

**Identification and Bioactivity Analysis of Endophytic Fungi Isolated
from Mangrove Plant (*Sonneratia caseolaris*)**

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Science (M.Sc.) in Biotechnology and Genetic Engineering

Submitted by

**Progha Promita Paul
Examination Roll No: MS-160712
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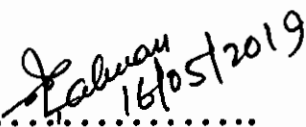
**Biotechnology and Genetic Engineering Discipline, Life Science School,
Khulna University, Khulna.
Khulna-9208, Bangladesh
September, 2018**

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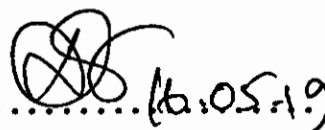
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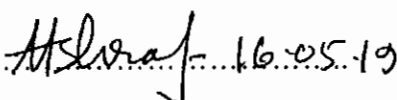
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Dedicated to

My beloved Brother

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Abstract

Endophytic fungi or endophytes exist widely inside the healthy tissues of living plants, and are important components of plant micro-ecosystems. The microorganism which lives within the intracellular tissue of a plant asymptotically with maintaining a symbiotic relationship are referred endophytic fungi. Endophytes and their host plants have established a special relationship with one and another, which can significantly influence the formation of metabolic products in plants, then affect quality and quantity of crude drugs derived from medicinal plants. The purpose of this study was to isolate and identification of endophytic fungi from the leaf tissue of *Sonneratia caseolaris* to determine their antibacterial antioxidant and cytotoxicity activities. Six endophytic fungi *wardomyces mosari*, *Simplicillum clavatum*, *Alternaria destruens*, *Alternaria porri*, *Diaporthe recaer*, *Apicoccum nigrum* were isolated from leaves of the mangrove plant *Sonneratia caseolaris*. Among all the extracts, CHL 01, CHL 03, CHL 04, CHL 05 EA₂OH(Ethyl acetate), CHL 08 MEOH were showed very good antioxidant activity with the lowest IC₅₀valu comparing with the value of the Ascorbic acid (10.9730 µg/ml).Which is used as standard. Other extracts showed mild to moderate activity with high value of IC₅₀. . All the crude extracts of the endophytic fungi from *Sonneratia caseolaris* plant tested for antibacterial activity against two gram positive and gram negative bacteria. Standard disc of Erythromycin (10µg/disc) was used for comparison purpose. The result of the concentration of (500µg/disc),(1000µg/disc) shows high zone of inhibition while the other concentration (100µg/disc) shows poor zone of inhibition. Among all the extracts, showed good activity with low LC₅₀ value against the nauplii.Among them CHL 01(*Wardomycesmoseri* spp.), CHL 03(*Alternariadestruens*), CHL 08 (*Apicoccumnigrum*) shows the maximum activity with low LC₅₀ value against the nauplii.

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Abbreviations Used in the Text

<i>et al.</i>	et alia= and others
etc.	et cetera= 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	Milliliter (s)
L	Liter
Conc.	Concentration (s)
g.	Gram
h.	Hour
mins.	Minutes
µg	Microgram (s) per milliliter
µg/ml	Microgram (s) per milliliter
MEGA	Molecular evolutionary genetics analysis
BLAST	Basic local alignment search tool
PL	Passur leaf

1. Introduction

Endophytes are microorganisms that live within plants for at least a part of their life cycle without causing any visible manifestation of disease (Bacon & White., 2000) "Endophytism" is, thus, a unique cost-benefit plant-microbe association defined by "location" (not "function") that is transiently symptomless, unobtrusive, and established entirely inside the living host plant tissues (Kusari *et al.*) It is widely considered in a conventional view that the quality and quantity of crude drugs originated from medicinal plants are largely affected by such factors as the genetic background of the concerned plants, ecological habitats where the plants live, and soil nutrients(Javey *et al.*, 2003; Sherameti *et al.*, 2005). However, in the recent years, it is gradually recognized that endophytic fungi or endophytes have played a very important role in affecting the quality and quantity of the crude drugs through a particular fungus-host interaction, indicating that more understanding on the particular relationships between endophytic fungi and medicinal plants is required for promoting crude drug production(Faeth & Fagan., 2002).

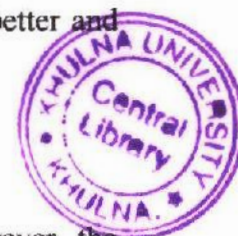
Although endophytic fungi are one of the most important elements in plant micro-ecosystems that should have significant influences on the growth and development of host plants, our knowledge about the exact relationships between endophytic fungi and their host plants is still very limited. Understanding and exploiting such relationships will facilitate the ideal production of better drugs by manipulating the growth conditions of medicinal plants by, for example, adding a particular group of endophytic fungi to the plants to improve the drug quality and quantity (FirÅ kovÅ *et al.*, 2007) Ideally, an alternative method can be developed to directly produce desired drugs through bioengineering of the selected medicinal plants and endophytic fungi under a certain cultural condition, if the fungus-host relationships and their metabolic mechanisms under cultural conditions are well understood(Li *et al.*, 2009)

Sonneratia caseolaris is a evergreen mangrove plant, commonly known as mangrove apple. The genus *Sonneratia* belongs to the family *Lythraceae*. *Sonneratia caseolaris* is an example of a successful mangrove plant with several highly active secondary chemical agents that are produced by its seeds, leaves and roots. This species is widespread and common in most of its range and is fast-growing, pioneering species. There has been an estimated 20% decline in mangrove area within this species range since 1980. Mangrove species are more at risk from

coastal development and extraction at the extremes of their distribution, and are likely to be contracting in these areas more than in other areas.

It is a commercially important medicinal plant that found in mangrove forest. It is a wild plant and its every part are full of medicinal use. The plant has long been used in traditional Indian medicine. Fruits yield 11% pectin (ZMB). Wood yields 52.7% brown pulp (8.5% lignin, 17.6% pentosan). Emodin and chrysophanic acid may be the coloring matter in the crude drug (Perry., 1980). Bark from Africa assayed at 17.1% tannin, of the pyrogallol class. Indian stem bark assayed 9–17%, twig bark 11-12%. Wood yields two coloring principles, archin ($C_{15}H_{10}O_5$) and archinin ($C_{15}H_{14}O_{12}$) (Upadhyay *et al.*, 1998)

Endophytic fungi or endophytes exist widely inside the healthy tissues of living plants, and are important components of plant micro-ecosystems. Over the long period of evolution, some co-existing endophytes and their host plants have established a special relationship with one and another, which can significantly influence the formation of metabolic products in plants, then affect quality and quantity of crude drugs derived from medicinal plants. On the other hand, the endophytic fungi can also confer profound impacts on their host plants by enhancing their growth, increasing their fitness, strengthening their tolerances to abiotic and biotic stresses, and promoting their accumulation of secondary metabolites. All the changes are very important for the production of bioactive components in their hosts. Hence, it is essential to understand such relationships between endophytic fungi and their host medicinal plants. Such knowledge can be well exploited and applied for the production of better and more drugs from medicinal plants (Jia *et al.*)



Endophytes do produce secondary metabolites when placed in culture. However the temperature, the composition of the medium, and the degree of aeration will affect the amount and kinds of compounds that are produced by an endophytic fungi. Currently, endophytic fungi are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological higher plants growing in so many unusual environments. Of the approximately 300 000 higher plant species that exist on the earth, each individual plant, of the millions that exist here, is host to one or more endophytes. It seems obvious that they are a rich and reliable source of genetic diversity. Assessment of genetic diversity based on phenotype has limitations, since most of

the morphological characters are greatly controlled by quantitative factors and affected by environmental modification.

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 Sundarban as well as find out the suggestion for effective marketing and health plan. The specific objectives of this study are show below:

- Isolation and identification of endophytic fungi from *sonneratia caseolaris*
- Finding the antioxidant, antimicrobial and cytotoxic properties of *sonneratia caseolaris*.

Chapter 2

Literature Review

2.1. Endophytic Fungi

During the past 30 years the terms 'endophyte' and 'endophytic fungi' have appeared frequently in the mycological literature to describe the internal mycota of living plants (Large, 1940). An important year in the history of endophyte research is 1977, when Charles Bacon and colleagues found the cause of fescue toxicosis, a syndrome suffered by cattle fed in pastures of the grass *Festuca arundinacea* (Bacon *et al.*, 1977). The fungal endophytes are a diverse group of organisms forming associations almost ubiquitously throughout the plant kingdom. The endophytes which provide indirect defense against herbivores may have come from a number of origins, including mutualistic root endophyte associations and the evolution of entomopathogenic fungi into plant-associated endophytes.

Endophytic fungi are described as fungi which live within plant tissue with no evidence of symptoms to the host plant. Some of these endophytes provide protection to their host plant from tissue invading pathogens by producing compounds that are involved in the host plant's defence mechanism. The fungi have been shown to produce similar compounds as their host plant. Endophytic fungi inhabit the internal tissues of a plant without causing any negative effects. They are considered a source of novel activities, compounds and biotechnological processes with great and underexplored potential. The estimated number of total fungal species is 1.5 million with approximately 712,000 described species. Currently, it is believed that all plant species host at least one endophyte. Many secondary metabolites from endophyte-plant interactions have also been isolated and used in raw or derived forms to produce a variety of drugs treating many conditions.

Endophytes are fungi which infect plants without causing symptoms. Fungi belonging to this group are ubiquitous, and plant species not associated to fungal endophytes are not known. In addition, there is a large biological diversity among endophytes, and it is not rare for some plant species to be hosts of more than one hundred different endophytic species (Sánchez *et al.*, 2007). Different mechanisms of transmission, as well as symbiotic lifestyles occur among endophytic species. Latent pathogens seem to represent a relatively small proportion of endophytic assemblages, also composed by latent saprophytes and mutualistic species. Some endophytes are generalists, being able to infect a wide range of hosts, while others are

specialists, limited to one or a few hosts. Endophytes are gaining attention as a subject for research and applications in Plant Pathology. This is because in some cases plants associated to endophytes have shown increased resistance to plant pathogens, particularly fungi and nematodes.

Endophytes are known to affect the interactions of plants with their environment, and to alter the course of their interactions with plant pathogens. In spite of this, fungal endophytes are still quite inconspicuous in plant pathology textbooks. This is likely to change soon. In addition to represent a source of organisms for disease control and plant improvement, the study of endophytes may have an important influence in the conceptual framework where plant-pathogen interactions are interpreted and investigated.

The procedures most commonly used for endophyte surveys are based on the surface disinfection of apparently healthy plant tissue samples to kill epiphytic fungi (Bills., 1996). The surface-disinfected plant samples are subsequently placed on a synthetic growth media and, when endophytic hyphae emerge from the plant tissue and start growing in the agar medium, isolations can be made. With this technique obligate biotrophs or fungi not growing well in the selected medium will not be isolated. As a result, the real number of endophytic species in a sample can be underestimated. Non culturable endophytes do exist, and fortunately some techniques allowing their detection in plant tissues have been developed (Neubert *et al.*, 2006; Gallery *et al.*, 2007)

An average of about 50 endophytic species per plant species was found in surveys done before the year 2000. When molecular methods for the identification of fungi began to be applied to endophyte research (Arnold., 2008) the number of fungal species identified per host plant species increased substantially. The reason for this is that an important proportion of fungal isolates may be sterile in laboratory cultures. Genotypic identification methods have allowed to identify, or at least to distinguish, among sterile cultures. If more plant samples would have been analyzed, more endophytic species would have been discovered. These results imply that most surveys of endophytic mycobiota underestimate the real number of species associated to a given plant species.

Seem to constitute an important fraction of endophyte assemblages, most endophytes do not cause symptoms on plants. Some studies have shown that seeds and seedlings are virtually endophyte-free, and the incidence of fungal endophytes increases as leaves or seeds grow

older (Arnold *et al.*, 2003; Gallery *et al.*, 2007). Some saprophytes which produce fructifications in dead plant parts have been identified as endophytes in healthy tissues (Sánchez *et al.*, 2007). Some endophytic species may induce plant defense mechanisms which counteract pathogen attack, others produce antibiotic substances which inhibit pathogen growth, competition for plant space and resources may also occur between resident endophytes and incoming pathogens; finally, some parasites of plant pathogens are known to behave as endophytes.

Many endophytic species produce antibiotic substances (Strobel., 2002; Schulz & Boyle., 2005). Liquid extracts from endophyte cultures have been found to inhibit the growth of several species of plant pathogenic fungi (Liu 2001; Park 2005) Kim *et al.*, 2005). If such compounds were produced by endophytes in planta, this could constitute a defense mechanism against fungal pathogens. Another type of plant protection mediated by endophytic fungi may come from nematophagous fungi which can inhabit plant roots as endophytes (Bordallo *et al.*, 2002). In this case, a phenomenon of systemic plant resistance induced by the endophyte appeared to be the mechanism of resistance to the nematode pathogen (Zabalgogezcoa., 2008). Bactericidal effects of endophyte culture extracts have been demonstrated and do not seem to differ from those observed for fungi or nematodes. In endophytes may have an important role in the adaptation of plants to some particular environments (Rodriguez *et al.*, 2004).

2.1.1 General Information

Sonneratia caseolaris is commonly known as Firefly Mangrove, Red-flowered Pomunpan Mangrove, or Mangrove Apple is an evergreen, tropical, medium-sized tree with columnar crown growing about 6-20 m tall and 30 cm in trunk diameter, commonly in East Asia. It has pencil-like pneumatophores, or aerial roots. The trunk is swollen at the base when young. The leaves are thick but narrow, opposite, and leathery. The flowers occur singly, with red, narrow petals, and green sepals. The fruits are round and hard containing many seeds. The fruits, known source of pectin, are cooked, or used for vinegar or beverages. Young fruits are used as flavoring. Young leaves can be consumed raw. The plant is a folk remedy for sprains, swellings, and worms. It is also used against coughs, hematuria, smallpox, and cuts and bruises. The plant is planted in coastal swamp areas to control soil erosion. The bark is a

tannin source. Vertical roots are used as floats for fish nets and in the manufacture of inner soles for shoes. The wood is used for piles, posts, poles, railway.

2.1.1.1 Taxonomy

Taxonomic classification of *S. caseolaris* is given below:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Magnoliales

Family: Sonneratiaceae

Genus: *Sonneratia* L. f. P

Species: *Sonneratia caseolaris* J.E. Sm. P

2.1.1.2 Synonyms of *S. caseolaris*

There other names of this plants are *sonneratia casiolaris* Engl (L), *Sonneratia obovata* blume, *Sonneratia ovalis* Korth, *Sonneratia acida* L, *Sonneratia neglecta* Blume, *Rhizophora caseolaris* L, *Sonneratia evenia* Blume

2.1.1.3 Common name of *S. caseolaris*

Sonneratia caseolaris, also known as mangrove apple or crabapple mangrove, in Bangladesh local name of *S. caseolaris* are Ora, Orali, Choila, Archaka, Shoila; Orcha (Sundarbans); Chhaila (Barisal). The *Sonneratia* are called 'berembang' in Malaya, 'mangrove apple' in English, and 'Mangrovenapfel' or 'Holzapfelmangrove' in German. In the Philippines,

particularly in the Bataan and Pampanga region, they are called 'Pagatpat'. In Malayalam also known as Blatti, Chakkarakandal, Puzhamunja, Thirala. The fruit is noted for its outward similarity to the persimmon fruit.

2.1.1.4 Etymology of *S.caseolaris*

Latin *Sonneratia*, after Pierre Sonnerat (1749-1841), French botanist and explorer; Latin *caseolaris*, small cheese, referring to this species' distinctive rounded shape of the fruit.



2.1.1.5 Origin and Geographic Distribution of *S. caseolaris*

S. caseolaris is native to South and South-East Asia and is distributed from India and Sri Lanka up to southern China, and throughout South-East Asia to northern Australia and the western Pacific islands. In the south Asia this species is presents in Bangladesh, Brunei, Indonesia, Malaysia Philippines, Myanmar, Cambodia, Thailand, Vietnam. In Australia it is found in northwest Australia, Papua New Guinea. This species show different morphology depending on geographical distribution.

2.1.1.6 Plant Morphology

It will be a sort about tree that grows On salt-marsh forests along the coasts for numerous nations. The mangrove tree fruit tree grows essential On salty areas along littoral riverbanks the place the tides climb and fall. Its abandons would oval. Those mangrove tree fruit tree blooms after those dry season only in front of the blustery season. The parts that would regularly utilized should settle on nourishments would the abandons Furthermore fruits. Two predominant sorts of the apples and oranges On Vietnam need aidthose saucer mangrove tree fruit (*Sonneratia caseolaris*) and the guava mangrove tree fruit (*Sonneratia giffithii*). Saucer mangrove tree apples develop along riverbanks, looking into islands, what's more at waterway ramifications. Its fruits are extensive What's more round like a saucer, Consequently its name. With respect to the guava mangrove tree fruit tree, it is planted close homes on facilitate picking its fruit, which is consumed seasonally. This apples and oranges is

medium over size. As far as compound composition, mangrove apples tree holds the shade operators archin (emodin) Also archicin (chrysophanic acid), which bring anti-oxidizing, laxative, also detoxifying impacts. They also reinforce blood vessels, cure stomach ulcers, lessen absorption about sugars Furthermore fat, also lessen pulse. As stated by accepted oriental medicine, the mangrove tree fruit need cooling properties, quells inflammation, further more diminishes agony. Those abandons bring harsh taste What's more styptic properties. Over India, individual's utilization those tree grown foods juices Toward aging them to make a solution that inhibits dying. To Malaysia, individuals bawl those abandons with rice to make pharmaceutical that remedies pee maintenance Also murders intestinal parasites. They consume ready mangrove apples tree on treat hacking. Done Myanmar, individuals utilization mangrove tree by grinding them under a glue mortar or glutinous paste, which would that point blended for salt and connected should creepy crawly bites, same time to congested bruises they mature those soil grown foods juice Similarly as homeostatic. For numerous nations basic society often grind those leaves, include salt, what's more utilized for wounds.

Despite the mangrove tree fruit might appear to be a touch plebeian, it will be element that is committed under a number wide open claim to fame dishes: mangrove tree fruit soup spoon soup, mangrove tree fruit shoots singed with field mice, ready mangrove tree apples braised for fish, mangrove tree apples Also fish hotpot, What's more mangrove tree fruit jam, and so on. The simplest will be to lift mangrove apples tree and dip them to salt Likewise a snake.

Leaves

The leaves by bring inverse arrangement, in any case at times would exchange. They are straightforward for smooth birch edges and pinnate venation. Stipules would normally reduce, showing up Likewise column for moment hairs, alternately absent.

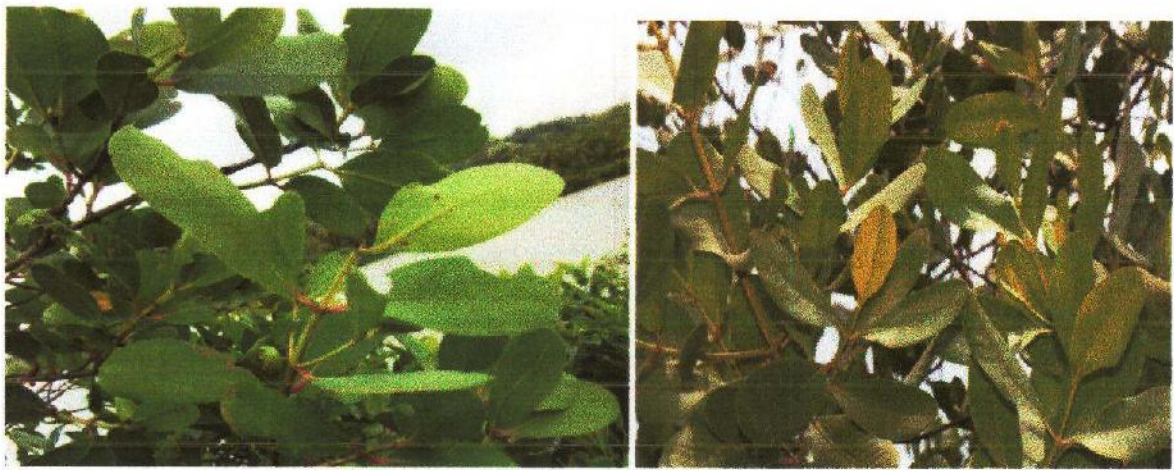


Figure 2.1: leaves of *S.caseolaris*

Flowers

The blossoms from claiming this plant would bisexual, radially alternately Sporadically bilaterally symmetric, Hosting a well-developed hypanthium. The blossoms would A large portion usually four-merous at could a chance to be six-merous, for four will eight sepals and petals. The sepals might make distinct, incompletely combined to structure a tube, or touching without covering. The petals would have folded in the bud and wrinkled toward maturity, also would normally dissimilar What'smore overlapping; they need aid Sporadically absent. There need aid normally twice as huge numbers stamens as petals, orchestrated over two whorls, and the stamens are often unequal long. Occasionally, those stamens need aid decreased to one whorl, alternately need aid more various with numerous whorls. That ovary may be normally superior, rarely semi-inferior, or infrequently mediocre. The two with Numerous carpels could be combined together with two will various ovules On every locule, for axile placentation of the ovules.



Figure 2.2: Flowers of *S.caseolaris*

Fruits and seeds

Those fruits need aid Typically a dry, dehiscent capsule, sporadically might a chance to be a berry. The seeds need aid normally smoothed, with more layered external integument and epidermal hairs extend Also ended up adhesive The point when wet would found done regarding half the genera.



Figure2.3: Fruits of *S.caseolaris*

2.1.1.7 Growth and development of *S.caseolaris*

➤ Germination is epigeal. It is a pioneer species of mangrove swamps, and seeds will not germinate easily in shade. Seedling leaves are narrowly lanceolate, 12-13 cm × 1-5 cm; adult tree leaves are elliptical. Flowering in South-East Asia is year-round, nocturnal, and pollination seems to be effected by nectar-drinking bats or large night-moths. Stamens and petals fall from the flowers within 12 hours of opening. At anthesis, the flower emits a sour, buttery odour. During the main flowering period in Vietnam (March - April) the plant attracts thousands of fireflies which illuminate areas of mangrove.

2.1.1.8 Ecology of *S.caseolaris*

➤ *S. caseolaris* is common in the inner parts of mangrove forests on deep muddy soils and extends inland along tidal creeks usually as far as the influence of salinity extends. Its characteristic habitat consists of river banks and tidal areas with mud banks, often in upstream estuarine positions of rivers subjected to large volumes of freshwater run-off. In some instances it has been found growing in fully fresh water without any connection with brackish water.

2.1.1.9 Agronomy of *S.caseolaris*

S. caseolaris is propagated by seed. These are small and tend to float. Reforestation is usually by means of seedlings, planted at a spacing of 1 m × 1 m. Threatening diseases and pests have not been reported except mangrove crabs, which feed on leaves and may destroy seedlings.

2.1.1.10 Cultivation of *S.caseolaris*

➤ A plant of coastal areas in the tropics. It grows best where the mean annual minimum and maximum temperatures are within the range 20 - 30°C, though it tolerates 10 - 38°C. It prefers

a mean annual rainfall of 1,500 - 2,500mm, tolerating 1,000 - 3,000mm, and succeeds in areas with no dry season as well as those with a dry season. Prefers a sunny position. Prefers a heavy soil, but tolerates most soil types. Grows in areas that are inundated by salt water at high tides. Prefers a pH in the range 6.7 - 7.3, but tolerates 6.5 - 7.5. Plants are tolerant of strong, salt-laden winds. The flowers are nocturnal, opening in the evening and closing in the early morning.

2.1.1.11 Uses of *s.caseolaris*

Medicinal uses

The plant is said to be homeostatic. Also used as folk remedy for swellings, and worms. The old fruit walls are used as a treatment for worms. Half-ripe fruits are a treatment for coughs. The fruits are used to make poultices. The pounded leaves are used as a treatment for haematuria, and smallpox. The leaves are crushed, mixed with salt and applied as a poultice onto cuts and bruises. All in all, it is also a very good antioxidant for the body and can help slow down the age related changes in the body.

Folk Medicine

Appeared for be hemostat, crabapple mangrove tree will be society cure for swellings, and worms. Malaysians utilization old tree grown foods dividers to worms, fruits to cough and smallpox.

Agroforestry Uses:

A very important tree in the coastal swamp community, helping to protect the soil from erosion and providing an important habitat for wildlife.

Other Uses

Those pneumatophores (vertical bases climbing over ground) need aid utilized as floats to fishnets and, being corky On texture, need aid utilized in the fabricate of inward soles for shoes.

Furthermore, camwood a chance to be utilized Likewise substitute to plug alternately pith. The bases would have bubbled preceding constantly utilized. Those bark is a hotspot from claiming tannins. Those heartwoods will be light tan should dark chocolate, the sapwood light greyish tan Also 3 - 8cm thick. At wet alternately under varnish, the heartwood of old develop trees takes a gander very nearly bootleg. Those grain may be straight or extremely marginally crossed; those compositions fine, altogether homogeneous, smooth, not glossy; it need a different salty taste and a fishy alternately swampy odor, particularly new. The wood may be reasonably difficult and reasonably overwhelming with overwhelming. It is not difficult to work; keeps up great in the ground Furthermore actually those sapwood is infrequently struck by insects. Those wood holds a little sum from claiming salt, settling on the utilization for copper nails Also screws important. It may be utilized for piles, posts, poles, track ties, clearing blocks; ship, bridge, furthermore wharf building; general solid construction; entryways; siding, sheathing, ceiling, flooring, also various sorts about inner part finish; boat planking Also decking; furniture What's more cabinetwork; What's more musical instruments. The wood will be utilized for fuel, however just when better forested areas need aid not accessible.

Chapter 3

Materials and method

3.1 Plant Materials

3.1.1 Collection of Plant Leaf

Samples (*S. caseolaris*) were collected from Sundarbans (Hodda forest station, Nalian range, Sunderban east Division, Bangladesh). Fresh and vigorous leaves of *Sonneratia caseolaris* were taken and kept for sterilization process to go into primary culture.

3.1.2 Processing of Plant Leaf

After collection of plant sample from mangrove forest were washed by running tap water. The samples were air dried and kept into a zip lock bag and stored 4°C for further use. Samples were already taken for next sterilization process and kept into laminar hood for isolation and primary culture of endophytic fungi from plants leaf.

3.1.3 Isolation of Endophytic Fungi

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

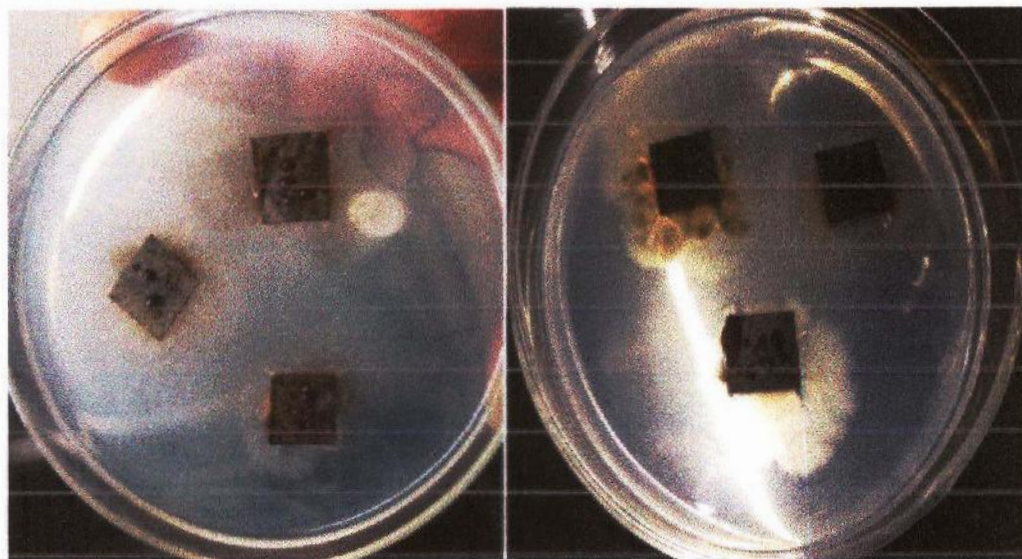


Figure 3.1 Isolation of endophytic fungi from plants leaf

3.1.4 Sterilization of Plant Leaf

The leafs of *S.caseolaris* cut into 1cm² sized small pieces. The small pieces of leaves were taken in an autoclaved petri dish. Small pieces of leaves washed by distilled water for 1 minute. Then pieces of leaves were washed by 70% ethanol for 1 minute. After that those pieces of leaves were washed by 5% NaOCl (Sodioum Hypochlorid) for 1-5 minute. Again the leaf pieces were washed with distilled water and water was removed by sterile filter paper. finally the leaf pieces were dried and prepared for isolation of endofhytic fungi from the sample, forceps and tips used in this technique were autoclave before use. All of the steps were done into laminar hood.

3.2 Primary Culture Preparation

Water agar medium were prepared for isolation endophytic fungi.

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
- Aluminium 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 Agar were measured by a digital balance machine and were added into the conical flask. Agar were dissolved by heating using microwave oven. After 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 autoclave at 121°C (15/1bs) for 15 mints. Finally, the medium was poured into petri dishes, cooled and stored for future use.

3.2.3 Inoculation

The leaf pieces were placed onto the water agar sterile medium using sterile forceps. Each agar plate were inoculated with three piece of leaf of the plant. After that, kept the agar plate into an incubator at 24°C. the procedure is performed in laminar hood (biosefty cabinet). Finally, the fungus were allowed to grow in incubator for 3-4 days and checked for growth regularly until it is transformed to pure culture.



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 500ml conical flask. Then, 9.9ml of PDA powder 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°C (15/1bs) for 15 mints. Finally, the medium was poured into petri dishes.

3.3.2 Inoculation

The prepared PDA media was poured into petri dishes where each of the petri dishes containing 20ml medium. Let them cool to semi solid medium in plate. Then the fungal growth cut from the primary culture from the growing edges using cork borer. After that it

was transferred into the PDA media containing. The procedure is performed in laminar hood (biosefty cabinet). Finally, the plates were kept in the incubator and checked for growth regularly until they from spores.

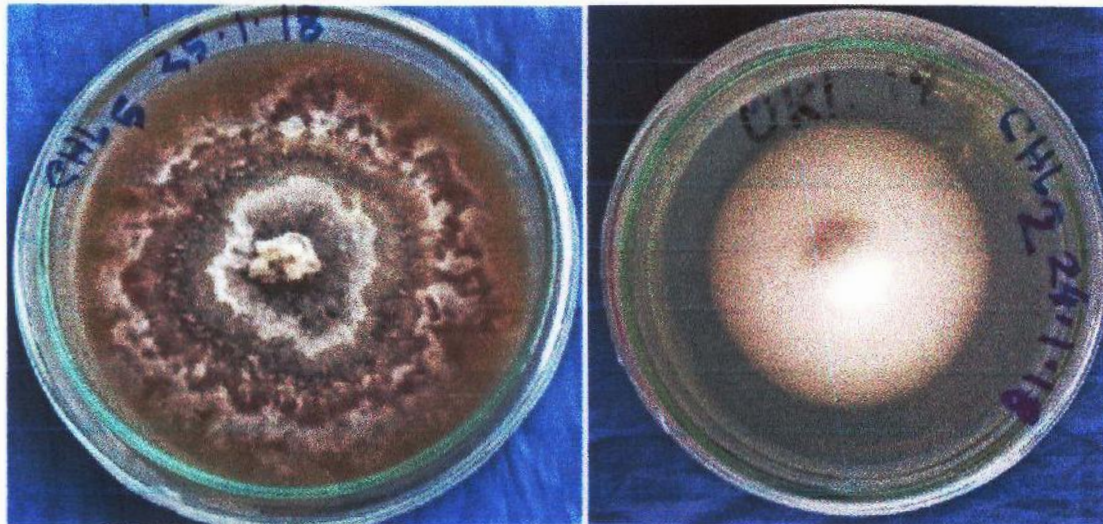


Figure 3.2 Pure culture of endophytic fungi

3.4 Preservation of Endophytic Fungi

3.4.1 Materials and instrument

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

3.4.2 Media preparation

At first, a clean cylinder was taken to measure 250 ml distilled water and poured it into a 500ml conical flask. Then, 9.9ml of PDA powder 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°C (15/lbs) for 15 mins. Finally, the medium was poured into falcon tubes. All process was occurred into laminar air flow

3.4.3 Preservation Process

For long-term storage, the pure fungal strains were transferred to 10-ml BD Falcon tubes containing ~5 ml preservation media. When growth can be observed (after ~3 d, depending on the fungal strain), the fungal strain was stored in freeze stepwise: first place it in a fridge at 4 °C for 2 h, then in a freezer at – 20 °C for 2 h and finally place it in a deep freezer at – 80 °C. Frozen pure fungal strains can be stored at – 80 °C. For recovery from – 80 °C, thaw the frozen culture quickly in a water bath at 37 °C and transfer a small piece with a sterile loop to a petri dish containing medium for pure culture.



Figure: 3.3 Preservation of endophytic fungi

3.5 Identification of Endophytic Fungi

Fungi were identified by means of macroscopic and microscopic observation.

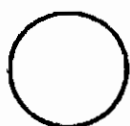
3.5.1 Morphological Identification

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.
- 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.

Form



Circular



Irregular



Filamentous



Rhizoid

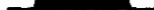
Elevation



Raised



Convex



Flat



Umbonate



Crateriform

Margin



Entire



Undulate



Filiform



Curled



Lobate

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.

3.6 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 28⁰C 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.

3.7 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.

3.7.1 Materials and Instruments Used for Cultivation

- Petridish
- Conical flask
- Micro-oven
- Measuring cylinder
- Cotton
- Cotton plug
- 70% Ethanol
- Loop
- Para film
- PD broth media
- Pure culture of fungi etc.

3.7.2 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 333.33gm potato was measured and sliced. Potato slices with distilled water were heated in a deck until potato extract were come out which took up to 60 minutes. Potato extract were taken in a conical flask and 33.33 gm of dextrose was added and dissolved using magnetic stirrer. After dissolved 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°C (15psi/lbs) for 15 minutes. Finally media was poured into conical flask, cooled and stored for future use.

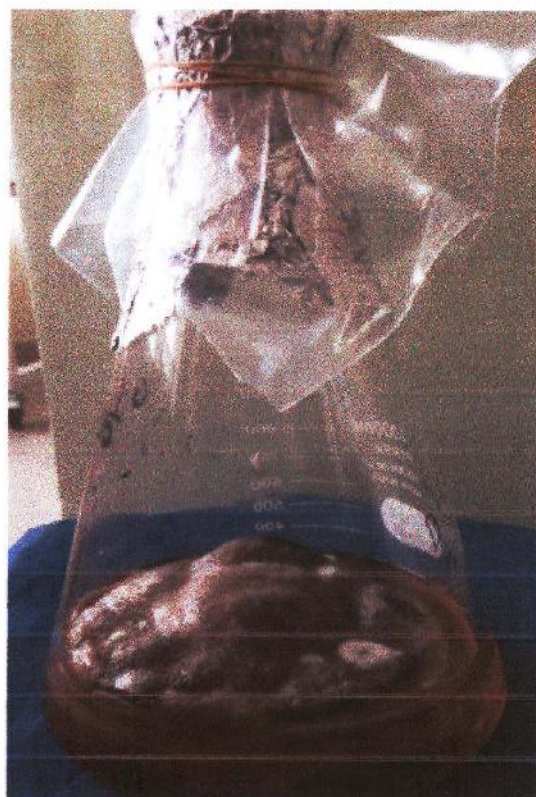


Figure: 3.5 Liquid PD medium

3.8 Transfer 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 inoculated petri dish into small pieces of $\sim 1.5 \text{ cm} \times 1.5 \text{ cm}$ and these pieces were transferred 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.

3.9Extraction of Secondary Metabolites

3.9.1Materials and Instruments Used for Extraction

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

3.9.2 Extraction

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. The broth was transferred to a separatory funnel and ethyl acetate was added in 2:1 ratio. Upper layer is separated from the separatory funnel. Then the secondary metabolite from the diffracted broth was separated by extracting with DCM, a medium polar solvent. Then the extracted liquid evaporated by rotary evaporator at 37⁰c.

3.10Genomic DNA Extraction

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

- 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.

3.11 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 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.

3.12 DNA confirmation by Agarose gel electrophoresis

- **Preparation of 1.5% Agarose gel**

1g of Agarose and 100ml distilled water were measured and taken in a conical flask with 500 μ l 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**

10 μ l of DNA sample was taken in a sterile PCR tube and 2 μ l 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 (7 μ l 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, 10 μ l 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 mins.

- **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.

3.13 DNA Quantification

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

3.14 DNA dilution

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

3.15 PCR Reaction

3.16 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 mins. The reactions was placed in a room temperature thermal cycler. PCR was performed by using standard parameters.

3.17 Amplification by PCR

3.17.1PCR profile

Denaturation

Initial denaturation at 95°C for 5 minutes

Annealing

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

Extension

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

3.18 Cycle Number

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

3.19 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.

3.20 DNA sequencing and phylogenetic tree construction

After getting the DNA band, purified DNA was ready for the sequencing. Phylogentic Analysis Using Parsimony (PAUP) software is used for sequencing.

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.

3.24 Preliminary bioassay

There are different test parameters to investigate bioactivity such as-

3.24.1 Antimicrobial test;

3.24.2 Antioxidant test;

3.24.3 Brine Shrimp Lethality Bioassay.

3.24.1 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)

3.241.1 Principle

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

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

3.24.1.2 Apparatus and reagents

- | | |
|-------------------------|------------------|
| ◆ Filter paper discs | ◆ sterile cotton |
| ◆ Micropipette | ◆ Refrigerator |
| ◆ Laminar air flow hood | ◆ Chloroform |
| ◆ Sterile forceps | ◆ Petri dishes |



- ♦ Screw cap test tubes
- ♦ Nutrient Agar Medium
- ♦ Inoculating loop
- ♦ Nose mask and Hand gloves
- ♦ Autoclave
- ♦ Spirit burner
- ♦ Ethanol
- ♦ Incubator etc.

3.24.1.3 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

Table 3.1: List of test microorganism

Gram-positive Bacteria	Gram-negative Bacteria
<i>Micrococcus</i>	<i>Escherichia coli</i>
<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>

3.25 Procedures

3.25.1. Culture medium selection

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

3.25.2Preparation 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 121⁰C for 20 minutes. The slants were used for making fresh culture of bacteria that were in turn used for sensitivity study.

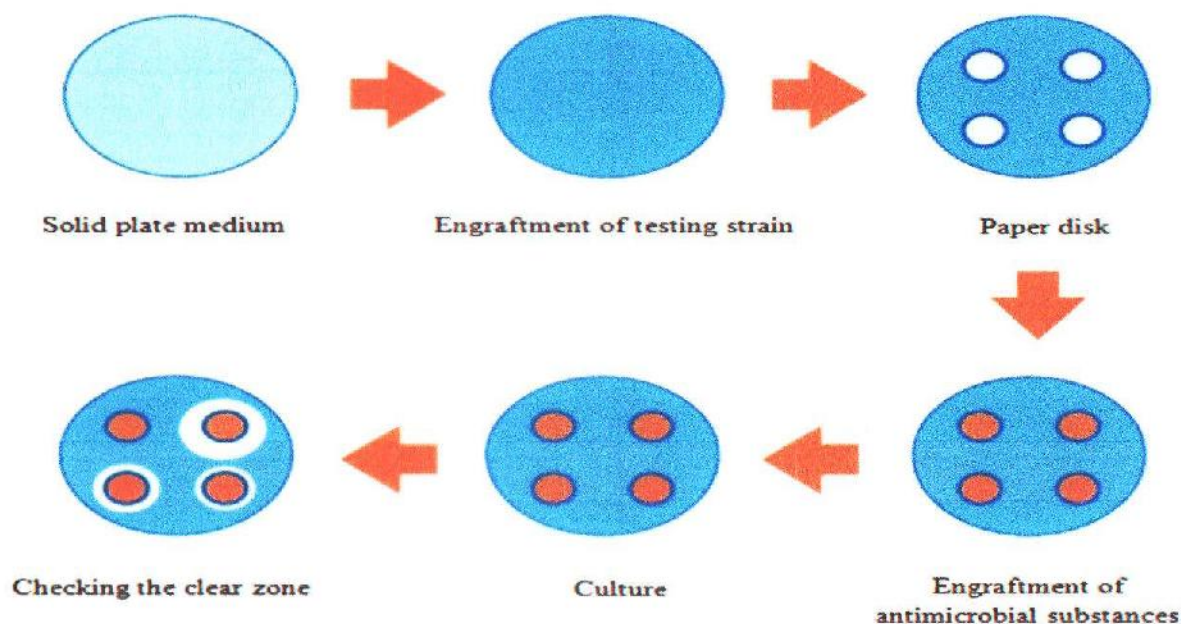


Figure 3.6: In vitro investigation of antimicrobial activity following the disk diffusion assay

3.25.3 Sterilization procedures

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

3.25.4 Preparation 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°C (for bacteria) and 25°C (for fungi) for their optimum growth.

3.25.5 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 (10µg/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.

3.25.6 Preparation of test sample discs

The extracts of endophytic fungi were tested for antimicrobial activity against a number of both gram positive and gram negative bacteria. The amount of sample per disc was 10µg of the endophytic fungi extracts.

3.25.7 Preparation of the test plates

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

3.25.8 Application of the test samples

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

3.25.9 Diffusion and incubation

The plates were then kept in a refrigerator at 4⁰C 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 37⁰C for 24 hours.

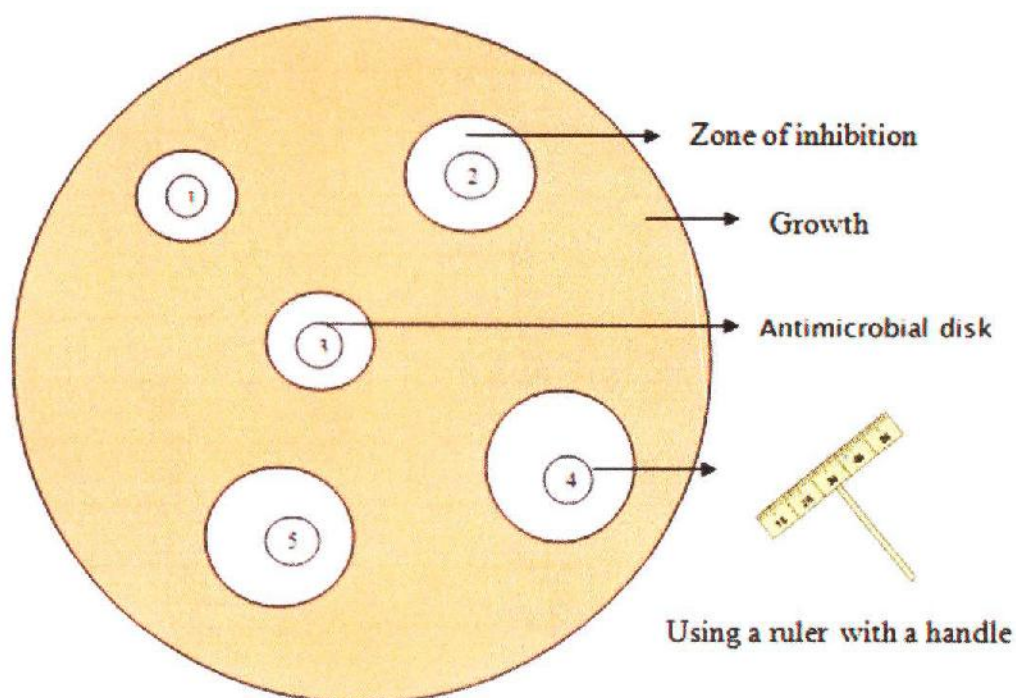


Figure 3.8: Measuring the zone of inhibition

3.25.10 Determination of antibacterial activity by measuring the zone of inhibition

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

3.24.2 Antioxidant activity: DPPH assay

3.24.2.1 Materials

- Apparatus
- Test tubes
- Beakers
- Vials
- Thermometer
- Micropipette

- UV spectrophotometer (Shimadji 1800)
- Electronic balance

3.24.2.2 Reagents

- Methanol
- 0.02 mcg/ml methanolic solution of DPPH
- Ascorbic acid(ASA)
- Distilled Water

3.24.2.3 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:

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

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.

3.24.3 Brine Shrimp Lethality Bioassay

Brine shrimp eggs are hatched in simulated sea water to get nauplii. Test samples are prepared by dissolving in dimethylsulfoxide (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 pre marked vials through micropipette. The vials are then left for 24 hours and then the nauplii are counted again to find out the cytotoxicity of the test agents.



Figure 3.9: *Artemia salina* leach (brine shrimp eggs)

3.24.3.1 Materials

- *Artemia salina* 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.

3.24.3.2 Methodology

3.24.3.2.1 Preparation of sea water

38g Sea salt (pure NaCl 20g and table salt 18g) was weighed accurately, dissolved in distilled water to make one liter and then filtered off to get a clear solution.

3.24.3.2.2 Construction of hatchery

The two-liter plastic cone shape bottle and cap was cleaned with detergent first and then with hot water. A sharp knife was used to cut one third portion of the bottle from bottom side. Then a small hole was made with drill machine or sharp knife to ensure that air pipe can enter into the bottle through the plastic cap. Glue was used to make the cap leak proof. An air stone was placed inside the bottle which was attached with the air pipe of the air pump machine. The threads on the cap and the two-liter bottle was compatible. Then the cap was screwed onto the two-liter bottle. This apparatus was used in inverted position with the help of a stand.

3.24.3.2.3 Hatching of brine shrimp

Sea water was taken in the bottle and shrimp eggs were added. Then the bottle was covered with plastic film. The shrimps were allowed for 18 to 24 hours to hatch and mature as nauplii (larvae). The hatched shrimps were attracted to light to collect them and they were taken for bioassay.

3.24.3.4 Preparation of stock solution

3.2mg of dried ethanol extract of bark and root of *Litsea polyanthaw* was taken in 10mL volumetric flask and volume of 10 mL was adjusted by sea water. The concentration of this solution became 320µg/mL & was considered as stock solution.

3.24.3.4.1 Application of test solution and brine shrimp nauplii into the 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 concentrations. Each test tube was accurately marked to indicate the 5mL volume. Then with the help of the micropipette 5 mL sample of concentration 10, 20, 40, 80, 160, 320, µg/mL were added to that 7 test tubes through serial dilution method and adjusted to 5 mL with saline water to get final concentration of 5, 10, 20, 40, 80, 160 µg/mL respectively. In standard test 7 test tubes were added with 10 alive brine shrimp nauplii and adjusted to 5 mL with saline water to make final concentration of 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05 µg/mL of standard (vincristine sulphate) through dilution method. The concentration of DMSO in these test tubes did not exceed 10 µl/mL. For the control, same volume of DMSO (as in the sample test tubes) were taken in the rest of the 7 test tubes and 10 alive brine shrimp nauplii were added ensuring final volume 5 mL.

3.24.3.4.2 Counting of nauplii

After 24 hrs the test tubes were observed and the number of survived nauplii in each test tube was counted and the results were noted. From this, the percentage of lethality of brine shrimp nauplii was calculated at each concentration for each sample.

$$\% \text{ Mortality} = \frac{[\text{Avg. no. of alive shrimp of control} - \text{Avg. no. of alive shrimp of sample}]}{\text{Avg. no. of alive shrimp of control}} \times 100$$

The test was performed in duplicate to avoid statistical error.

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18/12/19

Chapter: 4

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 *S. caseolaris*.
- Bioactivity test of the fungal extracts;

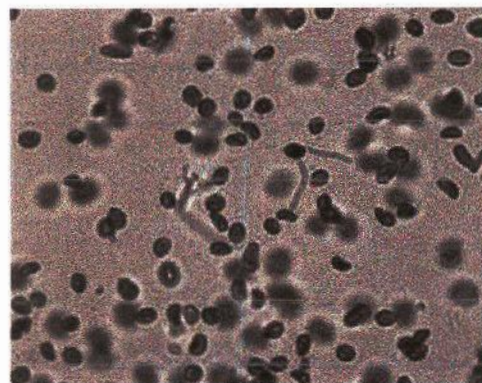
Isolation, cultivation and preparation of crude extracts from both of the mycelia and cultivated medium of fungal endophytes have been done following the published method (Kusari, 2008, Kjer, 2010).

4.2 Identification of the fungal strains

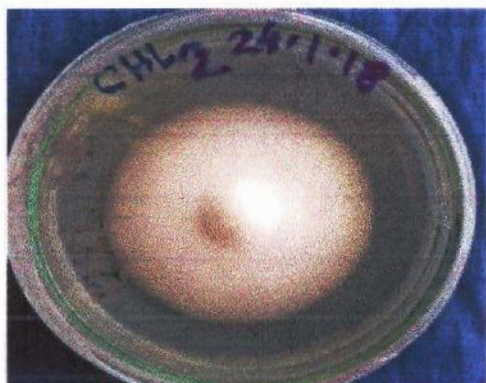
A total of six endophytic fungal strains was isolated and purified from the leaves of *S. caseolaris*. (Figure 4.1)



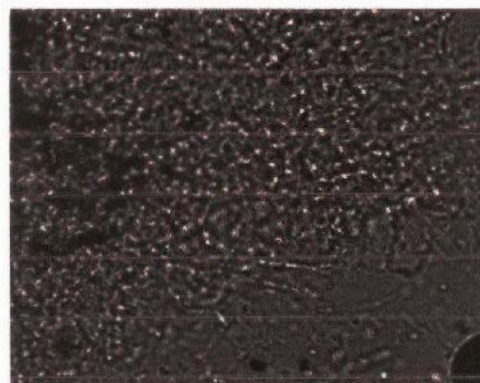
(a)



(b)



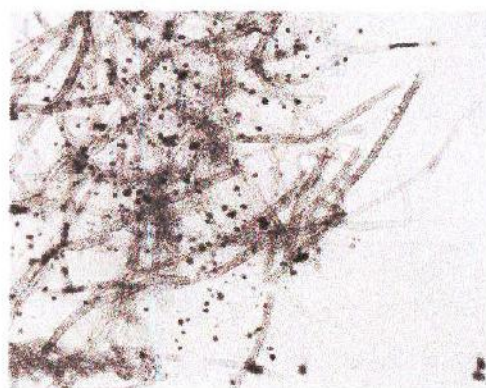
(c)



(d)



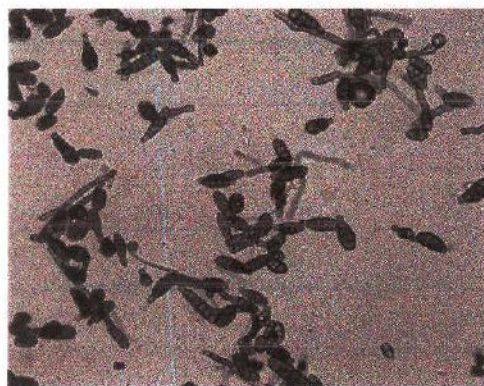
(e)



(f)



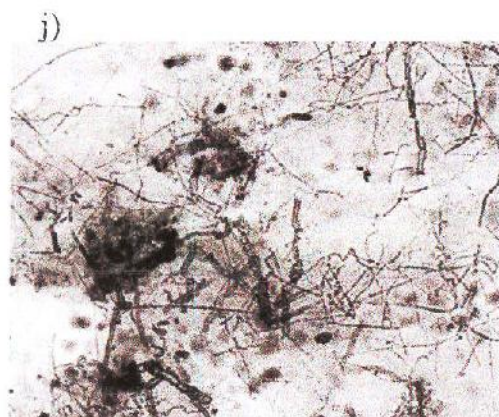
(g)



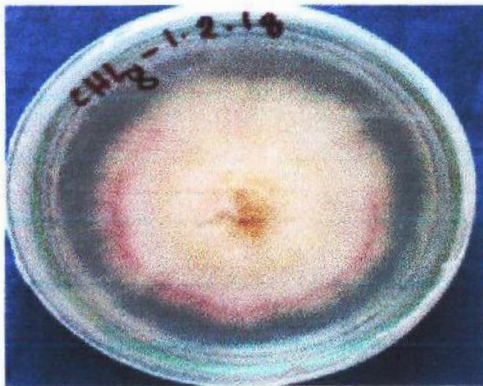
(h)



i)



j)



(k)



(l)

Figure 4.7: a,b,c,d,e,f,g,h,I,j,k,l no picture are showed the Macroscopic and microscopic colony morphologies of the endophytic fungi isolated from mangrove plant

4.2.1 Morphological Characteristics of Endophytic Fungi

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

- Macroscopic Characteristics;
- Microscopic Characteristics;

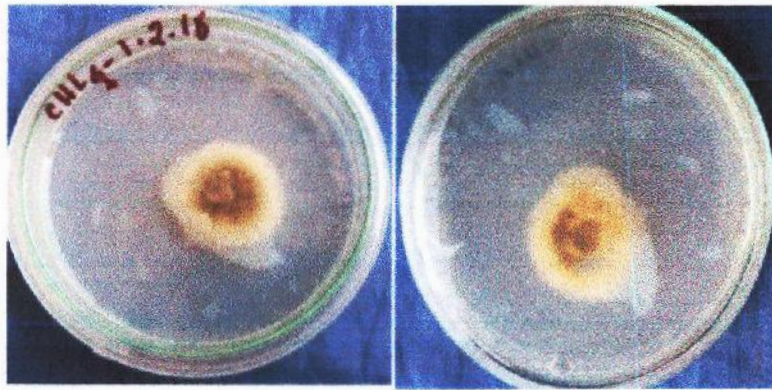
4.2.1.1 Macroscopic Characteristics of the Isolated Fungi

Macroscopic Characteristics

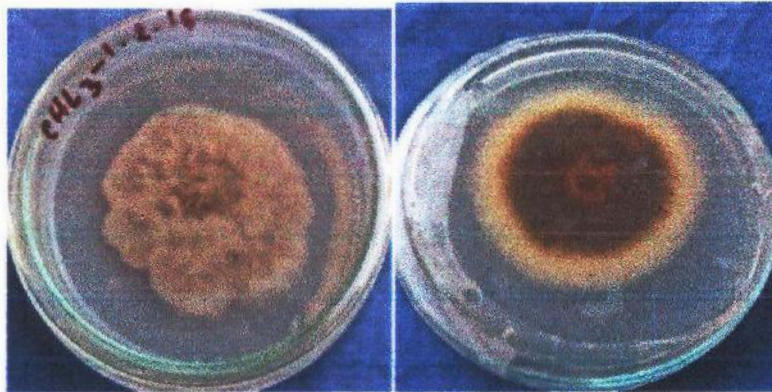
Macroscopic identification was done by visual observation. All the endophytic fungi exhibited characteristics colony and macroscopic morphology that could be used to differentiate them Fungi identification was done following the manual of Plant Pathology by G.N. Agrois (2005). Morphological characteristics were observed on the basis of growth rate of fungi, nature of growth, type of growth, diameter, hyphae, mycelium depth in agar ,morphology of colony, Color of the top view, ,texture of colony surface, side view of colony/ elevation and margin shape of colony .

Table 4.1: Morphology of CHL1

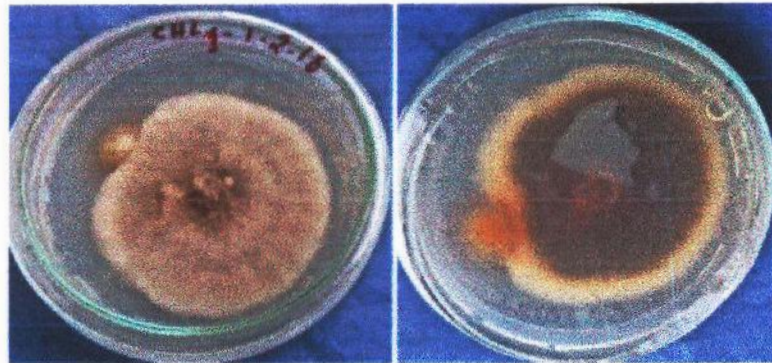
Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Moderate	Slow	Slow	Slow
Nature of growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Spore	Spore	Spore
Diameter	3.8cm	5.4cm	6.0cm	6.2cm
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Irregular	Irregular	Irregular	Irregular
Color of the top view	Blackish	Blackish	Blackish	Blackish
Color of the bottom view	Blackish	Creamy	Blackish	Blackish
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



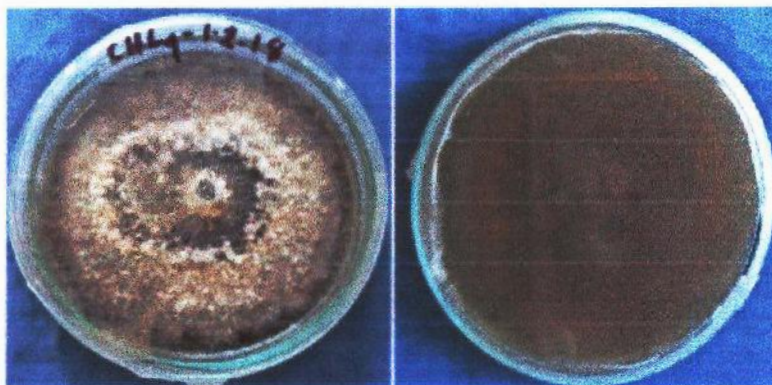
Growth after 3 days



Growth after 6 days



Growth after 9 days

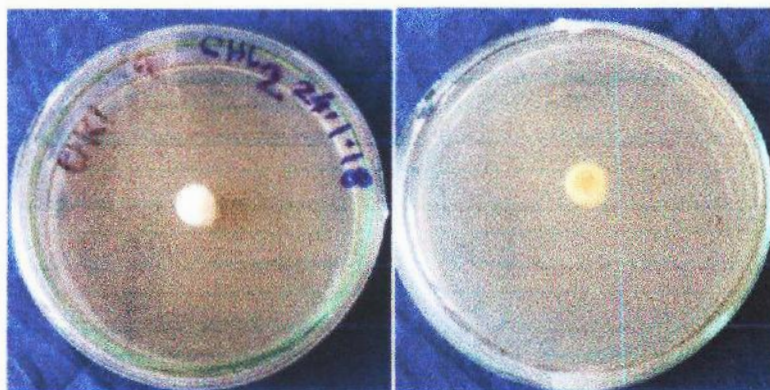


Growth after 12 days

Figure 4.8: Top and bottom view of the fungal strain CHL1 after 3days, 6days, 9 days and 12 days of small scale cultivation

Table 4.2: Morphology ofCHL-2

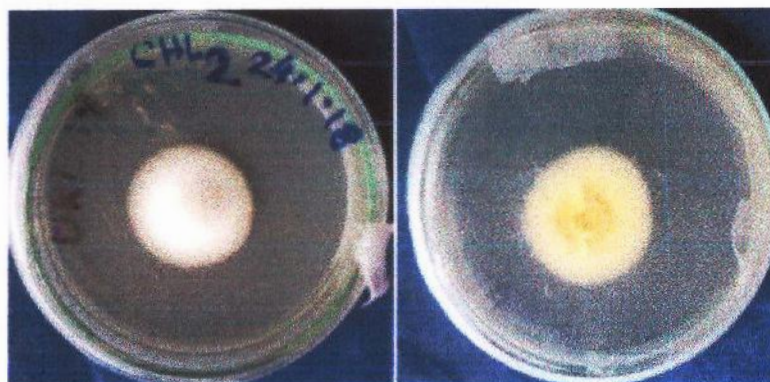
Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Slow	Slow	Slow	Slow
Nature of growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter	1.cm	3.85 cm	5.5 cm	7 cm
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Circular	Circular	Circular	Circular
Color of the top view	White	White	White	White
Color of the bottom view	White	White	White	White
Texture of colony surface	Wooly	Wooly	Wooly	Wooly
Side view of colony/ Elevation	Convex	Convex	Convex	Convex
Margin shape of colony	Entire	Entire	Entire	Entire



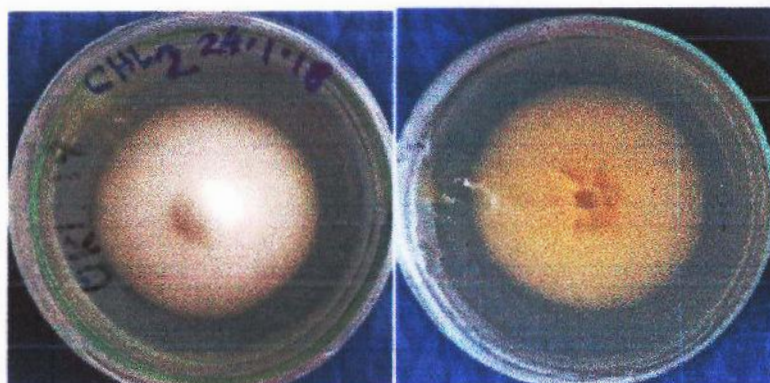
Growth after 3 days



Growth after 6 days



Growth after 9 days

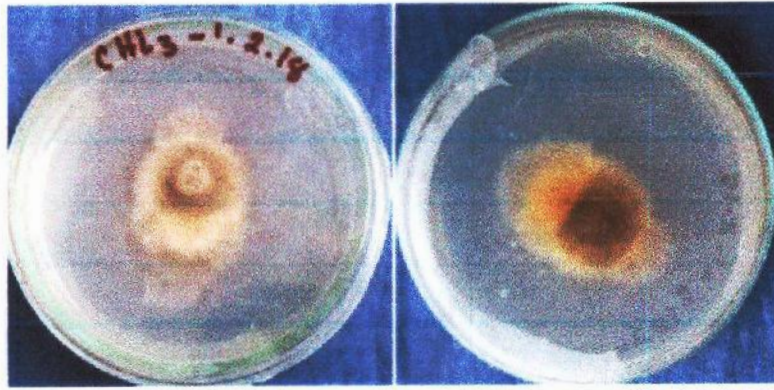


Growth after 12 days

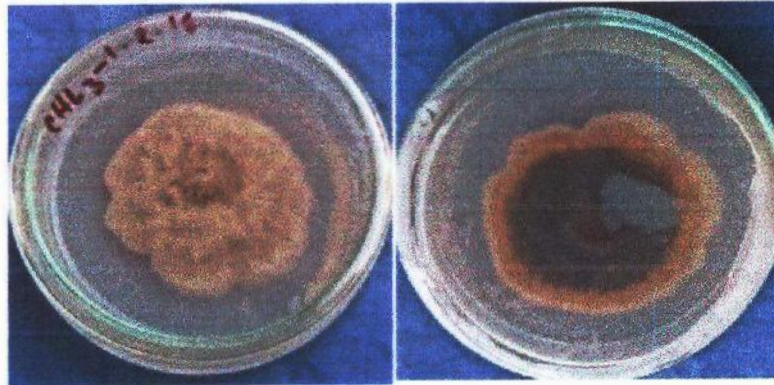
Figure 4.9: Top and bottom view of the fungal strain CHL2 after 3 days, 6 days, 9 days and 12 days of small scale cultivation

Table 4.3: Morphology of CHL-3

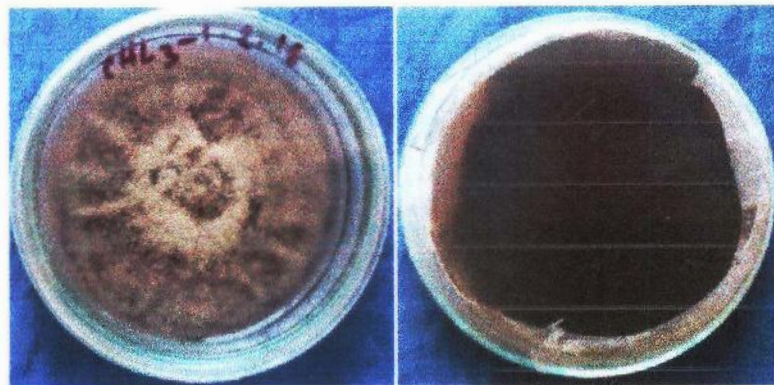
Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Slow	Rapid	Rapid	Rapid
Nature of growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter	3.25cm	4.65 cm	5.5 cm	7.8 cm
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Irregular	Irregular	Irregular	Irregular
Color of the top view	Whitish creamy	Gray	Blackish gray	Blackish gray
Color of the bottom view	Grey	Grey	Grey	Grey
Texture of colony surface	Smooth	Smooth	Smooth	Smooth
Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of colony	Entire	Entire	Entire	Entire



Growth after 3 days



Growth after 6 days



Growth after 9 days

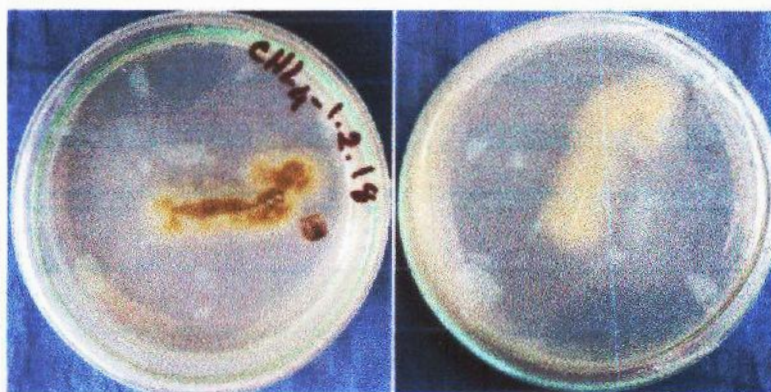


Growth after 12 days

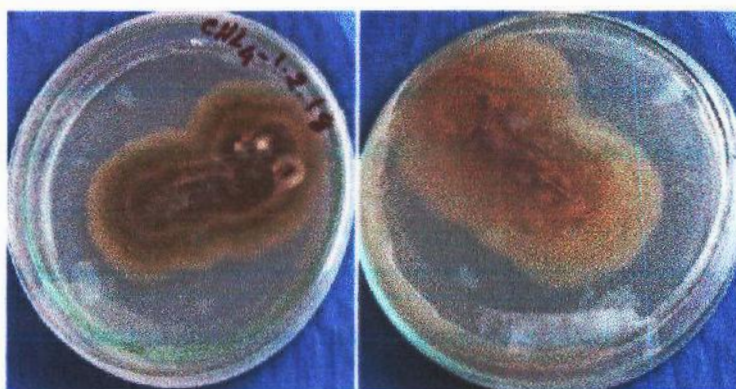
Figure 4.10: Top and bottom view of the fungal strain CHL3 after 3 days, 6 days, 9 days and 12 days of small scale cultivation

Table 4.4: Morphology of CHL-4

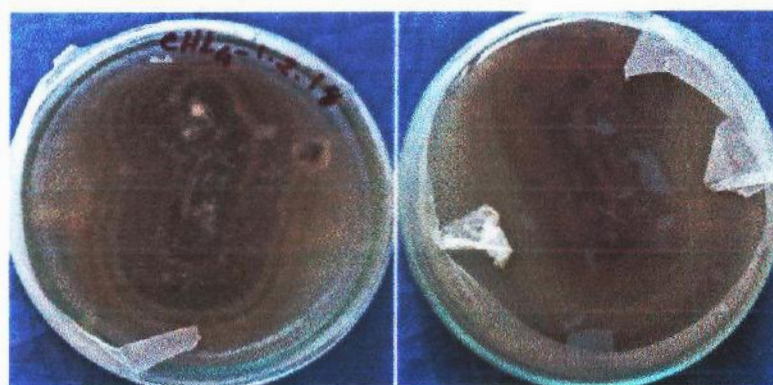
Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Rapid	Rapid	Rapid	Rapid
Nature of growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Spore	Spore	spore
Diameter	1 cm	3.85 cm	5.5 cm	7 cm
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Circular	Circular	Circular	Circular
Color of the top view	White	White	White	White
Color of the bottom view	White	White	White	White
Texture of colony surface	Wooly	Wooly	Wooly	Wooly
Side view of colony/ Elevation	Convex	Convex	Convex	Convex
Margin shape of colony	Entire	Entire	Entire	Entire



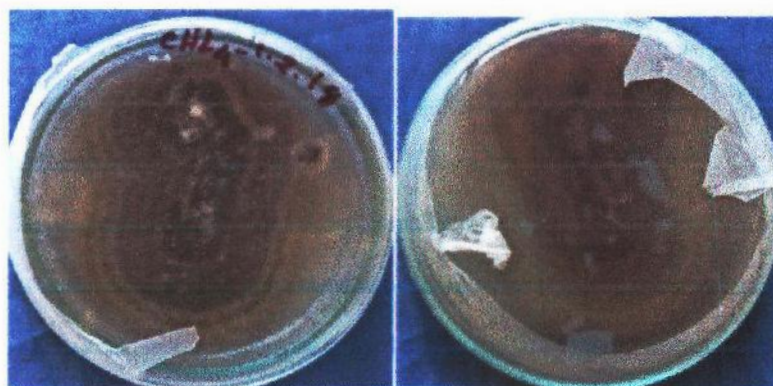
Growth after 3 days



Growth after 6 days



Growth after 9 days

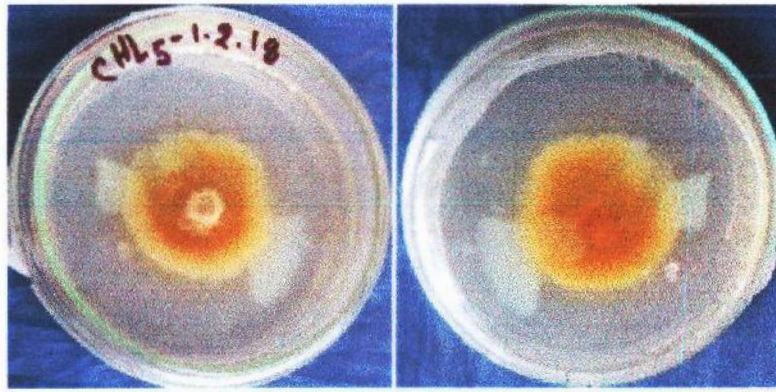


Growth after 12 days

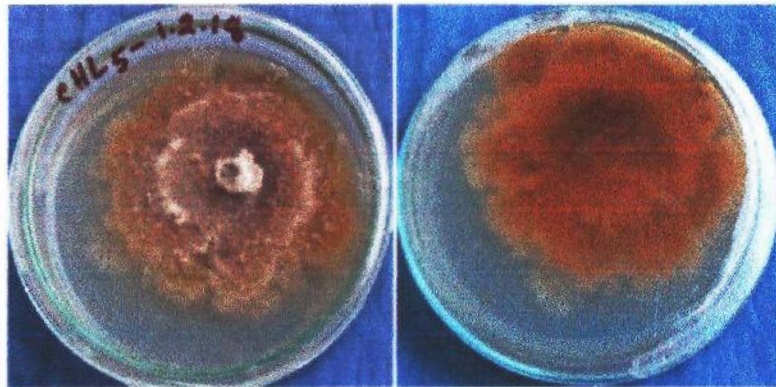
Figure 4.11: Top and bottom view of the fungal strain CHL4 after 3 days, 6 days, 9 days and 12 days of small scale cultivation

Table 4.5: Morphology of CHL-5

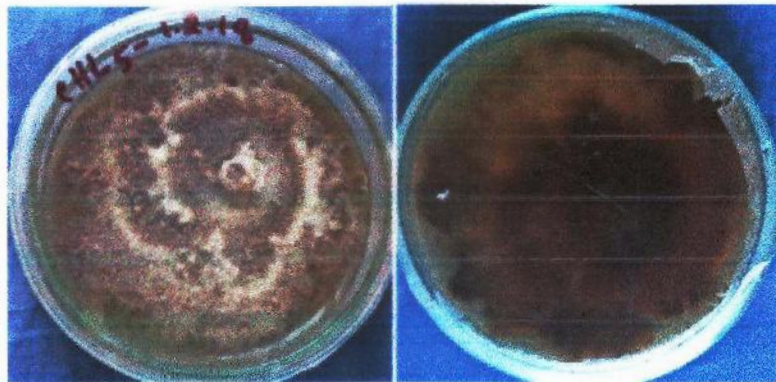
Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Slow	Rapid	Rapid	Rapid
Nature of growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Vegetative	Spore	Spore
Diameter	3.9cm	4.65 cm	5.5 cm	6.8 cm
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Irregular	Irregular	Irregular	Irregular
Color of the top view	Yellow	Gray	Blackish gray	Blackish gray
Color of the bottom view	Yellow	Grey	Grey	Grey
Texture of colony surface	Dray	Dray	Wrinkled	Wrinkled
Side view of colony/ Elevation	Flat	Flat	Flat	Flat
Margin shape of colony	Entire	Entire	Entire	Entire



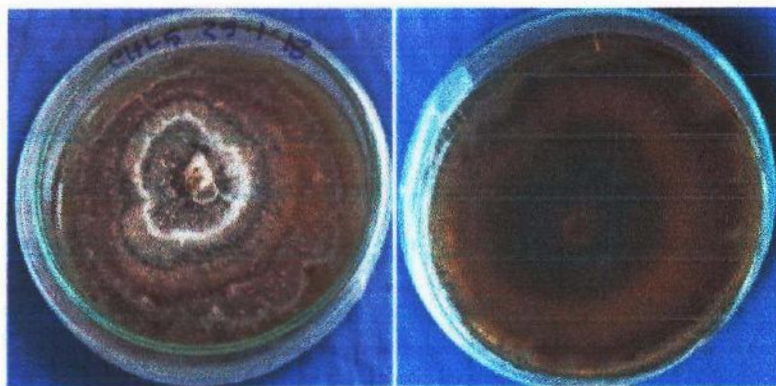
Growth after 3 days



Growth after 6 days



Growth after 9 days

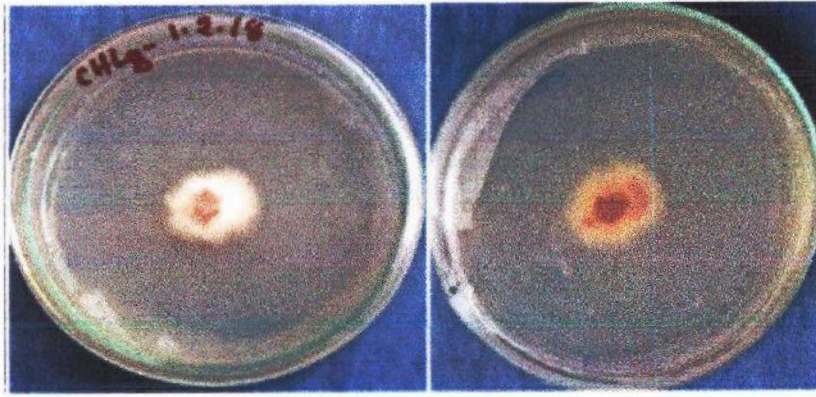


Growth after 12 days

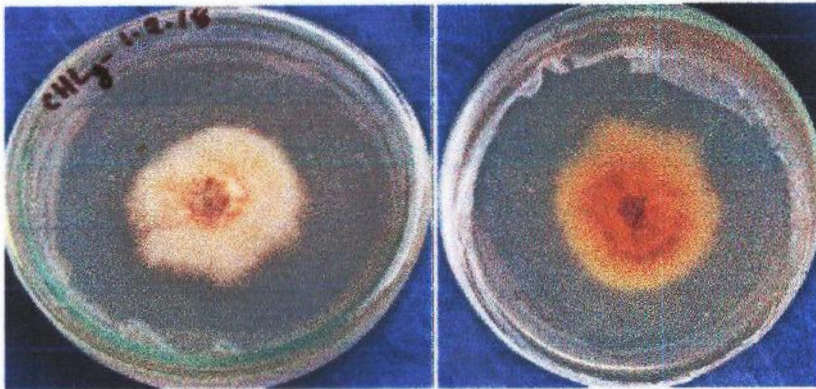
Figure 4.12: Top and bottom view of the fungal strain CHL3 after 3 days, 6 days, 9 days and 12 days of small scale cultivation

Table 4.6: Morphology of CHL-8

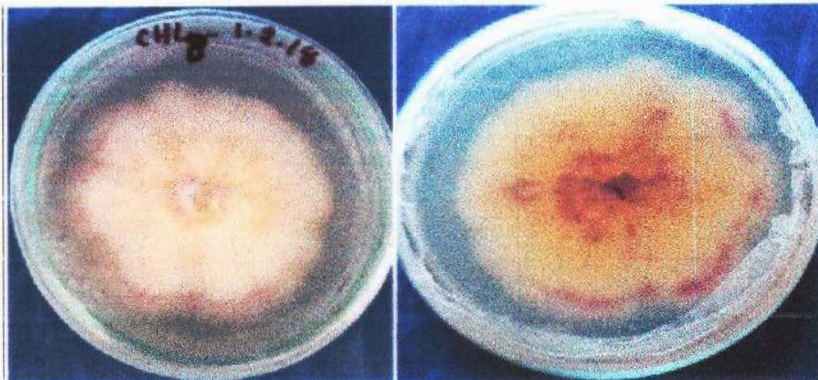
Characteristics	Observation (After 3 days)	Observation (After 6 days)	Observation (After 9 days)	Observation (After 12 days)
Growth rate of fungi	Slow	Slow	Slow	Slow
Nature of growth	Centre to edge	Centre to edge	Centre to edge	Centre to edge
Type of growth	Vegetative	Vegetative	Vegetative	Vegetative
Diameter	2.05cm	3.75cm	4.15cm	5.2cm
Hyphae	Aerial	Aerial	Aerial	Aerial
Mycelium depth in agar	Shallow	Shallow	Shallow	Shallow
Morphology of colony	Filamentous	Filamentous	Filamentous	Filamentous
Color of the top view	White	Whitish pink	Whitish pink	Whitish pink
Color of the bottom view	White	Pinkish	Pinkish white	Pinkish white
Texture of colony surface	Dray	Dray	Wrinkled	Wrinkled
Side view of colony/ Elevation	Raised	Raised	Raised	Raised
Margin shape of colony	Entire	Entire	Entire	Entire



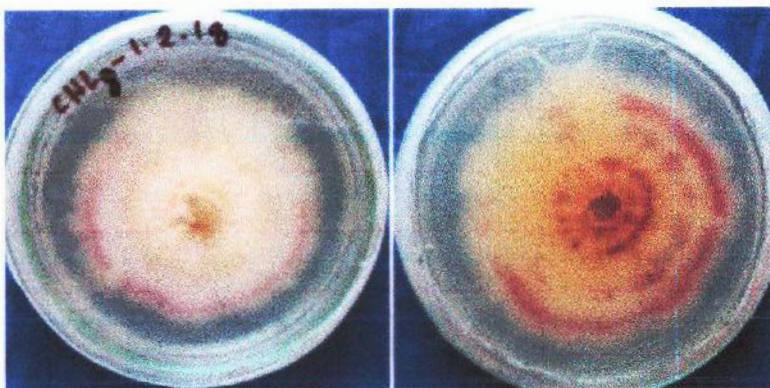
Growth after 3 days



Growth after 6 days



Growth after 9 days



Growth after 12 days

Figure 4.13: Top and bottom view of the fungal strain CHL3 after 3 days, 6 days, 9 days and 12 days of small scale cultivation

4.2.1.2 Microscopic characteristics of the Identified Fungi

Slides were prepared from cultures and stained with glycerine 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

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.

CHL1: The spherical structure consider as a spore.

CHL2: The mycelium was observed as long branched hyphe.

CHL3: In the culture plate, the mycelium was observed as long branched hyphe. The color of hyphe is gray when observed.

CHL4: Branched acropetal chains of multicellular conidia. Some conidiophores are branched, short or elongate. Conidia are obclavate, obpyriform, ovoid or ellipsoidal, often with a short conical or cylindrical beak, pale brown, smooth-walled or verrucose.

CHL 5: The mycelium was observed chains long filamentous hyphe. The color of hyphe is blackish gray and non spore when observed

CHL8: The mycelium was observed as long branched unsepted hyphe.

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

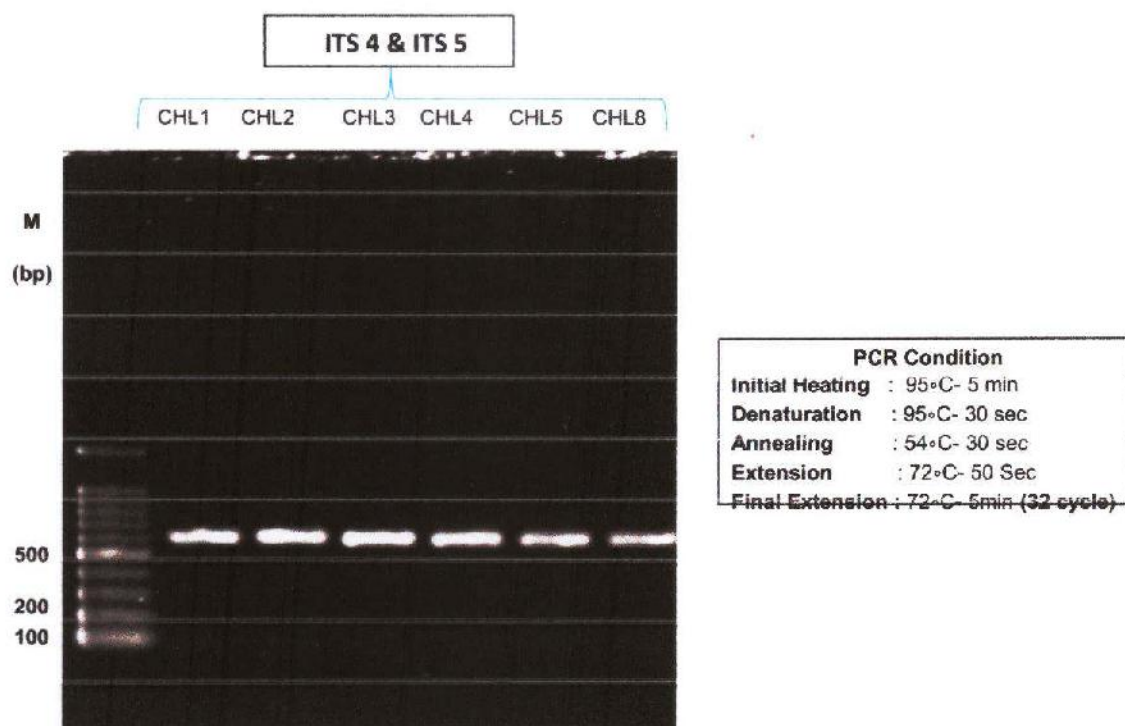


Fig4.14: ITS profiles of ITS 4 and ITS 5 primers generated from 6 different Plant CHL1, CHL2, CHL3, CHL4, CHL5, CHL6, M: denotes 100bp DNA ladder (Marker).

4.5 Genetic information of fungi from (National Center for Biotechnology Information)

After getting purified PCR product of DNA, sequencing was done and genetic information was found by input the BLAST and FASTA file in NCBI. That gave the genetic information and species name of fungi. The results showed the identity with the sample that is given below

Table: 4.6 Endophytic fungi isolate from *S.caseolaris* with NICB accession no

Internal Code	Name of the Fungi	Genbank Accession Number	BLAST		
			Match Sequence		
			Reference Accession Number	Query Length	Max Identity
CHL-1	<i>Wardomyces moseri</i>	MH 517570	Ln850995.1	569	100%
CHL-2	<i>simplicillium clavatum</i>	MH 510099	MG597047.1	609	99%
CHL-3	<i>Alternaria destruens</i>	MH 517571	MH037553.1	570	99%
CHL-4	<i>alternaria porri</i>	MH 517576	JN198481.1	572	99%
CHL-5	<i>diaporthe recaer</i>	MH 517577	KC343032.1	579	99%
CHL-8	<i>Apicoccum nigrum</i>	MN 517578	KX067870.1	545	99%

4.4 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 samples are given below

Table 4.7: Pair wise genetic distance of endophytic fungi isolated from *S.caseolaris*

	MH517570	MH510099	MH517571	MH517576	MH517577	MH517578
MH517570	-					
MH510099	0.75	-				
MH517571	0.75	0.76	-			
MH517576	0.74	0.68	0.74	-		
MH517577	0.70	0.73	0.75	0.72	-	
MH517578	0.74	0.75	0.75	0.76	0.72	-

UPGMA tree



Figure4.15: UPGMA dendrogram of six isolated fungi from *S.caseolaris*

4.5 Antioxidant 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 acited extract of CHL1

Concentration	Log con	Abs1	Abs2	Average	% inhibition	IC ₅₀
3.12	0.494155	0.443	0.476	0.459±0.02	23.28881	
6.24	0.795185	0.401	0.399	0.4±0.001	33.22204	
12.5	1.09691	0.366	0.365	0.3655±0.0007	38.98164	
25	1.39794	0.321	0.341	0.331±0.01	44.74124	22.830
50	1.69897	0.213	0.231	0.222±0.01	62.93823	
100	2	0.122	0.165	0.1435±0.03	76.04341	
200	2.30103	0.11	0.132	0.121±0.01	79.79967	
Blank		0.654	0.544	0.599±0.07		

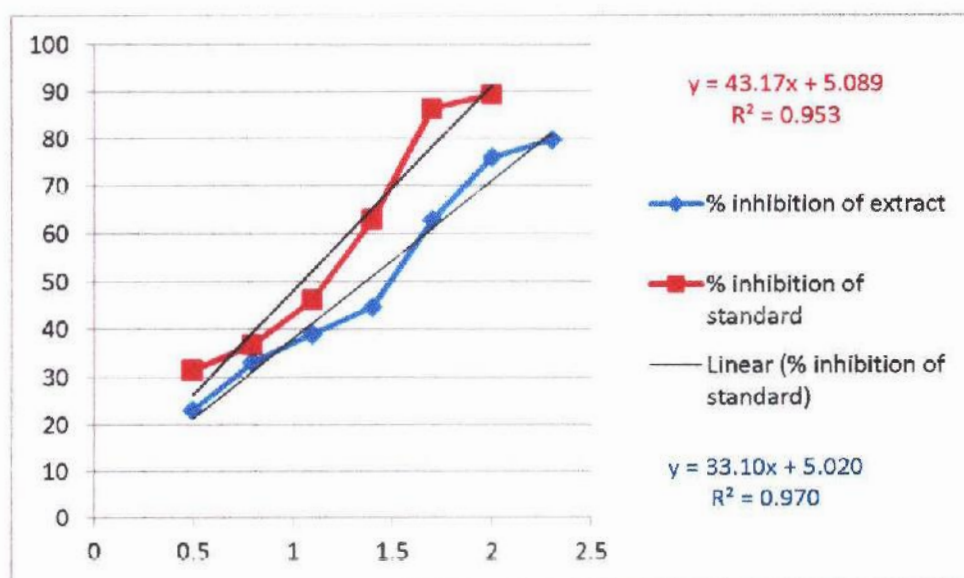


Figure 4.16: IC_{50} Value of Ethyl acited extract CHL1



Table 4.9: DPPH Scavenging Assay of methanol extract of CHL1

Concentration	Log con	Abs1	Abs2	Average	% inhibition	IC50
3.12	0.494155	0.297	0.302	0.2995±0.003	30.10502	
6.24	0.795185	0.289	0.289	0.289±0.00	32.55543	
12.5	1.09691	0.278	0.288	0.283±0.007	33.95566	
25	1.39794	0.203	0.234	0.2185±0.2	49.00817	28.973
50	1.69897	0.176	0.166	0.171±0.007	60.09335	
100	2	0.122	0.154	0.138±0.02	67.79463	
200	2.30103	0.189	0.105	0.147±0.05	65.69428	
Blank		0.455	0.402	0.42850±0.05		

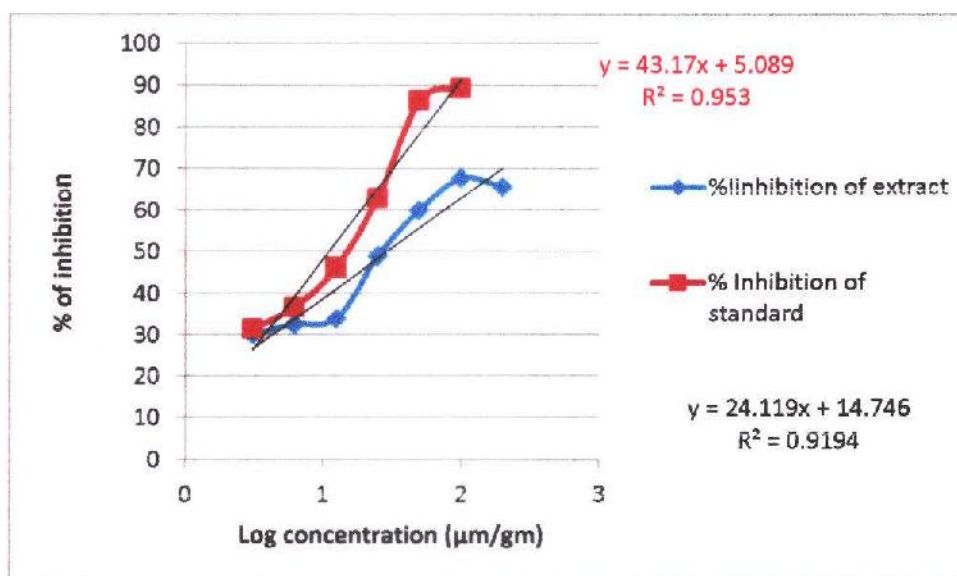


Figure 4.17: IC₅₀ value methanol extract of CHL1

Table 4.10: DPPH Scavenging Assay of Ethyl acited extract of CHL2

Concentration($\mu\text{g/ml}$)	Log con	Abs1	Abs2	Average	% Inhibition	IC50
3.12	0.494155	0.543	0.463	0.503 ± 0.05	4.64455	
6.24	0.795185	0.424	0.389	0.4065 ± 0.02	22.93839	
12.5	1.09691	0.399	0.377	0.388 ± 0.01	26.4455	
25	1.39794	0.365	0.304	0.3345 ± 0.04	36.58768	95.49
50	1.69897	0.278	0.299	0.2885 ± 0.01	45.30806	
100	2	0.267	0.275	0.271 ± 0.005	48.62559	
200	2.30103	0.227	0.235	0.231 ± 0.005	56.20853	
Blank		0.544	0.511	0.5275 ± 0.2		

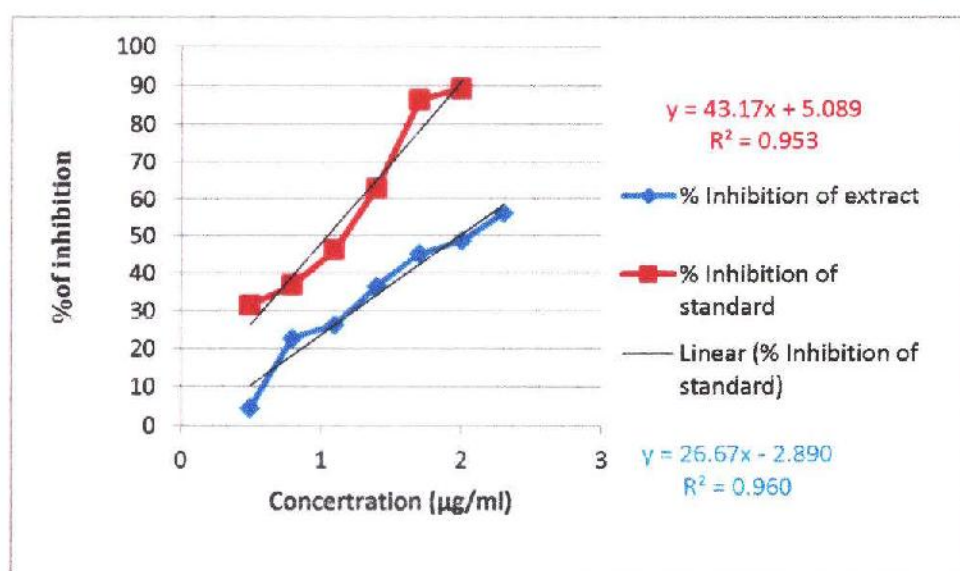


Figure 4.18: IC₅₀ Value of Ethyl acited extract CHL2

Table 4.11: DPPH Scavenging Assay of Methanol extract of CHL2

Concentration($\mu\text{g/ml}$)	Log con	Abs1	Abs2	Average	% inhibition	IC ₅₀
3.12	0.494155	0.502	0.485	0.4935 $\pm$ 0.01	4.913295	
6.24	0.795185	0.409	0.393	0.401 $\pm$ 0.01	22.73603	
12.5	1.09691	0.347	0.334	0.3405 $\pm$ 0.009	34.39306	
25	1.39794	0.377	0.303	0.34 $\pm$ 0.05	34.4894	158.48
50	1.69897	0.278	0.345	0.3115 $\pm$ 0.05	39.98073	
100	2	0.267	0.321	0.294 $\pm$ 0.03	43.3526	
200	2.30103	0.227	0.296	0.2615 $\pm$ 0.04	49.61464	
Blank		0.535	0.503	0.519 $\pm$ 0.02		

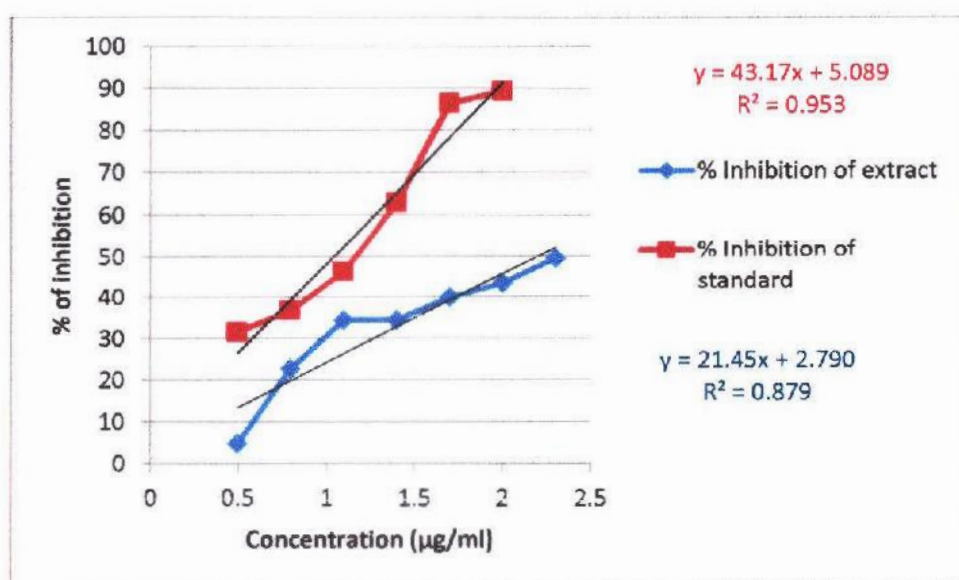


Figure 4.19: IC₅₀ value methanol extract of CHL2

Table 4.13: DPPH Scavenging Assay EAtoH extract of CHL3

Concentration($\mu\text{g/ml}$)	Log con	Abs1	Abs2	Average	% inhibition	IC50
3.12	0.494155	0.543	0.578	0.5605 ± 0.03	1.838879	
6.24	0.795185	0.354	0.354	0.354 ± 0.0	38.0035	
12.5	1.09691	0.288	0.287	0.2875 ± 0.0007	49.64974	
25	1.39794	0.266	0.203	0.2345 ± 0.04	58.9317	
50	1.69897	0.157	0.174	0.1655 ± 0.01	71.01576	19.95
100	2	0.166	0.134	0.15 ± 0.02	73.7303	
200	2.30103	0.099	0.11	0.1045 ± 0.007	81.69877	
Blank		0.544	0.598	0.571 ± 0.03		

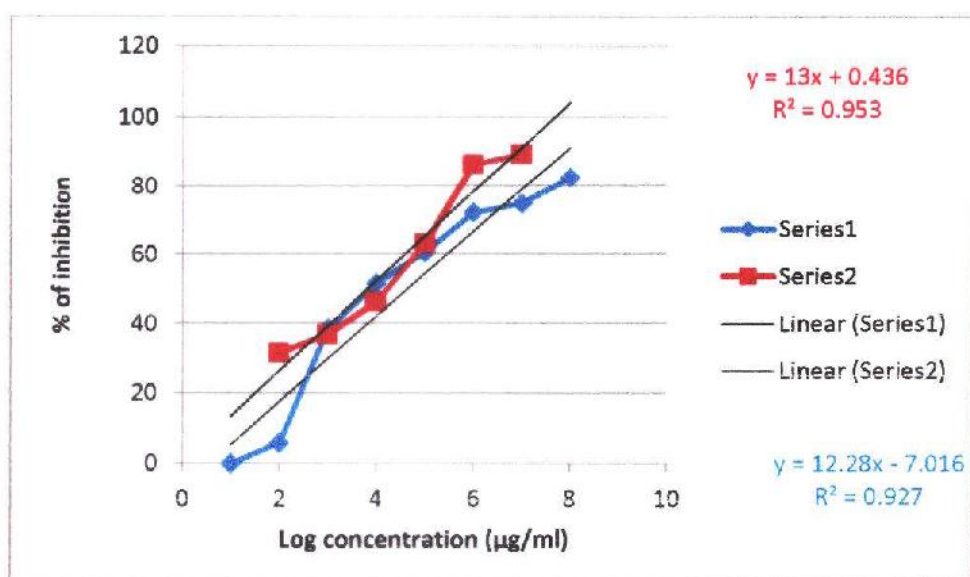


Figure 4.1: IC₅₀ Value of Ethyl acited extract CHL3

Table 4.14: DPPH Scavenging Assay of Methanol extract of CHL3

Concentration($\mu\text{g/ml}$)	Log con	Abs1	Abs2	Average	% inhibition	IC50
3.12	0.494155	0.574	0.521	0.5475 ± 0.03	4.031551	
6.24	0.795185	0.487	0.466	0.4765 ± 0.01	16.47677	
12.5	1.09691	0.421	0.454	0.4375 ± 0.02	23.31288	
25	1.39794	0.342	0.303	0.3225 ± 0.02	43.47064	31.62
50	1.69897	0.243	0.228	0.2355 ± 0.02	58.72042	
100	2	0.122	0.133	0.1275	77.65118	
200	2.30103	0.101	0.112	0.1065	81.33216	
Blank		0.552	0.589	0.5705		

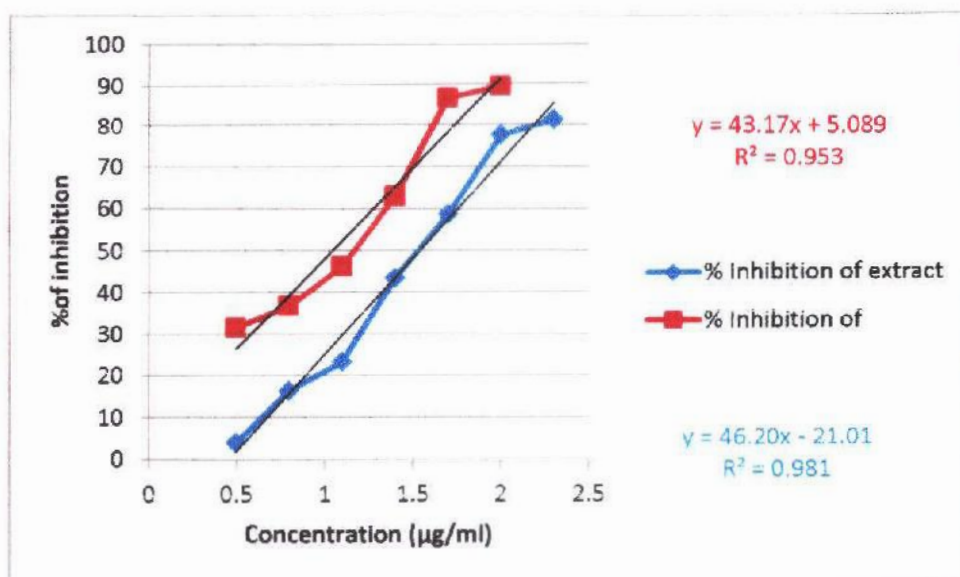


Figure 4.19: IC₅₀ value methanol extract of CHL3

Table 4.15: DPPH Scavenging Assay of Ethyl acited extract of CHL4

Concentration($\mu\text{g/ml}$)	Log con	Abs1	Abs2	Average	% inhibition	IC50
3.12	0.494155	0.356	0.321	0.3385	29.08901	
6.24	0.795185	0.3	0.344	0.322	32.5445	
12.5	1.09691	0.201	0.324	0.2625	45.00524	
25	1.39794	0.253	0.25	0.2515	47.3089	
50	1.69897	0.246	0.241	0.2435	48.98429	49.9684
100	2	0.228	0.224	0.226	52.64921	
200	2.30103	0.184	0.196	0.19	60.18848	
Blank		0.454	0.501	0.4775		

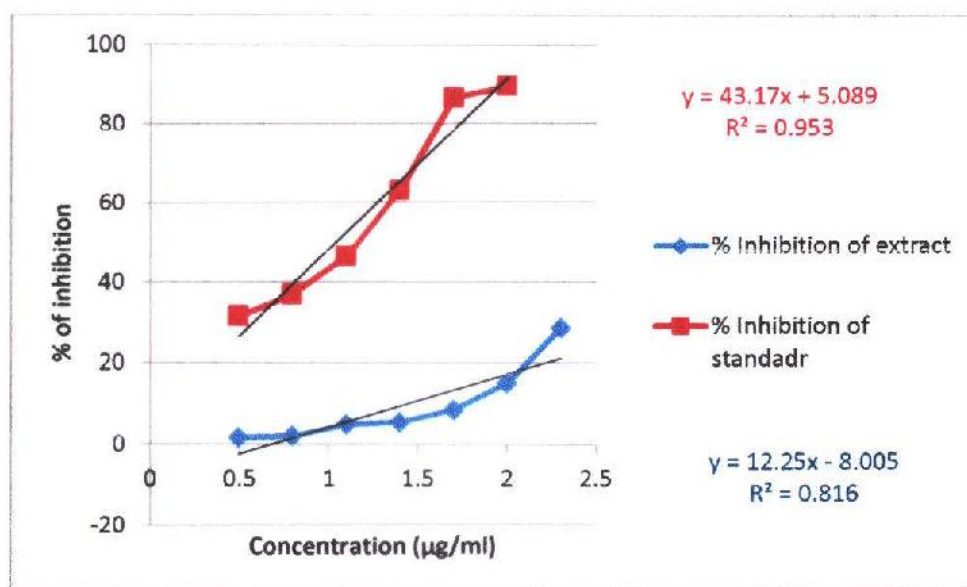


Figure4.20: IC₅₀ Value of Ethyl acited extract CHL4

Table 4.16: DPPH Scavenging Assay of Methanol extract of CHL4

Concentration($\mu\text{g/ml}$)	Log con	Abs1	Abs2	Average	% inhibition	IC50
3.12	0.494155	0.588	0.544	0.566	7.742461	
6.24	0.795185	0.443	0.405	0.424	30.88835	
12.5	1.09691	0.297	0.307	0.302	50.77425	
25	1.39794	0.264	0.256	0.26	57.62021	23.98
50	1.69897	0.27	0.249	0.2595	57.70171	
100	2	0.204	0.209	0.2065	66.34067	
200	2.30103	0.106	0.099	0.1025	83.29258	
Blank		0.603	0.624	0.6135		

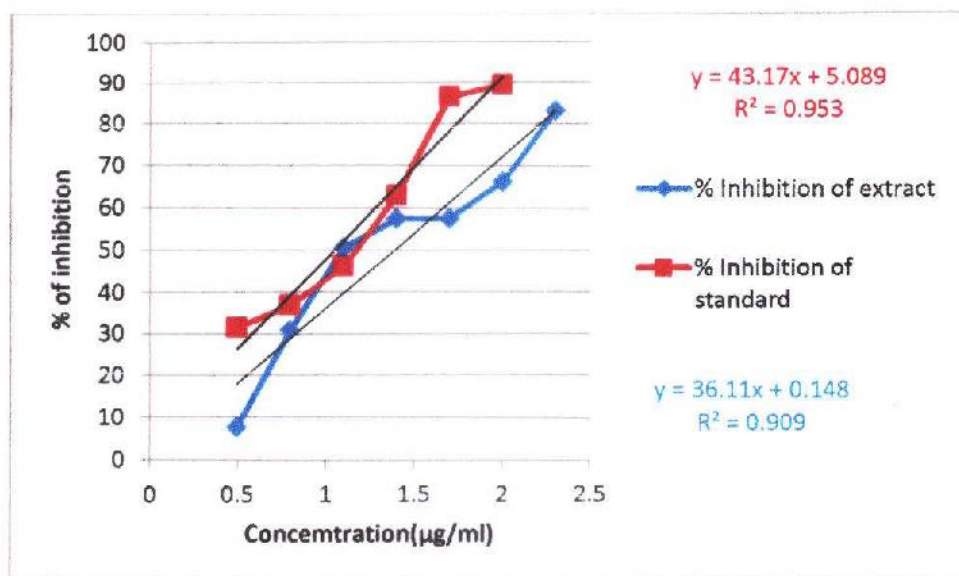


Figure 4.20: IC₅₀ value methanol extract of CHL4

Table 4.17: DPPH Scavenging Assay of Ethyl acited extract of CHI5

Concentration($\mu\text{g/ml}$)	Log con	Abs1	Abs2	Average	% inhibition	IC50
3.12	0.494155	0.399	0.418	0.4085	8.918618	
6.24	0.795185	0.387	0.399	0.393	12.37458	
12.5	1.09691	0.325	0.271	0.298	33.5563	
25	1.39794	0.181	0.233	0.207	53.84615	26.30
50	1.69897	0.119	0.082	0.1005	77.59197	
100	2	0.08	0.104	0.092	79.48718	
200	2.30103	0.11	0.106	0.108	75.91973	
Blank		0.453	0.444	0.4485		

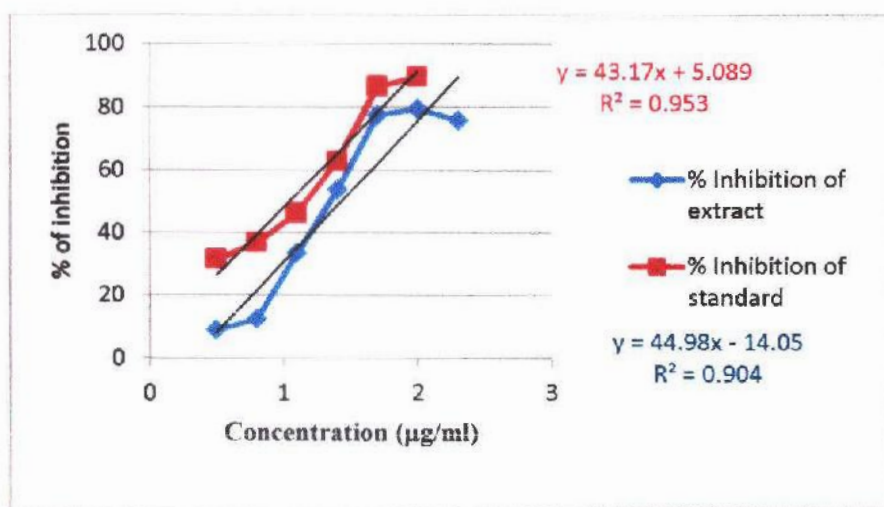


Figure 4.21: IC₅₀ Value of Ethyl acited extract CHL5

Table 4.18.: DPPH Scavenging Assay of Methanol extract of CHL5

Concentration($\mu\text{g/ml}$)	Log con	Abs1	Abs2	Average	% inhibition	IC50
3.12	0.494155	0.502	0.653	0.5775	14.0625	
6.24	0.795185	0.589	0.576	0.5825	13.31845	
12.5	1.09691	0.576	0.533	0.5545	17.48512	
25	1.39794	0.566	0.499	0.5325	20.75893	
50	1.69897	0.376	0.461	0.4185	37.72321	269.15
100	2	0.38	0.368	0.374	44.34524	
200	2.30103	0.355	0.357	0.356	47.02381	
Blank		0.689	0.655	0.672		

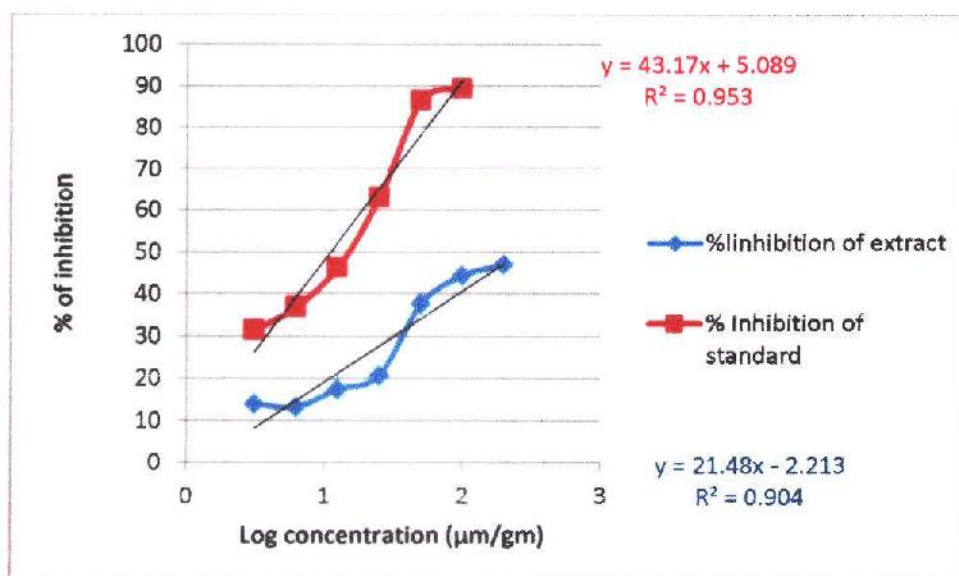


Figure 4.22: IC₅₀ Value of methanol extract CHL5

Table 4.19: DPPH Scavenging Assay of Ethyl acited extract of CHL8

Concentration($\mu\text{g/ml}$)	Log con	Abs1	Abs2	Average	% inhibition	IC50
3.12	0.494155	0.412	0.445	0.4285	11.00727	
6.24	0.795185	0.4	0.411	0.4055	15.78401	
12.5	1.09691	0.401	0.399	0.4	16.92627	
25	1.39794	0.365	0.398	0.3815	20.76843	478.63
50	1.69897	0.345	0.339	0.342	28.97196	
100	2	0.302	0.298	0.3	37.6947	
200	2.30103	0.226	0.291	0.2585	46.3136	
Blank		0.49	0.473	0.4815		

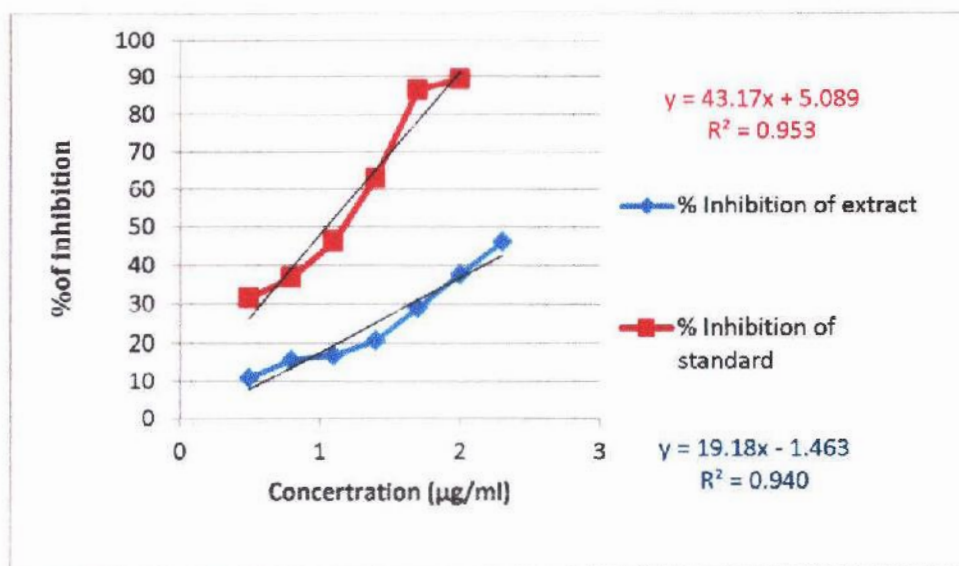


Figure 4.23: IC₅₀ Value of Ethyl acited extract CHL8

Table 4.20: DPPH Scavenging Assay of methanol extract of CHL8

Concentration($\mu\text{g/ml}$)	Log con	Abs1	Abs2	Average	% inhibition	IC ₅₀
3.12	0.494155	0.366	0.302	0.334	37.45318	
6.24	0.795185	0.299	0.278	0.2885	45.97378	
12.5	1.09691	0.287	0.233	0.26	51.31086	
25	1.39794	0.163	0.144	0.1535	71.25468	8.07
50	1.69897	0.147	0.146	0.1465	72.56554	
100	2	0.145	0.127	0.136	74.53184	
200	2.30103	0.134	0.121	0.1275	76.1236	
Blank		0.552	0.534	0.543		

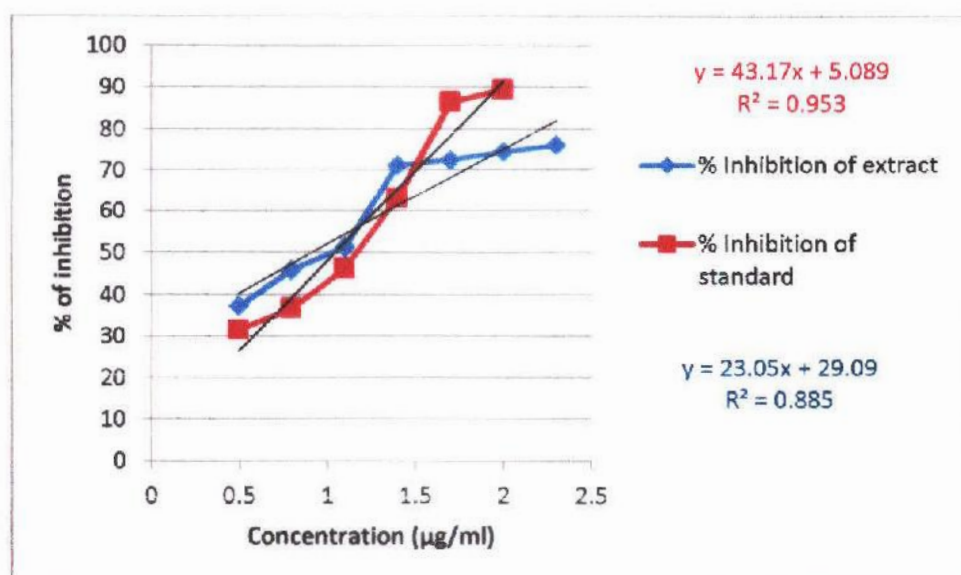


Figure 4.24: IC₅₀ Value of Mithanol extract CHL8

Table 4.21:IC50 value of Antioxident test

Name of sample	IC50 value
CHL1 EAtOH	22.83
CHL1 Methanol	28.97
CHL2 EAtOH	95.49
CHL2Methanol	158.48
CHL3 EAtOH	19.95
CHL3 Methanol	31.62
CHL4 EAtOH	49.968
CHL4 Methanol	23.98
CHL5 EAtOH	26.3
CHL5 Methanol	269.15
CHL8 EAtOH	478.63
CHL8 Methanol	8.07
Constant	10.964

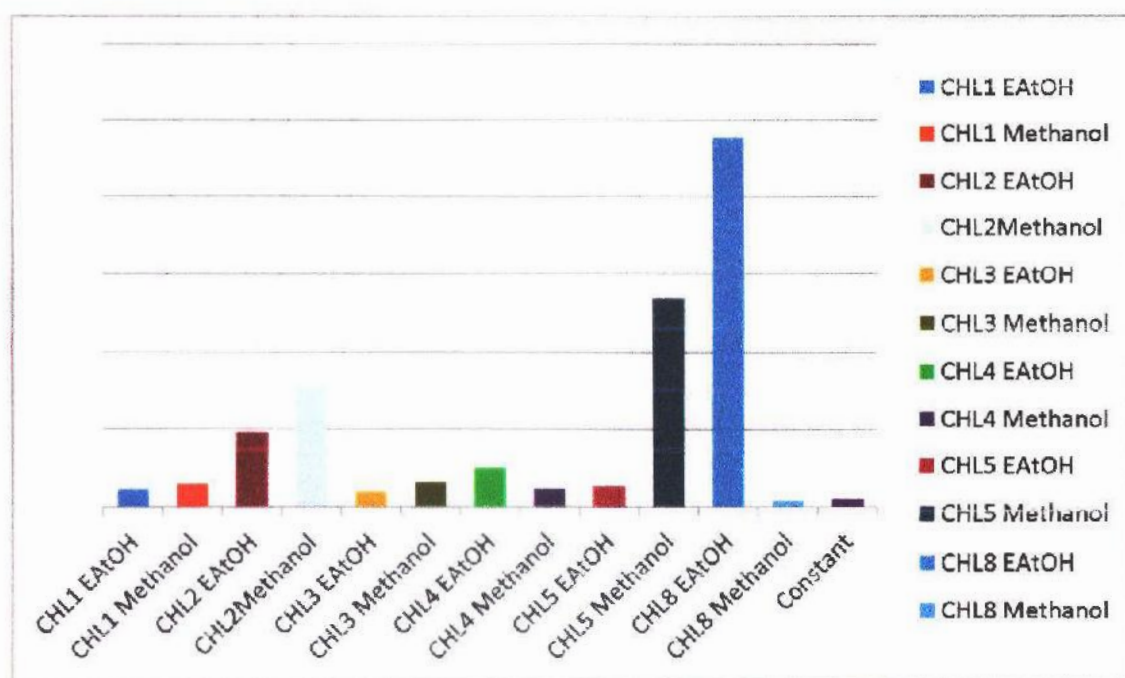


Figure 4.25: IC₅₀ value of the crude extracts from fungal endophytes

4.5 Antibacterial activity

Table 4.22: List of microbes used for screening of antimicrobial activity

Gram negative	Gram Positive
1. <i>Escherichia coli</i> 2. <i>Pseudomonas aeruginosa</i>	1. <i>Staphylococcus aureus</i> 2. <i>Micrococcus</i>

Table 4.23: Zone of inhibition against bacteria

Name of extract	Zone of inhibition(mm)			
	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Micrococcus</i>
CHL1 EAtoH extract(100 µg/disc)	-	-	-	-
CHL1 EAtoH extract(500 µg/disc)	5.6	3.4	10.3	7.9
CHL1 EAtoH extract(1000µg/disc)	7	12	13	11.25
CHL1 methanol extract(100 µg/disc)	7	4	0	0
CHL1 methanol extract(500 µg/disc)	10	11.75	12.75	8.75
CHL1 methanol extract(1000 µg/disc)	12	11.7	13.66	10.43
Blank	0	0	0	0
Erythromycin (10 µg/disc)	16.5	15.5	12.5	7.25

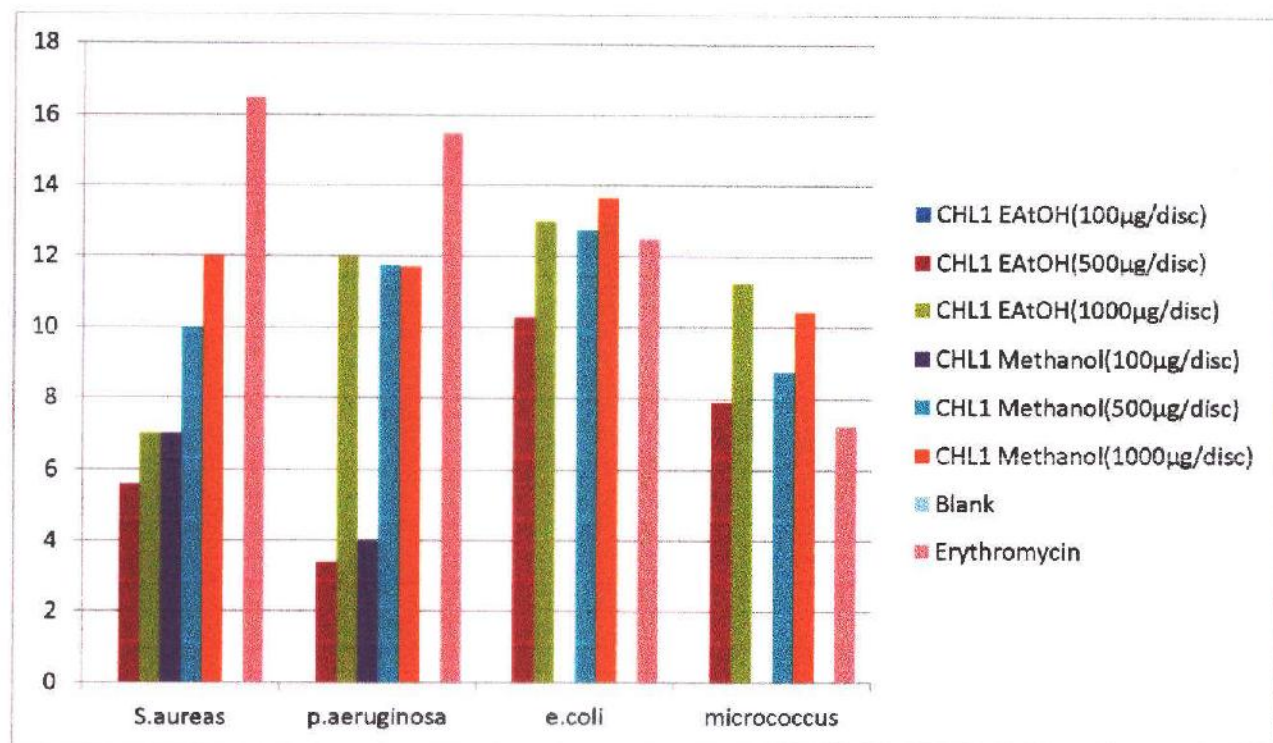


Figure 4.25: Zone of inhibition against bacteria of standard and samples

Table 4.24: Zone of inhibition against bacteria

Name of extract	Zone of inhibition			
	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherechia coli</i>	Micrococcus
CHL2 EAtoH extract(100 µg/disc)	-	-	-	-
CHL2 EAtoH extract(500 µg/disc)	4	3.0	-	5.7
CHL2 EAtoH extract(1000µg/disc)	8	10.5	8	12
CHL2 methanol extract(100 µg/disc)	-	-	-	-
CHL2 methanol extract(500 µg/disc)	13.5	11.75	15	10.75
CHL2 methanol extract(1000 µg/disc)	14	11.99	15.77	11.23
Blank	-	-	-	-
Erythromycin (10 µg/disc)	23	23	14.75	19.75

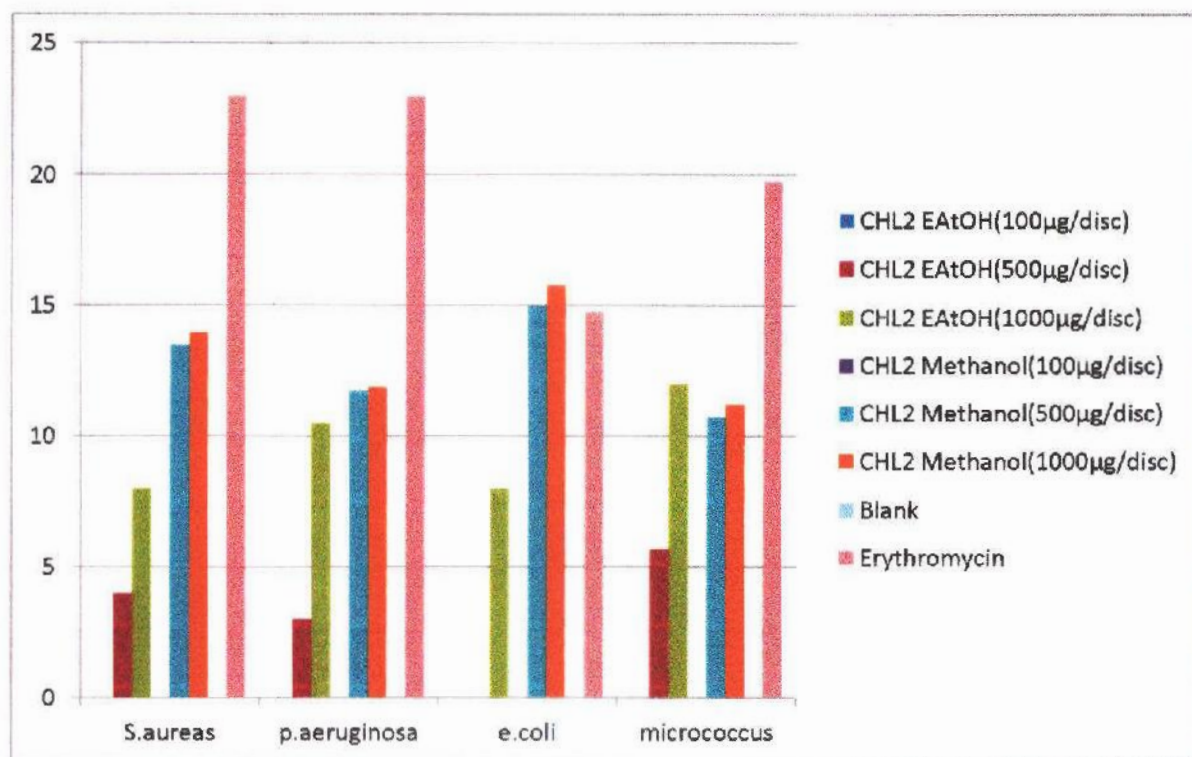


Figure 4.26: Zone of inhibition against bacteria of standard and samples

Table 4.25: Zone of inhibition against bacteria

Name of extract	Zone of inhibition			
	<i>Staphylococcus aureas</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherechia coli</i>	Micrococcus
CHL3 EAtoH extract(100 µg/disc)	-	-	-	-
CHL3 EAtoH extract(500 µg/disc)	3.80	2.43	5.1	0
CHL3 EAtoH extract(1000µg/disc)	7.75	8.5	6.75	8.25
CHL3 methanol extract(100 µg/disc)	8	-	-	-
CHL3 methanol extract(500 µg/disc)	3.5	-	7	-
CHL3 methanol extract(1000 µg/disc)	7	-	8.5	-
Blank	-	-	-	-
Erythromycin (10 µg/disc)	19	9.5	11	8.5

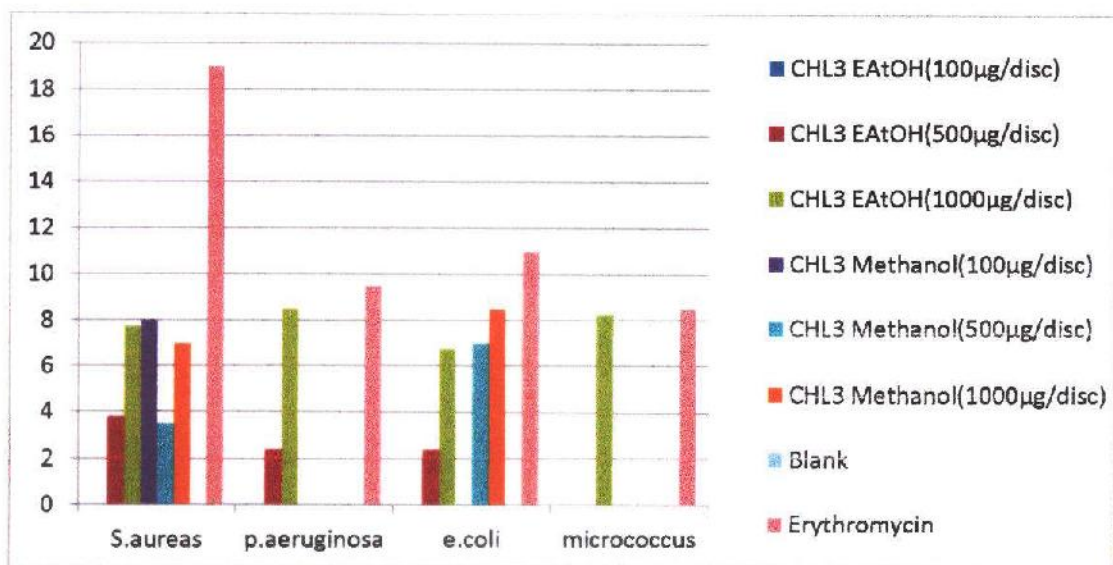


Figure 4.27: Zone of inhibition against bacteria of standard and samples

Table 4.26: Zone of inhibition against bacteria

Name of extract	Zone of inhibition			
	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Micrococcus</i>
CHL4 EAtoH extract(100 µg/disc)	-	-	-	-
CHL4 EAtoH extract(500 µg/disc)	3.80	2.43	5.1	0
CHL4 EAtoH extract(1000µg/disc)	7.75	8.5	6.75	8.25
CHL4 methanol extract(100 µg/disc)	10.5	9	8.5	8
CHL4 methanol extract(500 µg/disc)	3.5	9.9	8.9	10.2
CHL4methanol extract(1000 µg/disc)	8	11.5	9	13.25
Blank	-	-	-	-
Erythromycin (10 µg/disc)	19	9.5	11	8.5

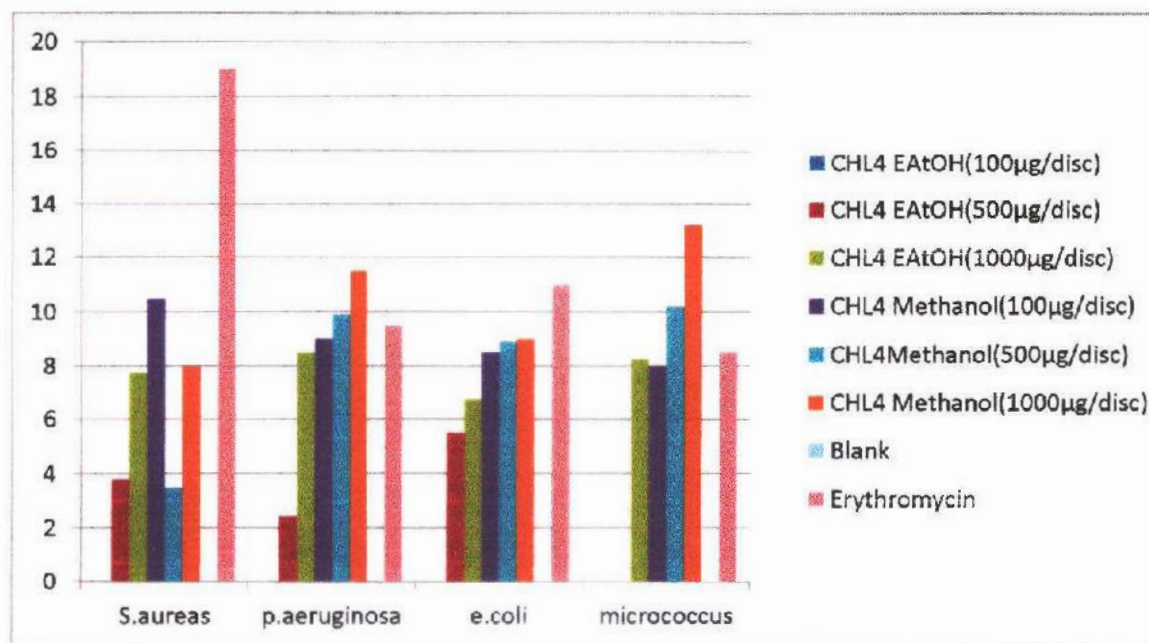


Figure 4.28: Zone of inhibition against bacteria of standard and sample

Table 4.27: Zone of inhibition against bacteria

Name of extract	Zone of inhibition			
	<i>Staphylococcus aureas</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherechia coli</i>	Micrococcus
CHL5EAtoH extract(100 µg/disc)	-	-	-	-
CHL5 EAtoH extract(500 µg/disc)	4.6	3.2	5	1.2
CHL5 EAtoH extract(1000µg/disc)	5.2	4.9	6.2	3.8
CHL5 methanol extract(100 µg/disc)	8	-	5.5	2
CHL5 methanol extract(500 µg/disc)	-	-	-	-
CHL5 methanol extract(1000 µg/disc)	-	-	-	-
Blank	-	-	-	-
Erythromycin (10 µg/disc)	16.5	15.5	12.5	7.25

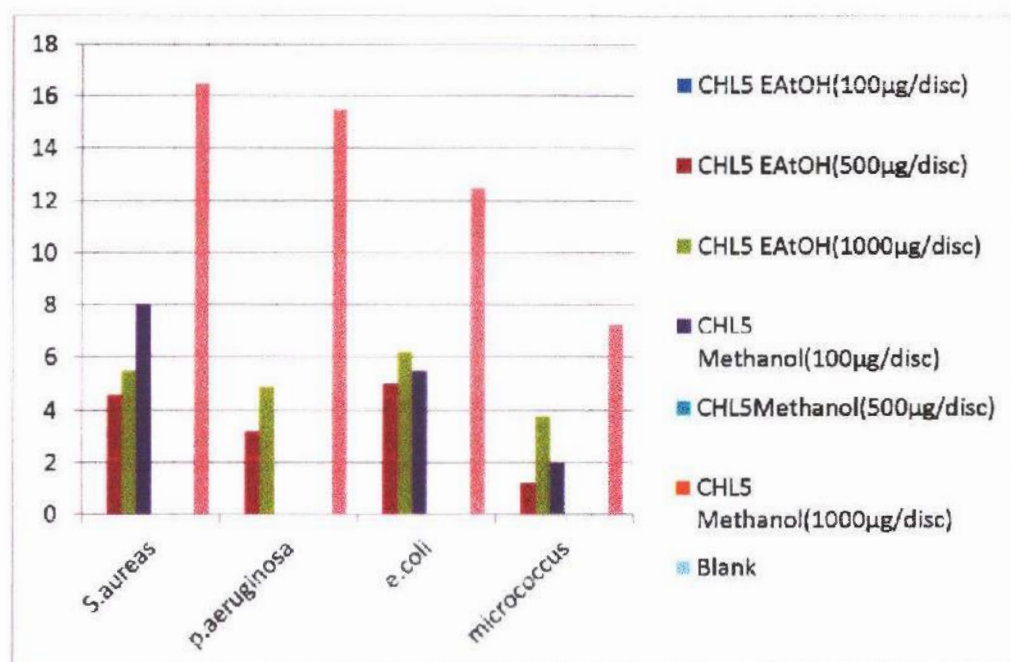


Figure 4.28: Zone of inhibition against bacteria of standard and samples

Table 4.28: Zone of inhibition against bacteria

Name of extract	Zone of inhibition			
	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Micrococcus</i>
CHL8 EAtoH extract(100 µg/disc)	-	-	-	-
CHL8 EAtoH extract(500 µg/disc)	-	-	-	-
CHL8 EAtoH extract(1000µg/disc)	-	-	-	-
CHL8 methanol extract(100 µg/disc)	3.5	6.5	8	4
CHL8 methanol extract(500 µg/disc)	5.2	7.1	8.9	6
CHL8 methanol extract(1000 µg/disc)	6.3	8.9	11.2	8.9
Blank	-	-	-	-
Erythromycin (10 µg/disc)	16.5	15.5	12.5	7.25

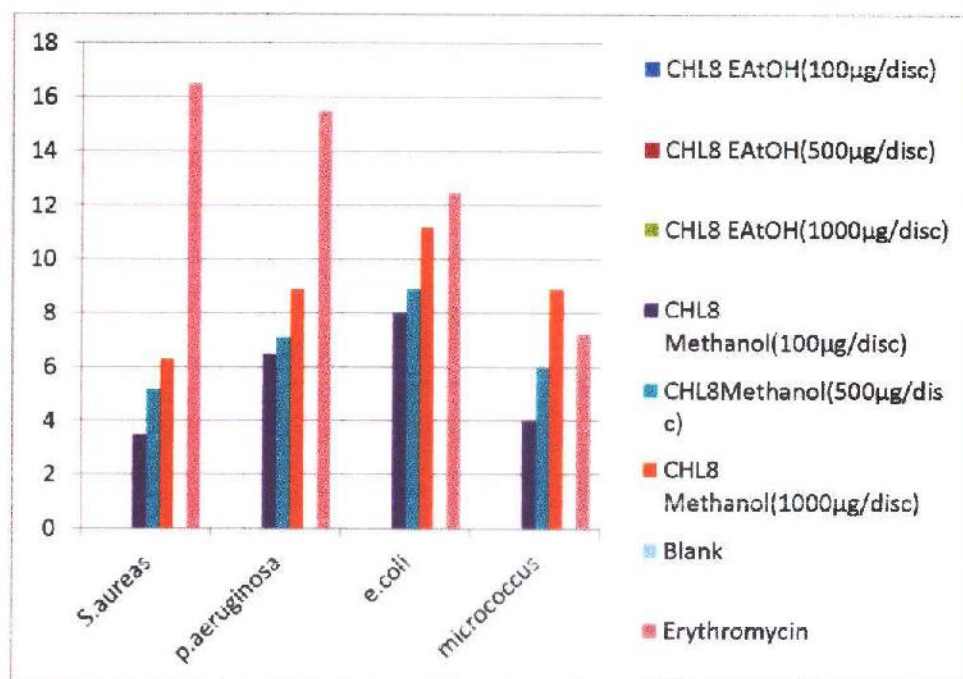


Figure 4.29: Zone of inhibition against bacteria of standard and samples



4.6 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 their associated endophytic fungal strain CHL1, CHL2, CHL3, CHL4, CHL5, CHL8 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 4.29: 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 4.30: Effects of standard drug vincristine sulphate 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)
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	

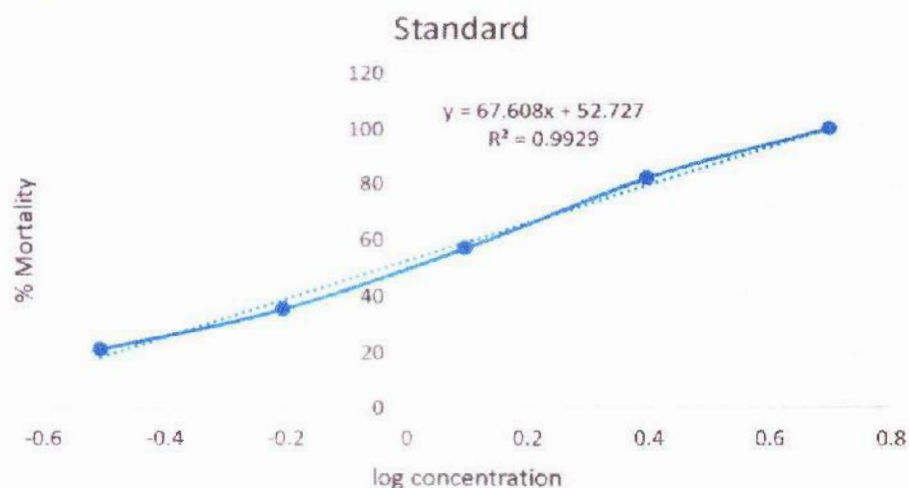


Figure 4.30: Graphical representation of brine shrimp lethality bioassay of standard (Vincristine sulphate)

Table 4.30: Effects of EAtoH extract of CHL1 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	9	9	9	9	9.33	3.54	
10	1	9	9	8	8.67		7.11	
20	1.301	9	8	8	8.33		10.68	
40	1.602	7	8	8	7.67		17.83	426.5
80	1.903	7	7	6	6.67		28.54	
160	2.204	5	7	6	6		35.69	
320	2.505	4	4	5	4.33		53.55	

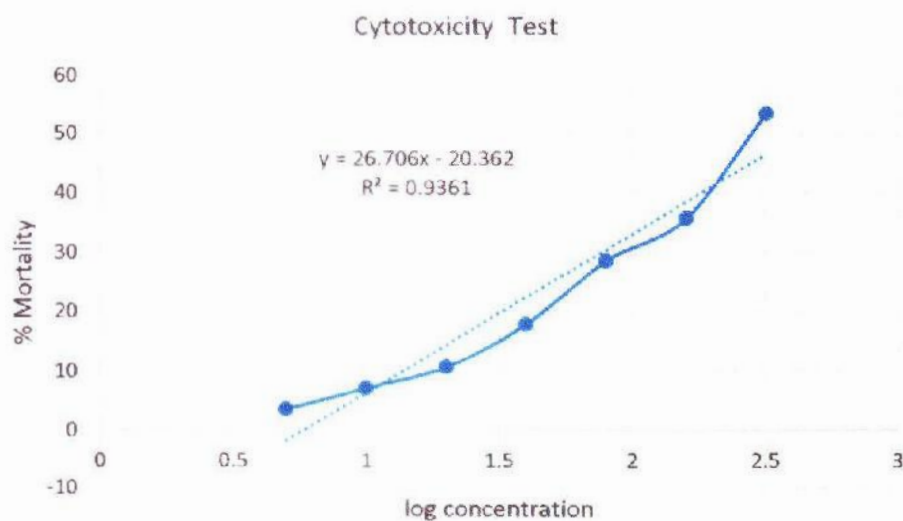


Figure 4.31: Graphical representation of brine shrimp lethality bioassay of EAtoH extract of CHL1

Table 4.31: Effects of Methanol extract of CHL1 on brine shrimp

Concentration ($\mu\text{g/ml}$)	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 ($\mu\text{g/ml}$)
5	0.699	8	8	9	8.33333333	9.33	10.68239	38.01
10	1	7	8	8	7.66666666		17.8278	
20	1.301	6	7	5	6		35.69132	
40	1.602	5	4	3	4		57.12755	
80	1.903	3	4	4	3.66666666		60.70025	
160	2.204	2	1	2	1.66666666		82.13648	
320	2.505	0	1	1	0.66666666		92.85459	

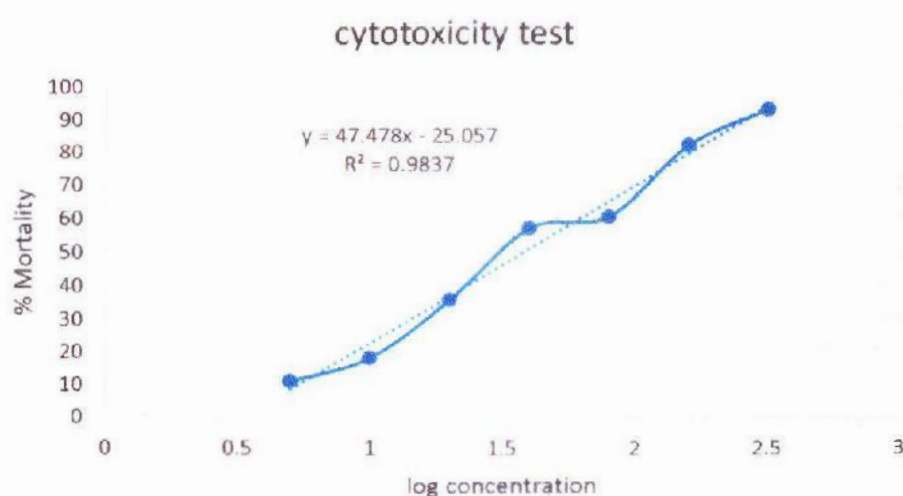


Figure 4.32: Graphical representation of brine shrimp lethality bioassay of methanol extract of CHL1

Table 4.32: Effects of EAtOH extract of CHL2 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	8	9	10	9	9.33	3.536977	80.59
10	1	9	9	8	8.6666667		7.109682	
20	1.301	8	9	7	8		14.25509	
40	1.602	7	5	7	6.3333333		32.11861	
80	1.903	4	4	5	4.3333333		53.55484	
160	2.204	3	3	3	3		67.84566	
320	2.505	2	3	2	2.3333333		74.99107	

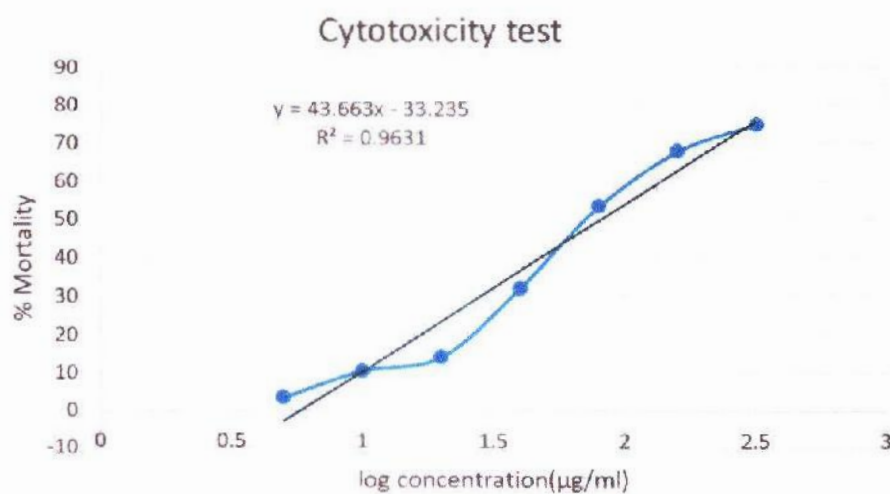


Figure 4.33: Graphical representation of brine shrimp lethality bioassay of EAtOH extract of CHL2

Table 4.33: Effects of methanol extract of CHL2 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	8	8	10	8.66667	9.33	7.109682	79.43
10	1	8	8	9	8.33333		10.68239	
20	1.301	8	9	7	8		14.25509	
40	1.602	5	7	6	6		35.69132	
80	1.903	5	4	5	4.66667		49.98214	
160	2.204	3	3	3	3		67.84566	
320	2.505	2	3	2	2.33333		74.99107	

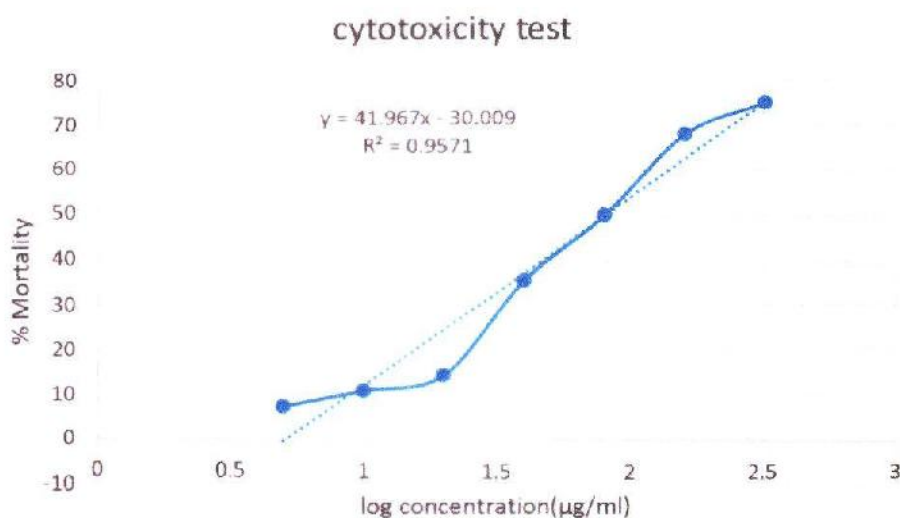


Figure 4.34: Graphical representation of brine shrimp lethality bioassay of methanol extract of CHL2

Table 4.34: Effects of EAtoH extract of CHL3 on brine shrimp

Concentration ($\mu\text{g/ml}$)	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 ($\mu\text{g/ml}$)
5	0.699	8	8	10	8.666666667	9.33	7.109682	60.25
10	1	8	6	9	7.666666667		17.8278	
20	1.301	5	6	7	6		35.69132	
40	1.602	5	6	5	5.333333333		42.83673	
80	1.903	4	5	5	4.666666667		49.98214	
160	2.204	4	2	1	2.333333333		74.99107	
320	2.505	3	2	3	2.666666667		71.41836	

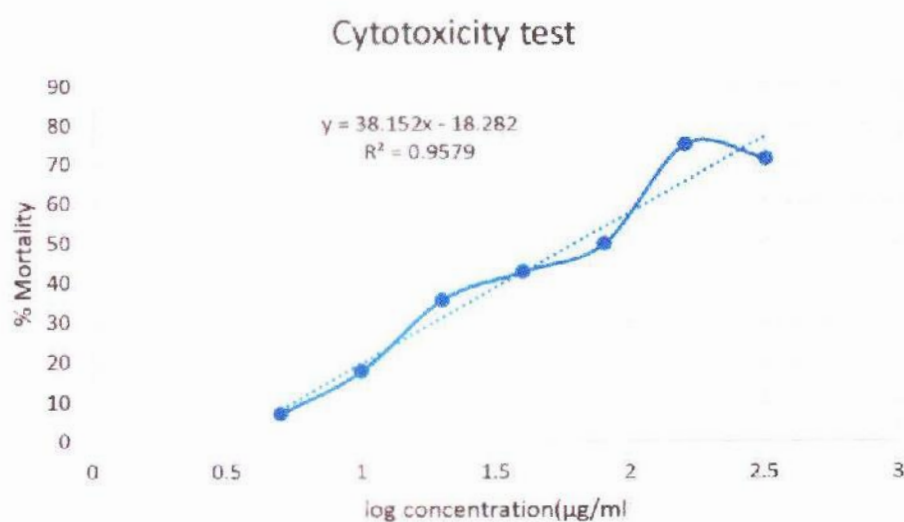


Figure 4.35: Graphical representation of brine shrimp lethality bioassay of EAtoH extract of CHL3

Table 4.35: Effects of methanol extract of CHL3 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	8	8	10	8.666666667	9.33	7.109682	47.86
10	1	8	5	9	7.333333333		21.4005	
20	1.301	5	5	7	5.666666667		39.26402	
40	1.602	5	4	7	5.333333333		42.83673	
80	1.903	4	4	5	4.333333333		53.55484	
160	2.204	4	2	1	2.333333333		74.99107	
320	2.505	2	0	2	1.333333333		85.70918	

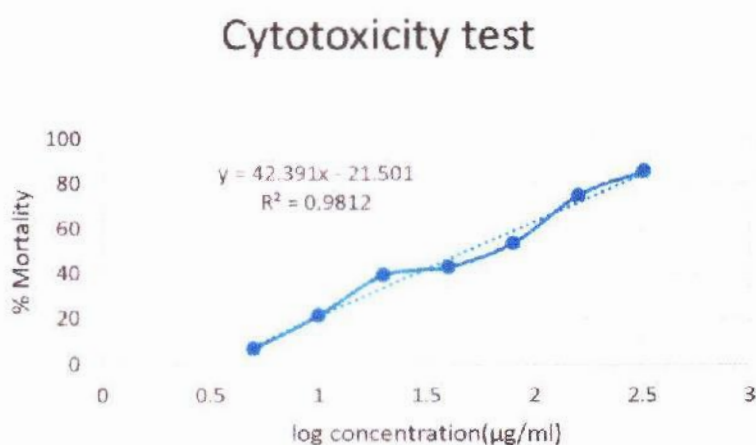


Figure 4.36: Graphical representation of brine shrimp lethality bioassay of methanol extract of CHL3

Table 4.36: Effects of EAtOH extract of CHL4 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	8	10	9	9	9.33	3.53698	57.54
10	1	8	8	9	8.333333333		10.6824	
20	1.301	5	8	7	6.666666666		28.5459	
40	1.602	5	4	6	5		46.4094	
80	1.903	4	4	5	4.333333333		53.5548	
160	2.204	4	3	2	3		67.8457	
320	2.505	2	0	2	1.333333333		85.7092	

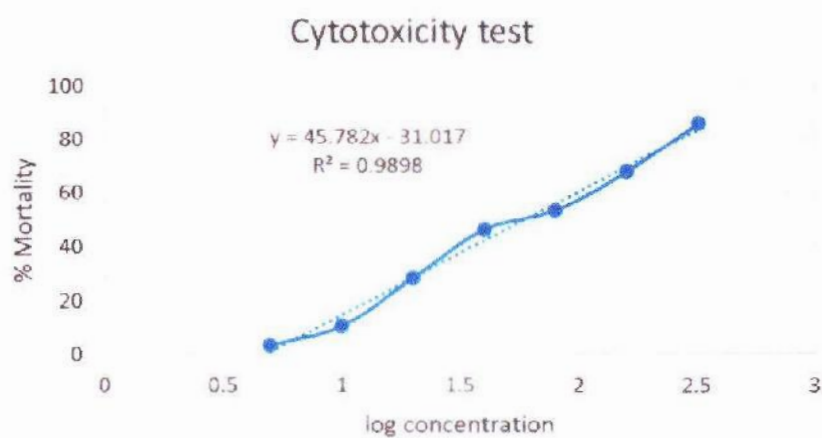


Figure 4.37.: Graphical representation of brine shrimp lethality bioassay of EAtOH extract of CHL4

Table 4.37: Effects of methanol extract of CHL4on 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	9	8	9	8.666666667	9.33	7.109682	228.87
10	1	9	8	9	8.666666667		7.109682	
20	1.301	8	7	8	7.666666667		17.8278	
40	1.602	6	8	7	7		24.9732	
80	1.903	7	6	6	6.333333333		32.11861	
160	2.204	5	6	6	5.666666667		39.26402	
320	2.505	3	3	4	3.333333333		64.27295	

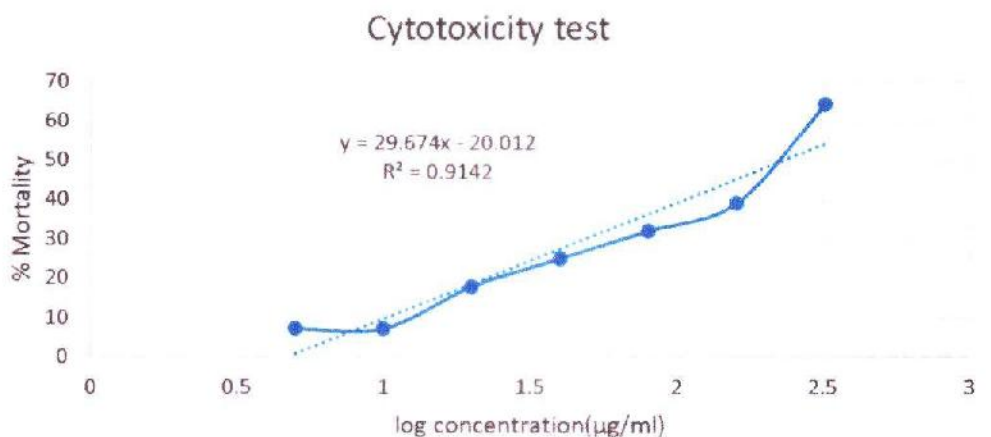


Figure 4.38: Graphical representation of brine shrimp lethality bioassay of methanol extract of CHL4

Table 4.39: Effects of methanol extract of CHL5 on brine shrimp

Concentration ($\mu\text{g/ml}$)	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 ($\mu\text{g/ml}$)
5	0.699	6	9	10	8.3333333333	9.33	10.68239	22.38
10	1	5	5	6	5.3333333333		42.83673	
20	1.301	3	5	4	4		57.12755	
40	1.602	3	3	5	3.6666666667		60.70025	
80	1.903	2	3	3	2.6666666667		71.41836	
160	2.204	1	2	1	1.3333333333		85.70918	
320	2.505	0	2	1	1		89.28189	

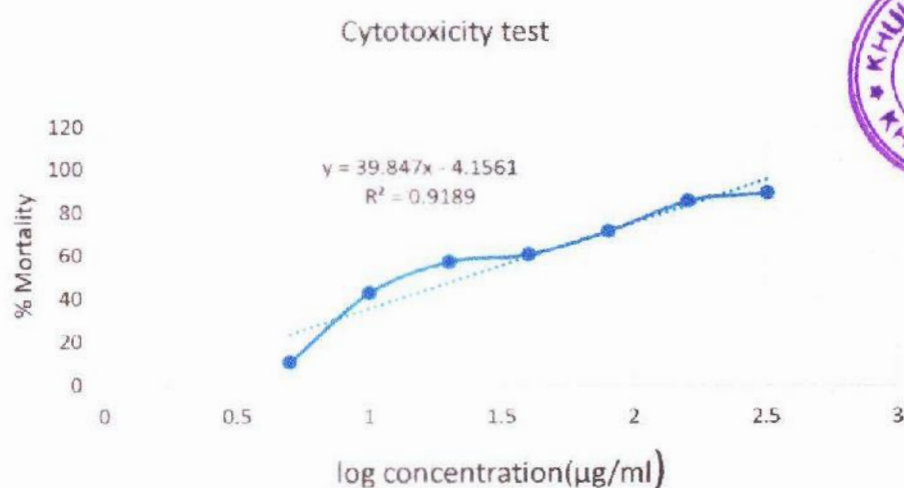


Figure 4.40: Graphical representation of brine shrimp lethality bioassay of methanol extract of CHL5

Table 4.40: Effects of EAtoH extract of CHL 8 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	4	5	5	4.66666667	9.33	49.98214	6.76
10	1	4	4	5	4.33333333		53.55484	
20	1.301	3	4	4	3.66666667		60.70025	
40	1.602	3	3	4	3.33333333		64.27295	
80	1.903	1	3	3	2.33333333		74.99107	
160	2.204	1	2	1	1.33333333		85.70918	
320	2.505	0	1	1	0.66666667		92.85459	

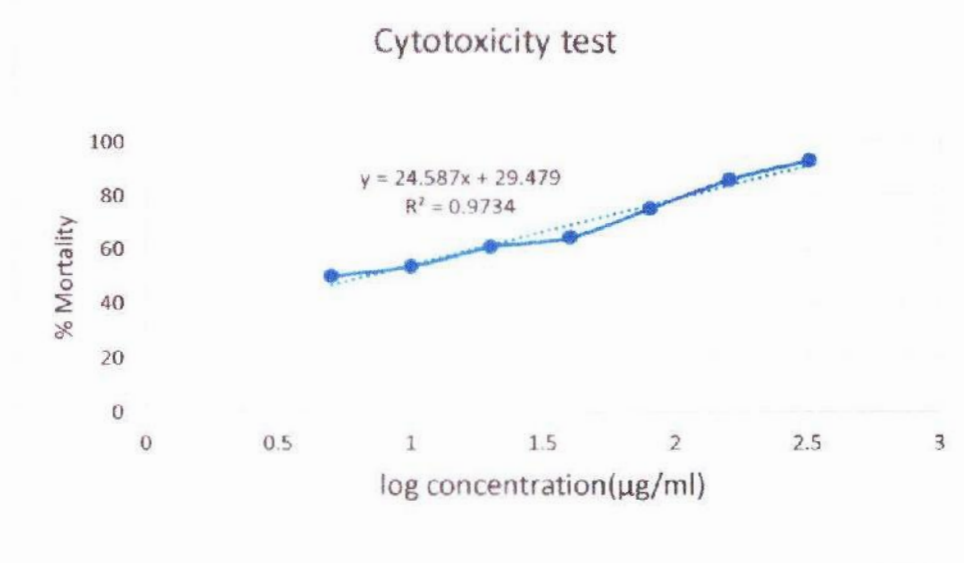


Figure 4.41: Graphical representation of brine shrimp lethality bioassay of EAtoH extract of CHL8

Table 4.41: Effects of methanol extract of CHL8 on brine shrimp

Concentration ($\mu\text{g/ml}$)	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	Lc50
5	0.699	10	8	9	9	9.33	3.536977	
10	1	8	7	8	7.666666667		17.8278	
20	1.301	8	5	7	6.666666667		28.54591	54.95
40	1.602	6	5	6	5.666666667		39.26402	
80	1.903	4	4	5	4.333333333		53.55484	
160	2.204	3	2	4	3		67.84566	
320	2.505	1	0	1	0.666666667		92.85459	

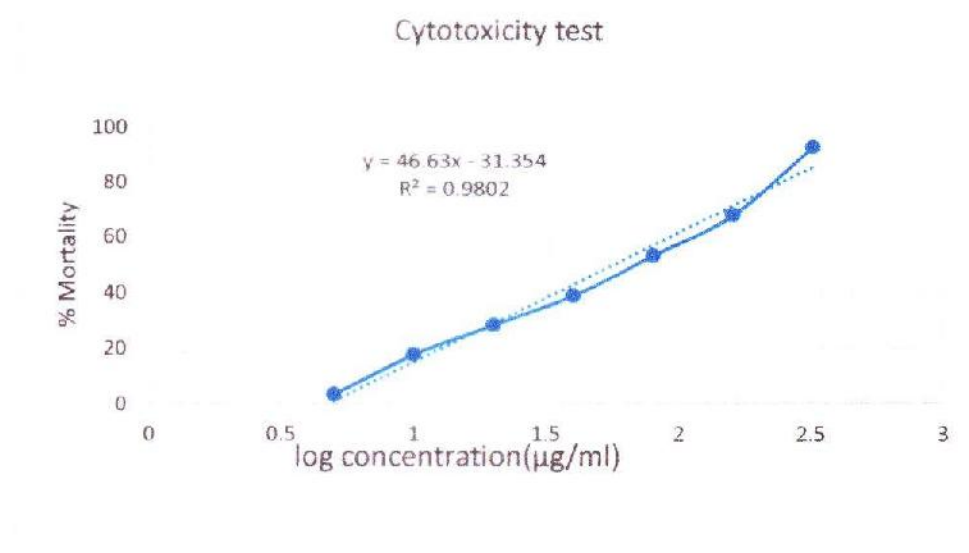


Figure 4.42: Graphical representation of brine shrimp lethality bioassay of methanol extract of CHL8

Table 4.42: values of LC50 of cytotoxicity test

Name of extract	LC50($\mu\text{g/ml}$)
Standard	1
CHL1 EAtoH	426.5
CHL1 Methanol	38.01
CHL2 EAtoH	80.59
CHL2 Metahnol	79.43
CHL3 EAtoH	60.25
CHL3 Methanol	47.86
CHL5 Methanol	22.38
CHL8 EAtoH	6.76
CHL8 Methanol	54.95

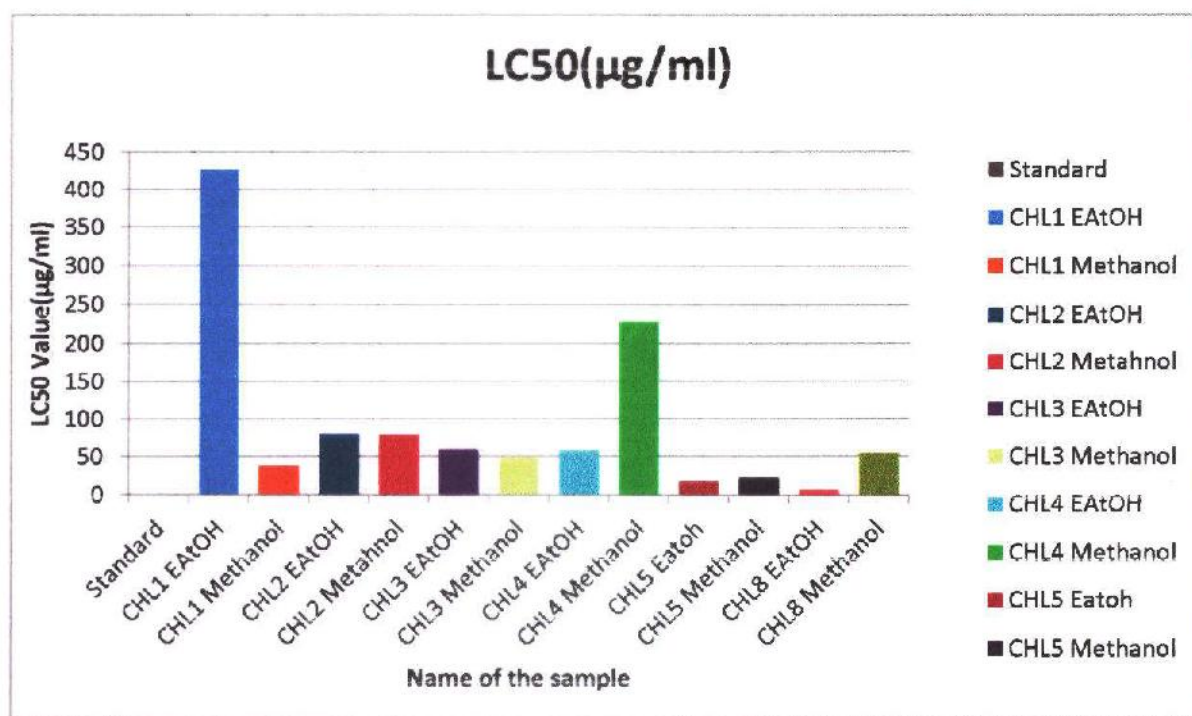


Figure 4.43: Graf of values of LC50 of cytotoxicity test

4.7 Discussion

Identification of fungi were 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. Agrios.. 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 (Lazzizeraaet *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, CHL 01, CHL 03, CHL 04, CHL 05 EAtoH, CHL 08 MEOH were showed very good antioxidant activity with the lowest IC₅₀valu comparing with the value of the standard (10.9730 µg/ml). Other extracts showed

mild to moderate activity with high value of IC₅₀. From the results, it can be concluded that extracts of the associated endophytic fungi from

Wardomycesmoseri spp.

Alternariadestruens spp.

Alternaria porri spp.

Diaporthe recaer spp.

Apicoccum nigrumspp 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 plant have cytotoxic nature. Among all the extracts, showed good activity with low LC₅₀ value against the nauplii. Among them CHL 01(*Wardomycesmoseri spp.*), CHL 03(*Alternariadestruens*), CHL 08 (*Apicoccumnigrum*) shows the maximum activity with low LC₅₀ value against the nauplii.

All the crude extracts of the endophytic fungi from *Sonneratia caseolaris* 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 antibacterial activity of the all the extracts exhibiting different zones of inhibition are shown in tabular and graph form.

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.



Chapter 6: Conclusion

Conclusion

This research focused on potential source to isolate bioactive secondary metabolites from a natural source of *Sonneratia casolaris* 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 leaf.

Endophytic fungi was isolated from mangrove plant of *Sonneratia casolaris*. 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 phylogenetic Analysis Using Parsimony (PAUP) version 4.0. From which, species was found for 10 regions that showed 99% identity with accession no. The sample CHL-1, CHL-2, CHL-3, CHL-4, CHL-5, CHL-8 represents *Wardomyces moseri*, *Simplicillium clavatum*, *Alternaria destruens*, *Alternaria porri*, *Diaporthe recaer*, *Apicoccum nigrum* were isolated from leaves of the mangrove plant *Sonneratia caseolaris*. The tree represents that all fungi are genetically similar with each other and dissimilar with other fungi.

Comparing the result from the tabular and graphical presentation of all the extracts, it can be said that endophytic fungi isolated from the plant have cytotoxic nature. Among all the extracts, showed good activity with low LC_{50} value against the nauplii. Among them CHL 01 (*Wardomyces moseri* spp.), CHL 03 (*Alternaria destruens*), CHL 08 (*Apicoccum nigrum*) shows the maximum activity with low LC_{50} value against the nauplii.

All the crude extracts of the endophytic fungi from *Sonneratia caseolaris* plant tested for antibacterial activity against a number of both gram positive and gram negative bacteria. Standard disc of Erythromycin (10 µg/disc) was used for comparison purpose. The results of antibacterial activity of all the extracts exhibiting different zones of inhibition are shown in tabular and graph form.

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.

6. References

1. Arnold A.E. (2008) Endophytic fungi: hidden components of tropical community ecology. *Tropical forest community ecology*, 178-88.
2. Arnold A.E., Mejía L.C., Kyllo D., Rojas E.I., Maynard Z., Robbins N. & Herre E.A. (2003) Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences* 100, 15649-54.
3. Bacon C.W., Porter J.K., Robbins J.D. & Luttrell E.S. (1977) *Epichloë typhina* from toxic tall fescue grasses. *Applied and environmental microbiology* 34, 576-81.
4. Bacon C.W. & White J. (2000) *Microbial endophytes*. CRC Press.
5. Bordallo J.J., López Llorca L.V., Jansson H.B., Salinas J., Persmark L. & Asensio L. (2002) Colonization of plant roots by egg parasitic and nematode trapping fungi. *New Phytologist* 154, 491-9.
6. Faeth S.H. & Fagan W.F. (2002) Fungal endophytes: common host plant symbionts but uncommon mutualists. *Integrative and Comparative Biology* 42, 360-8.
7. Firsirotu S., Firsirotu M. & Firsirotu M. (2007) Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia* 62, 251-7.
8. Gallery R.E., Dalling J.W., Wolfe B.T. & Arnold A.E. (2007) The influence of seed source, habitat and fungi on *Cecropia* seed survival in two Neotropical forests. *Theory and its seed dispersal, application in a changing world*. CABI Press, Wallingford, UK, 479-98.
9. Javey A., Guo J., Wang Q., Lundstrom M. & Dai H. (2003) Ballistic carbon nanotube field-effect transistors. *nature* 424, 654.

10. Jia M., Chen L., Xin H.-L., Zheng C.-J., Rahman K., Han T. & Qin L.-P. A friendly relationship between endophytic fungi and medicinal plants: a systematic review. *Frontiers in microbiology* 7, 906.
11. Kusari S., Hertweck C. & Spiteller M. Chemical ecology of endophytic fungi: origins of secondary metabolites. *Chemistry & biology* 19, 792-8.
12. Large E.C. (1940) The advance of the fungi. *The advance of the fungi*.
13. Li H.-y., Hao Z.-b., Wang X.-l., Huang L. & Li J.-p. (2009) Antioxidant activities of extracts and fractions from *Lysimachia foenum-graecum* Hance. *Bioresource technology* 100, 970-4.
14. Liu Y.-J. (2001) Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* 106, 259-62.
15. Neubert K., Mendgen K., Brinkmann H. & Wirsig S.G.R. (2006) Only a few fungal species dominate highly diverse mycofloras associated with the common reed. *Applied and environmental microbiology* 72, 1118-28.
16. Park K. (2005) Park's textbook of preventive and social medicine. *Preventive Medicine in Obstet, Paediatrics and Geriatrics*.
17. Sánchez S., Pumera M. & Fábregas E. (2007) Carbon nanotube/polysulfone screen-printed electrochemical immunosensor. *Biosensors and Bioelectronics* 23, 332-40.
18. Schulz B. & Boyle C. (2005) The endophytic continuum. *Mycological research* 109, 661-86.
19. Sherameti I., Shahollari B., Venus Y., Altschmied L., Varma A. & Oelmüller R. (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. *Journal of Biological Chemistry* 280, 26241-7.