Isolation and Identification of Endophytic Fungi from Mangrove Plant Goran (*Ceriops decandra*) for their bioactivity analysis

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Submitted by

Mousumi Kajol
Examination Roll No: MS-160717
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Biotechnology and Genetic Engineering Discipline
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September, 2018

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Declaration

This thesis paper is the original research work of the authors, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any university. The findings of the research has not been/ will not be published anywhere without the concurrence of the concerned supervisor.

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Dedicated to

My beloved parents

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Abstract

The microorganism which lives within the intracellular tissue of a plant asymptomatically with maintaining a symbiotic relationship are referred endophytic fungi. Endophytic fungi have considered as an outstanding source of secondary metabolites as their host. The purpose of this study was to isolate and identification of endophytic fungi from the leaf tissue of *Ceriops decandra* to determine their antioxidant, antimicrobial and cytotoxic activities. Five isolates were identified on the basis of morphological and DNA sequencing of ITS region. Four genera from five isolates viz *Curvularia sp.; Colletroticum sp.; Chaetomium sp.and Fusarium sp.* were identified. Phylogenetic analysis of sequenced DNA of ITS region revealed the relatedness of each of the isolated in species level. A dendrogram was constructed by Phylogenetic Analysis Using Parsimony (PAUP) version 4.0. Antibacterial study was carried out against two Gram-positive and two Gram-negative bacteria by disc diffusion method. Extract GRN-18 (methanolic extract) showed maximum zone of inhibition which was 17.5 mm against 3 bacteria (Micrococcus, E.coli, *Pseudomonas aeruginosa)*. For screening of antibacterial activity, all the extracts exhibited mild to moderate activity against some of the individual test bacteria. To evaluate the free radical scavenging activity of the fungal extracts, all the extracts were subjected to the DPPH scavenging activity test. Among them extract (GRN-16 methanolic extract) showed better IC_{50} value comparing with standard as Ascorbic acid (10.9730µg/ml). Endophytic fungi isolated from the *C.decandra* have lower cytotoxicity in nature. Extract GRN- 7 *(Colletroticum gloeosporioides spp.)* showed moderately good activity with low LC_{50} value against the nauplii when compared. This study could be taken as a good source of reference in the scientific world for the discovery of potential bioactive compounds.

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List of Tables

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List of Figures

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Abbreviations Used in the Text

et al.	et alia= and others
etc.	et cetra= and the others
gm	Gram (s)
gm/ml	Gram (s) per milliliter
i.e.	Id est= that is
Mg	Milligram (s)
mg/ml	Milligram (s) per milliliter
ml	Mililiter (s)
L	Liter
Conc.	Concentration (s)
g.	Gram
h.	Hour
mins.	Minutes
µg	Microgram (s) per mililiter
µg/ml	Microgram (s) per mililiter
MEGA	Molecular evolutionary genetics analysis
BLAST	Basic local alignment search tool
PL	Passur leaf

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CHAPTER ONE

INTRODUCTION

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1.0Introduction

Mangrove forests are fascinating and complex ecosystems (Feller *et al.*, 2010). Mangrove plants are salt-tolerant plants which act as primary producers in the estuarine food chain and they produce novel metabolites unique to the environment with various important economic and environmental functions (Bandarnayake 2002). Most mangrove plants do not consider the fact that plants in natural ecosystems have symbiotic associations with fungi.

Endophytic fungi that live inside the tissues of living plants are under-explored group of microorganisms. Recently they have been received considerable attention after they were found to protect their host against insect, pests, pathogens and even domestic herbivores. Almost all the plant species harbour one or more endophytic microorganisms. Endophytes are hidden within healthy host plants are a poorly investigated group of microorganisms, but they represented an abundant and dependable source of novel bioactive compounds with huge potential for exploitation in a wide variety of medical, agricultural, and industrial areas (Tan and Zou, 2001).These fungi are important to the structure, function, and health of plant communities and may be responsible for the adaptation of plants to environmental stresses (Clay and Holah, 1999). In addition, mutualistic symbiosis with mycorrhizal and endophytic fungi can confer salt tolerance to plants and decrease yield losses in cultivated crops grown in saline soils (Baltruschat *et al.*, 2008). Endophytic fungi have been recognized as possible sources of bioactive secondary metabolites (Schulz *et al.*, 2002, Strobel *et al.*, 2003). There is a need to search new ecological niches for potential sources of natural bioactive agents for different pharmaceutical, agriculture, and industrial applications.

Ceriops decandra (*C.decandra*) (Griff.) Ding Hou (Family: Rhizophoraceae) locally known as Goran and Cuttia (Bengali) is a medium-sized straight, columnar, ever green tree, under favorable conditions reaching up to 35 m in height distributed widely throughout the east coast of Bangladesh and India, southwestern Thailand and western part of the Malay peninsula.This mangrove species C. decandra has long been used as a traditional folk remedy for angina, diabetes, diarrhea, dysentery, wounds and boils (Watt and Breyer-Brandwijk ,1962; Duke and Wain, 1981; Kathiresan and Ramanathan, 1997). A new epoxy ent-kaurene diter penoid ceriopsin E was isolated from C. decandra (Anjaneyulu *et al.*, 2002). Two diterpenoids, ceriopsins F and G have been isolated from the root of C. decandra (Anjaneyulu and Rao, 2003). The leaf and pneumatophore of C. *decandra* possess antinociceptive and

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antidiabetic activities (Uddin *et al.*, 2005; Mannalamkunnath *et al.*, 2010). The anti-inflammatory drugs have not been used successfully in all cases due to adverse side effects suchas gastric lesions caused by non-steroidal anti-inflammatory drugs (NSAIDS). Therefore, new drugs lacking these side effects are searched for all over the world. During this process, the investigation of the efficacy of plant based drugs used in the traditional medicines for the treatment of pain, fever and inflammatory ailments have received attention because they are cheap and have little side effects (Kumara 2001).

Reactive oxygen species (ROS) are continuously produced during our normal physiologic events and removed by antioxidant defense mechanisms (Halliwell *et al.*, 1992). But an excessive generation of ROS and other radicals can damage proteins, carbohydrates, polyunsaturated fatty acids and DNA. This may thus lead to oxidative stress and to a variety of degenerative processes and diseases such as aging, immune deficiencies, neurologic disorders, inflammation,art ritis, ischemia, arteriosclerosis, coronary heart disease, stroke, diabetes mellitus, Parkinson's disease, Alzheimer's disease and certain cancers (Sies 1991; Gutteridge 1993; Aruoma 1994; Cook and Samman, 1996). Therefore, the great interest has been recently focused on the natural foods, medicinal plants and phytconstituents due to their well-known abilities toscaenge ROS induced free radicals (i.e. antioxidant power) (Hou *et al.*, 2003).The present research was thus designed to evaluate the antioxidant and anti-inflammatory activities of the ethanolic extract of C. *decandra* bark and scientifically justify its use as a traditional folk remedy (Kathiresan and Ramanathan, 1997).

Antimicrobial properties reported earlier in the tissue extracts of Ceriops decandra (Vadlapudi and Naidu, 2009; Chandrasekaran *et al.*, 2009; Ravikumar *et al.*, 2010; Simlai and Roy, 2012), have led us to investigate the partial structural nature and growth inhibitory potential of a semi-purified phytochemical fraction from this plant. The fraction named CD-3PM has been isolated from the wood tissue of this plant using chromatographic methods. Ceriopsdecandra (Griff.) Ding Hou is a shrubby mangrove species of the Rhizophoraceae family found in the Sundarban estuary in India. The species has been reported to be used by traditional healers as relief for wounds, boils, hepatitis, ulcers, angina, diabetes, diarrhea and dysentery (Watt and Breyer-Brandwijk, 1962; Duke and Wain, 1981; Kathiresan and Ramanathan, 1997; Bandaranayake, 1998; Simlai and Roy, 2013). Previously a number of phytochemicals have been isolated and identified from C. *decandra* (Wu *et al.*, 2008; Wang *et al.*, 2012; Nebula *et al.*, 2013), but these compounds have not been linked with the

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antimicrobial activities reported from the crude tissue extracts of this species (Vadlapudi and Naidu, 2009; Chandrasekaran *et al.*, 2009; Ravikumar *et al.*, 2010; Simlai and Roy, 2012). Vundru *et al.*, (2013) revealed the presence of a wide range of phytochemicals as indicated by gas chromatography–mass spectrometry (GC-MS) analysis in C. *decandra* crude leaf extracts with antifungal and larvicidal properties. A study by Sakagami *et al.*, 1998 is the only report in which the authors used fractionated leaf extracts of the plant containing ligninlikepolyphenolic structures demonstrating its protective effect in mice infected with lethal dose of E. coli. Apart from the observations made by Sakagami*et al.*, (1998), we have not come across any other scientific report that assigns the observed antimicrobial activities seen in this plant to one or more phytochemical components in a pure or semi-pure state (Wu *et al.*, 2008; Wang *et al.*, 2012; Nebula *et al.*, 2013). In this study, we have described the isolation and partial chemical characterization of a substantially purified phytochemical fraction named CD3PM from the wood tissue of C. *decandra.*

1.2 Objectives of the Study:

The main objective of the study is to gain practical knowledge and experience about laboratory work, medicinal facts, microbial facts, effectiveness and usefulness of *Ceriops decandra*in practical life. The broad objectives of this study are to find out major prospects of *Ceriops decandra*the Mangrove plant of Sundarbon as well as find out the suggestion for effective marketing and health plan. The specific objectives of this study are show below:

a. Isolation and identification of endophytic fungi from*C. decandra*.
b. Finding the antioxidant, antimicrobial and cytotoxic properties of *C. decandra.*

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CHAPTER TWO

LITERATUREREVIEW

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2.0 Literature Review

2.1 Literature Review of *Ceriops decandra*

2.1.1 General information of *Ceriops decandra*

Mangrove plant lives in tropical zone and in the subtropical zone. The living environment has the characteristic of strong reducibility, strong acid, high salt and rich nutrition (SU *et al* .,2012). *Ceriops decandra*is a common species of mangrove plant. It has a local name 'Goran'. *Ceriops decandra* is a genus of plants in the Rhizophoraceaefamily.The specific epithet *decandra* is from the Greek meaning "ten male", referring to the flower having ten stamens.

2.1.1.1 Taxonomy of *Ceriops decandra*

Kingdom	:	Plantae
Phylum	:	Angiosperms
Class	:	Eudicots
Subclass	:	Rosids
Order	:	Malpighiales
Family	:	Rhizophoraceae
Genus	:	*Ceriops*
Species:		*C. decandra*

2.1.1.2 Common Name of *Ceriops decandra*

Latin name: *Ceriops decandra*

Local name: Goran

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2.1.1.3 Available species of the plant

- *Bruguiera decandra*
- *Ceriops roxburghiana*
- *Rhizophora decandra*
- *Ceriops candolleana*

2.1.1.4 Geographical distribution of *Ceriops decandra*

Ceriops decandra grows naturally in India and Bangladesh (including the Sundarbans), Burma, Thailand and Peninsular Malaysia. Its habitat is mangrove swamps and tidal creeks.The genus *Ceriops* Arn.was once more widely distributed than it is today. For example, it was probably present in Europe in the Eocene; both *Ceriops* and some other Rhizophoraceous genera appear in the European fossil record before they appear in that of Southeast Asia. Clearly, the geographical ranges of the species have changed. Thus, although *C. decandra* is now centered in Southeast Asia, it is not certain that it originated in this region. Its current range extends from the Indus delta in Pakistan around the coast of India and across the Bay of Bengal to Burma and thence through Indo-China, Thailand and Southeast Asia to Papua New Guinea. It also occurs locally in north-eastern Australia. In Southeast Asia, it is found in Peninsular Malaysia, the Philippines, Borneo, Java, Sulawesi, the Lesser Sunda Islands, the Moluccas, and New Guinea. It has not yet been collected from Sumatra, but has been recently reported there.

2.1.1.5 Range Description

Recent research has shown that the range of *C. decandra* is restricted to the east coast of India and Bangladesh, southwestern Thailand and western part of the Malay Peninsula. The middle and southern part of its range is now considered to be *C. zippeliana* (Sheue*et al.* 2009).

2.1.1.5 Description of the plant

Ceriops decandra grows as a shrub or small tree up to 15 meters (50 ft) tall with a trunk diameter of up to 30 cm (12 in). Its bark is pale brown. The flowers are white. The ovoid to

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conical fruits measure up to 1.8 cm (0.7 in) long.*Ceriops decandra* is a straight, columnar tree with a relatively narrow crown; it usually grows up to 15 meters tall, but some specimens up to 35 meters have been recorded. The bowl is most commonly in the range 15 - 20cm in diameter, though some can reach 35cm; it has short basal buttresses which appear to develop from the fusion of clusters of stilt roots. Small pneumatophores (breathing roots) develop in wetsites.

The tree is commonly harvested from the wild as a source of wood and other materials for local use. In the past the bark was an important source of high quality tannin, and although its use for this purpose has waned in recent years, it is still used locally. This species is rare with a restricted distribution. Its large-scale exploitation for posts, poles, firewood and charcoal has been widespread, and still occurs in places. It is also threatened by habitat loss from coastal development throughout its range. Although the exact population reduction is unknown, it is estimated to be between 12 - 26% over the period 1980 - 2000. The plant is currently classified as 'Near Threatened' in the IUCN Red List of Threatened Species(2013), though may well qualify for a 'Threatened' category.

Ceriops decandra is a straight columnar tree, usually small to medium-sized. However, under favorable conditions, it attains a height of 35 m and trunk diameter of 35 cm, with a relatively narrow crown and short basal buttresses which appear to develop from the fusion of stilt roots clusters .The roots are superficial, radially spread, with small knobby and/or pneumatophores loop in wet sites. The bark is whitish or pale grey, and smooth but slightly fissured towards the base. It peels around the buttresses. The branches are conspicuously jointed with swollen nodes.

The leaves are oppositely arranged, clustered at the end of the twigs, coriaceous, obovate to elliptic-oblong, measuring 4.5-10 cm x 2.5-6 cm, wedge-shaped at the base, rounded or sub emarginated at the apex, hairless and glossy. The petiole is 1-2.5 cm long, with lance-shaped deciduous stipules at the base measuring 1.5-2.5 cm long .The flowers are in head-like, condensed, up to 5-flowered cymes in leaf axils at the upper part of a branch. They are 5-6-merous, measure 5-6 mm long and with deeply lobed sepal. The white petals are about 2.5 mm long and divided fringe-like at the apex. The stamens are twice the number of sepal lobes. The anthers are longer than the filaments. The ovary is semi-inferior and 3-celled.The fruit is an ovoid-conical berry, measures 1-1.8 cm long, with persistent, erect or ascending sepal lobes, blunt basally and warty at the apex. The seeds are viviparous. The hypocotyl is

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club-shaped, protruding below the fruit, measuring 9-15 cm long, occasionally longer (e.g. in New Guinea) and slightly fluted.

Figure 2.1 ***Ceriops decandra***

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2.1.1.6Cultivation Details

Ceriops decandra is most common in tidal forests in high rainfall regions, where characteristically, it grows in the middle to landward parts of the mangrove swamps. Here, it is commonest in sites flooded by virtually all high tides, i.e. where the soil surface is below mean water level of high tides. It develops best immediately behind the forest strip lining rivers, and on the slightly higher muddy tidal flats behind, between rivers and creeks. In these sites, fresh water is in regular supply and salinity never exceeds that of normal sea water. Locally, this species is gregarious; forming a slender pole forest, but it is most often associated with species of *Avicennia* L., *Bruguiera*Lamk and *Rhizophora* L. However, its locality is not constant, and it occurs on the landward fringes of some mangrove swamps.

A plant mainly of high rainfall areas in the tropics. The plant can grow in saline soils; it has a maximum tolerance of salinity at 67ppt and a salinity of optimal growth at15 ppt. A slow-growing species, though it can be tolerant of extreme environmental conditions. Trees tend to flower periodically and synchronously over wide areas, but seasonally under seasonal

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climates. Fruiting is often prolific. Seeds germinate and start to develop whilst still on the tree, with individual trees producing several thousands of seedlings at the same time. The seedlings take up to 12 months to develop, with shorter times in wet equatorial regions, and then fall. Subsequent development involves a seedling being stranded and lodged in the mud, followed by the rapid production of adventitious roots which serve to anchor it. Most seedlings are slender and small and cannot survive long periods while floating in the water, and consequently are not as successfully dispersed over long distances as those of other mangrove Rhizophoraceae. However, once 'planted' in the shade of other trees their rate of establishment is very high.

2.1.1.7 Medicinal Uses

The bark is astringent. A decoction is used to treat haemorrhages.

2.1.1.8 Other Uses

The bark yields around 25 - 37% of a high quality tannin. Both bark and leaves are used for tanning in South-East Asia and India. The bark of older trees has higher contents of tannin. The sap of the bark yields a black dye used in the 'batik' industry. The wood is a pale whitish-yellow when freshly cut, turning orange-brown on exposure to air. It is usually somewhat less heavy than the wood of the related Ceriopstagal, and is moderately resistant to decay, with a life in contact with the ground of about 2 years. The branches are used for tool handles, and bent ones for boat ribs. Some wood of this species has been chipped for pulp. The wood is used for fuel. When dry, the wood burns with a hotter flame than that of most other mangrove species

2.2 Endophytic fungi

The term 'endophytes' (Greek: endon-within; phyton-plant) was first coined by de Bary (1886) and endophytic is applied to fungi which live within plant tissue, for all or part of their life cycle and cause no apparent infections (Saikkonen *et al.*, 2004). It has no negative effect on cell. It colonizes and cause unapparent asymptomatic infections in healthy plant tissue (Wilson 1995; Hyde and Soytong, 2007).

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2.3 Biological properties of endophytic fungi

The endophytic fungus is having rich source of secondary metabolites which act as biological active agent in the higher plants. Endophytic fungi can protect their host plants from pathogens and pests (Arnold *et al.*, 2003). The fungal derivatives play an important part in human life. Most importantly their compounds are the source of drug for cancer, microbial and viral diseases. The natural compounds from the endophytes acts as growth inhibitor of plant pathogenic organism. Endophytic fungi are also capable of inducing resistance to diseases, and many mechanisms have been proposed for this resistance. The mechanisms of endophytic induced resistance are related to the nutritional status of the host, and to increase the fitness of plants by enhancing their tolerance to abiotic stress (Aguilar and Barea, 1996). Endophytes are rich sources of natural products which are used in agriculture (Sudha *et al.*, 2016). There is a friendly relationship between endophytic fungi and medicinal plants (Jia M *et al.*, 2016). Endophytes isolated from medicinal plants possess strong fungicidal, bactericidal and cytotoxic metabolites (Wang *et al.*, 2007). It produces enzymes which are used for various applications like degradation and biotransformation of organic compound (Firakova *et al.*, 2007; Pimentel *et al.*, 2011). Endophytic fungi isolated from mangrove plant and have antagonism role against fusarium wilt (Suciatmih and Rahmansyah, 2013)

2.4 Endophytic fungi from *Ceriops decandra*

Endophytic fungi that live inside the tissues of living plants are under-explored group of microorganisms. Recently they have been received considerable attention after they were found to protect their host against insect, pests, pathogens and even domestic herbivores. Almost all the plant species harbour one or more endophytic microorganisms. Endophytes are hidden within healthy host plants are a poorly investigated group of microorganisms, but they represented an abundant and dependable source of novel bioactive compounds with huge potential for exploitation in a wide variety of medical, agricultural, and industrial areas (Tan and Zou, 2001).These fungi are important to the structure, function, and health of plant communities and may be responsible for the adaptation of plants to environmental stresses (Clay and Holah, 1999). In addition, mutualistic symbiosis with mycorrhizal and endophytic fungi can confer salt tolerance to plants and decrease yield losses in cultivated crops grown in saline soils (Baltruschat *et al.*, 2008). Endophytic fungi have been recognized as possible sources of bioactive secondary metabolites (Schulz *et al.*, 2002, Strobel *et al.*, 2003). There is

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a need to search new ecological niches for potential sources of natural bioactive agents for different pharmaceutical, agriculture, and industrial applications.

2.5 Isolation of endophytic fungi from mangrove plants

Sukanyanee *et al.*, (2010) have isolated endophytic fungi from the leaves of mangrove forest trees growing at three different locations (*Chanthaburi Province, PrachuapKhiri Khan Province* and *Ranong Province*) in Thailand. Three thousand and nine-hundred leaf segments from 10 different hosts belonging to seven families, Rhizophoraceae (*Rhizophora apiculata, R. mucronata, Ceriops decandra*), Sonneratiaceae (*Sonneratia alba*), Combretaceae (Lumintzeralittorea), Avicenniaceae (*Avicenna alba*), Acanthaceae (*Acanthus ilicifolius*), Meliaceae (*Xylocarpus granatum* and *Xylocarpus moluccensis*) and Malvaceae (*Thespesia populneoides*) were screened for the presence of fungal endophytes.

Isabella *et .al.*, (2012) have done a survey in leaves of mangrove plants (*Avicennia schaueriana, Lagunculari aracemosa,* and *Rhizophora mangle*) that was performed at the Itamaraca Island, PE, Brazil. Forty taxa were isolated: 25 species representing 19 genera and 15 morphotypes determined as Mycelia sterilia. Leaves of *L. racemosa* hosted the highest number of colony forming units (CFU) and taxa.

Jia *et al.*, (2016) have isolated endophytic fungi from leaves and twigs of four mangroves *Aegiceras corniculatum, Avicennia marina, Bruguiera gymnorrhiza,* and *Kandelia candel* in Guangxi, Chaina. A total of 36 endophytic fungal taxa were identified based on morphological characteristics and molecular data, including 35 Ascomycota and 1 Basidiomycota, dominated by *phomopsis, Phyllosticta, Xylaria, Leptosphaerulina* and *Pestalotiopsis*.

Ananda *et al.*, (2002) have characterized for fungal communities from four mangrove plant species, *Acanthus ilicifolius, Avicennia officinalis, Rhizophora mucronata,* and *Sonneratiacaseolar is* from the mangroves of Udyavara (Karnataka) on the west coast of India to study the Diversity of endophytic fungi in the roots of mangrove species on the west coast of India.

Nurunnabi TR *et al.*, (2017) have isolated endophytic fungi from *Pestalotia* sp. of sundarbans mangrove plant Heritierafomes to study Anti-MRSA activity of oxysporone and xylitol.

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2.6 Morphological identification of endophytic fungi

Jia*et al* (2016) have identified 36 endophytic fungal taxa based on morphological characteristics and molecular data, including 35 Ascomycota and 1 Basidiomycota, dominated by *Phomopsis, Phyllosticta, Xylaria, Leptosphaerulina,* and *Pestalotiopsis* from four mangrove species in Southern China.

SU *et al.,* (2012) have isolated endophytic fungi from the Rhizophoraceae plant *Ceriops decardra* was investigated and 20 potential pure strains were isolated. According to microbial taxonomy and morphology method, 10 species Rhizoctonia DC of *Deuteromycotina*, *Penicillium* 6 species, 1 species of *Aspergillus*, 1 species *Trichoderma* and 2 species Mucor of *Zygomycotina* were identified with microscopic examination.

Paul *et al.,* (2012) have encountered a species of Heterobasidion during a diversity study of endophytic fungi from healthy root tissues of chili pepper (*Capsicum anmuum L.*) in Korea. Morphological descriptions of the endophytic isolate matched well with the previous references and supported the molecular identification. The fungus *Heterobasidion araucariae* CNU081069 is a new to Korea.

Boddington *et al.,* (2018) have isolated endophytes from roots of *Dendrobiumspeciosum*, a common epiphytic orchid in southern Queensland rainforests and used morphological criteria. Morphological analysis of the cultures obtained demonstrated a variety of non-mycorrhizal deuteron mycetes present in plant roots.

2.7 Molecular identification of endophytic fungi

Huang *et al.,* (2009) have selected thirty-four fungal isolates for molecular phylogenetic analysis using nuclear ribosomal DNA sequences, including both the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene region. The 34 endophytes were identified to various taxonomic levels, and some to the species level based on fungal sequences with known identifies in GenBank.

Gonzalez-Teuber M.*et al.,* (2017) focused on the Identification of the fungal endophytic community associated with the roots of quinoa plants (*Chenopodium quinoa*) growing near the salt lakes of the Atacama Desert, Chile. One hundred endophytic fungi were isolated

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healthy quinoa roots, and the internal transcribed spacer (ITS) region was sequenced for phylogenetic and taxonomic analysis.

Almedia *et al.*, (2015) have isolated fungal endophytes associated with the leaves of *Eichhorniaazurea* and *Eichhornia, crassipes*, native to the Upper Parana River floodplain, Brazil. The sequencing obtained was compared with those available in the GenBank database for the molecular identification of the isolates. The construction of phylogenetic trees was performed using the MEGA5 software.

Sette *et al.*, (2006) have characterized endophytic filamentous fungi taxonomically from coffee plants (*C. offeaarabica* and *C. robusta*) deposited in the Brazilian Collection of Environment and Industrial Microorganisms (CBMAI) by using molecular tools and investigated concerning their antimicrobial activity against different human pathogenic bacteria.

Fungal endophytes are micro fungi that colonize and live within healthy plant tissues without inducing symptoms of disease (Petrini *et al.*, 1991). These organisms are thought to be ubiquitous among terrestrial plants, having been recovered from all major plant lineages in a wide range of terrestrial communities. Additionally, numerous investigations over the past three decades have shown that endophytes are abundant in asymptomatic leaves of many conifers (Carroll 1978; Arnold *et al.*, 2007). Endophytes may offer significant benefits to their host plants by producing a plethora of substances that provide protection and survival value, such as enhancement of stress-, insect-, and disease-resistance, productivity improvement, and herbicide activity (Strobel *et al.*, 2004). Accordingly, endophytes likely play an important role in ecological communities (Gao *et al.*, 2005). However, few studies of the diversity, host plant affinities, and environmental and medicinal effects of fungal endophytes in Korea have been conducted to date (kil *et al.*, 2009; Kim *et al.*, 2012). Therefore, the present study was conducted to isolate and identify endophytic fungi from the leaves of coniferous trees on Bohyeon Mountain of Korea.

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CHAPTER THREE

MATERIALSAND METHODS

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3.0 Materials and Methods

3.1 Plant materials

3.1.1 Collection of plant leaf

Sample was collected from Sundarbans (Hodda forest station, Nalian Range, Sundarban East Division, Bangladesh). Fresh and vigor leaves of *Ceriops decandra* were taken and kept for sterilization process to go into primary culture.

3.1.2 Processing of plant leaf

Prior to surface sterilization, leaves were individually removed from shoots and thoroughly washed in, running tap water to remove dust particles on leaf surfaces, and then samples were air dried and kept into a zip lock bag and stored in refrigerator at 4^0C for further use. Remaining samples were already taken for next sterilization process and kept into laminar hood perform isolation and primary culture of endophytic fungi from plant's leaf.

3.1.3 Isolation of endophytic fungi

After sterilization, samples were ready for the isolation of endophytic fungi. Isolation of endophytic fungi from sample included some steps which are given below in step by step:

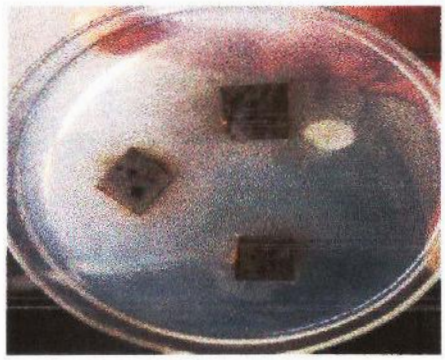

Figure 3.1 Isolation of endophytic fungi from plants leaf

3.1.3.1 Sterilization of plant leaf

All steps were done into laminar hood (biosafety cabinet, Esco, Singapore).At first the leaves of *C.decandra* cut into 1cm^2 sized small pieces. Those small pieces of leaves were taken in an autoclaved petri dish. Small pieces of leaves then thoroughly washed with distilled water (1

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min), surface-disinfected by soaking in 70% ethanol for 1 min followed by washing with 5% NaOCl (Sodium Hypochlorite) for 0.5-1 minute. Again the leaf pieces were washed with distilled water and water was removed by using sterile filter paper. Finally, the leaf pieces were dried and prepared for isolation of endophytic fungi from the sample. The scissors, forceps and tips used in this technique were autoclaved before use.

3.2 Primary culture preparation

Water agar medium was prepared for doing primary culture.

3.2.1 Instruments and vessel

- Beaker
- Petridish
- Large petridish
- Conical flask
- Micro oven
- Scissors
- Forceps
- Jar
- Measuring cylinder
- Scalpel
- filter paper
- Cotton
- Anti-cutter
- Aluminum foil
- Razor blade
- Polythene for covering or capping conical flasks and petri dishes
- Glass plate
- 70% Ethanol etc.

3.2.2 Water agar media preparation

At first, a clean cylinder was taken to measure 250 ml distilled water and poured it into a 500ml conical flask. Then, 4g agars were measured by a digital balance machine and were added into the conical flask. Agar was dissolved by heating using microwave oven. After

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dissolving agar, the medium was cooled to normal temperature and pH was adjusted to 5.6-5.8. The conical flask was covered with a cotton plug and then wrapped the cotton plug with aluminum foil. After that, the medium was autoclaved at 121^{0}C (15psi/Ibs) for 15 mins. Finally, the media was poured into petri dished, cooled and stored for future use.

3.3 Pure culture

3.3.1 PDA media preparation

At first, a clean cylinder was taken to measure 250 ml distilled water and poured it into a 550ml conical flask. Then, 9.9ml of PDA was weighted by a digital balance machine and added into the conical flask. The conical flask was covered with a cotton plug and then wrapped the cotton plug with aluminum foil. After that, the medium was autoclaved at 121^{0}C (15psi/Ibs) for 15 mins. Finally, the media was poured into petri dishes, cooled and stored for future use.

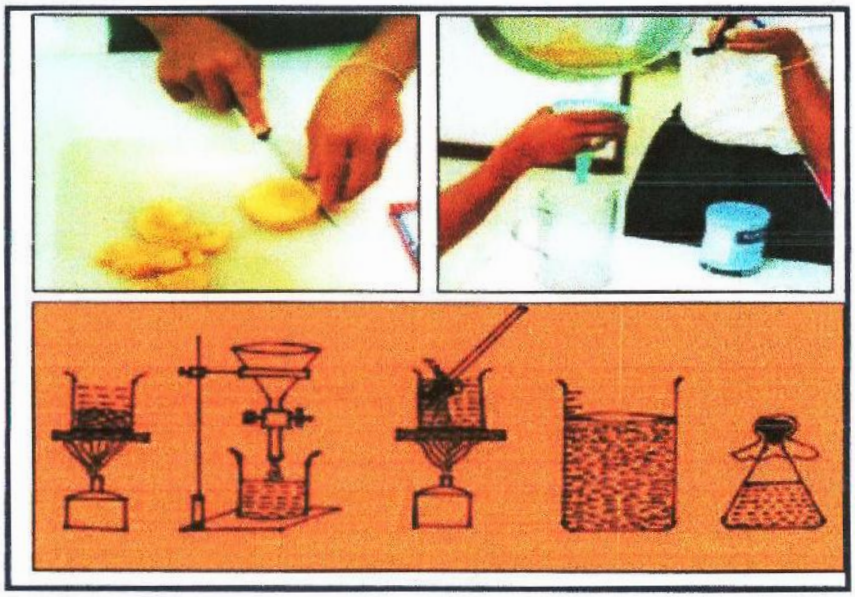

Figure: 3.2 PDA media preparation

3.3.2 Inoculation

The prepared PDA media was poured into petri dished where each of the petri dish containing 20ml medium. Let them cool to form semi solid media in plate. Then, the fungal growth was cut from primary culture from the growing edges using cork borer. After that, it was transferred into the PDA containing plate and each plate containing three pieces from one petri dish. The procedure is performed in laminar hood (biosafety cabinet). Finally, the plates were kept in an incubator and checked for growth regularly until they form spores.

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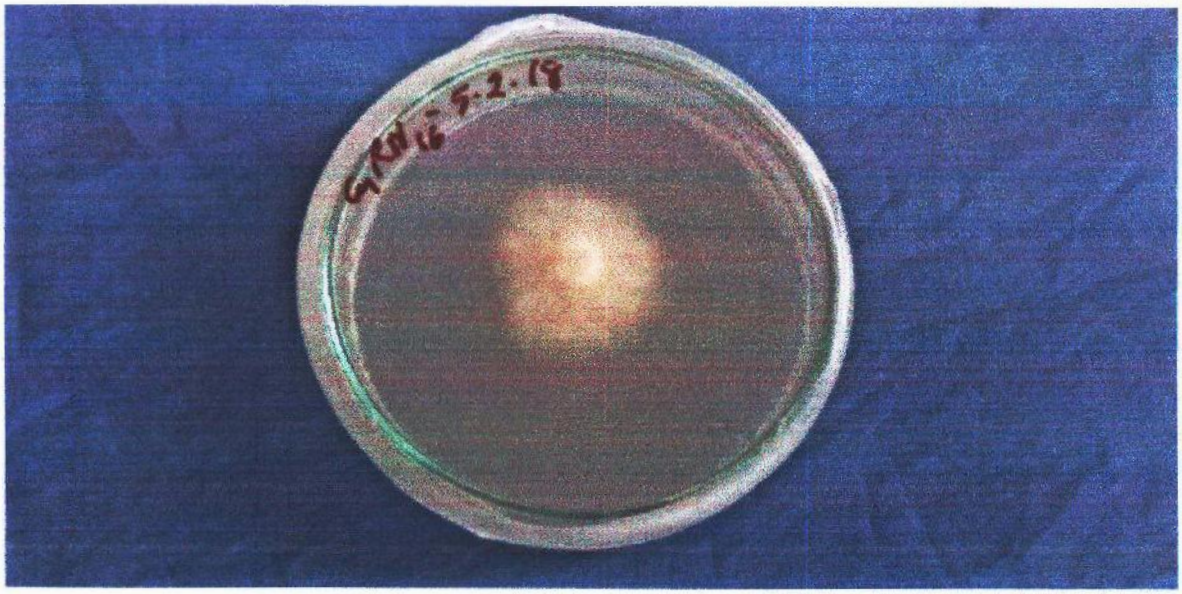

Figure 3.3 Pure cultures of endophytic fungi.

3.4 Identification of endophytic fungi

Fungi were identified by means of macroscopic and microscopic observation.

3.4.1 Morphological identification

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

- Form- the basic shape of the colony. For example, circular, filamentous etc.
- Size-the diameter of the colony. Tiny colony is referred to as punctiform.
- Elevation- this describes the side view of a colony.
- Margin/Border-the magnified shape of the edge of a colony.

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- Surface-the appearance of surface of the colony for example, smooth, rough or dull.
- Opacity-for example, transparent (clear), opaque, translucent (like looking)
- Color-means pigmentation for example white, buff, red, purple etc.

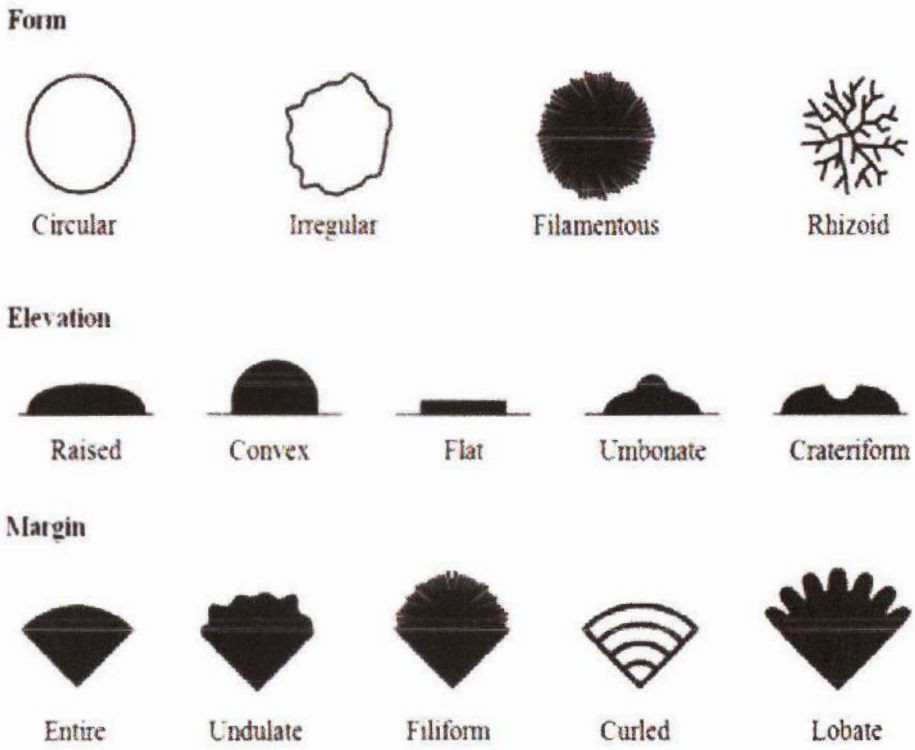

Figure 3.4: Morphological characteristics of fungi in petri dish

Morphological identification was conducted by the following parameter-

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

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3.5 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 the incubation at 28^{0}C on potato dextrose agar media, a small portion of the colony was taken into lacto-phenol cotton blue solution (0.05 gram cotton blue in 100 ml lacto-phenol). A drop of the sample was poured on a glass slide and spread with the help of a sterilized needle, then covered with a cover slip. It was then examined for a characteristic arrangement of spores under 10x, 40x and 100x objective lenses of a compound microscope. Identification was based on morphological characteristics such as growth pattern, hyphae, the color of the colony and medium, surface texture, margin character, aerial mycelium, sporulation and production of acervuli, coloration of the medium, and the size and coloration of the conidia using standard identification manuals (Devi *et al*, 2014). The fungi were identified using relevant keys and taxonomic notes from various standard manuals (Barnett, 1998). Total six endophytic fungi were isolated.

3.6 Cultivation of endophytic fungi for small scale

This procedure was carried out as described by Ghisalberti (2002) and kjer (2010). 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 PD broth.

3.6.1 Materials and instruments used for cultivation

- Petridish
- Conical flask
- Micro-oven
- Measuring cylinder
- Cotton
- Cotton plug
- 70% Ethanol
- Loop
- Para film
- PD broth media

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- Pure culture of fungi etc.

3.6.2 PD broth preparation

At first, a clean cylinder was taken to measure 600 ml distilled water and poured it into a 1000ml conical flask. Then, 333.33 gm potato was measured and sliced. Potato slices with dH_2O were heated in a deck until patato extracts were come out which took up to 60 minutes. Potato extracts were taken in a conical flask and 33.33 gm of dextrose was added and dissolved using magnetic stirrer. After dissolving dextrose pH was adjusted to 5.1. Then the conical flask was covered with a cotton plug and then with aluminum foil. After that medium was autoclaved at 121^0C (15psi/Ibs) for 15 mins. Finally media was poured into conical flask, cooled and stored for future use.

Figure 3.5: Liquid PD medium

3.6.3 Inoculation

Spores were collected from 8 different pure culture colonies them with cork borer and transferred to PD broth in conical flask. PD broth provides floating space and nutrient for fungal mycelia and extraction of mycelia is easy for DNA extraction.

3.7 Transfers to Small Scale

For small scale culture, Petridishes were prepared with PDA media and sub culture were done from pure culture. It was allowed to grow fungus for 18-21 days. After 21 days the culture obtained were ready for transfer to small scale. For small-scale cultivation in liquid media, a pure fungal strain was inoculated in a 1,000 ml Erlenmeyer flask containing either 300 ml of liquid PD media. For this purpose, a strain was cut that covers the surface of the

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inoculated petri dish into small pieces of ~1.5 cm × 1.5 cm and these pieces were transfered with a sterile loop into an Erlenmeyer flask containing the sterilized medium. Cultivation was carried out at room temperature under static conditions and daylight .Depending on the fungal growth; cultures on liquid medium are incubated for 3–4 weeks. To prevent the distribution of fungal fragments or spores, flasks should only be opened under a laminar air-flow.

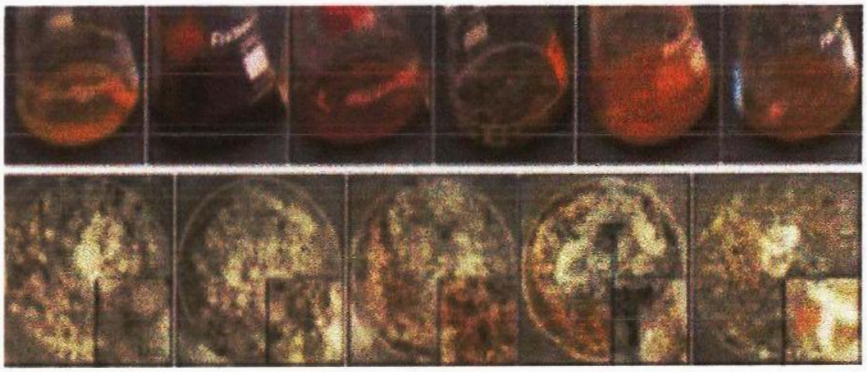

Figure: 3.6 Small Scale Culture

3.8 Extraction of secondary metabolites

3.8.1 Materials and instruments used for extraction

- Conical flask
- Funnel
- Separating funnel
- Beaker
- Cotton
- Filter paper
- Rotary evaporator
- Solvent (methanol, ethyl acetate) etc.

Extraction was done from small scale in two part. Firstly the mycelium part was separated from aqueous portion using filter paper. Secondly mycelium was grinded and soaked with methanol for 3 days or ethyl acetate for 5 days. Aqueous portion was mixed with chloroform in separation funnel. After that mycelium was separated from ethyl acetate using filter paper and filtered ethyl acetate was ready for evaporation to get extract.

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3.8.2 Preservation of identified fungi

Spores were collected from 8 different pure cultures of colonies that are transferred to PDA slant in test tubes. Each test tube containing 20ml media . Spores were taken twice from each colony and transferred to individual test tubes. Slants were kept in incubator and allowed to grow for 3-4 days. Finally, they were ready for preservation at 40C in freeze for further use if necessary.

Figure 3.7: Fungal growth in PDA slant for preservation

3.9 Genomic DNA Extraction

3.9.1 Instruments used for genomic DNA extraction, quantification, PCR, gel electrophoresis system, gel documentation and PCR clean-up

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- Automated DNA extractor, Model: Maxwell 16: Promega, USA.
- Homogenizer, Pro scientific
- Nano Drop Spectrophotometer, Model: ND2000, Origin: Thermo scientific, USA.
- Gene Atlas, Model: G2, Origin: Astec, Japan.
- Horizontal, Model: mini, Origin: CBS Scientific, USA.
- Alpa Imager, Model: mini, Origin: Protein Simple, USA.
- Centrifuge, Model: Kitman24, Origin: Tomy-Japan.

3.9.2 Process of genomic DNA Extraction

DNA extraction was done in automated machine by using magnetic beads. Each tubes contained magnetic beads. There was 5 tubes, one of them containing 20mg of spores

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collected from fungus plate. Another, new PCR tube was placed in the each column inside the machine to get the product. It took 45 minute to complete the whole process.

3.9.3 DNA confirmation by Agarose gel electrophoresis

- **Preparation of 1.5% Agarose gel**

1.5g of Agarose and 100 ml distilled water were measured and taken in a conical flask with 500 µl of 50X TAE buffer and dissolved with heating in microwave to make it crystal clear. Then the gel was poured in a gel casting tray and combs were set carefully. The gel was allowed to clot. Before proper clotting of the gel the comb was removed carefully.

- **Preparation of DNA samples for Electrophoresis**

10µl of DNA sample was taken in a sterile PCR tube and 2µl of 6X dye marker was added. The tube was vortex and centrifuged proper mixing.

- **Loading samples and running the gel**

The gel was prepared to place on electrophoresis chamber. Electrophoresis chamber was already full with gel running buffer (1X TAE) until the buffer covers the gel up to 2mm. Then, the gel was placed in the electrophoresis chamber. After that, the micropipette was used to load the sample in the gel slots (7µl of sample on each slot). After that, the lid was set on the gel box, connecting the electrodes correctly. The electrode wires were connected to the power supply, making sure the positive (Red) and the negative (Black) are correctly connected. The power was turned on. The gel run was monitored by tracking the movement of the blue loading dye. The power was switched off when the gel run was enough to visualize the DNA bands.

- **Staining the gel**

Gel staining solution was prepared by measuring 200ml of 1X TAE buffer and tracking into a gel staining tray. Then, 10µl of Diamond Dye was added into the solution. (Et-Br solution was avoided because it is carcinogenic). The gel was soaked in diamond dye solution for 5 mints.

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- **Visualization of DNA band under UV transilluminator**

After proper staining, the gel was placed on the UV transilluminator. The DNA bands were seen and photography by Gel doc.

3.9.4DNA Quantification

After DNA extraction, the concentration of the DNA was measured by using NanoDrop Spectrophotometer.

3.9.5 DNA dilution

Considering the DNA concentration, dilution was done to bring all the samples in the same concentration before PCR runs.

3.9.6 PCR Reaction

3.9.6.1 PCR Reaction procedure (Standard Application)

- **Supplied reagent**
    1. Template DNA
    2. Primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3')
    3. Primer ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')
    4. Mineral oil (optional)

The GoTaq® Hot Start Green Master Mix (Promega) was thawed at room temperature. The Master Mix was vortexed, and then centrifuged it briefly in a microcentrifuge to collect the material at the bottom of the tube. The following reaction mixture was prepared at room temperature for reaction.

The reactions were centrifuged in a micro centrifuge for 5 mints. The reactions was placed in a room temperature thermal cycler. PCR was performed by using standard parameters.

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3.9.6.2Amplification by PCR

3.9.6.2.1 PCR Profile

Denaturation

Initial denaturation at 95°c for 5 minute.

Annealing temperature:

The annealing temperature was optimized by performing the reaction starting approximately 5^0C below the calculated melting temperature of the primers and increasing the temperature in increments of 1^0C to the annealing temperature. The annealing step took 15 seconds to 1 minute at 45^0-60^0C.

The extension reaction was performed at the optimal temperature for Taq DNA polymerase, which was 72-74^0C. Allowed 1 minute for every 1kb of DNA to be amplified. Final extension was done of 5 minutes at 72-74^0C is recommended.

Generally, 25-30 cycles resulted in optimal amplification of desired products.

3.10 Purification

Equal volume of binding buffer and 2µl of PCR was taken (membrane binding solution). It is mixed well by pipetting, and then centrifuged and 700µl wash solution was added into silicon tube. The PCR product was centrifuged at 13,508 rpm for 1 minute. Centrifuge tube was containing silicon tube where water stored after centrifuge, and water was discarded and again silicon tube placed in centrifuge tube. Again, 500µl was solution was added in silicon tube and centrifuge was done at 13,508 rpm for 6 minutes. Water was discard like previous way and wash buffer was added again. Silicone tube placed in a sterilized PCR tube to evaporate the ethanol that was mixed with it. Then, 50µl nuclease free water was added. Finally, PCR product was purified and purity was checked in NanoDrop spectrophotometer.

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3.11 DNA sequencing and phylogenetic tree construction

After getting the DNA band, purified DNA was ready for the sequencing The sequencing is done by the software Phylogentic Analysis Using Parsimony (PAUP) version 4.0 .

DNA sequencing was done by containing with the commercial sequencing facilities provided by the Invent Technologies Ltd, House No-119, Flat No-B1, Road No-01, Dhaka-1213.

3.12 Preliminary bioassay

There are different test parameters to investigate bioactivity such as-

3.12.1Antimicrobial test.

3.12.2Antioxidant test.

3.12.3Brine Shrimp Lethality Bioassay.

3.12.1 Antimicrobial activity test

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

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

The bio-autographic and disc diffusion methods are known as qualitative techniques since these methods will give only an idea of the presence or absence of substances with antimicrobial activity. On the other hand, dilution methods are considered quantitative assays once they determine the minimal inhibitory concentration. The disc diffusion technique provide more suitable conditions for the microbial growth and is a widely accepted in vitro investigation which for preliminary screening of agents which may possess any antimicrobial activity. (Baucer *et al.*, 1996)

3.12.1.2 Principle

Solutions of known concentration (µg/ml) of the test samples are made by dissolving measured amount of the samples in definite volume of solvents. Discs containing the test

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material are placed on nutrient agar medium uniformly seeded with the test microorganisms. These plates are then kept at low temperature (4^0C) for 24 hours to allow maximum diffusion. During this time dried discs absorb water from the surrounding media and then the test materials are dissolved and diffused out of the media.

The plates are then incubated at 37^0C (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 zone of inhibition expressed in millimeter.

3.12.1.3 Apparatus and reagents

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

3.12.1.4 Test organisms

The bacterial strains used for the experiment were collected as pure cultures from the biochemistry lab (KU). Both gram positive and gram negative organisms were taken for the test and they are listed in the Table (3.1).

Table 3.1: List of test microorganism

Gram-positive Bacteria	**Gram-negative Bacteria**
Micrococcus	*Escherichia coli*
Staphylococcus aureus	*Pseudomonas aeruginosa*

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3.12.1.5Procedures

3.12.1.6Culture medium selection

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

3.12.1.7Preparation of medium

To prepare required volume of this medium, calculated amount of each of the constituents was taken in a conical flask and distilled water was added. The test tubes, petri-dish and media were sterilized by autoclaving at 15-lbs. pressure at 121^{0}C for 20 minutes. The slants were used for making fresh culture of bacteria that were in turn used for sensitivity study.

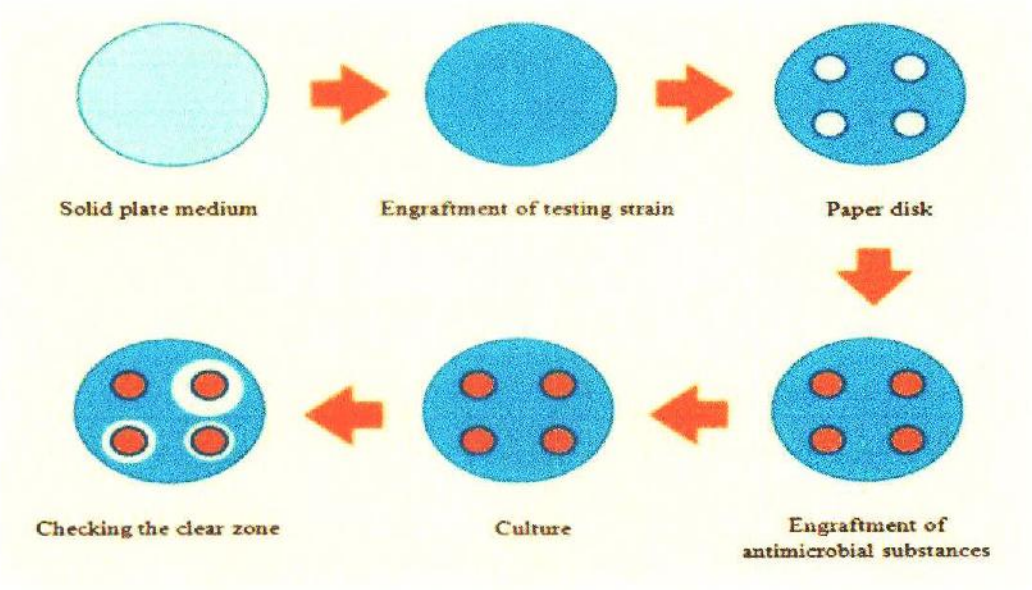

Figure 3.8:In vitro investigation of antimicrobial activity following the disk diffusion assay

3.12.1.8Sterilization procedures

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

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3.12.1.9Preparation of subculture

In an aseptic condition under laminar air cabinet, the test organisms were transferred from the pure cultures to the agar slants (for bacteria) and PDA slants (for fungi) with the help of a transfer loop to have fresh pure cultures. The inoculated strains were then incubated for 24 hours at 37^0C (for bacteria) and 25°C (for fungi) for their optimum growth.

3.12.1.10 Preparation of discs

Three types of discs were used for antibacterial screening.

- **Standard discs**

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

- **Blank discs**

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

- **Discs for solvent**

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

3.12.1.11 Preparation of test sample discs

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

3.12.1.12Preparation of the test plates

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

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3.12.1.13Application of the test samples

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

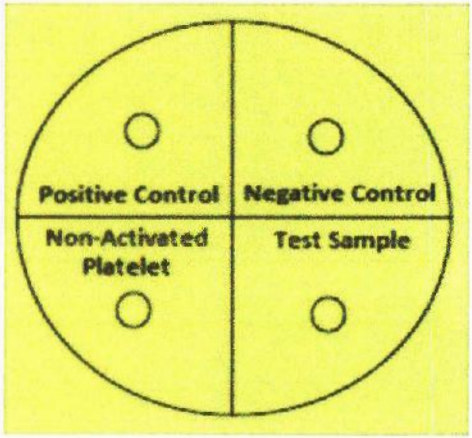

Figure 3.9:marked zones in the plates

3.12.1.14 Diffusion and incubation

The plates were then kept in a refrigerator at 4^0C 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 37^0C for 24 hours.

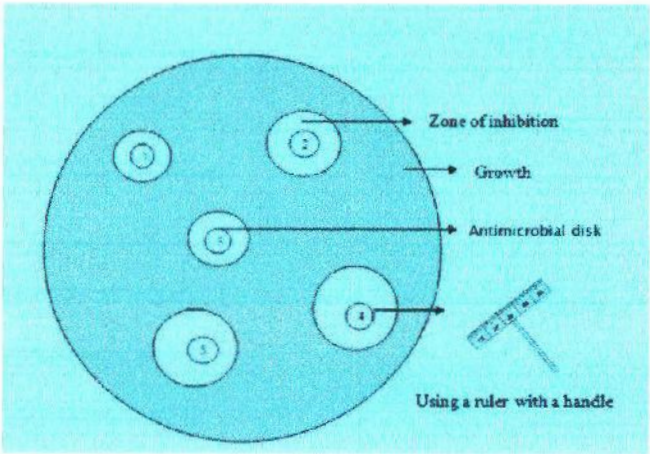

Figure 3.10: Measuring the zone of inhibition

3.12.15Determination of antibacterial activity by measuring the zone of inhibition

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

3.12.2 Antioxidant activity: DPPH assay

3.12.2.1 Materials

- Apparatus
- Test tubes
- Beakers
- Vials
- Thermometer
- Micropipette
- UV spectrophotometer (Shimadji 1800)
- Electronic balance

3.12.2.2 Reagents

- Methanol
- 0.02 mcg/ml methanolic solution of DPPH
- Standard : *tert- butyl-1-hydroxytoluene* (BHA) and Ascorbic acid(ASA)
- Distilled Water

3.12.2.3 Principle

The free radical scavenging activities (antioxidant capacity) of the plant extracts on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) were estimated by the method of Brand (Williams *et al* 1995). A stock solution of 1.6 mg of each extract in 0.4 ml methanol was prepared. The test solution was prepared at concentration of 3.125, 6.25, 12.5, 25, 50, 100, 200 µg/ml using methanol. 2.0 ml of methanol solution of the extract at different concentration were mixed with 2.0 ml of a DPPH methanol solution (20 µg/ ml).The mixture was properly mixed and kept in a dark place at room temperature for 30 min. The absorbance of the solutions was read at 517 nm against blank. The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of Ascorbic acid (ASA) by UV spectrophotometer.

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

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$$(I\%) = (1 - A_{sample}/A_{control}) \; X \; 100$$

Where $A_{control}$ is the absorbance of the control reaction (containing all reagents except the test material).

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

Figure 3.11:DPPH assy

3.12.3 Brine Shrimp Lethality Bioassay

Brine shrimp eggs are hatched in simulated sea water to get nauplii. Test samples are prepared by dissolving in dimethyl sulfoxide (DMSO) and by the addition of calculated amount of DMSO, desired concentration of the test sample is prepared. The nauplii are counted by visual inspection and are taken in vials containing 5ml of simulated sea water. Then samples of different concentrations are added to the premarked vials through micropipette. The vials are then left for 24 hours and then the nauplli are counted again to find out the cytotoxicity of the test agents.

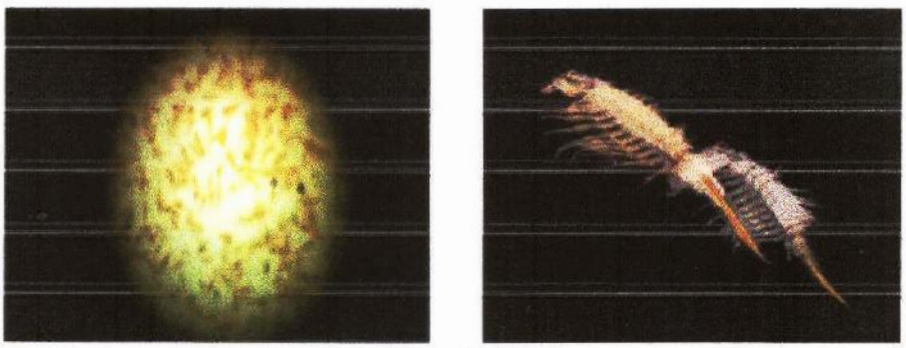

Figure 3.12: Brine shrimp eggs

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3.12.3.1 Materials

- *Artemiasalina* leach (brine shrimp eggs)
- Sea salt (NaCl)
- Small tank with perforated dividing dam to hatch the shrimp
- Lamp to attract shrimps
- Pipettes
- Micropipette
- Glass vials
- Magnifying glass etc.

3.12.3.2 Procedure of test

3.12.3.3 Preparation of seawater

38g sea salt (pure NaCl) was weighed, dissolved in one litter of distilled water and filtered off to get clear solution.

3.12.3.4 Hatching of brine shrimps

Artemia salina leach (brine shrimp eggs) collected from pet shops was used as the test organism. Seawater was taken in the small tank and shrimp eggs were added to one side of the tank and then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was carried out through the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and they were taken for experiment.

Figure: 3.13: Hatching of brine shrimps

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3.25mg of dried methanol extract of bark and root of *Litsea polyantha* was taken in 10ml volumetric flask and volume of 10ml was adjusted by sea water .The concentration of this solution became 320µg/ ml was considered as stock solution.

3.12.3.5 Application of test solution and brine shrimp nauplii into test tubes

26 clean test tubes were taken, 14 of which were for the samples in different concentrations, 7 for control test corresponding the amount of Dimethyl sulfoxide (DMSO) used in samples and 5 for standard in different concentration. Each test tube was accurately marked to indicate the 5ml volume. Then 2.5ml sample of concentration 10,20,40,80,160,320,640 µg/ ml were added to that 7 test tubes through serial dilution method and adjusted with 5ml with saline water to get final concentration of solution 5,10,20,40,80,160,320µg/ ml. 7 test tubes were added with 10 alive brain shrimps nauplii and adjusted to 5ml with saline water to make final concentration of 3.2,1.6,0.8,0.4,0.2,0.1,0.05 µg/ ml. The concentration of DMSO in this test tubes did not exceed 10 µg/ ml .For the control some volume of DMSO were taken in the rest of the 7 test tubes and 10 alive brain shrimps nauplii were added with the final volume of 5ml. With the help of a Pasteur pipette 10 living shrimps were added to each of the test tube containing 5 ml of seawater.

a. Counting of nauplii

After 24 hours the test tubes were observed and the number of survive nauplii in each test tube was counted and the results were noted.The percentage of lethality of brain shrimps nauplii was calculated at each concentration for each sample.

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CHAPTER FOUR

RESULTS AND DISCUSSION

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4.0 Results and Discussion

4.1 Introduction

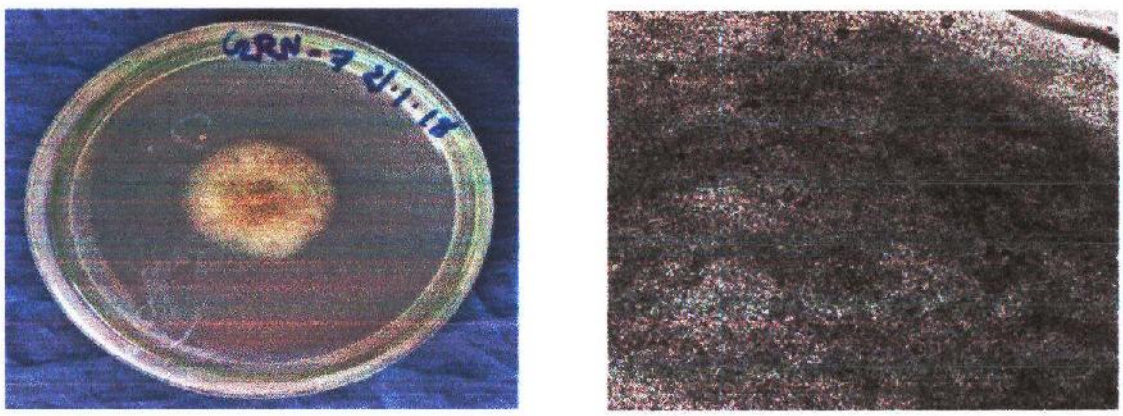
This chapter is designed to study the-

- Morphological and molecular identification of endophytic fungi isolated from the leaves of *C.decandra*.
- Bioactivity test of the fungal extracts;

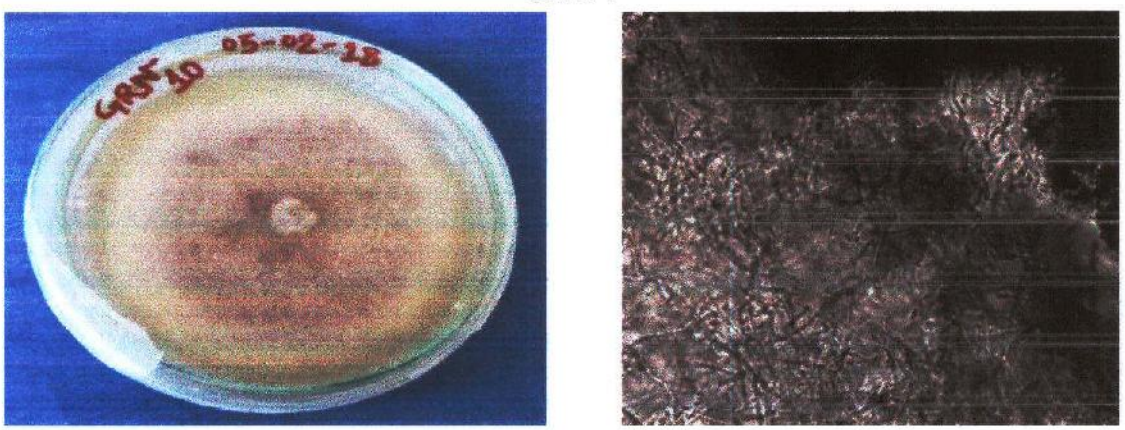
Isolation, cultivation and preparation of crude extracts from both of the mycelia and cultivated medium of fungal endophytes have been done following the published method (Kusari, 2008, Kjer, 2010).

4.2 Identification of the fungal strains

A total of five endophytic fungal strains was isolated and purified from the leaves of *C.decandra*. (Figure 4.1)



GRN 7



GRN10

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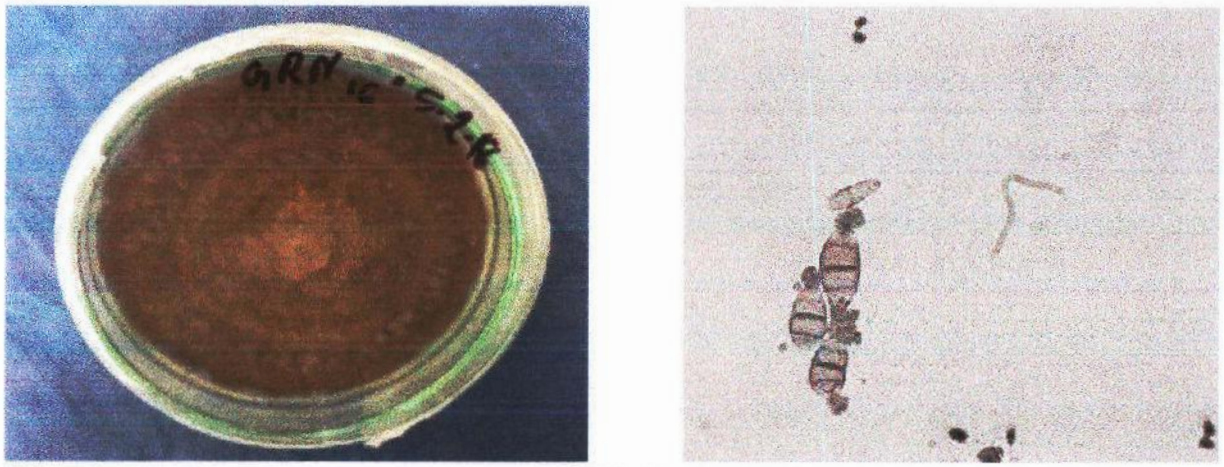
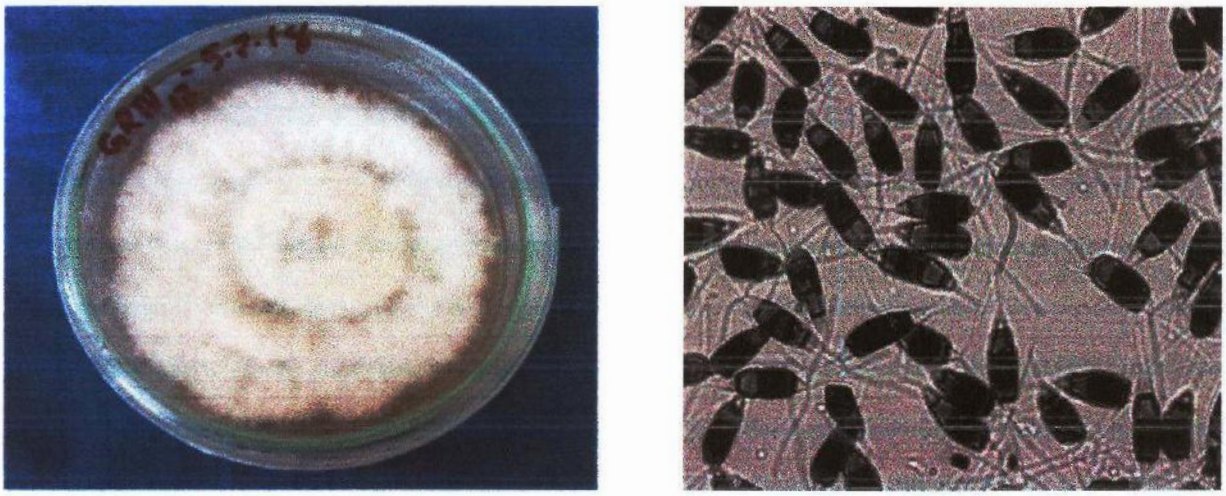
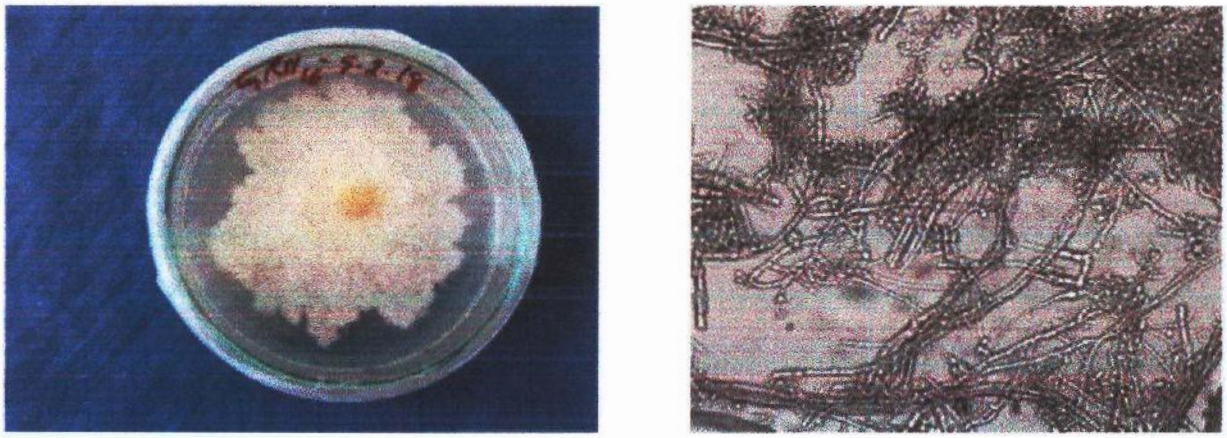


GRN 16



GRN 17



GRN18

Figure 4.1: Macroscopic and microscopic colony morphologies of the endophytic fungi isolated from *C. decandra*

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4.2.1Morphological Characteristics of endophytic fungi

The morphology of the isolated fungi is described in two parts-

- Macroscopic Characteristics;
- Microscopic Characteristics;

4.2.2Macroscopic Characteristics of the isolated fungi

All the endophytic fungi exhibited characteristics colony and macroscopic morphology that could be used to differentiate them. The macroscopic characteristics of the fungi are given in the following table and figure.

Table 4.1: Morphology of GRN7

Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Moderate	rapid	rapid	Slow
Nature of growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	vegetative	Spore	Spore
Diameter	3.8cm	6.4cm	6.9cm	7cm
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Filament	Filament	Filament	Filament
Color of the top view	Gray	Gray	Gray	Gray
Color of the bottom view	Gray	Gray	Gray	Gray
Texture of colony surface	Powdery	Powdery	Powdery	Powdery

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Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of colony	Filiform	Filiform	Filiform	Filiform

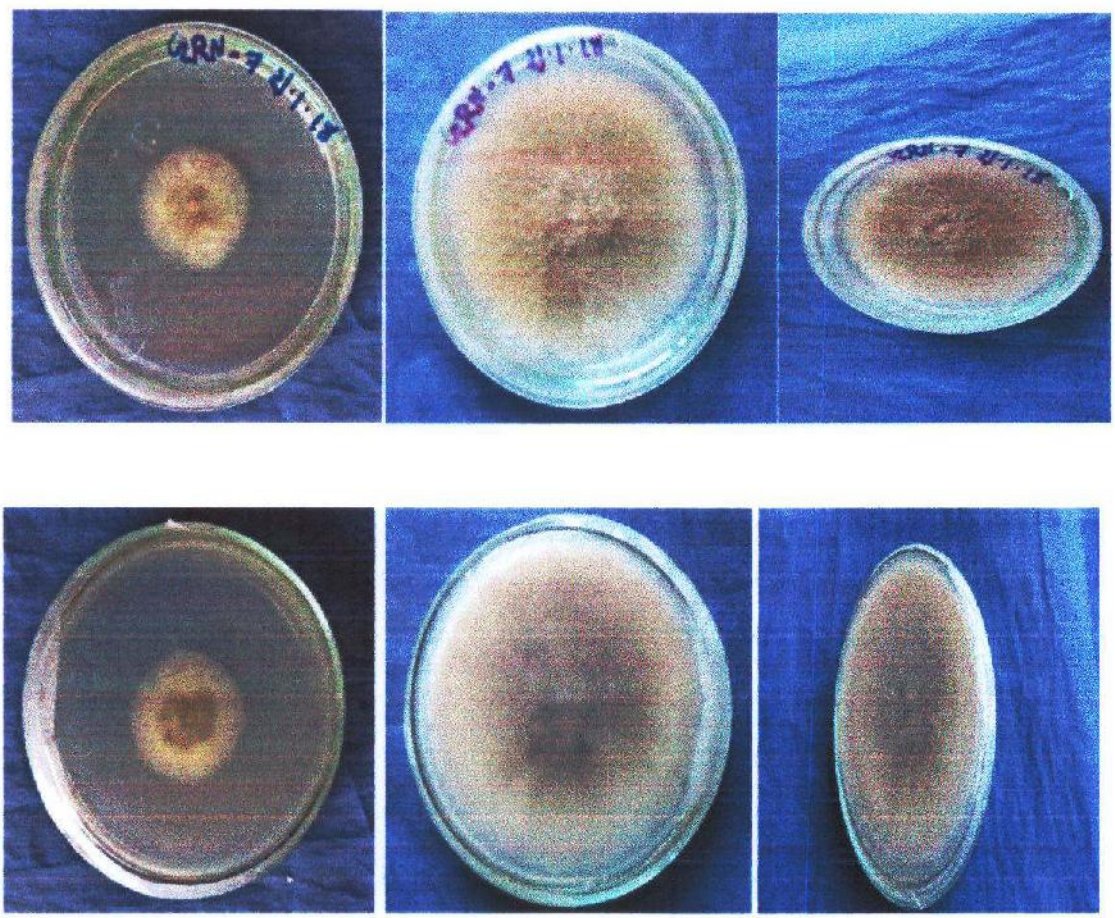

Figure 4.2: Top and bottom view of the fungal strain GRN7 after 3days, 6days And 9 days of small scale cultivation

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Table 4.2: Morphology of GRN10

Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Slow	moderate	Slow	Slow
Natureof growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Spore	Spore	Spore
Diameter	2.6cm	4.5 cm	6.5 cm	7 cm
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Filament	Filament	Filament	Filament
Color of the top view	Gray	Gray and White	Gray	Gray
Color of the bottom view	Gray and cream	Gray and cream	Gray and cream	Cream and black
Texture of colony surface	Dry	Dry	Dry	Dry
Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of colony	Filiform	Filiform	Filiform	Filiform

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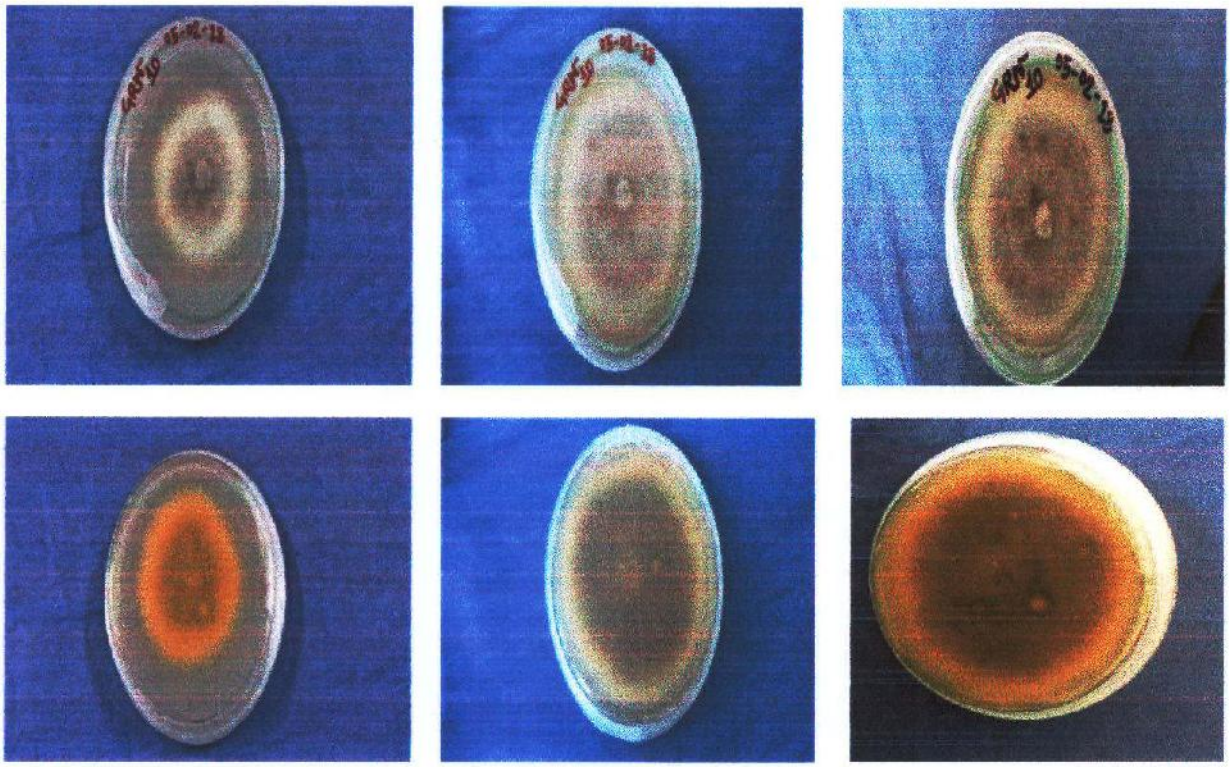

Figure 4.3: Top and bottom view of the fungal strain GRN 10 after 3days, 6days, and 9 days of small scale cultivation

Table 4.3: Morphology of GRN 18

Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Slow	Moderate	Slow	Slow
Natureof growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Spore	Spore	Spore
Diameter	2.5cm	5.4 cm	6.3 cm	6.8 cm
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium	Shallow	Shallow	Shallow	Shallow

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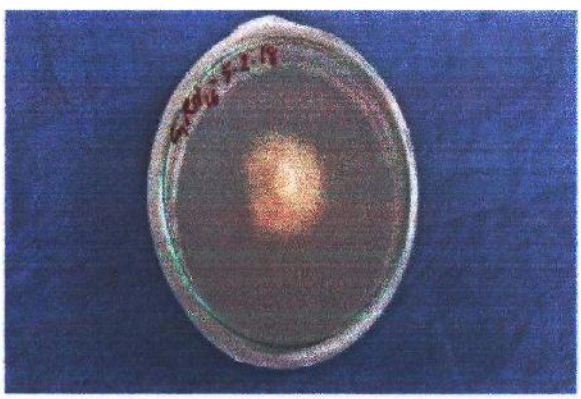
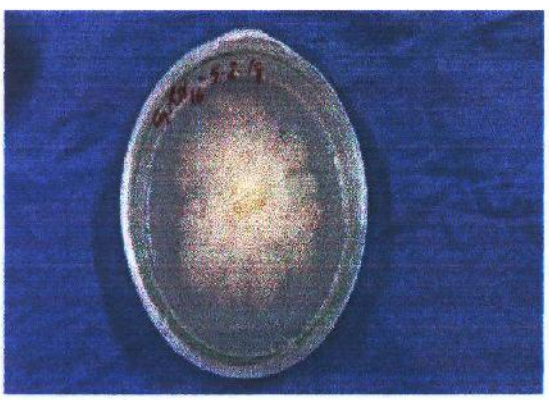
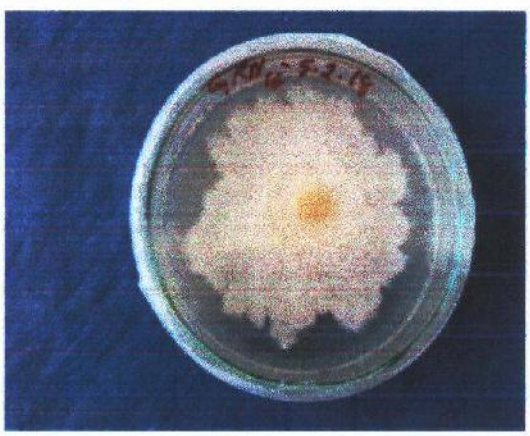
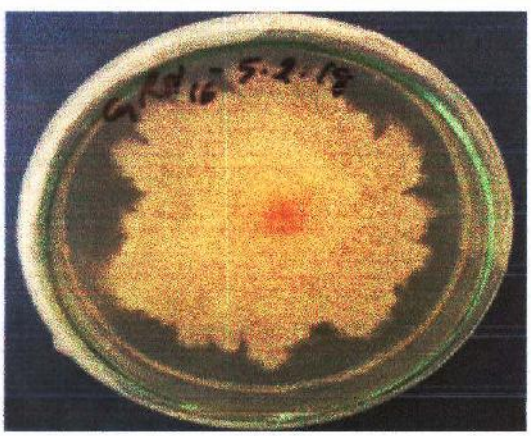
Morphology of colony	Rhizoid	Rhizoid	Rhizoid	Rhizoid
Color of the top view	White	White	White	White
Color of the bottom view	Pinkish white	Pinkish white	Pinkish white	Pinkish white and Cream
Texture of colony surface	Powdery	Powdery	Powdery	Powdery
Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of colony	Entire	Entire	Entire	Entire









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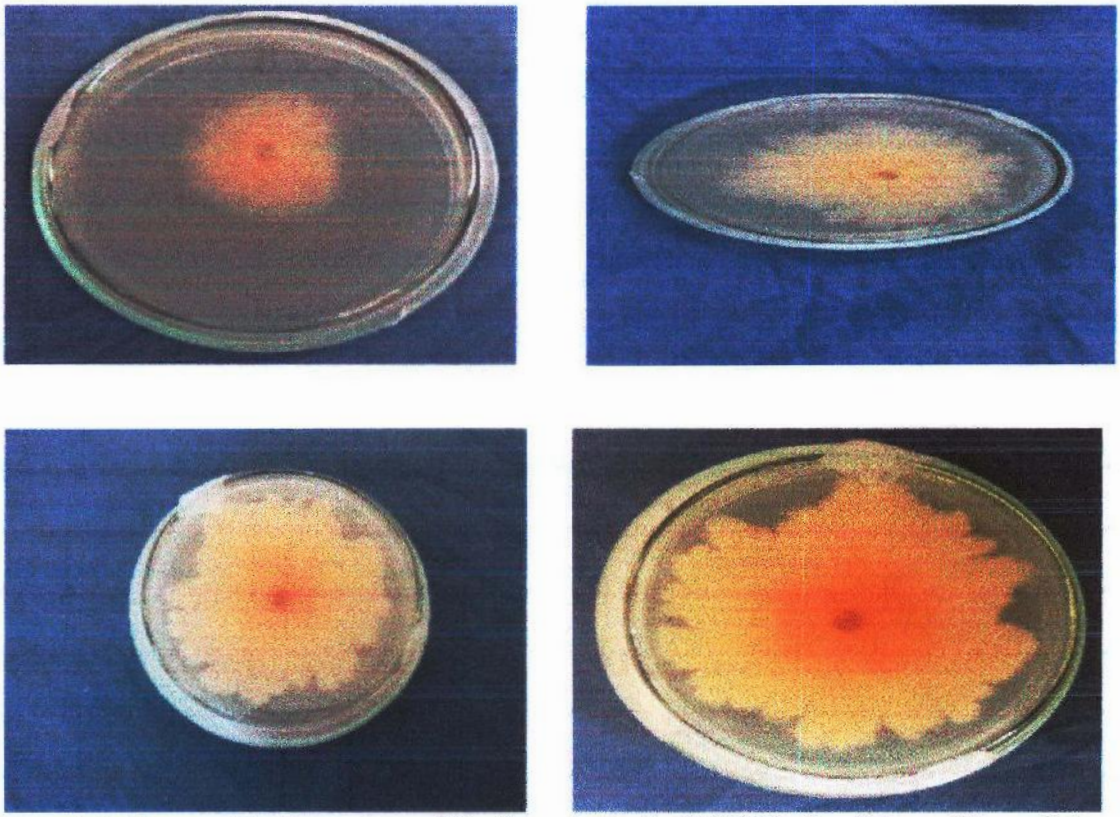

Figure 4.4: Top and bottom view of the fungal strain GRN18 after 3days, 6days, 9 days and 12 days of small scale cultivation

Table 4.4: Morphology of GRN17

Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Slow	Moderate	Slow	Slow
Natureof growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Spore	Spore	Spore
Diameter	2.4cm	5.8 cm	6.4 cm	7 cm
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Irregular	Irregular	Irregular	Irregular

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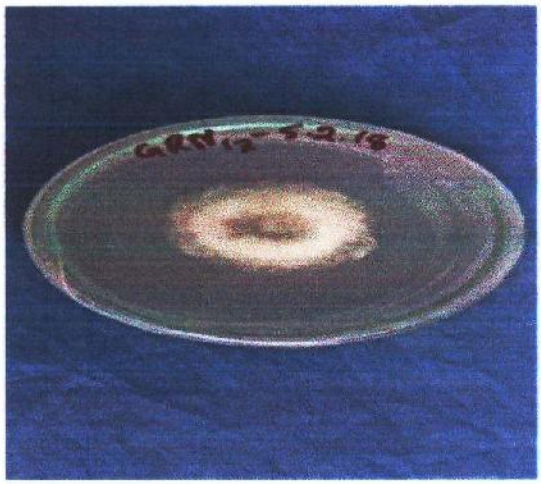
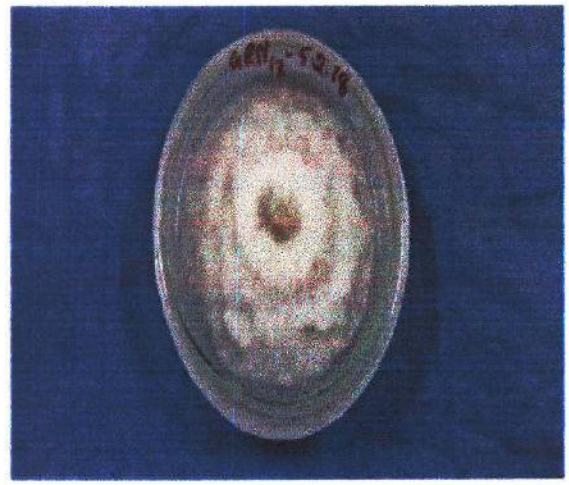
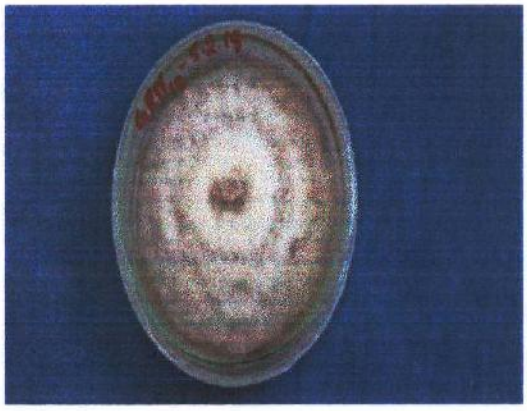
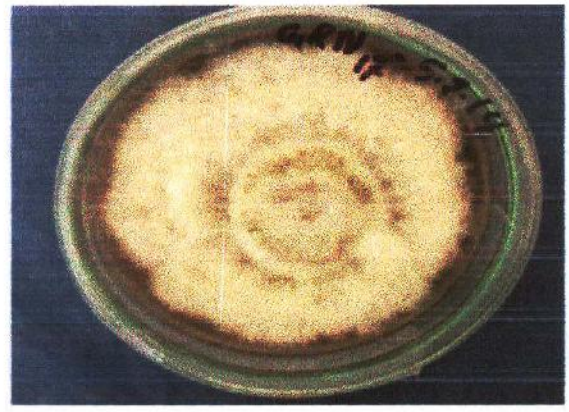
Color of the top view	White	White	White	White
Color of the bottom view	Cream	Cream	Cream	Yellowish
Texture of colony surface	Powdery	Powdery	Powdery	Powdery
Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of colony	Undulate	Undulate	Undulate	Undulate









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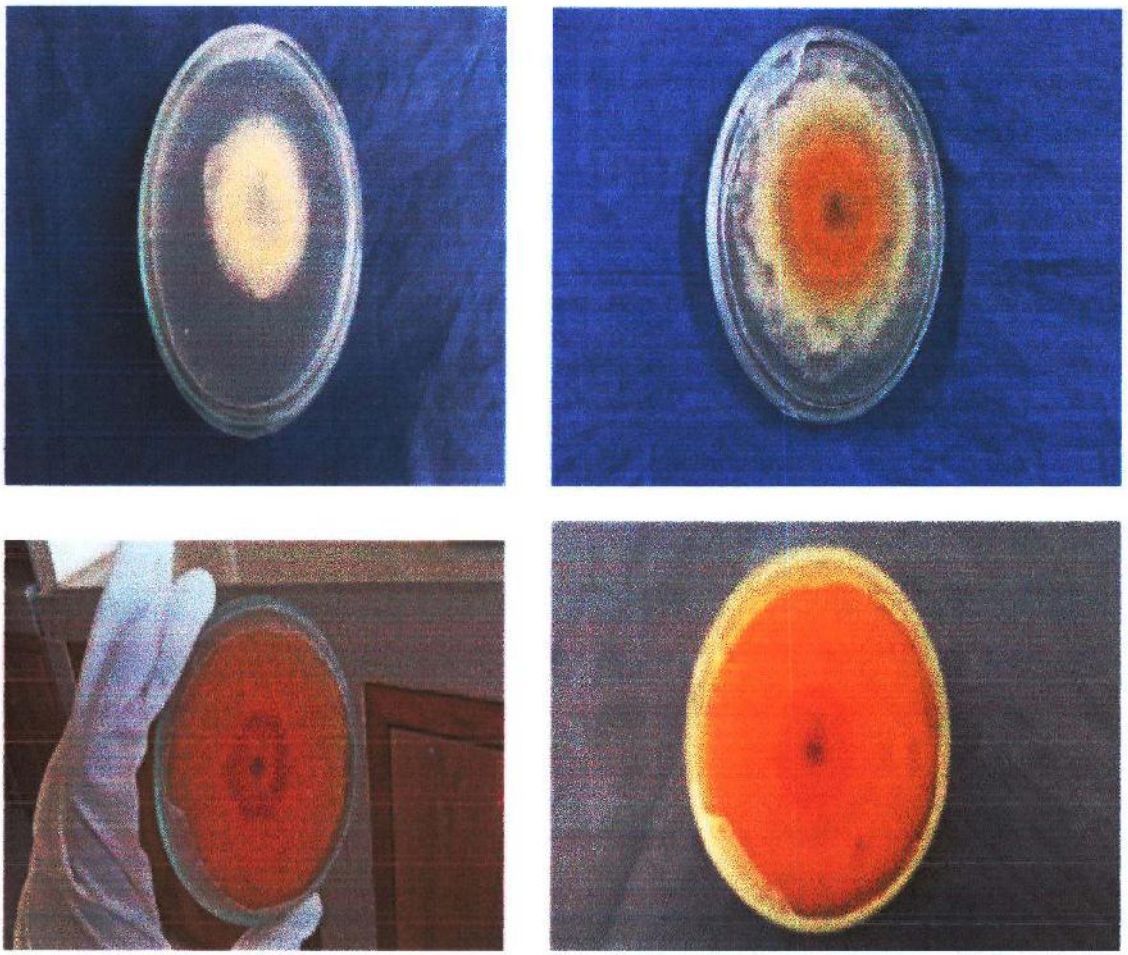

Figure 4.5: Top and bottom view of the fungal strain GRN17 after 3days, 6days, 9 days and 12 days of small scale cultivation

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Table 4.5: Morphology of GRN16

Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Rapid	Rapid	Moderate	Moderate
Natureof growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Spore	Spore	Spore
Diameter	3.5cm	5.65 cm	6.5 cm	6.9 cm
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	Gray	Gray	Blackish gray	Blackish gray
Color of the bottom view	Grey	Grey	Black	Black
Texture of colony surface	Dray	Dray	Wrinkled	Wrinkled
Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of colony	Curled	Curled	Curled	Curled

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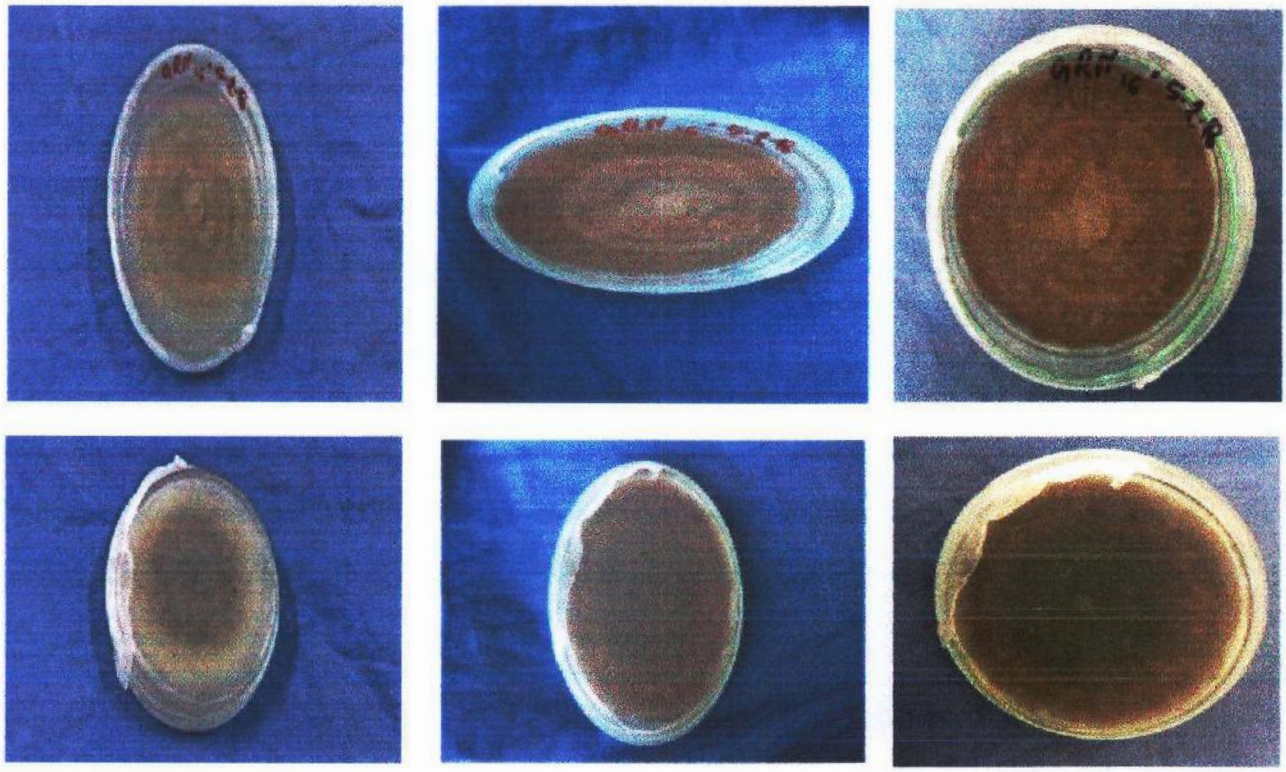

Figure 4.6: Top and bottom view of the fungal strain GRN 16 after 6days, 9 days and 12 days of small scale cultivation

4.2.3 Microscopic characteristics of the Identified Fungi

Slides were prepared from cultures and stained with glycerin and examined with a bright field and phase contrast microscope for morphological identification of endophytic fungi. The fungi were identified using relevant keys and taxonomic notes from various standard manuals (Barnettet al.1998). Fungi identified through conidia and spore characteristics.

Microscopic observation was done at 10X and 40X view of microscope to view spore and conidia to identify the fungi.

4.3 PCR amplification of intergenic spacer region

The quantified DNA sample subsequently diluted to 2ng/µl as per requirements for ideal PCR. The PCR did run using two primer and banding pattern was confirmed on 1.5 % agarose gel electrophoresis with 100bp DNA ladder. Expected size of ITS region in fungi is

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between 500-600 base pairs (bp) and observed PCR product size is within 550-705 base pairs.

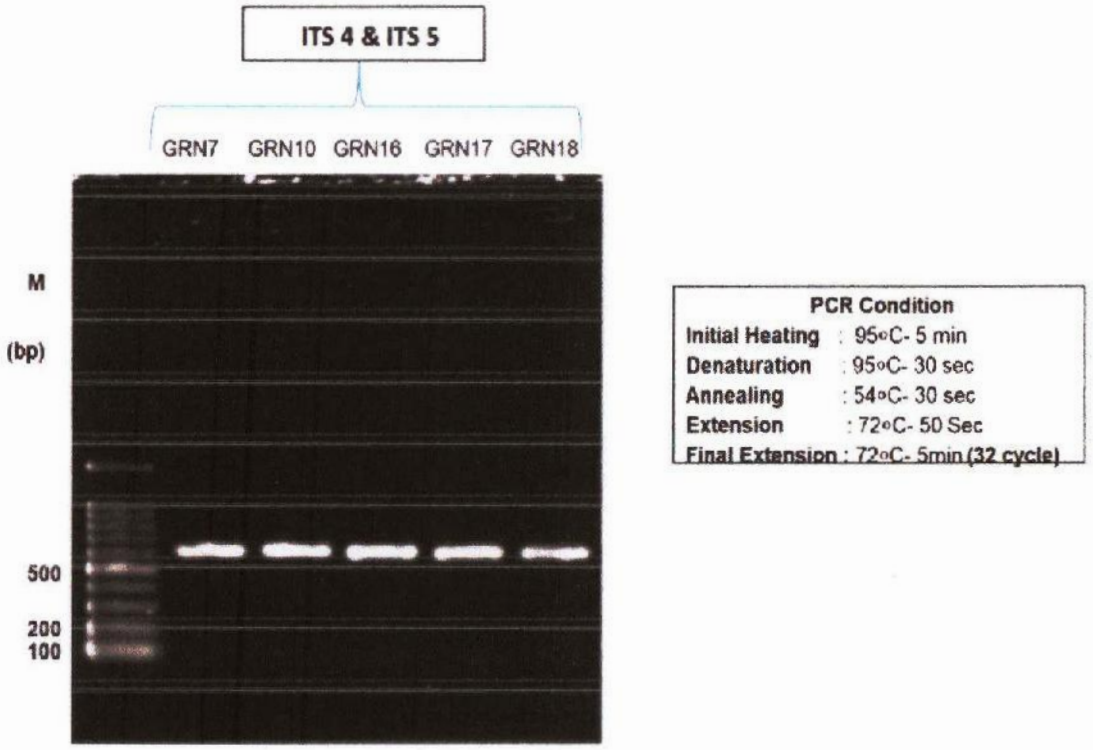

Figure 4.7: ITS profiles of ITS 5 primers generated from 5 different fungi GRN7, GRN10, GRN16, GRN17, GRN18 M: denotes 100bp ladder (Marker).

4.4 Sequencing result

Sequencing data was saved in FASTA format. It is given below in text format for five samples, GRN-7, GRN-10, GRN-16, GRN-17, and GRN-18.

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Table 4.6: Endophytic fungi isolated from *C. decandra* with Genbank accession number

Internal Code	Name of the Fungi	Genbank Accession Number	BLAST Match Sequence		
			Reference Accession Number	Query Length	Max Identity
GRN-16	*Curvularia jeniculate*	MH517581	KX022497.1	591	99%
GRN-7	*Colletroticum gloeospozioides*	MH517583	KX022505.1	575	99%
GRN-10	*Colletroticum horii*	MH517596	LC186039.1	575	99%
GRN-17	*Chaetomium globosum*	MH517676	KX926579.1	577	99%
GRN-18	*Fusarium solani*	MH517680	KF918580.1	548	99%

4.5 Phylogenetic analysis

Phylogenetics analysis is to infer evolutionary relationships for a set of taxa. Each taxon is typically represented using a sequence, and the phylogenetic tree describes the evolutionary relationship between different sequences. The phylogenetic or evolutionary tree of different sample showing the branching diagram or tree showing the inferred evolutionary relationships among the species. The results of phylogenetic tree for five samples are given below:

Table 4.7: Pair wise genetic distance of endophytic fungi isolated from *Ceriops decandra*

	MH517583	MH517596	MH517676	MH517680	MH515881
MH517583	-				
MH517596	0.69	-			
MH517596	0.70	0.015	-		
MH517680	0.71	0.69	0.69	-	
MH515881	0.73	0.76	0.76	0.69	-

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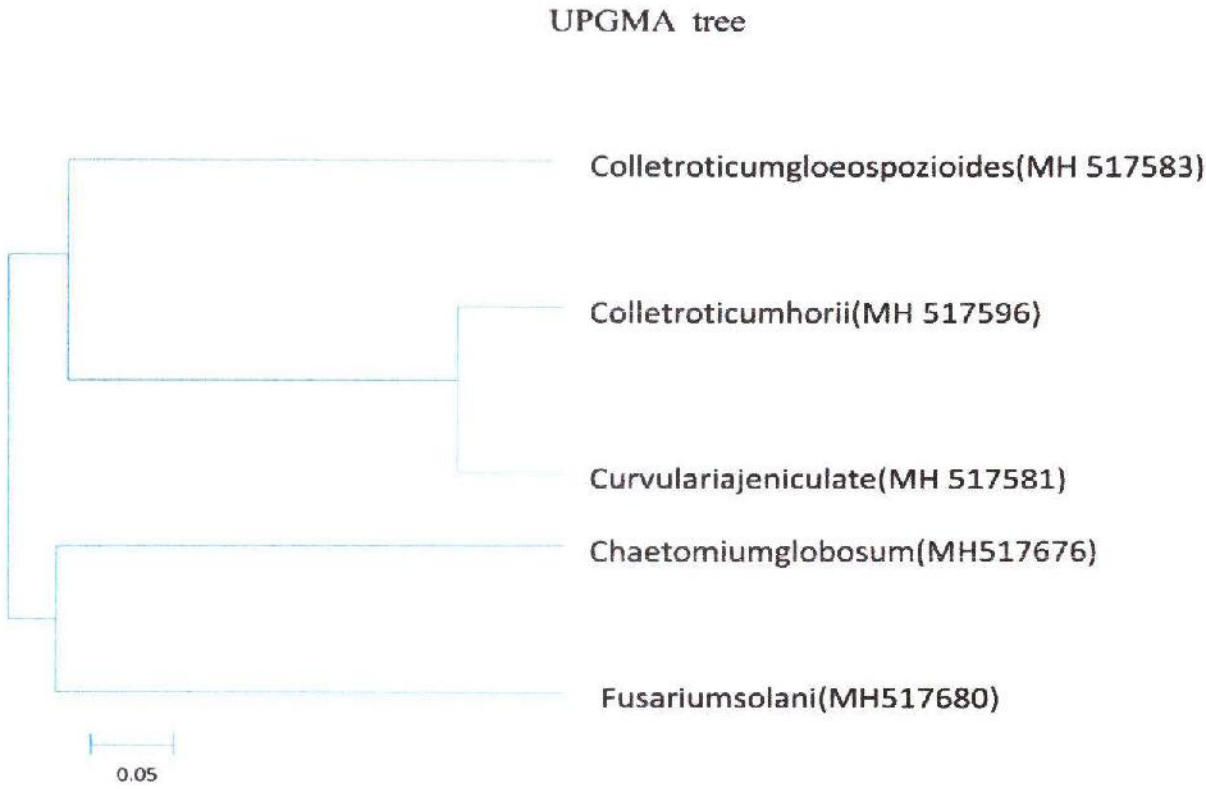

Figure 4.8: UPGMA dendrogram of five isolated fungi from *Ceriops decandra* .The dendrogram constructed using distance matrix of fungi.

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4.6Antioxidant activity by DPPH free radical scavenging Method

The endophytic fungal extracts of mangrove plant from culture media were subjected to free radical scavenging activity by the method of Brand-Williams et al. 1995. Here, Ascorbic acid was used as reference standard. Determination of the IC_{50} values signifies the capability to the nature of antioxidant activity.

Table 4.8: DPPH Scavenging Assay of Ethyl aceted extract of GRN 7

Concentration(µg/ml)	log con	Abs1	Abs2	Average	%inhibition	IC50
3.12	0.494155	0.302	0.368	0.335±.046	14.1025641	
6.24	0.795185	0.276	0.286	0.281±.007	27.9487179	
12.5	1.09691	0.233	0.255	0.244±.015	37.4358974	
25	1.39794	0.202	0.265	0.2335±.04	40.1282051	51.52
50	1.69897	0.179	0.188	0.1835±.006	52.9487179	
100	2	0.187	0.176	0.1815±.007	53.4615385	
200	2.30103	0.143	0.122	0.1325±.014	66.025641	
Blank		0.394	0.386	0.39±.005		

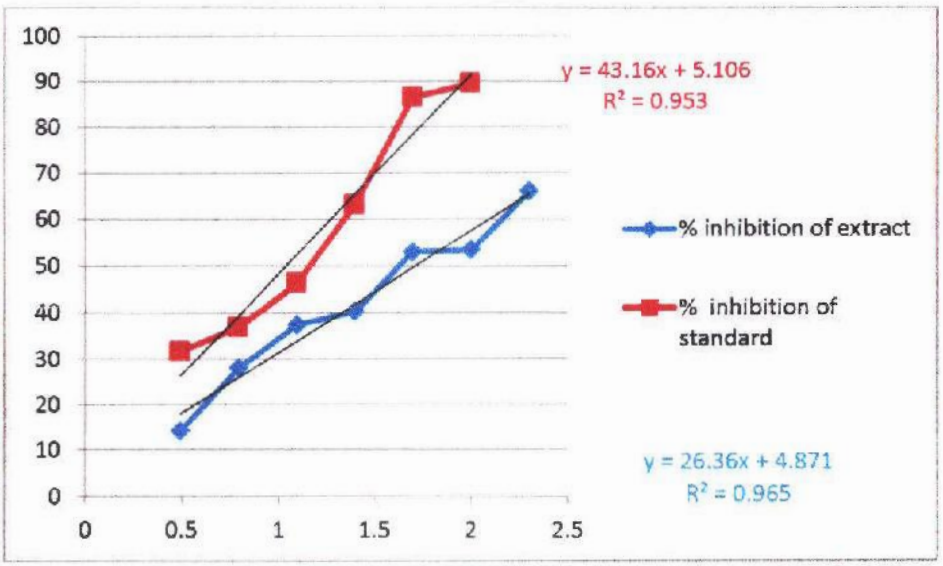

Figure 4.8 IC_{50} Value of Ethyl acited extract of GRN 7

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Table 4.6: DPPH Scavenging Assay of methanol extract of GRN 7

Concentration(µg/ml)	log con	ABS1	ABS2	Average	%inhibition	IC50
3.12	0.494155	0.322	0.356	0.339±.024	31.3069909	
6.25	0.79588	0.301	0.289	0.295±.008	40.2228977	
12.5	1.09691	0.234	0.254	0.244±.014	50.5572442	
25	1.39794	0.152	0.157	0.1545±.003	68.6930091	11.29
50	1.69897	0.142	0.138	0.14±.002	71.6312057	
100	2	0.114	0.12	0.117±.004	76.2917933	
200	2.30103	0.102	0.107	0.1045±.003	78.8247214	
Blank		0.489	0.498	0.4935±.006		

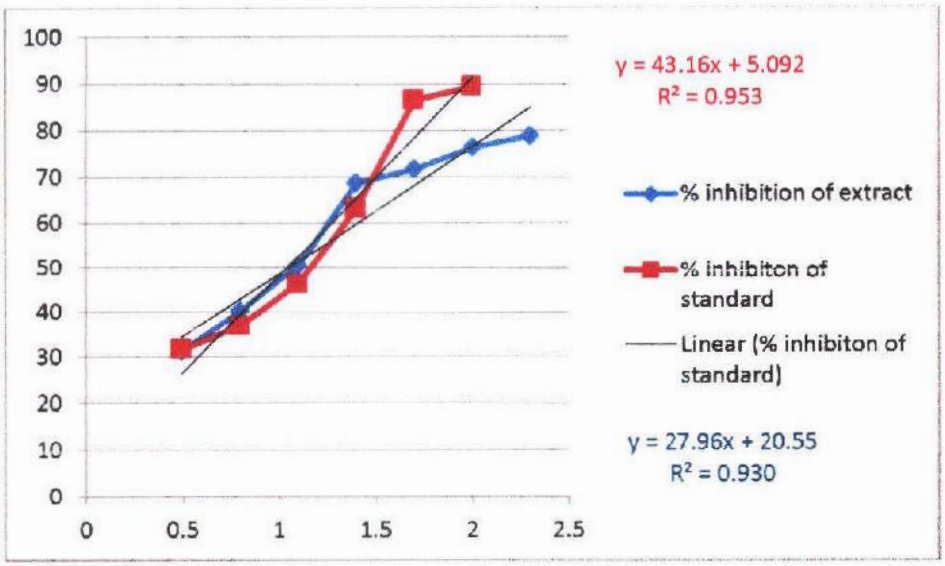

Figure 4.9 IC_{50} Value of methanol extract of GRN 7

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Table 4.7: DPPH Scavenging Assay of Ethyl acited extract of GRN 10

Concentration(µg/ml)	log con	Abs1	Abs2	Average	% of inhibition	IC50
3.12	0.494155	0.282	0.278	0.28±.002	42.74029	
6.24	0.795185	0.252	0.258	0.255±.004	47.85276	
12.5	1.09691	0.234	0.242	0.238±.005	51.32924	
25	1.39794	0.243	0.228	0.2355±.010	51.84049	12.73
50	1.69897	0.228	0.22	0.224±.005	54.19223	
100	2	0.196	0.198	0.197±.001	59.7137	
200	2.30103	0.182	0.156	0.169±.018	65.43967	
Blank		0.49	0.488	0.489±.001		

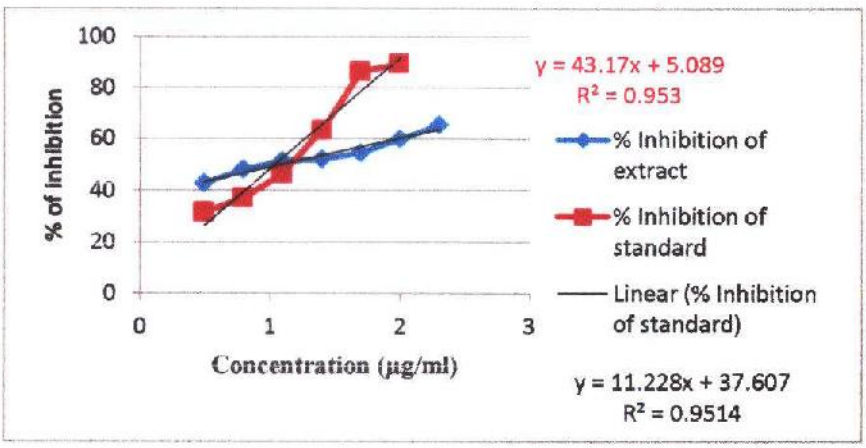

Figure 4.9 IC_{50} Value of Ethyl acited extract of GRN 10

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Table 4.8: DPPH Scavenging Assay of Methanol extract of GRN 10

Concentration(µg/ml)	log con	Abs1	Abs2	Average	% of inhibition	IC50
3.12	0.494155	0.385	0.386	0.3855±.0007	44.00871	
6.24	0.795185	0.388	0.377	0.3825±.007	44.44444	
12.5	1.09691	0.382	0.371	0.3765±.007	45.3159	
25	1.39794	0.363	0.366	0.3645±.002	47.05882	16.82
50	1.69897	0.341	0.339	0.34±.001	50.61728	
100	2	0.295	0.294	0.2945±.0007	57.22585	
200	2.30103	0.126	0.121	0.1235±.003	82.06245	
Blank		0.685	0.692	0.6885±.004		

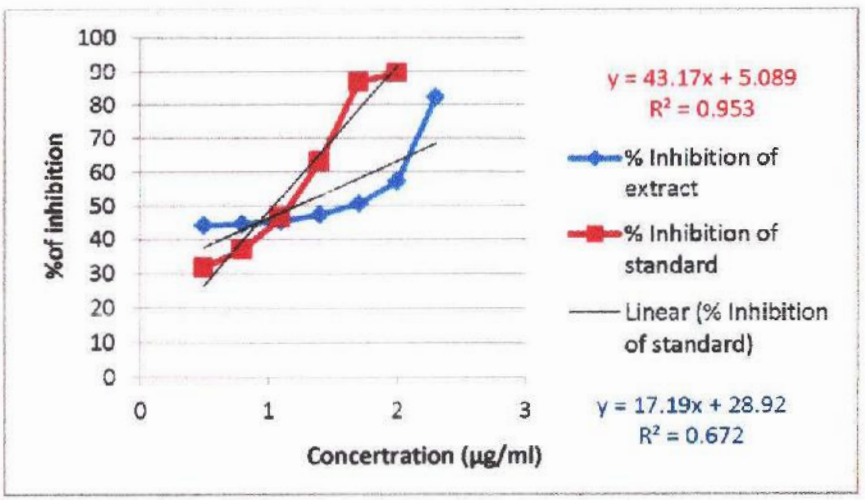

Figure4.10 IC_{50} Value of methanol extract of GRN10

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Table 4.9: DPPH Scavenging Assay of Ethyl acited extract of GRN 16

Concentration(µg/ml)	log con	Abs1	Abs2	Average	% of inhibition	IC50
3.12	0.494155	0.367	0.358	0.3625±.006	27.20884	
6.24	0.795185	0.338	0.349	0.3435±.007	31.0241	
12.5	1.09691	0.312	0.318	0.315±.004	36.74699	
25	1.39794	0.298	0.302	0.3±.002	39.75904	61.51
50	1.69897	0.285	0.272	0.2785±.009	44.07631	
100	2	0.277	0.272	0.2745±.003	44.87952	
200	2.30103	0.139	0.143	0.141±.002	71.68675	
Blank		0.502	0.494	0.498±.005		

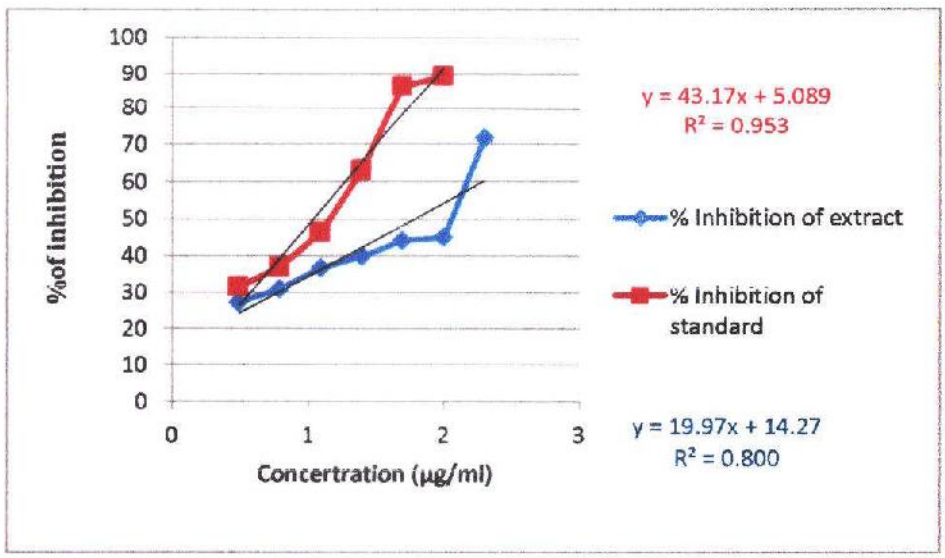

Figure 4.11 IC_{50} Value of Ethyl acited extract of GRN16

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Table 4.10: DPPH Scavenging Assay of Methanol extract of GRN 16

Concentration(µg/ml)	log con	Abs1	Abs2	Average	% of inhibition	IC50
3.12	0.494155	0.418	0.407	0.4125±.007	41.57224	
6.24	0.795185	0.301	0.388	0.3445±.004	51.20397	
12.5	1.09691	0.369	0.369	0.369±.061	47.73371	
25	1.39794	0.367	0.388	0.3775±.014	46.52975	10.81
50	1.69897	0.242	0.255	0.2485±.009	64.8017	
100	2	0.206	0.244	0.225±.026	68.13031	
200	2.30103	0.126	0.126	0.126±0	82.15297	
Blank		0.705	0.707	0.706±.001		

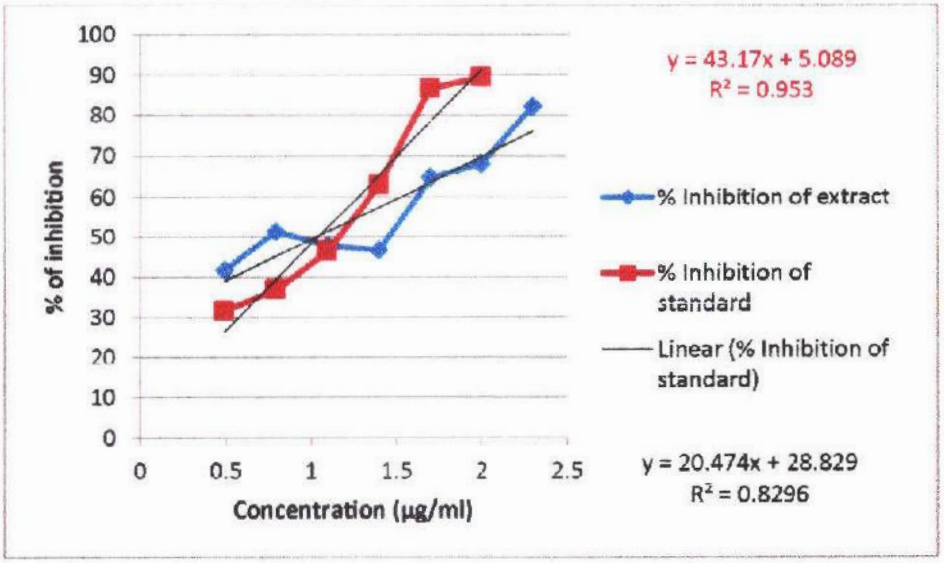

Figure 4.12 IC_{50} Value of methanol extract of GRN 16

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Table 4.11: DPPH Scavenging Assay of Ethyl acited extract of GRN 17

Concentration(µg/ml)	log con	Abs1	Abs2	Average	% of inhibition	IC50
3.12	0.494155	0.298	0.352	0.325±.038	12.0433	
6.24	0.795185	0.288	0.322	0.305±.024	17.45602	
12.5	1.09691	0.244	0.273	0.2585±.020	30.0406	
25	1.39794	0.167	0.154	0.1605±.009	56.56292	25.11
50	1.69897	0.11	0.12	0.115±.007	68.87686	
100	2	0.099	0.089	0.094±.007	74.56022	
200	2.30103	0.078	0.077	0.0775±.0007	79.02571	
Blank		0.369	0.37	0.3695±.0007		

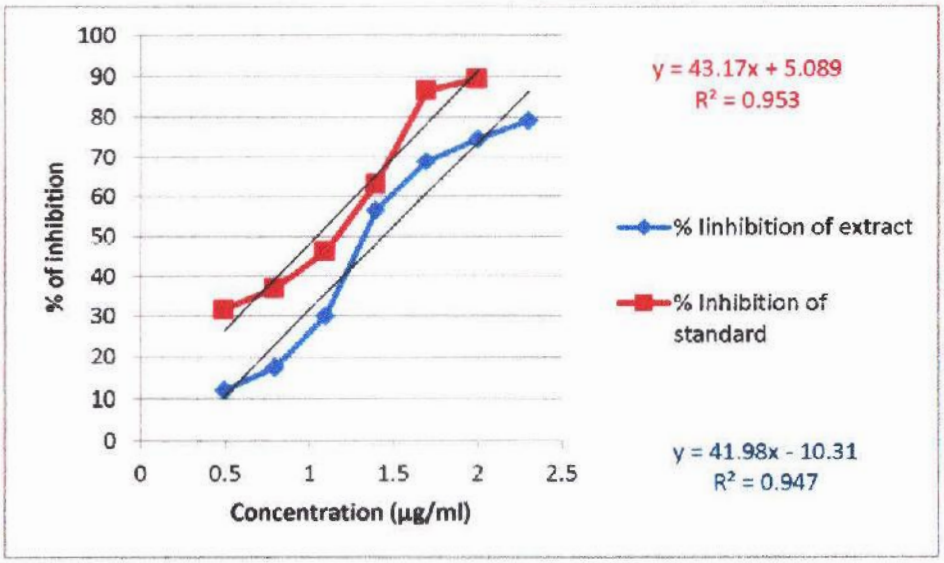

Figure 4.13 IC_{50} Value of Ethyl acited extract of GRN17

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Table 4.12: DPPH Scavenging Assay of Methanol extract of GRN 17

Concentration(µg/ml)	log con	Abs1	Abs2	Average	% of inhibition	IC50
3.12	0.494155	0.312	0.315	0.3135±.002	0.317965	
6.24	0.795185	0.309	0.306	0.3075±.002	2.225755	
12.5	1.09691	0.267	0.264	0.2655±.002	15.58029	
25	1.39794	0.237	0.243	0.24±.004	23.68839	131.21
50	1.69897	0.228	0.245	0.2365±.012	24.80127	
100	2	0.227	0.221	0.224±.004	28.77583	
200	2.30103	0.207	0.206	0.2065±.0007	34.34022	
Blank		0.31	0.319	0.3145±.006		

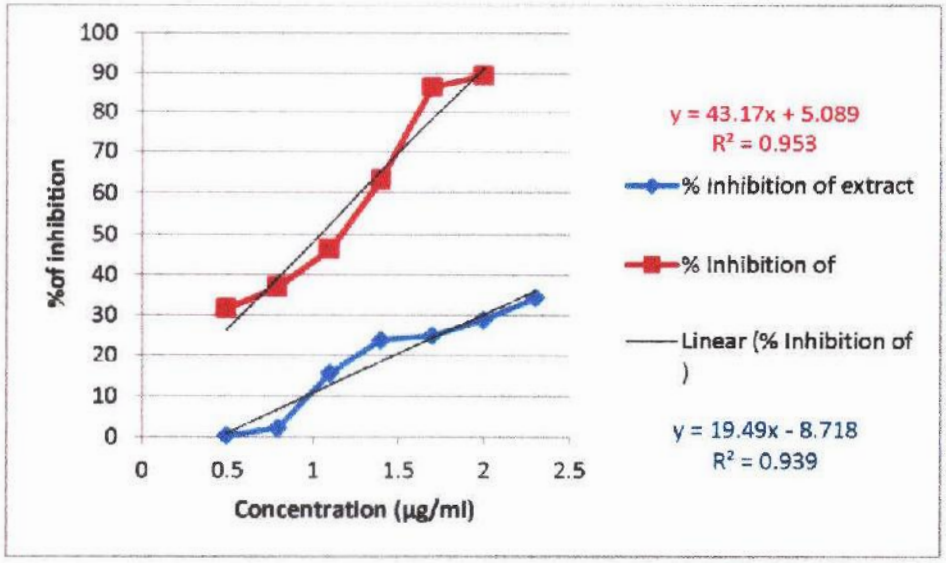

Figure 4.14 IC_{50} Value of methanol extract of GRN 17

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Table 4.13: DPPH Scavenging Assay of Ethyl acited extract of GRN 18

Concentration(µg/ml)	log con	Abs1	Abs2	Average	% of inhibition	IC50
3.12	0.494155	0.262	0.342	0.302±.056	27.22892	
6.24	0.795185	0.259	0.302	0.2805±.030	32.40964	
12.5	1.09691	0.266	0.298	0.282±.022	32.04819	
25	1.39794	0.253	0.276	0.2645±.016	36.26506	43.25
50	1.69897	0.179	0.201	0.19±.015	54.21687	
100	2	0.187	0.176	0.1815±.007	56.26506	
200	2.30103	0.102	0.134	0.118±.022	71.56627	
Blank		0.377	0.453	0.415±.053		

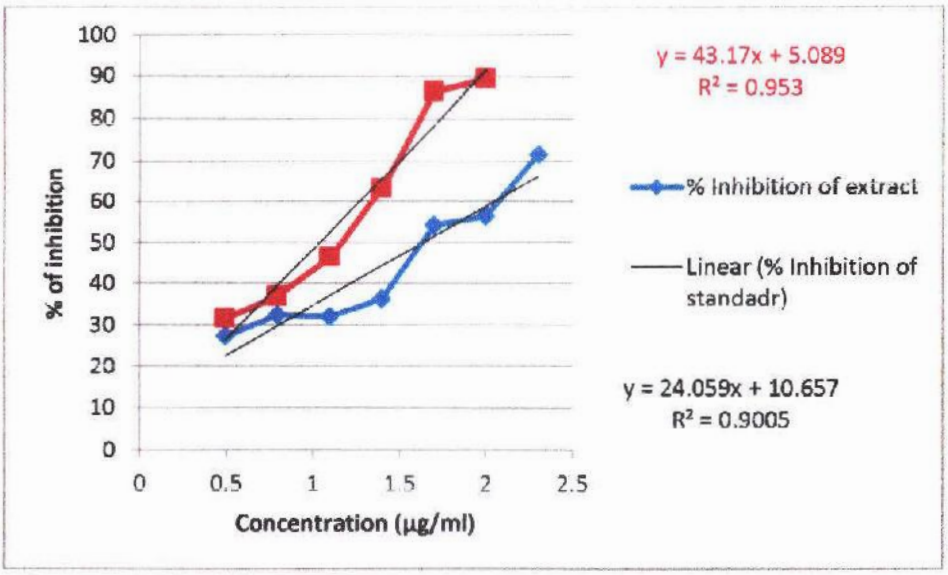

Figure 4.15 IC_{50} Value of Ethyl acited extract of GRN18

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Table 4.14: DPPH Scavenging Assay of Methanol extract of GRN 18

Concentration(µg/ml)	log con	Abs1	Abs2	Average	% of inhibition	IC50
3.12	0.494155	0.172	0.169	0.1705±.002	35.1711	
6.24	0.795185	0.153	0.156	0.1545±.002	41.25475	
12.5	1.09691	0.142	0.145	0.1435±.002	45.43726	
25	1.39794	0.134	0.138	0.136±.002	48.28897	44.66
50	1.69897	0.132	0.12	0.126±.008	52.09125	
100	2	0.122	0.126	0.124±.002	52.85171	
200	2.30103	0.116	0.118	0.117±.001	55.51331	
Blank		0.259	0.267	0.263±.005		

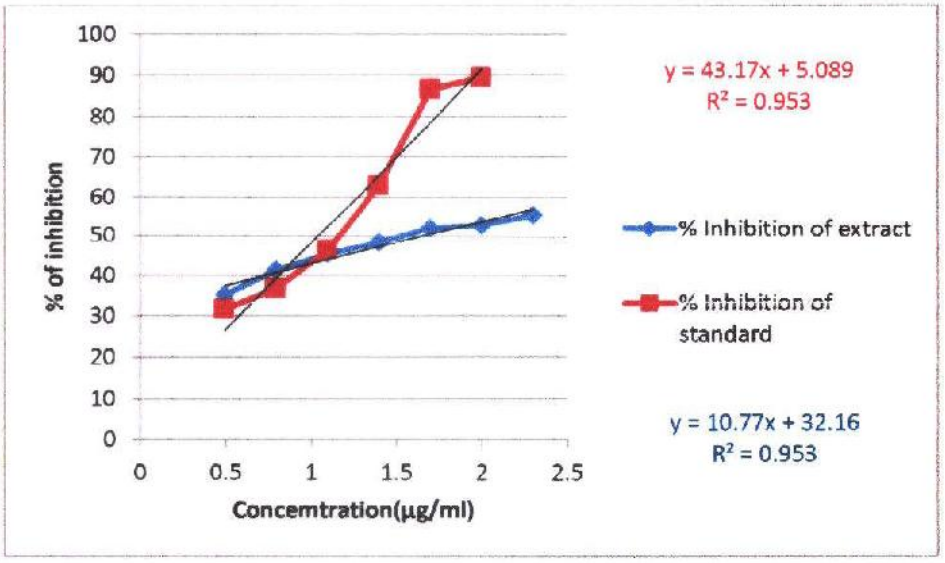

Figure 4.16 IC_{50} Value of methanol extract of GRN 18

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Table4.15: IC50 values of Antioxidant test

Name of the sample	IC50 values
Constant	10.96
GRN7 EatOH	51.52
GRN7 Methanol	11.2979
GRN10 EAtOH	12.735
GRN10 Methanol	16.826
GRN16 EAtOH	61.51
GRN16 Methanol	10.814
GRN17 EAtOH	25.1188
GRN17 Methanol	131.21
GRN18 EAtOH	43.25
GRN18 Methanol	44.66

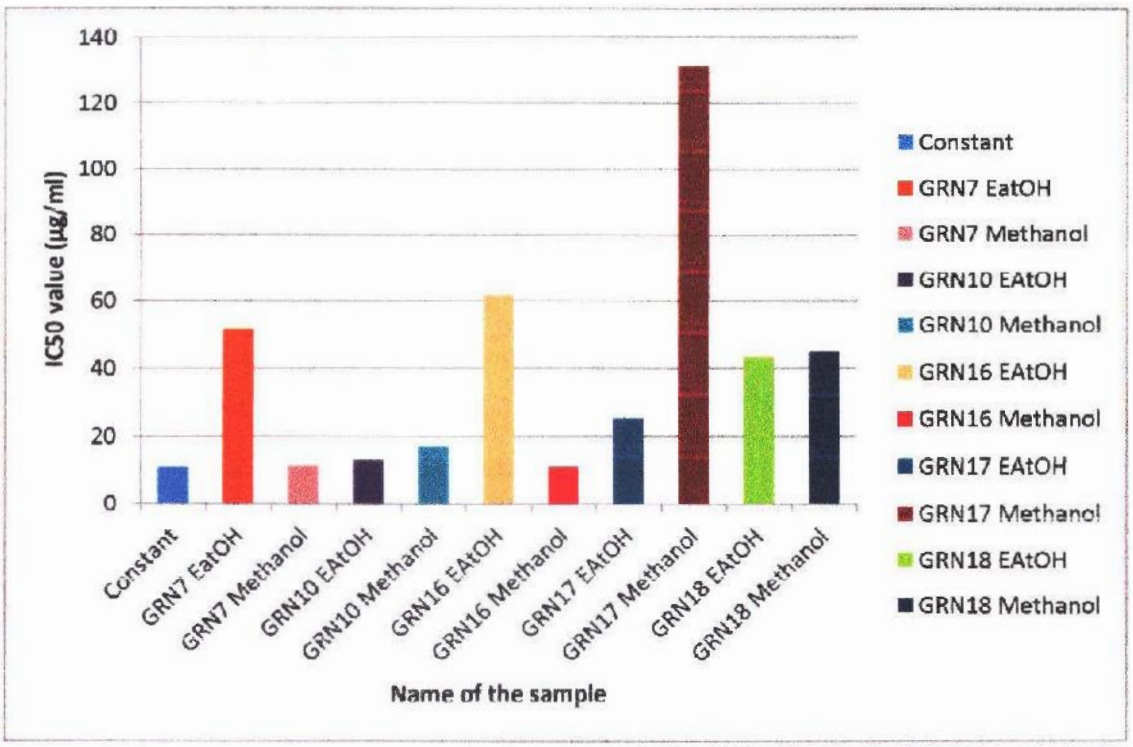

Figure 4.17 IC_{50} value of the crude extracts from fungal endophytes

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4.6.1 Antibactrerial activity

Table 4.16: List of microbes used for screening of antimicrobial activity

Gram negative	Gram Positive
1. *Escherichia coli*	1. *Staphylococcus aureus*
2. *Pseudomonas aeruginosa*	2. *Micrococcus*

The zone of inhibition in diameter are observed against all tested microorganisms are shown in table

Table 4.17: Zone of inhibition against bacteria

Name of extract	Zone of inhibition (mm)			
	Staphylococcus aureas	*Pseudomonas aeruginosa*	*Escherechia coli*	Micrococcus
GRN 7 EAtOH extract(100µg/disc)	-	-	-	-
GRN7 EAtOH extract(500µg/disc)	1.2	-	1.9	-
GRN7 EAtOH extract(1000µg/disc)	3.5	-	2.5	-
GRN7 methanol extract(100µg/disc)	-	-	-	-
GRN7 methanol extract(500µg/disc)	-	-	-	-
GRN7 methanol extract(1000µg/disc)	-	-	-	-
Blank	-	-	-	-
Erythromycin (10µg/disc)	18.5	20.5	14	15

"--" indicates No activity.

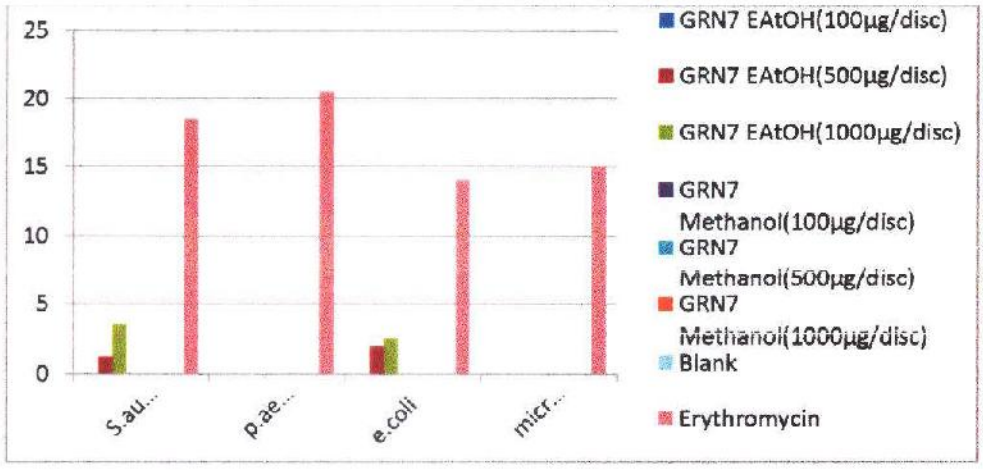

Figure 4.18: Zone of inhibition against bacteria of standard and samples

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Table 4.18: Zone of inhibition against bacteria

Name of extract	Zone of inhibition			
	Staphylococcus aureas	*Pseudomonas aeruginosa*	*Escherechia coli*	Micrococcus
GRN 10 EAtOH extract(100µg/disc)	-	3.7	-	-
GRN10 EAtOH extract(500µg/disc)	-	4.2	-	-
GRN10 EAtOH extract(1000µg/disc)	2.7	5.1	3.2	-
GRN10 methanol extract(100µg/disc)	-	-	-	-
GRN10 methanol extract(500µg/disc)	3.5	7.75	6.75	4
GRN10 methanol extract(1000µg/disc)	-	-	-	4
Blank	-	-	-	-
Erythromycin (10µg/disc)	18.5	20.5	14	15

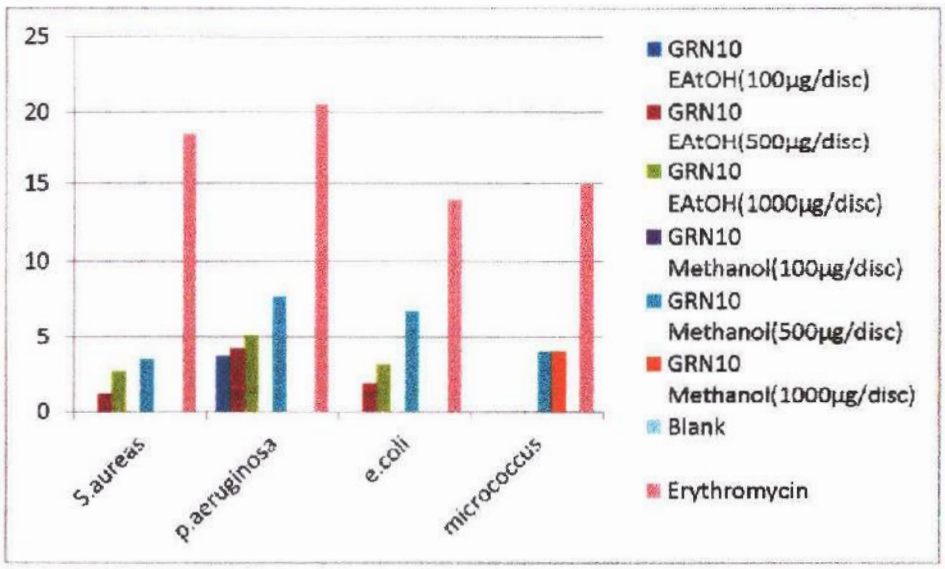

Figure 4.19: Zone of inhibition against bacteria of standard and samples

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Table 4.19: Zone of inhibition against bacteria

Name of extract	Zone of inhibition			
	Staphylococcus aureas	*Pseudomonas aeruginosa*	*Escherechia coli*	Micrococcus
GRN 16 EAtOH extract(100µg/disc)	5.21	-	6.51	-
GRN16 EAtOH extract(500µg/disc)	7.78	-	8.27	-
GRN16 EAtOH extract(1000µg/disc)	12.5	-	12.5	10.5
GRN16methanol extract(100µg/disc)	5.21	4.43	3.78	3.76
GRN16 methanol extract(500µg/disc)	8.75	10	7.75	8.75
GRN16 methanol extract(1000µg/disc)	10.5	11.5	11	10.75
Blank	-	-	-	-
Erythromycin (10µg/disc)	18.5	20.5	14	15

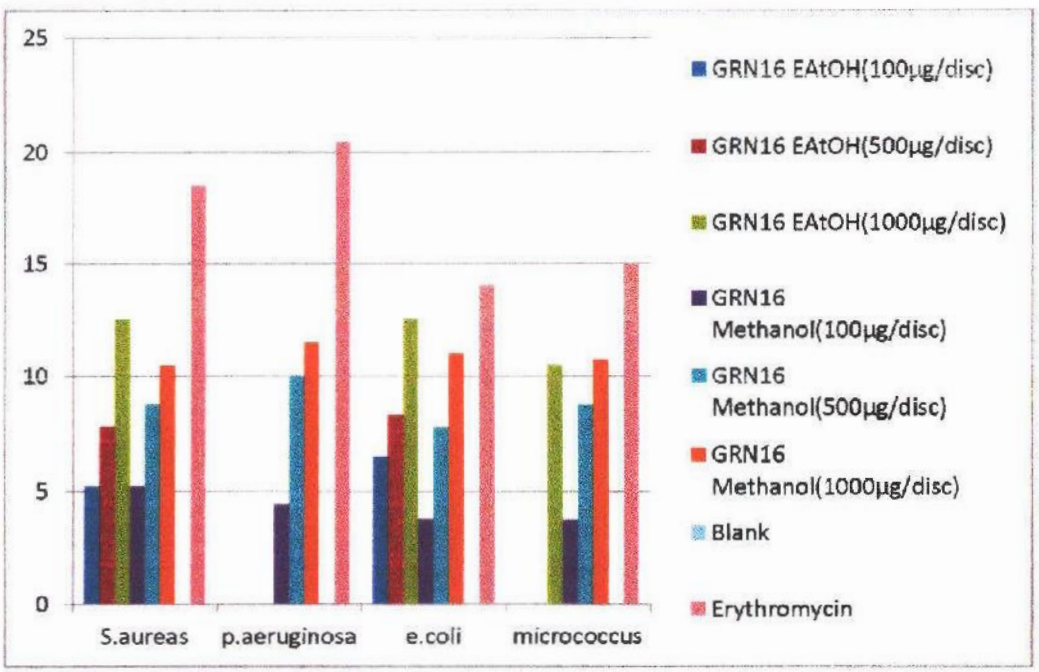

Figure 4.20: Zone of inhibition against bacteria of standard and samples

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Table 4.20: Zone of inhibition against bacteria

Name of extract	Zone of inhibition			
	Staphylococcus aureas	*Pseudomonas aeruginosa*	*Escherechia coli*	Micrococcus
GRN 17 EAtOH extract(100µg/disc)	-	-	-	-
GRN17 EAtOH extract(500µg/disc)	3.80	2.43	5.1	0
GRN17 EAtOH extract(1000µg/disc)	7.75	8.5	6.75	8.25
GRN17 methanol extract(100µg/disc)	10.5	9	8.5	8
GRN17 methanol extract(500µg/disc)	3.5	9.9	8.9	10.2
GRN17 methanol extract(1000µg/disc)	8	11.5	9	13.25
Blank	-	-	-	-
Erythromycin (10µg/disc)	19	9.5	11	8.5

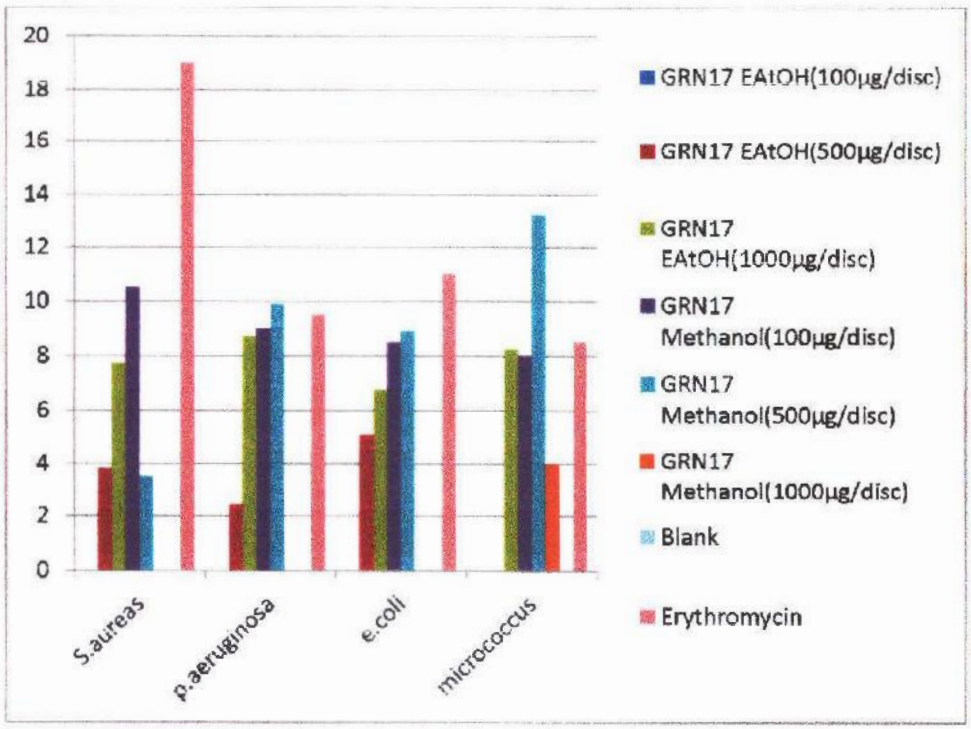

Figure 4.21: Zone of inhibition against bacteria of standard and samples

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Table 4.21: Zone of inhibition against bacteria

Name of extract	Zone of inhibition			
	Staphylococcus aureas	*Pseudomonas aeruginosa*	*Escherechia coli*	Micrococcus
GRN 18 EAtOH extract(100µg/disc)	-	-	-	-
GRN18 EAtOH extract(500µg/disc)	3.80	2.43	5.1	0
GRN18 EAtOH extract(1000µg/disc)	7.5	8.5	6	8.25
GRN18 methanol extract(100µg/disc)	10.5	9	8.5	8
GRN18 methanol extract(500µg/disc)	16	19	17	16.5
GRN18 methanol extract(1000µg/disc)	17	17.5	17.5	17.5
Blank	-	-	-	-
Erythromycin (10µg/disc)	16.5	19	19.5	13

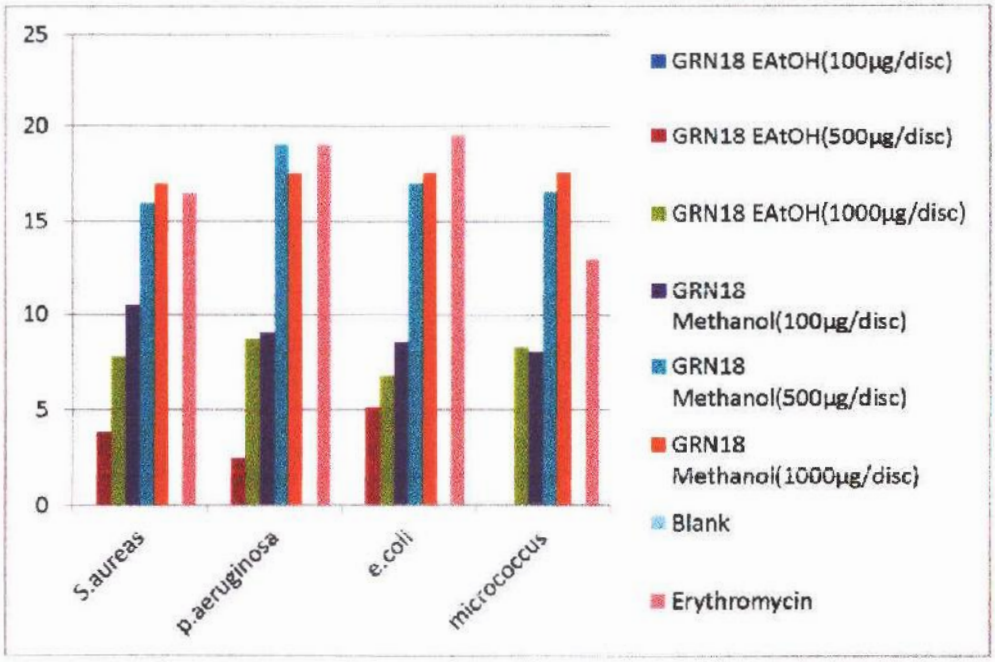

Figure 4.22: Zone of inhibition against bacteria of standard and samples

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4.6.2 Brine Shrimp Lethality Bioassay

Bioactive compounds are almost toxic at higher dose. Pharmacology is simply toxicology at higher dose, or toxicology is simply toxicology at lower dose. Thus, *in vivo* lethality in a simple zoological organism can be used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products.

Extracts of the leaves of mangrove plant and theirs associated endophytic fungal strain GRN7 GRN10, GRN16, GRN17, GRN18 were screened for probable cytotoxic activities using brine shrimp lethality bioassay.

The results are shown in the following table and figure mentioning the % of mortality and LC_{50} value of the extracts against the brine shrimp nauplii.

Table 4.22: Effect of Ethyl acited extract of GRN 7 on brine shrimp

Concentration (µg/ml)	log concentration	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
5	0.699	7	8	8	7.666666667	9.33	17.8278	
10	1	6	6	7	6.333333333		32.11861	
20	1.301	6	6	5	5.666666667		39.26402	
40	1.602	5	3	4	4		57.12755	27.54
80	1.903	3	3	2	2.666666667		71.41836	
160	2.204	2	1	1	1.333333333		85.70918	
320	2.505	1	0	1	0.666666667		92.85459	

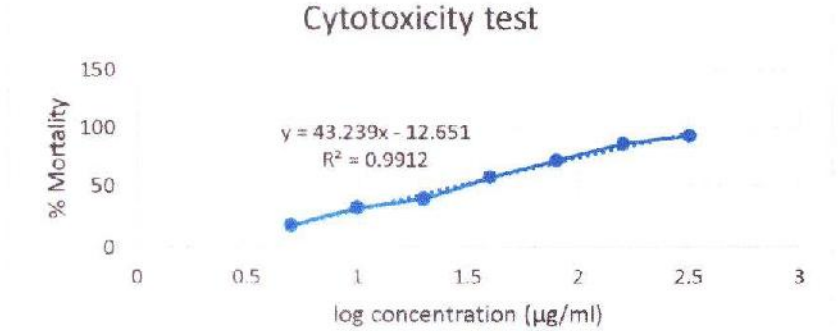

Figure 4.23:Graphical representation of brine shrimp lethality bioassay of ethyl acited extracts GRN7

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Table 4.23: Effect of Methanol of GRN 7 on brine shrimp

Con.(µg/ml)	log conc.	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
5	0.699	9	8	7	8	9.33	14.25509	
10	1	7	6	7	6.666666667		28.54591	
20	1.301	6	5	6	5.666666667		39.26402	
40	1.602	5	4	4	4.333333333		53.55484	30.90
80	1.903	2	3	2	2.333333333		74.99107	
160	2.204	2	1	2	1.666666667		82.13648	
320	2.505	1	1	1	1		89.28189	

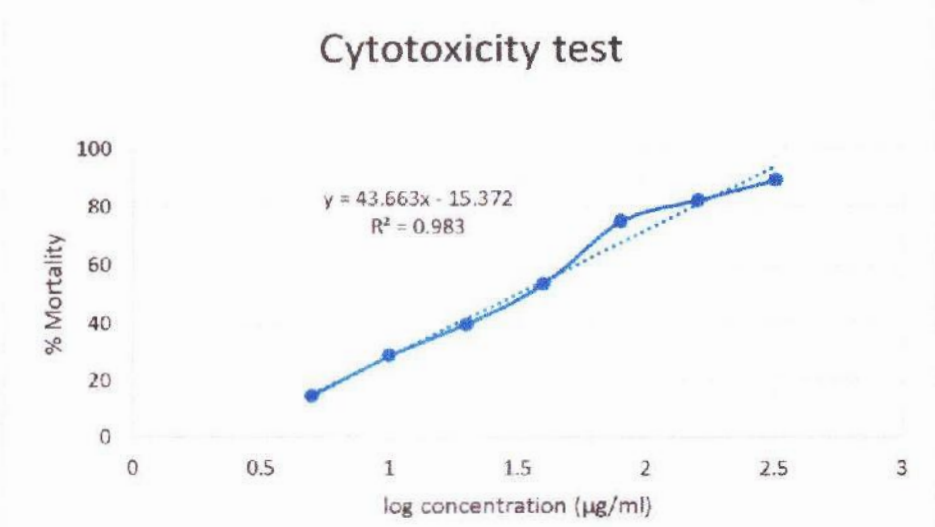

Figure4.24: Graphical representation of brine shrimp lethality bioassay of methanol extract of GRN7

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Table 4.24: Effect of Ethyl acited extract of GRN 10 on brine shrimp

Conc. (µg/ml)	log conc.	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
5	0.699	9	8	10	9		3.536977	
10	1	7	8	7	7.333333333		21.4005	
20	1.301	6	5	6	5.666666667		39.26402	
40	1.602	5	5	4	4.666666667		49.98214	36.30
80	1.903	4	3	2	3		67.84566	
160	2.204	1	1	2	1.333333333		85.70918	
320	2.505	0	1	0	0.333333333	9.33	96.4273	

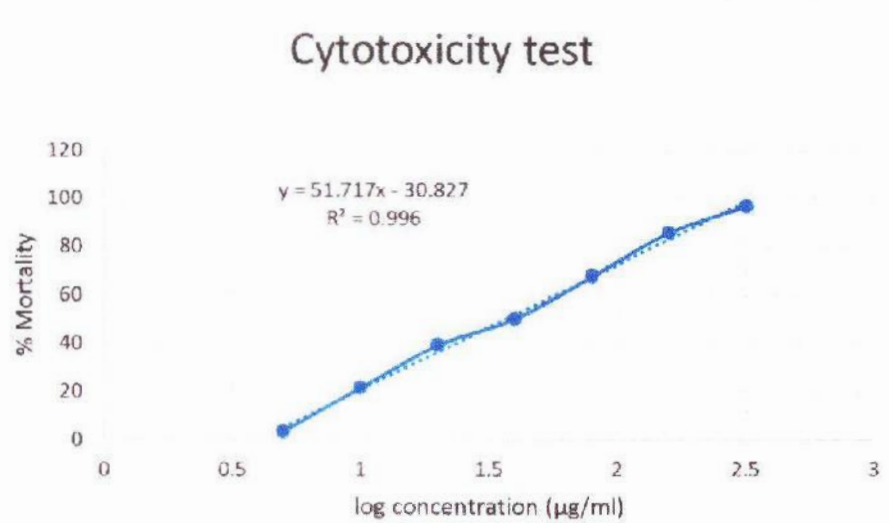

Figure 4.25: Graphical representation of brine shrimp lethality bioassay of GRN10 Ethyl acited extract

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Table 4.25: Effect of Methanol of GRN 10 on brine shrimp

Conc. (µg/ml)	log concentration	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
5	0.699	9	9	7	8.333333333		10.68239	
10	1	8	8	7	7.666666667		17.8278	
20	1.301	6	8	6	6.666666667		28.54591	
40	1.602	5	6	4	5		46.40943	51.28
80	1.903	4	5	2	3.666666667		60.70025	
160	2.204	3	3	2	2.666666667		71.41836	
320	2.505	2	1	2	1.666666667	9.33	82.13648	

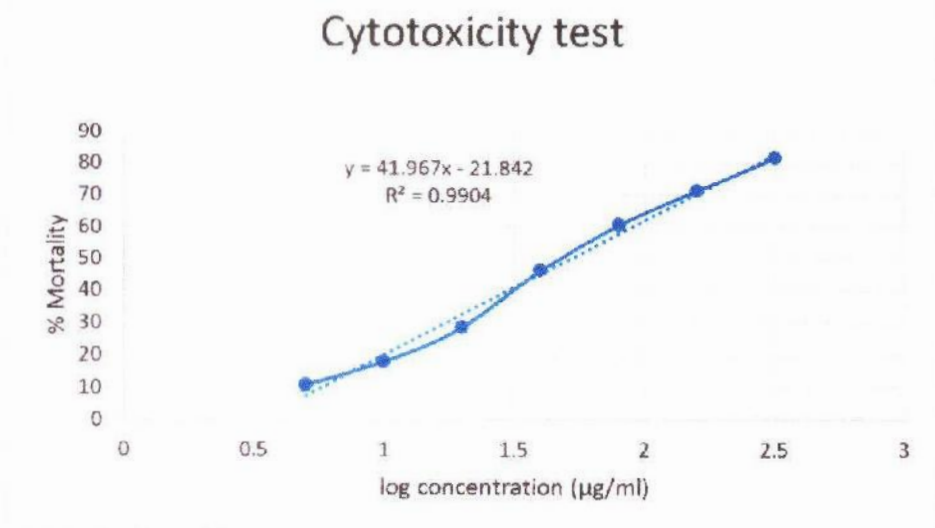

Figure 4.26: graphical representation of brine shrimp lethality bioassay of GRN10 of methanol extract

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Table 4.26: Effect of Ethyl acited extract of GRN 16 on brine shrimp

Conc. (µg/ml)	log concentration	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
5	0.699	8	9	8	8.333333333	9.33	10.68239	
10	1	8	8	7	7.666666667		17.8278	
20	1.301	5	8	8	7		24.9732	
40	1.602	5	6	5	5.333333333		42.83673	56.23
80	1.903	4	5	5	4.666666667		49.98214	
160	2.204	2	3	4	3		67.84566	
320	2.505	0	1	2	1		89.28189	

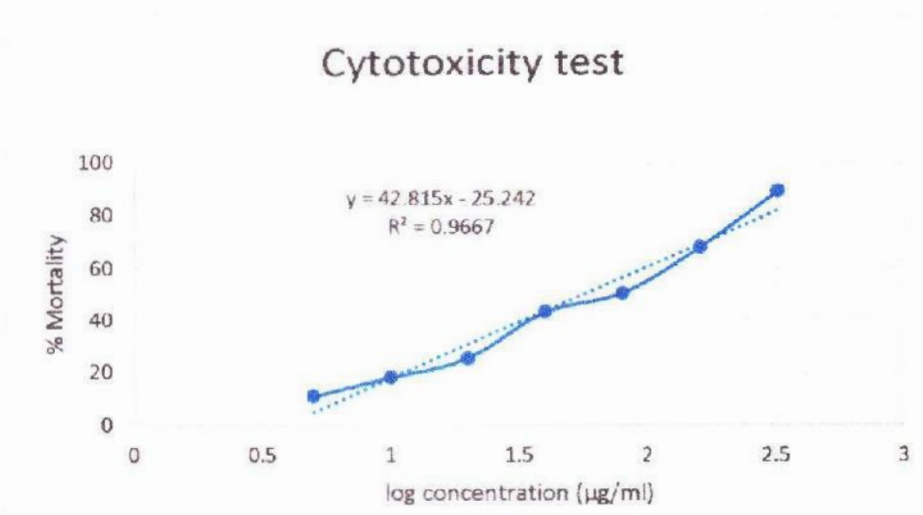

Figure 4.27: Graphical representation of brine shrimp lethality bioassay of GRN16 of Ethyl acited extract

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Table 4.27: Effect of methanol extract of GRN 16 on brine shrimp

Conc.(µg/ml)	log concentration	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
5	0.699	8	10	8	8.666666667		7.109682	
10	1	7	8	7	7.333333333		21.4005	
20	1.301	5	7	7	6.333333333		32.11861	
40	1.602	5	6	5	5.333333333		42.83673	38.01
80	1.903	4	3	2	3		67.84566	
160	2.204	2	1	0	1		89.28189	
320	2.505	0	1	0	0.333333333	9.33	96.4273	

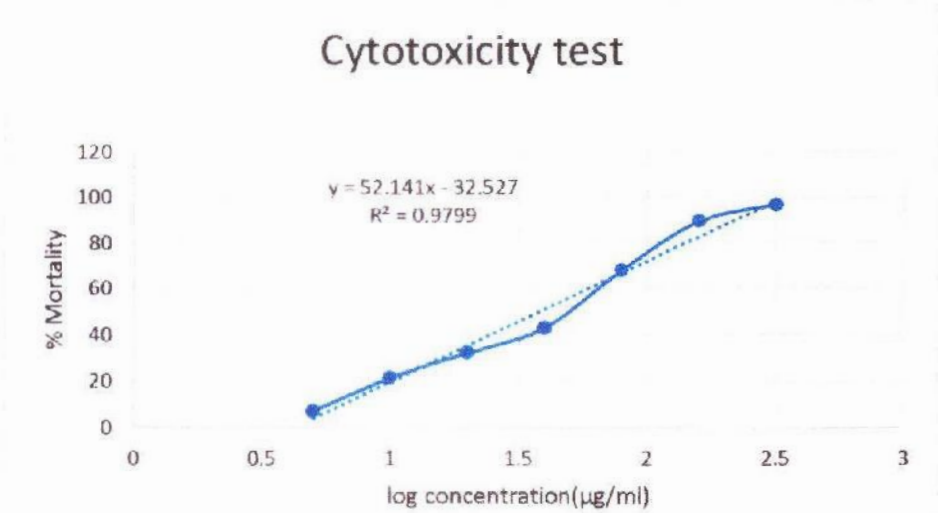

Figure 4.28: Graphical representation of brine shrimp lethality bioassay of GRN16 of methanol extract

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Table 4.28: Effect of Ethyl acited extract of GRN 17 on brine shrimp

Conc. (µg/ml)	log conc.	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
5	0.699	10	9	8	9	9.33	3.536977	
10	1	8	8	7	7.666666667		17.8278	
20	1.301	7	6	6	6.333333333		32.11861	
40	1.602	6	5	5	5.333333333		42.83673	38.01
80	1.903	4	3	2	3		67.84566	
160	2.204	1	1	0	0.666666667		92.85459	
320	2.505	0	0	0	0		100	

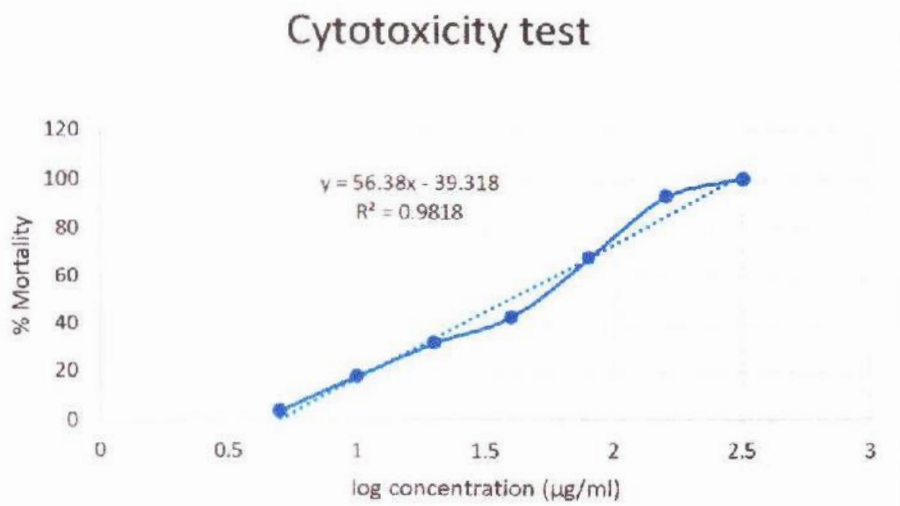

Figure 4.29: Graphical representation of brine shrimp lethality bioassay of GRN17 of Ethyl acited extract

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Table 4.29: Effect of methanol extract of GRN 17 on brine shrimp

Conc. (µg/ml)	log conc.	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
5	0.699	10	9	8	9	9.33	3.536977	
10	1	8	8	7	7.666666667		17.8278	
20	1.301	8	6	6	6.666666667		28.54591	51.28
40	1.602	6	4	5	5		46.40943	
80	1.903	4	3	4	3.666666667		60.70025	
160	2.204	3	2	3	2.666666667		71.41836	
320	2.505	1	2	1	1.333333333		85.70918	

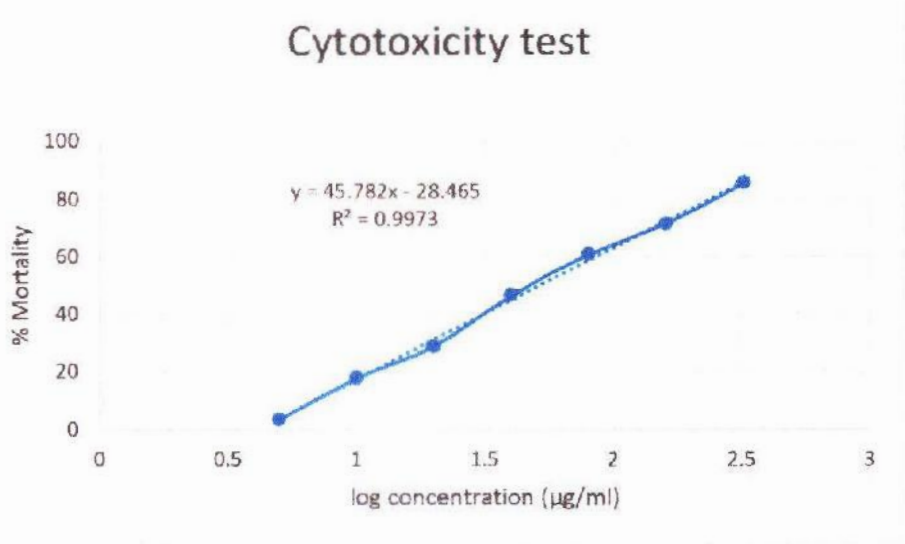

Figure 4.30: Graphical representation of brine shrimp lethality bioassay of GRN17 of Methanol extract

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Table 4.30: Effect of Ethyl acited extract of GRN 18 on brine shrimp

Conc. (µg/ml)	log conc.	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
5	0.699	8	9	8	8.333333333	9.33	10.68239	
10	1	6	8	6	6.666666667		28.54591	
20	1.301	5	6	5	5.333333333		42.83673	37.15
40	1.602	4	4	4	4		57.12755	
80	1.903	3	3	4	3.333333333		64.27295	
160	2.204	2	2	3	2.333333333		74.99107	
320	2.505	1	2	1	1.333333333		85.70918	

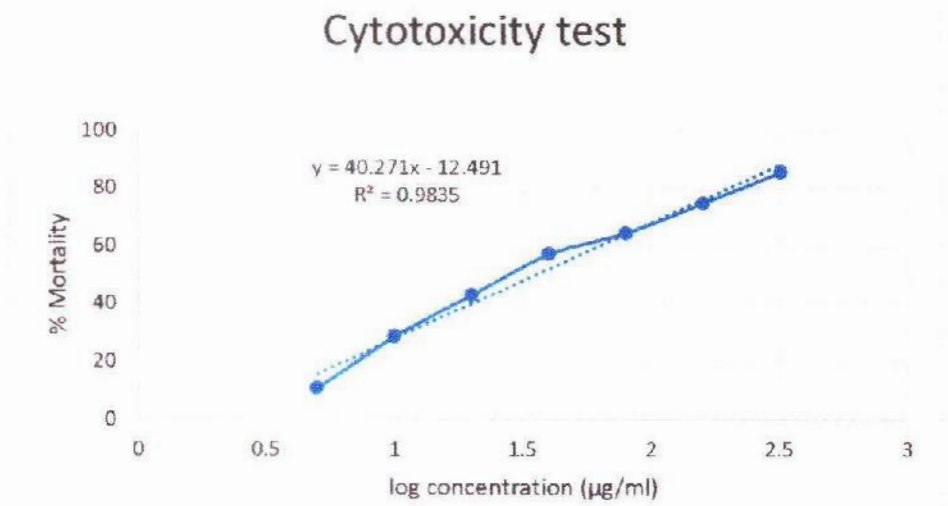

Figure 4.31: Graphical representation of brine shrimp lethality bioassay of GRN18 of Ethyl acited extract

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Table 4.31: Effect of methanol extract of GRN 18 on brine shrimp

Conc. (µg/ml)	log conc.	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
5	0.699	8	10	8	8.666666667	9.33	7.109682	
10	1	6	9	6	7		24.9732	
20	1.301	5	7	5	5.666666667		39.26402	37.15
40	1.602	4	5	4	4.333333333		53.55484	
80	1.903	2	3	3	2.666666667		71.41836	
160	2.204	2	2	3	2.333333333		74.99107	
320	2.505	1	2	1	1.333333333		85.70918	

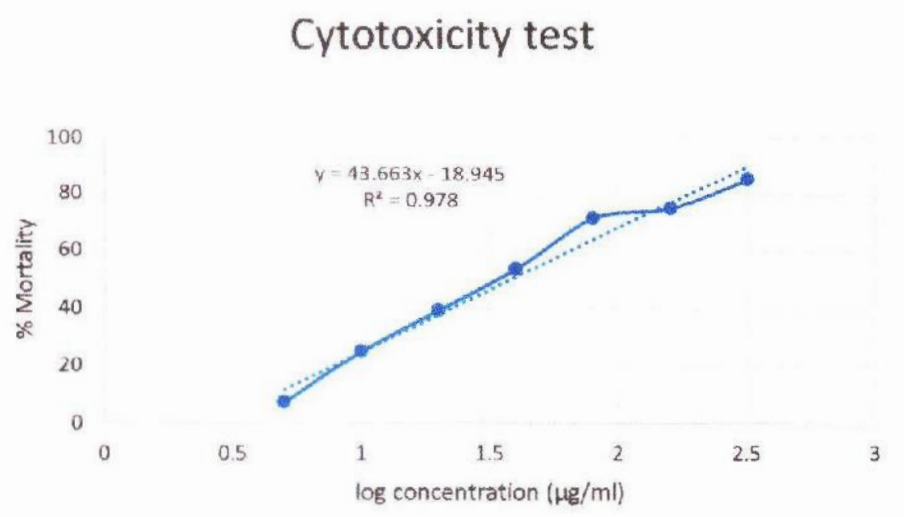

Figure 4.32: Graphical representation of brine shrimp lethality bioassay of GRN18 of Methanol extract

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Table 4.32: Alive shrimp counting for control

Control	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp
DMSO in sea water	9	10	9	9.33

Table 4.33: Effects of standard drug vincristine sulphate on brine shrimp

Conc. (µg/ml)	log conc.	No. of alive shrimp in test 1	No. of alive shrimp in test 2	No. of alive shrimp in test 3	Average alive shrimp	Average alive shrimp in Control	% Mortality	LC_{50} (µg/ml)
0.312	-0.506	7	7	8	7.33	9.33	21.40	1
0.625	-0.204	5	7	6	6		35.69	
1.25	0.097	4	5	3	4		57.13	
2.5	0.398	2	2	1	1.67		82.14	
5	0.699	0	0	0	0		100	

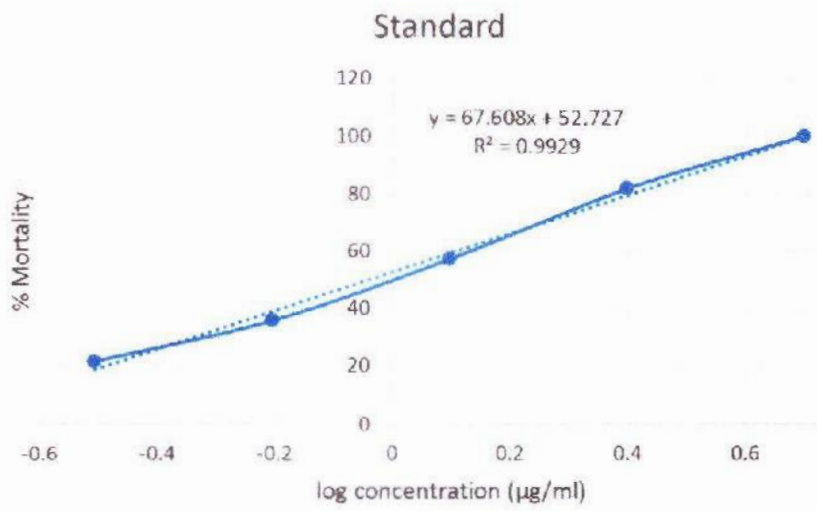

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Figure 4.33: Graphical representation of brine shrimp lethality bioassay of standard (Vincristine sulphate)

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Table 4.34: LC50 values of Cytotoxicity test

Name of extract	LC50(µg/ml)
Standard	1
GRN7 EAtOH	27.54
GRN7 Methanol	30.9
GRN10 EAtOH	36.3
GRN10 Metahnol	51.28
GRN16 EAtOH	56.23
GRN16 Methanol	38.01
GRN17 EAtOH	38.01
GRN17 Methanol	51.28
GRN18 Eatoh	37.15
GRN18 Methanol	37.15

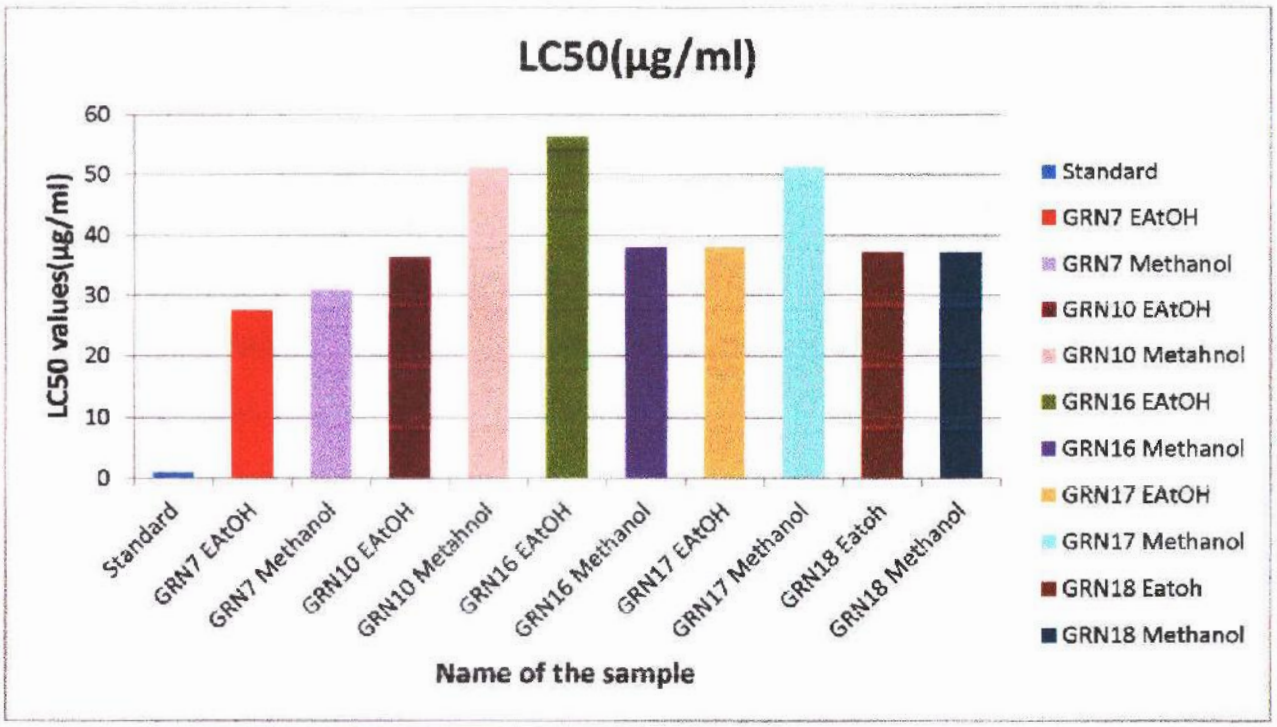

Figure 4.34: Values of LC50

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4.3 : Result Discussion

Identification of fungi ware done by two steps named macroscopic and microscopic. Both are under the procedure known as morphological identification. The morphological identification was done by the following manual of Plant Pathology by G.N. Agrois.. The molecular identification was done by PCR reactions for sequencing were carried out with universal primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White *et al.*, 1990). Sequence similarity searches were performed for each of the fungal sequences against the non-redundant database maintained by the National Center for Biotechnology Information using the BLAST algorithm (www.ncbi.nlm.nih.gov) (Huang *et al* 2009). The nucleotide sequences of reference taxa along with their GenBank accession numbers are given in charts. This process involves accurate identification and characterization of fungal endophytes. It shows the understanding of biology, ecology, evolutionary relationships among these organisms and their current and possible future uses in biotechnology. In order to obtain the most accurate name, the most up-to-date knowledge relating to correctly defining species is absolutely essential. The species concepts that is used for fungi have changed periodically. So, the knowledge of fungal morphology, biology, and evolutionary relationships developed (Rossman and PalmHernández 2008). Sequences of the strains isolated in this study were assembled with nucleotide. Sequences of isolated fungi retrieved from GenBank (Lazzizeraa*et al* 2008). This results can be further explored to understand the relationships between plant hosts and their fungal endophytes (Huang *et al* 2009).

The bioactivity known as antibacterial, antioxidant and cytotoxicity tests of isolated fungi were done by using their secondary metabolites. The result of each test is shown by charts and graphs. After gaining the results of every tests they were compared with constants or standards values to know the effectiveness and range of activity of fungi.

The results of free radical scavenging activity of the extracts have been compared in the following graph and table. Among all the extracts, GRN-7 methanolic extract and GRN-16 methanolic extract were showed better antioxidant activity with the lowest IC_{50}valu comparing with the value of the standard (10.9730 µg/ml). Other extracts showed mild to

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moderate activity with high value of IC50. From the results, it can be concluded that extracts of the associated endophytic fungi from can be a potential source of antioxidant.

Comparing the result from the tabular and graphical presentation of all the extracts, it can be said that endophytic fungi isolated from the *C. decandra* have lower cytotoxicity nature. Among all the extracts only Extract GRN- 7 *(Colletroticum gloeosporioides spp.)* showed moderately good activity with low LC_{50} value against the nauplii. Others are shown lower activity against the nauplii .

All the crude extracts of the endophytic fungi from *Ceriops decandra* plant tested for antibacterial activity against a number of both gram positive and gram negative bacteria. Standard disc of Erythromycin (10μg/disc) was used for comparison purpose. The results of antimicrobial activity of the all the extracts exhibiting different zones of inhibition are shown in tabular and graph form. Extract GRN-18 (methanolic extract) showed maximum zone of inhibition which was 17.5 mm against 3 bacteria (Micrococcus, E.coli, *Pseudomonas aeruginosa)*.

The results apparently show that the potentiality of the few fungal extracts to inhibit bacterial strain while the results also reflect the inactivity of the extracts from endophytic fungi against the fungal strains.

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5.0 Conclusion

This research focused on potential source to isolate bioactive secondary metabolites from a natural source of Goran (***Ceriops decandra***) from the mangrove plant found in Sundarban.

The study was designed in two parts. The first part described the isolation and identification of the endophytic fungi of the selected plant leafs.

Endopgytic fungi was isolated from mangrove plant of (***Ceriops decandra***).Isolated endophytic fungi was cultured and extracted to know the fungi name from the sample by ITS primer from PCR reaction. After that, a sufficient amount of DNA was found. Further, it was sequenced by software phylogentic Analysis Using Parsimony (PAUP) version 4.0 . From which, species was found fir 10 region that showed 99% identity with accession no. The sample GRN-7, GRN-10, GRN-16, GRN-17, GRN-18 represents *Curvularia geniculata* ,*Colletroticum gloeosporioides, Colletroticum horii* ,*Chaetomium globoum, Fusarium solani* . Phylogenetic tree represents that all fungi are genetically similar with each other and dissimilar with other fungi.

The second part of the thesis was involved in the preliminary chemical and biological screening of the extracts by small scale cultivation, detection of the presence of chemical constituents in the extract based on bioactivity testing and finally isolation and identification of the secondary metabolites. Biological study involves the evaluation of antioxidant, antimicrobial and cytotoxic activity. The extracts from isolated endophytic fungi culture were screened for their preliminary bioassay.

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