mg)**	**Chloroform/ Aqueous extract (mg)**
DgB1	27	123.5	11.5
DgB3	22	102.1	8.2
DgL3	25	110	10.3
DgL4	26	119.5	7.4

4.7 Antimicrobial screening of fungal extracts

Extracts of four isolated endophytic fungi from the plant of *Punica Granatum* – DgB1, DgB3, DgL3, and DgL4 were tested for antimicrobial activity against a number of both gram-positive (*Staphylococcus aureus, Bacillus megaterium)* and gram-negative (*Escherichia coli*, *Salmonella typhi)* bacterial strains. A Standard disc of Kanamycin (30 µg/disc) and a standard disc of Ketoconazole (30 µg/disc) were used as a standard against bacterial strains and fungal strains for comparison purposes. The antimicrobial activities were investigated by the method described by Bauer et al., 1966. The results of the antimicrobial activity of all the extracts exhibiting different zones of inhibition are shown in the following Table-

Table 12: Antibiogram

Bacterial strains	Fungal strains				Standard
	DgB1	DgB3	DgL3	DgL4	Kanamycin
Escherichia coli	11	---	---	---	33
Salmonella typhi	---	---	---	---	29
Staphylococcus aureus	20	---	---	---	31

Bacillus megaterium	9	7	---	---	28
Fungal strain					Ketoconazole
Aspergillus niger	---	---	---	---	28
Aspergillus flavus	---	---	---	---	35

“---” indicates No sensitivity. Zone of inhibition scale size = mm.

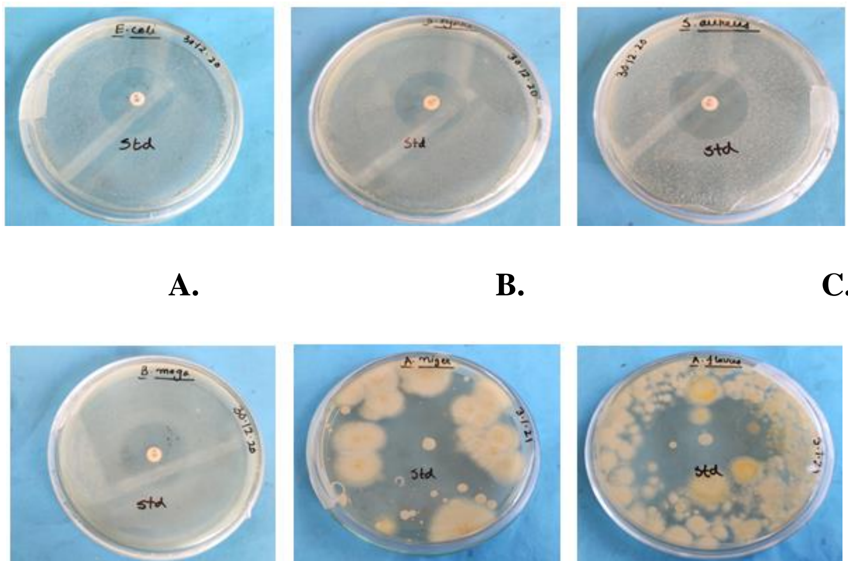
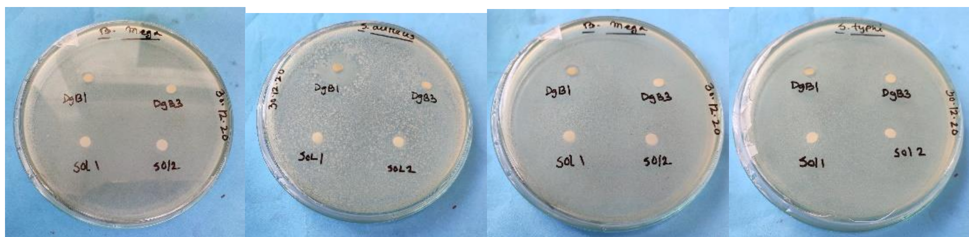


Figure 21: Antimicrobial activity of standard disc against A. *E. coli*, B. *S. typhi*, C. *S. aureus*, D. *B. megaterium*, E. *A. niger*, F. *A. flavus*

D. **E.** **F.**

Figure 22:.



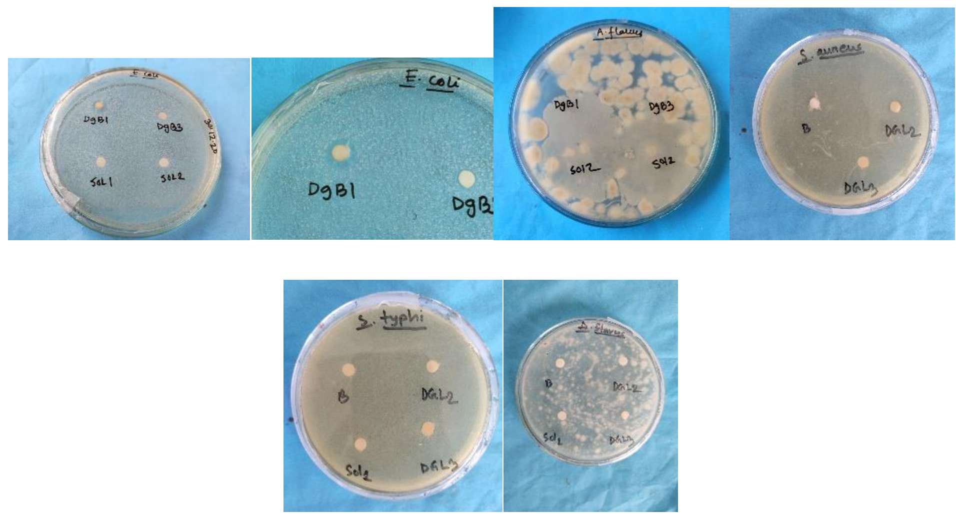


Figure 22: Antimicrobial activity of chosen samples

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4.8 Evaluation of antioxidant activity by DPPH free radical scavenging method

The fungal extracts DgB1, DgB3, DgL3, and DgL4 were subjected to free radical scavenging activity by the method of Brand-Williams et al., 1995. For, reference standard Ascorbic Acid (AA) and Butylated Hydroxy Anisole (BHA) were used.

4.8.1 Antioxidant activity of ascorbic acid (ASA)

At 25 µg/mL dose ASA showed about 95% free radicals scavenging activity. However, at the lowest dose of 3.125 µg/mL it also had more than 36 % activity. Its IC50 value was very low.

Table 13: IC50 value for ascorbic acid (ASA)

Absorbance of control	Concentration (µg/ml)	% of Inhibition	IC50 value (µg/ml)
0.274	50	96.20	2.32
	25	95.49	
	12.5	67.57	
	6.25	71.89	
	3.125	35.90	
	1.5625	13.71	
	0.78125	3.50	

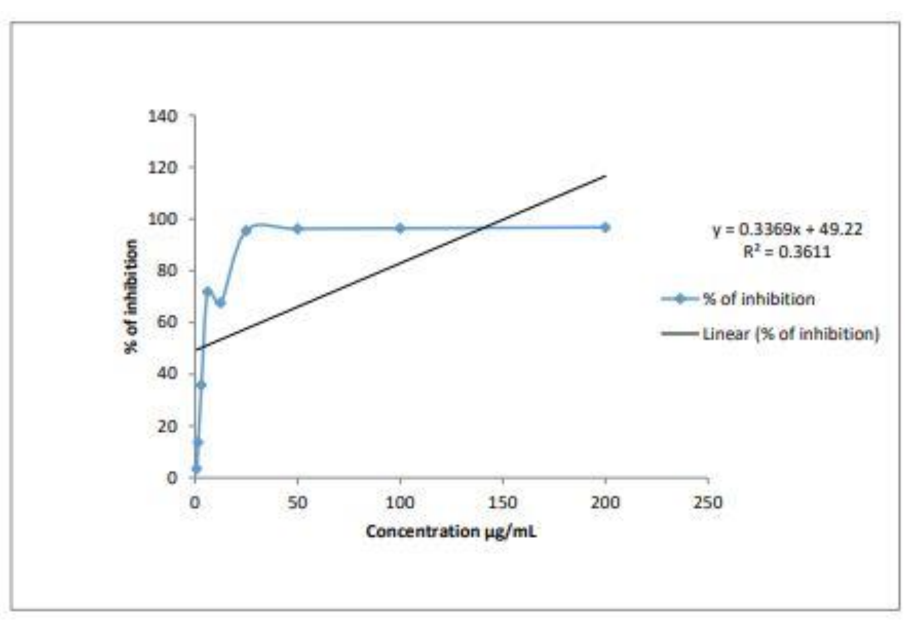


Figure 23: Dose-response relationship on free radical scavenging activity of ascorbic acid (ASA

4.8.2 Antioxidant activity of Butylated Hydroxy Anisole (BHA)

At 50 µg/mL dose BHA showed more than 91% free radicals scavenging activity. However, at the lowest dose of 0.78125 µg/mL, it also had about 10% activity. It's IC50 value was very low 11.6.

Table 14: IC50 value for Butylated Hydroxy Anisole (BHA)

Absorbance of control	Concentration (µg/ml)	% of Inhibition	IC50 value (µg/ml)
0.274	50	91.45	11.6
	25	86.71	
	12.5	70.52	
	6.25	43.33	
	3.125	37.70	
	1.5625	21.88	
	0.78125	9.8	

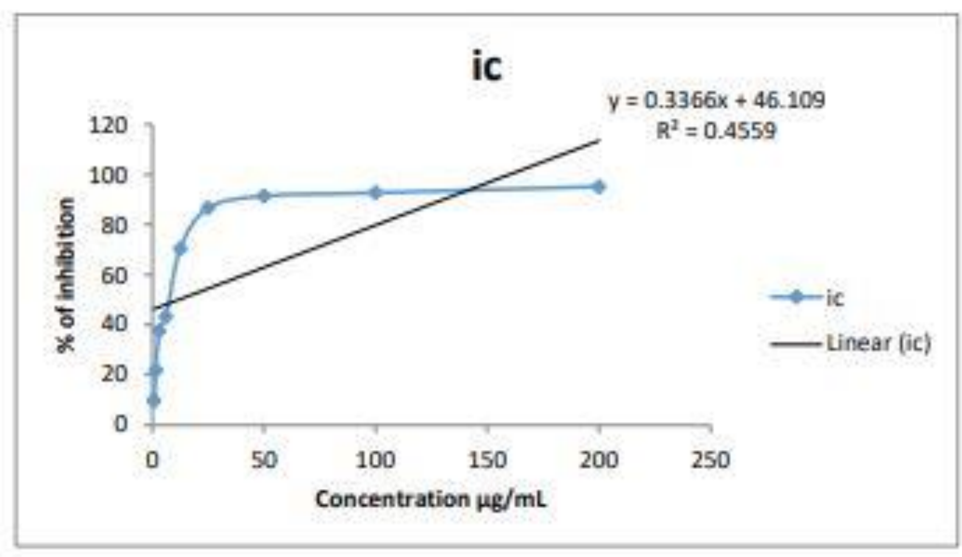


Figure 24: Dose-response relationship on free radical scavenging activity of Butylated Hydroxy Anisole (BHA).

4.8.3 Antioxidant activity of DgB1

The maximum dose (200µg/ml) of the DgB1, showed about 86.% free radical scavenging activity. The IC50 value of CFB-1 was 52.61µg/ml, where IC50 values of Ascorbic acid and Butylated Hydroxy Anisole (BHA) were 2.32µg/mL and 11.6µg/mL respectively. Therefore, comparing with the result of standard CFB-1 showed significant antioxidant activity.

Table 15: IC50 value for DgB1

Absorbance of control	**Concentration (µg/ml)**	**% of Inhibition**	**IC50 value (µg/ml)**
0.2474	200	86.01	52.61
	100	81.08	
	50	79.50	
	25	50.58	
	12.5	23.06	
	6.25	6.55	

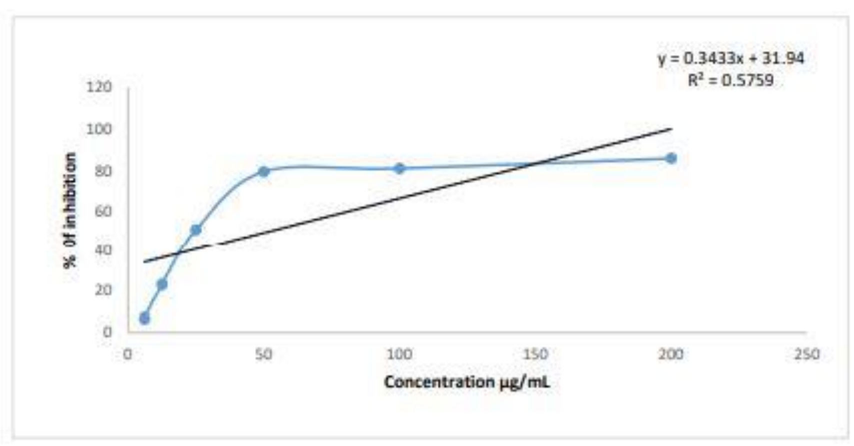


Figure 25: Dose-response relationship on free radical scavenging activity of DgB1

4.8.4 Antioxidant activity of DgB3

The maximum dose (200µg/ml) of the DgB3, showed about 18% free radical scavenging activity. IC50 value of DgB3 was 852.53µg/ml, where IC50 values of Ascorbic acid and Butylated Hydroxy Anisole (BHA) were 2.32µg/mL and 11.6µg/mL respectively. Therefore, comparing with the result of standard CFB-5 showed no antioxidant activity.

Table 16:IC50 value for DgB3

Absorbance of control	**Concentration (µg/ml)**	**% of Inhibition**	**IC50 value (µg/ml)**
0.2474	200	18.34	852.53
	100	17.08	
	50	11.99	
	25	10.78	
	12.5	10.45	
	6.25	9.59	

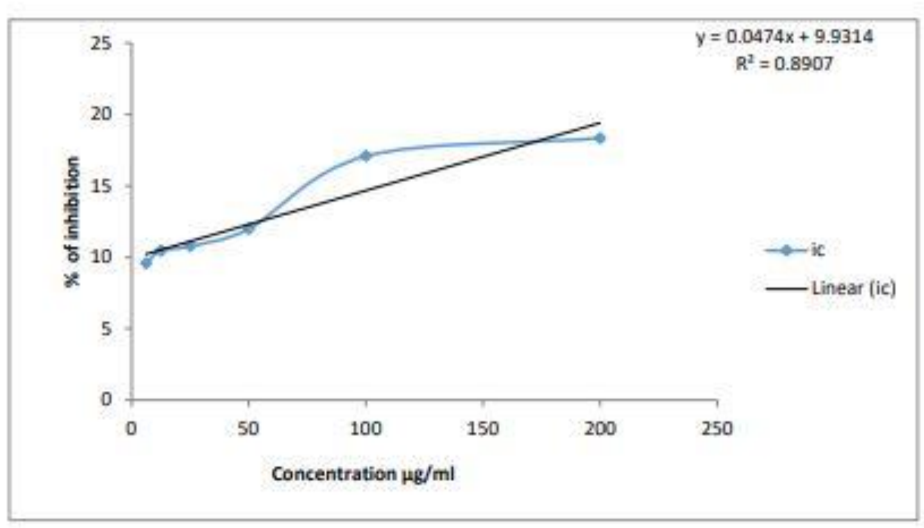


Figure 26: Dose-response relationship on free radical scavenging activity of DgB3

4.8.5 Antioxidant activity of DgL3

The maximum dose (200µg/ml) of the DgL3, showed about 52% free radical scavenging activity. IC50 value of CFB-7 was 184.09µg/ml, where IC50 values of Ascorbic acid and Butylated Hydroxy Anisole (BHA) were 2.32 µg/mL and 11.6µg/mL respectively. Therefore, comparing with the result of standard DgL3 showed moderate antioxidant activity.

Table 17: IC50 value for DgL3

Absorbance of control	Concentration (µg/ml)	% of Inhibition	IC50 value (µg/ml)
0.307	200	52.56	184.09
	100	33.59	
	50	13.62	
	25	7.28	
	12.5	6.91	
	6.125	4.39	

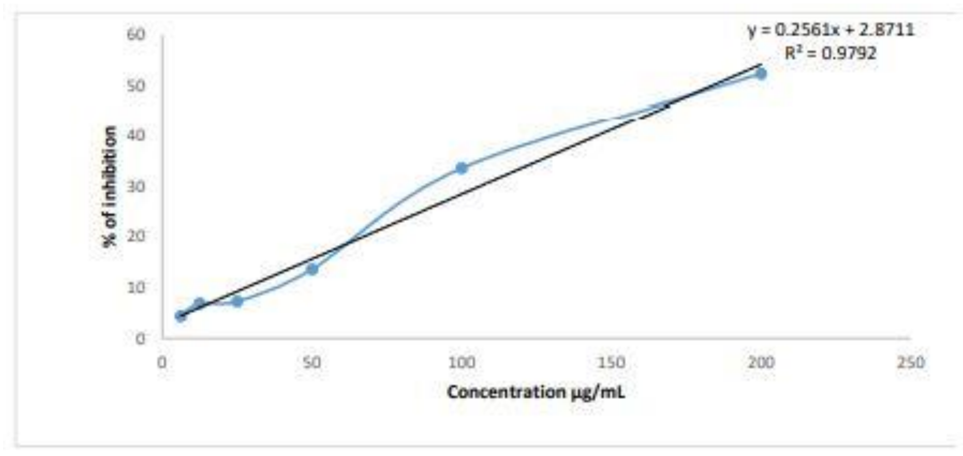


Figure 27: Dose-response relationship on free radical scavenging activity of DgL3

4.8.6 Antioxidant activity of DgL4

The maximum dose (200µg/ml) of the DgL4 extract, showed about 93% free radical scavenging activity. IC50 value of DgL4 was 21.08µg/ml, where IC50 values of Ascorbic acid and Butylated Hydroxy Anisole (BHA) were 2.32µg/mL and 11.6µg/mL respectively. Therefore, compared with the result of the standard the leaf extract showed significant antioxidant activity.

Table 18: IC50 value for DgL4

Absorbance of control	Concentration (µg/ml)	% of Inhibition	IC50 value (µg/ml)
	200	92.40	
	100	90.01	
0.2474	50	88.52	21.08
	25	83.67	
	12.5	71.75	
	6.25	46.40	
	3.125	27.16	
	1.5625	14.71	
	0.71825	6.91	

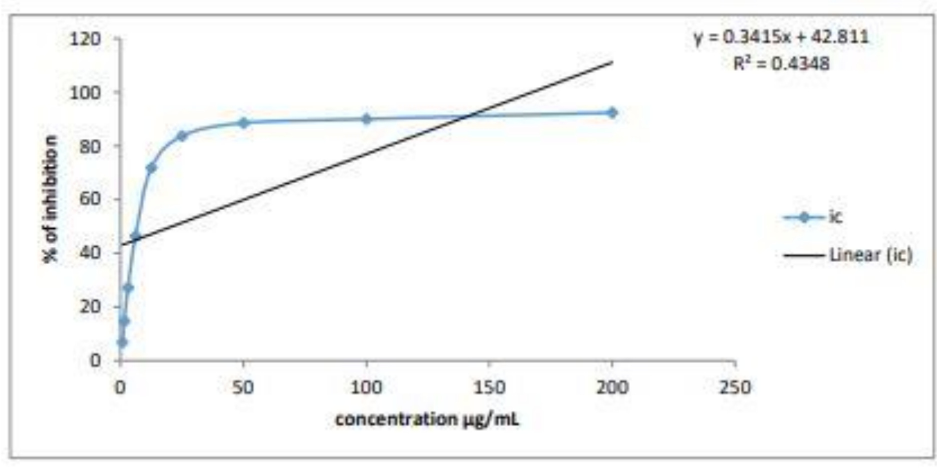


Figure 28: Dose-response relationship on free radical scavenging activity of DgL4

4.6.3 Summary of Antioxidant activity

DPPH is a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule. In the presence of a hydrogen donor, DPPH is reduced. It has been shown that the scavenging effects on the DPPH radical increased with the increasing concentration of the samples to a certain extent. For this work, each extract was measured for its ability to scavenge DPPH free radicals. The results were expressed as a percentage of inhibition of DPPH radical in comparison to BHA and Ascorbic acid. All samples showed moderate activity. The IC50 value of BHA and Ascorbic acid was 11.6µg/ml and 2.32µg/ml respectively. With the different concentrations of samples (6.125-200 µg\ml) the percent of inhibition for DgB1 was 6.55 to 86.01, DgB3 was 9.59 to 18.34, DgL3 was 4.39 to 52.26. Moreover, the percent of inhibition for DgL4 was 46.40 to 92.40. Therefore, the IC50 value of DgB1, DgB3, DgL3, and DgL4 was 52.61 µg/ml, 852.53 µg/ml, 184.09 µg/ml, and 21.08 µg/ml respectively.

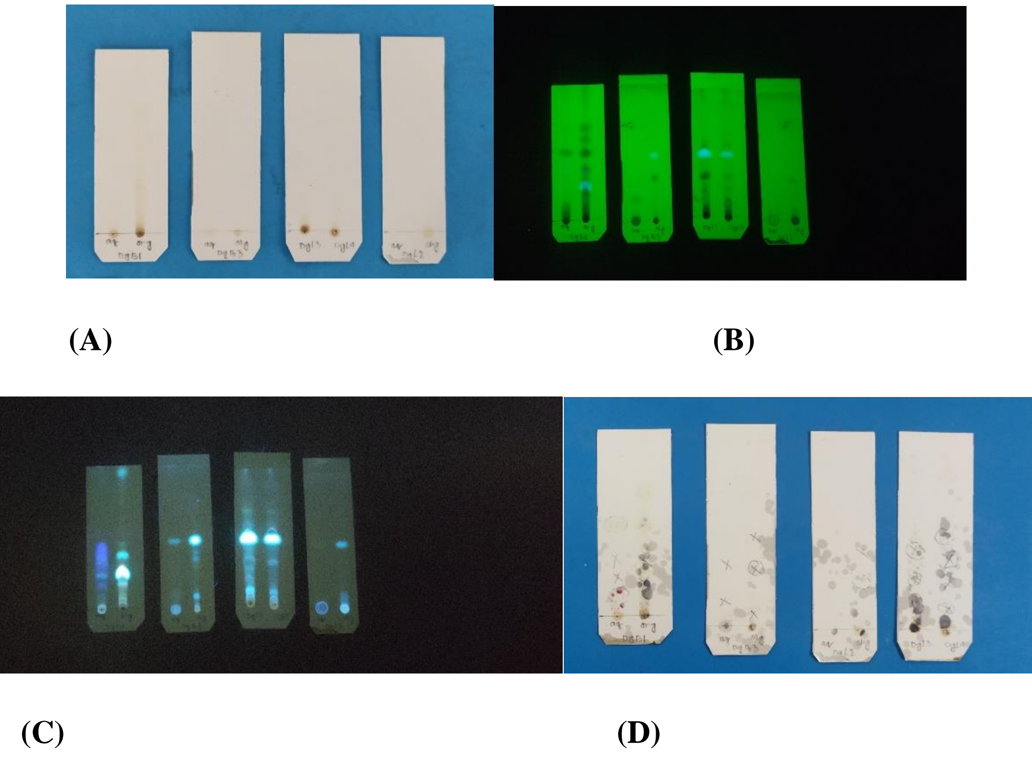
4.9 Preliminary chemical screening of fungal extracts for detection of compounds

Preliminary qualitative chemical tests for the identification of secondary metabolites of all the fungal extracts were carried out by the method described by Trease, 1989 and Harborne, 1973. All extracts were qualitatively tested for the presence of chemical constituents. This screening of extracts was performed by visual detection, UV light both in short at 254 nm and in long at 365 nm, and vanillin-H_2SO_4 spray reagent.

For the presence of different secondary metabolites in the ethyl-acetate or organic extracts of DgB1, DgB3, DgL3 and DgL4 was screened. Thin layer chromatographic technique was used for screening the extracts in different solvent systems. Distinctive compounds showed different color, thus by a preliminary chemical screening of extracts, the resence of different compounds was determined.

Table 19: Preliminary chemical screening of fungal extracts

Strain	Visual Observation		UV 254		UV 365		After Spray		Possible Compund
	Aqueos	Organic	Aqueos	Organic	Aqueos	Organic	Aqueos	Organi c	
DGB1	Nil	Pink Orange Brown Light Brown Brown	Light Green Brown	Blue Light Green Green	Purple Sky Blue Purple Blue	Sky Blue Yellow Sky Blue Dark quenchi ng Purple	Green Dark Brown	Purple Pink Brown	Flavonoid Flavonoid Cumarine Anthocyanine
DGB3	Nil	Brown Light Brown Brown	Light Green Brown	Sky Blue Green Dark Green	Sky Blue Blue	DQS Blue Blue Orange Blue	Purple Orange	Orange	Isocumarine
DGL3	Orange Light Brown	Nil	Green Sky Blue Dark Green Yellow Light Green Brown	Nil	Light Green Purple Blue Green Purple Sky Blue Purple	Nil	Purple	Purple Dark Purple Brown	Flavonoid Anthraquino ne Cumarine
DGL4	Nil	Nil	Green Sky Blue Yellow Orange Light Green Green Brown	Nil	Light Green Purple Dark quencing spot Blue Purple Blue Brown	Nil	Pink Orange	Orange Pink Purple Brown	Isocumarine Flavonoid Anthocyanine



(A) (B)

(C) (D)

Figure 29: Screening of fungal extracts by Thin Layer Chromatography (TLC) at Naked eye (A), at 254 nm (B), at 365 nm (C), and after spraying with spray reagent (D

4.10 Isolation of the compound DgL3

The culture of the strain DgL3 was suspended in ethyl acetate for 12 days for the purpose of cold extraction. The extract was filtered through a fresh cotton bed and finally with Whatman No. 1 filter paper. The volume of the filtrate was concentrated with a rotary evaporator at a low temperature (54˚C) and reduced pressure to yield fungal extract. The extract was then run into thin-layer chromatography for the detection of possible compounds. The compound DgL3 was light yellow to the naked eye. On the TLC plate under UV light at 254nm it appeared sky blue to green in color and appeared as a dark quenching spot, green, blue fluorescence at 365nm. The compound DgL3 was soluble in $CHCl_3$ and Methanol. The compound was then purified through solvent treatment using various mixtures of methanol and DCM. The data of the NMR spectrum is included below. From these data, it was proposed that the isolated compound could be ergosterol.

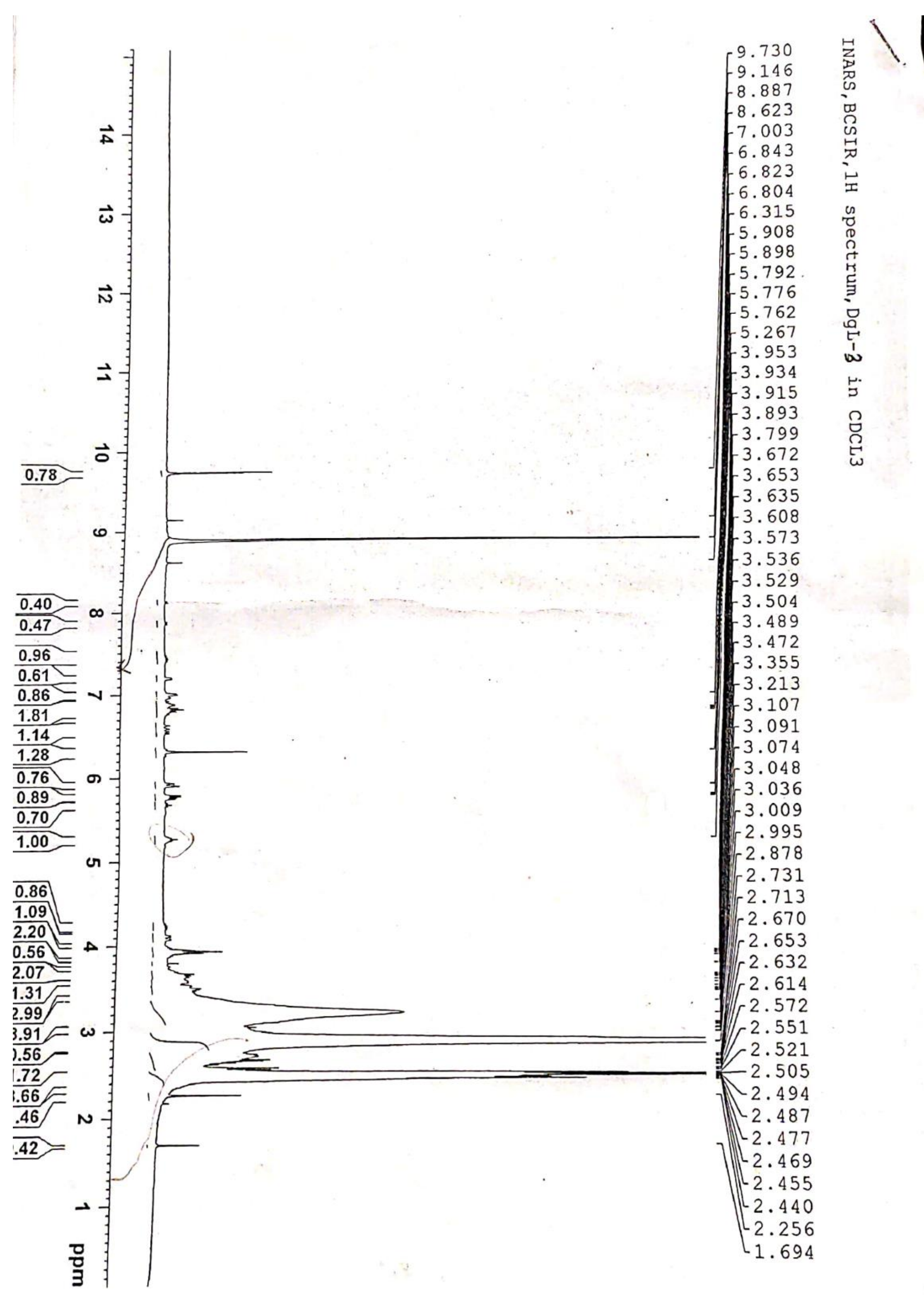


Figure 30: NMR spectrum (400 MHz, CDCl3 + 2 drops MeoD) of compound DgL3

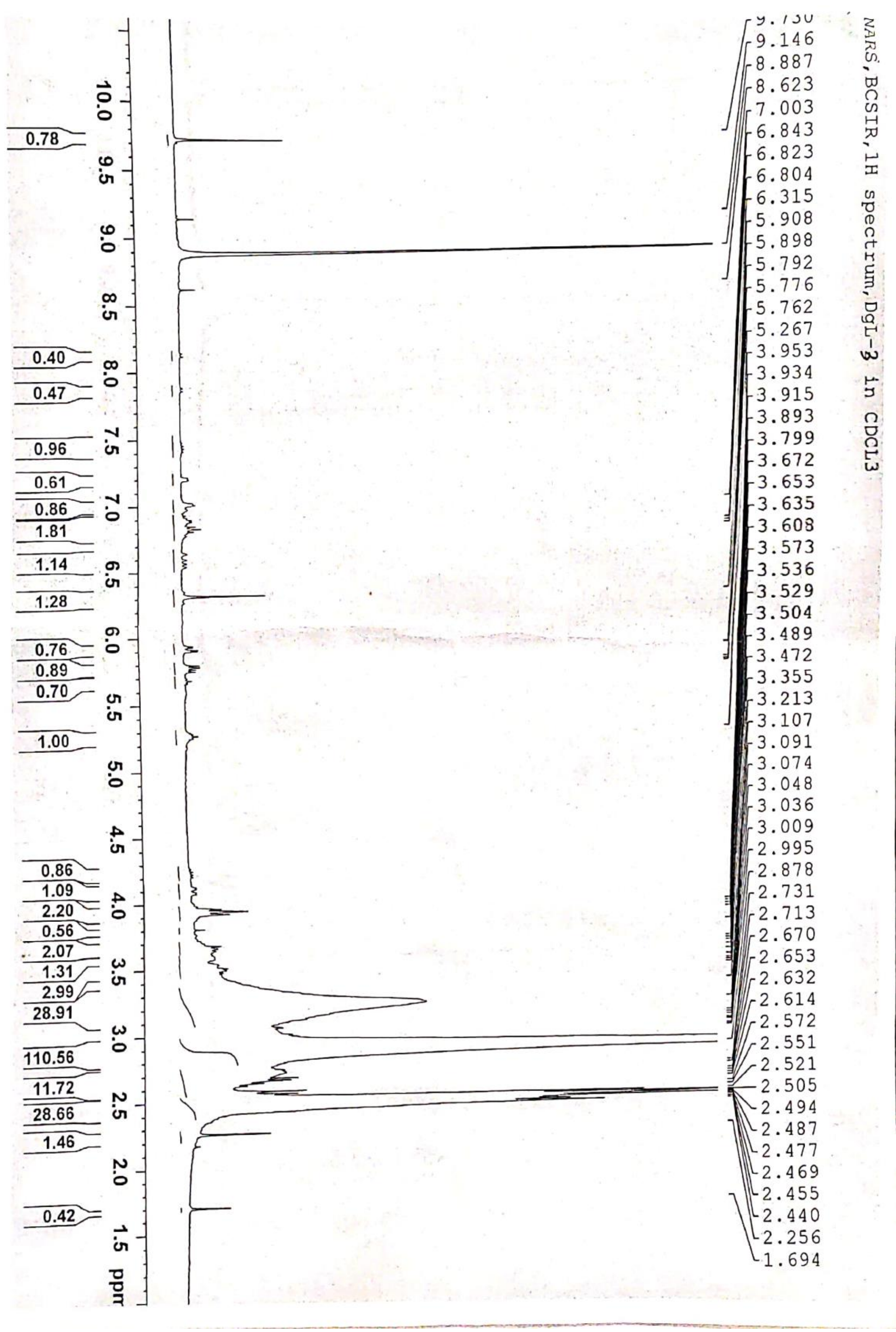


Figure 31: NMR spectrum (400 MHz, CDCl3 + 2 drops MeoD) of compound DgL3

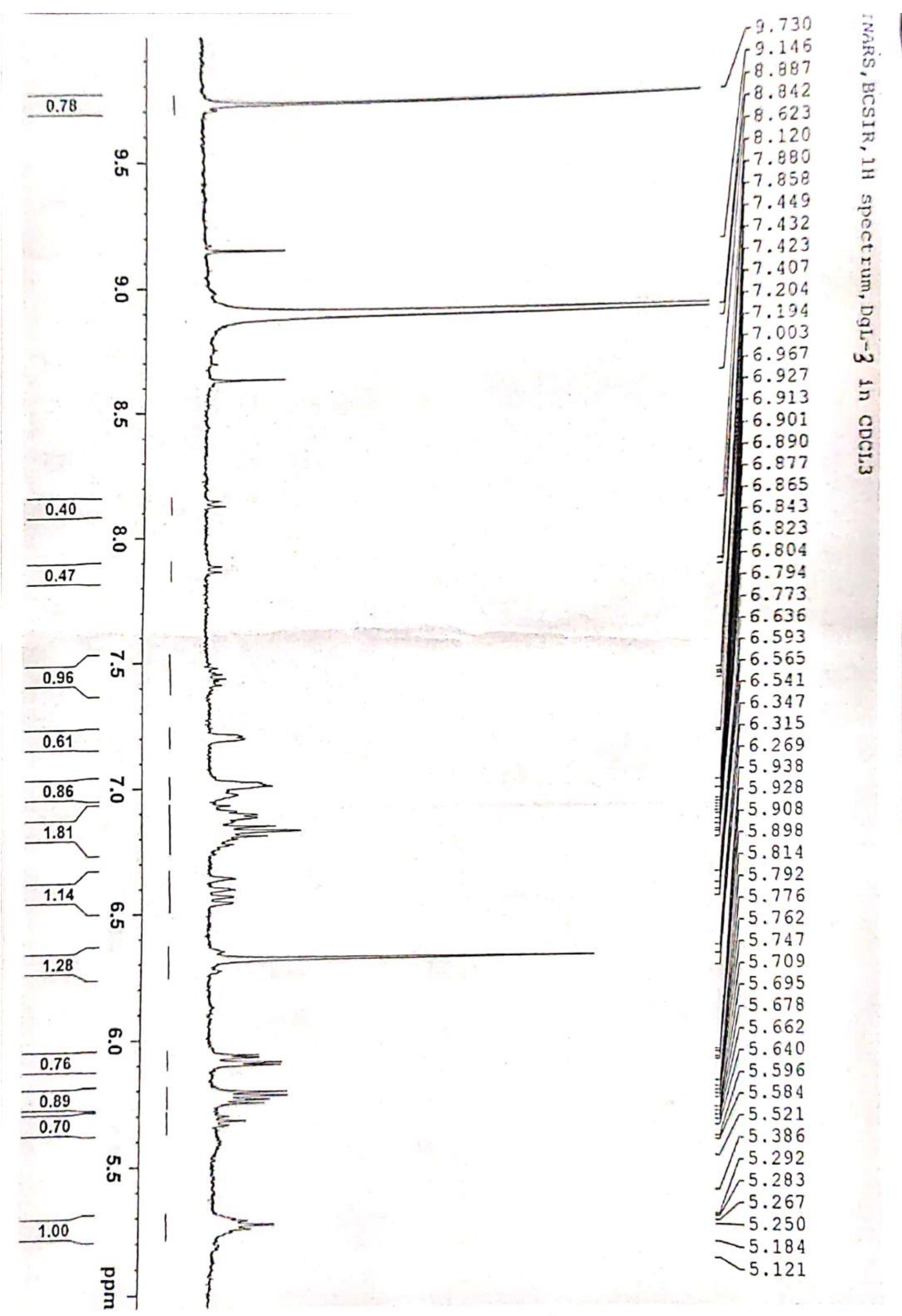


Figure 32: NMR spectrum (400 MHz, CDCl3 + 2 drops MeoD) of compound DgL3.

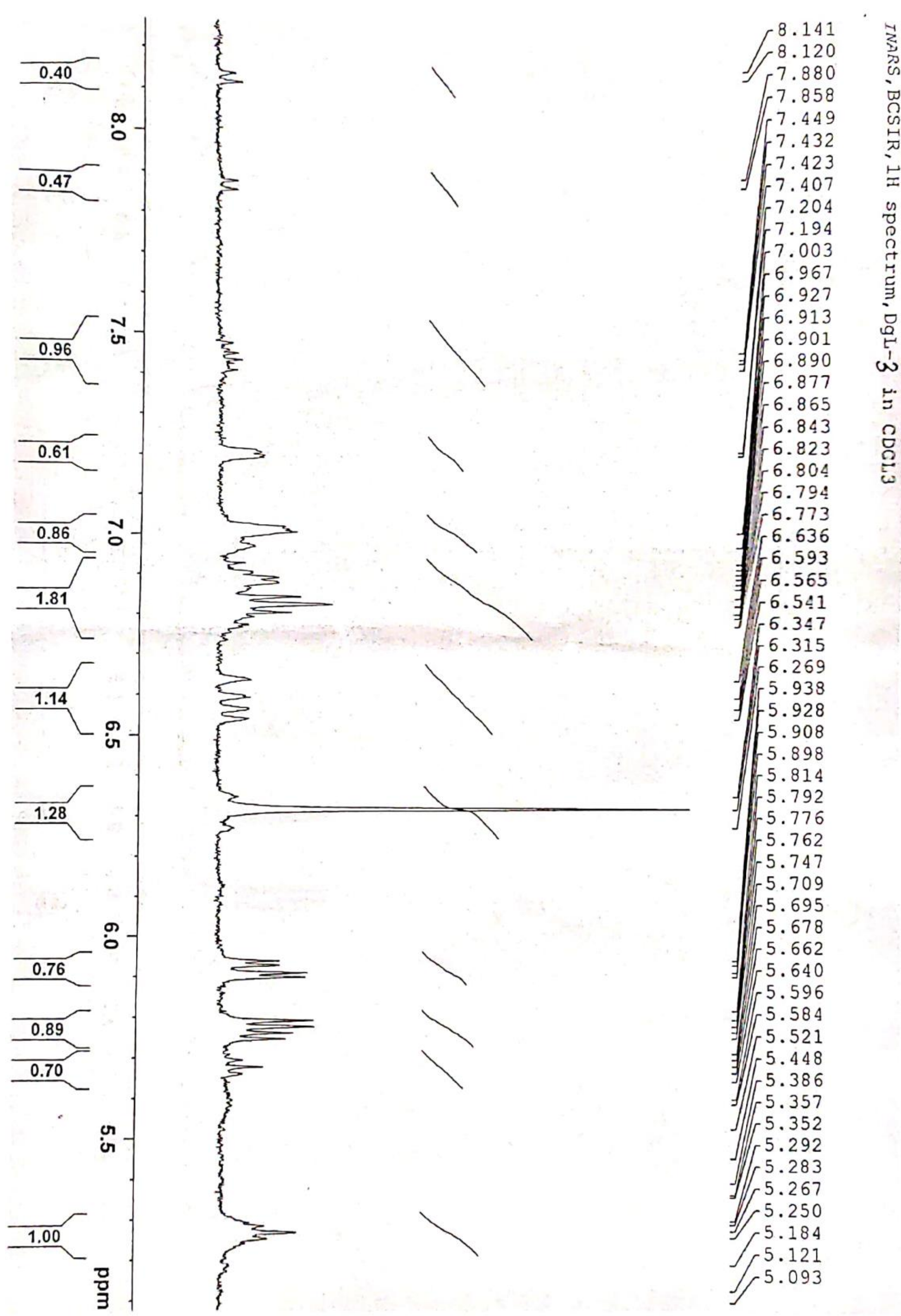


Figure 33: NMR spectrum (400 MHz, CDCl3 + 2 drops MeoD) of compound DgL3

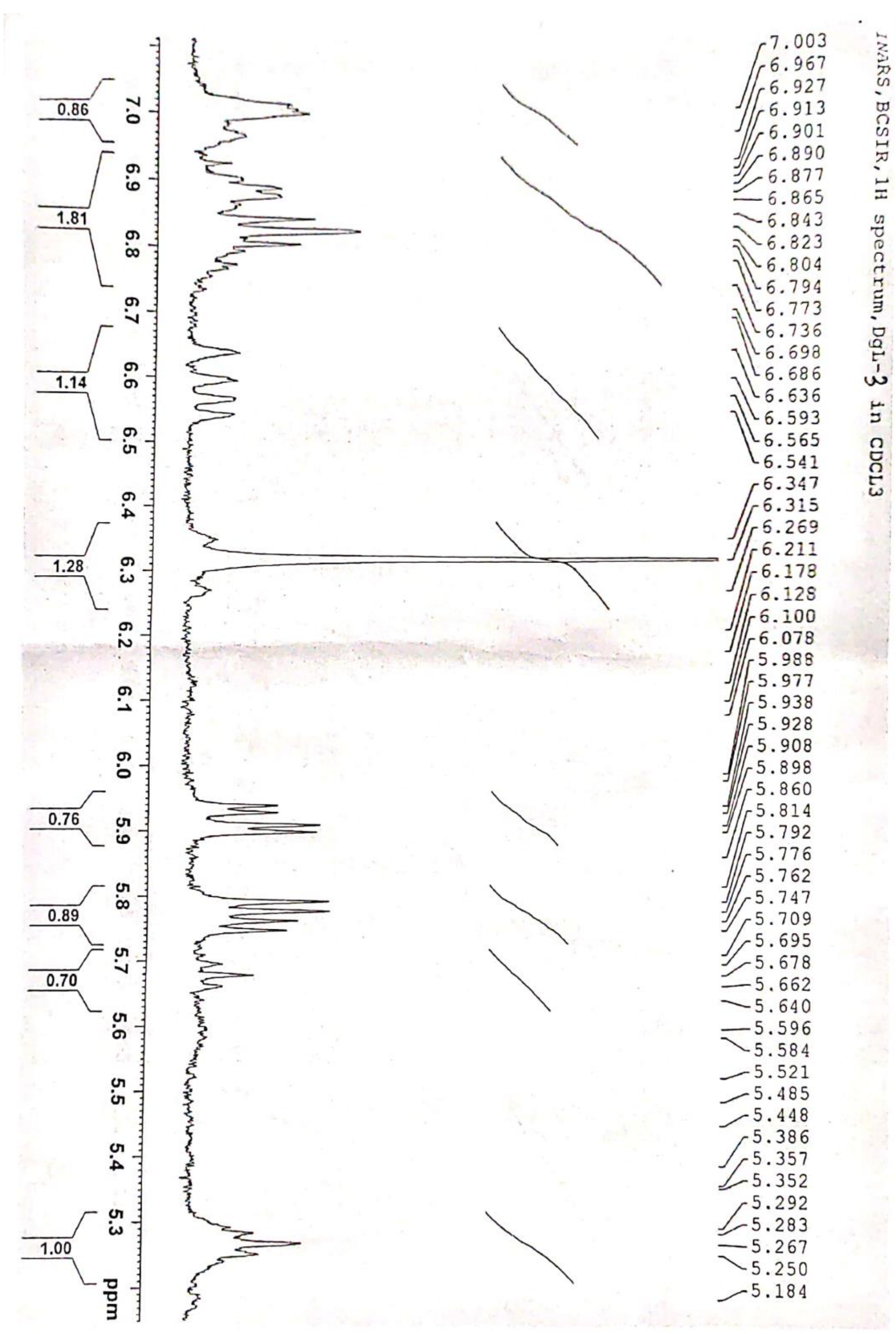


Figure 34: NMR spectrum (400 MHz, CDCl3 + 2 drops MeoD) of compound DgL3.

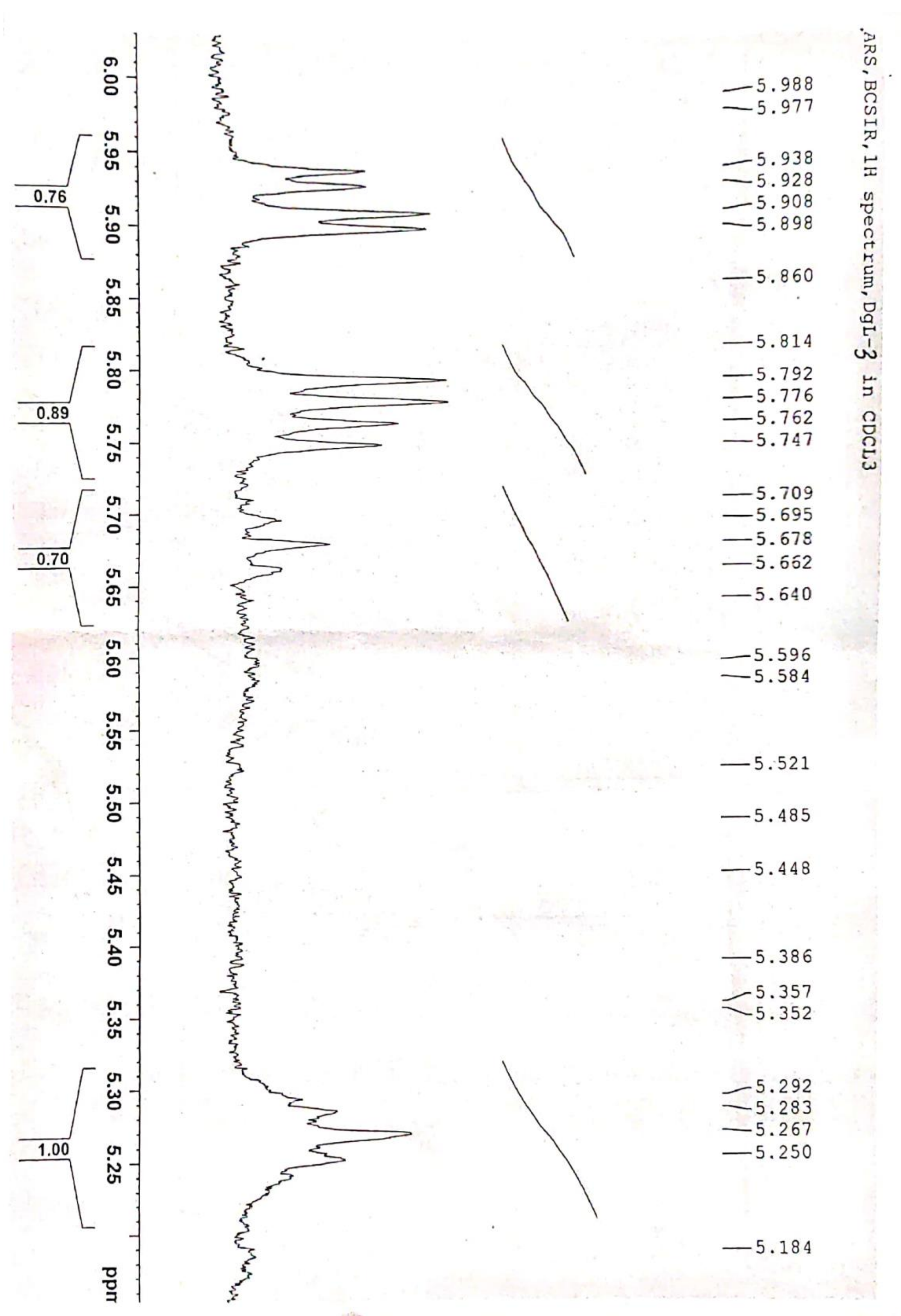


Figure 35: NMR spectrum (400 MHz, CDCl3 + 2 drops MeoD) of compound DgL3

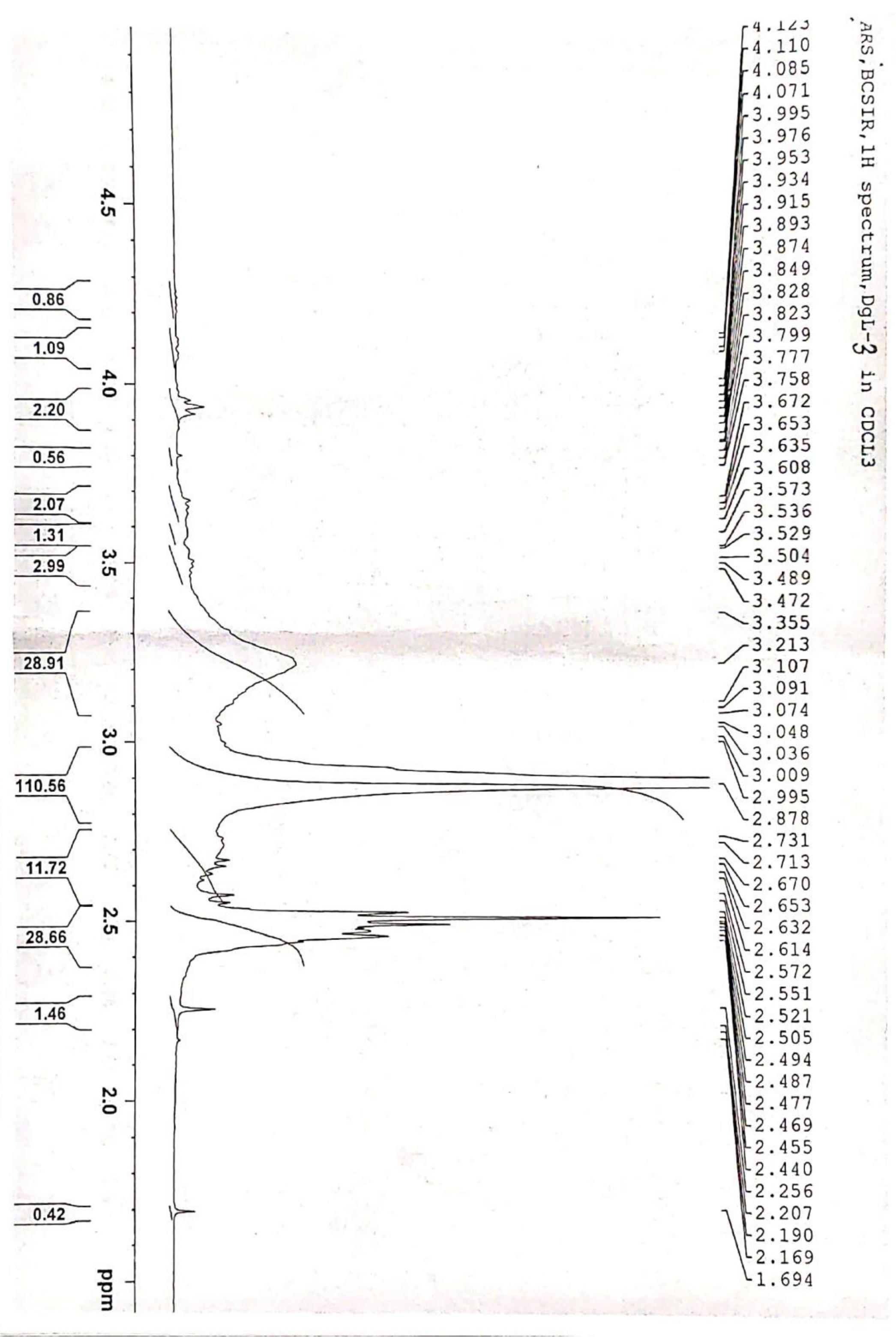


Figure 36: NMR spectrum (400 MHz, CDCl3 + 2 drops MeoD) of compound DgL3.

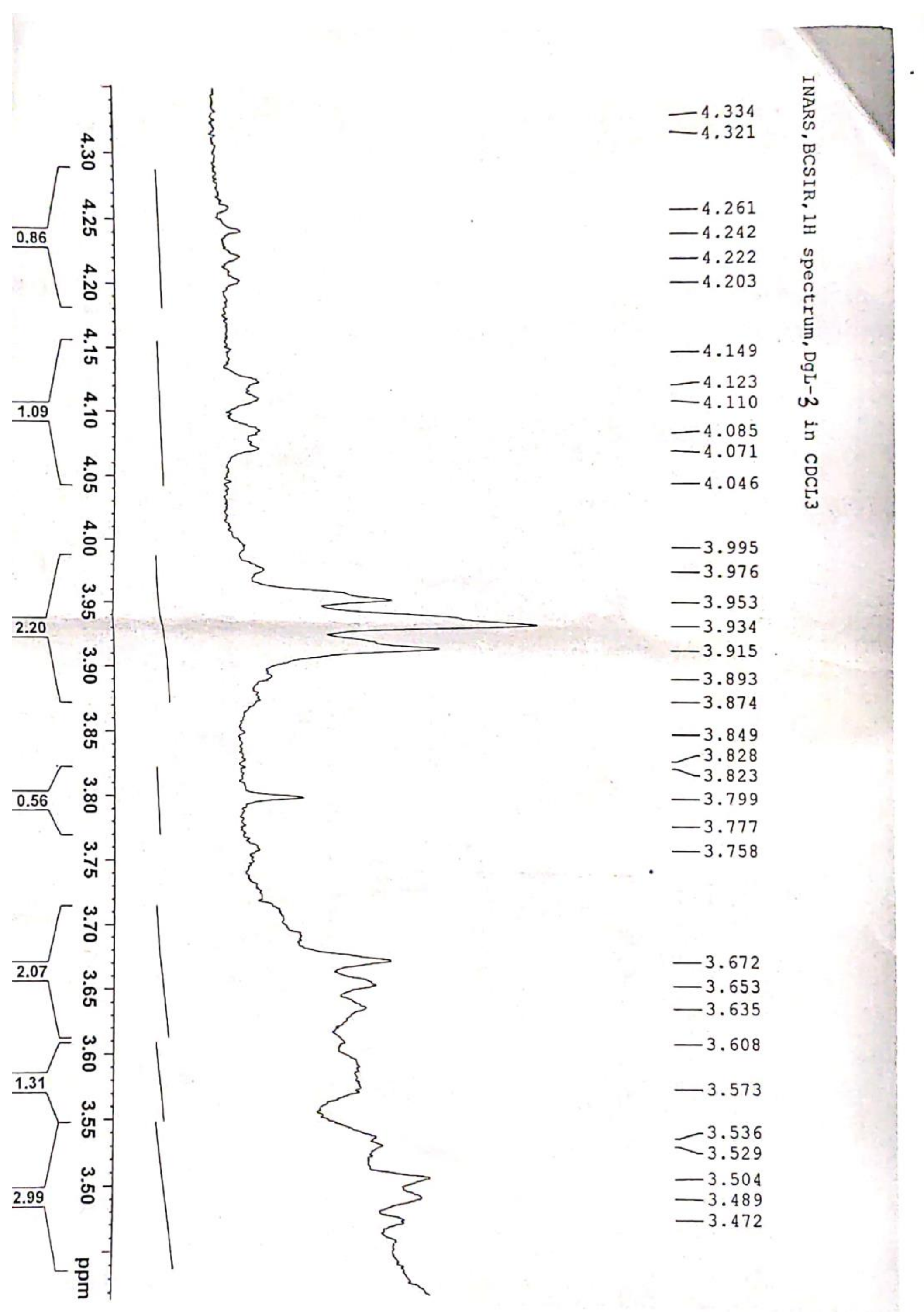


Figure 37: NMR spectrum (400 MHz, CDCl3 + 2 drops MeoD) of compound DgL3.

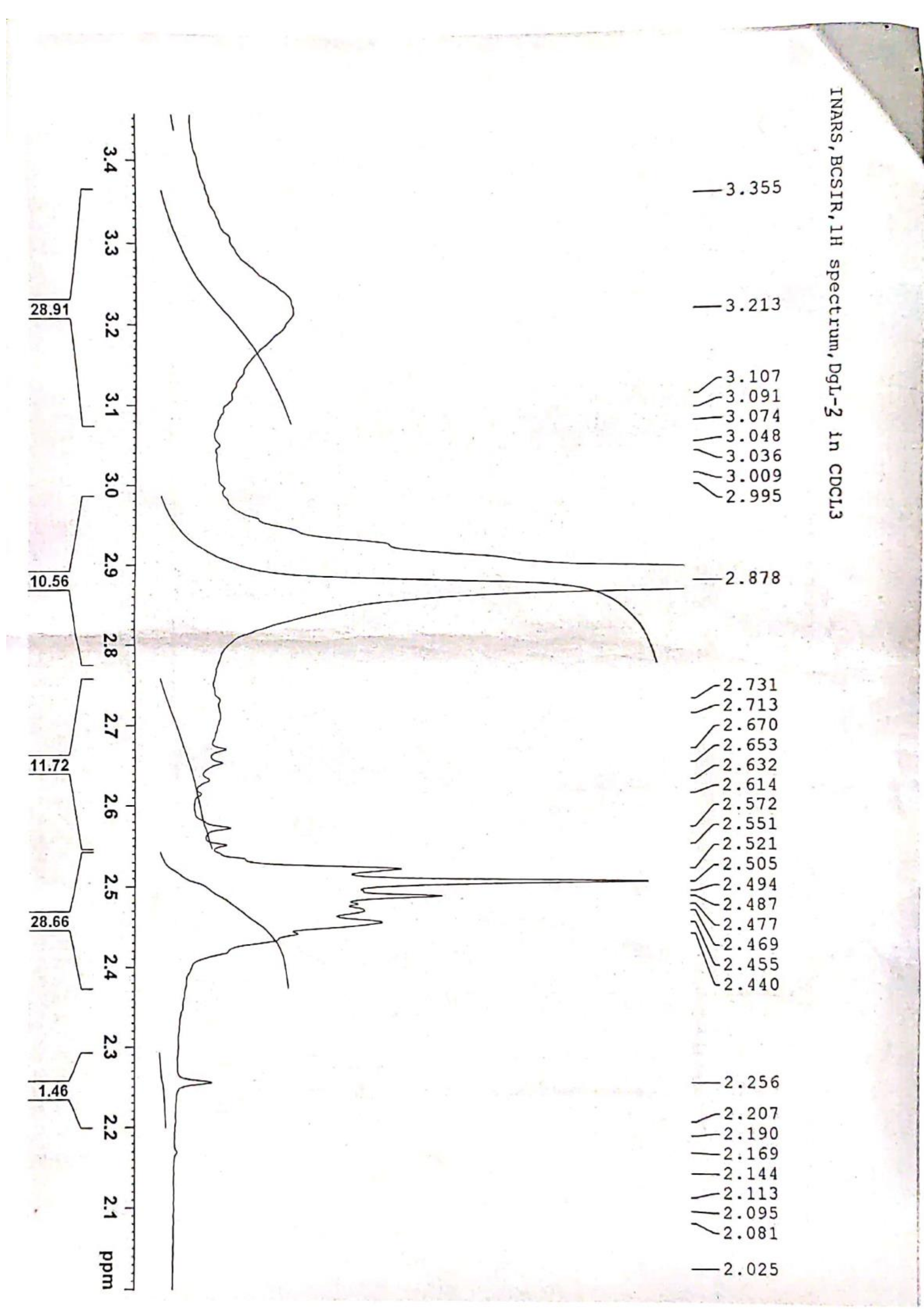


Figure 38:NMR spectrum (400 MHz, CDCl3 + 2 drops MeoD) of compound DgL3

Chapter 5

Discussion

Now a day‘s major concern of researchers is focused on the development of new novel drugs that could be useful in the treatment of various disease problems. These versatile medicinal plants are distinctive sources of various types of chemical compounds, which are accountable for the various activities of the plant. Hence, extensive exploration is required to utilize their therapeutic efficacy to combat diseases. Endophytic fungi are an important component of biodiversity, a source of natural products, and thus a potential source of drug discovery. Endophytic fungi are organisms that live in all healthy plant tissues without signs of disease or morphological changes for at least part of the whole life cycle of the plant (Sunitha et al., 2013). Most of endophytes are capable of producing active metabolites and some of these compounds are proven to have therapeutic values (Zhao et al., 2009). These microbes produce the same bioactive compounds as their host, which increases the interest in further study of this potential natural source. Common genera of Deuteromycetes, such as *Aspergillus, Fusarium, Penicillium* contain an endless number of species and have attracted much attention as a potential source of bioactive compounds. It has been reported that 51% of all biologically active substances have been isolated from endophytic fungi. Endophytic fungi from various plants that are claimed to have medicinal properties have been investigated and shown to produce bioactive metabolites including anticancer, antibacterial, antifungal, anti-malarial compounds. (Bhadury et al, 2006). Thus, endophytic fungi are the potential new leads for natural products.

Punica Granatum (pomegranate) is well known tropical fruit tree abundantly grown in our country. This plant has been used for medicinal purposes in our country as well as in many other countries. This plant is used for the treatment of diarrhea, dysentery, gastroenteritis, hypertension, diabetes, caries, and pain relief. The fruit pomegranate contains vitamins A, and C, iron, phosphorus and calcium, and minerals. It also has secondary metabolites such as antioxidants, polyphenols, antiviral compounds, and anti-inflammatory compounds. In short, the pomegranate plant itself and its extracts are a source of many secondary metabolites, showing a variety of bioactive abilities that could be used as a potential for new drug discovery. That is why for this research, the plant *Punica Granatum* was chosen. The objective of this study was to isolate

endophytic fungi, and their potential bioactive compounds and to explore the possibility of developing new drug candidates from the associated endophytic fungi of *Punica Granatum*. This research work was done at the Pharmaceutical Sciences Research Division, BCSIR Laboratories Dhaka. This work describes in detail the isolation and identification protocol of endophytic fungi originated from *Punica Granatum*.

Previously very little work was done on the topic of endophytic fungi isolated from *Punica Granatum* plant. So, this work might pour light onto something that was not discovered before.

A total of four endophytic fungi were isolated and purified from the plant *Punica Granatum.* Two endophytic fungal strains DgB1 and DgB3 were isolated from the bark of the plant and two strain DgL3 and DgL4 were isolated from the leaf of the plant.

Endophytic fungi were identified by means of morphology (macroscopic and microscopic observation) and molecular identification (DNA sequencing). From the plant *Punica Granatum,* four endophytic fungal strains were isolated and purified. Morphological identification was conducted by culturing the endophytic fungi in petri dishes using potato dextrose agar (PDA) media. The morphological features in 3rd, 6th, 9th, and 12th days of both the top and bottom view of culture were observed and data were noted down in tables. These data were then cross-referenced with probable strains identified by microscopic and molecular results. After cross-referencing the identified isolates were DgB1 as *Diaporthe phaseolorum,* DgB3 *Diaporthe phaseolorum* as *Diaporthe phaseolorum*, DgL3 as *Phomopsis tersa*, and DgL4 as *Colletotrichum siamense.* All four isolated fungal strains were examined under microscopic.
The fungal strains were identified on a molecular basis too. The purified amplified fungal DNA was then submitted for sequencing by a commercial service and the base sequence was compared with publicly available databases such as Gene Bank with the help of Blast-Algorithms. The sequences were aligned using the CLUSTAL W program and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6.0. The phylogenetic reconstruction was done using the neighbor-joining (NJ) algorithm, with bootstrap values calculated from 1,000 replicate runs, using the software routines included in the MEGA software. Each isolated strain was subjected to molecular identification.

Molecular results of DgB1 and DgB3 showed that partial sequencing of the ITS region for the sample resulted in 564 nucleic acid base pairs and showed 99% similarity with the endophyte

Diaporthe phaseolorum, accession number: MT043783.1 from the GenBank database. Therefore, this isolate was recognized as *Diaporthe phaseolorum.*

After molecular analysis of DgL3, showed that partial sequencing of the ITS region for the sample was 561 base pairs long and 100% similarity to the endophyte *Phomopsis tersa*, accession no- MG049670.1 from the GenBank database. Hence, the isolate was identified as *Phomopsis tersa.* Phylogenetic analysis of the 5.8S rRNA-ITS dataset consisted of 17 related taxa from *Phomopsis* species with type and outgroups taxa by maximum likelihood analysis.

The Molecular results of DgL4, showed that partial sequencing of the ITS region for the sample was 557 nucleic acid base pairs and 100% similar to the endophyte of *Colletotrichum siamense,* accession number: MT434640.1 from the GenBank database. So, this isolate was detected as of *Colletotrichum siamense.*

After the small-scale cultivation of all four endophytic fungi, the secondary metabolites were extracted with Ethyl-Acetate. Then, the fungal extracts were tested for their bioactivity.

All four isolates were tested for antimicrobial screening as a part of the bioactivity test. In this investigation antibacterial activity was tested against *Escherichia coli*, *Salmonella typhi, Staphylococcus aureus*, *Bacillus megaterium* and antifungal activity was tested against *Aspergillus niger* and *Aspergillus flavus.* The antibacterial activity can be classified into poor activity (7 – 9 mm), mild activity (10 – 12 mm), moderate activity (13 – 15 mm), good activity (16 - 18 mm), and above 18 mm is significant (Selvi and Balagengatharathilagam, 2014). After the test it was observed that the zones of inhibition for standard disc Kanamycin (30µg) were 33mm (*E. coli*), 29mm (*S. typhi*), 31 mm (*S. aureus)* and 28mm (*B. megaterium*). Hence, Kanamycin showed very significant antibacterial activity. As antifungal standard disc Ketoconazole (30µg) was used. It also exhibited very significant activity against both *A. niger* (28mm) and *A. flavus* (35mm).
To summarize the antibiogram result it can be said that the isolates exhibited poor activity against more or less all the tested bacterial strains. Among them, DgB1 showed good activity against *E. coli, Bacillus megaterium, and S. aureus*. Again, all the isolates were inactive against the fungal strains.

The free radical scavenging activities (antioxidant capacity) of the fungal extracts on the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) were estimated by the method of Brand –Williams

et al., 1995. A stock solution of 1.6 mg of each extract in 0.4 ml methanol was prepared. The test solution was prepared at a concentration of 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, and 200 (µg/mL) by using methanol.

2.0 ml of methanol solution of the extract at different concentration were mixed with 2.0 ml of a DPPH methanol solution (20 µg/ mL). The mixture was properly mixed and kept in a dark place at room temperature for 30 min. The absorbance of the solutions was read at 517 nm against blank. The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of Butylated hydroxyanisole (BHA) and Ascorbic acid (AA) by UV spectrophotometer. Extract concentration providing 50% inhibition (IC50) was calculated from the graph plotted inhibition percentage against extract concentration.

All four fungal extracts DgB1, DgB3, DgL3, and DgL4 were subjected to free radical scavenging activity the method of Brad-Williams et al, 1995. DPPH is a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule. In the presence of a hydrogen donor, DPPH is reduced. It has been showing that the scavenging effects on the DPPH radical increased with the increasing concentration of the samples to a certain extent. For this work, each extract was measured for its ability to scavenge DPPH free radicals. Here, Ascorbic acid (AA) and Butylated Hydroxy Anisole (BHA) were used as the reference standard. The IC50 values were compared to the IC50 value of the standards.

The percent of inhibitions of Butylated Hydroxy Anisole (BHA) for different concentrations (50-0.78125µg/ml) varied from 91% to 10% and the IC50 value was 11.6. In the case of Ascorbic acid (AA), the percent of inhibitions for different concentrations (50- 0.78125µg/ml) varied from 96% to 3.5%. The IC50 value for AA was 2.32. For both standards- BHA and AA, the IC50 values were very low. The low IC50 values were an indication of significant antioxidant activity.

If we look into the IC50 values of the extracts it can be clearly stated that the isolates DgB3 and DgL3 show low antioxidant activity. The isolate DgB1 shows moderate antioxidant activity with IC50 value of 52.61. However, the isolate DgL4 shows significant antioxidant activity with IC50 value of 21.08.

Preliminary qualitative chemical tests for the identification of secondary metabolites of all the fungal extracts were carried out by the method described by Trease, 1989 and Harborne, 1973.

Distinctive compounds showed different color, thus by a preliminary chemical screening of extracts, the presence of different compounds was determined. Light yellow color represents flavonoids, light pink and orange color represents anthraquinone, and pink, and violet are the representatives of steroids and terpenoids while blue and sky-blue color represent coumarin and isocoumarin groups (Harborne, J. B., 1973). Preliminary chemical screening of the plant and fungal extracts revealed the presence of various components such as flavonoids, anthraquinones, coumarins, isocoumarins, and steroids.

Solvent treatment is a process by which a compound consisting of the major portion of a mixture of compounds can be purified utilizing selective solvent washing. Initially, a solvent or a solvent mixture in which the desired compound is practically insoluble and other components are soluble is chosen. The undesired components are separated with repeated washing with this solvent or solvent mixture. If required other solvents or solvent mixtures can be used until a pure compound is obtained. To remove the undesired component, various mixtures of n-Hexane, methanol, and DCM were used. After, this was followed by TLC screening for purity. The isolated compound was denoted as DgL3. The compound DgL3 was light yellow to the naked eye. On the TLC plate under UV light at 254 nm it appeared purple to green in color and appeared as green, and blue at fluorescence at 365nm. The compound DgL3 was soluble in $CHCl_3$ and Methanol.
For the NMR spectrum, the compound was dissolved with chloroform and 2 drops of methanol. The spectral data of the compound DgL3 was not accurate due to the use of expired CHCI3. However, upon expert opinion taken, it was evident that the spectral features of compound DgL2 are characteristics for sterol types of compounds. From the literature and database, it is evident that the endophytic fungi produced steroids like ergosterol. Structure elucidation of compound DgL2 is underway.

Figure 39: Structure of Ergosterol

Chapter 6

Conclusion

We are entering the world of data science. There are lot of data to be gathered from endophytic related to the plants. They are a reliable source of novel natural compounds which have bioactive potentials and thus produce several compounds of pharmaceutical significance. The secondary metabolites produced by them are of great biotechnological importance. These unexplored ecological sources of microflora should be more studied. It is indeed very important to focus on the exploration of endophytic fungi from plant as the need for new natural products are ever increasing. The research on endophytic fungi would open opportunities in the field of agriculture, industry and medicine throughout the world.

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