" MORPHO-MOLECULAR IDENTIFICATION AND MYCO-CHEMICAL PROFILING OF ENDOPHYTIC FUNGI ISOLATED FROM *Psidium guajava*"

By

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

Department of Mathematics and Natural Sciences
BRAC University
December 2022

Declaration

It is hereby declared that

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2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
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The thesis "Morpho-Molecular Identification and Myco-Chemical Profiling of Endophytic Fungi Isolated from *Psidium guajava*" submitted by

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Ethics Statement

No human or animal model was used in this study.

Abstract

Endophytic fungi reside inside healthy living plants without causing any disease or morphological changes for the plant's part or whole life cycle. It increases the plant defense system and produces active secondary metabolites which have therapeutic abilities. These secondary metabolites have the potential for drug development. Isolation and identification of endophytic fungi and screening their associated secondary metabolites from the *Psidium guajava* plant grown in Bangladesh is a recent and new approach. Guava or *Psidium guajava* is used as a fruit and medicinal plant. A total of five endophytic fungi were isolated and purified from the leaf part – PgL1 and bark part – PgB1, PgB2, PgB3, and PgB4 of the plant. All the isolated fungal strains were identified up to genus level based on macroscopic and microscopic characteristics and up to species level based on molecular characteristics- PgB1 as *Phomopsis tersa*, PgB2 as *Diaporthe phaseolorum*, PgB3 as *Colletotrichum siamense*, PgB4 as *Nemania primolutea*, and PgL1 as *Daldinia eschscholtzii*. After small-scale cultivation, the antimicrobial study was carried out for all fungi extracts by disc diffusion method. They all exhibited poor to mild activity against all the tested Gram-negative and Gram-positive bacterial strains. Only PgB4 showed good antibacterial activity. On the other hand, PgB2 and PgB4 showed significant antifungal activity. The antioxidant test was done by the DPPH scavenging method. Among the fungal extracts, PgB3 showed the highest antioxidant activity, PgL1 showed significant antioxidant activity whereas PgB1, PgB2, and PgB4 showed moderate antioxidant activity. Preliminary chemical screening of the fungal extracts by thin layer chromatographic technique revealed the presence of various compounds such as flavonoids, anthraquinones, couramins, isocoumarins, steroids, etc. The extract PgB3 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). The spectral data of the extract suggests the compound was similar to sterol derivatives. The findings of this research indicate the presence of a variety of endophytic fungi in the medicinal plant Guava and that it could be an ideal target for the discovery of potentially bioactive compounds or lead structures for new drugs.

Keywords: Endophytic fungi; *Psidium guajava*; Fungal strains; Fungal extracts; Antimicrobial test; Antioxidant assay.

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May Allah bless and help us all to render something for the cause of mankind.

- Faizah Zaima

  December 2022

Dedicated to my parents

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Chapter 1: Introduction

1.1 Background

Fungi (fungus singular) are a kingdom of single-celled or multicellular eukaryotic heterotrophic organisms that have an important role in every ecosystem, associated with processes like decomposition, recycling, and transportation of nutrients in different environments. As of 2020, 148,000 species of fungi have been described, though the global biodiversity is not fully understood. In 2019, 1,882 new species of fungi were discovered, and also it was estimated that almost 90% of fungi remain unknown. They live in mutualistic, antagonistic, or neutral symbiosis relationships with a wide variety of autotrophic organisms (Saar *et al*., 2001).

Endophytes are an endosymbiotic group of microorganisms – often bacteria or fungi – that colonize the inter- and/or intracellular locations of plants (Pimentel et al., 2011; Singh and Dubey, 2015). For these organisms, all or part of their life cycle occurs within their hosts, without causing any apparent symptoms of the disease. They are ubiquitous and exhibit complex interactions with their hosts, which involve mutualism, antagonism, and rarely parasitism (Nair and Padmavathy, 2014). They produce phytohormones and other bioactive compounds of biotechnological interest (enzymes and pharmaceutical drugs) (Joseph and Priya, 2011; Parthasarathi et al., 2012). Most endophytes are capable of producing active metabolites and some of these compounds are proven to have therapeutic values (Santiago *et al*., 2012; Zhao *et al*., 2008). It has been reported that 51% of all biologically active substances have been isolated from endophytic fungi (Schulze *et al*., 2002). However, these symbiotic groups of organisms are not being exploited as they should. Very few of them are being researched. For the past few decades, it has become evident that the discovery rate of active novel chemical entities is declining.

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 compounds derived from natural products (Lahlou, 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). 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 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.

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). Many other examples abound that illustrate the value and importance of natural products from plants and microorganisms in modern civilization, and paclitaxel (Taxol) is the most recent example of an important natural product that has made an enormous impact on medicine (Wani et al., 1971, Bills et al., 2002). 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).

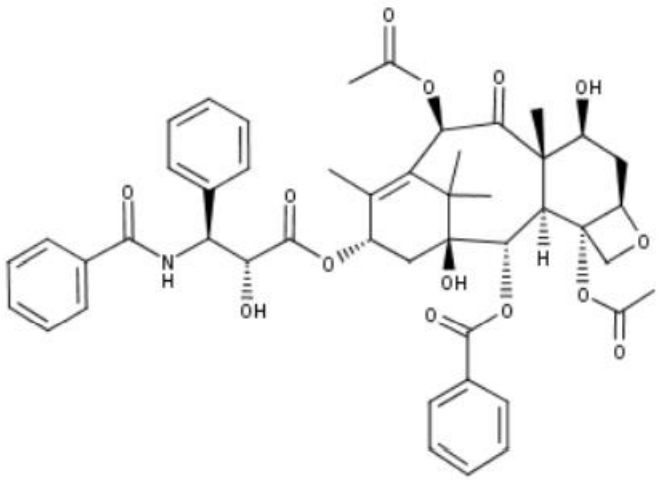


Figure 1. 1: Structure of Paclitaxel

1.3 Endophytic fungi

The presence of non-pathogenic organisms inside the plant was first observed by De Bary in 1866. He detected the presence of microbial cells while analyzing microscopic plant tissues through observation that remained unexplored until the end of the last century. De Bary (1866) provided the first definition of an endophyte, as "any organism that grows within plant tissues are termed as endophytes," Petrini provided the most suitable definition for endophytes in 1991, which means any organism that at some part of its life cycle, colonizes the internal plant tissues without causing any type of harm to the host plant. Another definition was given by Bacon and White in 2000, Endophytic fungi belong to mitosporic and meiosporic ascomycetes that "asymptomatically reside in the internal tissues of plants beneath the epidermal cell layer, where they colonize healthy and living tissue via quiescent infections."

Researchers have indicated the presence of one or more types of endophytes in every single plant studied to date (Strobel and Daisy, 2003). Endophytes can colonize in the stem, roots, petioles, leaf segments, inflorescences of weeds, fruit, buds, seeds, and also dead and hollow hyaline cells of plants (Hata and Stone, 2008; Specian et al., 2012; Stepniewska and Kuzniar, 2013). It is considered that a single plant species could possess thousands of microbes, categorized as epiphytes (microbial inhabitants of the rhizosphere and phyllosphere; those near or on plant tissue) or endophytes (microbes residing within plant tissues in leaves, roots, or stems), depending on their area of colonization in the plant species (Oldroyd et al., 2011; Turner et al.,2013; Andreote et al., 2014).

Numerous attempts have been made in recent years to discover the origin of endophytic organisms in different species (Hallmann et al., 1997; Mitter et al., 2013). At first, it was thought that the rhizosphere or the seed-born microbial communities as the major source of endophytes. Generally, it is known that the interaction of endophytes with plants, their transmission, and their existence should be connected with their genome structure. Researchers have reviewed the genome sizes and origins of endophytes by correlating the genome size with the bacterial lifestyle (Dini-Andreote et al., 2012). Endophytes normally live inside the plant species as the environment is more stable compared to nature. Some exceptional endophytes appear in the plant during a part of its lifecycle. Thus, the endophytic community is made up of organisms from distinct origins, with those with larger genomes likely to live in variable environments, such as soils, while those with

smaller genomes are likely to exist in a stable environment and are vertically transmitted (Mitter et al., 2013).

1.4 Endophytic fungi- host plant association

Endophytes can harmonize their growth with the host plant because there is a unique adaptation between endophytes and the host plant. Host plants produce chemotaxis, different types of secondary metabolites as a resistance mechanism to pathogens. Thus, endophytic fungi colonization is hindered by the secondary metabolites produced by host plants. As a result, endophytic fungi secrete matching detoxification enzymes- cellulases, xylanase, lactase, and protease to decompose secondary metabolites before they penetrate through the defense systems of the host plant. Once inside the tissues of a host-plant, the endophytic fungi assumed a quiescent (latent) state, either for the whole lifetime of the host plant (neutralism) or for an extended period (mutualism or antagonism) until environmental conditions are favorable for endophytic fungi or the ontogenetic state of the host changes to the advantage of the fungi.

1.5 Environmental and host plant factors influencing endophytic fungi

The population structure or distribution pattern of endophytic fungi depends on the variation in environments and the genetic background of host plants. Data from the reference analysis suggested that some environmental conditions, such as temperature, humidity, illumination, geographic location, and vegetation significantly affected the distribution pattern of endophytic fungi (Suryanarayanan et al., 2005; Song et al., 2007). For example, particular conditions determined the distribution ranges of host plants that in return determined the species of endophytic fungi and their spore germination, growth, reproduction, and metabolism during the entire life cycle. Similarly, results from the analyses suggested that the distribution of the certain endophytic fungal population was only restricted to particular host plant species (or families) and particular genetic backgrounds (genotypes) of a species (Dai et al., 2003; D'Amico et al., 2008).

1.5.1 Influence of environmental factors

Environmental conditions- temperature, humidity, and levels of soil nutrition determine the types and quantity of secondary metabolites the host plants would produce which indirectly affect the population structure of the endophytes. For example, under the conditions of a low mean annual sunshine hour and a high mean annual humidity, the host medicinal plants would produce more

nutrients that were suitable for the colonization, reproduction, and dissemination of the endophytic fungi (Wu et al., 2013). In contrast, under cold climatic conditions and inappropriate rates of respiration, oxygen concentration, and pH value, only certain types of host species could successfully grow.

1.5.2 Influences of the genetic background of host plant

The host plant's genetic background affects the expression of the endophytic fungal species parasite or mutualistic lifestyle. The fungal gene expression in response to host or host recognition in response to the fungi determines the directionality of the entire host-fungus symbiosis. Based on the facts indicated by the analyzed references that the fitness of the endophytic fungi largely depended on the fitness of the host plants, suggesting that the host plants largely determined the colonization and distribution of endophytic fungi in the host plants (Saikkonen et al., 2004). Furthermore, the phase disposition (age) of host plants and tissues may likewise influence the species composition of the endophytic community (Sieber, 2007). In addition, differential substrates utilized by different endophytic species demonstrated their resource distribution strategy when lived in the same organ of a host (Carroll and Petrini, 1983), reducing the competition between the endosymbionts.

1.6 Benefits from endophytic fungi to host plants

After colonization endophytic fungi confer some benefits to their host plants. The three types of beneficial aspects of the endophytic fungi-host relationship are-

1.6.1 Promoting the growth of host plants

Endophytic fungi could enhance the growth of host plants by increasing various hormones such as indole-3-acetic acid, and cytokines. Most hormones produced by endophytes are isolated from the roots of host plants. Again, endophytic fungi could also obtain nutritional elements- nitrogen, and phosphorus so that the host plants could use them for growth. For example, *Mycena dendrobii* could promote the seed germination and growth of the host plant *Gastrodia elata* by secreting indoleacetic acid (Guo and Wang, 2001).

1.6.2 Increasing the resistance to stresses of host plants

From several studies, it is found that some endophytic fungi could increase the resistance to biotic and abiotic stresses of host plants. In symbiotically conferred stress tolerance, the endophytic fungi were considered to act as a type of biological trigger that activated the defense systems of a host (Rodriguez and Redman, 2008). Some examples are- the endophytic fungi *Chaetomium* sp. produces abscisic acid which affects the motility of the second stage of juveniles of root-knot nematode *Meloidogyne incognita*, thus inhibiting the insects and increases the defense mechanism of the host plant *Cucumis sativus (*Yan et al., 2011).

1.6.3 Endophytic fungi as a source of secondary metabolites

Endophytes are reported to produce several bioactive metabolites in a single plant or microbe which served as an excellent source of drugs for treatment against various diseases and with potential applications in agriculture, medicine, food, and cosmetics industries (Strobel and Daisy, 2003; Jalgaonwala at al., 2011; Godstime et al., 2014; Shukla et al., 2014). Some endophytic fungi produce secondary metabolites. For example, podophyllotoxin (Eyberger et al., 2006; Puri et al., 2006), deoxypodophyllotoxin (Kusari et al., 2009a), hypericin and emodin (Kusari et al., 2008, 2009b), and azadirachtin (Kusari et al., 2012). Paclitaxol is the best-known example of anticancer compound taxol was found in the taxol-producing endophytic fungi *T. andreanae* that was isolated from *T. brevifolia* (Stierle et al., 1995).

1.7 Endophytes as a source of the drug against diseases

Infectious and parasitic diseases account for approximately half of the deaths worldwide (Menpara and Chanda, 2013). Medicinal plants and their endophytes are found to be an important source of more than 80% of natural drugs available in the market (Singh and Dubey, 2015). The emergence of multi-drug resistance microorganisms is another global problem. The discovery of novel antimicrobial secondary metabolites and bioactive compounds from different types of endophytic microorganisms is an important alternative to overcome the increasing levels of drug resistance to various pathogenic microorganisms (Godstime et al., 2014). Some broadly used antibiotics, antifungals, and antioxidants are produced by using the bioactive compounds of endophytic fungi. Some examples are – endophytic fungi *Cladosporium* sp. produces bioactive compounds Cardiac

glycosides, and phenolic compounds which are used as curative agents against pathogens *Klebsiella pneumonia, Proteus* sp. (Selvi and Balagengatharathilagam, 2014).

Fusarium proliferatum is effective against diseases caused by *Clostridium botulinum, Listeria monocytogenes* by producing bioactive agents Beauvericin, Kakadumycin (Meca et al., 2010, Golinska et al., 2015). *Ganoderma boninense* produces Rapamycin, Petalostemumol which are used against *Bacillus subtilis* (Parthasarathi et al., 2012)

1.8 Objectives of the study:

In this study, the associated endophytic fungi of *Psidium guajava* were isolated, identified and their biochemical activity was evaluated. There is no report regarding the isolation, identification, and search of novel bioactive compounds from endophytic fungi derived from *Psidium guajava* of Bangladesh.

Therefore, the objectives of the study are-

- To isolate pharmacologically active endophytic fungi from *Psidium guajava.*
- To identify the isolated endophytes.
- To isolate and characterize the bioactive metabolites of the study.
- To explore the possibility of developing new drug candidates from the associated endophytic fungi.

Chapter 2: Literature Review

The investigated plant for this research was Guava (*Psidium guajava*). A brief review of this plant is given below-

2.1 Habitat

Guava under the genus *Psidium* has about 133 genera and more than 3800 species all around the world. The Guava plant grows widely in the tropic areas because it is a plant that could grow on a big range of soils (Jimenez-Escrig et al., 2001). Guava is originally produced in tropical America. Though it can grow all over the tropics and subtropics, from Peru to Mexico. The major producing countries are India, Mexico, Brazil, Pakistan, Sri Lanka, Bangladesh, Burma, Thailand, Malaysia, Indonesia, Philippine etc.

The optimum temperature of growth for guava is 23-28 °C., but the mature trees can grow up to 45°C. Another important factor for guava production is rain. The ideal rain for growing guava is about 100 cm per annum distributed throughout the year. The best soil for guava is well-drained and rich in organic matter clay loam to sandy loam. The optimum pH of the soil should range from 4.2 - 8.2 and also should contain enough moisture.

2.2 Morphology

Guava is a medium-height evergreen shrub tree, ranging from 2 to 10m with shallow roots. The plant has a wide-spreading network of branches. Mostly its branches are curved and display opposite leaves with small petioles of about 3 to 16 cm. The leaves are wide and clear green in color and have clear and prominent veins (Arima and Danno, 2002; Rouseff et al., 2008). The plant produces white flowers with incurved petals having a nice fragrance. Flowers have four to six petals and yellow-colored anthers and pollination occurs by the insects (Naseer et al., 2018). Guava flowers arise in solitary or in cymes (2-3 flowers in a cluster) from the axils of the leaves. The flowers are hermaphrodites, pollinated by air or insects. About 80-86% of flowers set fruits but finally, 50-60% of fruits reached maturity as the initial shredding of flowers is usual.

The guava fruit is edible, round to pear-shaped, pale green to yellow at maturity in some species, pink to red in others in color. The size of the seeds is very small and they are easily chewable. They are arranged in regular patterns; their number ranges from 112 to 535 (Morton JF, 2004; Kumar et al., 2011). The guava fruit is important for the reproduction of plants.

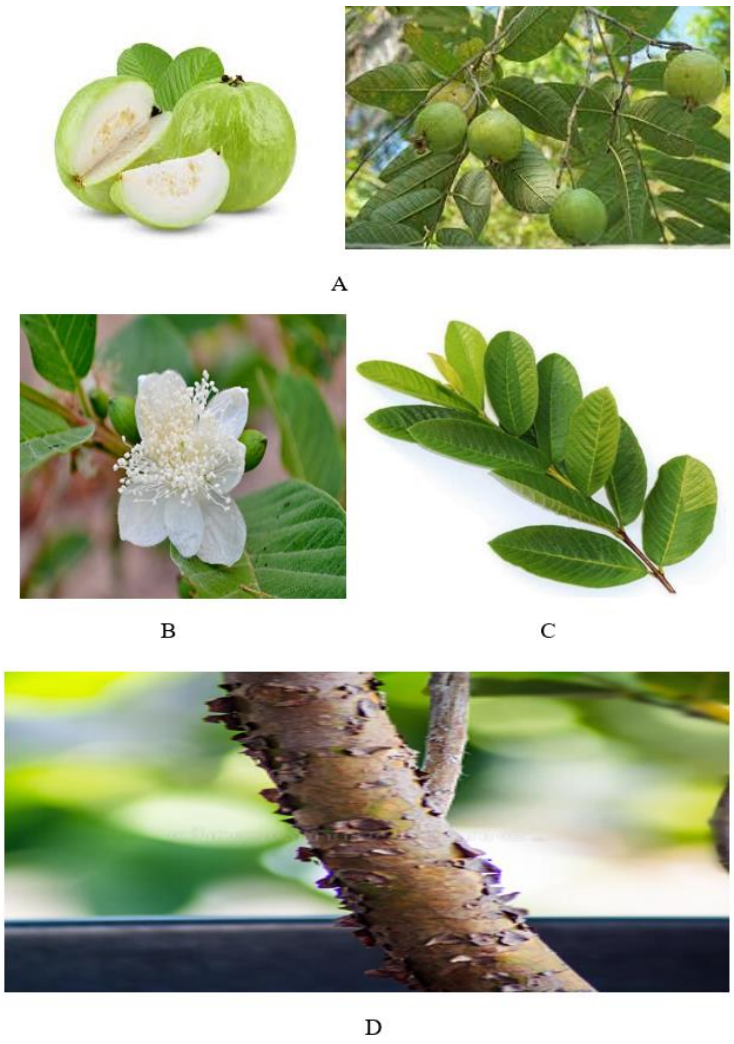


Figure 2. 1: Guava A) fruit, B) flower, C) leaves, and D) bark

2.3 Phytochemical Constituents

Guava has a high content of vitamin C and vitamin A, flavonoids, fructose sugar, and carotenoids (Das AJ, 2011; Khan MIH, 1985). It is also a very good source of important dietary fiber pectin. Guava contains a large number of antioxidants and phytochemicals including essential oils, polysaccharides, minerals, vitamins, enzymes, and triterpenoid acid alkaloids, steroids, glycosides, tannins, flavonoids, and saponins (Arima and Danno, 2002; Smith & Siwatibau, 1975).

Guava fruit contains terpenes, caryophyllene oxide, and p-selinene in large quantities which produce relaxation effects (Meckes et al., 1996). There are 41 hydrocarbons, 25 esters, 13 alcohols, and 9 aromatic compounds in guava (Vernin et al., 1991). Essential oil is present in leaves that contain α-pinene, limonene, β-pinene, isopropyl alcohol, menthol, terphenyl acetate,

caryophyllene, longicyclene and β-bisabolene. Oleanolic acid is also found in the guava leaves (Begum et al., 2004). Leaves have a high content of limonene about 42.1% and caryophyllene about 21.3% (Ogunwande et al., 2003)

The bark includes 12–30% of tannin and one source declares that it includes tannin 27.4%, or polyphenols, resin, and crystals of calcium oxalate. Tannin is also present in roots. Leukocyanidins, gallic acid, and sterols are also present in roots. Carbohydrates with salts are present in abundance. Tannic acid is also a part (Naseer et al., 2018).

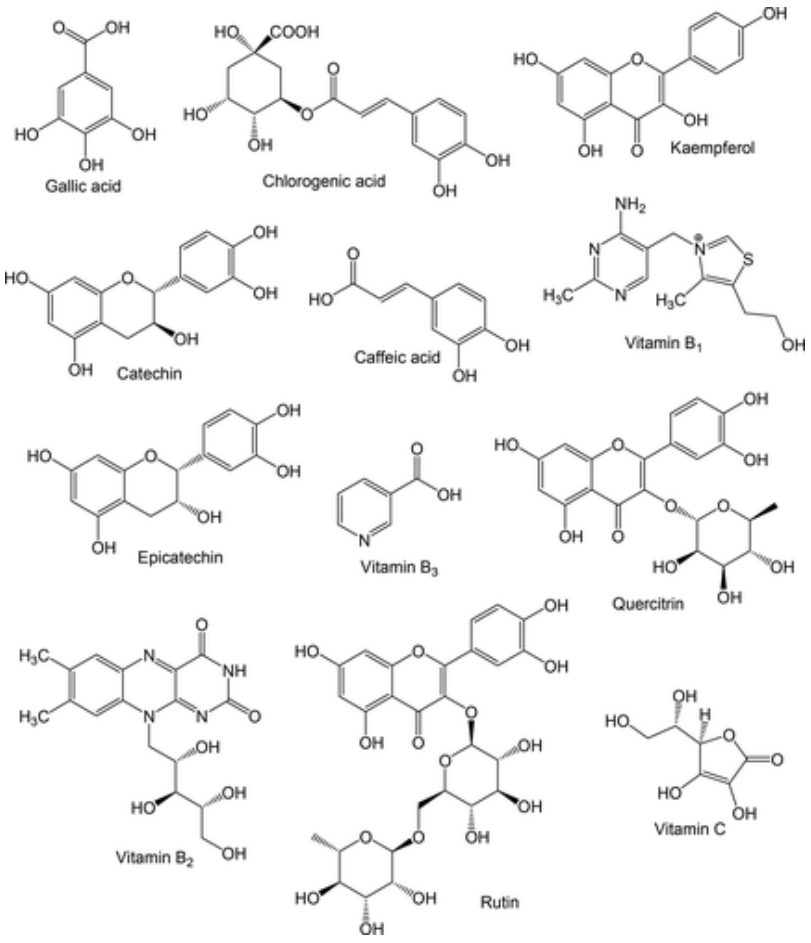


Figure 2. 2: Structures of various phytochemicals found in guava and its extracts

2.4 Medicinal Importance

Psidium guajava is used as a folk medicine all over the world due to its pharmacologic activities. Different parts of guava contain various secondary metabolites. These have different medical properties and pharmacological effects.

2.4.1 Antimicrobial activity

Guava extracts show high antimicrobial activity. The antibacterial activity of guava is high against gram-positive bacteria and moderate against gram-negative bacterial strains (Nair and Chanda, 2007). Guava leaves extract also inhibits the growth of S. aureus. Plant leaf and bark methanolic extracts of *P. guajava* have high antimicrobial activity. These extracts can inhibit *Bacillus* and *Salmonella* bacteria (Joseph and Priya, 2011). Terpinene and pinene are present in the aqueous extract of plant's leaves which shows antimicrobial activity. Due to bacteriostatic effects on pathogenic bacteria, it is also used as medicine in cough, diarrhea, oral ulcers, and in some swollen gums wounds (Dakappa et al., 2013; Rattanachaikunsopon and Phumkhachorn, 2010). In 2012 it was reported that guava leaves have many compounds which act as fungistatic and bacteriostatic agents. They can stop the growth of a lot of bacteria and act as anti-viral agents. They can control viral infections like- the influenza virus. They can hold and occupy the viral resistance. The actual reason for guava's anti-viral activity is the protein degradation ability of the guava extract (Banu and Sujatha, 2012). The essential oil of guava also has activity against *Salmonella* and *S. aureus (*Gonçalves et al., 2008).

2.4.2 Antidiarrheal activity

Diarrhea is a very common global health problem. It is estimated that about 2.2 million people die annually from diarrhea; most of them, are children or infants (Venkatesan et al., 2005). Guava extract has anti-diarrheal activity and it can be used for the treatment and prevention of diarrhea (Ojewole et al., 2008). Quercetin-3-arabinoside and quercetin are isolated from guava leaves. The extract also contains a compound that controls the muscular tone. Quercetin repressed intestinal contraction encouraged by enhanced absorption of calcium. It is thought that quercetin in guava leaf is responsible for its spasmolytic activity. Guava has high cytotoxicity (Teixeira et al., 2003). Guava can be used to treat diarrhea caused by *E. coli* or *S. aureus* toxins (Vieira et al., 2001).

2.4.3 Anti-inflammatory activity

Guava can alter the heme oxygenase-1 protein's work. And due to this reason, it can be used as an anti-inflammatory agent for the skin. Extract of guava in ethanol inhibits the lipopolysaccharide

from the manufacturing of nitric oxide. It suppresses the expression of E2. In this way, it works as an anti-inflammatory agent (Jeong et al., 2014). Guava extract also shows anti-nociceptive activity. It happened by acetic acid production. Phenol is an important compound that is present in guava and dependable for its anti-allergic and anti-inflammatory activity (Denny et al., 2013).

2.4.4 Antioxidant activity

Guava and its extracts contain a high quantity of antioxidants which can prevent the oxidation reaction. Guava is highly rich in antioxidants which help decrease the incidences of degenerative diseases such as brain dysfunction, inflammation, heart disease, cancer, arteriosclerosis, and arthritis (Feskanich et al., 2000). Guava has a high content of protocatechuic acid, quercetin, ferulic acid, ascorbic acid, quercetin, gallic acid, and caffeic acid which are important antioxidants. Some studies say that guava has a radio-protective ability with antioxidant activity (Jiminez-Escrig et al., 2001; He and Venant, 2004). Pink guava also has high antioxidant activity (Musa et al., 2011). Leaves of guava also have a high content of antioxidants which can be separated into extracts. Ascorbic acid an important antioxidant is present in leaves in excess (He and Venant, 2004; Thaipong et al., 2005). Quercetin, quercetin-3-O-glucopyranoside and morin can be isolated from leaves. These compounds show anti-oxidant activity. Quercetin has free radical balancing activity. Its reducing power is much higher than all other compounds. It is considered the most active and strong antioxidant in the leaves of guava (Nantitanon and Okonogi, 2012; Soman et al., 2010).

2.4.5 Anticancer/Antitumor Activity

Cancer is a complex health disorder that is identified by the development of cell proliferation or a decrease, causing apoptosis (Toyokuni, 2016). Guava leaf extracts are a good source of triterpenoids, sesquiterpenes, tannins, psiguadials, volatile oils, flavonoids, benzophenone glycosides, and miscellaneous quinones (Jiang et al., 2020). Psiguadial D and psiguadial C act as inhibitors of human hepatoma cells (HepG2) and protein tyrosine phosphatase 1B (PTP1B). Terpenoids and flavonoids present in guava leaf extracts exhibit antitumor effects by regulating the immune system, suppressing of signal transfer and tumor cell adhesion, and impediment to tumor angiogenesis and cell proliferation (Biswas et al., 2019).

A study was conducted to investigate the anticancer and antiangiogenic potential of guava leaf extracts against angiogenesis-dependent colorectal cancer (Lok et al., 2020). Guava leaf extracts rich in vitamin E, flavonoids (apigenin), and β-caryophyllene demonstrated strong antiproliferative activity against human colon carcinoma cell lines Caco-2, HT-29, and SW480.

2.4.6 Antidiabetic Activity

Diabetes is a major chronic disease and about 10% of the world's population suffers from blood glucose metabolic disorder, mainly characterized by a hyperglycemic condition (Mazumdar et al., 2015). The International Diabetes Federation (IDF) stated that 451 million people were affected by diabetes mellitus, resulting in 5 million deaths, in 2017 and the global prevalence of diabetes is projected to hit 693 million cases by 2045 (Cho et al., 2018). Guava leaf extracts have been widely used as ethnomedicine for diabetes management (Luo et al., 2019). Flavonoids and polysaccharides of guava leaf extracts have been reported for their antidiabetic potential in several studies (Zhu et al., 2020). Ethanolic extracts of the stem have a high anti-diabetic activity (Rai PK et al., 2007; Mukhtar et al., 2006).

2.5 Endophytic fungi from *Psidium guajava*

de Medeiros et al., 2011 discovered four new natural products and their antimicrobial effects from their research of endophytic fungi from *Psidium guajava*. The endophytic fungi *Cladosporium urendinicola* was isolated from the fruit of *P. guajava*. The new four depsides were identified from spectral data. The compounds were purified and studied and it was found that three out of the four compounds showed moderate antibacterial effects on *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtillis.*

Nonye T. et al., 2021, observed antioxidant and immunosuppressive activities of extracts from endophytic fungi isolated from *Psidium guajava* and *Newbouldia laevis*. The endophytic fungi were isolated from the stems and leaves of *N. leavis* and *P. guajava* and were identified as *Fusarium sp.* and *Cladosporium sp.* respectively. The compounds present in the extracts were protocatechic acid, p-hydroxybenzoic acid, nakijimol, chloramphenicol, orthosporin, chloramphenicol, citreohybridinol, nidulalin, and asterric acid. These compounds showed antioxidant and immunosuppressive effects.

Yang Z et al., 2019 investigated an endophytic fungus *Alternaria atrans* isolated from *Psidium guajava* which showed antifungal activity. Five known compounds and one new fusaric acid derivative- antrasfusarian were identified from spectral data. Among these, one compound had antifungal activity against *Alternaria solani*, *Colletotrichum gloeosporioides*, and *Phyricularia grisea*. The new natural metabolite antrasfusarian also showed inhibitory activity against the fungi *A. solani*, *C. gloeosporioides*, and *P. grisea*.

Sohini et al., 2022 discovered the bioactive potential of an endophytic fungus *Alternaria tenuissima* PE2 isolated from *Psidium guajava* from their research. The endophytic fungus *Alternaria tenuissima* was isolated from the leaves of the guava plant. The bioactive compounds produced by this isolate were thermostable, non-proteinacious antimicrobial substances that were effective against both gram-positive and gram-negative bacteria. This isolate also showed antifungal activity against the pathogenic yeast *Candida albicans*.

Festus et al., 2015 invented two new cytochalasins from an endophytic fungus, KL-1.1 isolated from *Psidium guajava* (Linn) leaves. The two new cytochalasin derivatives, 18-desoxy-19,20-epoxycytochalasin C and 18-desoxycytochalasin C along with five other known derivatives were identified by spectral data. These novel compounds could be further studied as potential anticancer agents.

Chutulo and Chalannavar (2021) detected antimicrobial activity of an endophytic fungus isolated from *Psidium guajava* L. *Fusarium oxysporum* was isolated and identified from the leaves of *P. guajava*. The secondary metabolite produced showed inhibitory activity against bacterial pathogens. The crude extract also showed inhibitory activity against Saccharomyces cerevisiae. Therefore, the fungus can be an ideal resource for the biological prospecting of antimicrobial agents.

Chutulo and Chalannavar (2020) isolated an endophytic fungus from *Psidium guajava* as an alternate source of bioactive secondary metabolites. *Daldinia eschscholtzii* was isolated from *Psidium guajava* leaves. The leaves extract showed strong antibacterial activity by the disc diffusion method and antioxidant activity. The total flavonoid and phenolic content estimates amounted to 27.4763 ± 0.68 quercetin equivalent per gram (QE/g) of the extract and 43.853056 ± 0.059 gallic acid equivalent per gram (GAE/g) of the extract, respectively. It showed potent

cytotoxicity against the lung adenocarcinoma human cancer cell line A519 (92.66 ± 0.56%). 18 major volatile organic compounds were present in the GC-MS analysis. This endophyte has the potential to be developed as an antimicrobial, antioxidant, and anticancer agent.

Manoharan et al., 2015 discovered endophytic fungi from *Mentha arvensis L*. and *Psidium guajava* L. and observed the production of silver nanoparticles and their antimicrobial effect. six endophytic fungi such as *Aspergillus fumigatus, Cladosporium, Cladosporioides, Colletrotrichum sp, Fusarium oxysporum,* and *Phomopsis sp.* were isolated from the healthy leaves and stem of *Mentha arvensis* L.and *Psidium guajava* L. and were screened for the production of silver nanoparticles. The presence of nanoparticles was confirmed by the change of color in fungal extracts and UV-Vis spectroscopy. The silver nanoparticle showed antimicrobial activity against *Escherichia coli, Salmonella typhi, Klebsiella pneumonia,* and *Staphylococcus aureus*. Therefore, it is confirmed that nanoparticles can be used as an antibacterial agent.

Janakiraman et al., 2019 stated the biosynthesis of silver nanoparticles by an endophytic fungus isolated from *Psidium guajava*. The endophytic fungus *Botryodiplodia theobromae* produced silver nanoparticles which showed cytotoxic effects on human MCF7 breast cancer cell lines and A549 human lung cancer cell lines. So, the silver nanoparticles produced by endophytic fungus could be used as a novel chemotherapeutic agent against human breast and lung cancers due to its powerful anti-proliferative action.

Chapter 3: Materials and Methods

3.1 Introduction

This study was designed to investigate endophytic fungi of *Psidium guajava*. The method was divided into two separate parts-

- Biological assay
- Chemical assay

This protocol consists of the following synchronized steps based on 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 *Psidium guajava* 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 *Psidium guajava*. The leaf and bark parts were sampled for the investigation of endophytic fungi and their bioactivity

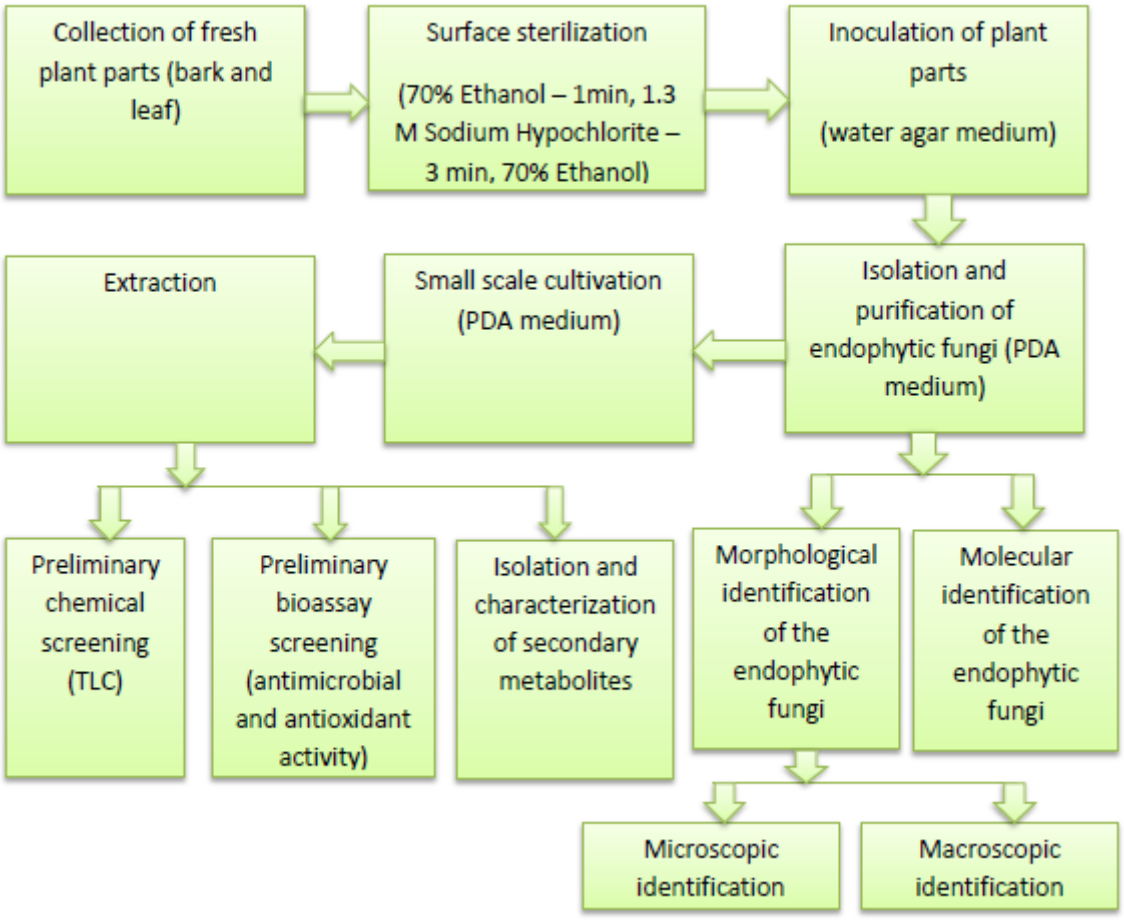


Figure 3. 1: A scheme for the procedure of isolation, purification, identification, cultivation, and extraction of endophytic fungi of *P. guajava*

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 ^{o}C 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.

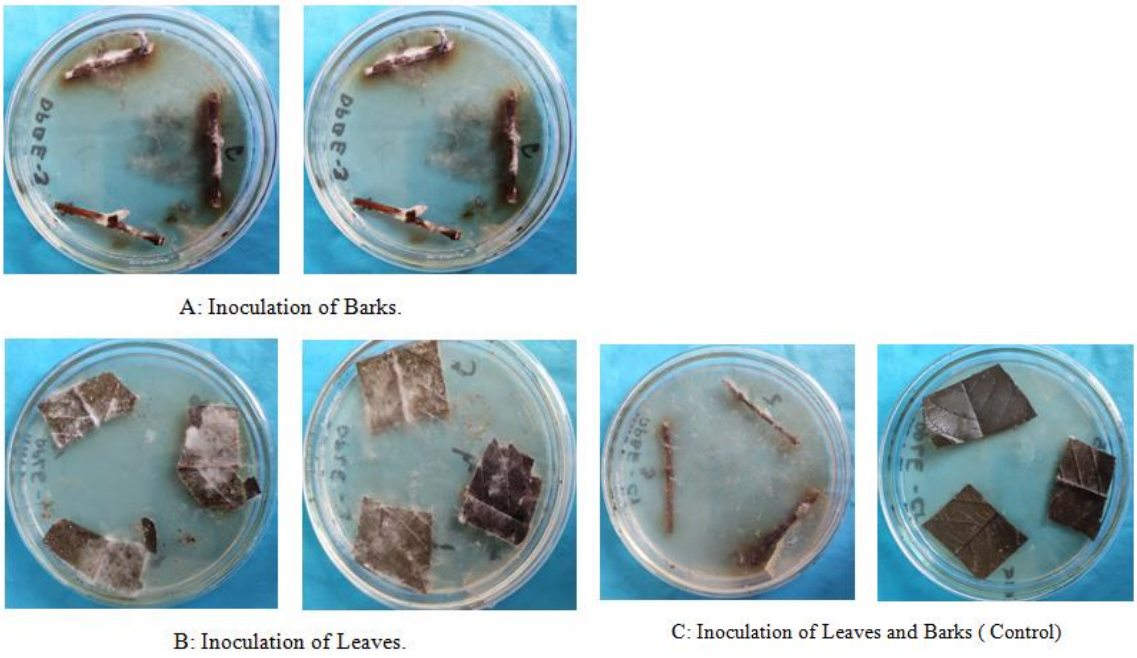


Figure 3. 2: Inoculation of plant parts; A: Leaves, B: Barks C: Leaves and Bark (control)

3.3.2 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 the 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 sub-cultured onto a rich mycological medium, Potato Dextrose Agar (PDA) medium, and brought into pure culture (Kusari, 2008).

3.4 Process of purification of endophytic fungi

- After isolation from the petri dish, the isolated pure fungal colony was transferred to slant onto PDA media purification media.
- The transfer process was careful as no bacterial contamination or different fungal mycelia don't touch the loop. It was transferred to one or a few fungal mycelia to avoid fungal contamination.
- Before transformation loop was burnt to 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℃ 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, some were circular, and others were irregular. Specific terminology is used to describe common colony types.

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^{o}C on potato dextrose agar media, a small portion of the colony was taken into lactophenol cotton blue solution. 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. Identification was based on morphological characteristics.

3.5.2 Molecular identification of fungal strain and their taxonomy

3.5.2.1 DNA extraction

Fungal mycelium (~100 mg) was scratched with liquid nitrogen, put into an Eppendorf tube, added 1.3 mL lysis buffer (100 mM Tris-HCl (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.

3.5.2.2 DNA quantification

DNA quantification was conducted through the NanoDrop 8000 (Thermo Scientific) by loading 2 µL of each sample for each detection (Elliott et al., 1997). The absorbance of a diluted RNA sample

was measured by the ratio of UV absorbance at 260 and 280 nm. The nucleic acid concentration is calculated using the Beer-Lambert law, which predicts a linear Change in absorbance with concentration through the machine's own software. The purification was considered more than 1.8 (260/280).

3.5.2.3 Evaluation of DNA quality

Maintaining DNA quality and quantity after subsequent DNA isolation is significant for the accuracy of PCR results. This procedure was done by gel electrophoresis (Magdeldin, 2012). The method for evaluating DNA is briefly described below. The gel container was tapped and put in the desired comb. 1.0 g (2.0 % w/v) Agarose was weighed out for a 50 ml 1X TBE (Tris-Borate EDTA) buffer. It was then heated in the microwave until the suspension was clear. The solution was left for cooling down to hand warmth and 2 µl of GelRedTM was added. The gel was poured into the container without producing air bubbles. Then the container was kept in the open air for approximately 30 minutes until it obtained a light grey color. A DNA sample was prepared by mixing 100-500 ng of DNA, 2 µl 6 X gel loading dye, and an appropriate volume of 1X TBE buffer to make up the total volume of 12 µL. DNA samples were then loaded into the gel and run for 30-40 min at 110 V in 1 X TBE buffer for 25-30 minutes before analysis. By running samples on a gel with electrophoresis, the isolated DNA can be checked by gel documentation (Applied Biosystem, USA) under the UV light following the manufacturer's instructions. The quality of the DNA was ensured after gel electrophoresis.

3.5.2.4 RNase treatment

The existence of genomic RNA in DNA preparations is a recurrent cause of false positive results in PCR-based assays due to PCR sensitiveness. This occurrence cannot be ignored when specific measures in the assay design are applied like intron-spanning primers design. Therefore, the removal of RNA present in the DNA solution by RNase digestion is an essential step. 1 µL 10X RNase1 reaction buffer (Invitrogen) and 1 µL RNase1 enzyme was added to the 1 µg DNA in 8 µL water, gently homogenized, and incubated at room temperature for 15 minutes. Then 1 µL RNase stop solution was added to the mixture, homogenized, and heated at 70^{o}C for 10 minutes to inactivate RNase1 activity for the reverse transcription step.

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.

Table 3. 1: 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 bases. 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 95ºC for 2 minutes to activate Promega Taq DNA polymerase
- Cycling steps were repeated 32 times.
- Denaturing: 30 second at 95ºC, annealing: 30 second at 54ºC, extension: 50 second at 72ºC
- Final extension for 5 minutes in 72ºC

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 50º 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 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 Small-scale cultivation

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

3.7 Process of extraction of secondary metabolites

- After the full growth of fungi on 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 100 ml 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 200 ml chloroform.
- After one week there 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 the weighted 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.

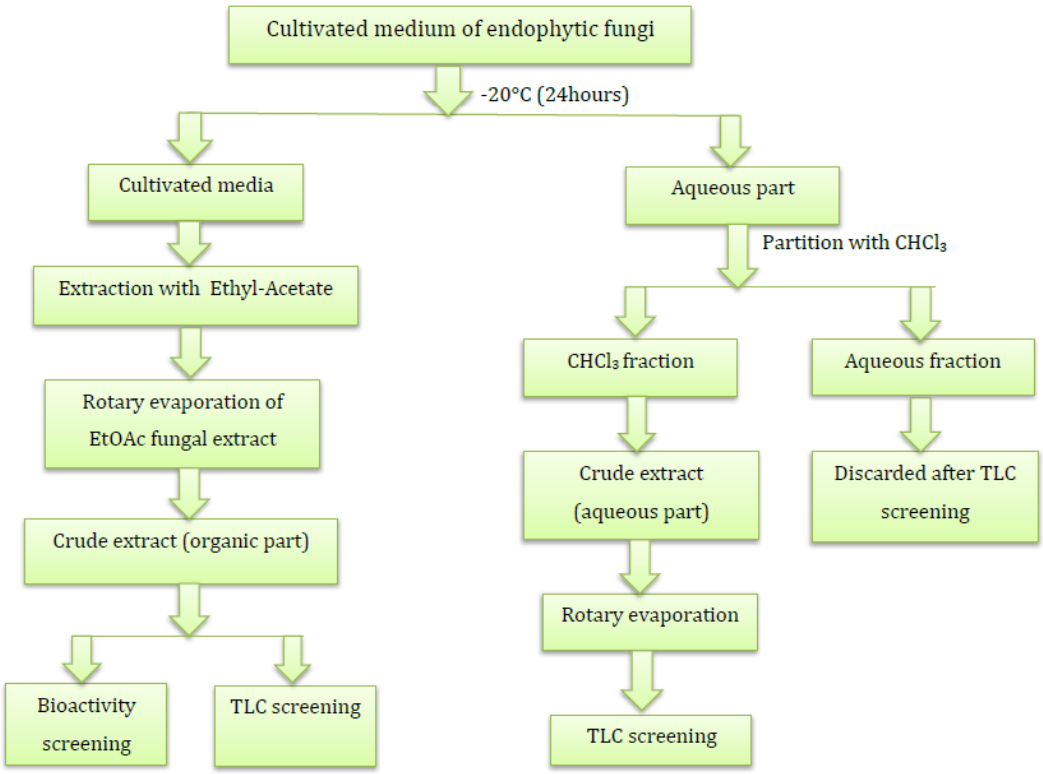


Figure 3. 3: A scheme for the extraction process of fungal metabolites

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

- **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. DPPH is a stable (in powder form) free radical with red color which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (HA) can be written as,

(DPPH) + (H-A) ---- DPPH-H + (A)

Antioxidants react with DPPH and reduce it to DPPH-H and as consequence, the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen-donating ability.

- **Procedure**

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.

0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100 ml of 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).

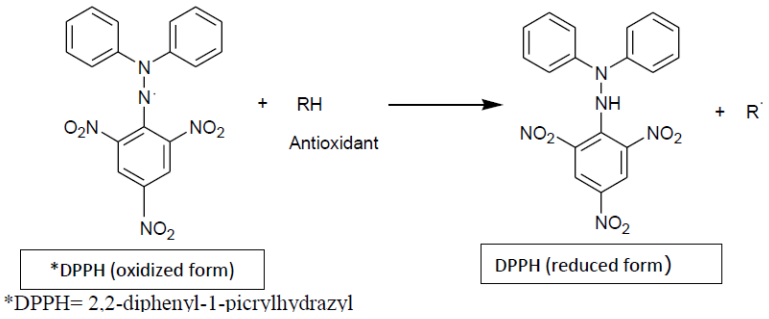


Figure 3. 4: Redox reaction of DPPH

Calculation of IC50:

The IC50 of each analytical sample was calculated according to the following procedure: Inhibition ratios (y) were plotted against the sample concentrations (x) at all points, and the respective regression line (y = ax + b) was drawn. The regression line was not required to pass through the origin. In fact, because the inhibition curve was not completely straight, but slightly curved, we can calculate the IC50 value using the interpolation method by joining the two points around the 50% inhibition with a straight line as follows: Two points enclosing a 50% inhibition ratio were selected, and a regression line (Y = AX + B) was drawn. X was calculated by substituting the value of Y with 50 in the regression equation of Y = AX + B.

3.8.2 Antimicrobial activity test: Disc diffusion method

- **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^{o} C) for 24 hours to allow maximum diffusion. The plates are then incubated at 37^{o} 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.

- **Test organisms**

The bacterial strains used for the experiment were collected as pure cultures from the Institute of Food Science & Technology (IFST), BCSIR.

Table 3. 2: 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*

- **Procedures**

- 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.
- 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 121o C for 20 minutes. The slants were used for making fresh cultures of bacteria that were in turn used for sensitivity study.
- 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.
- 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 370 C (for bacteria) and 25° C (for fungi) for their optimum growth.
- Three types of discs were used for antibacterial screening.

Positive control discs were used 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. Negative control discs were used to ensure that the residual solvents (left over from the discs even after air-drying) were not active themselves. 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.

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

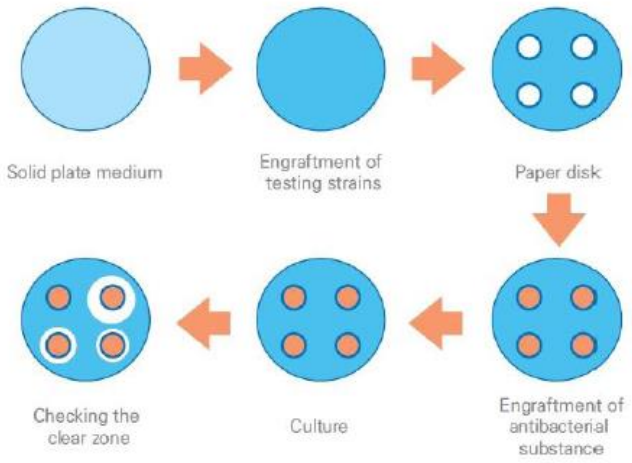


Figure 3. 5: In vitro investigation of antimicrobial activity following the disc diffusion assay.

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 - H_2SO_4 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. 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%). 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 was 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 are isolated from the crude and fractionated extracts using different chromatographic and other techniques.

3.9.2.1 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.

3.9.4 Isolation and purification of the compound from PgB3 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. 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 then sent for the NMR spectrum.

Chapter 4: Results

4.1 Introduction

This chapter is designed to study-

- The morphological and molecular characterization of endophytic fungi isolated from *Psidium guajava.* 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 five endophytic fungi were isolated and purified from the plant *Psidium guajava.* Among them, four endophytic fungal strains were isolated from the bark of *Psidium guajava* – PgB1, PgB2, PgB3, PgB4, and one strain was isolated from the leaf of *Psidium guajava* – PgL1. All the fungal strains were then identified by using macroscopic and microscopic morphological characteristics and molecular characterization.

Table 4. 1: Code of test samples

No.	Test sample	Sample code
1.	Endophytic fungal strain-1 from the bark of *Psidium guajava*	PgB1
2.	Endophytic fungal strain-2 from the bark of *Psidium guajava*	PgB2
3.	Endophytic fungal strain-3 from the bark of *Psidium guajava*	PgB3
4.	Endophytic fungal strain-4 from the bark of *Psidium guajava*	PgB4
5.	Endophytic fungal strain-1 from the leaf of *Psidium guajava*	PgL1

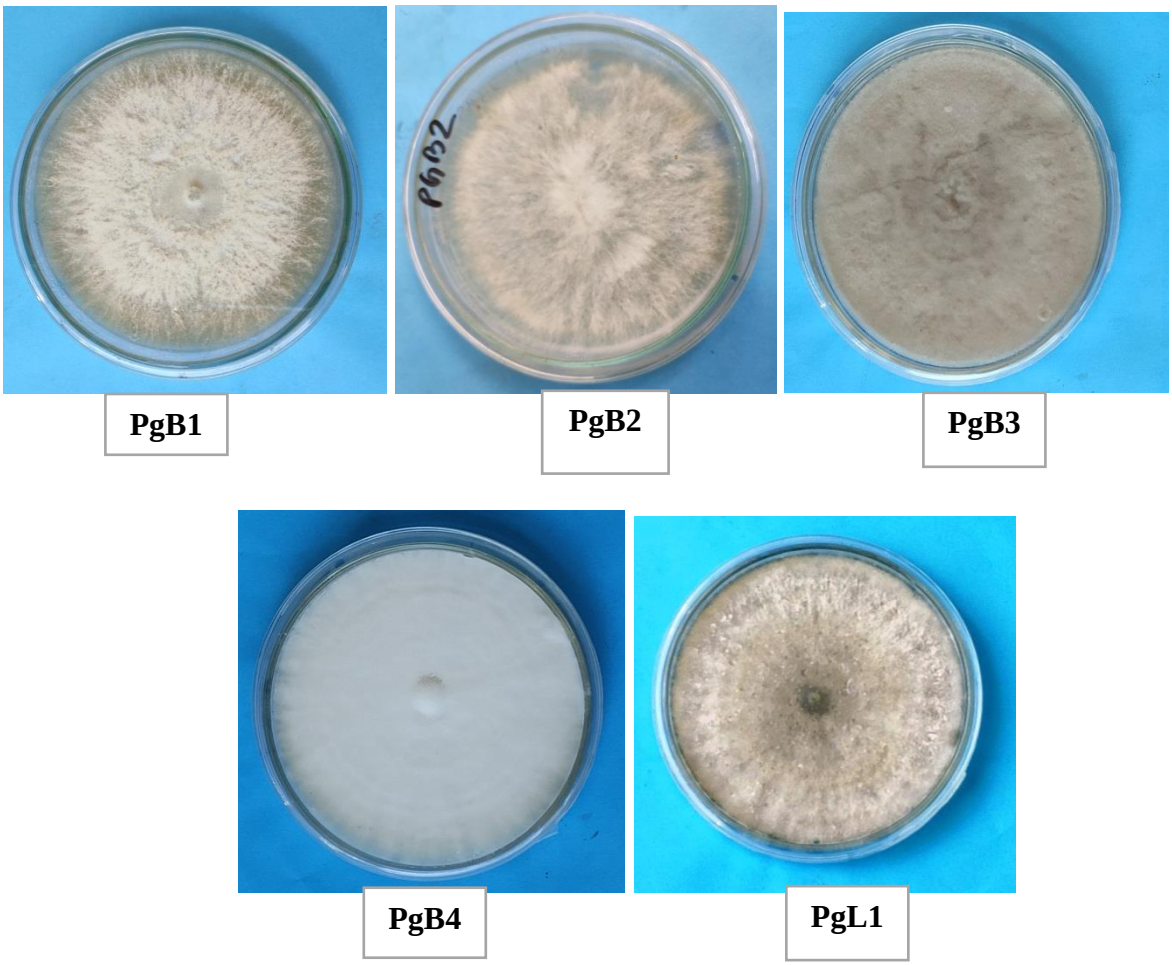


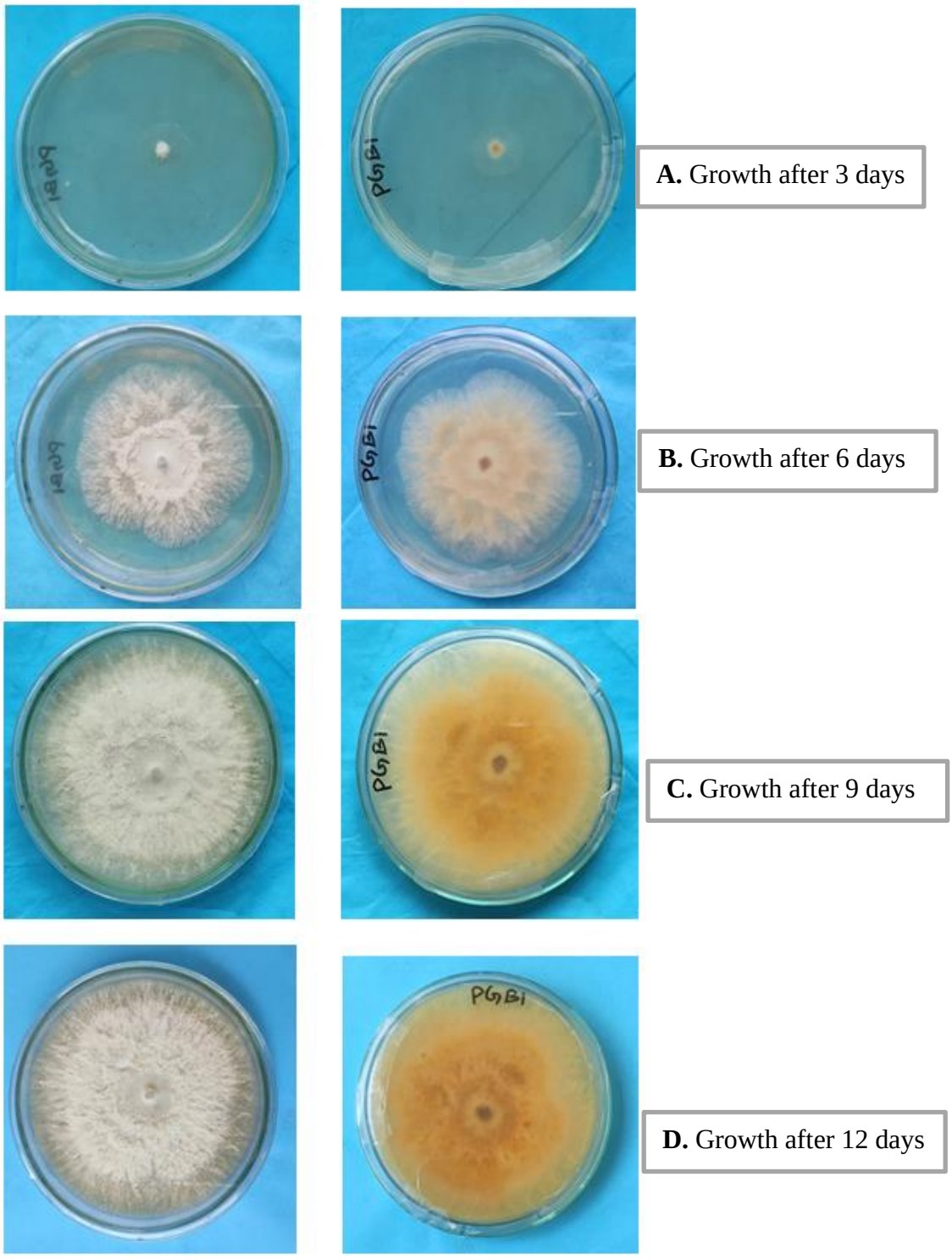
Figure 4. 1: Five endophytic fungal strains isolated from *Psidium guajava*

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 4. 2: Morphological features of fungal strain PgB1

Characteristics	Observation (after 3 days)	Observation (after 6 days)	Observation (after 9 days)	Observation (after 12 days)
Type of media	PDA	PDA	PDA	PDA
The growth rate of the fungi	Moderate	Moderate	Rapid	Rapid
Nature of growth	Center to edge	Center to edge	Center to edge	Center to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter	3 cm	7 cm	Full growth (9 cm)	Full growth (9 cm)
Surrounding temperature	28°C	28°C	28°C	28°C
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Irregular	Filamentous	Filamentous	Filamentous
Color of the top view	White	White	White	White
Color of the bottom view	White with an orange center	Light brown with an orange center	Light brown with an orange center	Pale brown with a black center
The texture of the colony surface	Wooly	Wooly	Wooly	Wooly
Side view of colony/ Elevation	Raised	Raised	Raised	Raised
Margin shape of colony	Entire	Filiform	Filiform	Filiform
Opacity	Opaque	Opaque	Opaque	Opaque



A. Growth after 3 days

B. Growth after 6 days

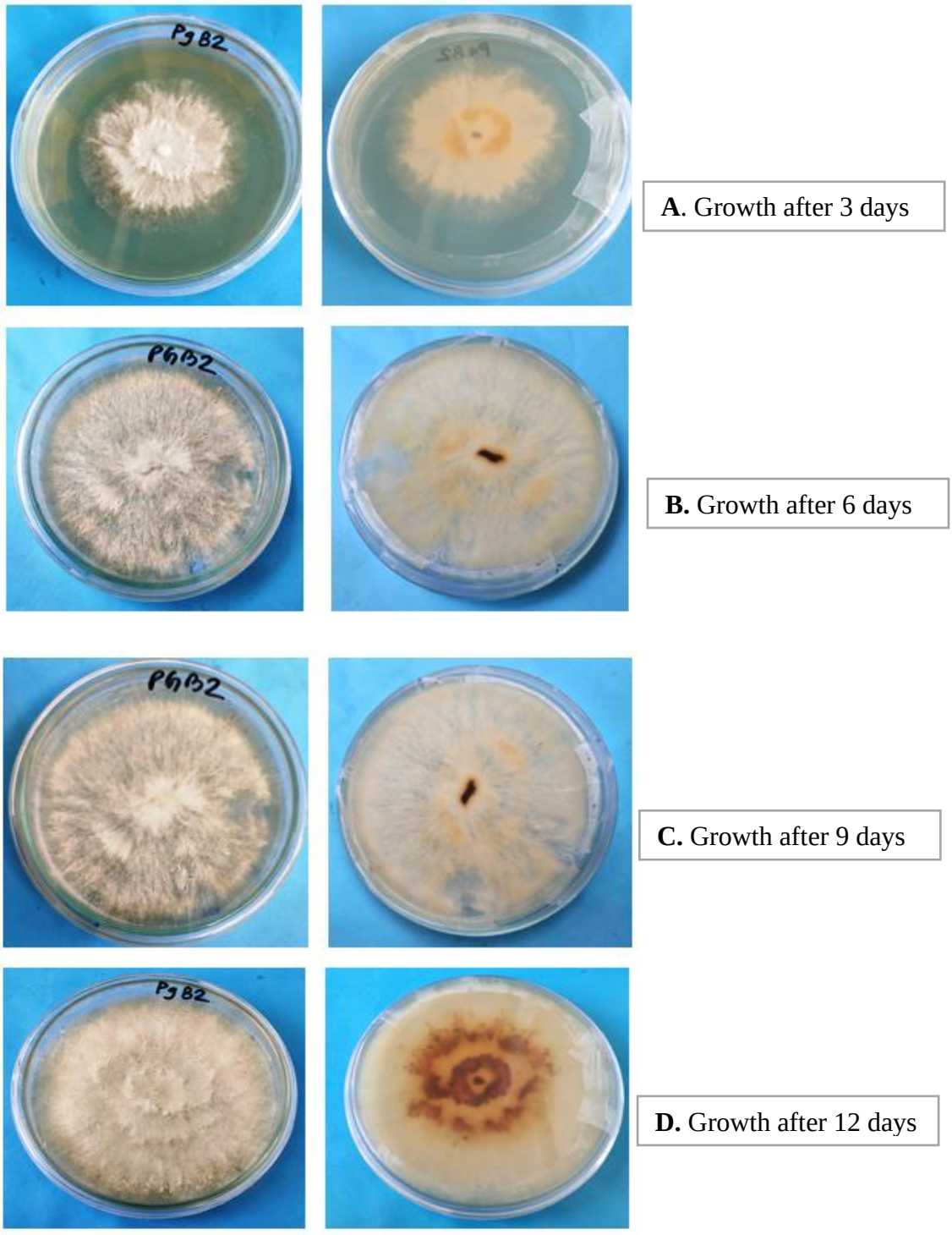
C. Growth after 9 days

D. Growth after 12 days

Figure 4. 2: Top and bottom view of the fungal strain PgB1 after (A) 3 days, (B) 6 days, (C) 9 days, and (D) 12 days of small-scale cultivation

Table 4. 3: Morphological features of fungal strain PgB2

Characteristics	Observation (after 3 days)	Observation (after 6 days)	Observation (after 9 days)	Observation (after 12 days)
Type of media	PDA	PDA	PDA	PDA
The growth rate of the fungi	Moderate	Rapid	Rapid	Rapid
Nature of growth	Center to edge	Center to edge	Center to edge	Center to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter	6 cm	Full growth (9 cm)	Full growth (9 cm)	Full growth (9 cm)
Surrounding temperature	28°C	28°C	28°C	28°C
Hyphae	Aerial	Surficial	Surficial	Surficial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Filamentous	Circular	Circular	Circular
Color of the top view	White	White	White	White
Color of the bottom view	White with an orange center	Orange with black center	Orange with black center	Orange with dark orange layers and a black center
The texture of the colony surface	Felty	Felty	Felty	Felty
Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of the colony	Filiform	Filiform	Filiform	Filiform
Opacity	Opaque	Opaque	Opaque	Opaque



A. Growth after 3 days

B. Growth after 6 days

C. Growth after 9 days

D. Growth after 12 days

Figure 4. 3: Top and bottom view of the fungal strain PgB2 after (A) 3 days, (B) 6 days, (C) 9 days, and (D) 12 days of small-scale cultivation

Table 4. 4: Morphological features of fungal strain PgB3

Characteristics	Observation (after 3 days)	Observation (after 6 days)	Observation (after 9 days)	Observation (after 12 days)
Type of media	PDA	PDA	PDA	PDA
The growth rate of the fungi	Moderate	Rapid	Rapid	Rapid
Nature of growth	Center to edge	Center to edge	Center to edge	Center to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter	3.5 cm	Full growth (9 cm)	Full growth (9 cm)	Full growth (9 cm)
Surrounding temperature	28°C	28°C	28°C	28°C
Hyphae	Surficial	Surficial	Surficial	Surficial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Circular	Circular	Circular	Circular
Color of the top view	White with black center	White with black center	Grey with black center	Grey with black center
Color of the bottom view	Off-white with black center	Off-white with black center	Light brown with black dots	Light brown with black dots
The texture of the colony surface	Wooly	Wooly	Wooly	Wooly
Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of the colony	Entire	Entire	Entire	Entire
Opacity	Opaque	Opaque	Opaque	Opaque

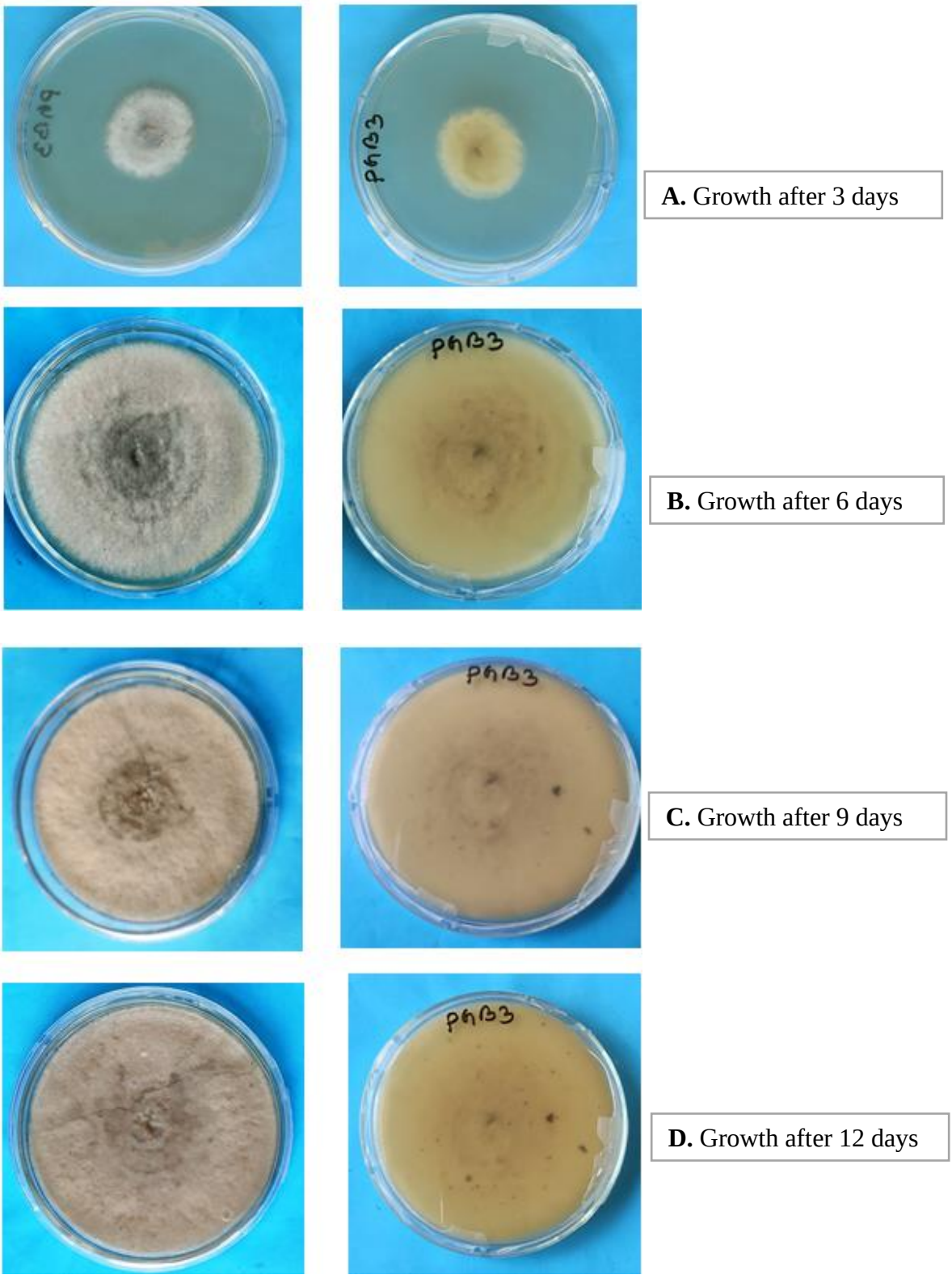
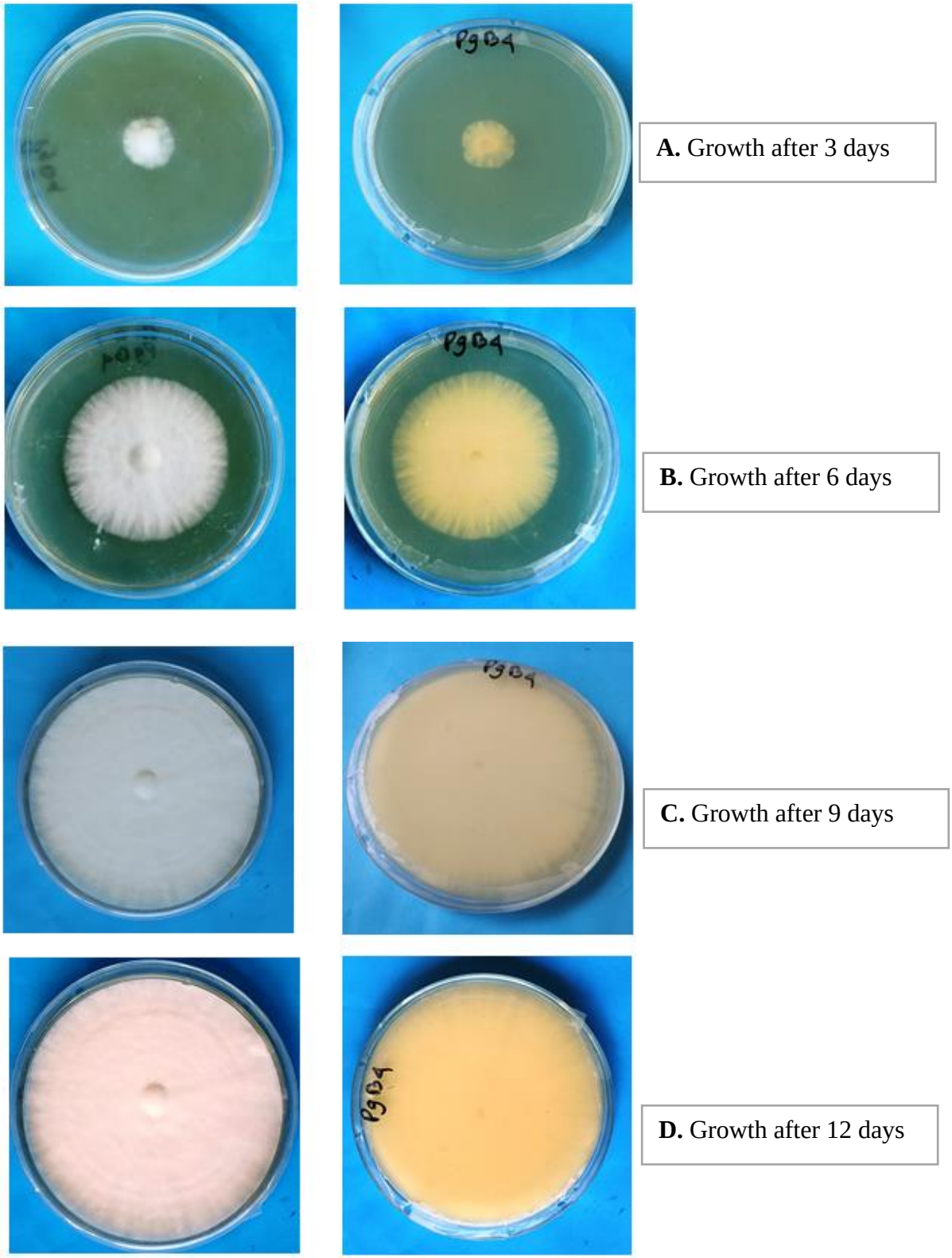


Figure 4. 4: Top and bottom view of the fungal strain PgB3 after (A) 3 days, (B) 6 days, (C) 9 days, and (D) 12 days of small-scale cultivation

Table 4. 5: Morphological features of fungal strain PgB4

Characteristics	Observation (after 3 days)	Observation (after 6 days)	Observation (after 9 days)	Observation (after 12 days)
Type of media	PDA	PDA	PDA	PDA
The growth rate of the 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	2 cm	6 cm	Full growth (9 cm)	Full growth (9 cm)
Surrounding temperature	28°C	28°C	28°C	28°C
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Filamentous	Filamentous	Filamentous	Filamentous
Color of the top view	White	Cotton white	Cotton white	Cotton white
Color of the bottom view	White	White	Yellowish	Yellowish
The texture of the colony surface	Wooly	Wooly	Wooly	Wooly
Side view of colony/ Elevation	Raised	Raised	Raised	Raised
Margin shape of the colony	Filiform	Filiform	Filiform	Filiform
Opacity	Opaque	Opaque	Opaque	Opaque



A. Growth after 3 days

B. Growth after 6 days

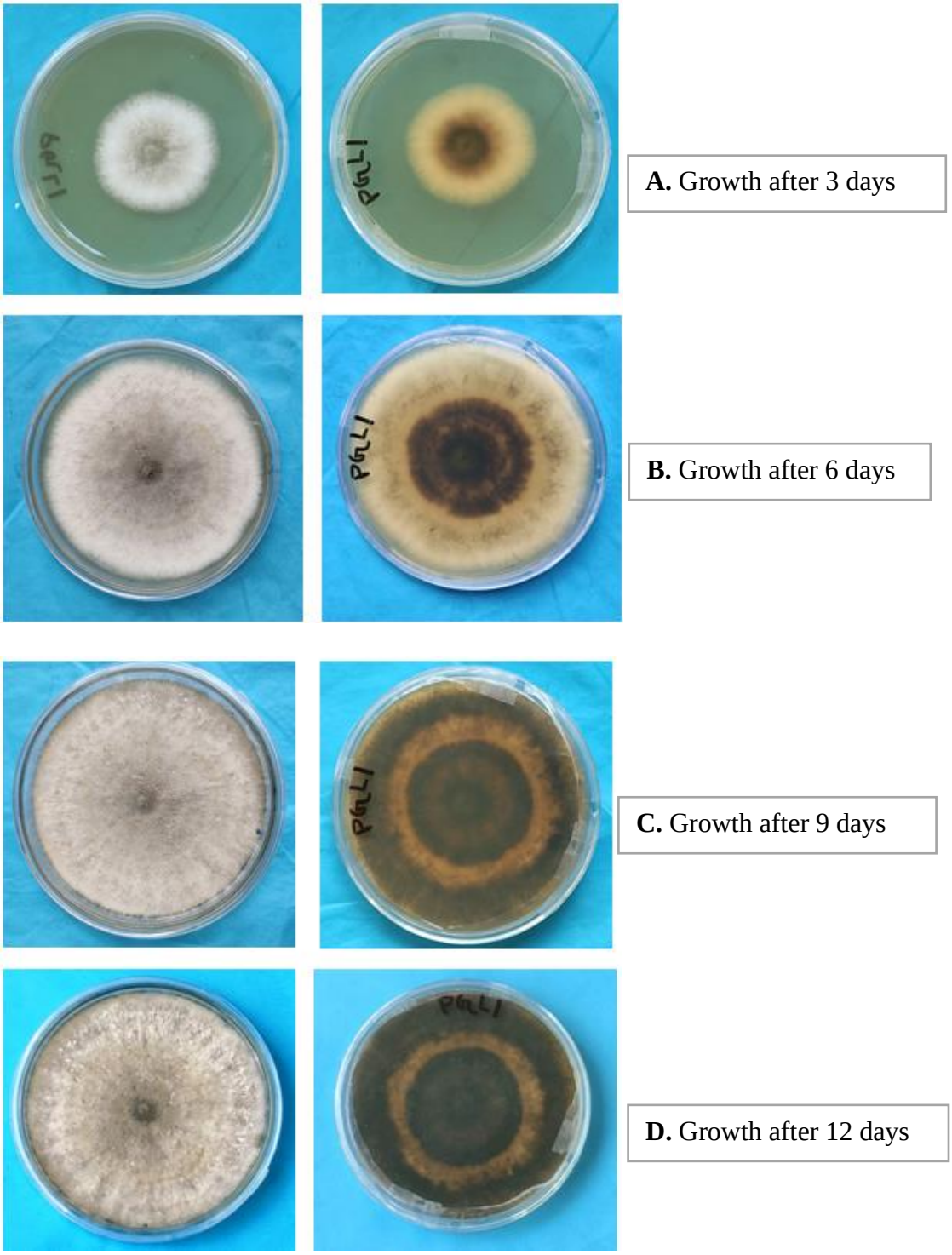
C. Growth after 9 days

D. Growth after 12 days

Figure 4. 5: Top and bottom view of the fungal strain PgB4 after (A) 3 days, (B) 6 days, (C) 9 days, and (D) 12 days of small-scale cultivation

Table 4. 6: Morphological features of fungal strain PgL1

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	Rapid	Rapid	Rapid
Nature of growth	Center to edge	Center to edge	Center to edge	Center to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter	4.5 cm	8.5 cm	Full growth (9 cm)	Full growth (9 cm)
Surrounding temperature	28°C	28°C	28°C	28°C
Hyphae	Surficial	Surficial	Surficial	Surficial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Circular	Circular	Circular	Circular
Color of the top view	White with black center	White with black center	Green-white with green-black center	Green-white with green-black center
Color of the bottom view	Off-white with black center	Off-white with black center	Black with a light brown layer	Black with a light brown layer
The texture of the colony surface	Wooly	Wooly	Wooly	Wooly
Side view of colony/ Elevation	Raised	Flat	Flat	Flat
Margin shape of colony	Entire	Entire	Entire	Entire
Opacity	Opaque	Opaque	Opaque	Opaque



A. Growth after 3 days

B. Growth after 6 days

C. Growth after 9 days

D. Growth after 12 days

Figure 4. 6: Top and bottom view of the fungal strain PgL1 after (A) 3 days, (B) 6 days, (C) 9 days, and (D) 12 days of small-scale cultivation

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 (Proukakis et al., 1998). The microscopic observation of the characteristic mycelium and conidiophores of the fungi is given below.

4.4.1 Identification of the fungal strain PgB1

From macroscopic examination revealed that the colonies were cottony, developing aerial mycelium, at first uniformly white, later becoming whitish with black patches. The reverse side of the petri dish was whitish, turning light brown with scattered dark brown spots. Under the microscope, conidia were hyaline, unicellular, filiform, and mostly slightly curved at the end. The characteristics are similar to the fungal strain *Phomopsis sp.* The following figure represented the microscopic view of PgB1.

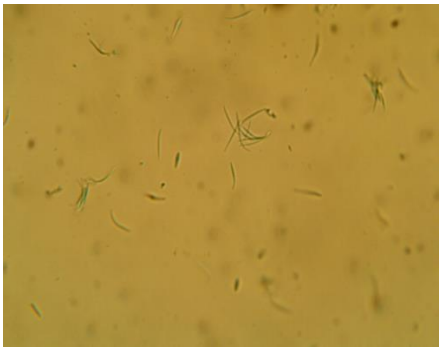


Figure 4. 7: Microscopic view of isolated endophytic fungi PgB1

4.4.2 Identification of the fungal strain PgB2

Macroscopic observation showed that the mycelium was white and less abundant, which filled out the petri dish within 9 days. The reverse side of the culture plate was white mixed with orange, later turned into orange with dark brown scattered spots. Conidia were filiform, straight, or slightly

curved at one end. The characteristics indicate that the fungal strain could be from *Diaporthe sp*. The microscopic view of PgB2 was represented by the following figure-

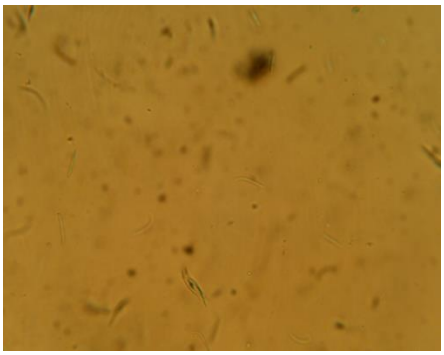


Figure 4. 8: Microscopic view of isolated endophytic fungi PgB2

4.4.3 Identification of fungal strain PgB3

Macroscopic observation of strain PgB3 revealed that on PDA media, the fungus has sparse, cottony, white with black center mycelium, then turned grey with a black center. The reverse side of the plate was whitish and turned light brown with black spots. The fungus grew very quickly, covering the whole surface of the petri dish within 6 days. The conidiospores were septated in two parts, cylindrical, and straight with a smooth round end, and showed profuse sporulation on PDA media. These characteristics suggested that the fungal strain could be from *Colletotrichum sp.* The microscopic view of PgB3 was represented by figure 4.9-

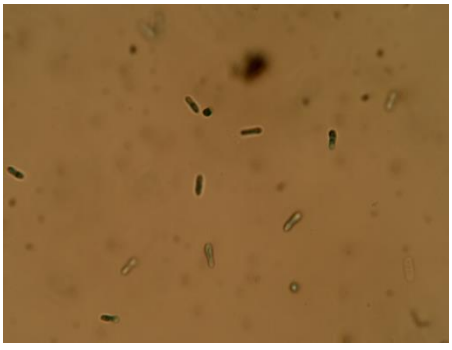


Figure 4. 9: Microscopic view of isolated endophytic fungi PgB3

4.4.4 Identification of fungal strain PgB4

Macroscopic observation revealed that the fungus was white, cottony, filamentous, and aerial hyphae. The reverse side of the petri dish was white and later turned yellowish. Under the microscope, it was observed that the mycelium was long, smooth, and branched. No conidium was found. The fungus growth rate was slow at first, but later it was moderate, covering the whole surface within 9 days. These character traits indicated that the fungal strain is from *Nemania sp.* The microscopic view of the isolate PgB4 was-

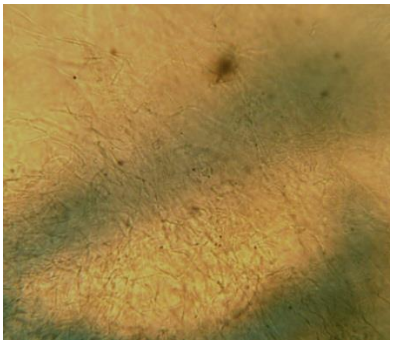


Figure 4. 10: Microscopic view of isolated endophytic fungi PgB4

4.4.5 Identification of fungal strain PgL1

From macroscopic observation, it could be said that the fungus showed moderately rapid growth spots. The culture colonizes the agar plate from the point of inoculation at the center, producing initial whitish septate hyphae. This hypha, later on, forms a mycelium and becomes greenish, finally turning grayish. The reverse side of the plate was off-white with a black center turned black with a light brown layer. Under microscopic observation, mycelia were branched, and thin. No conidium was found. These characteristics pointed to the fungal strain *Daldinia sp.* Figure 4.11 represented the microscopic view of PgL1.

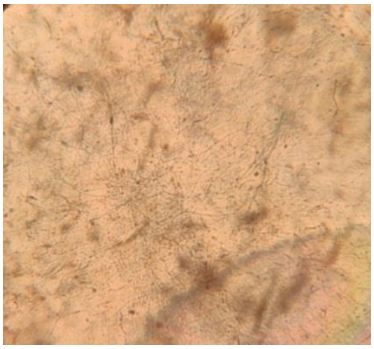


Figure 4. 11: Microscopic view of isolated endophytic fungi PgL1

4.5 Molecular identification of endophytic fungi

The isolated five endophytic fungi from the plant *Psidium guajava* were sub-cultured on PDA media for 7 days. The mycelium from these five 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.

4.5.1 Molecular identification of fungal strain PgB1

4.5.1.1 The nucleotide sequence analysis of PgB1

Molecular analysis of the endophytic fungus PgB1 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 PgB1 resulted in a 561 bp long sequence:

TCTCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCCCCAGGCGCACCC
AGAAACCCTTTGTGAACTTATACCTTACTGTTGCCTCGGCGTACGCTGGCCCCCAGG
GGTCCCTCTGTCTACAGAGGAGCAGGCACGCCGGCGGCCAAGTTAACTCTTGTTTTT
ACACTGAAACTCTGAGAAAAAAACACAAATGAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCCGGTATTCCGGA
GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGTTGGGGCAC
TGCTTCTCTCGCGGGAAGCAGGCCCTCAAATCTAGTGGCGAGCTCGCCAGGACCCCG
AGCGTAGTAGTTAAACCCTCGCTTTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACC
CCCAACTCTTGAAAATTGAACCTCGGATCAGGTAGGAATACCCGCTGAACT

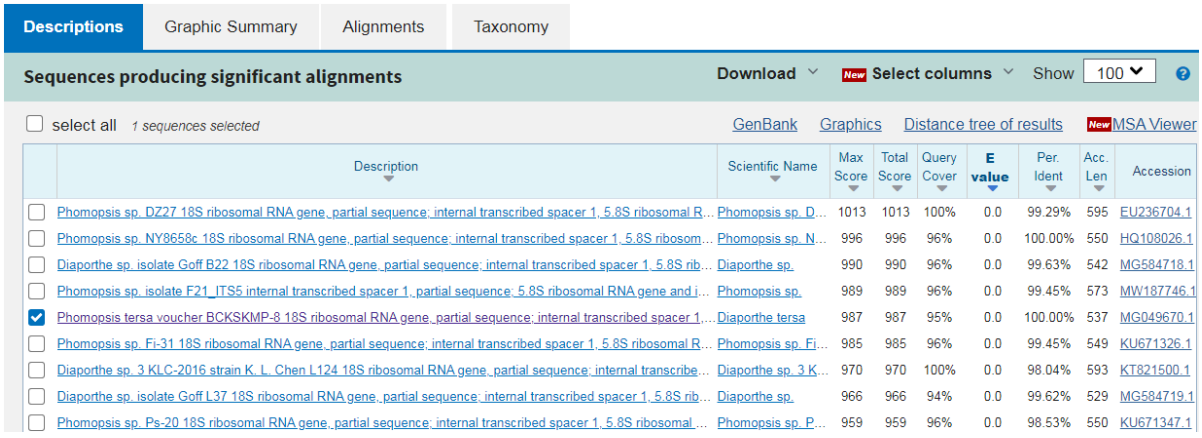
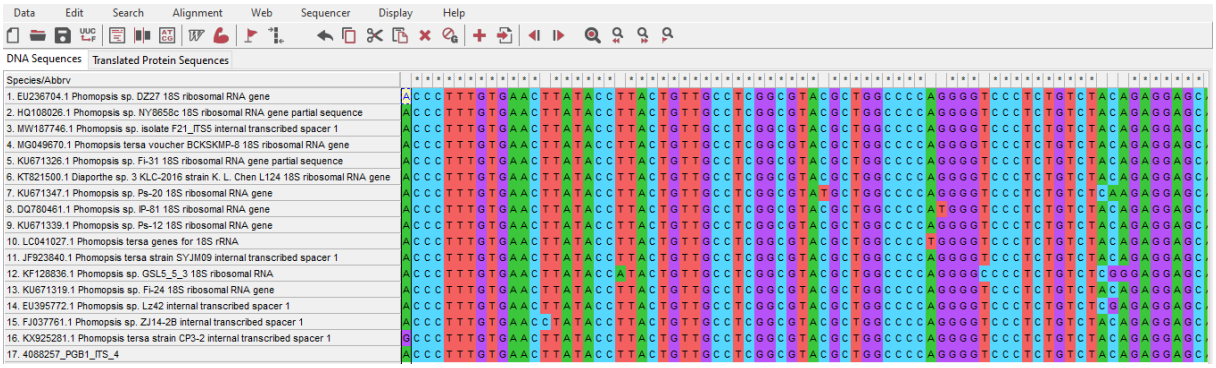


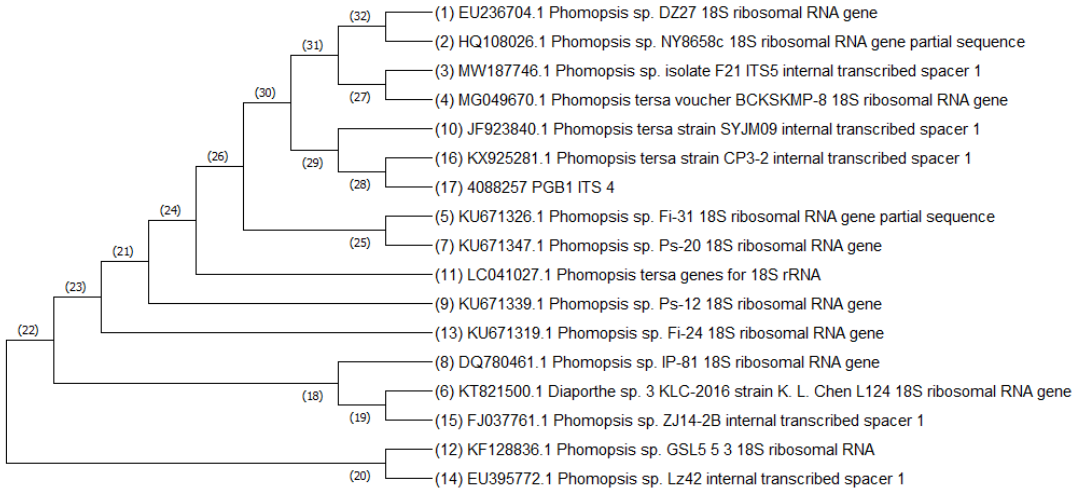
Figure 4. 12: Blast analysis of PgB1

4.5.1.2 Molecular identification of PgB1 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 4. 13: DNA taxonomy of fungal strain PgB1. (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

4.5.2 Molecular identification of fungal strain PgB2

4.5.2.1 The nucleotide sequence analysis of PgB2

Molecular analysis of the fungus PgB2 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 PgB2 resulted in a 564 bp long sequence:

ACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCCTCGGCG
CACCCAGAAACCCTTTGTGAACTTATACCTTACTGTTGCCTCGGCGCAGGCCGGCCT
CTTAGCTGAGGCCCCCCGGAGACGGGGAGCAGCCCGCCGGCGGCCAACCAAACTCT
TGTTTCTACAGTGAATCTCTGAGTAAAAAACATAAATGAATCAAAACTTTCAACAAC
GGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTAT
TCCGGAGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGTTG
GGGCACTGCTTCGAGAGAAGCAGGCCCTGAAATCTAGTGGCGAGCTCGCTAGGACC
CCGAGCGTAGTAGTTATATCTCGTTCTGGAAGGCCCTGGCGGTGCCCTGCCGTTAAA
CCCCCAACTTCTGAAATTTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTA

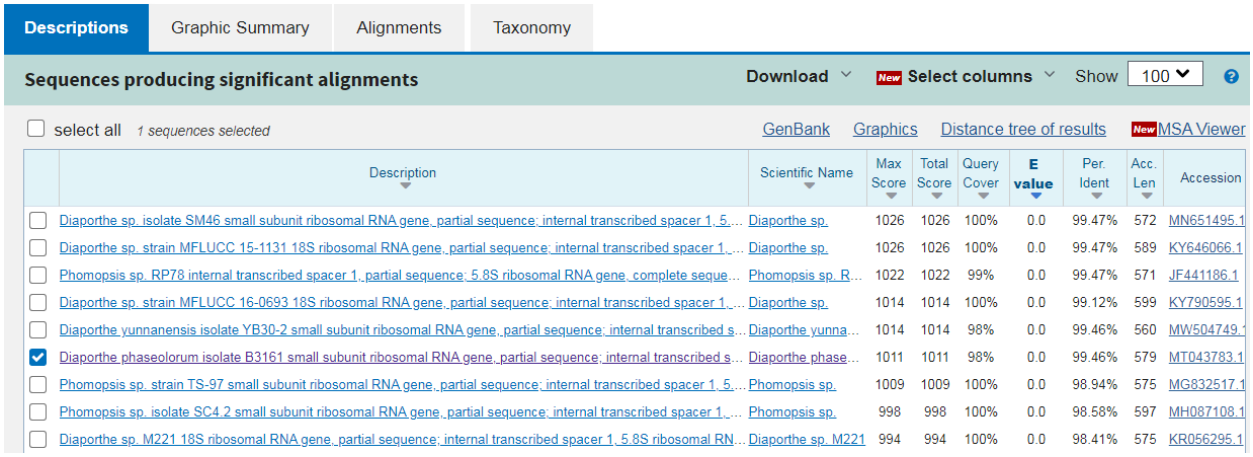
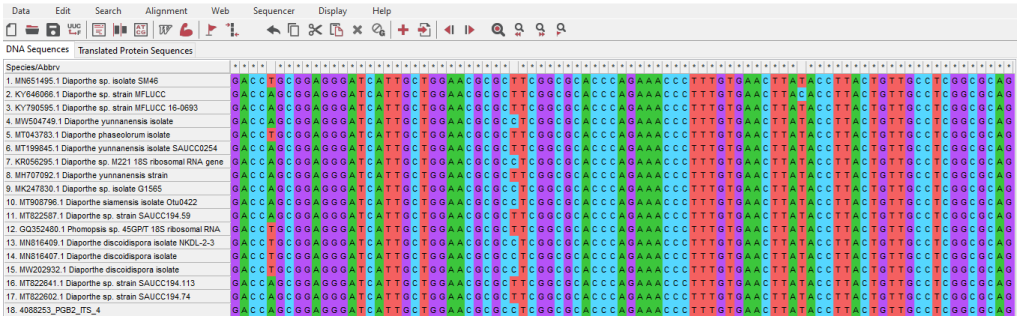


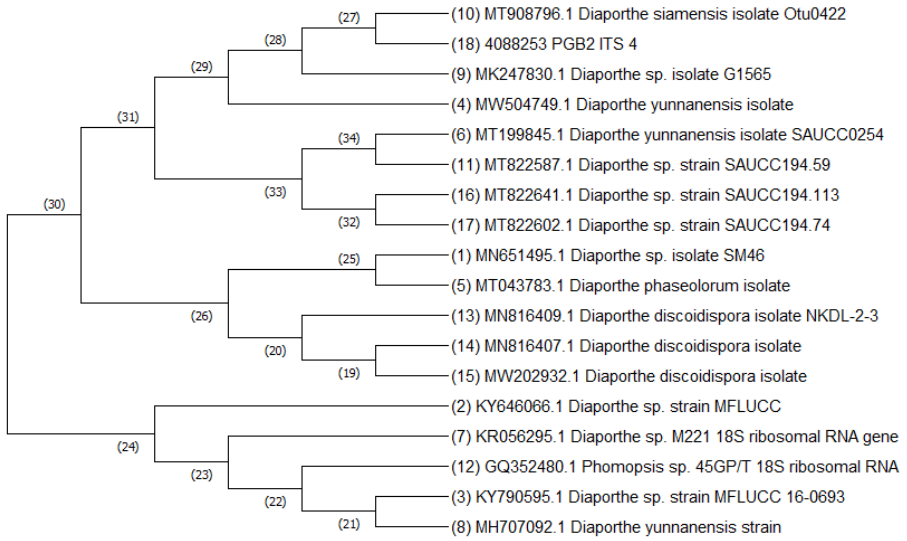
Figure 4. 14: Blast analysis of PgB2

4.5.2.2 Molecular identification of PgB2 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 4. 15: DNA taxonomy of fungal strain PgB2. (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

4.5.3 Molecular identification of fungal strain PgB3

4.5.3.1 The nucleotide sequence analysis of PgB3

Molecular analysis of the fungus PgB3 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 PgB3 resulted in a 557 bp long sequence:

GGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTTACGCTCTACAACCCT
TTGTGAACATACCTATAACTGTTGCTTCGGCGGGTAGGGTCTCCGCGACCCTCCCGG
CCTCCCGCCTCCGGGCGGGTCGGCGCCCGCCGGAGGATAACCAAACTCTGATTTAAC
GACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTAACAACGGATCTCTT
GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGG
CATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTACA
GCTGATGTAGGCCCTCAAAGGTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGT
AACTTTACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCAATTTT
CCAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTA

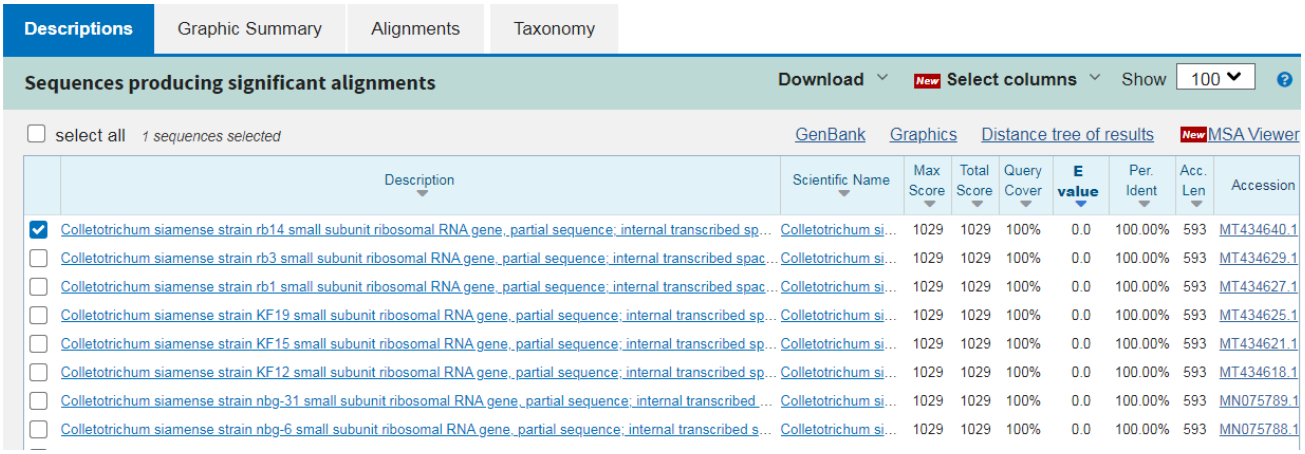
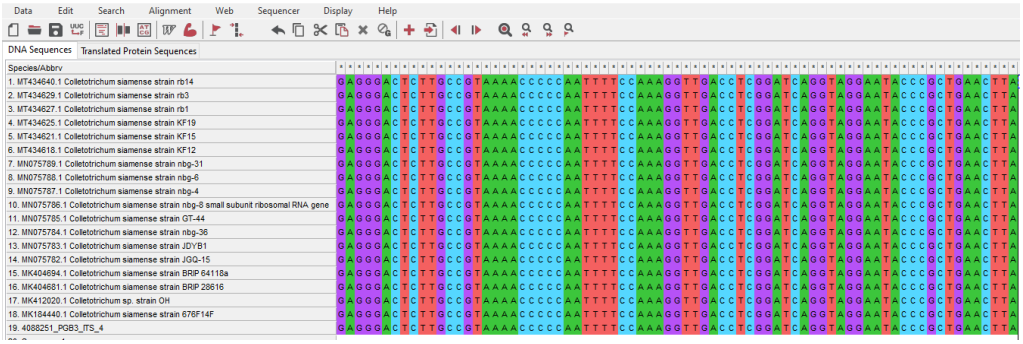


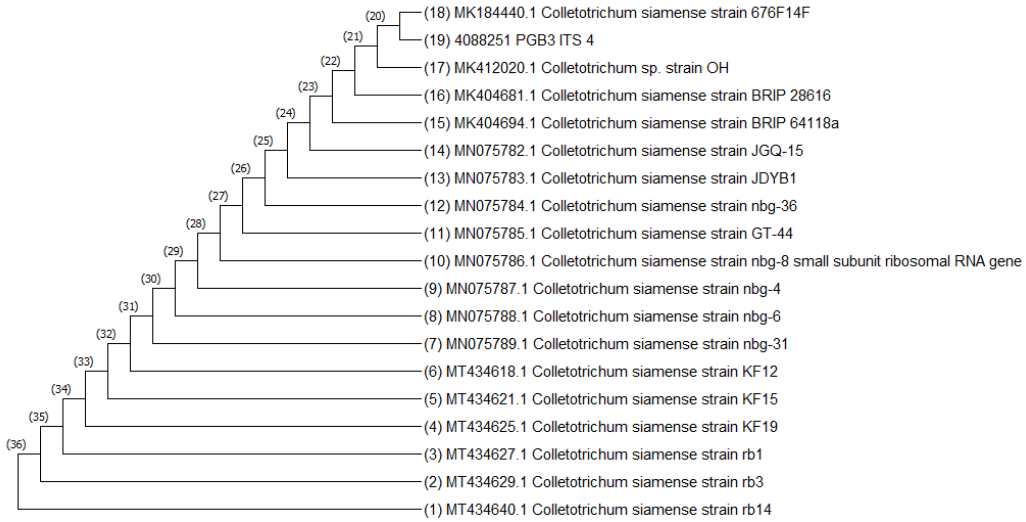
Figure 4. 16: Blast analysis of PgB3

4.5.3.2 Molecular identification of PgB3 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 4. 17: DNA taxonomy of fungal strain PgB3. (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 PgB4

4.5.4.1 The nucleotide sequence analysis of PgB4

Molecular analysis of the fungus PgB4 based on 5.8s rRNA gene revealed 99.64% similarity with another fungal isolate of *Nemania primolutea*, accession number: MG881830.1, deposited in the

U.S National Center for Biotechnology Information (NCBI). Partial sequencing of the ITS region for sample PgB4 resulted in a 559 bp long sequence:

CTCCGTTGGTGAACCAGCGGAGGGATCATTAAAGAGTTTTCTACAACTCCCAAACCC
CTGTGAACATACCTTCTGTTGCCTCGGCAGGCCTCGCCTACCCTCGTAGCCCCCTAC
ACCGTAGGGCCTACGCCGGGTGGTGCGCGGACCCTGCCGGCGGCCCGCGAAACTCT
GTTTAGCACTGAATCTCTGAACATATAACTAAATAAGTTAAAACTTTCAACAACGGA
TCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTA
GTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGG
GAGCCTACGGCAGCGTAGCTCCCCAAAGTTAGTGGCGTGGTCGGTTCACACTCCAG
ACGTAGTAGATTTTCGTCTCGCCTGTAGTTGGACCGGTCCCCTGCCGTAAAACACCC
CAATTCTAAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAAC

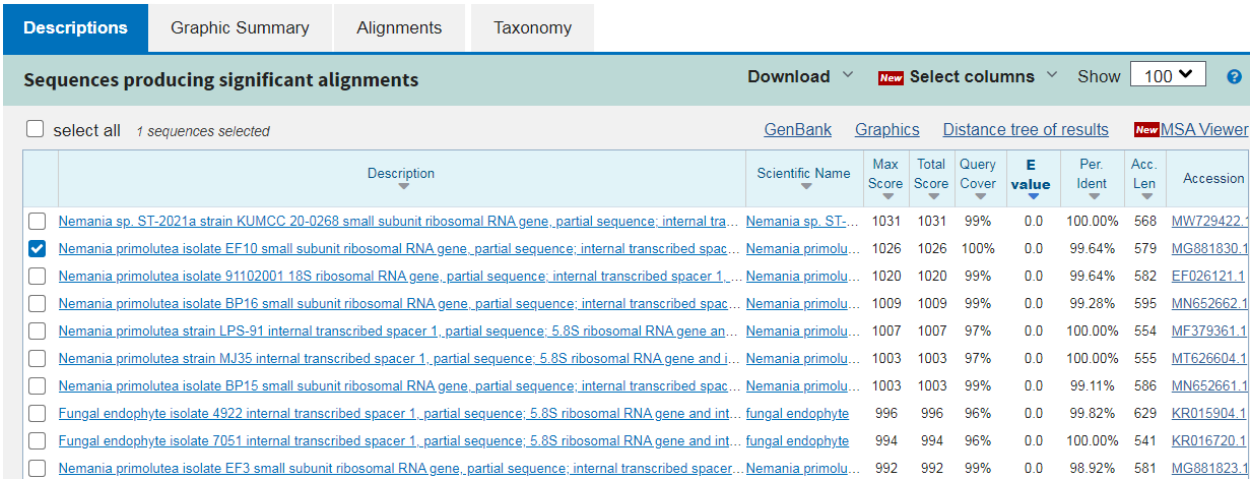
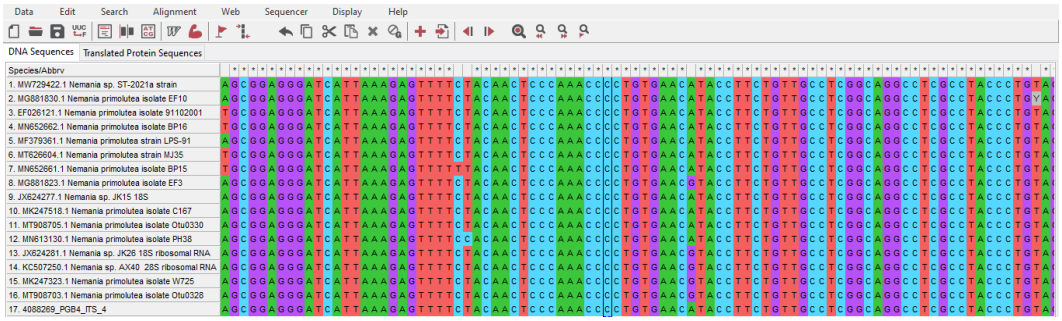


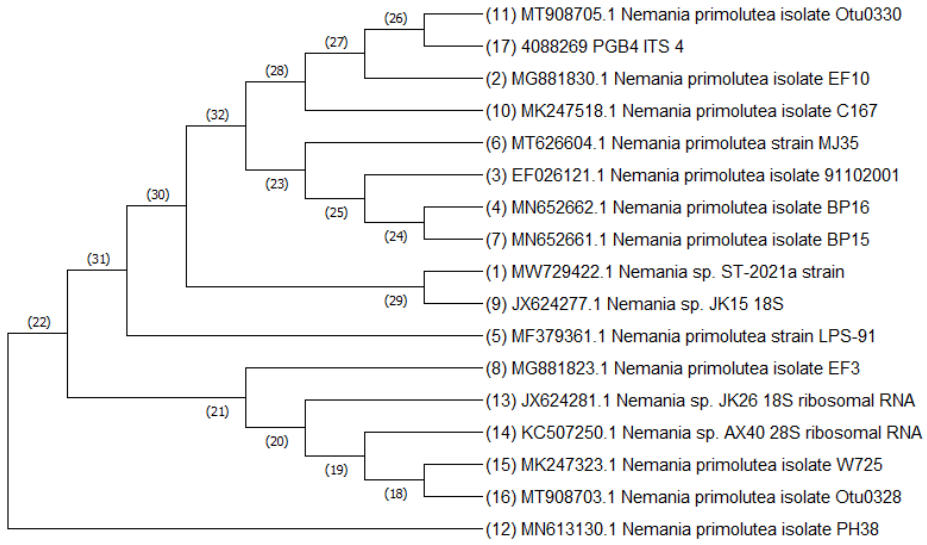
Figure 4. 18: Blast analysis of PgB4

4.5.4.2 Molecular identification of PgB4 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 4. 19: DNA taxonomy of fungal strain PgB4. (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

4.5.5 Molecular identification of fungal strain PgL1

4.5.5.1 The nucleotide sequence analysis of PgL1

Molecular analysis of the fungus PgL1 based on 5.8s rRNA gene revealed 100% similarity with another fungal isolate of *Daldinia eschscholtzii*, accession number: MN341731.1, deposited in the

U.S National Center for Biotechnology Information (NCBI). Partial sequencing of the ITS region for sample PgL1 resulted in a 554 bp long sequence:

AGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTATCTAAACTCCAACC
CTATGTGAACTTACCGCCGTTGCCTCGGCGGGCCGCGTTCGCCCTGTAGTTTACTAC
CTGGCGGCGCGCTACAGGCCCGCCGGTGGACTGCTAAACTCTGTTATATATACGTAT
CTCTGAATGCTTCAACTTAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTG
TTCGAGCGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAATCTAGGTCTC
CAGGGCCTAGTTCCCCAAAGTCATCGGCGGAGTCGGAGCGTACTCTCAGCGTAGTA
ATACCATTCTCGCTTTTGCAGTAGCCCCGGCGGCTTGCCGTAAAACCCCTATATCTTT
AGTGGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACT

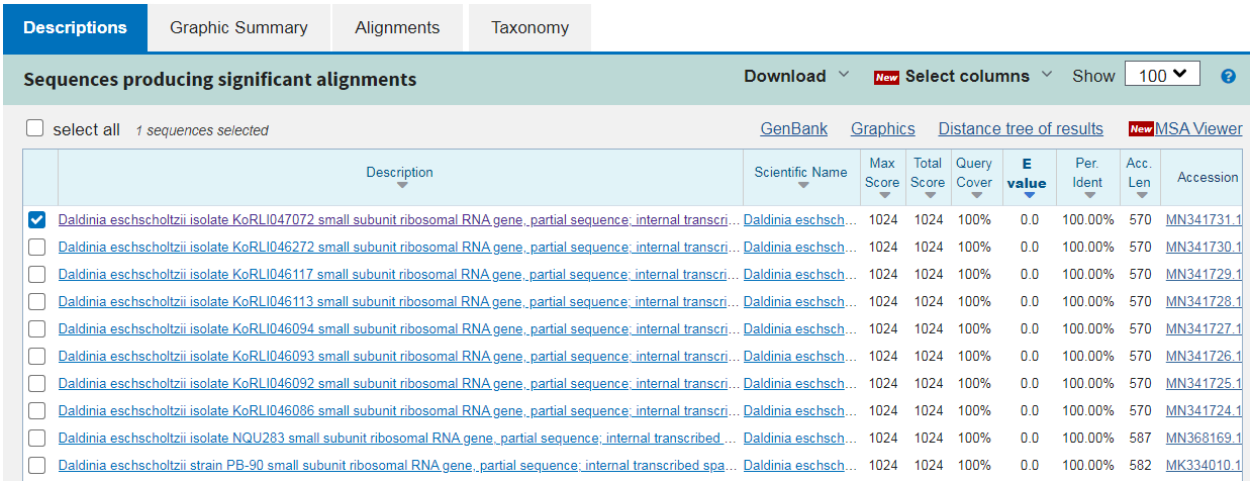
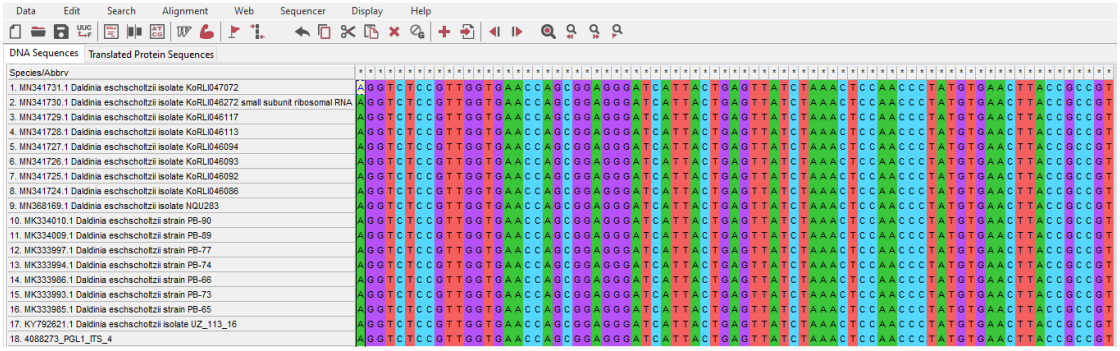


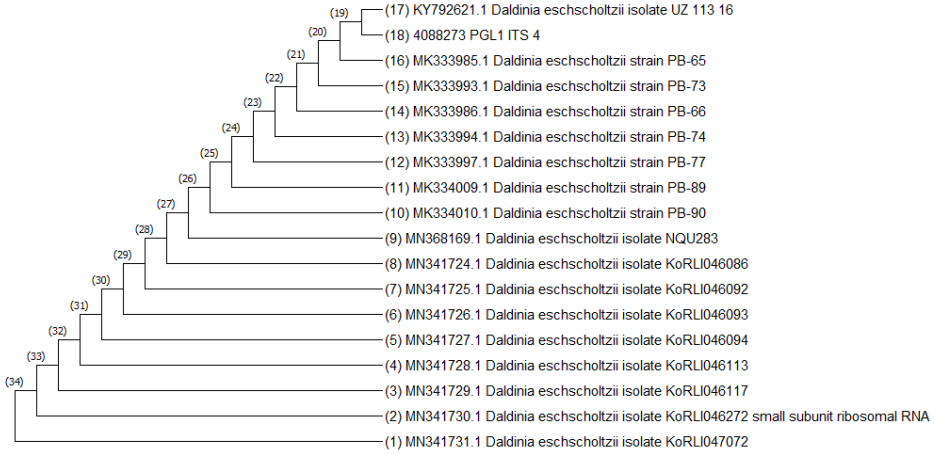
Figure 4. 20: Blast analysis of PgL1

4.5.5.2 Molecular identification of PgL1 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 4. 21: DNA taxonomy of fungal strain PgL1. (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 five endophytic fungal strains from *Psidium guajava* were cultivated on small scale (25-30 Petri dishes) at 27 °C for 21 days on 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.

4.7 Antimicrobial screening of fungal extracts

The results of the antimicrobial activity of all the extracts exhibiting different zones of inhibition are shown in the following Table-

Table 4. 7: Antimicrobial activity of fungal extracts

Bacterial strain	PgB1	PgB2	PgB3	PgB4	PgL1	Kanamycin
Escherichia coli	7	7	7	8	8	33
Salmonella typhi	---	9	8	16	9	29
Staphylococcus aureus	7	7	8	8	---	31
Bacillus megaterium	---	7	8	7	7	28
Fungal strain						**Ketoconazole**
Aspergillus niger	---	50	---	25	---	28
Aspergillus flavus	---	---	---	---	---	35

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

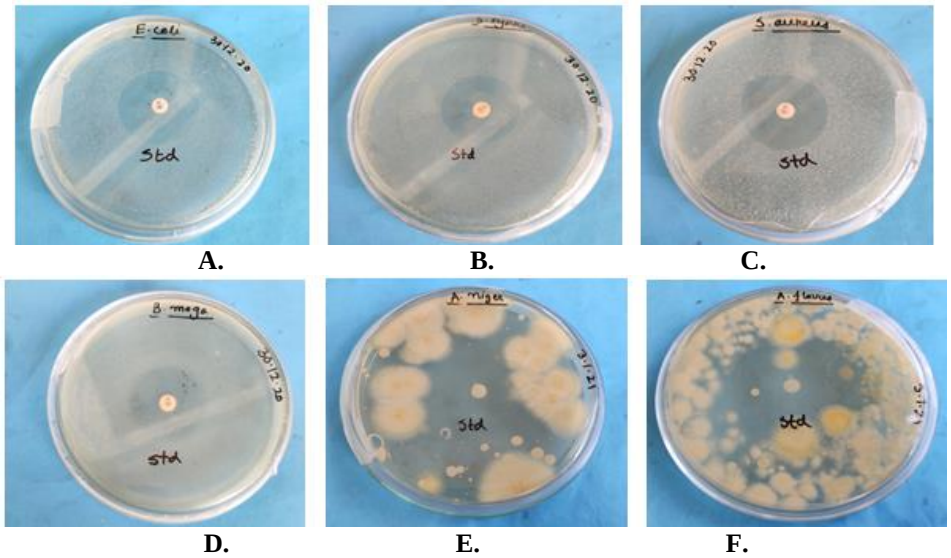
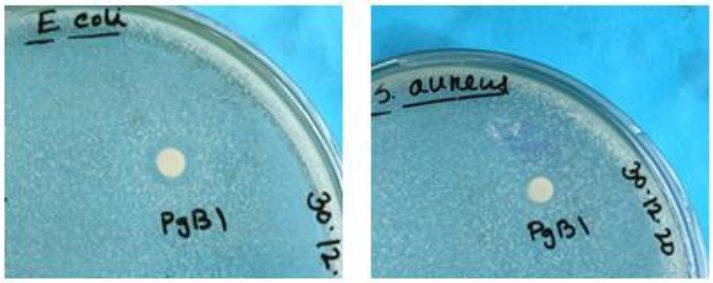
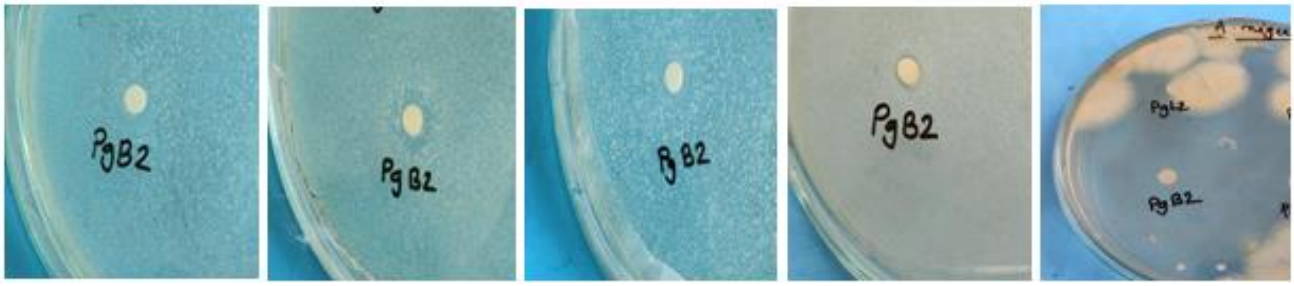


Figure 4. 22: Antimicrobial activity of standard disc against A. *E. coli,* B. *S. typhi,* C. *S. aureus,* D. *B. megaterium,* E. *A. niger,* F. *A. flavus.*



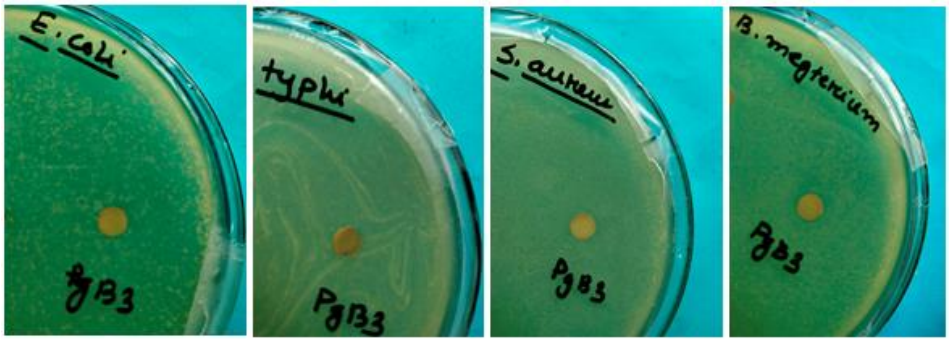
A. B.

Figure 4. 23: Antimicrobial activity of PgB1 against *E. coli* and *S. aureus*



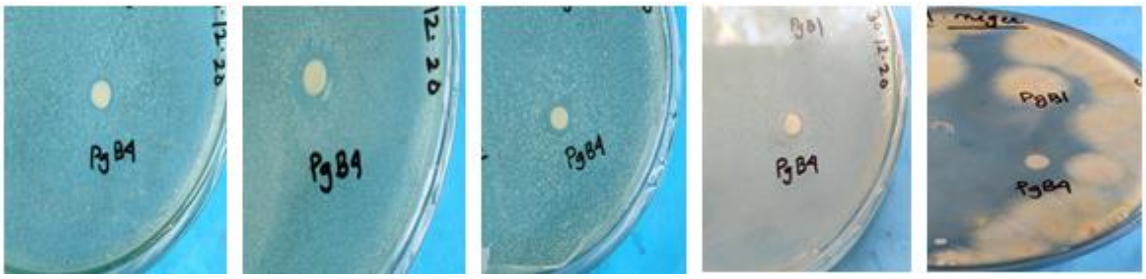
A. B. C. D. E.

Figure 4. 24: Antimicrobial activity of PgB2 against A. *E. coli,* B. *S. typhi,* C. *S. aureus,* D. *B. megaterium* and E. *A. niger.*



A. B. C. D.

Figure 4. 25: Antimicrobial activity of PgB3 against A. *E. coli,* B. *S. typhi,* C. *S. aureus,* and D. *B. megaterium*



A. **B.** **C.** **D.** **E.**

Figure 4. 26: Antimicrobial activity of PgB4 against A. *E. coli,* B. *S. typhi,* C. *S. aureus,* D. *B. megaterium* and E. *A. niger.*

A. **B.** **C.**

Figure 4. 27: Antimicrobial activity of PgL1 against A. *E. coli,* B. *S. typhi,* and C. *B. megaterium.*

4.8 Evaluation of antioxidant activity by DPPH free radical scavenging method

The fungal extracts of PgB1, PgB2, PgB3, PgB4, and PgL1 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 Butylated Hydroxy Anisole (BHA)

From the calculation, at a 50 µg/ml dose, BHA showed about 85.32% free radical scavenging activity and at a lower dose 0.78125µg/ml, free radical scavenging activity was 17%. After analyzing the data in a graph, the IC50 value was found 8.84µg/ml.

Table 4. 8: IC50 value for Butylated Hydroxy Anisole

Absorbance of control	Concentration (µg/ml)	% of Inhibition	The absorbance of the extract	IC50 value (µg/ml)
0.307	50	85.32	0.0242	8.84
	25	83.421	0.0279	
	12.5	71.217	0.0912	
	6.25	60.125	0.0253	
	3.125	45.332	0.0503	
	1.5625	30.678	0.1292	
	0.78125	17.418	0.3039	

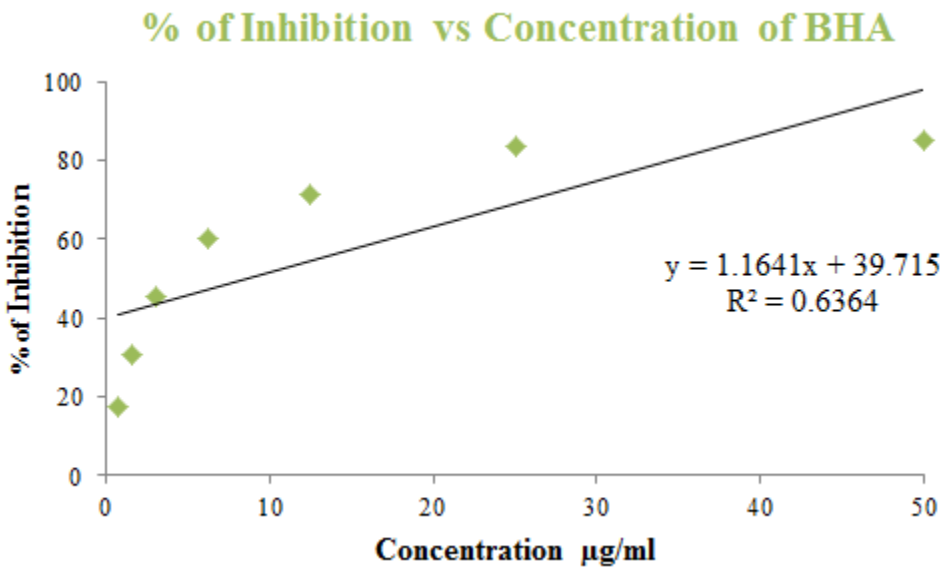


Figure 4. 28: Dose-response relationship on free radical scavenging activity of Butylated Hydroxy Anisole (BHA)

4.8.2 Antioxidant activity of ascorbic acid (AA)

After calculation, the free radical scavenging activity of ascorbic acid was 91% at 50 µg/ml dose and 5.41% at the lowest dose of 0.78125µg/ml. From the plotted graph, the IC50 value of ascorbic acid was 11.41 µg/ml.

Table 4. 9: IC50 value for Ascorbic Acid (AA)

Absorbance of control	Concentration (µg/ml)	% of Inhibition	The absorbance of the extract	IC50 value (µg/ml)
0.307	50	91.04	0.0098	11.41
	25	93.88	0.1878	
	12.5	78.63	0.0656	
	6.25	60.43	0.1214	
	3.125	37.21	0.1927	
	1.5625	14.17	0.2634	
	0.78125	5.41	0.2903	

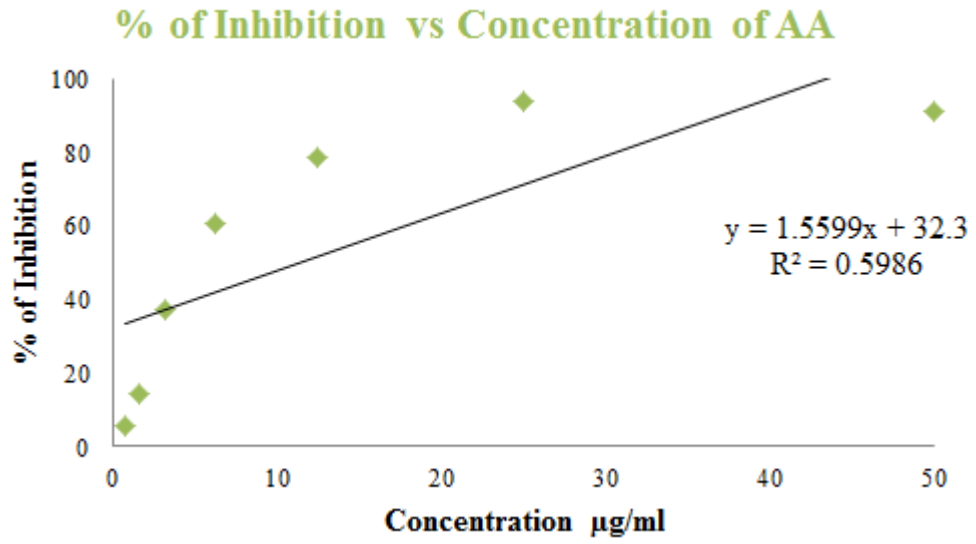


Figure 4. 29: Dose-response relationship on free radical scavenging activity of Ascorbic Acid (AA)

4.8.3 Antioxidant activity of PgB1

From the calculation, at 200 µg/ml dose, PgB1 extract showed about 86.41% free radical scavenging activity and at a lower dose of 0.78125µg/ml, free radical scavenging activity was 33.75%. After analyzing the data in a graph, the IC50 value was found 56.02 µg/ml.

Table 4. 10: IC50 value for PgB1

Absorbance of control	Concentration (µg/ml)	% of Inhibition	The absorbance of the extract	IC50 value (µg/ml)
0.307	200	86.41	0.05	56.02
	100	73.32	0.0982	
	50	60.24	0.1463	
	25	60.46	0.1455	
	12.5	30.27	0.2566	
	6.25	26.3	0.2712	
	3.125	18.91	0.2984	
	1.5625	27.26	0.2677	
	0.71825	33.75	0.2438	

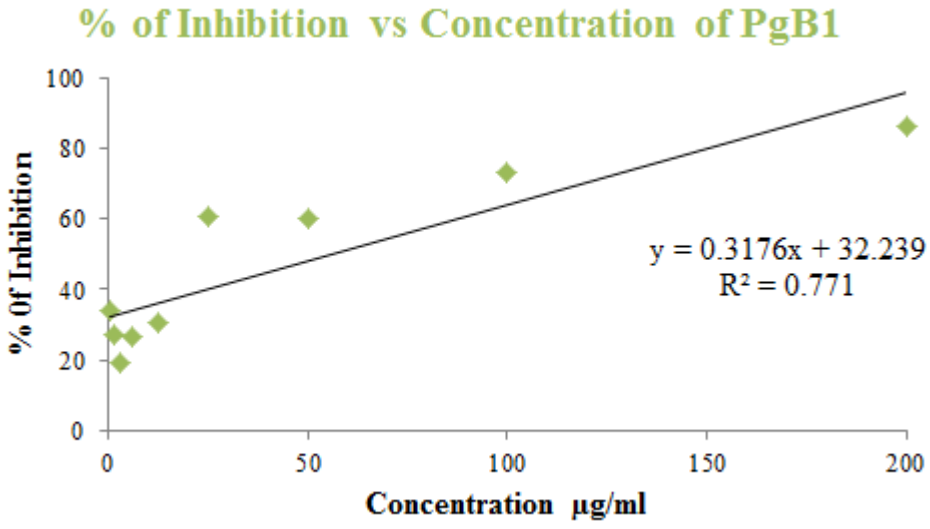


Figure 4. 30: Dose-response relationship on free radical scavenging activity of PgB1

4.8.4 Antioxidant activity of PgB2

After calculation, the free radical scavenging activity of PgB2 was 70% at 200 µg/ml dose and 27.98% at the lowest dose 0.78125µg/ml. From the plotted graph, the IC50 value of PgB2 was 83.9µg/ml.

Table 4. 11: IC50 value for PgB2

Absorbance of control	Concentration (µg/ml)	% of Inhibition	The absorbance of the extract	IC50 value (µg/ml)
0.307	200	70.12	0.0917	83.9
	100	56.94	0.1322	
	50	47.17	0.1622	
	25	43.71	0.1728	
	12.5	34.07	0.2024	
	6.25	33.19	0.2051	
	1.5625	30.87	0.2122	
	0.71825	27.98	0.2211	

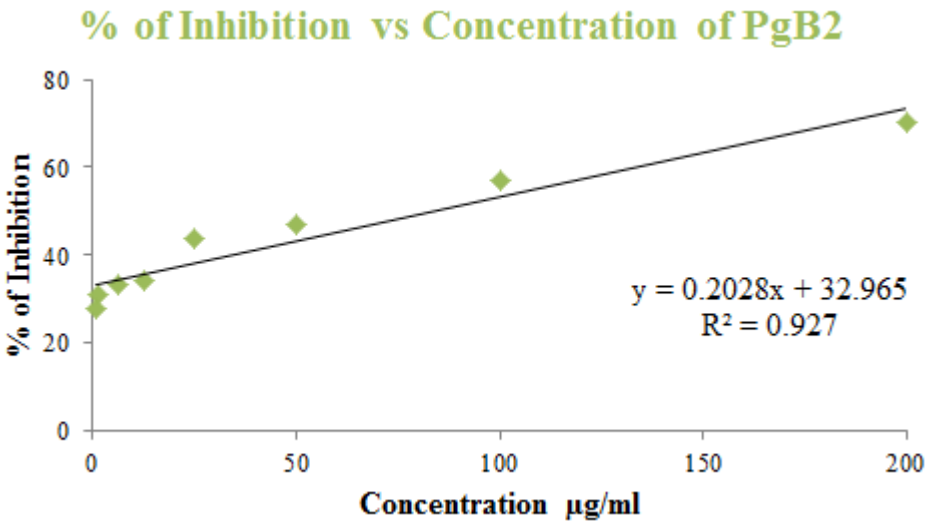


Figure 4. 31: Dose-response relationship on free radical scavenging activity of PgB2

4.8.5 Antioxidant activity of PgB3

From the calculation, at a 50 µg/ml dose, PgB3 extract showed about 73% free radical scavenging activity and at a lower dose 0.78125µg/ml, free radical scavenging activity was 42%. After analyzing the data in a graph, the IC50 value was found 3.4µg/ml.

Table 4. 12: IC50 value for PgB3

Absorbance of control	Concentration (µg/ml)	% of Inhibition	The absorbance of the extract	IC50 value (µg/ml)
0.307	50	73.09	0.0826	3.4
	25	68.69	0.0961	
	12.5	60.49	0.1213	
	6.25	55.18	0.1376	
	3.125	49.93	0.1537	
	1.5625	45.15	0.1684	
	0.71825	42.02	0.178	

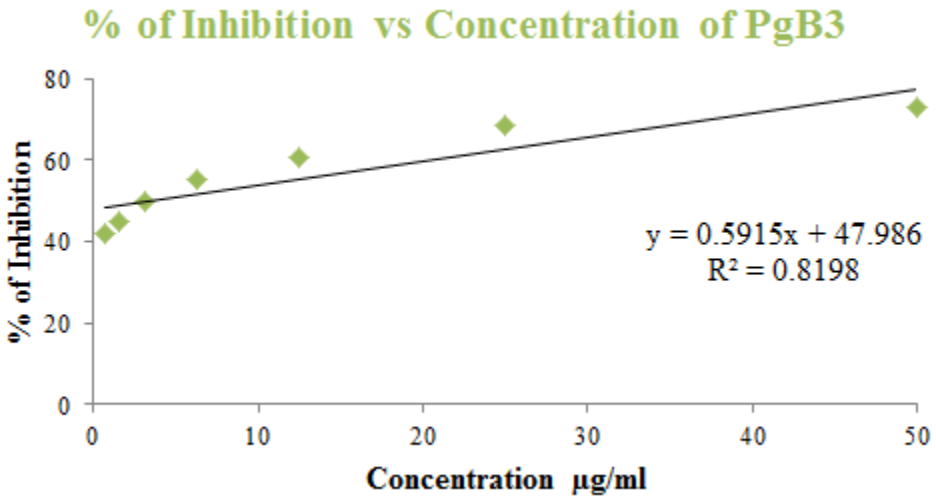


Figure 4. 32: Dose-response relationship on free radical scavenging activity of PgB3

4.8.6 Antioxidant activity of PgB4

After calculation, the free radical scavenging activity of PgB4 was 30.98% at 50 µg/ml dose and 8% at the lowest dose of 0.78125µg/ml. From the plotted graph, the IC50 value of PgB4 was 87.77µg/ml.

Table 4. 13: IC50 value for PgB4

Absorbance of control	Concentration (µg/ml)	% of Inhibition	The absorbance of the extract	IC50 value (µg/ml)
0.307	50	30.98	0.2724	87.77
	25	27.46	0.2824	
	12.5	25.09	0.272	
	6.25	24.76	0.2769	
	3.125	15.33	0.2895	
	1.5625	9.08	0.3346	
	0.71825	7.53	0.3403	

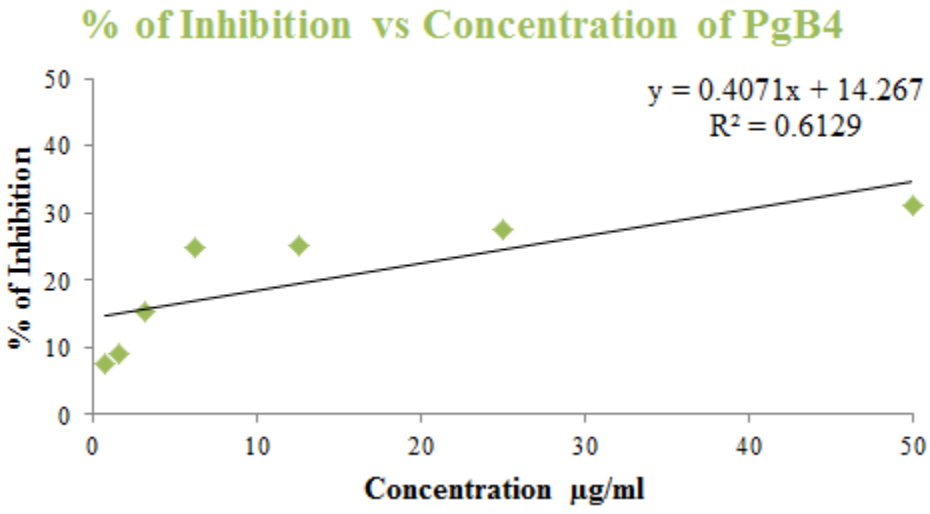


Figure 4. 33: Dose-response relationship on free radical scavenging activity of PgB4

4.8.7 Antioxidant activity of PgL1

From the calculation, at a 50 µg/ml dose, PgL1 extract showed about 71% free radical scavenging activity and at a lower dose of 0.78125µg/ml, free radical scavenging activity was 15.98%. After analyzing the data in a graph, the IC50 value was found 15.7µg/ml.

Table 4. 14: IC50 value for PgL1

Absorbance of control	Concentration (µg/ml)	% of Inhibition	The absorbance of the extract	IC50 value (µg/ml)
0.307	50	71.09	0.1064	15.7
	25	75.38	0.0906	
	12.5	64.02	0.1324	
	6.25	59.13	0.1504	
	3.125	31.87	0.2507	
	1.5625	21.66	0.2883	
	0.71825	15.98	0.3092	

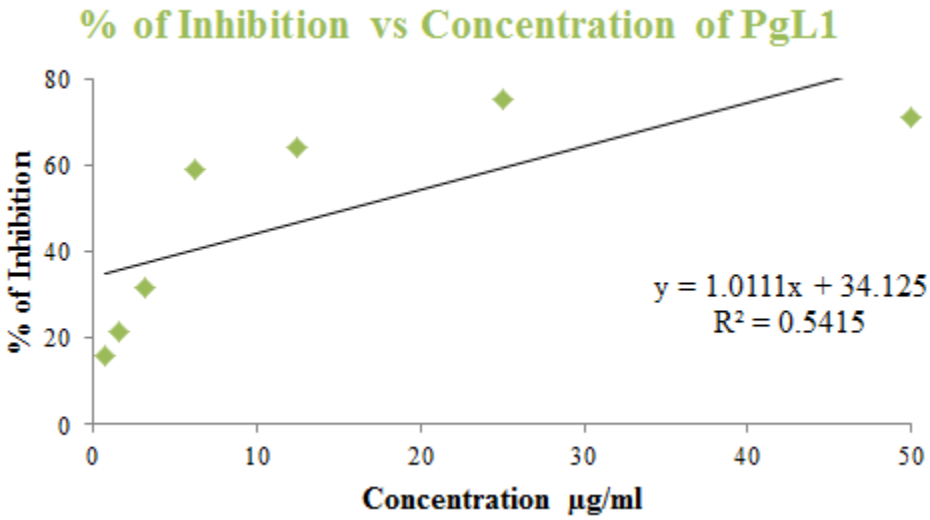


Figure 4. 34: Dose-response relationship on free radical scavenging activity of PgL1

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-H2SO4 spray reagent.

For the presence of different secondary metabolites in the ethyl-acetate or organic extracts of PgB1, PgB2, PgB3, PgB4, and PgL1 was screened. Thin layer chromatographic technique was used for screening the extracts in different solvent systems. Distinctive compounds showed different colors, thus by a preliminary chemical screening of extracts, the presence of different compounds was determined.

Table 4. 15: Preliminary chemical screening of fungal extracts

Test sample	Visual Observation	UV light at 254 nm	UV light at 365 nm	After Spray	Remarks on probable compounds
PgB1	Green Pink Orange	Sky Blue Orange Green	DQS Purple Green Purple Sky Blue	Purple Pink Brown	Isocumarins, Couramins, Steroids,
PgB2	Nil	Sky Blue Green	DQS Sky Blue Purple	Orange	Isocumarins, Couramins, Anthaquinones
PgB3	Light yellow	Sky Blue Green	DQS Green Purple Blue	Purple Dark Purple Brown	Isocumarins, Couramins, Steroids

PgB4	Light Brown Brow	Sky Blue Green Green Purple Green	Purple Green Blue DQS Purple Sky Blue	Orange Pink Purple Brown	Isocumarins, Couramins, Steroids
PgL1	Brown Yellow Brown	Orange Light Green Light Green Yellowish Green Light Green	Purple Sky Blue DQS Green Brown	Pink Orange Light Green Brown	Anthraquinones, Steroids, Flavonoids

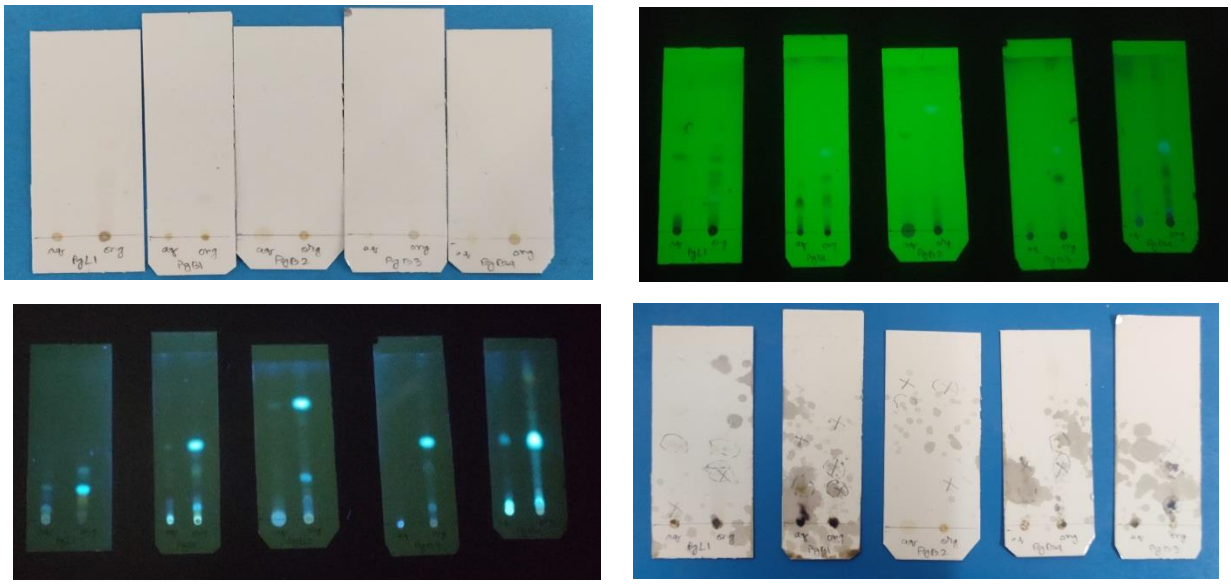


Figure 4. 35: Screening of fungal extracts by Thin Layer Chromatography (TLC) at Naked eye (A), at 254 nm (B), at 365 nm (C), and after spraying with spray reagent (D)

4.10 Isolation of the compound from PgB3:

The culture of the strain PgB3 was suspended in ethyl acetate for 12 days for 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 TLC for the detection of possible compounds. The compound from PgB3 was light yellow to the naked eye. On the TLC plate under UV light at 254 nm, it appeared sky blue to green in color and appeared as a dark quenching spot, green, blue fluorescence at 365nm. The compound from PgB3 was soluble in CHCl3 and Methanol. The compound was then purified through solvent treatment using various mixtures of methanol and DCM. The data of NMR spectrum is included below. From these data, it was proposed that the isolated compound could be of sterol derivatives.

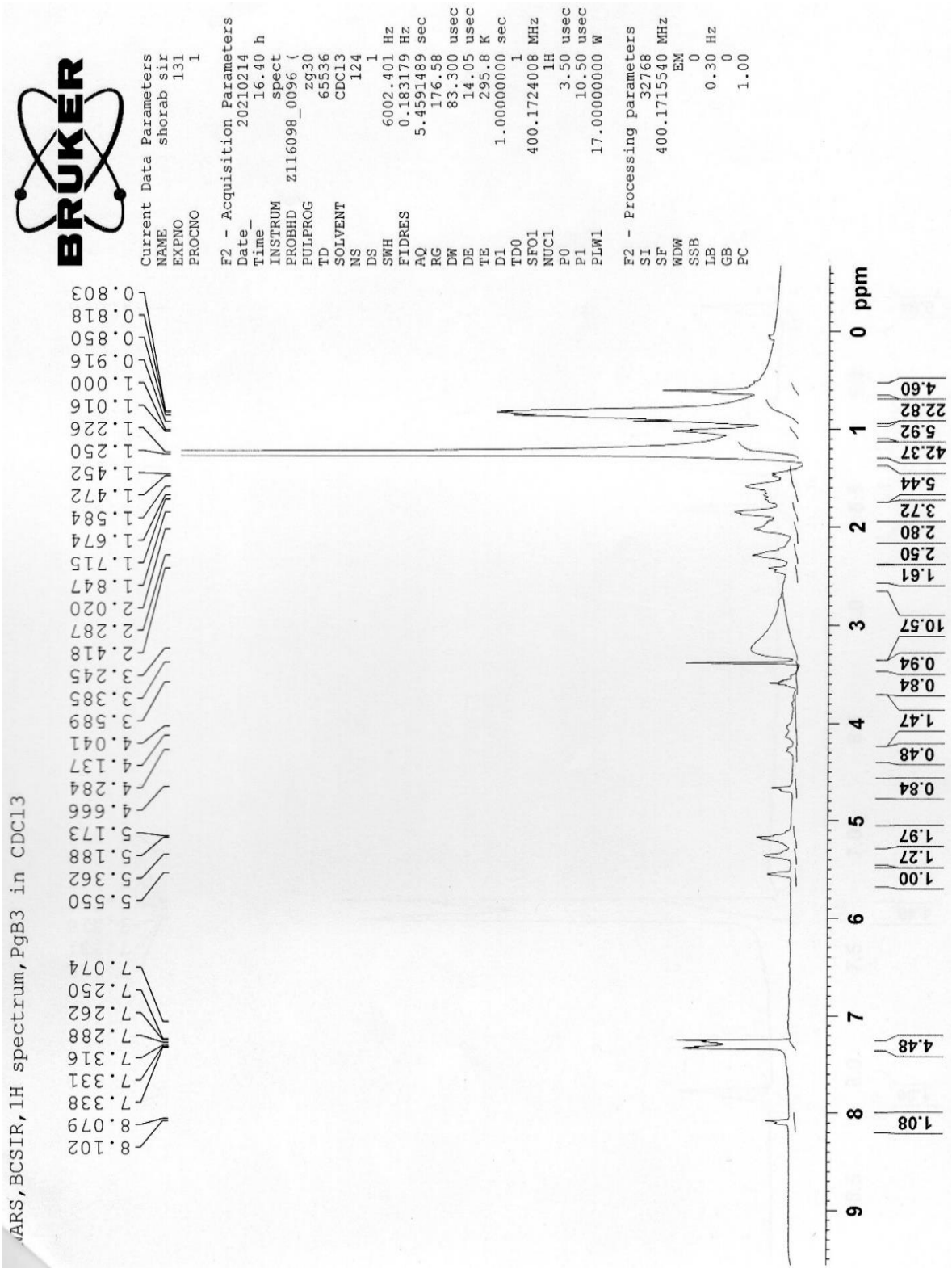


Figure 4. 36: NMR spectrum (400 MHz, $CDCl_3$ + 2 drops MeoD) of compound from PgB3

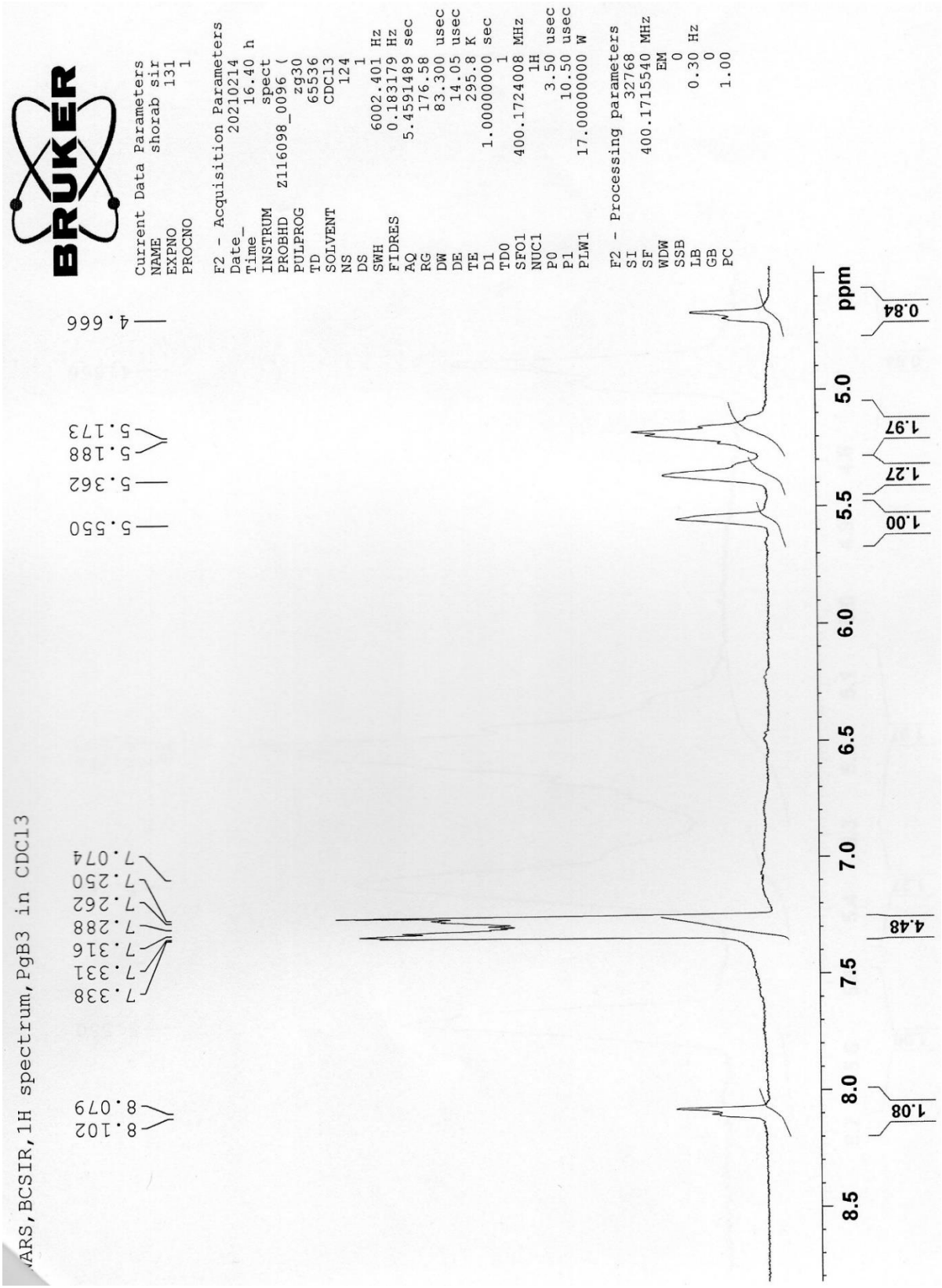


Figure 4. 37: NMR spectrum (400 MHz, $CDCl_3$ + 2 drops MeoD) of compound from PgB3

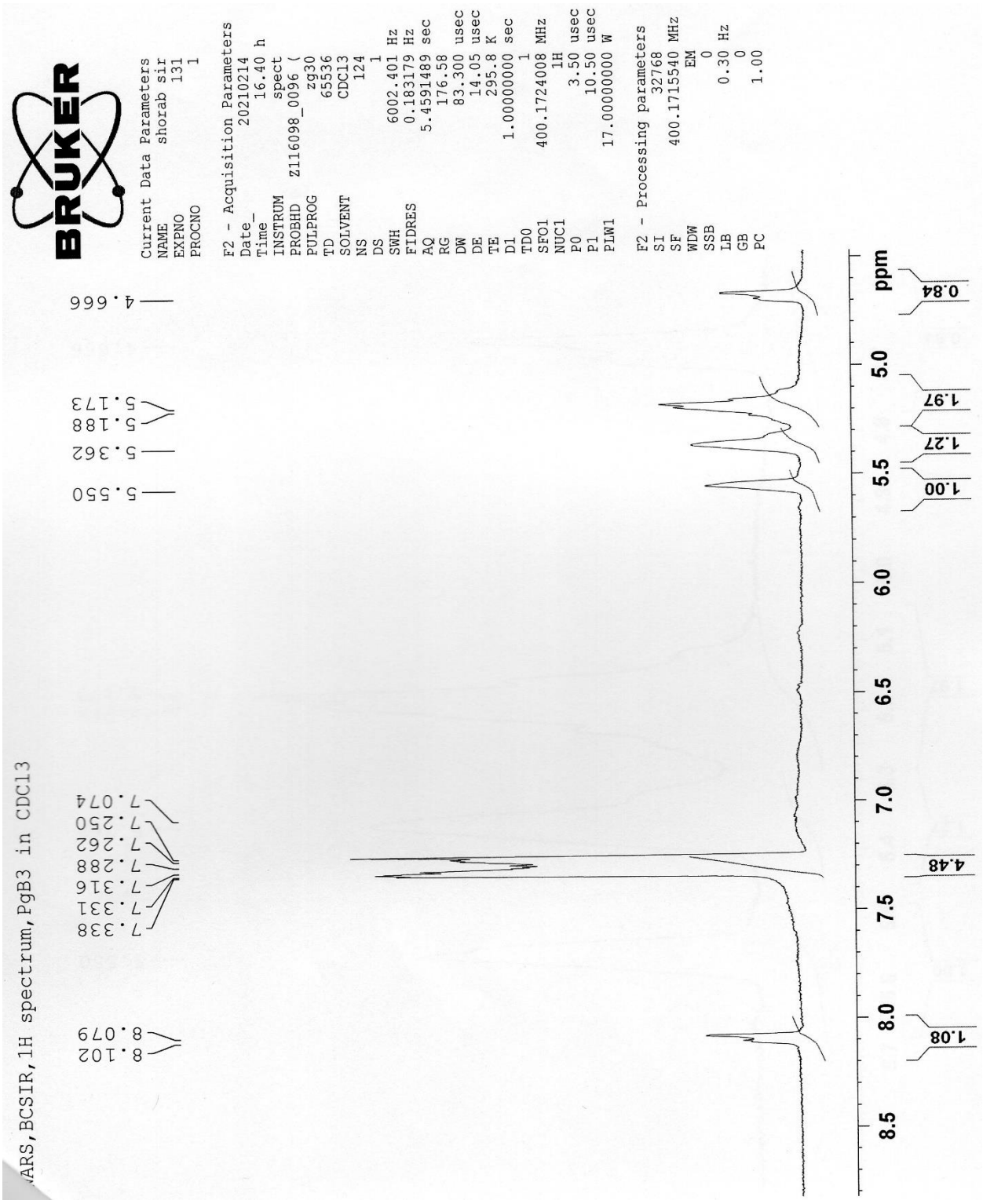


Figure 4. 38: NMR spectrum (400 MHz, $CDCl_3$ + 2 drops MeoD) of compound from PgB3

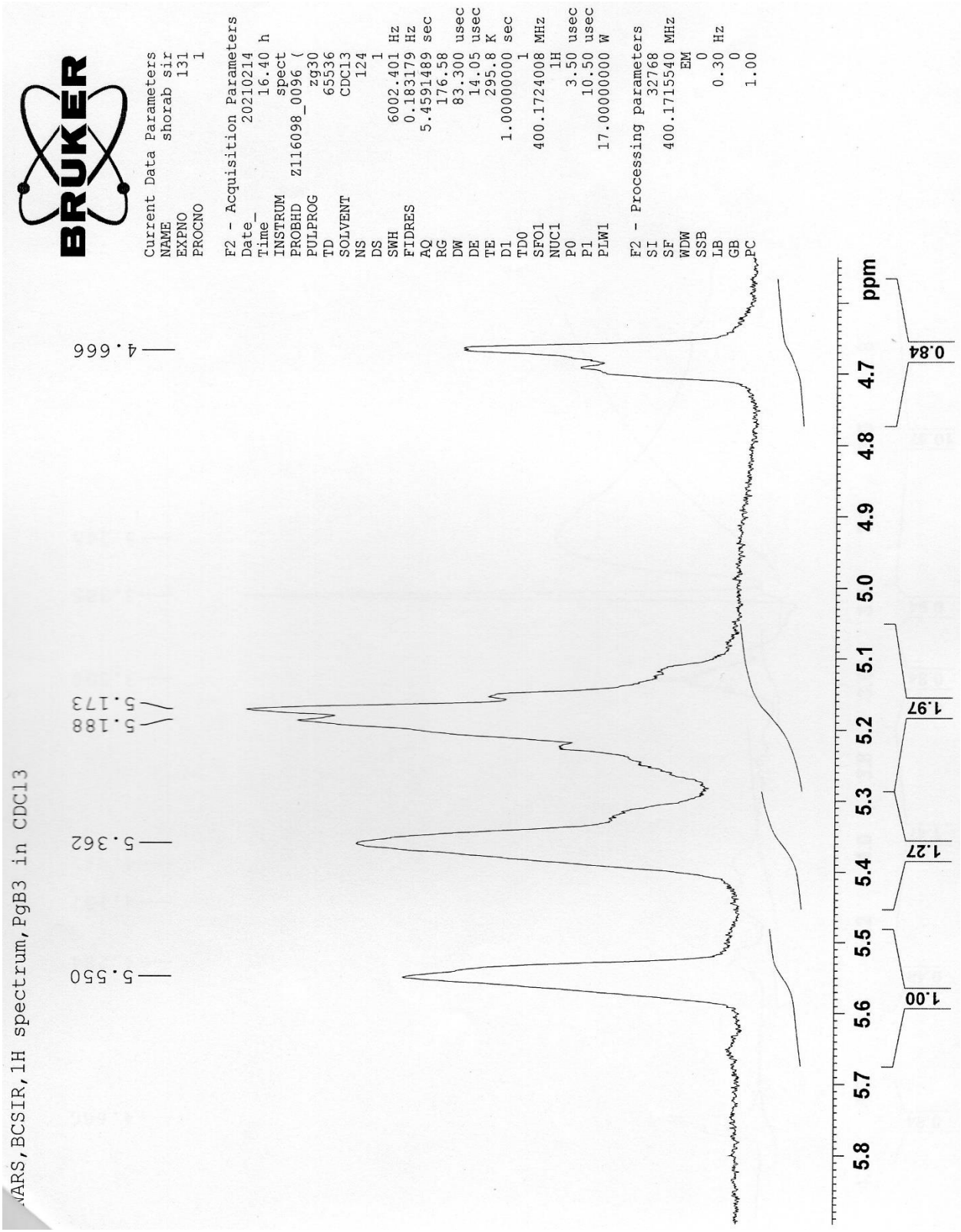


Figure 4. 39: NMR spectrum (400 MHz, $CDCl_3$ + 2 drops MeoD) of compound from PgB3

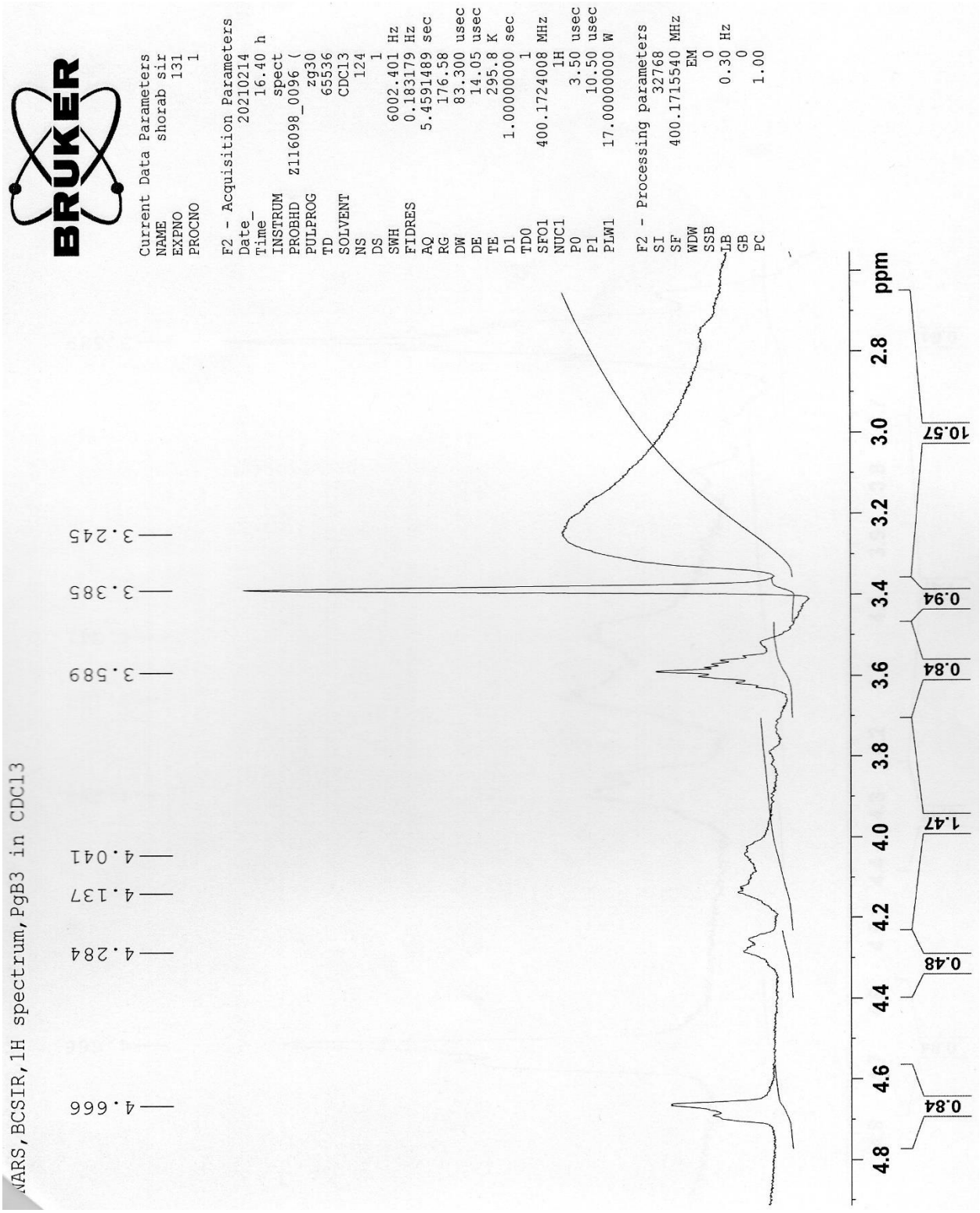


Figure 4. 40: NMR spectrum (400 MHz, $CDCl_3$ + 2 drops MeoD) of compound from PgB3

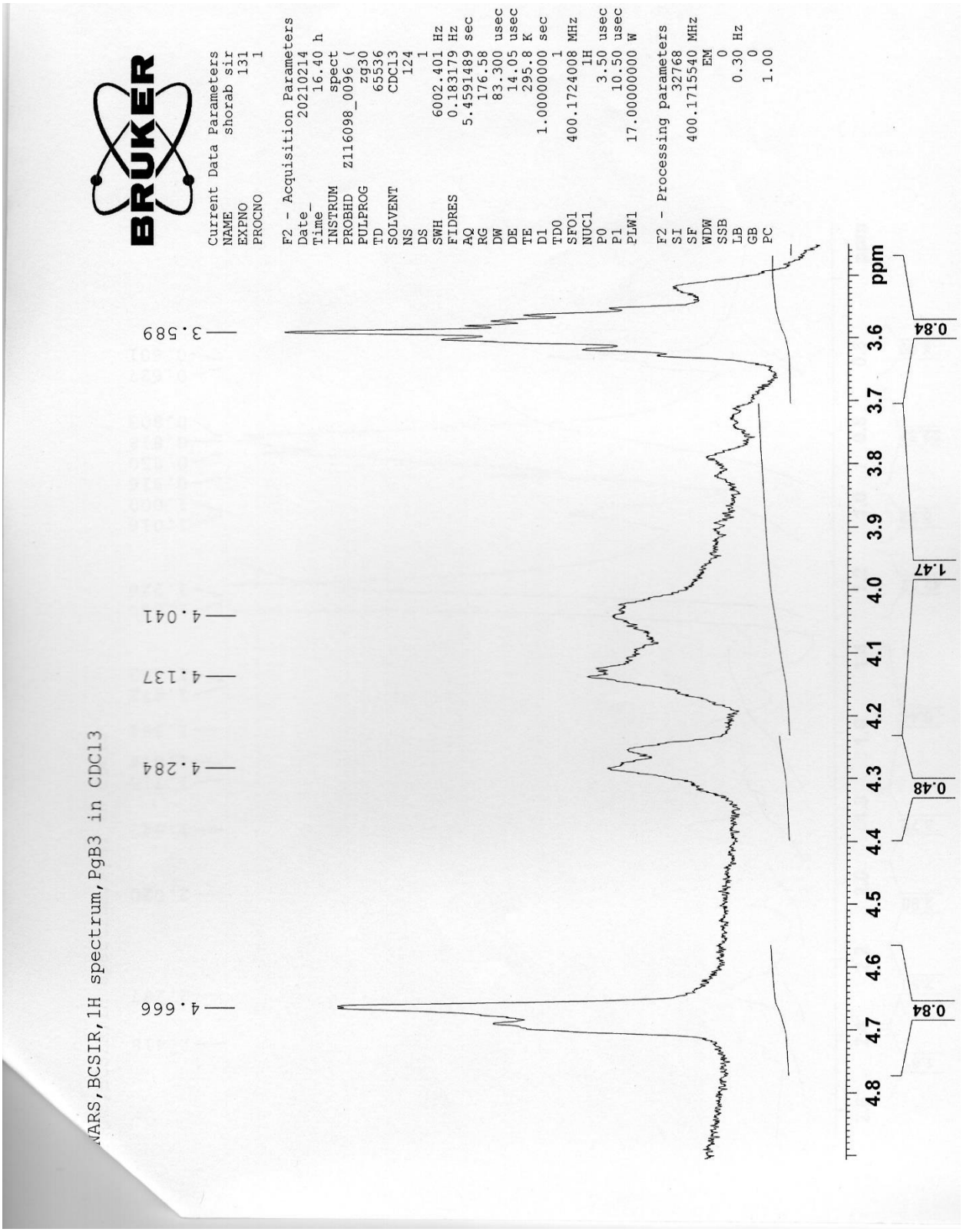


Figure 4. 41: NMR spectrum (400 MHz, $CDCl_3$ + 2 drops MeoD) of compound from PgB3

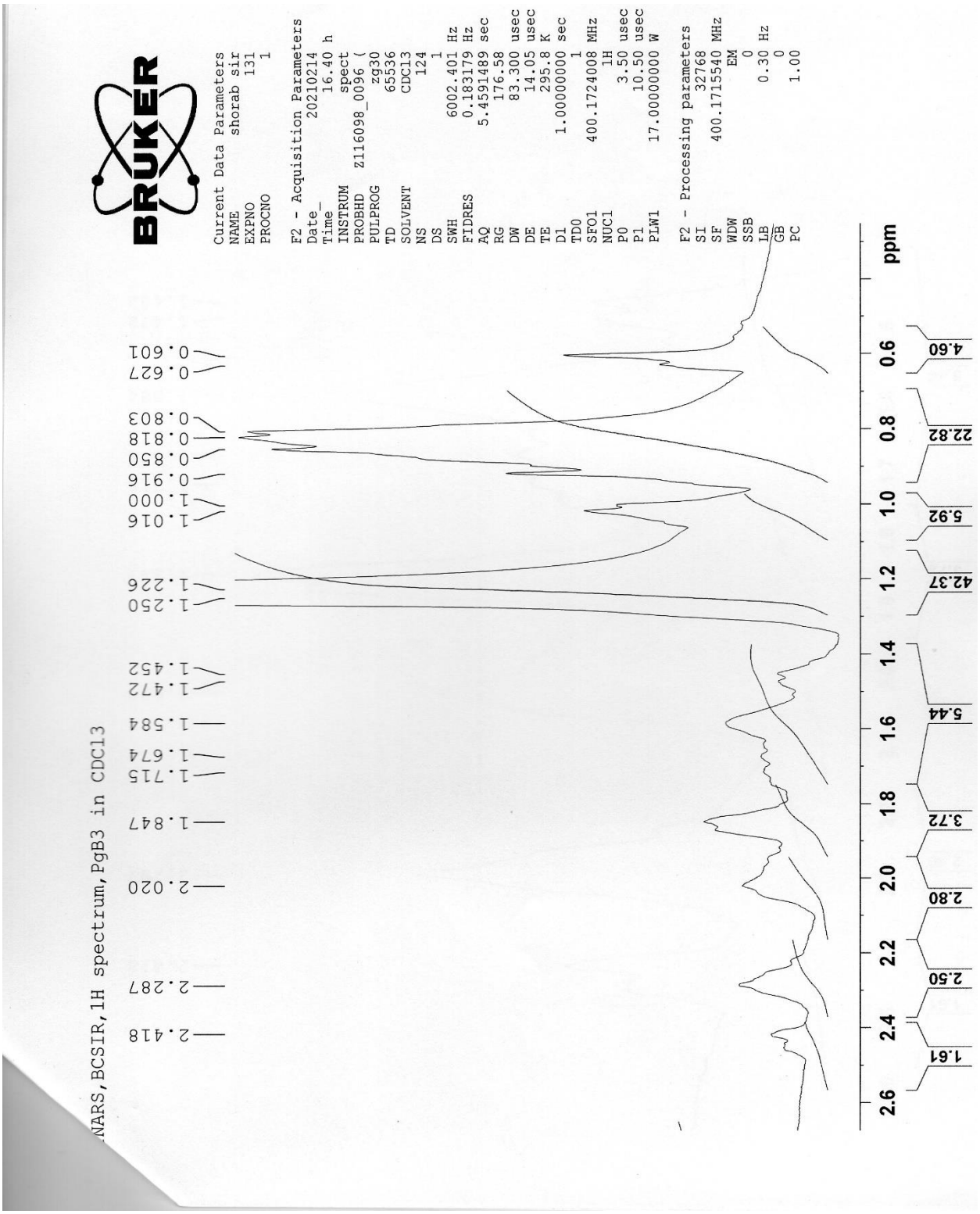


Figure 4. 42: NMR spectrum (400 MHz, $CDCl_3$ + 2 drops MeoD) of compound from PgB3

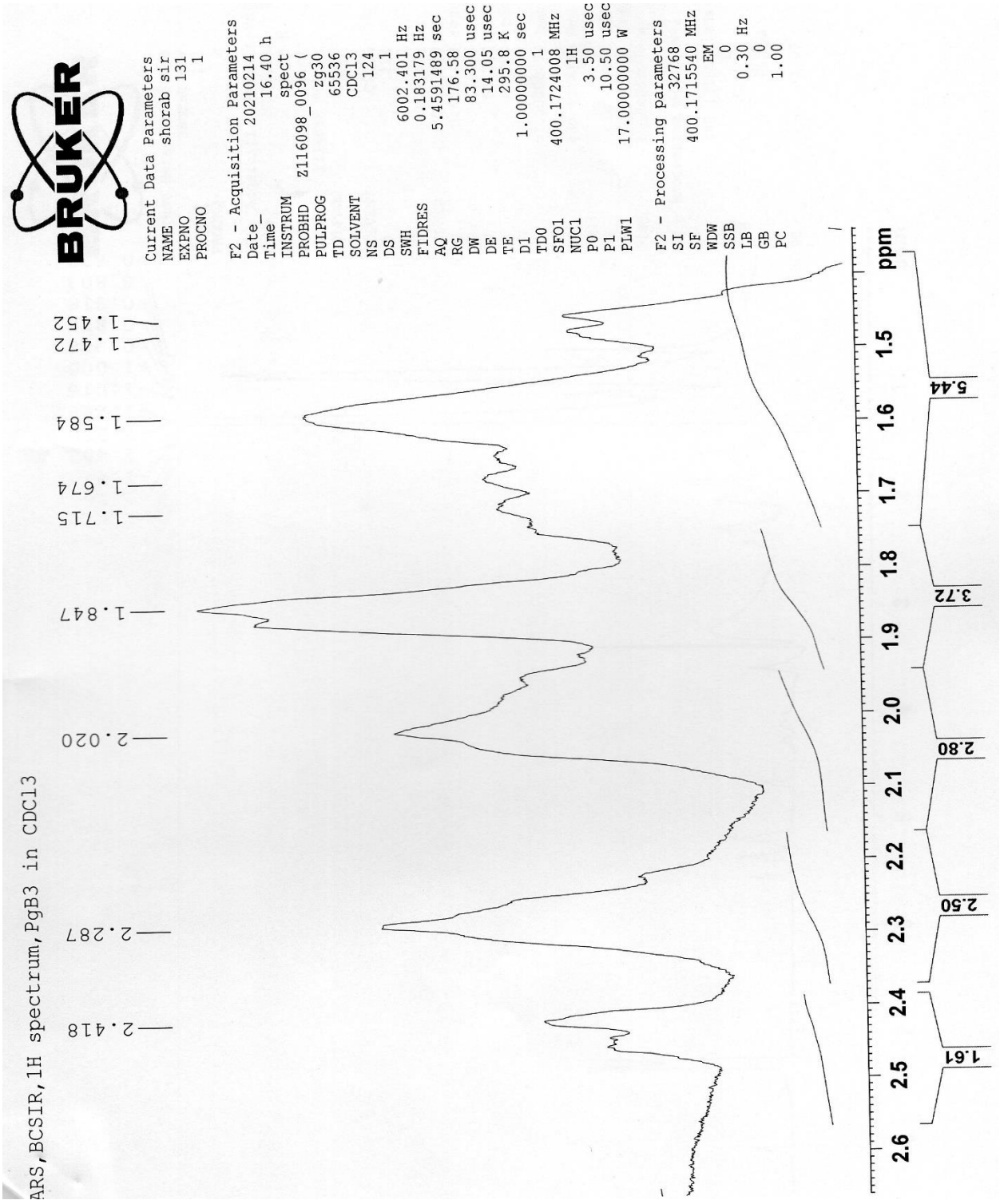


Figure 4. 43: NMR spectrum (400 MHz, $CDCl_3$ + 2 drops MeoD) of compound from PgB3

Chapter 5: Discussion

Humans have always relied on nature from the beginning for food, cloth, shelter, and medicine. Plant-natural products have been recognized as the most successful source of potential drug discovery. The search for new natural products as a potential source of drugs is ever-growing due to the emergence of multidrug-resistant infections, the emergence of new pathogens and new diseases, the availability of effective drugs and vaccines, etc against deathly 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). 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. 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, and anti-malarial compounds. (Aly et al, 2010; Bhadury et al, 2006). Thus, endophytic fungi are the potential new leads for natural products.

Psidium guajava (guava) 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 *Psidium guajava* 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 *Psidium guajava*. 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 originating from *Psidium guajava*.

A total of five endophytic fungi were isolated and purified from the plant *Psidium guajava.* Four endophytic fungal strains PgB1, PgB2, PgB3, PgB4 were isolated from the bark of the plant and one strain PgL1 was isolated from the leaf of the plant.

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

From the plant *Psidium guajava,* five 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 the 3rd, 6th, 9 th, and 12th days of both the top and bottom view of culture were observed and data were noted down in tables 4.2, 4.3, 4.4, 4.5, and 4.6 as well as figures 4.2, 4.3, 4.4, 4.5 and 4.6. These data were then cross-referenced with probable strains identified by microscopic and molecular results. After cross-referencing the identified isolates were PgB1 as *Phomopsis sp.,* PgB2 as *Diaporthe sp.,* PgB3 as *Colletotrichum sp.,* PgB4 as *Nemania sp.,* and PgL1 as *Daldinia sp.*

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. It was then examined for a characteristic arrangement of spores under 10X, 40X, and 100X objective lenses of a compound microscope. All five isolated fungal strains were examined under microscopic.

From microscopic observation of PgB1 (Figure 4.7), distinct conidia were seen which are associated with the *Phomopsis sp.* Therefore, it could be said that the endophyte PgB1 was from *Phomopsis sp.* When PgB2 (Figure 4.8) was observed under the microscope, conidia specific to the *Diaporthe sp.* were found. Thus, the endophyte PgB2 was identified as *Diaporthe sp.* After examining PgB3 (Figure 4.9) under the microscope, conidia were observed which could be only found in *Colletotrichum sp.* As a result, the endophyte PgB3 was detected as *Colletotrichum sp.* PgB4 was also subjected to microscopic observation (Figure 4.10) and from the characteristic traits, the endophyte PgB4 was recognized as *Nemania sp.* After a microscopic examination of PgL1 (Figure 4.11), the discerning characters of the mycelium indicated that the endophyte PgL1 was *Daldinia sp.*

All five fungal strains were identified on a molecular basis. 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. The DNA of five strains was extracted, quantified, amplified, and purified.

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 PgB1 showed that partial sequencing of the ITS region for sample PgB1 resulted in a 561 bp long sequence and 100% similar to the endophyte *Phomopsis tersa*, accession no- MG049670.1 from the GenBank database.

After molecular analysis of PgB2, it was revealed that partial sequencing of the ITS region for sample PgB2 resulted in a 564 bp long sequence 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.*

From the Molecular results of PgB3, it could be said that partial sequencing of the ITS region for sample PgB3 resulted in a 557 bp long sequence and was 100% similar to the endophyte of *Colletotrichum siamense,* accession number: MT434640.1 from the GenBank database. So, this isolate was detected as *Colletotrichum siamense.*

Molecular analysis showed that the partial sequencing of the ITS region for sample PgB4 resulted in a 559 bp long sequence and had a similarity of 99% with the endophyte *Nemania primolutea,* accession number: MG881830.1 from the GenBank database. As a result, this isolate was determined as *Nemania primolutea.*

From molecular observation of PgL1, it was found that partial sequencing of the ITS region for sample PgL1 resulted in a 554 bp long sequence and 100% similarity with the isolate *Daldinia eschscholtzii,* accession number: MN341731.1 from the GenBank database. Thus, the isolate was discovered as *Daldinia eschscholtzii.*

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

All five isolates were tested for antimicrobial screening as a part of the bioactivity test. In this investigation antibacterial activity by disc diffusion method was tested against *Escherichia coli, Salmonella typhi, Staphylococcus aureus, Bacillus megaterium,* and antifungal activity was tested against *Aspergillus niger,* and *Aspergillus flavus.* In this investigation, kanamycin (30 µg/disc) and ketoconazole (30 µg/disc) standard disc were used as standard discs.

Yee Yee Thu et al., 2018, isolated *Phomopsis sp*. from the leaves of *Psidium guajava* L. Three compounds were isolated from the methanol extract and the compounds had antimicrobial activity. Among those, compound I exhibited high activity against *Bacillus subtilis* (18mm) and *Candida albicans* (17mm), and *Escherichia coli* (19mm). Compound II showed very high activity against *B. subtilis* (38mm) and *Xanthomonas oryzae* (25mm) but weak activity on *C. albicans* (13mm), and *E.coli* (14mm). Compound III indicated very high activity against B. subtilis (27mm) and *X.oryzae* (18mm) but weak activity on *C. albicans* (13mm) and *E. coli* (15mm). The crude extract of the endophytic *D. eschscholtzii* from *Psidium guajava* leaves has shown the bacterial inhibition zone diameter from 6.0±1.0 mm to 16.67±2.08 mm. The extract exhibited a wider inhibition zone diameter to *B. subtilis* (16.67 mm) and less inhibition zone to *S. aureus* (12 mm) and *E. coli* (13 mm) (Chutulo and Chalannavar, 2020)

According to Table 4.7, it could be said that the zones of inhibition for standard disc Kanamycin (30µg) were 33 mm (*E. coli*), 29 mm (*S. typhi*), 31mm (*S. aureus)* and 28 mm (*B. megaterium*). Hence, Kanamycin showed very significant antibacterial activity. As an antifungal standard disc Ketoconazole (30µg) was used. It also exhibited very significant activity against both *A. niger* (28mm) and *A. flavus* (35 mm).

The isolate PgB1 produced a zone of inhibition of 7 mm against *E. coli* and *S. aureus.* Thus, PgB1 showed poor antimicrobial activity against both *E. coli* and *S. aureus*. Nonetheless, this isolate also did not show any antifungal activity. PgB2 exhibited poor antibacterial activity against all the test bacterial strains by producing zones of inhibition of 7mm against *E. coli, S. aureus* as well as *B. megaterium* and 9 mm against *S. typhi*. Furthermore, this isolate produced a zone of 50 mm against *A. niger* which was larger than the zone of inhibition produced by ketoconazole (28 mm). Therefore, PgB2 showed very significant antifungal activity. Though PgB3 was inactive against fungal strains, it exhibited poor antimicrobial activity against all the tested microorganisms. The size of zones of inhibition produced by isolate PgB3 were 7 mm against *E. coli* and 8mm against *S. typhi, S. aureus* as well as *B. megaterium*. For the isolate PgB4, Table 4.7 indicated that this

isolate showed good antibacterial activity against *S. typhi* by producing a 16 mm zone of inhibition. It also exhibited significant antifungal activity against *A. niger* as it produced a zone of inhibition of 25mm. PgB4 induced zones of inhibition of 8mm against *E. coli* as well as *S. aureus* and 7 mm against *B. megaterium*. So, it had poor resistance against these three bacteria. In the case of PgL1, there was no antifungal activity observed. This isolate showed poor activity against *E. coli* (7mm), *S. typhi* (8 mm), and *B. megterium* (7mm).

In summary, all the isolates exhibited poor to mild activity against more or less all the tested bacterial strains. Among them, PgB4 showed good activity against *S. typhi*. Again, all the isolates were inactive against fungal strain with exception of PgB2 and PgB4. However, PgB2 was very significantly active and PgB4 was significantly active against *A. niger*.

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 study conducted by Nonye T. et al., 2021 reported endophytic fungi isolated from *Psidium guajava* possessed antioxidant activities attributed to the bioactive compounds present in their metabolites. A 500 µg/mL solution of fungal endophyte extract showed 69.84 inhibition, respectively compared to 99.5% inhibition of quercetin at the same concentration. The extract gave an IC50 of the value of 44.07 µg/ml. The antioxidant activity of *C. madrasense* isolated from soil showed a good scavenging effect amounting to 70.21% on DPPH radicals (Abo-Elmagd, 2014). Chutulo and Chalannavar, 2020 reported that the crude extract of the endophytic *D. eschscholtzii* extracted from *Psidium guajava* has shown high DPPH free radical scavenging activity. At low concentrations, the inhibition was 52.646 ± 0.848% whereas the inhibition observed by ascorbic acid at low concentration was 8.997 ± 3.903%. At high concentrations, the extract has shown strong inhibition of 80.298 ± 0.0608% of the free radicals, which was almost closer to the standard antioxidant 91.0451 ± 1.384%.

All five fungal extracts PgB1, PgB2, PgB3, PgB4, and PgL1 were subjected to free radical scavenging activity the method of Brad-Williams et al, 1995. Here, Ascorbic acid (AA) and Butylated Hydroxy Anisole (BHA) were used as reference standards. The IC50 values of the fungal extracts were compared to the IC50 value of the standards.

Table 4.8 showed the percent of inhibitions of Butylated Hydroxy Anisole (BHA) for different concentrations (50- 0.78125µg/ml) varied from 86% to 17% and the IC50 value was 8.84 µg/ml. According to Table 4.9, in the case of Ascorbic acid (AA), the percent of inhibitions for different concentrations (50- 0.78125µg/ml) varied from 91% to 5%. The IC50 value for AA was 11.41 µg/ml. For both standards- BHA and AA, the IC50 values were very low. The low IC50 values were an indication of significant antioxidant activity. Table 4.10 indicated that the IC50 value of PgB1 was 56.02 µg/ml. Compared to the IC50 values of the standards, the free radical scavenging activity of PgB1 was moderate. At different concentrations (200- 0.78125µg/ml), the percent of inhibitions was 86% to 33%. For PgB2, Table 4.11, revealed that at different concentrations (200- 0.78125µg/ml), the percent of inhibitions was 70% to 27%. The IC50 value was 83.9 µg/ml. In comparison with standards, PgB2 showed poor antioxidant activity. As per Table 4.12, the percent of inhibitions varied from 73% to 42% for different concentrations (50- 0.78125µg/ml) and the IC50 value was 3.4 µg/ml for PgB3. The IC50 value was very low, even lower than both BHA and A.A. Hence, PgB3 showed very significant antioxidant activity. In the case of PgB4, table 4.13 showed different concentrations (50- 0.78125µg/ml), the percent of inhibitions was 30% to 7% and the IC50 value was 87.77 µg/ml. So, from the IC50 value, it could be said that PgB4 had poor antioxidant activity. From Tale 4.14, it was found that the percent of inhibitions of PgL1 for different concentrations (50- 0.78125µg/ml) varied from 71% to 15% and the IC50 value was 15.7 µg/ml. The IC50 value was slightly higher than the standards, indicating significant antioxidant activity.

The results of the free radical scavenging activity of the extracts have been compared in the following graph. The IC50 values for BHA and AA were 8.84 µg/ml and 11.41 µg/ml. Among all the extracts, the fungal extract PgB3 showed the highest antioxidant activity with the lowest IC50 value of 3.4 µg/ml, including both the standards in this investigation. The fungal extract PgL1 also showed significant antioxidant activity with an IC50 value of 15.7 µg/ml slightly higher than the standards. Other fungal extracts PgB1 showed moderate antioxidant activity with an IC50 value of 55.92 µg/ml, whereas PgB2 and PgB4 showed poor antioxidant activity with IC50 values of 83.9 µg/ml and 87.77 µg/ml. Therefore, PgB4 showed the lowest antioxidant activity with the highest IC50 value. After comparing all the IC50 values, the increasing order of antioxidants was PgB4 < PgB2 < PgB1< PgL1< AA < BHA < PgB3.

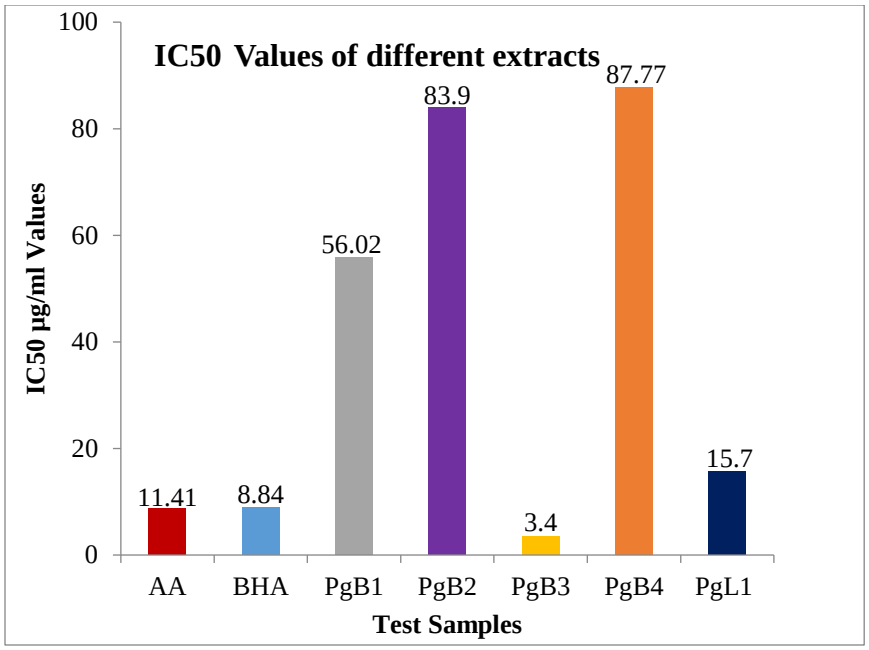


Figure 5. 1: IC50 values of different extracts and standards

All the fungal extracts were qualitatively tested for the presence of chemical constituents. For the presence of different secondary metabolites in the ethyl-acetate or organic extracts of PgB1, PgB2, PgB3, PgB4, and PgL1 was screened. Thin layer chromatographic technique was used for the initial screening of the fungal extracts in different solvent systems. Distinctive compounds showed different colors, 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 fungal extract from PgB3 revealed the presence of various components such as 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. 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 from PgB3 was light yellow to the naked eye. On the TLC plate under UV light at 254 nm it appeared sky blue to green in color and appeared as a dark quenching spot, green, blue fluorescence at 365nm. The compound from PgB3 was soluble in CHCl3 and Methanol.

For the NMR spectrum, the compound was dissolved with chloroform and 2 drops of methanol. The spectral data of the compound from PgB3 was not accurate due to the use of expired CHCI3. However, from the NMR data, it was observed that the structure of the isolated compound was similar to the structure of ergosterol. When the spectral image of the compound was compared with the image of ergosterol, it was seen that the compound was very similar to those of ergosterol derivatives with slight impurities. It was proposed that the compound from PgB3 was of sterol derivatives by direct comparison.

Conclusion and Future directions

Nowadays endophytic fungi are gaining scientific interest for their isolation and identification because of their therapeutic potential. They are a reliable source of novel natural compounds having interesting bioactivity and thus produce several compounds of pharmaceutical significance. Their secondary metabolites have huge potential for a biotechnological promise. They are relatively unexplored ecological sources seeing only very few plants are studied for their endophytic microflora. So, it is very important to focus on the exploration of endophytic fungi from the plant as the need for new natural products is ever-increasing. The research on endophytic would open opportunities in the field of agriculture, industry, and medicine throughout the world. Hence, further research on endophytic fungi is necessary as they could open the possibility for a new world where no disease will be untreatable.

References

1. Abo-Elmagd, H. I. (2014). Evaluation and optimization of antioxidant potentiality of *Chaetomium madrasense* AUMC 9376. *Journal of Genetic Engineering and Biotechnology*, *12*(1), 21–26.
2. Aly, A.H., Debbab, A., Kjer, J. et al. (2010). Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Diversity 41*, 1–16.
3. Andreote, F. D., Gumiere, T., & Durrer, A. (2014). Exploring interactions of plant microbiomes. *Scientia Agricola*, *71*(6), 528–539.
4. Arima, H., & Danno, G. (2002). Isolation of Antimicrobial Compounds from Guava (*Psidium guajava* L.) and their Structural Elucidation. *Bioscience, Biotechnology, and Biochemistry*, *66*(8), 1727–1730.
5. Atanasov, A. G., Zotchev, S. B., Dirsch, V. M., & Supuran, C. T. (2021). Natural products in drug discovery: advances and opportunities. *Nature Reviews Drug Discovery*, *20*.
6. Bacon C. W., White J. (2000). *Microbial Endophytes*. New York, NY: CRC.
7. Banu MS., & Sujatha K. (2012). Antimicrobial screening of leaf extract of *Psidium guajava* and its isolated fraction against some pathogenic microorganisms. *Drug Invent Today*, *4*(3):348–50.
8. Begum S, Hassan SI, Ali SN, Siddiqui BS. (2004). Chemical constituents from the leaves of *Psidium guajava*. *Nat Prod Res*, *18*(2):135–40.
9. Bhadury, Punyasloke & Austen, Melanie & Bilton, David & Lambshead, P John & Rogers, Alex. (2006). Development and evaluation of a DNA-barcoding approach for the rapid identification of nematodes. *Marine Ecology Progress Series*. 320. 1-9.
10. Bills G, Dombrowski A, Pelaez F, Polishhook J, An Z (2002) In: Watling R, Frankland JC, Ainsworth AM, Issac S, Robinson CH (eds) Tropical mycology: micromycetes, vol 2. *CABI Publishing, New York*, pp 165–19
11. Biswas, S.; Talukdar, P. Talapatra, D.S.N. (2019). Presence of phytochemicals in fruits and leaves of guava (*Psidium guajava* Linn.) for cancer prevention: *A mini review. J. Drug Deliv. Ther*, *9*, 726–729.
12. Brand-Williams, W., Cuvelier, M.E. and Berset, C.L.W.T. (1995) Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT-Food Science and Technology*, *28*, 25-30.
13. Carroll, G., & Petrini, O. (1983). Patterns of substrate utilization by some fungal endophytes from coniferous foliage. *Mycologia (USA)*.

14. Cho, N. H., Shaw, J. E., Karuranga, S., Huang, Y., da Rocha Fernandes, J. D., Ohlrogge, A. W., & Malanda, B. (2018). IDF Diabetes Atlas: Global estimates of diabetes prevalence for 2017 and projections for 2045. *Diabetes Research and Clinical Practice*, *138*(1), 271–281.
15. Chutulo, Eyob & Chalannavar, Raju. (2021). Antimicrobial activity of *Fusarium oxysporum*, endophytic fungus, isolated from *Psidium guajava* L. (White fruit). *International Journal of Pharmaceutical Sciences and Research, 2021*, 11. 5844-5855. 10.13040
16. Chutulo, Eyob. (2020). *Daldinia eschscholtzii*: an endophytic fungus isolated from *Psidium guajava* as an alternative source of bioactive secondary metabolites. *Asian Journal of Mycology*, *3*. 10.5943
17. D'Amico, M., Frisullo, S., & Cirulli, M. (2008). Endophytic fungi occurring in fennel, lettuce, chicory, and celery--commercial crops in southern Italy. *Mycological Research*, *112*(Pt 1), 100–107.
18. Dai, C., Yu, B., Xu, Z., & Yuan, S. (2003). Effect of environmental factors on the growth and fatty acid composition of five endophytic fungi from *Sapium sebiferum*. *Ying Yong Sheng Tai Xue Bao = the Journal of Applied Ecology*, *14*(9), 1525–1528.
19. Dakappa SS, Adhikari R, Timilsina SS, Sajjekhan S. (2013). A review on the medicinal plant *Psidium Guajava* Linn. (Myrtaceae). J *Drug Deliv Ther*, *3*(2):162–8.
20. Das, A. J. (2011). Review on Nutritional, Medicinal and Pharmacological Properties of *Centella asiatica* (Indian pennywort). *Journal of Biologically Active Products from Nature*, *1*(4), 216–228.
21. De Bary, A. (1866). Morphologie und Physiologie der Pilze, Flechten, und Myxomyceten. *Hofmeister's Handbook of physiological Botany*, Vol. 2, Leipzig.
22. de Medeiros, L.S., Murgu, M., de Souza, A.Q.L. and Rodrigues-Fo., E. (2011), Antimicrobial Depsides Produced by *Cladosporium uredinicola*, an Endophytic Fungus Isolated from *Psidium guajava* Fruits. *HCA*, *94*: 1077-1084.
23. Denny C, Melo PS, Franchin M, Massarioli AP, Bergamaschi KB, Alencar SM De. (2013) Guava pomace: a new source of anti-inflammatory and analgesic bioactives.
24. Dini-Andreote, F., Andreote, F. D., Araújo, W. L., Trevors, J. T., & van Elsas, J. D. (2012). Bacterial Genomes: Habitat Specificity and Uncharted Organisms. *Microbial Ecology*, *64*(1), 1–7.
25. Eyberger, A. L., Dondapati, R., & Porter, J. R. (2006). Endophyte fungal isolates from *Podophyllum peltatum* produce podophyllotoxin. *Journal of Natural Products*, *69*(8), 1121–1124.
26. Feskanich D, Ziegler RG, Michaud DS, Giovannucci EL, Speizer FE, Willett WC, Colditz GA. (2000). Prospective study of fruit and vegetable consumption and risk of lung cancer among men and women. *J Natl Cancer Inst*, *92*:1812–23.

27. Festus B.C. Okoye, Chukwuemeka S. Nworu, Abdessamad Debbab, Charles O. Esimone, Peter Proksch. (2015). Two new cytochalasins from an endophytic fungus, KL-1.1 isolated from *Psidium guajava* leaves, *Phytochemistry Letters*, *Volume 14*, Pages 51-55, ISSN 1874-3900.
28. Godstime O. C., Enwa F. O., Augustina J. O., Christopher E. O. (2014). Mechanisms of antimicrobial actions of phytochemicals against enteric pathogens – a review. *J. Pharm. Chem. Biol. Sci.* 2 77–85.
29. Golinska, P., Wypij, M., Agarkar, G., Rathod, D., Dahm, H., and Rai, M. (2015). Endophytic actinobacteria of medicinal plants: diversity and bioactivity. *Antonie Van Leeuwenhoek 108,* 267–289.
30. Gonçalves FA, Andrade Neto M, Bezerra JNS, et al. (2008). Antibacterial activity of guava, *Psidium guajava* Linnaeus, leaf extracts on diarrhea-causing enteric bacteria isolated from seabob shrimp, *Xiphopenaeus kroyeri* (Heller) *Revista do Instituto de Medicina Tropical de Sao Paulo. 50*(1):11–15.
31. Guo, S., & Wang, Q. (2001, January 1). *Character and action of good strain on stimulating seed germination of Gastrodia elat*. Jun Wu Xi Tong = Mycosystema.
32. Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Can. J. Microbiol. 43,* 895–914.
33. Harborne, J.B. (1973) Phytochemical Methods: A Guide to Modern Technique of Plant Analysis. *Chapman and Hall, London,* 107-150.
34. Hata, K., & Sone, K. (2008). Isolation of endophytes from leaves of *Neolitsea sericea* in broadleaf and conifer stands. *Mycoscience, 49*(4), 229–232.
35. He Q., & Venant N. (2004). Antioxidant power of phytochemicals from Psidium guajava leaf. J Zhejiang Univ *Sci A, 5*(6):676–83.
36. Jalgaonwala R. E., Mohite B. V., Mahajan R. T. (2011). Natural products from plant associated endophytic fungi. *J. Microbiol. Biotechnol. Res.* 1 21–32.
37. Janakiraman, V., Govindarajan, K. & C R, M. (2019). Biosynthesis of Silver Nanoparticles from Endophytic Fungi, and its Cytotoxic Activity. *BioNanoSci, 9,* 573–579.
38. Jeong S, Cho SK, Ahn KS, Lee JH, Yang DC, Kim J. (2014). Anti-inflammatory effects of an ethanolic extract of guava (*Psidium guajava* L.) leaves in vitro and in vivo. *J Med Food, 17*(6):678–85.

39. Jiang, Lirong & Lu, Junyu & Qin, Yue & Jiang, Weirong & Wang, Yanping. (2020). Antitumor effect of guava leaves on lung cancer: A network pharmacology study. *Arabian Journal of Chemistry, 13*. 7773-7797.
40. Jiménez-Escrig, A., Rincón, M., Pulido, R., & Saura-Calixto, F. (2001). Guava fruit (*Psidium guajava* L.) as a new source of antioxidant dietary fiber. *Journal of Agricultural and Food Chemistry*, *49*(11), 5489–5493.
41. Jiménez-Escrig, Antonio & Rincón, Mariela & Pulido, Raquel & Saura-Calixto, Fulgencio. (2001). Guava Fruit (*Psidium guajava* L.) as a New Source of Antioxidant Dietary Fiber. *Journal of agricultural and food chemistry*, *49*. 5489-93.
42. Joseph, B., and Priya, R. M. (2011). Bioactive compounds from endophytes and their potential in pharmaceutical effect: a review. *Am. J. Biochem. Mol. Bio,* 1, 291–309.
43. Khan MIH, Ahmad JA. (1985). Pharmacognostic study of Psidiurn guajuva L. J Med Plant Res.23(2):95–103.
44. Kumar KPV, Pillai MSN, Thusnavis GR. (2011). Seed extract of *P sidium guajava* as ecofriendly corrosion inhibitor for carbon steel in hydrochloric acid medium. *J Mater Sci Technol, 27*(12):1143–9.
45. Kusari, S., Lamshöft, M., and Spiteller, M. (2009a). *Aspergillus fumigatus* Fresenius, an endophytic fungus from *Juniperus communis* L. Horstmann as a novel source of the anticancer pro-drug deoxypodophyllotoxin. *J. Appl. Microbiol. 107,* 1019–1030.
46. Kusari, S., Lamshöft, M., Zühlke, S., and Spiteller, M. (2008). An endophytic fungus from *Hypericum perforatum* that produces hypericin. *J. Nat. Prod. 71,* 159–162.
47. Kusari, S., Verma, V. C., Lamshoeft, M., and Spiteller, M. (2012). An endophytic fungus from *Azadirachta indica* A. Juss. that produces azadirachtin. *World J. Microbiol. Biotechnol. 28,* 1287–1294.
48. Kusari, S., Zühlke, S., Kosuth, J., Cellárová, E., and Spiteller, M. (2009b). Light-independent metabolomics of endophytic *Thielavia subthermophila* provides insight into microbial hypericin biosynthesis. *J. Nat. Prod. 72,* 1825–1835.
49. Lahlou, M. (2007). Screening of natural products for drug discovery. *Expert Opinion on Drug Discovery*, *2*(5), 697–705.

50. Lok, B.; Sandai, D.; Baharetha, H.; Nazari, V.; Asif, M.; Tan, C.; Majid, A. (2020). Anticancer effect of *Psidium guajava* (guava) leaf extracts against colorectal cancer through inhibition of angiogenesis. *Asian Pac. J. Trop. Biomed, 10*, 293.
51. Luo, Y.; Peng, B.; Wei, W.; Tian, X.; Wu, Z. (2019). Antioxidant and anti-diabetic activities of polysaccharides from guava leaves. *Molecules*, *24*, 1343.
52. *Magdeldin, S. (2012) Gel Elektrophoresis-Principles and Basics. Intech, Rijeka. - References - Scientific Research Publishing*.
53. Manoharan, Senthamarai & Krishnan, Navaneetha & Senthilkumar, Vinitha & Manivannan, Sowmiya & Veerababhu, Vijayalekshmi & Kilavan Packiam, Kannan. (2015). Green synthesis of silver nanoparticles and their antimicrobial property of endophytic fungi isolated from *Mentha arvensis* L. and *Psidium guajava* L. *Journal of Chemical and Pharmaceutical Research, 2015*, 7(9):340-346.
54. Mazumdar, S.; Akter, R.; Talukder, D. (2015). Antidiabetic and antidiarrhoeal effects on ethanolic extract of *Psidium guajava* (L.) Bat. leaves in Wister rats. *Asian Pac. J. Trop. Biomed, 5*, 10–14.
55. Meca, G., Sospedra, I., Soriano, J. M., Ritieni, A., Moretti, A., and Manes, J. (2010). Antibacterial effect of the bioactive compound beauvericin produced by *Fusarium proliferatum* on solid medium of wheat. *Toxicon 56*, 349–354.
56. Meckes, M., Calzada, F., Tortoriello, J., González, J. L., & Martínez, M. (1996). Terpenoids Isolated from *Psidium guajava* Hexane Extract with Depressant Activity on Central Nervous System. *Phytotherapy Research*, *10*(7), 600–603.
57. Menpara, D., and Chanda, S. (2013). "Endophytic bacteria - unexplored reservoir of antimicrobials for combating microbial pathogens," in Microbial Pathogens and Strategies for Combating them: Science, Technology and Education, ed. A. Méndez-Vilas (Badajoz: Formatex Research Center), 1095–1103.
58. Mitter, B., Petric, A., Shin, M. W., Chain, P. S. G., Hauberg-Lotte, L., Reinhold-Hurek, B., Nowak, J., & Sessitsch, A. (2013). Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. *Frontiers in Plant Science*, *4*.
59. Morton JF. (2004). Fruits of Warm Climates; p. 425–8.

60. Mukhtar HM, Ansari SH, Bhat ZA, Naved T, Singh P. (2006). Antidiabetic activity of an ethanol extract obtained from the stem bark of *Psidium guajava* (Myrtaceae). *Die Pharmazie, 2006*; 61:725–7.
61. Musa KH, Abdullah A, Jusoh K, Subramaniam V. (2011). Antioxidant activity of pink-flesh guava (*Psidium guajava* l.): effect of extraction techniques and solvents. *Food Anal Methods, 4:*100–7.
62. Nair, D. N., & Padmavathy, S. (2014). Impact of Endophytic Microorganisms on Plants, Environment and Humans. *The Scientific World Journal*.
63. Nair, S.S.; Kavrekar, V.; Mishra, A. (2013). In vitro studies on alpha amylase and alpha glucosidase inhibitory activities of selected plant extracts. *Eur. J. Exp. Bio, 3,* 128–132.
64. Nantitanon W., & Okonogi S. (2012). Comparison of antioxidant activity of compounds isolated from guava leaves and a stability study of the most active compound. *Drug Discov Ther, 6*(1):38–43.
65. Naseer, S., Hussain, S., Naeem, N., Pervaiz, M., & Rahman, M. (2018). The phytochemistry and medicinal value of *Psidium guajava* (guava). *Clinical Phytoscience, 4*(1).
66. Newman, D. J., Cragg, G. M., & Snader, K. M. (2003). Natural Products as Sources of New Drugs over the Period 1981−2002. *Journal of Natural Products*, *66*(7), 1022–1037.
67. Nonye T. Ujam, Daniel L. Ajaghaku, Festus B.C. Okoye, Charles O. Esimone, (2021). Antioxidant and immunosuppressive activities of extracts of endophytic fungi isolated from *Psidium guajava* and *Newbouldia laevis*, *Phytomedicine Plus, Volume 1, Issue 2, 100028*, ISSN 2667-0313,
68. Ogunwande IA, Olawore NO, Adeleke KA, Ekundayo O, Koenig WA. (2003). Chemical composition of the leaf volatile oil of *Psidium guajava* L. growing in Nigeria. *Flavour Fragr, J. 18*:136–8.
69. Ojewole J, Awe EO, Chiwororo WDH. (2008). Antidiarrhoeal activity of *Psidium guajava* Linn. (Myrtaceae) leaf aqueous extract in rodents. *J Smooth Muscle Res, 44*(6):195–207.
70. Oldroyd, G. E. D., Murray, J. D., Poole, P. S., & Downie, J. A. (2011). The rules of engagement in the legume-rhizobial symbiosis. *Annual Review of Genetics, 45*, 119–144.
71. Parthasarathi S., Sathya S., Bupesh G., Samy D. R., Mohan M. R., Selva G. K., et al. (2012). Isolation and characterization of antimicrobial compound from marine *Streptomyces hygroscopicus* BDUS 49. *World J. Fish Mar. Sci.* 4 268–277.
72. Parthasarathi, S., Sathya, S., Bupesh, G., Samy, R. D., Mohan, M. R., Kumar, G. S., Manikandan, M., Kim, C.-J., & Balakrishnan, K. (2012). Isolation and characterization of antimicrobial compound

from marine *Streptomyces hygroscopicus* BDUS 49. *World Journal of Fish and Marine Sciences*, *4*(3), 268–277.

73. Petrini O. 1991. Fungal Endophyte of Tree Leaves, p 179-197. In microbial ecology of leaves, Andrews J, Hirano SS (eds.). *Spring-Verlag*, New York.
74. Pimentel, M. R., Molina, G., Dionísio, A. P., Maróstica Junior, M. R., & Pastore, G. M. (2011). The Use of Endophytes to Obtain Bioactive Compounds and Their Application in Biotransformation Process. *Biotechnology Research International*, *2011*, 1–11.
75. Proukakis, N. P., Burnett, K., & Stoof, H. T. C. (1998). Microscopic treatment of binary interactions in the nonequilibrium dynamics of partially Bose-condensed trapped gases. *Physical Review A*, *57*(2), 1230–1247.
76. Puri S. C., Nazir A., Chawla R., Arora R., Riyaz-Ul-Hasan S., Amna T. (2006). The endophytic fungus *Trametes hirsuta* as a novel alternative source of podophyllotoxin and related aryl tetralin lignans. *J. Biotechnol.* 122, 494–510.
77. Rai PK, Rai NK, Rai AK, Watal G. (2007). Role of LIBS in elemental analysis of *Psidium guajava* responsible for glycemic potential role. *Instrum Sci Technol, 35*:507–22.
78. Rattanachaikunsopon, P., & Phumkhachorn, P. (2010). Contents and antibacterial activity of flavonoids extracted from leaves of *Psidium guajava*. *Journal of Medicinal Plants Research*, *4*(5), 393–396.
79. Rodriguez, R., & Redman, R. (2008). More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *Journal of Experimental Botany*, *59*(5), 1109–1114.
80. Rouseff, R. L., Onagbola, E. O., Smoot, J. M., & Stelinski, L. L. (2008). Sulfur Volatiles in Guava (*Psidium guajava* L.) Leaves: Possible Defense Mechanism. *Journal of Agricultural and Food Chemistry*, *56*(19), 8905–8910.
81. Saar, D. E., Polans, N. O., Sørensen, P. D., & Duvall, M. R. (2001). Angiosperm DNA contamination by endophytic fungi: Detection and methods of avoidance. *Plant Molecular Biology Reporter*, *19*(3), 249–260.
82. Saikkonen, K. (2004). Evolution of endophyte plant symbioses. *Trends in Plant Science*, *9*(6), 275–280.
83. Santiago, C., Fitchett, C., Munro, M. H. G., Jalil, J., & Santhanam, J. (2012). Cytotoxic and Antifungal Activities of 5-Hydroxyramulosin, a Compound Produced by an Endophytic Fungus

Isolated from *Cinnamomum mollisimum*. *Evidence-Based Complementary and Alternative Medicine*, *2012*, e689310.

84. Schulz, B., Boyle, C., Draeger, S., Römmert, A.-K., & Krohn, K. (2002). Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research*, *106*(9), 996–1004.
85. Selvi, B. K., and Balagengatharathilagam, P. (2014). Isolation and screening of endophytic fungi from medicinal plants of Virudhunagar district for antimicrobial activity. *Int. J. Sci. Nat. 5,* 147–155.
86. Shoeb, M., Haque, M., & Nahar, N. (2014). Bioactive compounds from endophytic fungus *Penicillium thiomii* isolated from *Terminalia chebula* Retz. *J. Nat. Prod. Plant Resour, 4*(3), 65-70.
87. Shukla, S., Pharmaceutical, S. D., Habbu, P., Kulkarni, V., Jagadish, K., Pandey, A., & Sutariya, V. (2014). *Endophytic microbes: A novel source for biologically/pharmacologically active secondary metabolites*.
88. Sieber T. N. (2007). Endophytic fungi in forest trees: are they mutualists? *Fung. Biol. Rev.* 21, 75–89. 10.1016/j.fbr.2007.05.004
89. Singh, R., & Dubey, A. (2015). *Endophytic Actinomycetes as Emerging Source for Therapeutic Compounds*.
90. Smith, R. M., & Siwatibau, S. (1975). Sesquiterpene hydrocarbons of fijian guavas. *Phytochemistry*, *14*(9), 2013–2015.
91. Sohini Chatterjee, Suvranil Ghosh, Narayan Chandra Mandal. (2022). Potential of an endophytic fungus *Alternaria tenuissima* PE2 isolated from Psidium guajava L. for the production of bioactive compounds, *South African Journal of Botany, Volume 150*, Pages 658-670, ISSN 0254-6299.
92. Soman S, Rauf AA, Indira M, Rajamanickam C. (2010). Antioxidant and Antiglycative potential of ethyl acetate fraction of *Psidium guajava* leaf extract in Streptozotocin-induced diabetic rats. *Plant Foods Hum Nutr, 65*:386–91.
93. Song, S., Otkur, M., Zhang, Z., and Tang, Q. (2007). Isolation and characterization of endophytic microorganisms in *Glaycyrrhiza* inflat Bat. from Xinjiang. *Microbiology 5*, 867–870.
94. Specian, V., Sarragiotto, M. H., Pamphile, J. A., & Clemente, E. (2012). Chemical characterization of bioactive compounds from the endophytic fungus *Diaporthe helianthi* isolated from *Luehea divaricata*. *Brazilian Journal of Microbiology*, *43*(3), 1174–1182.
95. Stępniewska, Z., & Kuźniar, A. (2013). Endophytic microorganisms—promising applications in bioremediation of greenhouse gases. *Applied Microbiology and Biotechnology*, *97*(22), 9589–9596.

96. Stierle, A., Strobel, G., Stierle, D., Grothaus, P., & Bignami, G. (1995). The Search for a Taxol-Producing Microorganism Among the Endophytic Fungi of the Pacific Yew, *Taxus brevifolia*. *Journal of Natural Products*, *58*(9), 1315–1324.

97. Strobel, G., & Daisy, B. (2003). Bioprospecting for Microbial Endophytes and Their Natural Products. *Microbiology and Molecular Biology Reviews*, *67*(4), 491–502.

98. Strobel, G., Daisy, B., Castillo, U., & Harper, J. (2004). Natural Products from Endophytic Microorganisms⊥. *Journal of Natural Products*, *67*(2), 257–268.

99. Sunitha, V.H. and Nirmala Devi, D. and Srinivas, C. (2013) Extracellular Enzymatic Activity of Endophytic Fungal Strains Isolated from Medicinal Plants. *World Journal of Agricultural Sciences, 9* (1). pp. 1-9. ISSN 1817-3047

100. Suryanarayanan, T. S., Wittlinger, S. K., & Faeth, S. H. (2005). Endophytic fungi associated with cacti in Arizona. *Mycological Research, 109*(5), 635–639.

101. Teixeira RO, Camparoto ML, Mantovani MS & Vicentini VEP (2003). Assessment of two medicinal plants *Psidium guajava* L. and *Achillea millefolium* L., in in vitro and in vivo assays. *Genetics and Molecular Biology 26*: 551-555

102. Thaipong K, Boonprakob U, Cisneros-zevallos L, Byrne DH, Pathom N. (2005). Hydrophilic and lipophilic antioxidant activities of guava fruits. *Southeast Asian J Trop Med Public Health, 36*:254–7.

103. Toyokuni, S. (2016). Oxidative stress as an iceberg in carcinogenesis and cancer biology. *Arch. Biochem. Biophys*, *595*, 46–49.

104. Trease GE, Evans WC. (1989). *Pharmacognosy*. 11th edition. London, UK: Macmillan.

105. Turner, T. R., James, E. K., & Poole, P. S. (2013). The plant microbiome. *Genome Biology*, *14*(6).

106. Venkatesan N, Thiyagarajan V, Narayanan S, Arul A, Raja S, Kumar SGV, Rajarajan T, Perianayagam JB. (2005). Antidiarrheal potential of *Asparagus racemous* wild root extracts in laboratoire animals. *J Pharm Pharmaceut Sci, 8*:39–45.

107. Vernin G, Vernin E, Vernin C, Metzger J. (1991). Extraction and GC-MS-SPECMA data bank analysis of the aroma of *Psidium guajaua* L. fruit from Egypt. *Flavour Fragr,J. 6*:143–8.

108. Vieira RHSF, Rodrigues DP, Gonçalves FA, Menezes FGR, Aragoo JS, Sousa OV. (2001) Microbicidal effect of medicinal plant extracts (*Psidium guajava* linn. And *Carica papaya* linn.) upon

bacteria isolated from fish muscle and known to induce dirrhea in children. *Rev Inst Med trop S Paulo, 43*:145–8.

109. Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P., & McPhail, A. T. (1971). Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia. Journal of the American Chemical Society*, *93*(9), 2325–2327.

110. Wu, L., Han, T., Li, W., Jia, M., Xue, L., Rahman, K., & Qin, L. (2013). Geographic and tissue influences on endophytic fungal communities of *Taxus chinensis* var. mairei in China. *Current Microbiology*, *66*(1), 40–48.

111. Yan, X., Sikora, R. A., & Zheng, J. (2011). Potential use of cucumber (Cucumis sativus L.) endophytic fungi as seed treatment agents against root-knot nematode Meloidogyne incognita. *Journal of Zhejiang University SCIENCE B*, *12*(3), 219–225.

112. Yang Z, Dan W-J, Li Y-X, Peng G-R, Zhang A-L, Gao J-M. (2019). Antifungal Metabolites from *Alternaria atrans*: An Endophytic Fungus in Psidium guajava. *Natural Product Communications*, *14*(5).

113. Yee, Y., Mhon, H., & Mon, M. (2018). *BIOACTIVE COMPOUNDS OF AN ENDOPHYTIC FUNGUS PHOMOPSIS SP. ISOLATED FROM PSIDIUM GUAJAVA L.*

114. Zhao, G., Li, G., Zhou, X., Matsuo, I., Ito, Y., Suzuki, T., Lennarz, W. J., & Schindelin, H. (2008). Structural and mutational studies on the importance of oligosaccharide binding for the activity of yeast PNGase. *Glycobiology*, *19*(2), 118–125.

115. Zhu, X., Ouyang, W., Lan, Y., Xiao, H., Tang, L., Liu, G., Feng, K., Zhang, L., Song, M., & Cao, Y. (2020). Anti-hyperglycemic and liver protective effects of flavonoids from *Psidium guajava* L. (guava) leaf in diabetic mice. *Food Bioscience*.

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