Isolation and characterization of endophytic fungi from *Elaeocarpus sphaericus* (Gaertn.) K. Schum. and *Quassia indica* (Gaertn.) Nooteb. for the production of secondary metabolites

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By

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CERTIFICATE

This is to certify that the thesis entitled **"Isolation and characterization of endophytic fungi from *Elaeocarpus sphaericus* (Gaertn.) K. Schum. and *Quassia indica* (Gaertn.) Nooteb. for the production of secondary metabolites"** is a report of the original work carried out by **Ms. Deepthi V.C**, under my supervision and guidance in the Department of Biotechnology, University of Calicut, Kerala and that no part thereof has been presented for the award of any other degree. The candidate has exempted from the course work of Ph.D. programme in accordance with the UGC regulations.

C.U. Campus
21.01.2019

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Isolation and characterization of endophytic fungi from *Elaeocarpus sphaericus* (Gaertn.) K. Schum. and *Quassia indica* (Gaertn.) Nooteb. for the production of secondary metabolites"** submitted to the University of Calicut, as partial fulfilment of Ph.D. programme for the award of the degree of Doctor of Philosophy in Biotechnology is original and carried out by me under the supervision of Dr. Elyas, K.K, Professor, Department of Biotechnology, University of Calicut. This has not been submitted earlier either in part or in full for any degree or diploma of any University.

C.U. Campus **DEEPTHI V.C**

21.01.2019

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Deepthi V.C

Dedicated to

My loving daughter Ishani

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ABBREVIATIONS

µg	:	Microgram
µl	:	Microliter
µM	:	Micromolar
cm	:	Centimeter
mg	:	Milligram
min	:	Minutes
ml	:	Millilitre
mm	:	Millimeter
mM	:	Millimolar
mM	:	Millimolar
nM	:	Nanomolar
v/v	:	Volume/volume
w/v	:	Weight/volume
ANS	:	Anisaldehyde sulphuric acid
ATCC	:	American type culture collection
BEA	:	Broth ethyl acetate
BLAST	:	Basic local alignment search tool
bp	:	Base pair
CDB	:	Czapek Dox broth
CD	:	Czapek Dox
CF	:	Colonization frequency
CFU	:	Colony forming unit
CH	:	Chloroform
CTAB	:	Cetyl trimethyl ammonium bromide
DMEM	:	Dulbecco's Modified Eagle Media
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
E. coli	:	*Escherichia coli*
E. faecalis	:	*Enterococcus faecalis*
EA	:	Ethyl acetate
EDTA	:	Ethylenediaminetetraacetic acid
ESEF 1	:	*Elaeocarpus sphaericus* endophytic fungus 1
ESEF 10	:	*Elaeocarpus sphaericus* endophytic fungus 10

ESEF 11	:	*Elaeocarpus sphaericus* endophytic fungus 11
ESEF 12	:	*Elaeocarpus sphaericus* endophytic fungus 12
ESEF 2	:	*Elaeocarpus sphaericus* endophytic fungus 2
ESEF 3	:	*Elaeocarpus sphaericus* endophytic fungus 3
ESEF 4	:	*Elaeocarpus sphaericus* endophytic fungus 4
ESEF 5	:	*Elaeocarpus sphaericus* endophytic fungus 5
ESEF 6	:	*Elaeocarpus sphaericus* endophytic fungus 6
ESEF 7	:	*Elaeocarpus sphaericus* endophytic fungus 7
ESEF 8	:	*Elaeocarpus sphaericus* endophytic fungus 8
ESEF 9	:	*Elaeocarpus sphaericus* endophytic fungus 9
FBS	:	Fast blue salt
GA	:	Gibberellic acid
GAE	:	Gallic acid equivalent
HPLC	:	High performance liquid chromatography
HP-TLC	:	High performance thin layer chromatography
HR LCMS	:	High resolution liquid chromatography mass spectrometer
HR-MS	:	High resolution mass spectrometer
IAA	:	Indole-3-acetic acid
ITS	:	Internal transcribed spacer
K. pneumoniae	:	*Klebsiella pneumoniae*
L. monocytogenes	:	*Listeria monocytogenes*
LB	:	Luria bertani
LC-MS	:	Liquid chromatography mass spectrometry
MEA	:	Mycelia ethyl acetate
MEB	:	Malt extract broth
MH	:	Mycelia hexane
MIC	:	Minimum inhibitory concentration
MM	:	Mycelia methanol
MTT	:	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl tetrazolium bromide
NCBI	:	National centre for biotechnology information
OD	:	Optical density
P. aeruginosa	:	*Pseudomonas aeruginosa*
PCR	:	Polymerase chain reaction
PDA	:	Potato dextrose agar
PDB	:	Potato dextrose broth

PE	:	Petroleum ether
QIEF 1	:	*Quassia indica* endophytic fungus 1
QIEF 10	:	*Quassia indica* endophytic fungus 10
QIEF 2	:	*Quassia indica* endophytic fungus 2
QIEF 3	:	*Quassia indica* endophytic fungus 3
QIEF 4	:	*Quassia indica* endophytic fungus 4
QIEF 5	:	*Quassia indica* endophytic fungus 5
QIEF 6	:	*Quassia indica* endophytic fungus 6
QIEF 7	:	*Quassia indica* endophytic fungus 7
QIEF 8	:	*Quassia indica* endophytic fungus 8
QIEF 9	:	*Quassia indica* endophytic fungus 9
rDNA	:	Ribosomal DNA
Rf	:	Rate of flow
RPMI	:	Roswell park memorial institute
S. aureus	:	*Staphylococcus aureus*
SD	:	Standard deviation
TAE	:	Tris acetate EDTA
TE	:	Tris- EDTA
TLC	:	Thin layer chromatography
Trp	:	Tryptophan
UV	:	Ultra violet
V	:	Voltage

ABSTRACT

Endophytes are endosymbiont, which reside in the tissues beneath the epidermal cell layers within the plants for at least a part of its life cycle without causing apparent diseases. Two medicinal plants *Elaeocarpus sphaericus* and *Quassia indica* were selected in the study for the isolation of endophytic fungi. 12 morphologically different endophytic fungi were selected from 203 isolates of 122 leaf segments from *Elaeocarpus sphaericus* and the isolates were given unique code ESEF 1 to ESEF 12. 10 morphologically different endophytic fungi were selected from 139 isolates of 110 leaf segments from *Quassia indica* and the isolates were given unique code QIEF 1 to QIEF 10. Endophytic fungi were identified by microscopic and molecular techniques using internal transcribed spacer polymerase chain reaction (ITS PCR). The identified endophytes are *Xylaria* sp., *Diaporthe* sp., *Fusarium* sp., *Endomelanconiopsis* sp., *Nigrospora* sp., *Pestalotiopsis* sp., *Daldinia* sp., *Phyllosticta* sp., *Colletotrichum* sp. and *Meyerozyma* sp. The highest colonization frequency was observed in *Diaporthe* sp. (ESEF 4 and QIEF 2) as 20.49 % and 20.90 % respectively. The endophytic fungus**,** ESEF 6 (*Xylaria* sp.) have good antibacterial and cytotoxic activities, further analysis was done for the isolation and characterization of endophytic fungi producing metabolites with antibacterial and cytotoxic activities. The solvent extracts- mycelial ethyl acetate (MEA), broth ethyl acetate (BEA) extracts and their corresponding partially purified active fractions (after column chromatography followed by TLC scrapping out) have cytotoxic activity on K562 and HCT 116 cell lines and antibacterial activities against both gram positive and gram negative bacteria. The extracts also possess phenolic content and antioxidant activity. Metabolite analysis of the extracts and active fractions were evaluated. Partially purified active fractions were characterized by HPLC, HR-MS and HR-LCMS/MS profiling. HR-LCMS/MS analysis detected 3 compounds in partially purified cytotoxic active fraction based on similar m/z value in HR-MS analysis. The compounds are neral, dihydrospatheliachromone, and a derivative of vitamin D3 (1 alpha, 25-dihydroxy-26, 27-dimethyl-20, 21, 22, 22, 23, 23-hexadehydro-24a-homovitamin D3). Among the isolates, QIEF 5 (*Colletotrichum* sp.) has the highest IAA production at different concentrations of tryptophan. IAA production by QIEF 5 was characterized by TLC, HPLC and HR-MS analysis. Moreover, incubation with QIEF 5 influenced the growth of rice seedlings.

Chapter 1

INTRODUCTION

INTRODUCTION

Endophytes are the new source of convenient bioactive compounds and they are expounding as the microorganisms that lives inside the tissues of plants either intracellularly or intercellularly. Endophytes live in the plant as partially in their entire life span without developing any visible symptoms to the host plant (Jiang et al., 2013). The word endophyte was coined by Heinric Anton de Bary in 1884, which includes bacteria, fungi, algae and are considered as the major constituents of plant microbiomes with potential applications in agriculture, medicine and food industry (Newman and Cragg, 2012; Selim et al., 2012; Wu et al., 2015). Endophytes are endosymbiont; forming symbiotic association with the host plants. The colonization of endophytes in to the tissues of host plants is asymptomatic (Aly et al., 2011) and it is the balanced antagonism between the endophyte and the host, where the endophytes get nutrients from the plant and they protect the plant from environmental stress. Endophytes are beneficial to the host by promoting host plant growth, increasing plant nutrient uptake, inhibiting pathogen growth on plants and reduces the intensity of diseases (Zhang et al., 2006; Bae et al., 2009).

Among the endophytes, fungi have the broad spectrum of biologically active natural products. They are the abundant and diverse group of organisms found in plants (Huang et al., 2008; Naik et al., 2008; Pawlowska et al., 2014) including the seed plants, gymnosperms, algae, ferns, lichens and mosses (Naik et al., 2014). Each plant holds one or more endophytic fungi. The entry of endophytes to the host is by penetrating the plant tissues through roots, aerial parts like stomata, cotyledons and flowers (Kobayashi and Palumbo, 2000). Fungal endophytes are involved in two modes of transmission, either directly from parent to offspring via the host seeds as vertical transmission or among individuals by sexual or asexual spore transfer as horizontal transmission (Saikkonen et al., 2004). Endophytic fungi are classified functionally in to two as clavicipitaceous or grass endophytes (C-endophytes) and non clavicipitaceous (NC-endophytes) based on the taxonomy, plant hosts, relationship within the organisms and ecological functions of endophytic fungi. The NC-endophytes includes the endophytes of non-vascular plants, corns, angiosperms

and ferns (Rodrigues et al., 2009). Endophytes are grouped in to two, the group I that do not form external structures and group II form the structures that derived from host as the mycorrhizae, the special type of endophyte and N_2-fixing bacteria.

The distribution of endophytic fungi depends upon some of the environmental factors like humidity, temperature, geographic location, vegetation and illumination (Song et al., 2007) which influenced the spore germination, reproduction, growth and metabolism throughout the entire life cycle of endophytic fungi. Some endophytes were also particular to specific host plants (or families), genetic background of species (D' Amico et al., 2008) and age of the host tissues (Sieber, 2007). The endophyte-host relationship depends up on the host-specificity, host selectivity, host preference, or host recurrence (Cohen, 2006). The interactions/associations between the fungus and plant were preceded by the physical encounter where the endophytes possess diverse virulence factors and plants have the protection mechanism. If both the virulence factors and defence mechanism were balanced, the interactions maintain asymptomatic. However, the environmental factors influence the balanced mechanisms and it counteract each other, either the fungus perish or the plants cause diseases. Thus the host-endophyte interactions may later turn into latent pathogens, mutualists, commensals, temperory residents or latent saprotrophs (Davis and Shaw, 2008; Purahong and Hyde, 2011).

Endophytic fungi identify traditionally based on the morphological characteristics i.e, colour of the colony, hyphae, spores and sexual reproduction structures, but large number of endophytes is non- sporulating known as 'sterile mycelia' which are difficult to identify. Hence molecular techniques are used as an alternative method for taxonomic studies to reveal the hidden identities and diversity or sterile mycelia (Fierer, 2008). Internal transcribed spacer (ITS) is the spacer DNA present at the 18S and 5.8S ribosomal DNA (rDNA) coding the ITS1 region and 5.8S and 28S rDNA coding ITS2 region (Bakker et al., 1992). It is the extensively used identification tool for fungal endophytes (Gherbawy and Hussien, 2010; Pecoraroa et al., 2012; Gherbawy and Gashgari, 2013).

Endophytic fungi have beneficial relationship with their host plants. They have the potential to produce large number of bioactive secondary metabolites

predominantly as natural products which belong to the types of alkaloids, phenolics, flavonoids, steroids, lactones and terpenoids derivatives (Yu et al., 2010; Gutierrez et al., 2012; Deshmukh et al., 2014). The bioactive secondary metabolites conceivably used as therapeutic agents against various diseases (Kusari and Spitteler, 2012; Debbab et al., 2013; Mousa and Raizada, 2013) which have also been found to be novel anticancer agents and antibiotics (Strobel, 2015). These metabolites are mainly involved in antibacterial, antitumor, antifungal and anti-inflammatory activities. According to WHO's GLOBOCAN, about 14.1 million new cancer cases were reported and that are anticipated to rise about 22 million in the next two decades (Torre et al., 2015). Endophytic fungi are the new source of antimicrobial agents. They are the low molecular weight organic natural substances active against other microorganisms at low concentrations (Guo et al., 2008) and are applied as the major alternative to overcome the high level of drug resistance by plant and human pathogens (Yu et al., 2010). As well endophytes produce antioxidants as bioactive compounds, the substances that guard the cells against the damage due to free radicals and ROS that causes various effects including degeneration of cells, damage occurring in nucleic acids and oncogenesis (Huang et al., 2007; Seifried et al., 2007) leads to the cancer and alzheimer's diseases.

Natural products as sources of new drugs derived from endophytes are in clinical development as anticancer and anti-infective agents. One of the most important natural products produced by endophytic fungus is the multibillion dollar anticancer drug taxol (Paclitaxel). It was first isolated from the pacific yew tree *Taxus brevifolia* in 1971 and is the traditional medicinal plant used by Native Americans since 1945. Some endophytic fungi *Taxomyces andreanae* of the same plant also produced the same compound. This provides the rationale for the use of traditional medicinal plants as the initial source of endophyte isolation for the production of bioactive compounds. Camptothecin, Podophyllotoxin, Vincristine, vinblastine are some of the major plant derived anti-cancer drugs, which were also produced by the fungal endophytes isolated from the specific plants (Demain and Sanchez, 2009; Zhao et al., 2011).

Plants produce phytohormones; the natural substances for its growth and development. Apart from plants, endophytes mainly bacteria (Apine and Jadhav, 2011) fungi (Waqas et al., 2012; Hoffman et al., 2013) and yeast (Xin et al., 2009) have also been known to produce different classes of phytohormones; the signalling molecules that control growth and development of plants (Davies, 2010; Rashid et al., 2012). One of the prime phytohormone is indole -3-acetic acid (IAA), a principle auxin that plays a vital role in cell division, elongation and enlargement (Ishida et al., 2013; Kumla et al., 2013). It is mainly produced by L-tryptophan metabolism which is necessary for embryogenesis, seedling growth and developmental processes. Besides auxins, endophytes produce gibberllins, abscisins and ethylene responsible for the developmental processes (Firakova et al., 2007) and they involved in nitrogen fixation, phosphate solubilisation, siderophore and ACC deaminase production, enhance phosphorous uptake and provide essential vitamins to the host plants (Yadav et al., 2016).

Endophytes also have the potential to produce various extra cellular enzymes like amylases, cellulases, proteinases, laccases, pectinases and lipases. These enzymes play the vital role in hydrolysis and biodegradation processes, the remarkable mechanisms against the pathogenic infection and to acquire nutrition from host plants (Sunitha et al., 2013). Endophytes play the major role in agriculture industry particularly in biotic and abiotic stress responses including drought, high salinity, heat, and pathogen attack that inversely affect the plant morphology and physiology (Hardoim et al., 2015). The stress responses increased with the possible mechanism of antioxidant pathways due to the production of antioxidant compounds or by the effectors from endophytic fungi that stimulate the antioxidant pathway of host plant (Egamberdieva et al., 2017). Moreover the biological activities of endophytic fungi are also involved in anti-diabetic, anti-malarial, immune suppressant, anti-nematodes and insecticidal agents. Plant pathogenic fungi are one of the serious threats to the production yield and health of the plant that causes diseases to the plants. The use of chemical pesticides in the agricultural field extensively leads to the development of pesticide resistant strains (Liu et al., 2016). Endophytic fungi increase resistance to the pathogenic invaders and protect the host plants (Yu et al., 2017).

Plants with good medicinal values have been considered as the most promising sources of natural products and were utilized in ancient times as therapies for many diseases. Medicinal plants produce a plethora of biologically active compounds; about 6 % of existing higher plants were explored for their biological activities from a total of 10 to 15 % of the medicinal plants investigated (Bisht et al., 2006). Natural products, especially distinct bioactive compounds derived from plants are being used as paramount drugs in different countries. Twenty five percentages of authorized drugs worldwide were originated from plants, considering 11 % of the basic and vital drugs recognized by the WHO (Dubey et al., 2012). However, the necessity of bioactive molecules from plants unfortunately leads to the disadvantages like low yield due to varying environmental conditions, overharvesting of plants due to the limited supply of plant derived drugs. Favourably endophytes alleviate the problem faced from plant derived bioactive molecules. Endophytes from the medicinal plants are potential to produce the novel bioactive secondary metabolites, it also accumulate the secondary metabolites as the important medicinal drugs originally produced from the host plants (Shwab and Keller, 2008).

Rudraksha is the common name of *Elaeocarpus sphaericus*. It is an evergreen tree found in tropical and subtropical areas. It is known as the king of herbal medicine and it is applied in traditional medicine as a therapy to treat various diseases including the common mental problems such as hypertension, stress and anxiety, degenerative joint disease; besides it is used to cure liver diseases (Khare, 2004). *Quassia indica*, the tree of 10 to 15 meter height that distributed in coastal, tropical and subtropical forests. It is used as the traditional medicine by local folklore practitioners and tribal people in India to treat various diseases. Bark of *Quassia indica* is used for the treatment of fever and skin diseases, the macerated bark is used as tonic and stomachic. Quassia is used for the treatment of various diseases like vata, kapha, arthritis, constipation and skin diseases like scabies, pruritus, leprosy, erysipelas (Viswanad et al., 2011).

1.1. AIM AND OBJECTIVES

Owing to the above mentioned knowledge and considering endophytic fungi as the new resource with potential in biotechnology**,** the present investigations were carried out to isolate and identify endophytic fungi from the two medicinal plants namely *Elaeocarpus sphaericus* (Gaertn.) K. Schum. and *Quassia indica* (Gaertn.) Nooteb. Endophytic fungi from these two plants were hitherto unexplored. Subsequently, bioprospecting of endophytic fungi insight to the production of secondary metabolites for future medicinal, industrial and agricultural applications were explored. The phytochemicals, antibacterial and cytotoxic activities of the plants *Elaeocarpus sphaericus* and *Quassia indica* were evaluated.

OBJECTIVES

1. Isolation and identification of endophytic fungi from *Elaeocarpus sphaericus* (Gaertn.) K. Schum. and *Quassia indica* (Gaertn.) Nooteb.
2. Screening of endophytic fungal extracts for antibacterial and cytotoxic activities.
3. Characterization of active secondary metabolites produced by the selected endophytic fungus and their antibacterial and cytotoxic activities.
4. Evaluation of phytochemical constituents, antibacterial and cytotoxic activities of the host plants, *Elaeocarpus sphaericus* and *Quassia indica*.
5. Characterization of plant growth regulator from the selected endophytic fungus and the effect of fungus on growth of rice seedlings.

Chapter 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Endophytes are the microorganisms that colonize the internal tissues of the plants without generating an apparent disease to the host. It could be either a fungus or a bacterium that inhabit the plants (Strobel and Daisy, 2003) and they are omnipresent as predominance in plant species till today. While, a greater amount of the foliar endophytic fungi are yet to be discovered (Hyde et al., 2007, Rodriguez et al., 2009). Anton de Bary, the father of plant pathology introduced the term endophyte to describe the colonization of microorganisms that penetrate the internal tissues of the host plants like roots, aerial parts like stomata, cotyledons and flowers (Kobayashi and Palumbo, 2000). The word endophyte is as broad as its definition, it also used for pathogenic bacteria, pathogenic endophytic algae, parasitic endophytic plants, fungi in latent developmental stages and microorganisms in symbioses or commensals (Sturz and Nowak, 2000; Bai et al., 2002). Other than endophytes the fungi related to plants are classified to different groups as mycorrhizae, epiphyte, pathogens and saprotrophs but most of these endophyte- plant relationships are limited (Faeth, 2009).

2.1. Endophytic fungi

Endophytic fungi are the major group of microorganisms present in the tissues of plants. They are principal component in plant- microbial ecosystems mainly belong to ascomycete's class with mitosporic and meiosporic reproduction. In general endophytic fungi are beneficial to the host plants by promoting host plant growth, inhibit pathogen growth in plants, increase the nutrient uptake of plants and decrease the intensity of the diseases to plants (Zhang et al., 2006; Bae et al., 2009). Endophytic fungi were applied as new sources of metabolites in agriculture, medicine and pharmaceutical industry.

2.2. Classification of endophytic fungi

Two major classes of endophytes are clavicipitaceous or grass endophytes (C-endophytes or class I) and non clavicipitaceous (NC-endophytes) based on the taxonomy, host plants, relationship within the organisms and ecological functions of

endophytic fungi. Clavicipitaceous types hold the unique properties different from NC-endophytes. NC-endophytes are further classified in to type II, III, IV as interactions leads to symbiotic to pathogenic. The NC-endophytes includes the endophytes of non-vascular plats, corns, angiosperms and ferns as they represents three distinct functional groups as class II, class III, class IV (Rodrigues et al., 2009). Class II colonize both above and below ground tissues that consists of mainly ascomycetes and a minor group of basidiomycetes that comes under the group of dikarya based on the colonization and transmission in the hosts and within the plant biodiversity. Class I and II transmit either vertically or horizontally; but only horizontal transmission was observed in Class III and Class IV. Class III endophytes were limited to the growth in plant tissues below the ground and they found in localized areas of plant tissues while the forth class were colonized in many of the plant tissues and developed below the ground (Rodrigues et al., 2009). Endophytic fungi were classified in to three ecological groups as mycorrhizae, pasture and non-pasture endophytic fungi (Feath and Fagan, 2002).

2.3. Diversity and occurrence of endophytic fungi

Endophytic fungi are the diverse and unexplored prime groups of microorganisms. About 10,000 fungal species of the estimated 1.5 million endophytic fungi were characterized so far while, remaining were unexplored. Endophytic fungi comprise a large, while hidden constituent of fungal biodiversity (Arnold et al., 2000; Rodrigues et al., 2009). Plants serve as the host for different communities of endophytes as internal tissues (Sun et al., 2008); one or more endophytic fungi were present in almost all plant species and different parts of the plants have been exploited for endophyte isolation (Rodriguez et al., 2009, Currie et al., 2014). To date a few plants in the environment have been explored for their endophytic biodiversity. More than one species of specific endophytic fungi were found in single leaves, for example in soybean (Impullitti and Malvick, 2013) in tree leaves of tropical area (Vaz et al., 2014). Same species of fungal isolates from different leaves of same plants were also exhibited the systematic growth within their hosts. The distribution of endophytic fungi depends on the environmental factors like geographic location, temperature, humidity, illumination and vegetation (Suryanarayanan et al., 2005; Song et al., 2007).

Endophyte diversity is observed high in tropical forests as the diversity of host plants high, colonization rates of endophytic fungi vary in tropical forest trees (Hyde and Zoytong, 2008). Leave, stem and root of a single plant usually diverse in their dominant isolates. Reports showed that the *Bouteloua gracilis* plant had a diverse pattern of root endophytes and may also produce functional differences (Chaverri and Gazis, 2010; Herrera et al., 2010). Distribution of endophytic fungi in the medicinal plant *Adecalymma alliaceum* Miers and the diversity of the isolated endophytic fungi studied for the first time, stems, leaves and petioles of *A. alliaceum* exhibited varieties group of organisms (Kharwar et al., 2011). The evidences showed that the diversity of endophytic fungi in endemic medicinal plants of Tirumala hills reported for the first time (Anitha et al., 2013) and *Spilanthes acmella*- a promising medicinal plant scanned out for their diversity (Lakshman and Jayshree, 2013). The diversity was also observed in different leaf stages of same plant; the important medicinal plant *Calotropis procera* was studied for their biodiversity in different leaf stages (Nascimento et al., 2015).

2.4. Endophyte-host plant interaction

Endophytic fungi manifest the systematic growth within the host plants which revealed a symbiotic to pathogenic relationship; they can be mutualists, latent pathogens, temporary residents and latent saprotophs (Davis and Shaw, 2008) till the favourable environmental factors of endophytic fungi and the host plants changed (Sieber, 2007). The type of interaction between fungi and host is showed in figure 2.1. These interactions were influenced by the phylogenetic relationship, abiotic factors, and geographic location. The metabolic interactions of endophytes with its host favoured the synthesis of biologically active secondary metabolites. Some endophytic fungi colonize to their host plants exhibited the beneficial interactions by producing different bioactive compounds like alkaloids, flavonoids, terpenoids and steroids to increase abiotic and biotic stress resistance (Firakova et al., 2007; Rodrigues et al., 2009). Endophytes are able to produce different phytohormones that upgraded their host plant growth (Waqas et al., 2012) and they could assist the accumulation of secondary metabolites including the essential medicinal components or drugs originally produced by their host plants (Shwab and Keller, 2008).

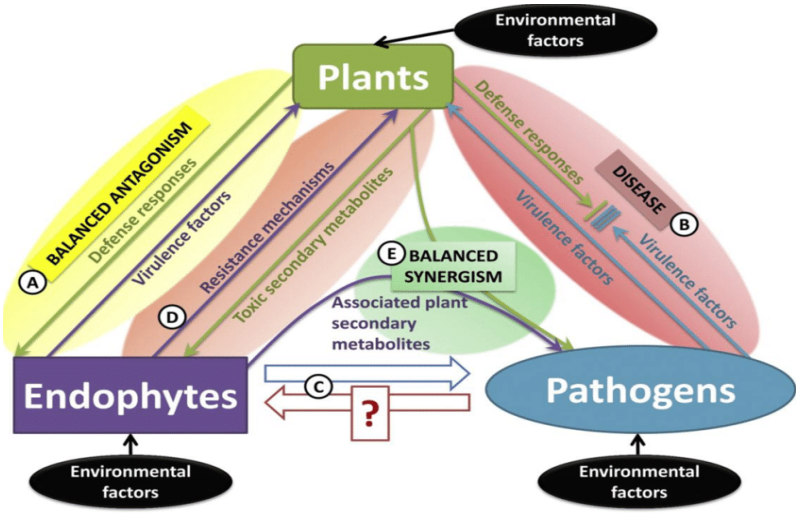


Figure 2.1. Interactions between endophytes and host plants. (A) Balanced antagonism concept, (B) disease caused by endophytic fungi to host plants was shown, (C) demonstrated endophyte- pathogen mutualism, (?) pointed out that the process not happening universally; advance research needed for the mechanism (D) illustrated the resistance mechanism of endophyte, (E) indicated balanced synergism.(Kusari et al., 2012)

2.5. Techniques used for the identification of endophytic fungi

Identification of endophytic fungi by traditional method was mainly done based on morphological features and the sporulating characteristics by microscopic observations (Sette et al., 2006; Crous et al., 2007). Microscopic identification required the hyphal characteristics and the sexual or asexual reproduction structures of the spores. Spore production may depend upon on the provided cultural conditions of the fungus (Guo et al., 2000). The isolates that failed to produce the spores are sectioned in to separate morphotypes as mycelia sterilia (Lecap et al., 2003). The non-sporulating or low-sporulating characteristics were frequently observed in endophytic fungi. Endophytic fungi isolated and identified from *Pilanthes acmella* Linn. based on the morphology of spores, the identified fungal species were *Aspergillus flavipes*, *Aureobasidium pullulans*, *Aspergillus niger*, *Bipolaris nodulosa*, *Cladosporium epiphyllum*, *Colletrichum corda*, *Hymenula affinis*, *Rhizopus nodusus* (Lakshman and Jayshree, 2013). Endophytic fungus isolated from Tirumala hills were identified by sporulating features and the identified isolates are *Colletotrichum falcatum*, *Fuzarium oxysporum*, *Aspergillus fumigates*, *Pestalotiopsis* sp., *Aspergillus flavipes*, *Penecillum senticosum*, Sterile mycelia, *Gliocladium rosium*, *Phomopsis jaquiniana*, *Nigrospora sphaerica*, *Leptosphaeria* sp., *Phomopsis archeri*, *Alternaria alternata, Aspergillus*

niger (Anitha et al., 2013). Similar microscopic identification was used to identify four genera of endophytic fungi from the leaves of Makassar fruit and the identified cultures are *Trichoderma* sp., *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp. (Amin et al., 2015).

2.5.1. Molecular method

Molecular techniques, the highly sensitive and specific tool have been used as the identification for taxonomic characterization of endophytic fungi. The non-sporulating fungi which are difficult to identify may use molecular techniques to identify the isolates. The rDNA gene complex region in fungi are 18S rDNA coding region, ITS1, sequence coding for 5.8S rDNA gene, ITS2 region and 28S coding region are shown in figure 2.2. The coding regions are conserved between fungi that give the molecular base for phylogenetic relationship (White et al., 1990) and the internal transcribed regions present in between the coding regions develop rapidly and form the variation of fungi in their genus and species identity. Both the rRNA coding and the ITS regions present as tandem repeats with thousands copies long, each was separated by the intergenic spacer (IGS) region. ITS region sequencing is the extensively used technique for the taxonomic classification of fungi and it is approved as the universal barcode sequence for fungi (Schoch et al., 2012).

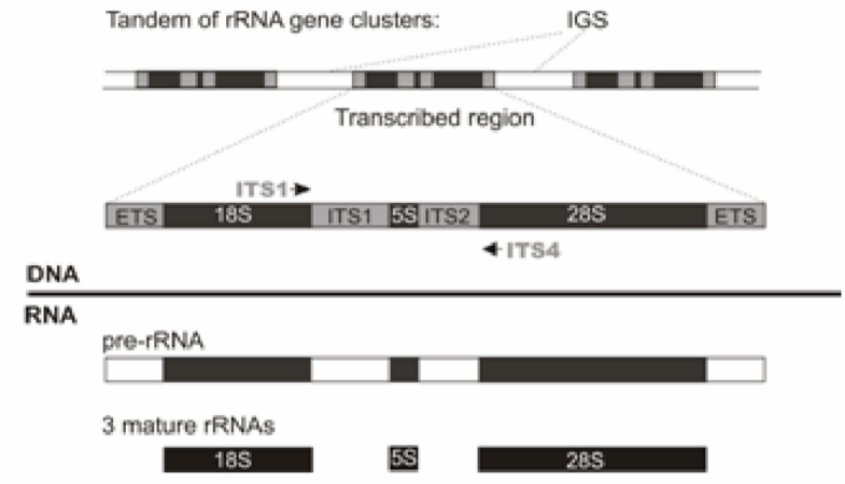


Figure 2.2. The location of rDNA gene complex region. The location of rDNA, the spacer regions ITS1 and ITS2 and the position of primers ITS1 and ITS4 for the PCR amplification. ETS- external transcribed spacer, IGS- non transcribed intergenic spacer (Shahid et al., 2014).

Several studies used ITS regions for the taxonomic identification of endophytic fungi. Sette et al., 2006, taxonomically characterized the filamentous fungi and established the phylogenetic relationship between the isolates of coffee plants (*C. arabica* and *C. robusta*). Thirty three taxonomically different isolates were identified from leaves of *Calotropis procera* in Taif Region based on ITS sequencing. The frequently identified isolates are *Penicillium chrysogenum*, *Aspergillus flavus*, *Cochliobolus lunatus*, *F. oxysporum*, *Penicillium chrysogenum*, *Chaetomium globosum* and *Fusarium dimerum* (Gherbawy and Gashgiri, 2013). The medicinal herb *Salvia miltiorrhiza* have endophytic fungi in their roots, which were taxonomically classified by the morphological and ITS rRNA gene sequencing. Among the isolates, *Fusarium* sp. and *Alternaria* sp. are the two common fungi present in their roots (Lou et al., 2013). Fungal endophyte community related with *Carapa guianensis*, the Amazonian medicinal plant identified 35 different genuses by using ITS region sequencing. The fungal isolates are *Xylaria*, *Colletotrichum*, *Diaporthe*, *Endomelanconiopsis*, *Aspergilllus*, *Fusarium*, *Pestalotiopsis*, *Phomopsis*, *Guignardia*, *Beltrania*, *Pilidiella, Trichoderma*, and *Botryosphaeria*. The identified taxa provide molecular similarity and phylogenetic analysis of fungal sequences (Ferreira et al., 2015). Morphological and molecular techniques were also used to identify the isolate, *Colletotrichum gloeosporioides* from the leaves of *Camellia sinensis* (Rabha et al., 2016). Identically, the isolates *Diaporthe* sp. *Phomopsis* sp. and *Alternaria* sp. were isolated and identified from *Annona muricata* by ITS sequencing and the phylogeny revealed identity between the isolates (Minarni et al., 2017).

2.6. Chromatographic techniques to study the endophytic fungal metabolites

Chromatographic techniques were used to isolate and purify the metabolites produced by endophytic fungi. Thin layer chromatography is an important method for analysis of metabolites and it is employed to track the metabolites of endophytic fungi. Ethyl acetate extract of fungal endophytes isolated and characterized from *Centella asiatica* were employed for TLC analysis to determine the presence of alkaloid and flavonoids. Ethyl acetate-methanol-water (10:1.3:1) was used as mobile phase for alkaloid and ethyl acetate-formic acid-acetic acid-water (10:1.1:1.1:2.6) for flavonoids. Derivatization with appropriate reagents confirmed the presence of

these secondary metabolites in fungal extracts (Devi et al., 2012). Thin layer chromatography to separate components within extracts of endophytic fungi isolated from *Coleus amboinicus* Lour studied by Astuti et al., 2014. The metabolites were separated on TLC plates and were kept above the agar plate inoculated with microorganisms to reveal the presence of antimicrobial compounds by TLC bioautography technique. Further chemical contents were visualized using TLC detection reagents. Isolation of endophytic fungi from *Artemisia annua* L. and the identification of its antimicrobial compounds by using contact bioautography method were described by Purwantini et al., 2015. They developed the TLC plate using toluene: ethyl acetate: formic acid (6:3:1 v/v) as mobile phase. The result showed that the extract of endophytic fungi had an antimicrobial compound (Rf value = 0.383) and the active compound was dimethyl cladosporine.

Antibacterial compounds from rare endophytic actinomycetes *Micrococcus yunnanensis* were isolated and characterized by chromatography based purification. The crude extract were purified by analytical TLC with chloroform-methanol (24:1 v/v), chloroform-Methanol (7.5:17.5 v/v), methanol-dichloromethane-water (1:1:1 v/v), benzene-acetic acid-water (4:1:5 v/v) and acetonitrile-water (92.5:7.5 v/v) successively. Bands showed in TLC plates were scraped off and dissolved in methanol for the evaluation of antibacterial activity. The solvent system was also optimised to separate antibacterial compound on bulk scale using preparative TLC (Ranjan and Jadeja, 2017). A study by Rante et al., 2017, identified the bioactive compounds from endophytic fungi of the medicinal plant *Mellochia umbellate*. The active extracts were run on TLC for identification of their metabolites using hexane: ethyl acetate (2:1) and the results were observed under UV at 254nm, 366 nm and visible light. Metabolite identification by TLC and corresponding spraying techniques revealed that the active extracts contained alkaloids, flavonoids and steroids. The antibacterial and antioxidant activity was also analysed by TLC. Active extracts were developed with CH_2Cl_2: MeOH (10:1) and the plates were sprayed with p-iodonitrotetrazolium violet reagent. Growth inhibition of bacteria was observed by clear zone formation around the extract. The plates was dried and sprayed with 0.02 % DPPH in methanol where a yellow spot on purple background indicated the antioxidant activity (Praptiwi et al., 2018).

Chromatographic techniques like HPLC and LC-MS analysis were used for the identification of compounds from endophytic fungi. Purification and identification of the compound camptothecine produced by three endophytic fungi, *Fomitopsis* sp., *Alternaria alternata* and *Phomposis* sp. isolated from the seeds and fruits of *Miquelia dentata* was done by using the chromatographic techniques like HPLC, LC-MS and ESI-MS/MS (Shweta et al., 2013). HPLC and mass spectrometry analysis was used to identify the biologically active steroidal alkaloids- peimisine, peimine and imperialine-3β-D-glucoside from the endophytic fungus *Fusarium redolens* 6WBY3 (Pan et al., 2015). Similarly, the compounds- vincamine, tabersonine and ethyl-vincamine isolated from endophytic fungi of *Nerium indicum* were analyzed by using HPLC analysis, amount of each compound was calculated by the regression equation of standard curve. Further, LC-MS analysis of these compounds displayed the m/z values that indicated the molecular mass of the compounds (Na et al., 2016). Semi preparative HPLC was used to separate the fractions and also to purify the compounds campyridones A and B and campyridones C and D from *Campylocarpon* sp. HDN13-307, a mangrove endophytic fungus (Zhu et al., 2016). The fungus, *Periconia* sp. F-31 extracted and subjected different chromatographic techniques like column chromatography, preparative HLPC to purify the compounds Periconones B-E (Liu et al., 2017).

2.7. Endophytic fungi and its metabolites isolated from medicinal plants

Endophytic fungi are capable of producing the bioactive secondary metabolites from medicinal plants. Due to the long duration in co-evolution, the fungus and the host plants gradually develop a friendly relationship. This may benefit both the fungus and host, where the host can provide the abundant nutrients and habitat for the growth and survival of its endophytes. In other way, the endophytic fungi generate numerous metabolites to prevent the hosts from the environmental stresses, which successively lead to the growth of the host plants (Silvia et al., 2007; Rodriguez et al., 2009). Endophytic fungi evolved the potential to generate similar or same bioactive secondary metabolites that are originally generated from the hosts (Gunatilaka, 2006; Zhou et al., 2009). However, the increased necessity of plant related bioactive molecules leads to the disadvantages like low yield due to the changes in the environmental factors and the overharvesting of medicinal plants due to the least availability of specific

metabolites. Thus endophytic fungi from medicinal plants were selected as the probable and alternative sources of secondary metabolites with high impact on bioactivity.

Researchers focussed on the isolation of endophytic fungi and their metabolites particularly from medicinal plants. The leaves of Neem tree hold the endophytic fungus *Fusarium avenaceum* (Rajagopal and Suryanarayanan, 2000). Western Ghats region, India has largest diversity in plants and correspondingly diversity of endophytic fungi. Medicinal plants (*Adhatoda zeylancia*, *Bauhinia phoenicea*, *Callicarpa tomentosa*, *Clerodendrum serratum* and *Lobelia nicotinifolia*) in Kudremukh Range Western Ghats own the dominant fungi *Curvularia clavata*, *C. lunata*, *C. pallescens* and *F. oxysporum* (Raviraja, 2005). Endophytic fungi were isolated from endemic medicinal plants of Tirumala hills and identified different endophytic fungal species. viz. *Colletotrichum falcatum*, *Fuzarium oxysporum*, *Aspergillus fumigates*, *Pestalotiopsis species*, *Aspergillus flavipes*, *Penecillum senticosum*, *Sterile mycelia*, *Gliocladium rosium*, *Phomopsis jaquiniana*, *Nigrospora sphaerica*, *Leptosphaeria species*, *Phomopsis archeri*, *Alternaria alternata, Aspergillus niger* (Anitha et al., 2013). Mycoflora of *Terminalia arjuna*, the Indian medicinal plant have the endophytes *Aspergillus flavus*, *Diaporthe arengae*, *Lasiodiplodia theobromae* and *Aspergillus flavus*, *Alternaria* Sp.. The leaves of the balloon plant, *Cardiospermum halicacabum* have the endophytic fungi *Penicillium finiculosum* and *Trichoderma viride* (Chathurdevi and Gowrie, 2016).

Camptothecin, the important anticancer drug was also isolated from the endophytic fungi *Entrosphospora infrequens* from inner bark of *Nothapodytes foetida* in India. Similar works were also performed by Shweta et al., 2010. They isolated 10-hydroxy camptothecin and 9-methoxycampthothecin apart from camptothecin from *Fusarium solani*, the endophytic fungi of *Apodytes diminata*. *Azadirachta indica*, one of the important medicinal herbs used to isolate the endophytic fungi *Chloridium* sp. and identified the compound Javanicin, a napthoquinone representing strong antibacterial agents (Kharwar et al., 2009). Deshmukh and co researchers in 2009, isolated endophytic fungus from the medicinal plant *Mimosops elengi* and further isolated the metabolite ergoflavin, the pigment having anti-inflammatory and

anticancer activities. The important drug taxol was also isolated from different endophytes such as *Fusarium* sp. from *Taxus wallichiana* (Gogoi et al., 2008), *Fusarium solani* from *Taxus celebina* (Chakravarthi et al., 2008) and *Gliocladium* sp. from *Taxus baccata* (Sreekanth et al., 2011) in India. Functional metabolites produced by endophytic fungi are listed in table 2.1.

Table 2.1. Functional metabolites produced by endophytic fungi

Compound	Endophytic Fungus	Host plant	Biological activity	References
Phomodione	*Phoma* sp.	*Saurauia scabbrrinae*	Antimicrobial	Hoffman et al., 2008
Eremophilanolide	*Xylaria* sp.	*Licuala spinosa*	Anticancer	Isaka et al., 2010
Chlorogenic acid	*Sordariomycete* sp. B5	*Eucommia ulmoides*	Antibacterial Anticancer Antioxidant	Chen et al., 2010
Benquoine	*Phomopsis* sp.	*Alpinia malaccensis*	Antimicrobial	Adelin et al., 2011
Tyrosol	*Diaporthe helianthi*	*Leuhea divaricata*	Antimicrobial	Specian et al., 2012
Chaetoglobosin X	*Chaetomium globosum*	*Curcuma wenyujin*	Anticancer	Wang et al., 2012
Rhein	*Fusarium solani*	*Rheum palmatum* L.	Antimicrobial Anticancer	You et al., 2013
Botryorhodine E	*Botryospheria obtuse*	*Bidens pilosa*	Anticancer	Abdou, 2013
Camptothecin	*Trichoderma atroviride*	*Camptotheca acuminata*	Anticancer	Pu et al., 2013
	Fomitopsis sp.	*Miquelia dentata*	Anticancer	Shweta et al., 2013
Cryptosporioptide	*Cryptosporiopsis* sp.	*Viburnum tinus*	Antimicrobial	Saleem et al., 2013
Polyketide	*Penicillium citrinum*	*Ocimum tenuiflorum*	Anticancer	Lai et al., 2013
Botryorhodine A–D	*Botryosphaeria rhodina*	*Bidens pilosa*	Anticancer	Abdou et al., 2010
Sanguinarine	*Fusarium proliferatum*	*Macleaya cordata*	Antimicrobial	Wang et al., 2014
Colletonoic acid	*Colletotrichum* sp.	*Salsola oppositifolia*	Antimicrobial	Hussain et al., 2014

Compound	Endophytic Fungus	Host plant	Biological activity	References
Atrovenetinone	*Phoma* sp.	*Senecio kleinii*	Antimicrobial	Hussain et al., 2015
Kaempferol	*Mucor fragilis*	*Sinopodophyllum hexandrum*	Antimicrobial	Huang et al., 2014
Vincristine	*Talaromyces radicus*	*Catharanthus roseus*	Anticancer	Palem et al., 2015
Vinblastine	*Talaromyces radicus*	*Catharanthus roseus*	Anticancer	Palem et al., 2015
Taxol	*Paraconiothyrium* sp.	Yew trees	Anticancer	Soliman et al., 2015
Harzianone	*Trichoderma* sp. *Xy24*	*Xylocarpus granatum*	Anticancer	Zhang et al., 2016
Meleagrin	*Penicillium chrysogenum*	*Olea europea*	Anticancer	Mady et al., 2016
Ilicicolin H	*Campylocarpon* sp.	*Sonneratia caseolaris*	Anticancer	Zhu et al., 2016
Periconones E	*Periconia* sp.		Anticancer	Liu et al., 2017
Vinblastine	*Nigrospora sphaerica*	*Catharanthus roseus*	Anticancer	Ayob et al., 2017
Vinblastine	*Nigrospora sphaerica*	*Catharanthus roseus*	Anticancer	Ayob et al., 2017
Monocerin	*Exserohilum rostratum*	*Bauhinia guianensis*	Antimicrobial	Pinheiro et al., 2017
Compound Rz	*Polyporales* sp.	*Rheum emodi*	Anticancer	Dar et al., 2017
Gallic acid	*Fusarium* sp. JZ-Z6 *Fusarium* sp. JZ-Z7	*Fritillaria unibracteata*	Antioxidant	Pan et al., 2017
oxo-Agarospirol	*Arthrinium* sp. 0042 *Colletotrichum* sp. 0047/0048 *Diaporthe* sp. 0051	*Aquilaria subintegra*	Antioxidant	Monggoot et al., 2017
Indole-3-acetic acid	*Pleurotus ostreatus*	*Jatropha seedcake*	Growth promoting	Bose et al., 2013
	Alternaria alternata	*Asclepias sinaica*		Fouda et al., 2015
	Alternaria sp.	*Brassica napus*		Shi et al., 2017

2.8. Endophytic fungi as the producers of antimicrobial compounds

Endophytic fungi from medicinal plants are the promising sources of antimicrobial agents. Antimicrobial activity had provided the basis for its application in pharmaceutical industry in the form of traditional and folk medicine. Plants used in folk medicine have played important role in isolation of bioactive metabolites of endophytic fungi. Bio prospecting of fungal endophytes from *Vitex negundo* L. was studied and screened for their antimicrobial potential could justify the traditional use of *Vitex negundo* L. against human pathogenic bacteria (Desale and Bodhankar, 2013). The ethyl acetate extracts of endophytic fungi from the roots of the medicinal herb *Salvia miltiorrhiza* Bunge were screened for antimicrobial activity on pathogenic bacteria and fungi. Among the isolates, *Alternaria* sp. and *Sarocladium kiliense* were found to have strong antibacterial and antifungal activities (Lou et al., 2013). *Calotropis procera* is an important medicinal plant and some of the properties may be mediated by its fungal endophytes that revealed the presence of potential antimicrobial sources (Nascimento et al., 2015). Investigations for antimicrobial potential of fungal endophytes from three popular medicinal plants (*Alstonia boonei*-Ahun, *Enantia chlorantha*- Awopa and *Kigelia africana*-Pandoro) having ethno botanical history were screened against the human pathogens *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi* and *Candida albicans* (Tolulope et al., 2015). Various endophytic fungi were inhabit the different tissues of *C. procera* have capability of producing bioactive secondary metabolites with significant antibacterial activity. The antibacterial activity of the crude ethyl acetate extracts of 20 different fungal endophytes isolated from different tissues of *Calotropis procera* by using agar well diffusion method against nine bacterial strains. The endophytes belong to *Aspergillus* and *Fusarium* genus exhibited good antibacterial activity (Rani et al., 2017). The fungal extracts *Colletotrichum* sp., *Alternaria* sp., *Pestalotiopsis* sp. of *Madhuca longifolia* bark found to have highest activity against the pathogens *Bacillus subtilis, Staphylococcus aureus, Salmonella typhi and Vibrio cholera* (Kuralarasi and Linkakumar, 2018).

The consciousness of scientific community has been drawn to endophytes due to their potential to produce novel bioactive compounds as the necessity for new

bioactive compounds in medicine, industry, and agriculture had been increasing. Fungal endophytes from *C. acuminata* could be used as an alternative source for the production of 9-Methoxy Camptothecin (9-MCPT), a well known anticancer drug and other natural antimicrobial compounds. Metabolites of endophytic fungi exhibited good biological activities. *Plectophomella* sp., *Physalospora* sp. and *Crataegus monogyna* of were characterized for the identification of the compounds (-)-mycorrhizin A, cytochalasins E and K and radicinin, which showed significant antibacterial, antifungal and herbicidal activities (Hussain et al., 2014). Secondary metabolite equisetin of the endophytic fungus *Fusarium* sp. was isolated from *Opuntia dillenii* exhibited antibacterial property. The compound was isolated through bioassay guided chromatography; the chemical structure was identified as the tetramic acid derivative, equisetin by ESIMS and NMR. The minimum inhibitory concentrations (MICs) for equisetin against *Bacillus subtilis*, *Staphylococcus aureus* and Methicillin resistant *Staphylococcus aureus* were identified (Ratnaweera et al., 2015). Endophytic fungi of an aquatic plant *Nmphaea nouchali* were valuable potential sources for the isolation of bioactive metabolites. Investigations of the antimicrobial activities of an endophytic fungus *Chaetomium globosum* isolated from *N. nouchali* were capable of producing antimicrobial substances. Bioassay- guided fractionation and structure elucidation by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) identified the known compounds chaetoglobosin A and chaetoglobosin C. (Dissanayake et al., 2016). The compound Polyketide monocerin isolated from the methanolic extract of the fungus *Exserohilium rostratum* from *Bauhinia guianensis* was identified by NMR and MS displayed the broad spectrum antimicrobial activity (Pinheiro et al., 2017).

2.9. Endophytic fungi as producers of anticancer compounds

The search for novel anticancer compounds by scientific community have shifted their focus from plants to microorganisms especially to fungal endophytes since they are the wide source of unexplored and uncharacterized microorganisms secreting novel bioactive metabolites. Secondary metabolites from endophytic fungi have gained recognition as potential source of anticancer compounds (Schulz et al., 2002) since the discovery of fungal paclitaxel from an endophytic

fungus of Pacific yew *Taxomyces andreanae* (Stierle et al., 1993). Vincristine and Podophyllotoxin (Puri et al., 2006) were also used identified as the anti-cancer compounds. Similarly 9-methoxycamptothecin and 10-hydroxycamptothecin, two analogues of pentacyclicquinoline alkaloid camptothecin isolated from an endophytic fungus of the medicinal plant *Camptotheca acuminata*, exhibits similar potency as the unmodified camptothecin (Kusari et al., 2009).

Researchers have continued to screen for many other endophytic fungi associated with medicinal plants. As a result, they have come up with many compounds possessing anti cancer activity. An alkaloid 12-demethyl-12-oxo-eurotechinulin B, isolated from *Eurotium rubrum* growing in the inner tissues of *Hibiscus tiliaceus* is cytotoxically active on human hepatocarcinoma (SMMC-7721) cell lines (Yan et al., 2012). Photipyrone B is Pyrones isolated from *Pestalotiopsis photiniae* living in *Roystonea regia* showing inhibition on breast cancer cell lines (MDA-MB-231 cells) (Ding et al., 2012). Epicocconigrone A (Polyketide) from the leaves of *Epicoccum nigrum* from *Mentha suaveolens* showed 50% inhibition of proliferation on Raji cells (El Amrani et al., 2013). Periconiasin A and periconiasin B are two polyketides isolated from the endophytic fungus *Periconia* sp. F-31 of *Annona muricata*. The metabolites showed anticancer activity on adenocarcinoma (HCT-8) and human gastric (BGC-823) cell lines (Zhang et al., 2013). Alkaloids-chaetomugilide A, B and C were isolated from *Chaetomium globosum* TY1 living in the bark of *Ginkgo biloba* showed potent anticancer activity on liver heptocellular carcinoma (HepG-2) cells (Li et al., 2013). Mycoleptodiscin B, a nitrogen containing compound secreted by *Mycoleptodiscus* sp. from leaves of *Desmotes incomparabilis* showed activity on lung cancer (H460), prostate cancer (PC-3), lung myo fibroblast (IMR-90) cell lines (Ortega et al., 2013).

The compound polyketide dothiorelone F isolated from *Dothiorella* sp. harboured in bark of the black mangrove, *Aegiceras corniculatum* was investigated by Du and Su, 2014, and the compound have cytotoxic activity on Raji cells (Du and Su, 2014). Myrotheciumone A, belongs to lactones isolated from endophytic fungi *Myrothecium roridum* living in the stems of *Ajuga decumbens* showed anticancer activity on liver heptocellular carcinoma, hepatocarcinoma, breast cancer, liver

cancer, and normal cell lines (Lin et al., 2014). Phomopsidone A, a lactone and a novel depsidone metabolite identified from *Phomopsis* sp. A123 from leaves of *Kandelia candel* was studied by Zhang et al., 2014, and the compound showed cytotoxic activity on MDA-MB-435 at 63 μM. Lai et al., 2013, studied the chemical investigation of endophytic fungus *Penicillium citrinum* from *Ocimum tenuiflorum* and isolated two new alkaloids (3,4,7-trimethylisoquinoline-6,8-diol and s(E)-7-(3-methyl-4-oxo-6,7,8,8a-tetrahydro-4H-pyrrolo[2,1-b][1,3]oxazin-2-yl)hept- 2-enoic acid. with four known alkaloids and fourteen known polyketides and the structures of these compounds were determined by extensive analysis of the 1D, 2D NMR and MS data. The compounds were evaluated for their cytotoxic and antimicrobial activities, two compounds showed significant cytotoxicity against the murine lymphoma cell lines.

2.10. Endophytic fungi as antioxidants

Antioxidants are the free radical scavengers that interact with the free radicals and neutralize them to prevent the damage caused to the organism (Bharathidasan and Panneerselvam, 2012). Screening of endophytic isolates had exhibited the presence of antioxidants. The endophytic isolate, *Pestalotiopsis* sp. from *Terminalia arjuna* produce 1, 3-dihydro isobenzofurans- Pestacin and Isopestacins; the first antioxidant compounds to come in to spotlight (Harper et al., 2003). Thereafter the hunt for antioxidant producing endophytic fungi gained acceptance worldwide. Song et al., 2005, assayed the extracts from endophytic fungal strains growing on *Trachelospermum jasminoides* for the presence of antioxidant and free radical-scavenging agents using various methods. These included DPPH and hydroxyl radical assays, the antioxidant actions on linoleic acid and human low-density lipoproteins. Extensive research works led to the isolation of graphislactone A from the endophytic *Cephalosporium* sp. IFB-E001. Graphislactone A showed dose-dependent antioxidant activities in vitro and was more potent than butylated hydroxytoluene (BHT) and ascorbic acid, the two standards used for antioxidant studies.

The ethanolic extract of an endophyte, *Phyllosticta* sp. from *Guazuma tomentosa* have potent antioxidant activity in ABTS and DPPH radical assay

(Srinivasan et al., 2010). The endophyte *Chaetomium* sp., isolated from *Triticum durum* showed 38% antioxidant activity compared to the β-carotene/ linoleic acid system (Sadrati et al., 2013). Related research on *Chaetomium* sp. isolated from *Nerium oleander* revealed highest antioxidant capacity and phenolic content (Huang et al., 2007). The metabolites from fungal isolate *Cephalosporium* sp. AL031 of *Sinarundinaria nitida* after bioassay-guided fractionation isolated four different compounds which include three known compounds (4,5,6-trihydroxy-7-methyl-1, 3-dihydroisobenzofuran, 4, 6-dihydroxy-5-methoxy-7-methyl-1, 3- dihydroisobenzofuran and 4, 5, 6-trihydroxy-7-methylphthalide) and a new isobenzofuranone derivative, 4, 6-dihydroxy-5-methoxy-7-methylphthalide. The isolated compounds have antioxidant activity by DPPH radical-scavenging assay (Huang et al., 2012).

2.11. Endophytic fungi as plant growth regulators

Endophytes including fungi, bacteria, and algae have the capability to produce different types of secondary metabolites as plant growth regulators which include auxins that may utilized by the plants for its prominent growth and development. Endophytic fungi are normally safe to plant and its growth promoting activities unfold the new array in organic agriculture. Indole acetic acid (IAA) is the prime group of auxins produced in plants mainly by tryptophan dependent pathway, which is necessary for development of plants like seedling growth, embryo genesis (Stepanaova et al., 2008). Plant related microorganisms also produces the plant growth regulators including gibberellins, cytokines, jasmonic acid abscisic acid and ethylene, which influences on tissue differentiation, growth and reproduction of the hosts (Spaepen et al., 2007; Sirrenberg et al., 2007). Phytohormones takes part a major role in plant growth especially by inducing cell elongation and cell division and increased root length (Bhattacharyya and Jha, 2012). Plant hormones and biochemicals produced by the microbes were depending upon the incubation period, pH, temperature, growth dynamics and internal physiology (Khan et al., 2012; Wei et al., 2013). Research on indole acetic acid producing fungi promotes plant growth and protection in sustainable agriculture is underway (Fu et al., 2015). Endophytic fungi like *Fusarium oxysporum, Aspergillus Niger*, *Aspergillus flavus*, *Penicillium*

corylophilum, *and Rhizopus stolonifer* from different plants had the capability to produce phytohormones like GA and IAA for plant growth promotion (Waqas et al., 2012). Plant growth promoting properties of endophytic fungi were reported in different plants; the leaves stem and root of *Camellia sinensis* (tea plant) of Assam showed the increased plant growth up to certain period and later it got decreased (Nath et al., 2015). Medicinal plant *Asclepias sinaica* was identified as the host of distinct endophytic fungal strains *Penicillium chrysogenum*, *Alternaria alternate* and sterile mycelia which are capable of producing IAA and ammonia and were having the influence on promotion of plant growth via several strategies (Fouda et al., 2015). Endophytic fungi of *Echinacea purpurea* and *Lonicera japonica* revealed the significant growth and improved nutrient uptake in pea seedling. Besides, these endophytes have a substantial future for phytopathogen control and their employment as bio control agents (Gupta et al., 2016). Reports on IAA and GA producing capacity of fungal isolates associated with *Zea mays* L. assist the host plant growth (Ismail et al., 2016).

IAA produced by yeast (Xin et al., 2009) and bacteria (Sachdev et al., 2009; Apine and Jadhav, 2011) were also reported. *Klebsiella* strains isolated from rhizosphere of wheat seedlings induced the production of IAA and which was confirmed by GC-MS and IR spectrum analysis (Sachdev et al., 2009). Various endophytic bacterial strains of *Oryza sativa* L. screened for the production of IAA and different plant growth promoting (PGP) traits that were effective in colonizing rice plant hence they assist rice seedling growth (Etesami et al., 2014). Endophytic diazotrophic bacteria from different varieties of rice cultivars in Korea (Ji et al., 2014), different cultivars of tomato and tomato hybrids (Abbamondii et al., 2016) and some rice varieties (Sev et al., 2016) were associated with the promotion of plant growth. Endophytic bacteria, *Enterobacter* sp. (PnB 11) and *Klebsiella* sp. (PnB 10) isolated from *Piper nigrum* were producing IAA and they proved the plant growth promotion in *Vigna radiata* seedlings (Jasim et al., 2013). Bacterial strains associated with the bark of *Moringa peregrine* were analyzed and quantified the IAA producing capacity and were characterized by UPLC-MS/MS, the isolated strains exhibited growth of tomato plants (Khan et al., 2016). IAA producing capability depends upon different concentration of the tryptophan supplemented.

Bacterial root endophytes of *Brown sarson* showed production of IAA at different concentrations of tryptophan, displayed growth of the plants (Padder et al., 2017).

2.12. Other applications of endophytic fungi

Natural products from endophytic fungi possess biological activities against many diseases. Endophytic fungi are known to produce the antibiotic products, which were also the inhibitors of viral growth. Metabolites produced by the endophytic fungi isolated from the desert plants were act as the inhibitors of HIV-1 replication (Wellensiek et al., 2013). Endophytic fungus, *Muscodor albus* produce volatile organic compounds that are harmful to plant or human pathogenic fungi and were also effective on specific insects and nematodes (Strobel, 2006). Endophytic fungi from *Salvadora oleoides* exhibited anti diabetic activity induced with glucose loaded, fasting and alloxan in wistar albino rats (Dhankhar et al., 2013). Actinomycetes isolated from the stem and leaves of *Rauwolfia densiflora* and *Leucas ciliata* produce α-amylase inhibitor that delays glucose adsorption and release of glucose from carbohydrates (Akshatha et al., 2014). Metabolites, subglutinol A and B were produced from *Fusarium subglutinans*, the isolate of *T. wilfordii* were used as immunosuppressive agents. These were used as drugs for the prevention of allograft rejection in transplant patients and in future these drugs may used for the treatment of autoimmune diseases like rheumatoid arthritis and insulin dependent diabetes (Kumar et al., 2005; Padhi et al., 2013).

2.13. The endophytic fungus- *Xylaria* sp.

The fungus *Xylaria* sp. (Xylaria Hill ex Schrank) is the member of the Xylariaceae family and ascomycetes genera having pigments in their cultures and stromata (Stadler et al., 2004). *Xylaria* sp. is widely distributed from temperate region to the tropical zones of the earth. Normally, *Xylaria* sp. found as wood destroyers or saprotrophs which had emended, as it was also visualized as the endophytes found ubiquitously in vascular plants (Petrini et al., 1995). Other than Xylaria, the entire Xylariaceae family plays the major ecological role. They also associated with seed plants which may be due to the long period of co-evolution (Fournier et al., 2011). It has become the important endophytic fungus because of

the secondary metabolites present in them. Secondary metabolites produced by different *Xylaria* sp. are listed in table 2.2. The prime chemical constituents of *Xylaria* sp. contain the compounds like sesquiterpenes and N- containing compounds. Different species of Xylaria possess diverse structural and functional metabolites with various pharmacological uses such as antimicrobial, antifungal, cytotoxic, antiviral, anti malarial and antihelmintic activities (Song et al., 2014). In addition to this the biological activities also include acetylcholine esterase inhibitory, anti-obesity, l-calcium channel blocking.

Xylaria carpophila cultures hold the biologically active compounds xylocarpins A-E and five new sesquiterpenes (Ramesh et al., 2012). Sesquiterpenes were identified as the main secondary metabolites with biological activities. Eremophilanes, a type of sesquiterpenes were isolated from different types of *Xylaria* sp. including *X. persicaria*. Besides, the N-containing compounds cytochalasins were isolated from *Xylaria* sp. mainly from *X. hypoxylon* and *X. obovata* that possess the cytotoxic activities (Song et al., 2014). α-Glucosidase Inhibitors were identified from *Xylaria feejeensis* isolated from *Hintonia latiflora.* Two new compounds 3S,4R-(+)-4-hydroxymellein and pestalotin 4′-O-methyl-β-mannopyranoside along with some known compounds 3S,4S-(+)-4-hydroxymellein, 3S-(+)-8-methoxymellein, quinine derivatives were isolated from *X. feejeensis*. Among the compounds, 3S,4R- (+) - 4 -hydroxymellein and 3S, 4S - (+) - 4 - hydroxymellein inhibited α-glucosidase of *Saccharomyces cerevisiae* comparable with the positive control acarbose. Additionally, the docking studies of these compounds predicted that it bind to α-glucosidase in the site other than catalytic domain with an allosteric type of inhibition (Rivera-Chavez et al., 2015). *Xylaria feejeensis* SM3e-1b isolated from the *Sapium macrocarpum* produced novel coriloxine derivatives and quinine derivatives. The compounds inhibited the seed germination, root growth and oxygen uptake by the seedlings of *Panicum miliaceum*, *Medicago sativa*, *Amaranthus hypochondriacus* and *Trifolium pratense* (Garcia-Mendez et al., 2016). Further studies in the same compounds of coriloxine derivatives 4, 7 can use as the prototype structure to develop the new herbicides (Macias-Rubalcava et al., 2017).

Table 2.2. List of some secondary metabolites isolated from different *Xylaria* sp.

Compound Name	Fungus	References
Kolokoside A-D	*Xylaria* sp. NRRL40192	Deyrup et al., 2007
Xylarenolide	*Xylaria* sp. 101	Li et al., 2010
Xylarenic acid	*Xylaria* sp. 101	Li et al., 2010
Mairetolide F	*Xylaria* sp. BCC21097	Isaka et al., 2010
Xylaranol A-B	*Xylaria* sp. 101	Li et al., 2010
Xylaric acid A	*Xylaria* sp.	Yan et al., 2011
Xylcarpin A-C	*Xylaria carpophila*	Yin et al., 2011
Xylaropyrone	*Xylaria feejeensis* MU 18	Siriwach et al., 2011
Ergosterin	*Xylaria* sp. 7S-1 – 3-1	Wu et al., 2011
Cytochalasin H and H2	*Xylaria* sp. A23	Li et al., 2012
Xyolide	*Xylaria feejensis*	Baraban et al., 2013
Xylarellein	*Xylaria* sp. PSUG12	Rukachaisirikul et al., 2013
isocoumarin glycoside	*Xylaria* sp. CFCC 87468	Wang et al., 2014
phenylethanol glycoside	*Xylaria* sp. CFCC 87468	Wang et al., 2014
Zofimarin	*Xylaria* sp. Acra L38	Chaichanan et al., 2014
Xylabisboein A	*Xylaria* sp. SNBGTC2501	Sorres et al., 2015
5-Methylmellein	*Xylaria* sp. SNBGTC2501	Sorres et al., 2015
Gliocladic acid	*Xylaria* sp. NC1214	Wei et al., 2015
Cytochalasin C and R	*Xylaria* sp. NC1214	Wei et al., 2015
Pestalotin 4'-*O*-methyl-*β*-mannopyranoside	*Xylaria feejensis*	Rivera-Chavez et al., 2015
3,7-dimethyl-9-(-2,2,5,5-tetramethyl-1, 3-dioxolan-4-yl)nona-1,6-dien-3-ol,	*Xylaria* sp.	Lin et al., 2016
Tyrosol	*Xylaria papulis*	Chen et al., 2016

2.14. The endophytic fungus- *Colletotrichum* sp.

Colletotrichum sp. are the group of fungus that are classified under Glomerellaceae family and Deuteromycotinia division. The genus Colletotrichum is the plant symbionts as endophytes or phytopathogen while some *Colletotrichum* sp. form mutualistic relation with the host plants (Rodriguez and Redman, 2008). As

plant pathogens *Colletotrichum* sp. form the anthracnose diseases like seedling blight, necrotic wound on parts of the plants and also crown and stem rots (Agrios, 2005). *Colletotrichum* sp. also form a biotrophic strategy that may provide the importance of the fungus as endophytic fungi of living pants without causing apparent symptoms (Lu et al., 2004; Joshee et al., 2009).

Colletotrichum sp. was identified as an endophyte from the *Artemisia annua* stems isolated ergosterol, indole-3-acetic acid and antimicrobial metabolites. The metabolites (3b,5a-dihydroxy-6b-acetoxy-ergosta-7,22-diene, 3b,5a-dihydroxy-6b-phenylacetyloxy-ergosta-7,22-diene, -isoprenylindole-3-carboxylic acid, 3b-hydroxy-ergosta-5-ene, 3-oxo-ergosta4,6,8,22-tetraene) were identified as fungistatic to the pathogenic fungi like *Phytophthora capsici*, *Rhizoctonia cerealis*, *Gaeumannomyces graminis* var. *tritici*, *Helminthosporium sativum* in crops (Lu et al., 2000). *C. gloeosporioides f.* sp. *aeschynomene* fungus produces indole-3-acetic acid (IAA) by using the external tryptophan supplement. IAA production in plants was higher during the biotrophic stages of infection (Maor et al., 2004). The fungus, *Colletotrichum fructicola* isolated from *Coffea arabica* produces IAA with L-tryptophan supplementation at in vitro condition by incubating the fungus for 30°C for 26 days. Further, fungal crude IAA was able to stimulate the elongation of coleoptiles of rice, rye and maize (Numponsak et al., 2018). *Colletotrichum gloeosporioides* was isolated from the medicinal plant *Vitex negundo*, exhibited the antibacterial activity against the penicillin, methicillin and vancomycin resistant *S. aureus* strains (Arivudainambi et al., 2011). *Colletotrichum* sp. derived from *Tragia insuavis* were capable of producing antibacterial activity and the compounds, anthraquinones were also isolated *from Colletotrichum* sp. of *Tragia insuavis* (Nyamboki et al., 2017). Colletotricones A, the cytotoxic active compound was isolated and identified from the endophytic fungus, *Colletotrichum gloeosporioides* A12, of *Aquilaria sinensis* and the cytotoxic activity was evaluated against the lung, liver and breast cell lines (Liu et al., 2018). *Colletotrichum* sp. was used as the bio control agents. The species *Colletotrichum gloeosporioides* f. sp. *salsolae* was suggested as the biocontrol agent to the invasive weed in North America (Berner et al., 2009). A report of Chithra et al., 2014, identified piperine production by *Colletotrichum gloeosporioides* isolated from *Piper nigrum* L.; and it was proved by HPLC and LCMS analysis.

2.15. Medicinal plants used in the study

2.15.1. *Elaeocarpus sphaericus* (Gaertn.) K. Schum.

Classification

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnolipsida
Subclass	:	Dilleniidae
Order	:	Malvales
Family	:	Elaeocarpeaceae

Figure 2.3. *Elaeocarpus sphaericus* plant and the leaves

Elaeocarpus sphaericus (Gaertn.) K. Schum. (Synonym- *Elaeocarpus ganitrus* Roxb.) is the evergreen tree inhabit in tropical and subtropical regions (Figure 2.3). Rudraksha is the common name of *Elaeocarpus sphaericus*. In Hindu mythology Rudraksha means the "Tears of Lord Shiva". It is found in Manila, Myanmar, Philippines, Bangladesh, Bhutan, Nepal and India (Zmarzty, 2001). In India it occupies the regions of Himalaya, Bihar, Madhya Pradesh, Arunachal Pradesh and Konkan Ghats. The tree is about 15-200 meter height with broad leaves of 10-15 cm length (Kumar, 2008). The parts of the plant was used in herbal medicine for the treatment of nerve pain, palpitation, asthma, depression, migraine, stress, anxiety, arthritis, hypertension, epilepsy and liver diseases (Khare, 2004) and it is labelled a the king of herbal medicine. Apart from this the leaves and fruit extract holds the analgesic,

antihypertensive, antiepileptic, anticonvulsive properties. The chemical constituents of *Elaeocarpus sphaericus* include the alkaloids, steroids, flavonoids (quercetin), glycosides, tannins, fatty acids, proteins, carbohydrates. Alkaloids mainly epielaeocarpiline, isoelaeocarpine, alloelaeocarpiline epiisoelaeocarpiline, and pseudoepiisoelaeocarpilline were also obtained from *E. sphaericus* (Ray et al., 1979).

Researchers investigated the biological properties of *Elaeocarpus sphaericus* and the related species. The different solvent extracts (petroleum ether, benzene, chloroform, acetone and ethanol) from fruits of *E. sphaericus* exhibited antibacterial activity against the bacterial strains *E. coli*, *S. aureus*, *B. subtilis K. pneumoniae*, *Pseudomonas* sp.(Singh and Nath, 1999). The leaf, stem, bark and fruit of *Elaeocarpus serratus* L. were extracted with acetone, methanol and water and investigated for antimicrobial activity by agar well diffusion method against *Staphylococcus aureus, Shigella sonnei, Salmonella typhi, Klebsiella pneumoniae* and *Candida albicans.* The acetone and methanol extracts of leaf, stem and bark showed maximum activity against all the tested strains (Jayashree et al., 2014). The epicarp and endocarp of *Elaeocarpus ganitrus* possess antimicrobial activity in their methanolic and acetone extracts (Dalei and Sahoo, 2016). Similar research was carried out by Pandey et al., 2016, in *Elaeocarpus ganitrus* Roxb. and identified phytochemicals terpenoids, steroids, saponins, flavonoids, alkaloids and tannins in solvent extracts and these extracts have antimicrobial activity against *S. aureus*, *Pseudomonas*, *E. coli*, *B. subtilis and Rhizopus oryzae*. Rudraksha seeds also possess antifungal activity in different solvent extracts against the fungi *Candida albicans*, *Candida tropicallis* and *Aspergillus niger* (Singh et al., 2010).

Elaeocarpus ganitrus possess antioxidant properties in ethanolic extracts. The antioxidant activity of ethanolic extract was 24.18 mg ascorbic acid equivalents at 500 µg/ml and the reducing power assay was between 1.112 to 1.973 concentrations. Antioxidant capacity was in connection with the total phenolics and flavonoids present in the extract of *E. ganitrus* (Kumar, 2008). Anticancer activities from the chloroform extract of *Elaeopcarpus mastersil*, the related species of *Elaeocarpus sphaericus* showed cytotoxicity against the oral epidermoid carcinoma cell lines (Ito et al., 2002).

The later research on chloroform extract of the bark of *Elaeocarpus mastersii* showed significant anticancer activity on several human cancer cell lines tested. The cytotoxic potential of ethanolic extract was evaluated by brine shrimp lethality bioassay. Three compounds isolated from *Elaeocarpus mastersii* viz. cucurbitacins, cucurbitacin D and cucurbitacin F exhibited cytotoxic effect against cervical cancer cell lines (Dadhich et al., 2013). The methanolic and aqueous extracts from the leaves of *E. sphaericus* exhibited the promising anti-inflammatory properties performed on carrageenan-induced paw oedema in rats (Jaspreet et al., 2012). Antihypertensive activity was also studied in aqueous extracts of *E. ganitrus* by using Swiss albino mice and male wister rat as models (Sakat et al., 2009).

2.15.2. *Quassia indica* (Gaertn.) Nooteb.

Classification

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Mangoliopsida
Subclass	:	Rosidae
Order	:	Sapindales
Family	:	Simaroubaceae

Figure 2.4. *Quassia indica* plant and the leaves

The plant *Quassia indica* (Earlier name- *Samadera indica*) is a locally available medicinal plant belongs to Simaroubaceae family distributed in India (Figure 2.4). There are three species of *Quassia* was identified from Simaroubaceae family, the other species are *Q. amara* from tropical America and *Quassia africana* in Congo. *Quassia indica* used as folk medicine by tribal people in India for various treatments and skin diseases like pruritus, scabies, erysipelas and leprosy (Viswanad et al., 2011). In detail, the leaves after extracting was used to recover cough and it used externally to cure skin diseases. The macerated bark was used to relieve stomachic (Chopra et al., 1986). The plant Quassia contains a major phytoconstituents quassinoids grouped under the class of diterpenoids, which considered as prime compound of Simaroubaceae family (Saraiva et al., 2006). Four types of quassinoids (samaderins B3, C2 and A4, indaquassin C1) were isolated from the bark and seeds of *Quassia indica*. The biological properties of Quassia plants includes antioxidant, antimalarial, antiplasmodial and antihelmintic activities (Raja and Ravidranadh, 2014). The methanolic extracts of *Samadera indica* displayed antimicrobial activities against the gram positive (*B. Subtilis*, *S. aureus*), gram negative (*P. aeruginosa*, *E. coli* and *P. vulgaris*) microbial pathogens and fungal pathogen *C. albicans*. In addition, methanolic extracts possess potential antioxidant activity than the standard Quercetin (Viswanad et al., 2011). Identical research was carried out by Jolly et al., 2014. Here, the methanolic extract from the leaves of *Quassia indica* was showed significant antioxidant activity (DPPH radical scavenging) and anti-inflammatory activity tested in wistar albino rats induced with carrageenan induced paw edema).

Phytochemicals present in bark and leaf extracts of in *Q. indica* are alkaloids, flavonoids, phytosterols, saponins, phenols, terpenoids, fats and fixed oils and carbohydrates. Majority of phytochemicals were present in chloroform, methanol and aqueous extracts. Antimicrobial activity of leaf and bark extracts had significant antibacterial activity against the pathogens *S. aureus*, *E. coli*, *A. niger* and *C. albicans* at a concentration of 1 mg/ml (Anusha and Sudha Bai, 2017). Similarly, acetone, petroleum ether, methanol and aqueous extracts of *Q. indica* leaves displayed antibacterial activity against the bacteria strains *Serratia* sp. and *E. coli* by disc diffusion method (Aiswarya and Pushpalatha, 2017).

The literature review provides information regarding the general account of endophytic fungi with special reference to classification, diversity and interaction

with the host plants, a brief overview of endophytic fungi and its metabolites as bioactive compounds isolated from medicinal plants and a brief summary of the endophytic fungi as a rich repository of lead compounds with promising future medicinal, industrial and agricultural applications. Moreover it provides report on medicinal uses of the two host plants selected in the study.

Chapter 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Collection of plant samples

The leaves of the two medicinal plants *Elaeocarpus sphaericus* (Gaertn.) K. Schum. and *Quassia indica* (Gaertn.) Nooteb. were collected from the Botanical Garden, University of Calicut. The Plants were identified and authenticated by Dr. A. K. Pradeep, Assistant Professor, Department of Botany, University of Calicut, Kerala. Voucher specimens with accession numbers 6935 for *Elaeocarpus sphaericus* and 148232 for *Quassia indica* has been deposited in the herbarium of Department of Botany, University of Calicut, Kerala. Fresh and symptomless leaves were collected in pre sterilized polythene bags and were processed within 24 hrs of collection.

3.2. Isolation of endophytic fungi

Endophytic fungi were isolated from the leaves as described by Ezra et al., 2004, with minor modifications. Samples were washed thoroughly with running tap water for 20 mins. Subsequently the leaves were surface sterilized by treating it with 1 % sodium hypochlorite for 3 to 4 mins, followed by 70 % ethanol wash for 1 min and then rinsed three times with sterile distilled water. The moisture content was blotted in a sterile filter paper. The last rinsed water was streaked on to the PDA medium and also the sterilized segments were imprinted on the medium to ensure the complete surface sterilization. Surface sterilized segments were cut in to 1 x 1 cm length and placed in the petri dishes containing medium supplemented with 100 mg/l of chloramphenicol. Petri dishes were monitored every day for the growth of fungal colonies from the segments. Hyphal tips that emerged from the edges of the segments were transferred separately onto fresh PDA medium. The pure cultures were used for further analysis and were stored in cryovials on PDA with 15% glycerol (v/v) at $^{-}$80°C in an ultra-low temperature freezer (New Brunswick, eppendorf) at the Department of Biotechnology, University of Calicut, Kerala.

3.3. Identification of endophytic fungi

Morphological characteristics of endophytic fungi were observed. Endophytic fungi were identified based on microscopic and molecular methods.

3.3.1. Morphological and microscopic observation of endophytic fungi

Identification of fungi was based on the morphology of the isolates and characteristics of the spores produced. Morphological characteristics were studied by plating the fungi on PDA and incubating for 21 to 28 days. The growth appearances were noticed for both the top and reverse sides of the culture plates. For tentative identification, microscopic slides of endophytic fungi were prepared by tease mount method using lactophenol cotton blue staining (Dugan, 2006) and observed under microscope (Olympus BX43, Japan).

3.3.2. Molecular identification of endophytic fungi using ITS-PCR

Molecular identification of the endophytic fungi was done by fungal DNA extraction followed by ITS-PCR amplification. The amplified products were sequenced; blast similarity search was done for the identification of endophytic fungi. Phylogenetic analysis used to reveal the evolutionary relation.

3.3.2.1. Fungal DNA extraction

The fungal DNA isolation was done by using the method described by Pich and Schubert, 1993. About 0.5 gm of fungal mycelium grown on PDA medium was cut from the cultures and homogenized with liquid nitrogen in a motor and pestle. The homogenized mycelium was grinded with 1 ml of extraction buffer and incubated at 65°C for 10 mins followed by centrifugation at 12000 rpm for 5 mins. The supernatant was transferred to a fresh tube and equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. The solution was mixed and centrifuged at 12000 rpm for 5 mins. The aqueous layer was transferred to a fresh tube and equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12000 rpm for 5 mins. To the supernatant collected, two volumes of ice cold isopropanol was added and kept at $^{-}$ 20°C for 10 mins. Following this, it was centrifuged for 5 mins at 12000 rpm. Pellet

was washed with 500 µl of 70 % ethanol. The solution was centrifuged at 10000 rpm for 5 min and the pellet was air dried. The air dried pellet was resuspended in Tris EDTA buffer. To this, 1 µl of RNase A was added and kept for one hour at 37°C. After incubation 400 µl of TE was added and mixed, followed by 500 µl of phenol: chloroform:isoamyl alcohol solution and then centrifuged at 12000 rpm for 5 mins at room temperature. To the collected upper layer, equal volume of chloroform:isoamyl alcohol was added and mixed. It was centrifuged at 12000 rpm for 10 mins at room temperature. The upper layer was removed and 1 volume of ice cold isopropanol was added and kept overnight at ⁻20°C, then centrifuged at 12000 rpm for 10 mins. The pelleted DNA was washed with 70 % ethanol and again centrifuged at 12000 rpm for 10 mins. The pellet was air dried and dissolved in 20 µl of TE buffer and stored at ⁻20°C for further analysis.

3.3.2.2. Quantification of DNA

The isolated DNA was quantified by using a nanodrop (Biospectrometer, Eppendorf). 1 µl of the DNA sample was placed in the slot and the absorbance was read at A_{260} and A_{280} nm. The purity of DNA was calculated by using the A_{260}/A_{280} ratio and which would be 1.8. (The O.D. at 260 nm with value 1 is considered to be equal to 50µg of dsDNA).

3.3.2.3. Internal transcribed spacer polymerase chain reaction (ITS-PCR)

The ITS region was amplified by using PCR with ITS1 and ITS4 as forward and reverse primers (White et al., 1990) is given in table 3.1. The total reaction volume of PCR amplification was 20 µl, which was carried out containing 2X PCR master mix of volume 10 µl, 1 µl of 0.5µM of both forward and reverse primers, 25 ng of template DNA. Amplification was done in the thermocycler (Thermo Fisher Scientific, 2720, USA) with the following conditions with initial denaturation of 94°C for 5 min followed by 30 amplification cycles of 94°C denaturation for 1 min 30 sec, 56°C annealing for 1 min 30 sec, 72°C extension for 2 min and the final extension step of 72°C for 5 min.

Table 3.1. Primers used for ITS amplification

ITS1	5'-TCCGTAGGTGAACCTGCGG-3'
ITS4	5'- TCCTCCGCTTATTGATATGC-3'

3.3.2.4. Agarose gel electrophoresis

Following amplification, the PCR amplified products were separated by using agarose gel electrophoresis (Genei, Merck specialities Pvt Ltd) with 1 % agarose gel (low EEO grade; HiMedia, India) containing 20 µl of ethidium bromide (0.5 mg/ml final concentration). The amplified products were mixed with the 6X gel loading buffer to obtain 1X final concentration and the gel was run in tris-acetate EDTA buffer pH 8.0 for 2 hrs at 50 V (Sambrook et al., 1989). A 100 bp DNA marker was used as the reference (Genei, Merck specialities Pvt Ltd). The bands were visualized in a gel documentation system (Medicare-Chemiluminesence gel sctan 8X chemi). Molecular weight of the obtained DNA bands was compared with the standard 100 bp DNA marker.

3.3.2.5. ITS sequencing and phylogenetic analysis

Sequencing of the amplified products was done by using an automated DNA sequence (ABI 3730xl 96 capillary system, Thermo Fisher Scientific) and was submitted to GenBank in NCBI website (http://www.ncbi.nlm.nih.gov). The sequences were subjected to BLAST analysis using the software (http: //www. ncbi.nlm. nih.gov/BLAST/).

Multiple Sequence Alignment: Forward and Reverse sequences were combined and edited using BioEdit software (Hall, 1999). Sequences were aligned using Clustal W (Thompson et al., 1994) incorporated in MEGA6 (Tamura et al., 2013) software. Alignments were thoroughly checked and manually edited using MEGA 6 (Tamura et al., 2013).

Phylogenetic analysis: Model of sequence evolution was calculated using jModelTest 2.0 (Darriba et al., 2012) and GTR+1+G was obtained as best fit model. Bayesian inference was performed using MrBayes version 3.2 (Ronquist and

Huelsenbeck, 2003) for the model of sequence evolution obtained from jModelTest 2.0. Standard DNA sequence data were used for phylogenetic tree construction. In these analysis gaps were coded as missing.

3.4. Colonization frequency of endophytic fungi

Percentage of colonization frequency of the identified endophytic fungi was calculated by using the formula given below as described by Kumar and Hyde, 2004.

$$CF\ \% = \frac{\text{Number of segments colonized by endophytes}}{\text{Total number of segments analyzed}} \times 100$$

3.5. Species diversity index

Diversity index of the endophytic fungi by using Shannon diversity index (*H'*) and evenness index was calculated (Zar, 2004) by using the formula given below.

$$H' = \sum_{i=1}^{k} - (P_i \times \ln P_i)$$

3.6. Fermentation and extraction of endophytic fungi

Endophytic fungi were incubated in PDB at room temperature for 21 to 28 days. After the incubation period the solvent extraction of endophytic fungi was performed as given below:

a). Crude endophytic fungal extracts: The fungal extracts were prepared by using the method described by Wang et al., 2012, with minor modifications. The mycelium was dried and powdered; 1 gm of powdered mycelium was extracted with ethyl acetate. The broth was extracted using three times the equal volume with ethyl acetate solvent in the separating funnel and the ethyl acetate layer was collected. Both the mycelia and ethyl acetate extracts were pooled together.

b). Fungus extract ESEF 6: The dried and powdered mycelium (5 gm) was extracted sequentially with 50 ml of solvents (hexane, ethyl acetate and methanol) based on the increasing polarity to obtain the mycelia hexane (MH), mycelia ethyl

acetate (MEA) and mycelia methanol (MM) extracts. Simultaneously, the broth was extracted with three times with equal volume of ethyl acetate solvent by using the separating funnel and the ethyl acetate layer was collected (Arivundainambi et al., 2011).

c). Preparation of leaves extracts: The leaves of the *Elaeocarpus sphaericus* and *Quassia indica* were shade dried for 2 weeks and were powdered. The powdered leaf (25 gm) was sequentially extracted with solvents petroleum ether, chloroform, ethyl acetate, methanol and water based on the increasing polarity in shaking conditions for 48 hrs (Sharma and Paliwal, 2013)

All the extracts were filtered through Whatmann No. 1 filter paper and concentrated under reduced pressure in a rotary evaporator below 50^{o}C.

3.7. Phytochemical analysis

The fungus and plant extracts were analysed for the presence of metabolites/ phytochemicals. Phytochemical analysis includes the preliminary phytochemical screening, thin layer chromatography and high performance thin layer chromatography.

3.7.1. Qualitative phytochemical analysis

Chemical tests were carried out on the extracts using standard procedures for identifying the phytoconstituents. The phytochemical tests are as mentioned below:

***Alkaloids*:** Small amount of the extract was dissolved in few drops of dilute HCl and filtered. To this freshly prepared Dragendorff's reagent was added. The formation of reddish brown precipitate indicated presence of alkaloids (Kokate et al., 2001).

***Flavonoids* (*Shinoda* test):** About 0.5g of plant extracts were dissolved in 5ml of ethanol. Few drops of concentrated HCl was added along with 0.5 g of magnesium turning and observed for pink colour development (Kokate et al., 2001).

***Phenols*:** The extract was dissolved in water and treated with 2ml of 2% ferric chloride solution. Observation of green or blue colour indicates positive result (Gibbs, 1974).

***Saponins*:** To 0.5g of extract, 5ml of distilled water was added and vigorously shaken to observe for a stable persistent froth (Kokate et al., 2001).

***Tannins*:** The extract was boiled with distilled water and then filtered. Few drops of 0.1% of ferric chloride was added and thoroughly mixed. Observation of brownish green or bluish black colour shows the presence of tannins (Trease and Evans, 1985).

***Glycosides*:** Extracts were shaken with distilled water and glacial acetic acid containing a few drops of ferric chloride was added, followed by H_2SO_4 along the side of the test tube. The formation of brown ring at the interface shows presence of cardiac glycoside and a violet ring may appear below the brown ring (Ayoola et al., 2008).

***Carbohydrates*:** Extracts were mixed with distilled water and was filtered. The filtrate was mix with two drops of ethanol (5%) solution of alpha-naphthol and few drops of H_2SO_4were added. Formation of violet coloured ring at the junction indicates the presence of carbohydrates (Nandagopal and Ranjithaumari, 2007).

***Steroids* (*Liebermann Buchard* test):** The plant extract was dissolved in chloroform and few drops of acetic anhydride and concentrated sulphuric acid was added. The formation of green colour in the upper layer indicates positive result (Joshi et al., 2013).

3.7.2. Thin layer chromatography (TLC) analysis

Thin layer chromatography is an indispensable method in the study of constituents or secondary metabolites present in organic extracts of various living entities. Being easy, inexpensive and sensitive, this method is widely employed in both qualitative and quantitative analysis. TLC also aids in determining the number of components contained in a mixture, the identity of compounds, and the purity of a compound. Usually non-volatile samples are analysed to characterize them according to Rf value, visualisation under UV light, derivatization with various spray reagents etc.

Preliminary phytochemical analyses of the antibacterial and cytotoxic extracts were evaluated using thin layer chromatography (TLC) (Harborne, 1998).

Manually prepared and precoated TLC Silica gel 60 F_{254} aluminium backed sheets (Merck) were used in the study. TLC plates (silica gel G) were manually prepared by mixing 30 to 35 gm silica gel G in 75 ml water and evenly spread on glass plates. Activation was done in an oven for 1 hr at 110^{o}C prior to use. Using a micro capillary tube the extract is applied as a single spot to the TLC plate and is allowed to dry thoroughly before being placed in to the vapour saturated developing chamber containing the solvent/solvent system. The extract was developed with the appropriate solvent system and the spots were labelled, their retention factor (Rf value) were also calculated. The Rf values were calculated according to the following formula:

$$\text{Rf value} = \frac{\text{Distance from the baseline moved by the solute}}{\text{Distance from the baseline moved by the solvent (solvent front)}}$$

a). **Fungus extracts (ESEF 6):** The plates were developed with various solvent systems in different combinations and the one giving better separation was selected. The solvent system chloroform:methanol (7:3 and 9:1) gave better separation and thus, it was selected for further analysis. Visualization was done under UV light and spraying with anisaldehyde sulphuric acid reagent and fast blue salt reagent followed by drying in the oven at 105°C.

b). P**lant extracts:** The plates were developed with various solvent systems in different combinations and the one giving better separation was selected. The solvent system petroleum ether:ethyl acetate (8:2) gave better separation and thus, it was used for further analysis.

3.7.3. HPTLC analysis

HPTLC profile of both fungus (MEA and BEA) and leaves extracts were done according to the method (Stahl, 1969; Wagner et al., 1996). The detection was done by using CAMAG (Switzerland) HPTLC system with a sample applicator. The solvent system such as chloroform:methanol (7:3 and 9:1) for fungus extracts and petroleum ether:ethyl acetate (8:2) for plant extracts was used to obtain the peaks with high resolution. The sample was loaded using Hamilton syringe with the help of Linomat 5 applicator attached to the CAMAG HPTLC

system through WIN CATS software (Stahl, 2005; Karthika et al., 2014). The developed HPTLC chromatogram was captured in a photo documentation chamber (CAMAG REPROSTAR-3) under visible light (550 nm) and UV (254 nm and 366 nm). The number of peaks and its corresponding area of percentage, Rf values were noted for spectral comparison. Post chromatographic derivatization of solvent extracts from endophytic fungus was performed by 2 % ethanolic ferric chloride ($FeCl_3$) and anisaldehyde reagents (ANS).

3.8. Purification of secondary metabolites from ESEF 6 by column chromatography

To purify the active compound from the solvent extracts, the column chromatography with silica gel 60-120 mesh was performed separately for both the MEA and BEA extracts. Silica gel of 60-120 mesh was mixed thoroughly with the chloroform to degas and then the slurry was used to pack the column. The solvent extracts (0.5 gm) were mixed with silica gel 60-120 mesh and were dried and powdered. The extract-silica mixture was loaded above the packed column and a piece of cotton was kept on top to avoid any disturbance. The column was eluted with chloroform: methanol in the following combinations (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100). The fractions were screened for antibacterial and cytotoxic activities. Further, the active fractions were again chromatographed on TLC plates and the spots were scraped off. The spots were also screened for the antibacterial and cytotoxic activity for the identification of bioactive metabolites.

3.9. Antibacterial activity

3.9.1. Microorganisms used in the study

The bacterial cultures used for the study were collected from American type culture collection (ATCC). The bacterial strains used in the study are *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Listeria monocytogenes*

(clinical isolate) and *Enterococcus faecalis* (ATCC 29212). The bacterial cultures were maintained as 15 % glycerol stock and stored at $^{-}80^{o}$C and the slants were prepared on luria bertani agar (Himedia, Mumbai) and stored at 4^{o}C.

3.9.2. Antibacterial activity by disc diffusion method

Antibacterial activity of the endophytic extracts, purified active fraction and plant extracts were done by using agar disc diffusion method (NCCLS, 2003). All the strains *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Enterococcus faecalis* were revived and subcultured from the stock and 24 hr old culture from the bacterial suspension was used for antibacterial activity. The turbidity of bacterial strains were made equivalent to 0.5 McFarland standards and that contain 1.5 × 10^{8} CFU/ml approximately. 100 µl of bacterial suspension was inoculated on the surface of the LB agar plates. The extracts and positive control were dissolved in DMSO to make up the concentration and DMSO was used as the negative control. The extract, positive control and negative control, 20 µl of each was pippeted to the sterile discs (Himedia) and were kept on LB agar plates. All the plates were incubated at 37°C for 24 hrs and the zone of inhibition in diameter ± SD was measured. The concentration of extracts and positive control used is given below.

a) Fungal extracts and its purified active fraction- 5 mg/ml concentration and 100 µg/disc
b) Plant extracts- 50 mg/ml concentration and 1 mg/disc
c) Positive control (Chloramphenicol) - 1mg/ml concentration and 20 µg/disc
d) Negative control (DMSO) - 20 µl.

3.10. Cytotoxic activity

3.10.1. Cell lines used for the study

The K562 (chronic myelogenous leukemia), HCT 116 (human colon carcinoma) and L929 (mouse fibroblast) cell lines were purchased from NCCS, Pune. K562 cells were cultured in RPMI-1640 (Roswell Park Memorial Institute) media while HCT-116 cells and L929 were grown in DMEM (Dulbecco's Modified Eagle Medium).

K562 leukemia cell line: These cells are non-adherent and rounded. Being chronic myelogenous leukemia, it is characterized by the presence of a constitutively active tyrosine kinase, known as the BCR/ABL (oncoprotein) due to the reciprocal translocation between chromosomes 9 and 22 (Deininger et al., 2000).

HCT 116 cell line: HCT116 cells are adherent human colon cancer cells possessing a mutation in codon 13 of the KRAS proto-oncogene. These cells have an epithelial morphology (Ashwani et al., 2008).

L929 Mouse fibroblast cell line: These are adherent mouse fibroblast cell lines established from the normal subcutaneous areolar and adipose tissue of a male C3H/a mouse. The markers present in L929 cells are murine hypertriploid karyotype - 61-67 and 12-16 centric fusion markers.

3.10.2. Cell viability assay

MTT assay was performed according to the previously described protocol (Mosman, 1983) with slight modifications. All the extracts to be tested for cytotoxic potential were dissolved in DMSO and stored in $4^{o}C$ as stock. The stock was appropriately diluted with growth media before addition to the 96 well plates. 150 µl of medium containing 10^5 cells were seeded in to each well of the micro titre plate. Appropriate controls were also maintained. Different concentrations of the extracts were then added to the respective wells and the final volume was made up to 200 µl with respective media. The culture plates were incubated for 48 hours in CO_2 incubator at $37^{o}C$ and 5% CO_2. 20µl of the MTT solution (5 mg/ml) was added to each well after removing the plant extract and the plates were incubated for further 4 hrs in CO_2 incubator at $37^{o}C$. At the end of incubation, spent medium was pippeted out and the formazan crystals thus formed were dissolved in 200 µl of DMSO. Optical density was read at 570 nm against a blank containing DMSO in an automated plate reader. Percentage of viability was calculated by the equation

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of extract} \times 100}{\text{Absorbance of control}}$$

IC_{50} was calculated to compare and analyse the cytotoxic potential of the extracts and the fractions. It is the concentration of the extract needed to inhibit the

cell viability by 50%. These values are determined by extrapolation from percentage of viability versus concentration curves. The experiment was repeated thrice and the average IC_{50} ± SD was reported.

3.11. Total phenolic content and antioxidant activity

Total phenolic content and antioxidant activities like DPPH radical scavenging activity, reducing power assay and total antioxidant capacity my phosphomolybdenum methods were determined by using the protocol has given below.

3.11.1. Total phenolic content of fungus extracts

Total phenolic content of the fungus extracts was determined by Folin-Ciocalteau method (Singelton et al., 1999; Gupta and Prakash, 2009). The fungus extracts were (2ml) mixed with 200 µl Folin- Ciocalteau's reagent and were kept for 10 min at room temperature followed by the addition of 300 µl of 15% Na_2CO_3. The solution was mixed thoroughly and allowed to stand at 25^oC for 2 hrs. The absorbance was measured at 765 nm by using spectrophotometer. Gallic acid was used as the standard. Total phenolic content of the extracts of endophytic fungi were expressed as gallic acid equivalent in milligram/gram of dry weight of the extract.

3.11.2. Determination of DPPH free radical scavenging activity

DPPH radical scavenging activity of the fungus extracts was estimated by the method (Hasan et al., 2006; Alam et al., 2008; Chen et al., 2008). Ascorbic acid was used as positive control. The fungus extracts (0.2 ml) was mixed with 1ml of 100 mM methanolic DPPH and the mixture was incubated in dark for 30 mins. After incubation, the absorbance was read at 517 nm and the activity was determined using the formula:

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

3.11.3. Reducing power activity of fungus extracts

Reducing power of the fungus extracts was determined by the following method (Oyaizu, 1986; Chen et al., 2008). The fungus extracts (1 ml), was dissolved

with equal volume (2.5ml) of sodium phosphate butter and potassium ferricyanide (1%) and was for 20 mins at 50^{o}C. To the solution 2.5 ml of trichloroacetic acid (10%) was mixed and it was further centrifuged for 10mins at 3000 rpm. The upper layer (2.5 ml) was collected and mixed with equal amount of distilled water and to this 0.5 ml of 0.1 % $FeCl_3$ was added. The absorbance was measured at 700 nm against the blank. Ascorbic acid was used as the standard.

3.11.4. Determination of total antioxidant capacity by phosphomolybdenum method

Total antioxidant capacity of fungus extracts were determined by phosphomolybdenum method (Prieto et al., 1999). To 0.1 ml fungus extract 1 ml of the reagent was mixed (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 mins in boiling water bath. After cooling the absorbance was measured at 695 nm against blank. Ascorbic acid was used as the standard and total antioxidant activity was expressed as ascorbic acid equivalents (AAE).

3.12. HPLC, HR-MS and HR-LCMS/MS Analysis

The antibacterial and cytotoxic active fractions after column chromatography were further chromatographed on TLC plates and the spots were scraped off. The active spots characterized for HLPC, HR-MS analysis. The HPLC system (Shimadzu LC Prominent) with C18 column of 250 x 5 mm (length and diameter), pore size 4.6 µM, the binary pump and PDA detector was used for the analysis. The sample injection volume 20 µl (sample concentration- 0.1mg/ml), mobile phase methanol: water (60:40) v/v; with a flow rate of 1ml/min and detected at 280 nm. Ultra membrane filter (pore size 0.45 µM; Merck, Germany) was used for filtering the sample and the Winchrom integrator was used to calculate the peak area. HR-MS analyses of the samples were performed on C18 column-Hypersil with the mobile phase chloroform: methanol and for QIEF 5 the mobile phase was chloroform:methanol. The gradient elution of 97 % methanol and 3 % water for 5 mins. The sample injection volume is 2 µl with a flow rate of 150 µl/ min. The source was operated in both positive and

negative mode at an ion spray voltage of 3KV. HR LCMS/MS analysis of partially purified active fraction of MEA was performed on HR LCMS/MS (Aligent technologies, USA, model: 1290 infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube couple with 6550 iFunnel Q-TOFs). The column used was Hypersil gold 3micron (100 x 2.1 mm). The mobile phase consists of a gradient of 0.1% formic acid in water and 0.1% formic acid + 10% water + acetonitrile given at a flow rate of 0.3 ml/min for 30 min. Other specifications include nitrogen gas flow rate (13 ml/min at 25°C), sheath gas flow rate (11 ml/min at 30°C), nebulizer pressure (35 psi), capillary voltage (3500 V), nozzle voltage (1000 V) and fragmentation energy (175 V). The data was acquired in Acquisition Software Version Column4 6200 series TOF/6500 series Q-TOF B.05.01 (B5125.1).

3.13. Isolation and characterization IAA from endophytic fungus and the effect of fungus on seedling growth

Endophytic fungi were screened for the production of IAA and further characterized the IAA produced with reference to the standard IAA. Further, the effect of endophytic fungus on growth of rice seedlings was studied.

3.13.1. Screening of IAA Production by endophytic fungi

The IAA production ability of the endophytic fungi was determined (Ehmann, 1977; Ahmad et al., 2005) with slight modifications. The endophytic fungi (1x 1 cm diameter block) was inoculated into 20 ml of Czapek Dox broth containing different concentrations of tryptophan (0, 1, 2 and 5mg/ml) and incubated at room temperature for 10 days. Media without fungal inoculation was used as the control. After incubation, 5 ml from each broth was collected and transferred to the fresh tube, centrifuged at 6000 rpm for 30 mins. 2 ml of salkowski's reagent and one drop of ortho-phosphoric acid was mixed with 1 ml of the supernatant and the development of a pink colour indicated the presence of IAA production. The OD was measured at 530 nm by using as spectrophotometer (Perkin Elmer, Singapore). The amount of IAA production was determined by using standard IAA graph (Ahmad et al., 2005).

3.13.2. Extraction and optimization of QIEF 5 for IAA production

Endophytic fungus QIEF 5 was grown in different media with the supplementation of tryptophan (1 mg/ml). The effect of different parameters like time, pH and temperature was studied on one factor at a time basis. The broth was acidified to pH 3.0 with IN HCl and extracted using three times the equal volume with ethyl acetate solvent in separating funnel and the ethyl acetate layer was collected. The extract was concentrated under reduced pressure in a rotary evaporator below 50^{o}C (Ahmad et al., 2005).

3.13.3. Thin layer chromatography analysis of QIEF 5

Thin layer chromatography was performed on TLC Silica gel 60 F_{254} aluminium backed sheets (Merck). The method was described in section 3.7.2. The fungus extract QIEF 5 was spotted on the TLC plate and it was developed with the solvent system chloroform:acetic acid glacial (9.5:0.5) and the plate was visualized under UV at 254 nm (Bentley, 1962), followed by spraying it with salkowski reagent and visualized in visible light (Ahmad et al., 2005).

3.13.4. HPLC analysis and HR-MS analysis of QIEF 5

The HPLC analysis was performed as mentioned in the section 3.12. Glacial acetic acid (1%) was used along with the mobile phase methanol:water (60:40, v/v). HR-MS analyses of the samples were performed on C18 column-Hypersil with the mobile phase chloroform:methanol and for QIEF 5 the mobile phase was chloroform: methanol with 0.1 % formic acid. The gradient elution of 97 % methanol and 3 % water for 5 mins. The sample injection volume is 2 µl with a flow rate of 150 µl/ min. The source was operated in both positive and negative mode at an ion spray voltage of 3KV.

3.13.5. Effect of endophytic fungus on promotion of growth of rice seedlings

The effect of endophytic fungus inoculation on growth of rice seedlings was evaluated on rice (*Oryza sativa* L.) seedlings (Fouda et al., 2015). The rice seeds were surface sterilized with 2.5% sodium hypochlorite by socking it for 3 mins and

further the seeds were washed with sterile distilled water for 5 times. The endophytic fungi culture was inoculated in to the PDB and incubated it at room temperature for 10 days. After the incubation, the surface sterilized seeds were transferred to the PDB containing the fungal strains and kept at room temperature for 24 hrs. PDB without fungal inoculation was used as control treatment. After 24 hrs of incubation, the soaked seeds were transferred to a sterilized wet whatman paper kept in a sterilized beaker and the set up was incubated at room temperature for 7 days in dark to measure the growth of the seedlings.

3.14. Statistical analysis

The data obtained from the experiments in the study were analyzed by independent t-test and analysis of variance (ANOVA) using SPSS software version 20 for the determination of significant differences and variations. The *P* value ($P< 0.05$) was taken in to consideration as statistically significant.

Chapter 4

RESULTS

RESULTS

The experimental results were divided into five subsections; (1) isolation and identification of endophytic fungi from *Elaeocarpus sphaericus* and *Quassia indica*, (2) screening of endophytic fungal extracts for antibacterial and cytotoxic activities, (3) characterization of active secondary metabolites produced by the selected endophytic fungus and their antibacterial and cytotoxic activities, (4) evaluation of phytochemical constituents, antibacterial and cytotoxic activities of the host plants, *Elaeocarpus sphaericus* and *Quassia indica*, (5) characterization of plant growth regulator indole acetic acid (IAA) from the selected endophytic fungus and the effect of fungus on growth of rice seedlings.

4.1. Isolation and identification of endophytic fungi from *Elaeocarpus sphaericus* (Gaertn.) K. Schum. and *Quassia indica* (Gaertn.) Nooteb.

From *Elaeocarpus sphaericus*, 203 endophytic fungi from 122 segments of leaves were isolated and 139 endophytic fungi were isolated from 110 leaf segments of *Quassia indica.*

4.1.1. Morphological and microscopic observation of endophytic fungi from *Elaeocarpus sphaericus*

12 morphologically different endophytic fungal cultures were selected from 203 isolates of 122 leaf segments from *Elaeocarpus sphaericus* and each fungal isolates were given unique code ESEF 1 to ESEF 12 (*Elaeocarpus sphaericus* endophytic fungus 1 to 12). The morphological and microscopic views of isolated fungi are shown in figure 4.1, 4.2 and 4.3 and the observations are described in table 4.1. Among these, ESEF 12 exhibited the sporulating structure on microscopic observation and it was identified as *Pestalotia* sp. whereas the remaining isolates failed to produce spores.

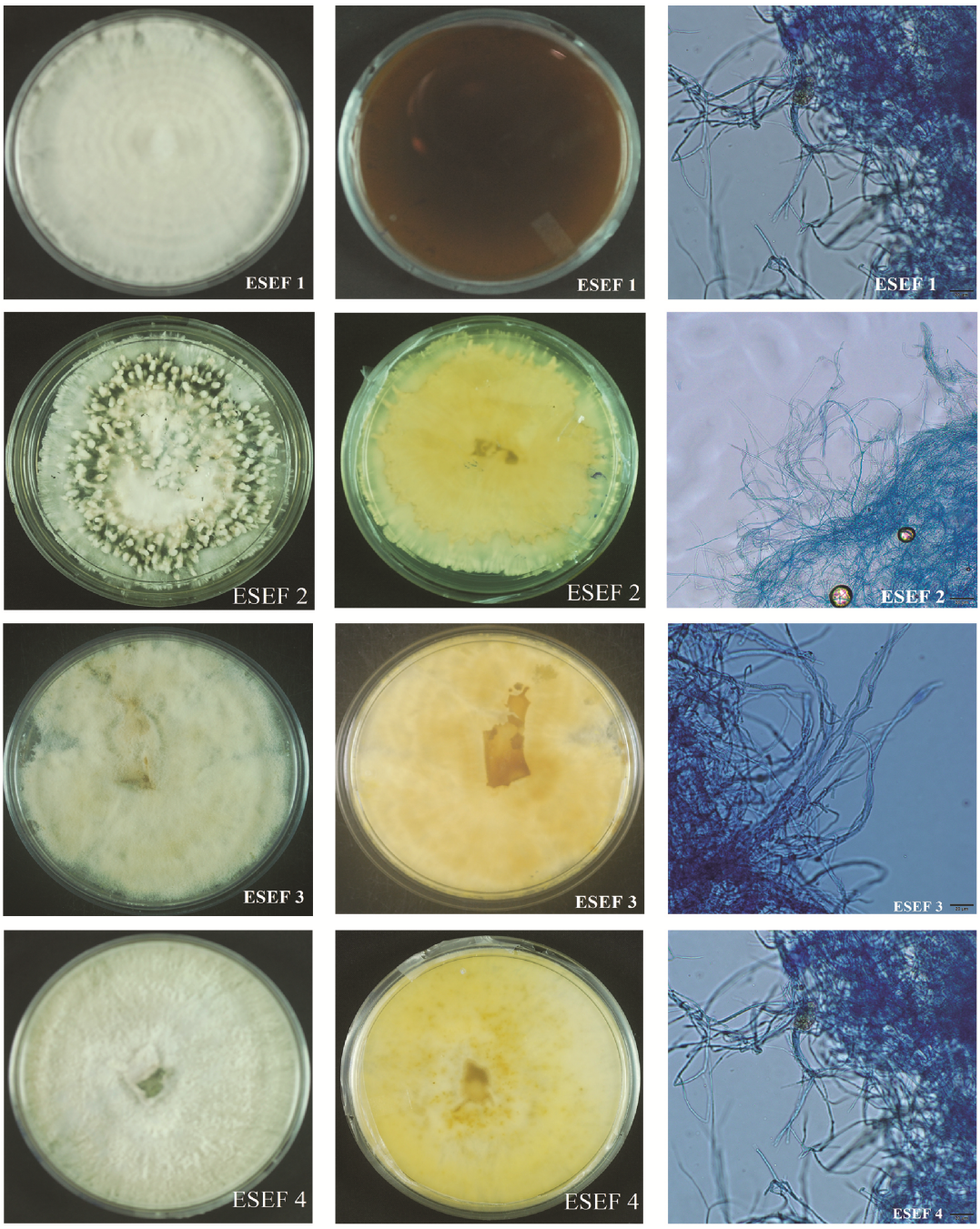


Figure 4.1. Morphological and microscopic observation of endophytic fungi ESEF 1 to ESEF 4 isolated from *Elaeocarpus sphaericus*. The top view, reverse view and microscopic view (magnification 40X) of endophytic fungi in potato dextrose agar incubated for 21 to 28 days.

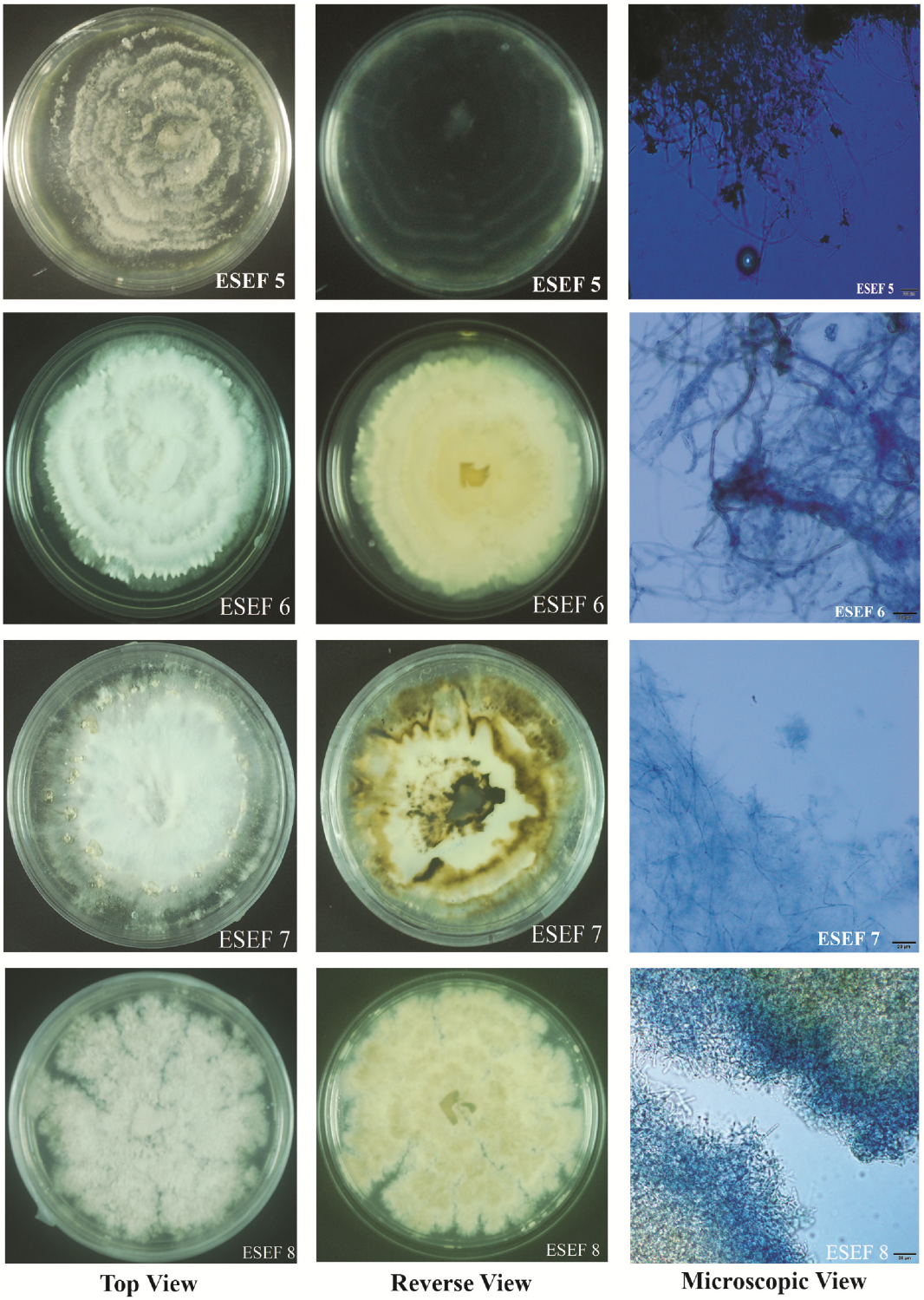


Figure 4.2. Morphological and microscopic observation of endophytic fungi ESEF 5 to ESEF 8 isolated from *Elaeocarpus sphaericus*. The top view, reverse view and microscopic view (magnification 40X) of endophytic fungi in potato dextrose agar incubated for 21 to 28 days.

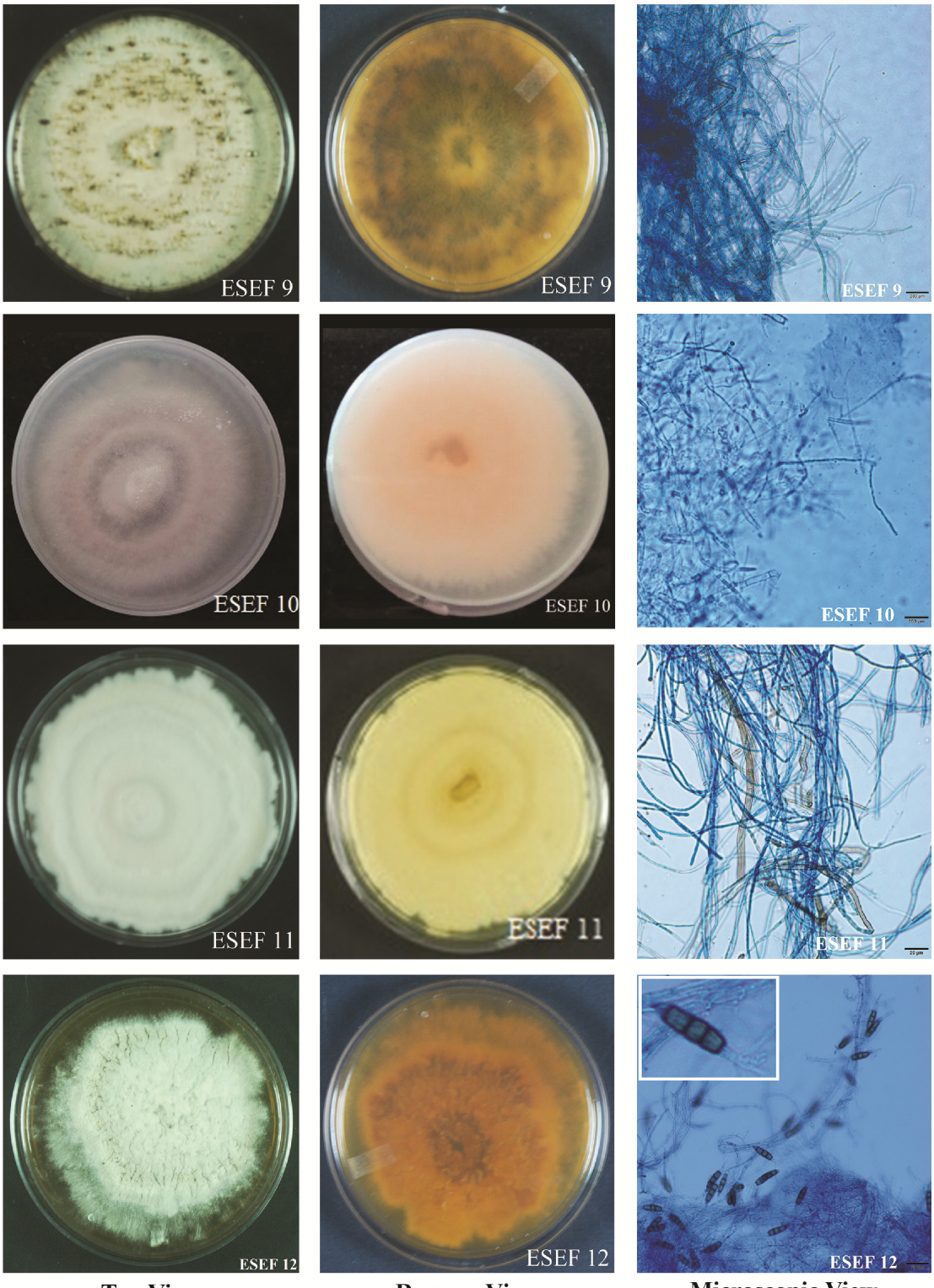


Figure 4.3. Morphological and microscopic observation of endophytic fungi ESEF 9 to ESEF 12 isolated from *Elaeocarpus sphaericus*. The top view, reverse view and microscopic view (magnification 40X) of endophytic fungi in potato dextrose agar incubated for 21 to 28 days. Inset view of the spore produced by ESEF 12.

Table 4.1. Morphological and microscopic observation of endophytic fungi isolated from *Elaeocarpus sphaericus*

Sl. No.	Fungal isolate	Morphological observation	Microscopic observation
1	ESEF 1	White colour colonies with dark pink colour on reverse view. The media colour changed to pink. The fungus showed moderate growth with filamentous form and flat elevation.	Sterile mycelia
2	ESEF 2	White colour colonies which later turned into black colour at centre. Mycelium is filamentous with undulate margin, white and black appendages arised from the mycelium.	Sterile mycelia
3	ESEF 3	Cream colour colonies. Mycelium is filamentous with undulate margin, white and black appendages arised from the mycelium.	Sterile mycelia
4	ESEF 4	White colour colonies having circular form and crateriform elevation. Reverse view cream in colour. Sclerotia formation above the surface.	Sterile mycelia
5	ESEF 5	Black colour colonies with filamentous form, raised elevation and curled margin.	Sterile mycelia
6	ESEF 6	White colour colonies with circular form and raised elevation. Black colour developed above the surface and appendages arised from it.	Sterile mycelia
7	ESEF 7	White colour colonies having moderate growth, circular form, flat elevation and entire margin. Reverse side brown in colour.	Sterile mycelia
8	ESEF 8	White to light gray coloured colonies with yellow colour on reverse view. The fungus grows in irregular form with flat elevation and undulate margin.	Sterile mycelia
9	ESEF 9	White coloured colonies which later turned in to cream colour with brown colour on reverse view. The culture is having flat elevation with circular form and curled margin.	Sterile mycelia
10	ESEF 10	White coloured colonies which turned to pink in colour. The isolate showed crateriform elevation with a filamentous form and a filiform margin.	Sterile mycelia

Sl. No.	Fungal isolate	Morphological observation	Microscopic observation
11	ESEF 11	White colour colonies with cottony appearance, cream colour on reverse view. The culture is having umbonate elevation with irregular form and undulate margin.	Sterile mycelia
12	ESEF 12	White coloured colonies with a light brown on reverse view. The fungi have filamentous with flat elevation.	Conidia are multi-celled pigmented cells at centre and pointed cells at the end; appendages arise from the end cells.

4.1.2. Morphological and microscopic observation of endophytic fungi from *Quassia indica*

Ten morphologically different endophytic fungal cultures were selected from 139 isolates of 110 leaf segments from *Quassia indica* and fungal isolates were given unique code QIEF 1 to QIEF 10 (*Quassia indica* endophytic fungus 1 to 10). The morphological and microscopic views of isolated fungi are shown in figure 4.4, 4.5 and 4.6 and the observations are also described in table 4.2. Two fungi coded as QIEF 8 and QIEF 10 showed the sporulating characteristics and was identified as *Pestalotia* sp. and *Fusarium* sp. respectively. All other isolates could not be identified due to the lack of spore formation.

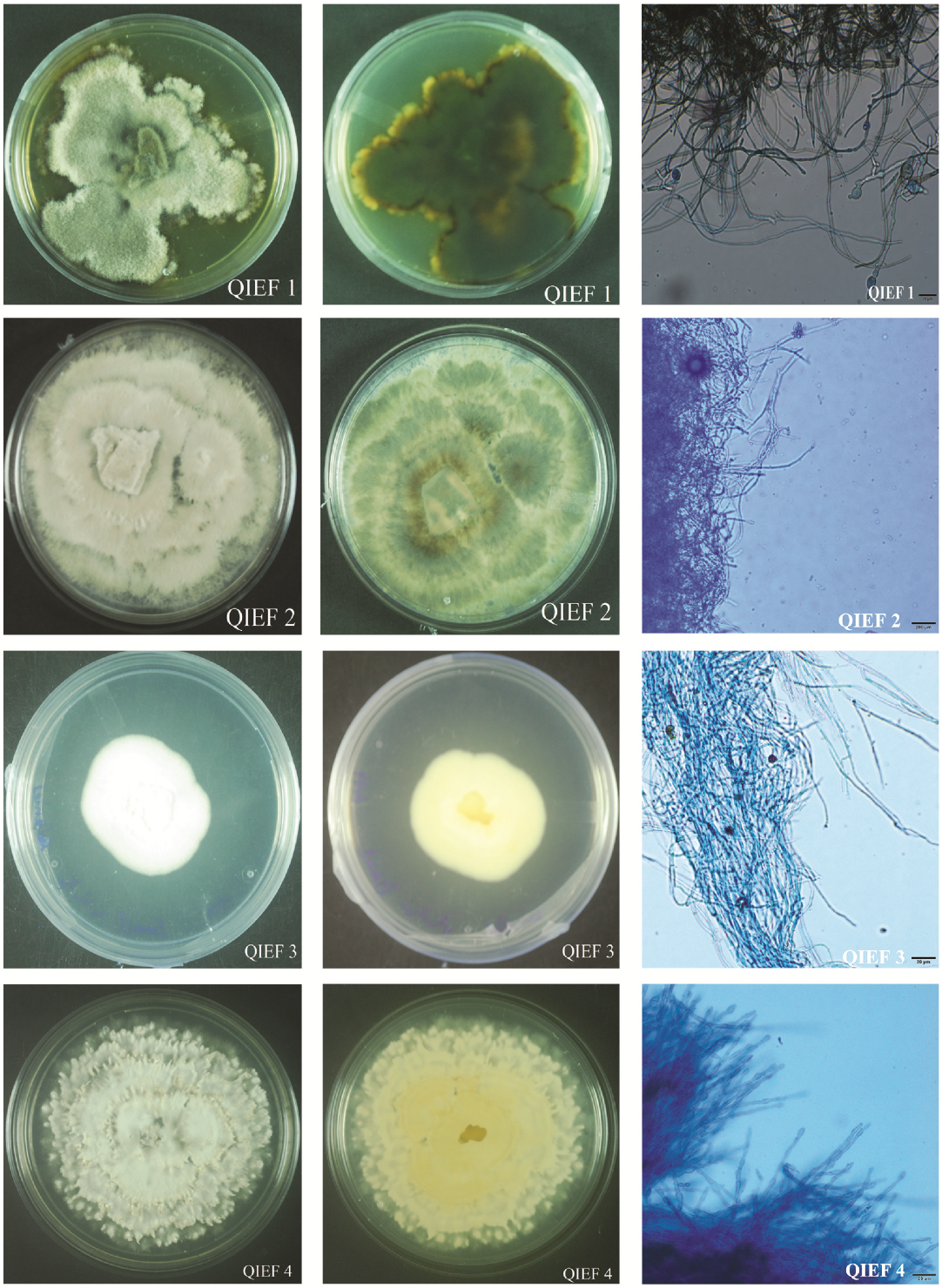


Figure 4.4. Morphological and microscopic observation of endophytic fungi QIEF 1 to QIEF 4 isolated from *Quassia indica.* The top view, reverse view and microscopic view (magnification 40X) of endophytic fungi in potato dextrose agar incubated for 21 to 28 days.

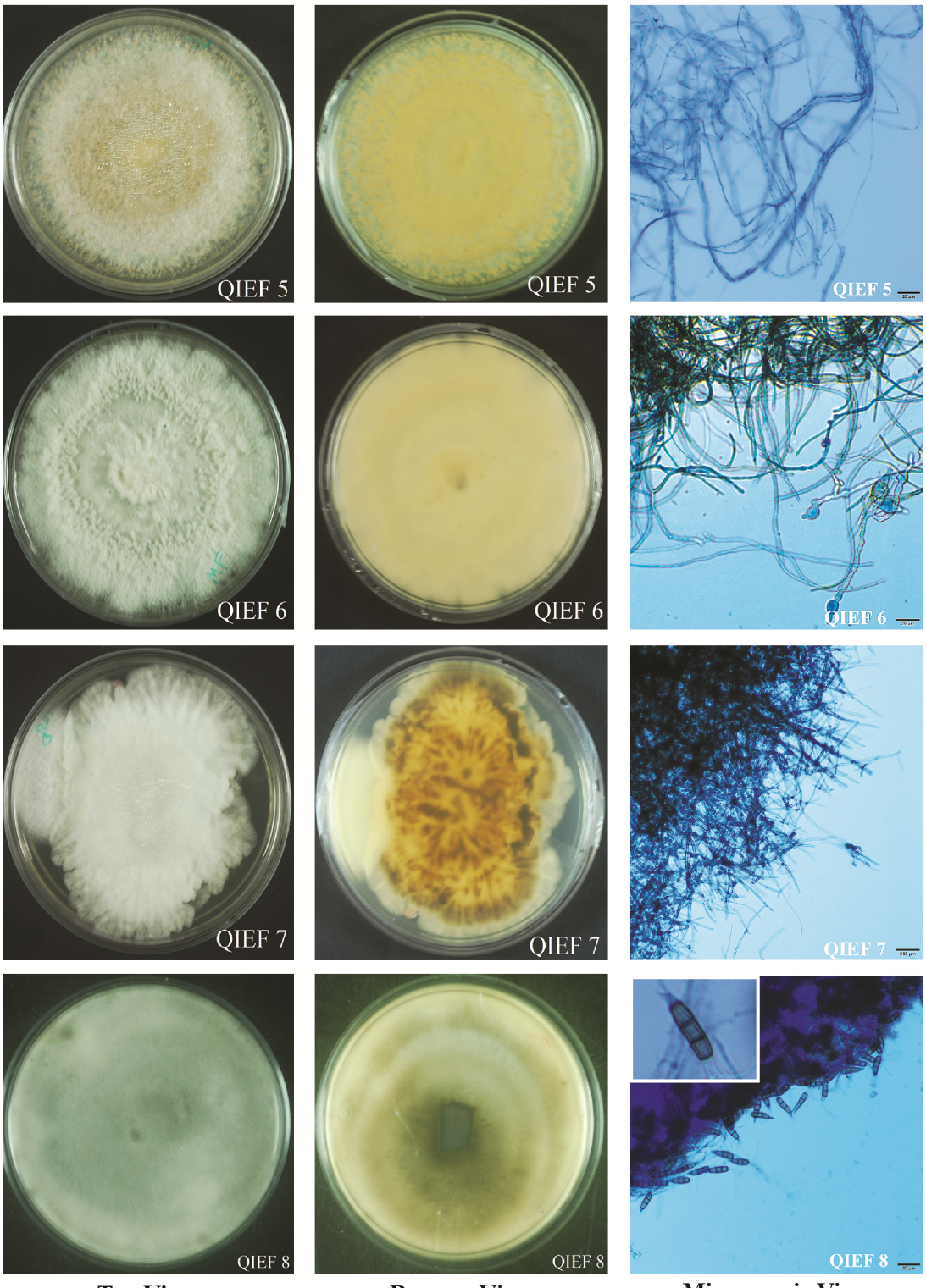


Figure 4.5. Morphological and microscopic observation of endophytic fungi QIEF 5 to QIEF 8 isolated from *Quassia indica*. The top view, back view and microscopic view (magnification 40X) of endophytic fungi in potato dextrose agar incubated for 21 to 28 days. Inset view of the spore produced by QIEF 8.

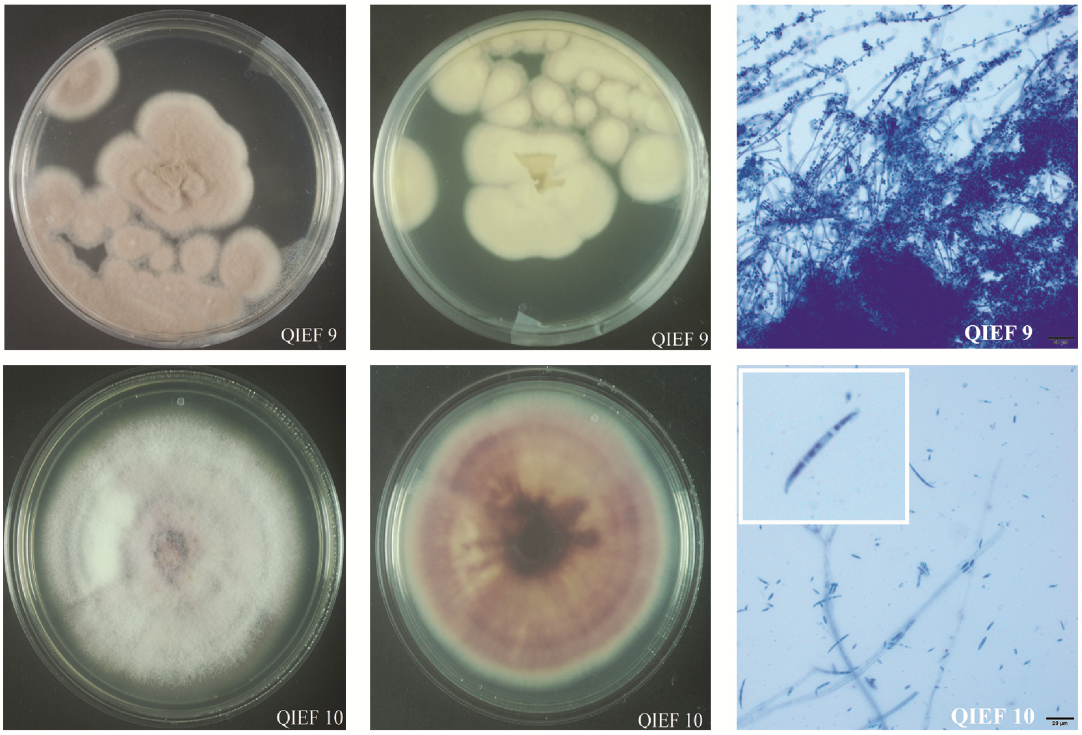


Figure 4.6. Morphological and microscopic observation of endophytic fungi QIEF 9 and QIEF 10 isolated from *Quassia indica.* The top view, back view and microscopic view (40X) of endophytic fungi in potato dextrose agar incubated for 21 to 28 days. Inset view of the spore produced by QIEF 10.

Table 4.2. Morphological and microscopic observation of endophytic fungi isolated from *Quassia indica*

Sl. No	Fungal isolate	Morphological observation	Microscopic observation
1	QIEF 1	White colour colonies later turned in to grey, reverse side black in colour. Fungus grows as irregular form with raised elevation and undulate margin.	Sterile mycelia
2	QIEF 2	Cream colour colonies later turned in to grey colour. Fungus grows with flat elevation, rhizoid form and filiform margin	Sterile mycelia
3	QIEF 3	White colour colonies with circular form, raised elevation, filiform margin and slow growth.	Sterile mycelia

Sl. No	Fungal isolate	Morphological observation	Microscopic observation
4	QIEF 4	Colonies are white in colour, reverse side cream to yellow and appendages arose from the mycelia. Isolate grows with filamentous form, raised elevation and filiform margin.	Sterile mycelia
5	QIEF 5	White colour mycelium secretes reddish orange colour on the surface. Isolate grow with corvex elevation, entire margin and circular form.	Sterile mycelia
6	QIEF 6	Colonies are white in colour with filamentous form, raised elevation and filiform margin having fast growth.	Sterile mycelia
7	QIEF 7	White colour colonies with yellow colour reverse view. The culture is having flat elevation with filamentous and undulate margin.	Sterile mycelia.
8	QIEF 8	White colour colonies with black colour on reverse view. The fungus is having raised elevation with circular form and entire margin.	Conidia were multi-celled having the pigmented cells at centre and pointed cells at the end; appendages arise from the end cells.
9	QIEF 9	Pink colour colonies with filamentous form, raised elevation and white colour edge.	Sterile mycelia
10	QIEF 10	White colour colonies later turned in to violet colour, the bottom of the agar plate turned to dark violet colour. The culture has a flat elevation with filamentous form and filiform margin.	The microscopic characteristics showed that the macroconidia were ovoid, 1 to 2 celled and slightly curved.

4.1.3. Molecular identification of endophytic fungi

Molecular methods were used to authenticate and identify the isolates from *Elaeocarpus sphaericus* and *Quassia indica.* All the 12 endophytic fungi from *Elaeocarpus sphaericus* and 10 from *Quassia indica* were amplified by using ITS-PCR with ITS1 and ITS4 primers. A single band of approximately 600 bp was observed in all lanes on 1% agarose gel (Figure 4.7 and Figure 4.8). The amplified products were sequenced and all the sequences were submitted to GenBank to obtain the accession numbers. The closely related endophytic fungi from *Elaeocarpus sphaericus* were identified by BLAST search analysis. The genus of the identified endophytic fungi is mentioned in the study. *Xylaria* sp. (ESEF 1, ESEF 2, ESEF 6 and ESEF 7), *Fusarium* sp. (ESEF 3), *Diaporthe* sp. (ESEF 4 and ESEF 8), *Endomelanconiopsis* sp. (ESEF 5), *Nigrospora* sp. (ESEF 9, ESEF 10 and ESEF 11), *Pestalotiopsis* sp. (ESEF 12) are the endophytic fungi identified from *Elaeocarpus sphaericus*. Colonization frequencies of fungal isolates of *Elaeocarpus sphaericus* varied from 3.27 % to 20.49 % and the highest colonization frequency was observed in ESEF 4.

The closely related Endophytic fungi identified from *Quassia indica* by BLAST analysis are *Daldinia* sp. (QIEF 1), *Diaporthe* sp. (QIEF 2), *Phyllosticta* sp. (QIEF 3), *Lecanicillium* sp. (QIEF 4), *Colletotrichum* sp. (QIEF 5 and QIEF 6), *Fusarium* sp. (QIEF 7 and QIEF 10), *Pestalotiopsis* sp. (QIEF 8), and *Meyerozyma* sp. (QIEF 9). Colonization frequencies of fungal isolates of *Quassia indica* ranged from 2.72 % to 20.90 % and the highest colonization frequency was observed in QIEF 2. Colonization frequency percentage of endophytic fungi from *Elaeocarpus sphaericus* and *Quassia indica*, their closely related fungus based on the similarity data from BLAST analysis and the accession numbers obtained are given in table 4.3 and 4.4.

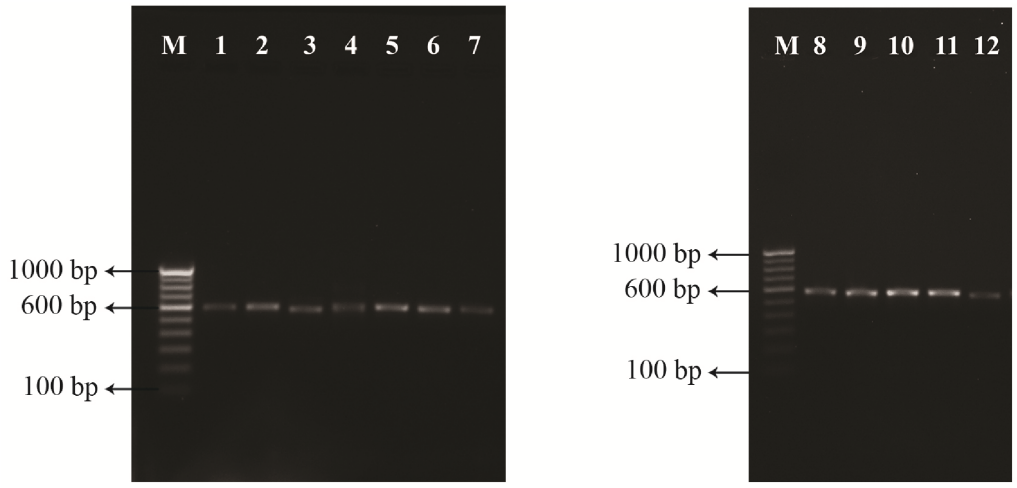


Figure 4.7. PCR amplified products of endophytic fungi from *Elaeocarpus sphaericus.* The primers used - ITS1 and ITS4. Lane M: DNA marker of 100 bp, lane 1 to 7, 8 to 12: ESEF 1 to 12 amplified product approximately 600 bp.

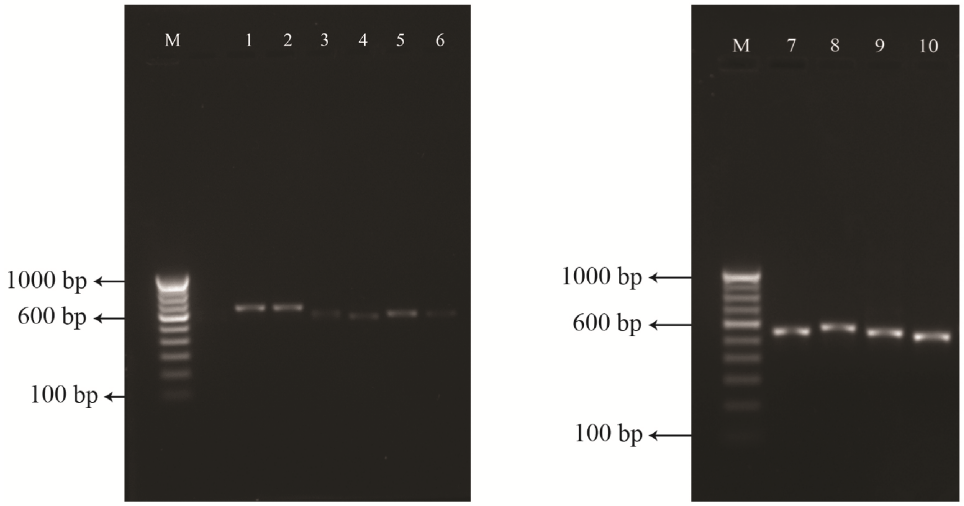


Figure 4.8. PCR amplified products of endophytic fungi from *Quassia indica.* The primers used- ITS1 and ITS4. Lane M: DNA marker of 100 bp, lane 1 to 6, 7 to 10: QIEF 1 to 10 amplified product approximately 600 bp.

Table 4.3. Colonization frequency percentage of endophytic fungi from *Elaeocarpus sphaericus* and their closely related fungus based on the similarity search from BLAST analysis

Sl. No.	Fungal isolate code	Accession Numbers	Colonization frequency percentage	Closely related fungal sequence	Blast Similarity (%)
1	ESEF 1	MH819666	5.73 %	*Xylaria psidii* (KJ767111.1)	97 %
2	ESEF 2	MH819667	19.67 %	*Xylaria feejeensis* (KY951907.1)	100 %
3	ESEF 3	MH045725	7.37 %	*Fusarium equiseti* (KR866143.1)	86 %
4	ESEF 4	MK050996	20.49 %	*Diaporthe discoidispora* (MH371249.1)	99 %
5	ESEF 5	MH835348	13.93 %	*Endomelanconiopsis endophytica* (GQ469966.1)	99 %
6	ESEF 6	MH836377	12.29 %	*Xylaria feejeensis* (MH469506.1)	98 %
7	ESEF 7	MK045721	4.09 %	*Xylaria* sp. (MG372015.1)	98 %
8	ESEF 8	MK045723	9.01 %	*Diaporthe hongkongensis* (MH371251.1)	98 %
9	ESEF 9	MH910671	9.83 %	*Nigrospora* sp.(MH345896.1)	99 %
10	ESEF 10	MH909796	3.27 %	*Nigrospora oryzae* (MH619725.1)	97 %
11	ESEF 11	MK045718	8.19 %	*Nigrospora* sp. (MK299416.1)	99 %
12	ESEF 12	MH915546	8.19 %	*Pestalotiopsis mangiferae* (KM998724.1)	99 %

Table 4.4. Colonization frequency percentage of the endophytic fungi from *Quassia indica* and their closely related fungus based on the similarity search from BLAST analysis

Sl. No.	Fungal isolate code	Accession numbers	Colonization frequency percentage	Closely related fungal sequence	Blast Similarity (%)
1	QIEF 1	MH819669	8.18 %	*Daldinia eschscholtzii* (KC895542.1)	99 %
2	QIEF 2	MK045724	20.90 %	*Diaporthe* sp. (KU671315.1)	98 %
3	QIEF 3	MH910044	3.63 %	*Phyllosticta elongata* (KX424992.1)	98 %
4	QIEF 4	MH910098	12.72 %	*Lecanicillium saksenae* (MH976712.1)	99 %
5	QIEF 5	MH910090	10.90 %	*Colletotrichum siamense* (KP748194.1)	92 %
6	QIEF 6	MK207056	6.36 %	*Colletotrichum acutatum* (KX347475.1)	97 %
7	QIEF 7	MK050999	2.72 %	*Fusarium proliferatum* (FR682078.1)	99 %
8	QIEF 8	MH915547	8.18 %	*Pestalotiopsis microspora* (KJ787111.1)	98 %
9	QIEF 9	MK050997	2.72 %	*Meyerozyma guilliermondii* (KP281457.1)	87 %
10	QIEF 10	MK050998	4.54 %	*Fusarium* sp. (HQ846580.1)	97 %

4.1.4. Phylogenetic analysis of endophytic fungi

Phylogenetic tree of endophytes from both *Elaeocarpus sphaericus* and *Quassia indica* were constructed by using MrBayes version 3.2. Inference of Bayesian analysis of the isolated endophytic fungi from *Elaeocarpus sphaericus* is

shown in figure 4.9. The tree consists of 8 taxa. ESEF 1 (*Xylaria* sp.) rooted separately. ESEF 2 and ESEF 6 (*Xylaria* sp.) were positioned in one subclade; the posterior probability value is 1 and was rooted from the taxa *Xylaria aff. curta* and *Xylaria bertani*. ESEF 3 (*Fusarium* sp.) positioned in 3rd taxa and that displayed closest evolutionary relation with *Fusarium equiseti* (posterior probability- 0.95). ESEF 4 and ESEF 8 (*Diaporthe* sp.) present in 4th taxa and they grouped into a subclade which showed closest similarity each other with the posterior probability value 0.94. ESEF 5 (*Endomelanconiopsis* sp.) grouped in 6th clade that and had the evolutionary relation with *Endomelanconiopsis endophytica* (0.69). The 7th clade included ESEF 9, ESEF 10 and ESEF 11, where in ESEF 9 and ESEF 10 with posterior probability value 0.98, the isolates had close relation with *Nigrospora aurantiaca*. ESEF 11 was grouped with *Nigrospora chinensis*. ESEF 12 (*Pestalotiopsis* sp.) in 8th clade showed posterior probability- 0.69 and form closest similarity with *Pestalotiopsis cinchonae*. High posterior density (HPD) values got in bayesian analysis are tabulated (Table 4.5).

Table 4.5. 95 % High posterior density interval of bayesian analysis of endophytic fungi from *Elaeocarpus sphaericus*

			95% HPD Interval					
Parameter	Mean	Variance	Lower	Upper	Median	min ESS*	avg ESS	PSRF+
TL	9.819706	0.359752	8.747844	11.080430	9.795057	553.88	789.09	1.000
r(A<->C)	0.115761	0.000126	0.093327	0.137204	0.115461	1215.26	1426.95	1.000
r(A<->G)	0.180243	0.000192	0.152742	0.206937	0.179804	1248.47	1326.64	1.000
r(A<->T)	0.175874	0.000185	0.150626	0.203101	0.175616	1081.68	1301.09	1.000
r(C<->G)	0.094297	0.000103	0.074940	0.114138	0.093987	979.96	1271.03	1.000
r(C<->T)	0.326767	0.000314	0.293211	0.361885	0.326685	1258.18	1371.69	1.000
r(G<->T)	0.107058	0.000114	0.086610	0.127906	0.106839	1423.58	1717.33	1.001
pi(A)	0.246444	0.000072	0.229697	0.262704	0.246368	1380.08	1456.83	1.000
pi(C)	0.252642	0.000074	0.235616	0.269547	0.252277	1496.51	1659.78	1.000
pi(G)	0.267489	0.000086	0.249991	0.286479	0.267325	1473.12	1558.45	1.000
pi(T)	0.233425	0.000068	0.216169	0.248524	0.233392	1502.97	1629.19	1.000
alpha	0.773918	0.003535	0.664179	0.894029	0.770464	2100.05	2171.78	1.000
pinvar	0.020336	0.000230	0.000001	0.049034	0.017404	1735.21	1988.83	1.000

* Convergence diagnostic (ESS = Estimated Sample Size); min and avg values correspond to minimal and average ESS among runs.
ESS value below 100 may indicate that the parameter is undersampled.
\+ Convergence diagnostic (PSRF = Potential Scale Reduction Factor; Gelman and Rubin, 1992) should approach 1.0 as runs converge.

Bayesian analysis of the isolated endophytic fungi from *Quassia indica* is shown in figure 4.10. The isolates were grouped into 8 clades. QIEF 1 (*Daldinia* sp.) formed separate, but that originated from the same root of

Daldinia bambusicola and *Daldinia caldariorum*. QIEF 2 (*Diaporthe* sp.) grouped into 2nd clade. The phylogeny showed and evolutionary relation with *Daldinia* sp. QIEF 4 (*Lecanicillium* sp.) and QIEF 6 (*Colletotrichum* sp.) in the subclade showed the posterior probability 0.79. QIEF 3 (*Phyllosticta* sp.) and QIEF 9 (*Meyerozyma* sp.) were from the same clade represent these isolates were consists of a common ancestor. QIEF 9 has evolutionary relation with *Meyerozyma* sp. with a posterior probability 1. QIEF 5 (*Colletotrichum* sp.) with the probability value 0.59 was grouped into 4th clade it had the closest evolutionary relation with *Colletotrichum boninense*. QIEF 7 and QIEF 10 grouped in to the subclade with posterior probability 0.94. QIEF 8 (*Pestalotiopsis* sp.) grouped in to a clade having the probability value 0.75 and the isolate had the similarity with *Pestalotia diospyri*. High posterior density (HPD) values got in bayesian analysis are tabulated (Table 4.6).

Table 4.6. 95 % High posterior density interval of bayesian analysis of endophytic fungi from *Quassia indica*

			95% HPD Interval					
Parameter	Mean	Variance	Lower	Upper	Median	min ESS*	avg ESS	PSRF+
TL	10.935818	0.325257	9.827382	12.066880	10.919680	730.33	893.73	1.000
r(A<->C)	0.130764	0.000109	0.111387	0.152956	0.130568	1651.59	1699.56	1.000
r(A<->G)	0.193469	0.000147	0.170236	0.217723	0.193418	1032.05	1323.33	1.000
r(A<->T)	0.141367	0.000108	0.119843	0.160819	0.141082	1591.05	1591.50	1.002
r(C<->G)	0.121297	0.000095	0.103170	0.141029	0.121105	1234.39	1297.28	1.001
r(C<->T)	0.326471	0.000237	0.297645	0.357392	0.326225	1211.78	1355.72	1.000
r(G<->T)	0.086631	0.000071	0.070428	0.103676	0.086516	1857.89	1959.06	1.000
pi(A)	0.252001	0.000058	0.236953	0.266246	0.252074	1768.02	1773.52	1.000
pi(C)	0.225908	0.000051	0.212391	0.240046	0.225922	1663.45	1777.50	1.000
pi(G)	0.280062	0.000068	0.263960	0.296050	0.279880	1717.04	1770.32	1.000
pi(T)	0.242028	0.000055	0.227026	0.256230	0.241968	1522.48	1542.00	1.001
alpha	1.056753	0.005289	0.921327	1.197636	1.051969	1545.09	1687.41	1.000
pinvar	0.008496	0.000048	0.000003	0.022072	0.006751	2325.05	2380.88	1.000

* Convergence diagnostic (ESS = Estimated Sample Size); min and avg values correspond to minimal and average ESS among runs.
ESS value below 100 may indicate that the parameter is undersampled.
\+ Convergence diagnostic (PSRF = Potential Scale Reduction Factor; Gelman and Rubin, 1992) should approach 1.0 as runs converge.

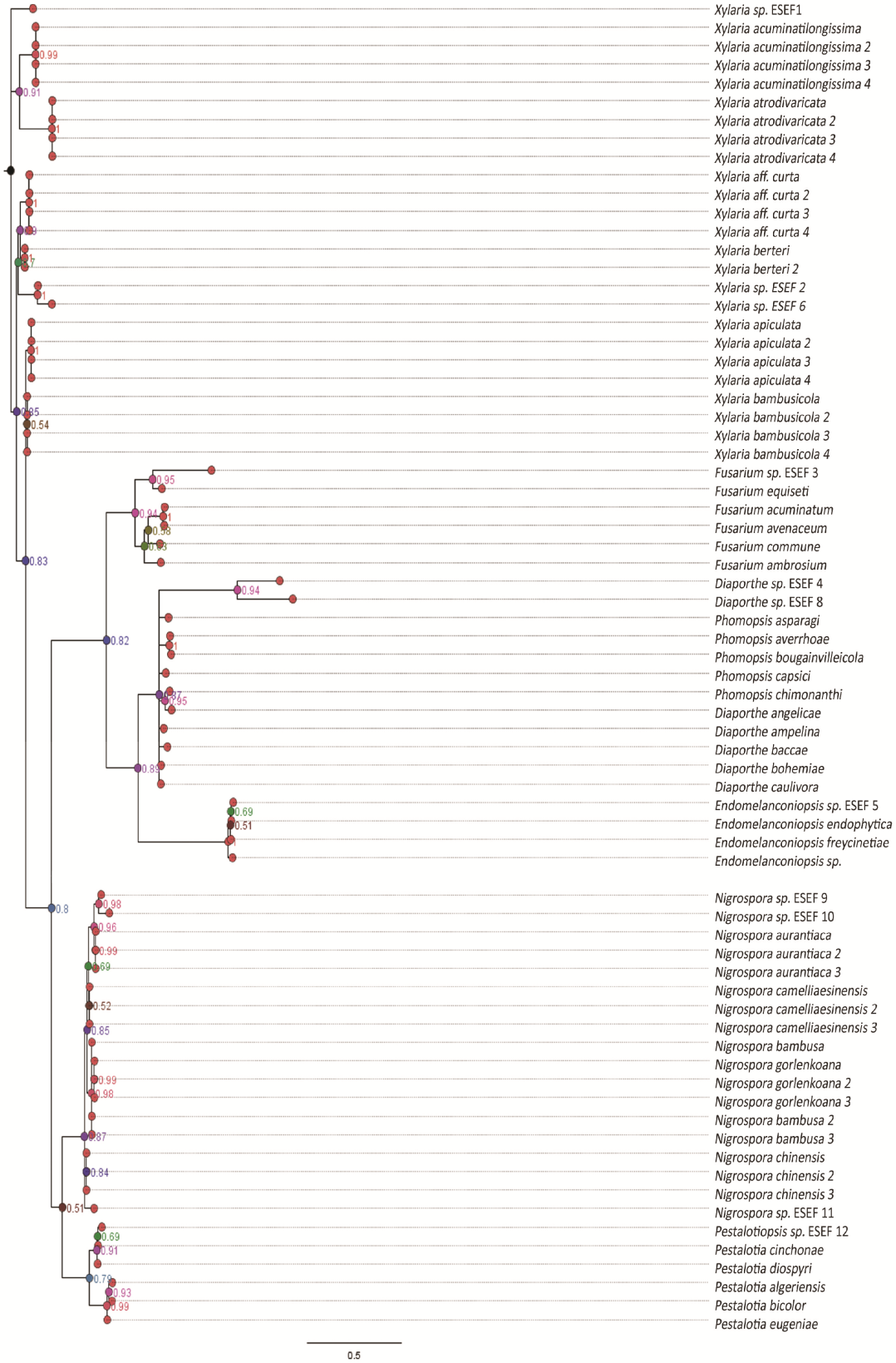


Figure 4.9. Bayesian analysis of endophytic fungi from *Elaeocarpus sphaericus*. Majority value consensus based on bayesian analysis of ITS rDNA sequences.

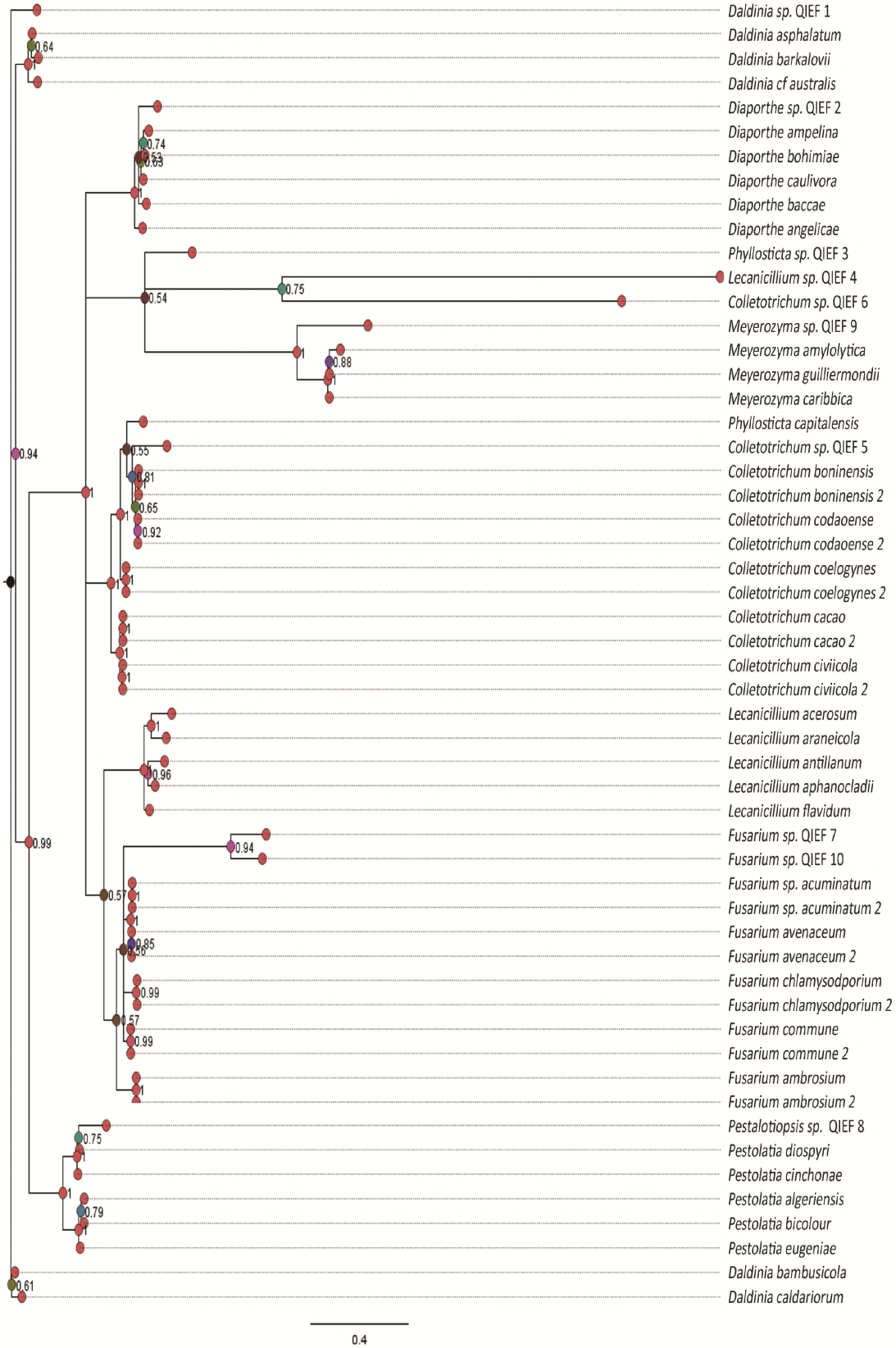


Figure 4.10. Bayesian analysis of endophytic fungi from *Quassia indica.* Majority value consensus based on bayesian analysis of ITS rDNA sequences.

4.1.5. Diversity index of *Elaeocarpus sphaericus* and *Quassia indica*

Diversity index of *Elaeocarpus sphaericus* and *Quassia indica* were done by Shannon-wiener diversity index. *Elaeocarpus sphaericus* exhibited the diversity index *H′* 2.248 having evenness 0.90 and *Quassia indica* showed the diversity index *H′* 1.943 with the evenness of 0.84 (Table 4.7).

Table 4.7. Diversity index and evenness of *Elaeocarpus sphaericus* and *Quassia indica*

Sl. No.		*Elaeocarpus sphaericus*	*Quassia indica*
1.	Shannon-weiner diversity index (*H′*)	2.248	1.943
2.	Evenness	0.90	0.84

4.2. Screening of endophytic fungal extracts for antibacterial and cytotoxic activities

All the selected endophytic fungi from both *Elaeocarpus sphaericus* and *Quassia indica* were incubated for 21 to 28 days. The mycelium and broth were extracted and pooled together. The fungal extracts were used for antibacterial and cytotoxic activities.

4.2.1. Antibacterial activity of extracts of endophytic fungi isolated from *Elaeocarpus sphaericus*

Extracts of 12 endophytic fungi that isolated from *Elaeocarpus sphaericus* were screened for their antibacterial activities against the selected gram positive and gram negative bacteria by disc diffusion method (Figure 4.11 (i) and (ii)). Of the 12 isolates subjected for evaluation, ESEF 4 and ESEF 8 (*Diaporthe* sp.), ESEF 5 (*Endomelanconiopsis* sp.), ESEF 6 and ESEF 7 (*Xylaria* sp.), ESEF 9 and ESEF 10 (*Nigrospora* sp.) showed zone of inhibition against *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*. ESEF 6 and ESEF 7 (*Xylaria* sp.), ESEF 8 (*Diaporthe* sp.), ESEF 10 (*Nigrospora* sp.) inhibited the *Listeria monocytogenes*.

Among these ESEF 6 (*Xylaria* sp.) showed comparatively good inhibition, the zone of inhibition was 13 mm, 13.66±0.57 mm, 13.66±0.5 mm and 14.33±0.57 mm respectively for *E. coli*, *K. pneumoniae*, *S. aureus* and *L. monocytogenes*. All the fungal extracts showed no zone of inhibition against *Pseudomonas aeruginosa* and *Enterococcus faecalis*. Table 4.8 showed the zone of inhibition in mm ± SD.

Further statistical analysis was conducted to identify the level of significance in each fungal extracts with the tested microorganisms. Independent t-test revealed that the antibacterial activities of fungal endophytes were significantly different in comparison with the control ($P< 0.05$). ANOVA showed significant differences and variations between the endophytic fungi against the microorganisms *E. coli*, *K. pneumoniae*, *S. aureus* and *L. monocytogenes* (F= 303.67, 285.75, 237.93, 63.67; $P< 0.001$) (Figure 4.12). The multiple comparison analysis (Dunnett t-test) of the differences revealed that all the endophytic fungal extracts were significantly lower ($P< 0.001$) than the positive control (Figure 4.12). Significant differences and variations were also found among the microorganisms in different endophytic fungi. Antibacterial activity of ESEF 8 and ESEF 10 showed significant variations (F= 33.00, F= 41.00; $P< 0.001$) against the tested organisms. Whereas ESEF 4, ESEF 5, ESEF 6 and ESEF 7 showed no significant variations among the microorganisms (F= 0.00; $P= 1.00$, F= 1.00; $P= 0.422$, F= 3.56, $P= 0.067$, F= 3.33; $P= 0.077$).

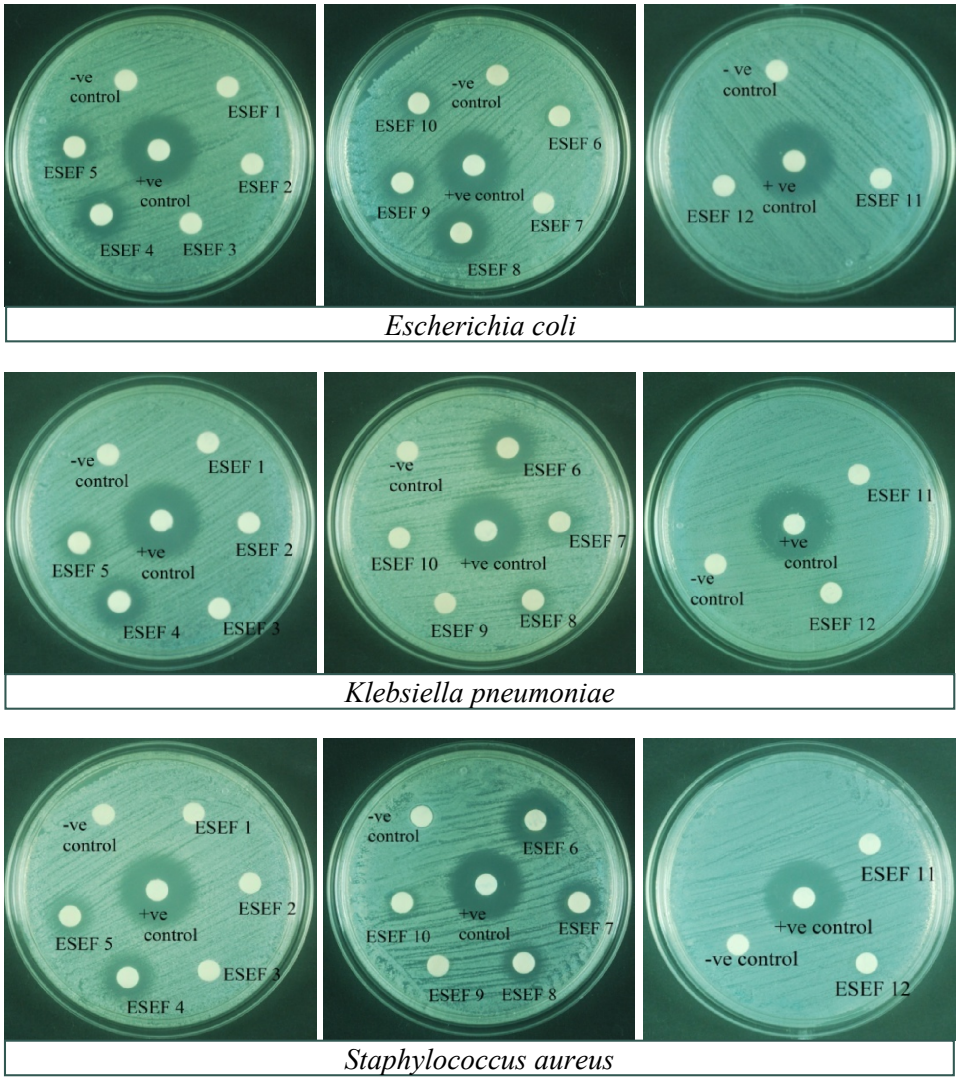
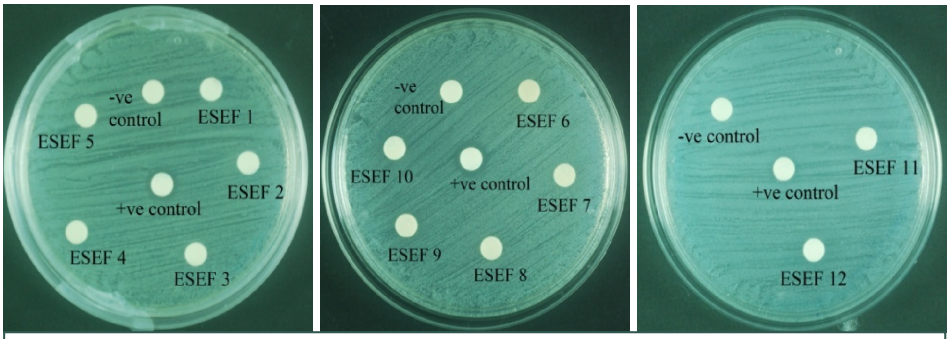
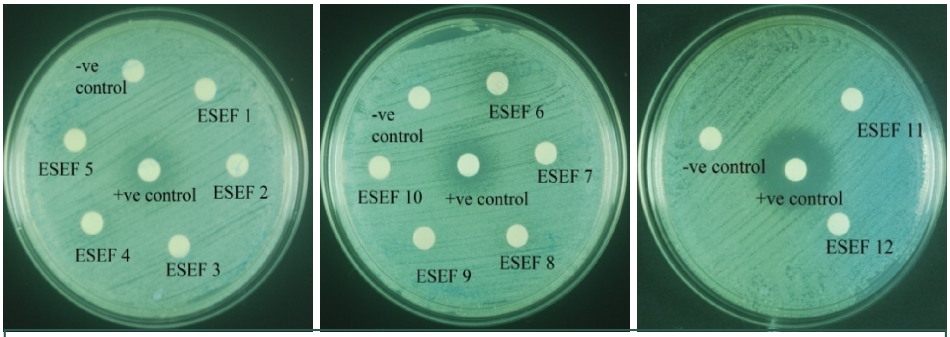


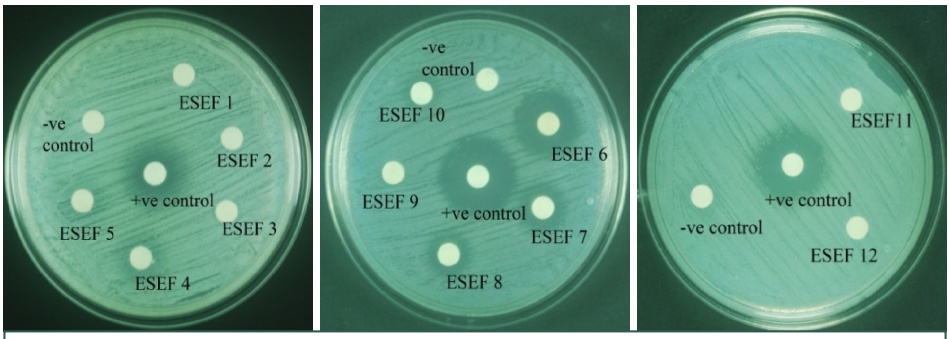
Figure 4.11 (i). Antibacterial activities of extracts of endophytic fungi isolated from *Elaeocarpus sphaericus*. Positive control (Chloramphenicol)- 20µg/Disc, fungal extracts- 100µg/Disc and negative control- DMSO.



Pseudomonas aeruginosa



Enterococcus faecalis



Listeria monocytogenes

Figure 4.11 (ii). Antibacterial activity of extracts of endophytic fungi isolated from *Elaeocarpus sphaericus*. Positive control (Chloramphenicol)- 20µg/Disc, fungal extracts- 100µg/Disc and negative control- DMSO.

Table 4.8. Antibacterial activity of extracts of fungal endophyte from *Elaeocarpus sphaericus*

		Diameter of zone of inhibition (mm)					
Sl. No	**Fungal isolate**	***E. coli***	***K. Pneumoniae***	***S. aureus***	***P. aeruginosa***	***L. monocytogenes***	***E. faecalis***
1	ESEF 1	–	–	–	–	–	–
2	ESEF 2	–	–	–	–	–	–
3	ESEF 3	–	–	–	–	–	–
4	ESEF 4	11.66±0.57	11.66 ± 0.57	11.66 ± 0.57	–	–	–
5	ESEF 5	10	10	10.33 ±0.57	–	–	–
6	ESEF 6	**13**	**13.66 ± 0.57**	**13.66 ± 0.57**	–	**14.33 ± 0.57**	–
7	ESEF 7	9.66±0.57	9	11	–	10.33 ± 1.52	–
8	ESEF 8	9	7.66 ± 0.57	9	–	10	–
9	ESEF 9	–	–	–	–	–	–
10	ESEF 10	9	7	6.66 ± 0.57	–	7	–
11	ESEF 11	–	–	–	–	–	–
12	ESEF 12	–	–	–	–	–	–
13	$^{+}$ve control	19.66±0.57	19. 33 ± 0.57	20.33 ± 0.57	7	17 ± 1	17 ± 4.61
14	$^{-}$ve control	–	–	–	–	–	–

Diameter of zone of inhibition in mm ± SD, (n=3). '–' = No zone of inhibition.

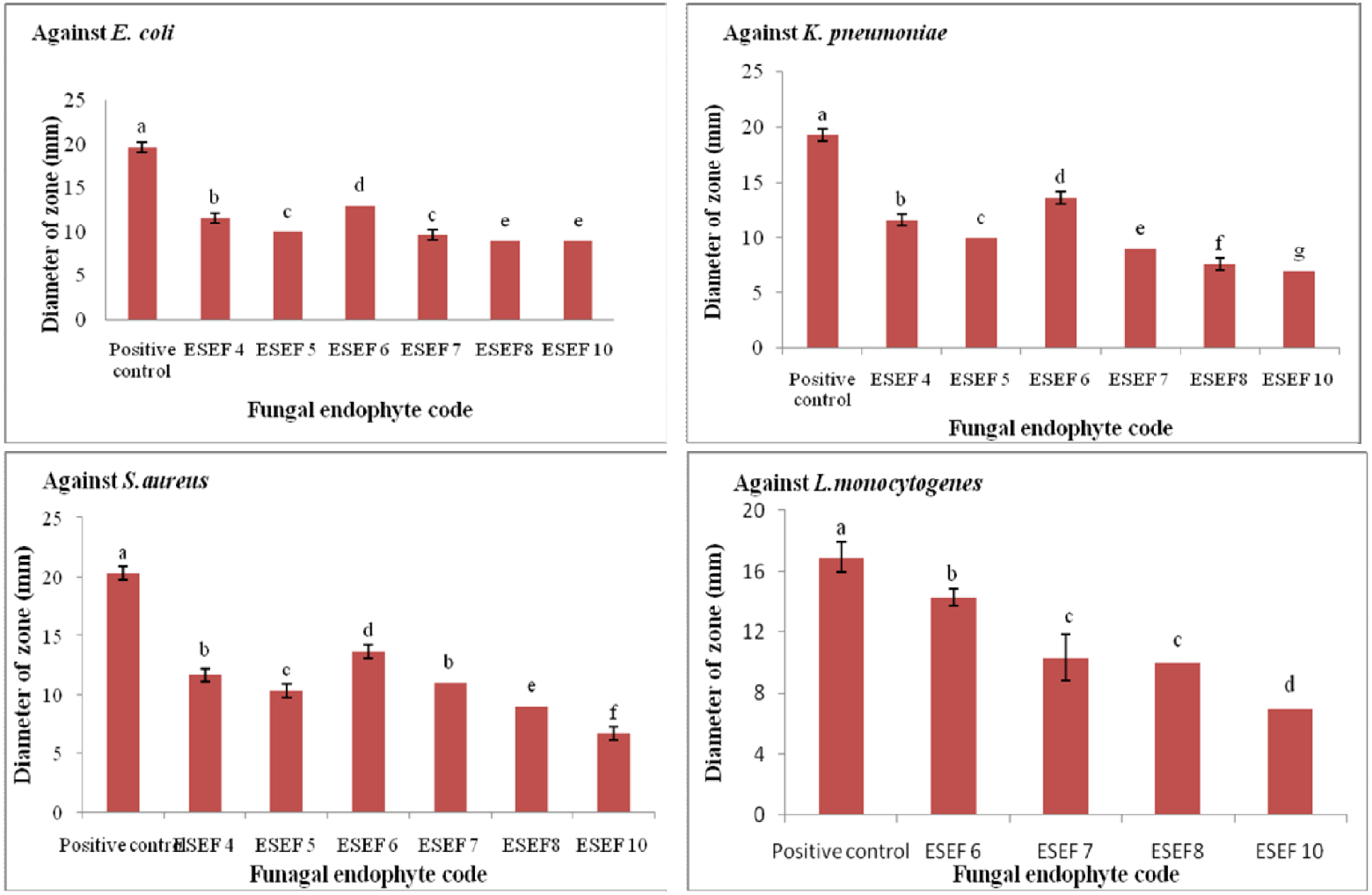


Figure 4.12. Antibacterial activity of extracts of endophytic fungi from *Elaeocarpus sphaericus* against the tested bacteria. Diameter zone of inhibition in mm ± SD, (n=3). Distinct letters on bars designated that mean values have significant differences ($P< 0.001$).

4.2.2. Antibacterial activity of extracts of endophytic fungi isolated from *Quassia indica*

Antibacterial activities of the crude extract of 10 endophytic fungi from *Quassia indica* were tested against the selected gram positive and gram negative bacteria by disc diffusion method (Figure 4.13 (i) and (ii)). Out of the 10 isolates, QIEF 2 (*Diaporthe* sp.), QIEF 3 (*Phyllosticta* sp.) showed inhibition against *E. coli*, *K. pneumoniae*, *S. aureus*, *L. monocytogenes* and *E. faecalis*. Similarly, QIEF 6 (*Colletotrichum* sp.) and QIEF 7 (*Fusarium* sp.) showed inhibition against *E. coli*, *K. pneumoniae*, *S. aureus* and *E. faecalis*. Among these isolates, QIEF 2 showed significant antibacterial activity and the zone of inhibition was 15.33±0.57 mm for *E. coli*, *K. pneumoniae* and *S. aureus*, 14.33±0.57 mm and 16 mm for *L. monocytogenes* and *E. faecalis* respectively. The remaining cultures showed no activity against these bacteria. Crude extracts of all the isolates failed to inhibit the growth of *Pseudomonas aeruginosa* at the tested concentrations. Table 4.9 showed the zone of inhibition in mm ± SD.

Statistical analysis exhibited that the antibacterial activities of fungal extracts have significant difference in comparison with control ($P< 0.05$). ANOVA test between fungal isolates in each bacteria have the significant difference in antibacterial activity (F=246.17, 209.75, 238.38, 10.24, 655.39; $P< 0.01$ respectively for *E. coli*, *K. pneumoniae*, *S. aureus*, *L. monocytogenes* and *E. faecalis*) (Figure 4.14 (i) and (ii)). One way analysis of variance in endophytic fungi QIEF 2 and QIEF 6 showed significant variations between the microorganisms (F=4.00, F=7.56; $P< 0.05$). While in QIEF 3 (F= 2.17; $P= 0.147$) and QIEF 7, there is no significant variations shown among the microorganisms.

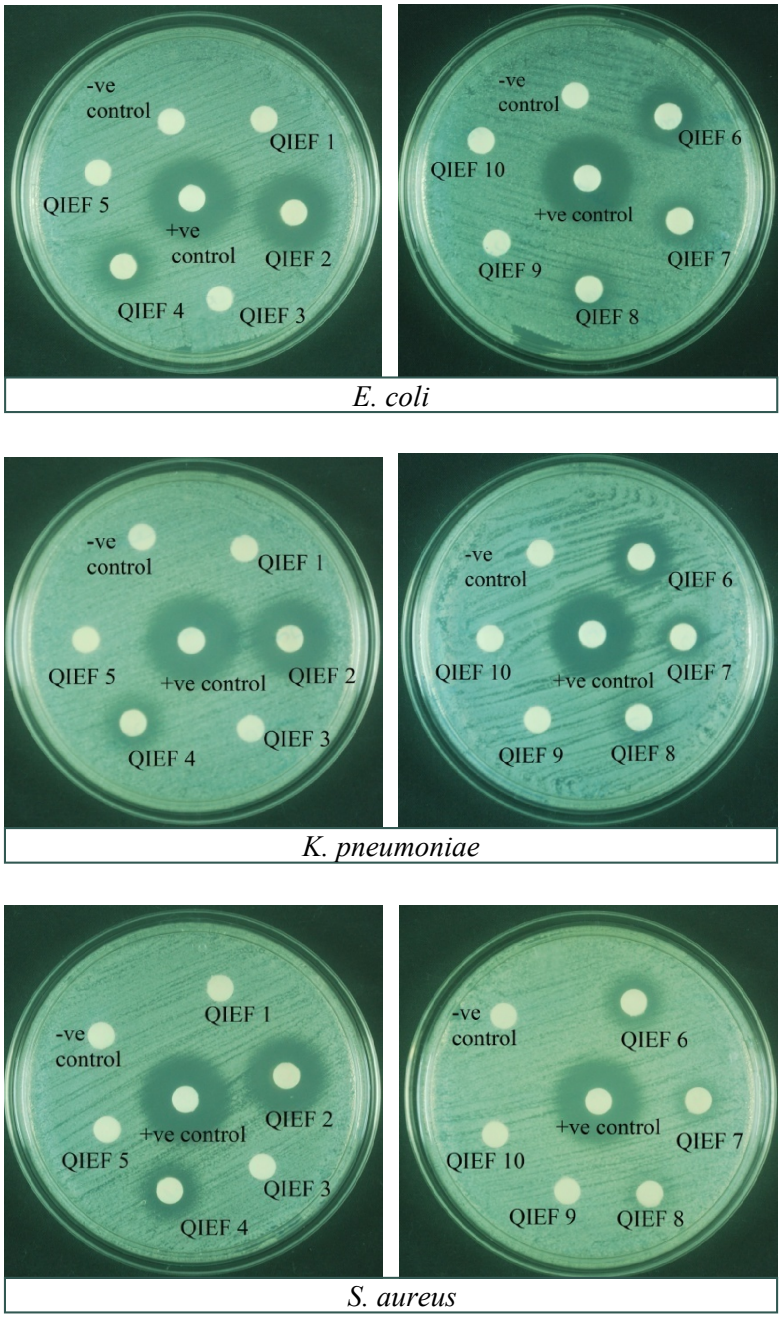
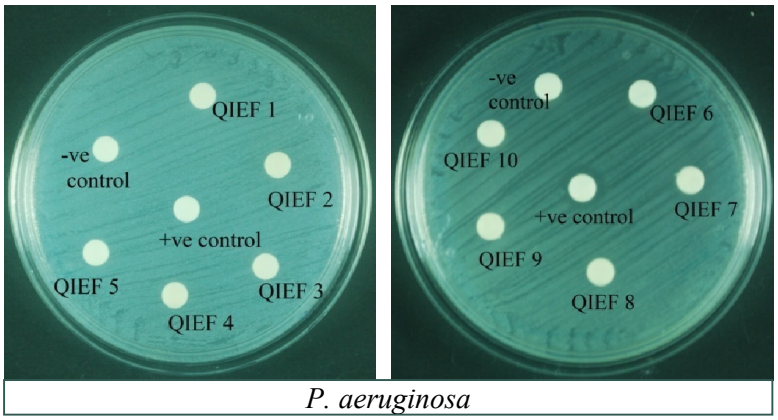
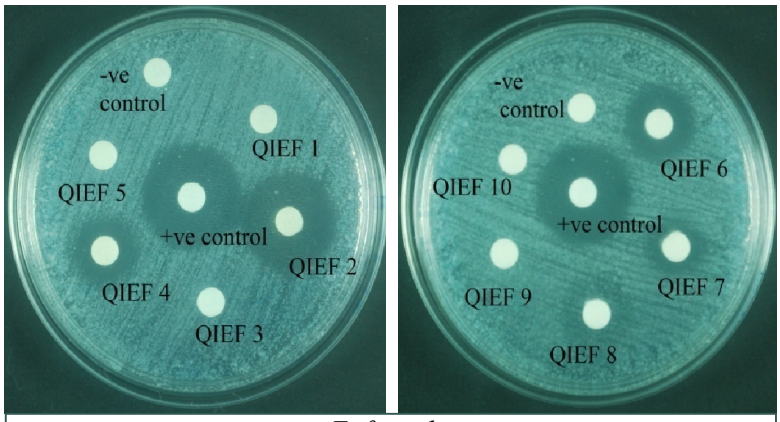


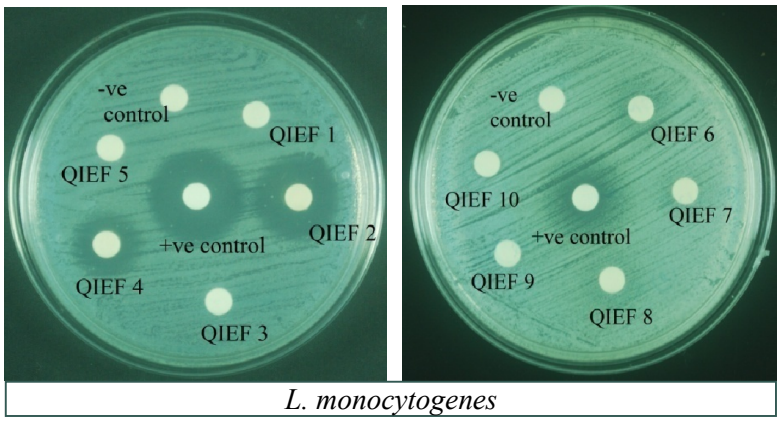
Figure 4.13 (i). Antibacterial activity of extracts of endophytic fungi isolated from *Quassia indica*. Positive control (Chloramphenicol)- 20µg/Disc, fungal extracts- 100µg/Disc and negative control- DMSO.



P. aeruginosa



E. faecalis



L. monocytogenes

Figure 4.13 (ii). Antibacterial activity of extracts of endophytic fungi isolated from *Quassia indica*. Positive control (Chloramphenicol)-20µg/ Disc, fungal extracts- 100µg/Disc and negative control- DMSO.

Table 4.9. Antibacterial activity screening of endophytic fungi isolated from *Quassia indica*

		Diameter of zone of inhibition (mm)					
Sl. No	**Fungal isolate**	***E. coli***	***K. Pneumoniae***	***S. aureus***	***P. aeruginosa***	***L. monocytogenes***	***E. faecalis***
1	QIEF 1	_	_	_	_	_	_
2	QIEF 2	**15.33 ±0.57**	**15.33±0.57**	**15.33±0.57**	_	**14.33 ± 0.57**	**16**
3	QIEF 3	10.66 ±0.57	10.33±0.57	10.33±0.57	_	11	10
4	QIEF 4	_	_	_	_	_	_
5	QIEF 5	_	_	_	_	_	_
6	QIEF 6	11.33 ±0.57	11.66±0.57	11.33±0.57	_	_	13
7	QIEF 7	9	9	9	_	_	10
8	QIEF 8	_	_	_	_	_	_
9	QIEF 9	_	_	_	_	_	_
10	QIEF 10	_	_	_	_	_	_
11	$^{+}$ve control	19	19.66±0.57	20.33±0.57	7	17.33 ± 2.51	19.33±0.57
12	$^{-}$ve control	–	–	–	–	–	–

Diameter of zone of inhibition in mm ± SD (n=3). '–' = No zone of inhibition.

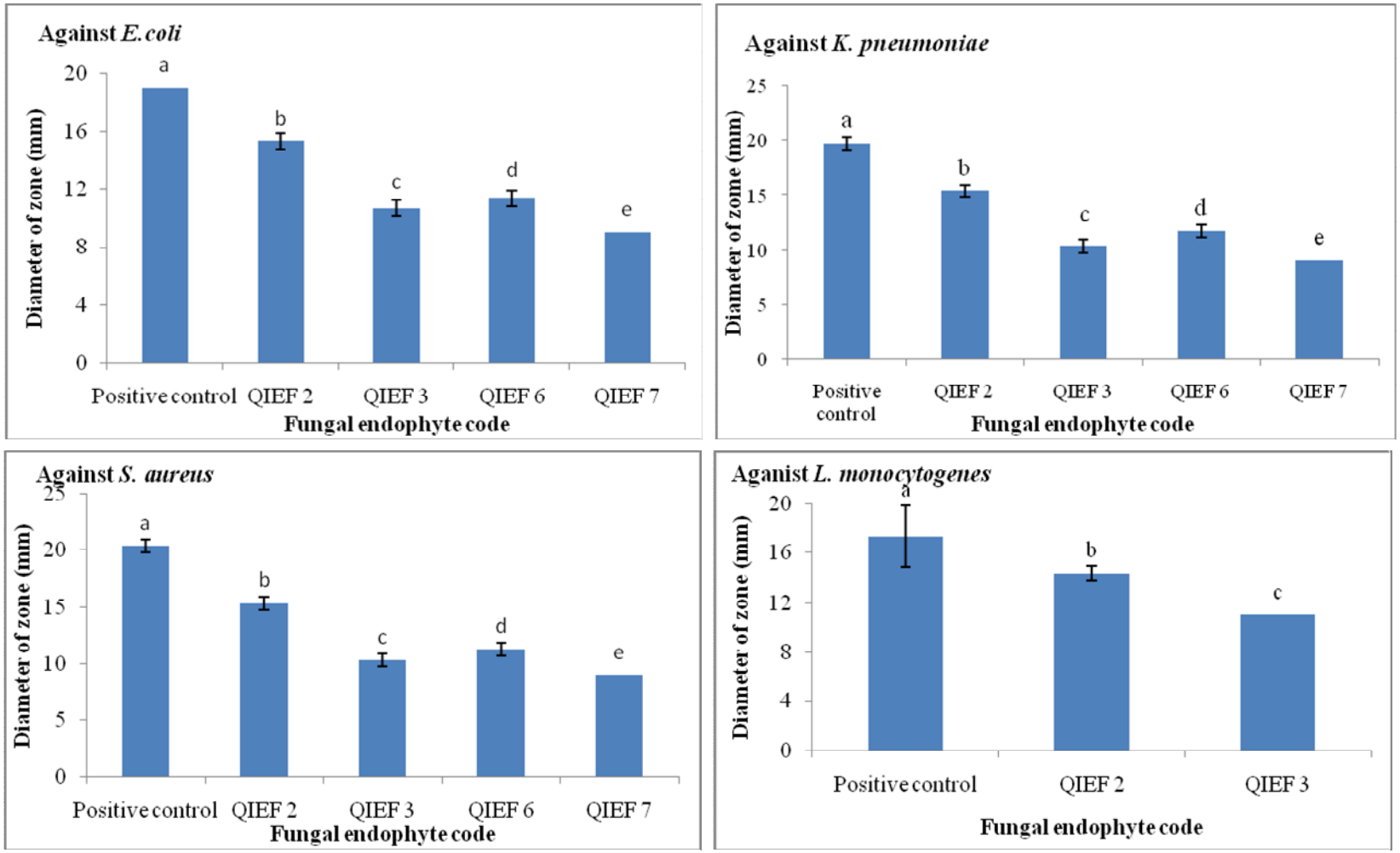


Figure 4.14 (i). Antibacterial activity of extracts of endophytic fungi from *Quassia inidica* against the tested bacteria. Diameter zone of inhibition in mm ± SD, (n=3). Distinct letters on bars indicate that mean values have significant differences ($P< 0.01$).

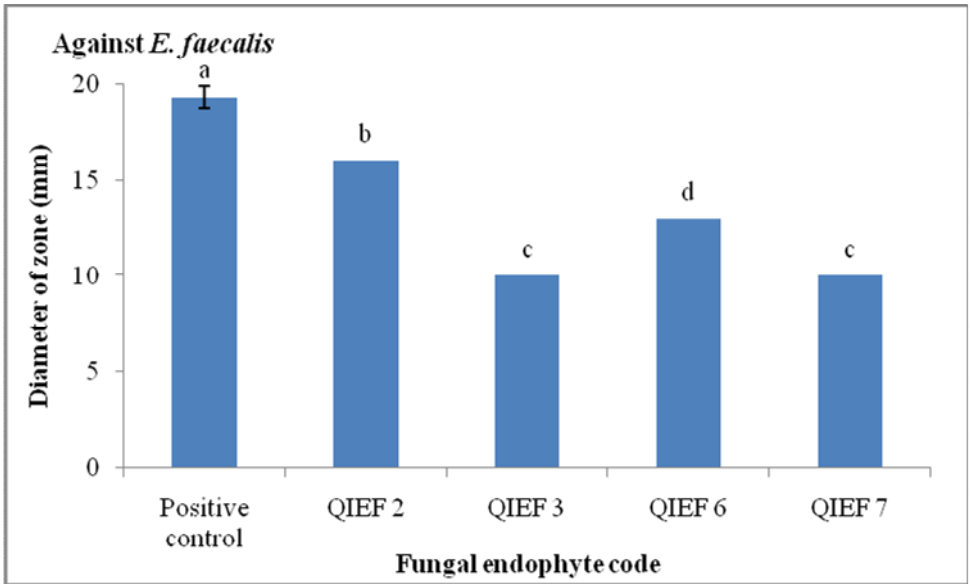


Figure 4.14 (ii). Antibacterial activity extratcs of endophytic fungi from *Quassia indica* against the tested bacteria. Diameter zone of inhibition in mm ± SD, (n=3). Letters on error bars indicate that mean values have significant differences ($P< 0.01$).

4.2.3. Cytotoxic activity of extracts of endophytic fungi from *Elaeocarpus sphaericus* and *Quassia indica*

All the endophytes of *Elaeocarpus sphaericus* and *Quassia indica* were screened for cytotoxic activity using MTT assay on K562 cell line treated with various concentrations of the extracts (0 to 200µg/ml) for 48 hrs. The graph showing the percentage of viability with different concentrations is shown in figure 4.15 and the IC_{50} values are tabulated (Table 4.10). The isolates, ESEF 6 and ESEF 7 (*Xylaria* sp.) showed the IC_{50} values 7.8±0.18 µg/ml and 54.5±3.54 µg/ml. The IC_{50} value of ESEF 9 (*Nigrospora* sp.) was 84±2.83 µg/ml and ESEF 12 (*Pestalotiopsis* sp.) was 25.75±3.19 µg/ml from *Elaeocarpus sphaericus*. Among the isolates of *Quassia indica*, QIEF 1 (*Daldinia* sp.) and QIEF 4 (*Lecanicillium* sp.) showed IC_{50} values 140±3.19 µg/ml and 83.8±1.77 µg/ml respectively. Other extracts of fungal isolates showed no cytotoxic activity at given concentrations. The IC_{50} value of curcumin is 10.66±0.57 µg/ml.

Analysis of variance found that there are significant differences between the endophytic fungal extracts in cytotoxic activity on K562 cell line ($F= 773.41$; $P< 0.001$); but the comparison between the extracts of endophytic fungi showed that ESEF 9 and QIEF 4 had no significant differences in cytotoxic activity ($P> 0.05$).

Multiple comparison analysis (Dunnett t- test) showed that the fungal extract of ESEF 6 was not significantly different from the curcumin ($P > 0.05$), whereas all the other fungal extracts were significantly different from curcumin ($P < 0.001$) (Table 4.10).

Table 4.10. IC_{50} values of extracts of fungal endophyte from *Elaeocarpus sphaericus* and *Quassia indica* on K562 cell line

Sl. No	Fungal isolate	K562 Cell line (IC_{50} values in µg/ml)
1	ESEF 6	7.8 ± 0.18^{a}
2	ESEF 7	54.5 ± 3.54^{b}
3	ESEF 9	84 ± 2.83^{c}
4	ESEF 12	25.75 ± 3.19^{d}
5	QIEF 1	140 ± 3.19^{e}
6	QIEF 4	83.8 ± 1.77^{c}
7	Curcumin	10.66 ± 0.57^{a}

Same superscript letter (a-e) represent no significant difference ($P > 0.05$) between the IC_{50} values from different endophytic fungi and curcumin.

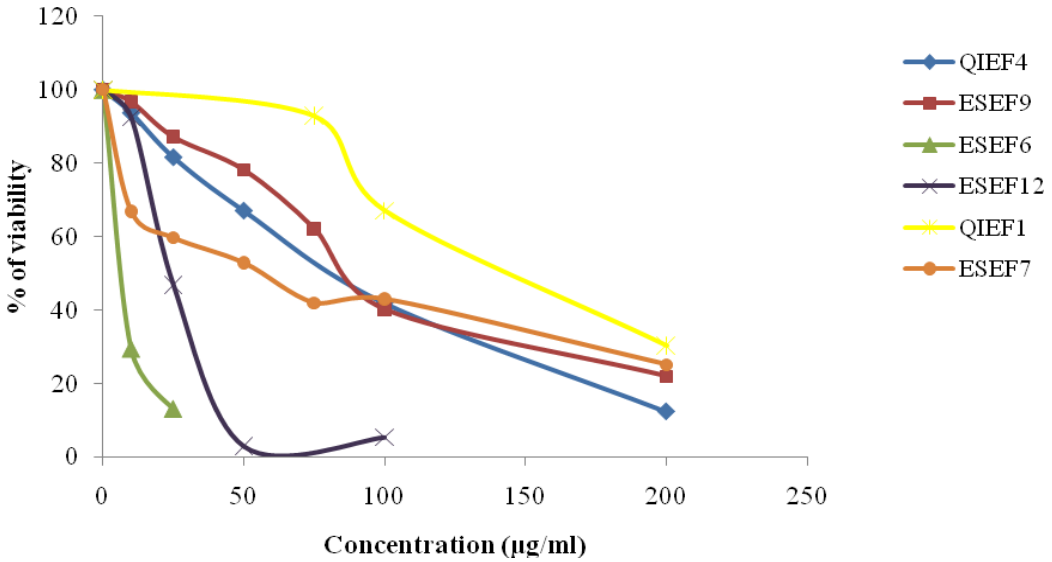


Figure 4.15. Cytotoxic activity of extracts of endophytic fungi from *Elaeocarpus sphaericus* and *Quassia indica*.

4.3. Characterization of active secondary metabolites produced by the selected endophytic fungus and their antibacterial and cytotoxic activities

ESEF 6 (*Xylaria* sp.) extract has comparatively good antibacterial and cytotoxic activities. The fungus was selected for further isolation and characterization of metabolites produced and their antibacterial and cytotoxic activities.

4.3.1. Fermentation and extraction of ESEF 6

The endophytic fungus ESEF 6 (*Xylaria* sp.) was grown in PDB and incubated at room temperature for 21 to 28 days; the broth and mycelia were separated by filtration. The mycelium was dried and powdered, sequentially extracted with solvents hexane, ethyl acetate and methanol. Broth was extracted three times with equal volume of ethyl acetate and the extracted sample was evaporated for the broth extract. Different fungal extracts were designated as mycelia hexane (MH), mycelia ethyl acetate (MEA), mycelia methanol (MM) and broth ethyl acetate (BEA) extracts. The percentage yield of fungal extracts is displayed in table 4.11.

Table 4.11. Extractive values of different solvents

Solvent extracts	Percentage yield
Mycelia Hexane (MH)	0.70
Mycelia Ethyl acetate (MEA)	9.72
Mycelia Methanol (MM)	4.39
Broth Ethyl acetate (BEA)	2.36

4.3.2. Antibacterial activity of solvent extracts of ESEF 6

Antibacterial activities of the solvent extracts of ESEF 6 were tested against the selected gram positive and gram negative bacteria by disc diffusion method (Figure 4.16). Out of the four solvent extracts, BEA extract showed zone of inhibitions against *E. coli*, *K. pneumoniae*, *S. aureus*, *L. monocytogenes* and *E. faecalis*. The zone of inhibition was 12.66±0.57 mm, 15 mm, 15 mm, 13.33±1.52 mm, and 13.66±1.15 mm

respectively. Remaining extracts showed no zone of inhibition against the organisms tested. The zone of inhibition in mm ± SD is given in table 4.12. Based on the data obtained after antibacterial activity, BEA extract was selected for further characterization. BEA extract exhibited significant difference in antibacterial activity while comparing with the control ($P< 0.05$) (Table 4.12). Analysis of variance showed significant differences in antibacterial activity between the microorganisms in BEA extract ($F= 23.67$, $P< 0.001$).

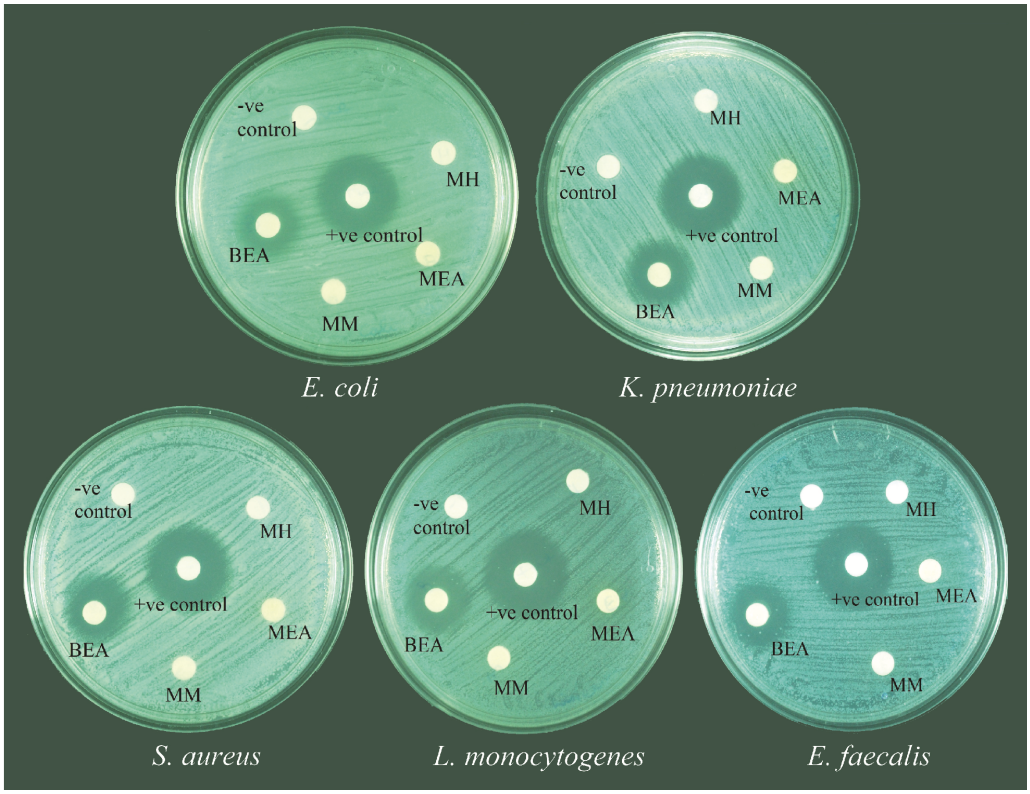


Figure 4.16. Antibacterial activity of different solvent extracts of ESEF 6. MH-Mycelia hexane extract, MEA- Mycelia ethyl acetate extract, MM- Mycelia methanol extract, BEA-Broth ethyl acetate extract. Positive control (Chloramphenicol)- 20µg/Disc, solvent extracts-100µg/Disc and negative control- DMSO.

Table 4.12. Antibacterial activity of solvent extracts of ESEF 6

		Diameter of zone of inhibition (mm)				
Sl. No	**ESEF 6 Extracts**	***E. coli***	***K. pneumoniae***	***S. aureus***	***L. monocytogenes***	***E. faecalis***
1	MH	–	–	–	–	–
2	MEA	–	–	–	–	–
3	MM	–	–	–	–	–
4	**BEA**	**12.66±0.57**[a]	**15** [a]	**15** [a]	**13.33 ±1.52** [a]	**13.66±1.15**[a]
5	[+]ve control	20 [b]	20 [b]	20.33±0.57[b]	18 ± 1.73 [b]	20 [b]
6	[-]ve control	–	–	–	–	–

Diameter of zone of inhibition in mm ± SD (n=3), '–' = No zone of inhibition. Different superscript letter (a and b) indicates significant difference ($P< 0.05$) between zone of inhibition from different endophytic fungi against bacteria (same column).

4.3.3. Cytotoxic activity of solvent extracts of ESEF 6

The cytotoxic activity of the solvent extracts of ESEF 6 on K562, HCT 116 and L929 cell lines were evaluated by MTT assay. The cells were treated with different concentrations of solvent extracts from 0 to 10 µg/ml for 48 hrs. The percentage of viability with different concentrations is shown in figure 4.17 and the IC_{50} values are tabulated (Table 4.13). Mycelia hexane (MH) and mycelia ethyl acetate (MEA) extracts showed the IC_{50} values 8.5±1.32 µg/ml and 3.5±0.08 µg/ml on K562 cell line whereas on HCT 116 cells, the IC_{50} values are 5.5±1.5 µg/ml and 2.65±0.70 µg/ml respectively. Both the extracts were non toxic to L929 cell lines at the concentrations tested. The other solvent extracts showed no cytotoxic activity at given concentrations. The IC_{50} values of curcumin are 12.5±0.08 µg/ml, 21.83±1.60 µg/ml and 10±0.003 µg/ml for K562, HCT116 and L929 cell lines respectively. From these results, MEA extract was selected for further characterization owing to its least IC_{50} values.

Independent t-test was employed to analyse the cytotoxic activity of mycelial hexane and mycelial ethyl acetate extracts in different cell lines. Compared to control, MH extracts have significant difference in cytotoxic activity on HCT 116 cell line ($P< 0.001$) and had no significant difference on K562 cell line ($P> 0.05$). MEA extracts had significant difference in cytotoxic activity on both K562 and HCT 116 cell lines ($P< 0.05$). While comparing between the fungal extracts, the significant

difference in cytotoxic activity was found on K562 ($P< 0.05$) and HCT 116 ($P< 0.05$) cell lines (Table 4.13). MEA extract has significantly lower IC_{50} values which indicate that the extract has more cytotoxic activity while comparing with MH and curcumin.

Table 4.13. IC_{50} values of MH and MEA extracts on K562, HCT 116 and L929 cell lines

Solvent extracts	Cell line (IC_{50} values in µg/ml)		
	K562	HCT 116	L929
MH	8.5 ± 1.32^{a}	5.5 ± 1.5^{a}	Non toxic
MEA	3.5 ± 0.08^{b}	2.65 ± 0.70^{b}	Non toxic
Curcumin	12.5 ± 0.08^{a}	21.83 ± 1.60^{c}	10 ± 0.003

Same superscript letter (a-c) indicates no significant difference ($P> 0.05$) between the IC_{50} values from different fungal extracts and curcumin (same column).

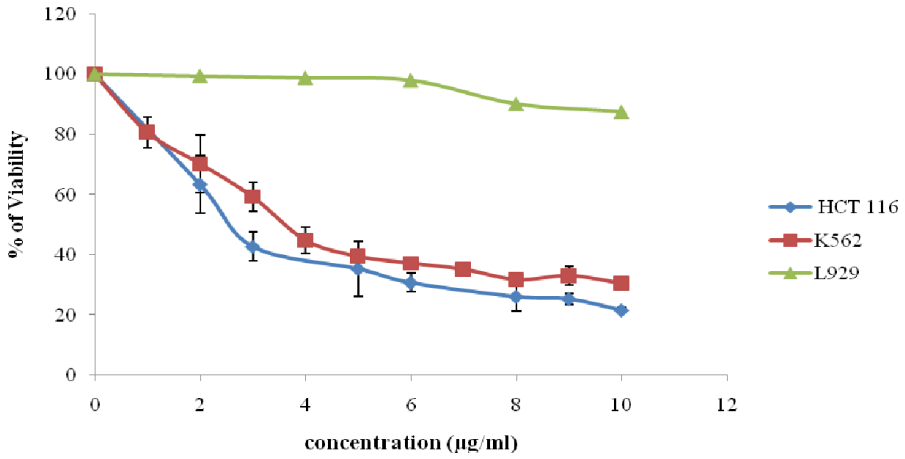


Figure 4.17. Cytotoxic activity of mycelia ethyl acetate (MEA) extract on K562, HCT 116 and L929 cell lines. Data represented as mean ± SD, (n=3).

4.3.4. Total phenolic content and antioxidant activities of solvent extracts of ESEF 6

Based on antibacterial and cytotoxic activities MEA and BEA extracts were used for further analysis. Both the extracts were screened for the total phenolic content and antioxidant activities. DPPH radical scavenging activity, reducing power assay and total antioxidant capacity by phosphomolybdenum methods were used to detect the antioxidant activities.

4.3.4.1. Determination of total phenolic content

The total phenol content in MEA and BEA extracts of QIEF 6 was determined using gallic acid as the standard. It was calculated as 9.8±1.2 and 99.48±6.1 mg Gallic acid equivalent (GAE)/g dry weight of the extract for MEA and BEA extracts respectively (Table 4.14).

Table 4.14. Total phenolic content of solvent extracts

Sample	Total Phenolic Content (mg GAE/g dry weight of the extract)
MEA extract	9.8 ± 1.2
BEA extract	99.48 ± 6.1

4.3.4.2. DPPH free radical scavenging activity

MEA and BEA extracts showed increasing scavenging effect with increasing concentrations. The BEA was equally potent as the standard ascorbic acid at a concentration of 1 mg/ml. Similarly, MEA also showed significant and moderate activity compared to the BEA and the standard. The neutralising activity exhibited by these extracts against the DPPH radical reveals that these extracts possess substances with hydrogen donating ability and the endophytic isolate secretes them in to the media during its growth. The comparison of the activities of both the extracts with the standard is depicted in figure 4.18.

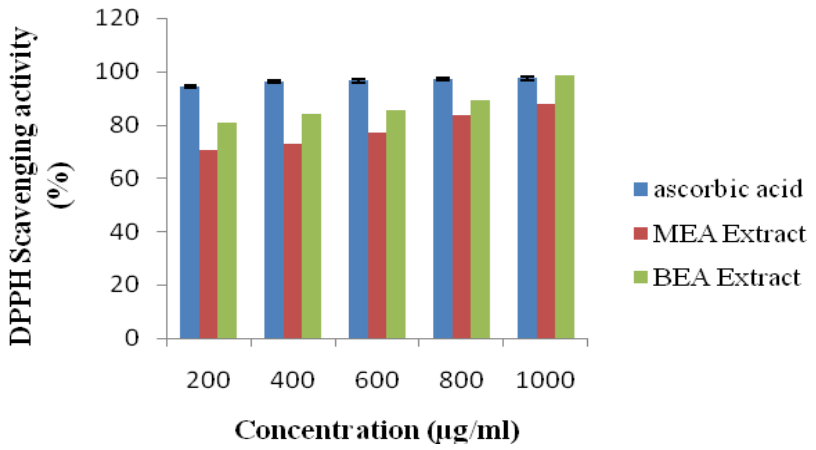


Figure 4.18. DPPH radical scavening activity of MEA and BEA extracts. Data represented as mean ± SD, (n=3).

4.3.4.3. Reducing power assay

Reducing power of the ethyl acetate extracts MEA and BEA was moderate and found to increase with increasing concentrations. The activity of MEA at a concentration of 500 µg/ml was comparable to the activity of the standard at 10 µg/ml whereas the activity of BEA at the same concentration (500 µg/ml) is greater than MEA and is as potent as ascorbic acid at 25 µg/ml. The comparison of the activities of both the extracts with the standard is depicted in figure 4.19.

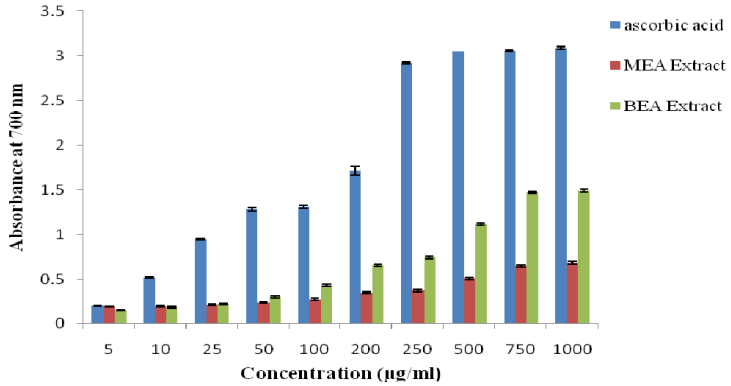


Figure 4.19. Reducing power activity of MEA and BEA extracts. Data represented as mean ± SD, (n=3).

4.3.4.4. Total antioxidant capacity (phosphomolybdenum method)

Total antioxidant activity of solvent extracts was measured by phosphomolybdenum assay. The extracts showed mild activity compared to the standard ascorbic acid. However BEA exhibited almost double the activity of MEA at all the concentrations tested. Comparison of activities of both the extracts with the standard is depicted in figure 4.20.

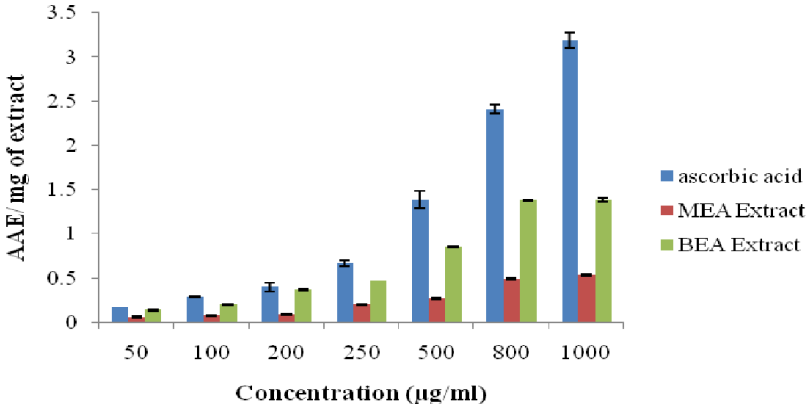


Figure 4.20. Total antioxidant activity of MEA and BEA extracts. Data represented as mean ± SD, (n=3).

4.3.5. Qualitative phytochemical analysis of solvent extracts of ESEF6

The preliminary screening of metabolites from the solvent extracts of ESEF 6 displayed in table 4.15. Solvent extracts of ESEF 6 showed the presence of phenolics, carbohydrates and steroids in MEA extract whereas the presence of flavonoids and carbohydrates were observed in BEA extracts.

Table 4.15. Phytochemical screening of the solvent extracts

Phytochemical tests	MEA Extract	BEA Extract
Alkaloids	−	−
Flavonoids	−	+
Phenol	+	−
Saponins	−	−
Tannin	−	−
Terpenoids	−	−
Glycosides	−	−
Carbohydrates	+	+
Steroids	+	−

+ = Positive Result, - = Negative Result

4.3.6. Thin layer chromatogram of solvent extracts of ESEF 6

The thin layer chromatogram of all the solvent extracts separated by using chloroform: methanol (7:3) as mobile phase was observed under UV at 254 nm and 365 nm (Figure 4.21). The Rf values of MEA and BEA extracts are given in table 4.16. The MEA extract showed 5 bands observed under UV at 254 nm and 6 bands at 365 nm. Whereas BEA extract showed 4 bands at 254 nm and 6 bands at 365 nm.

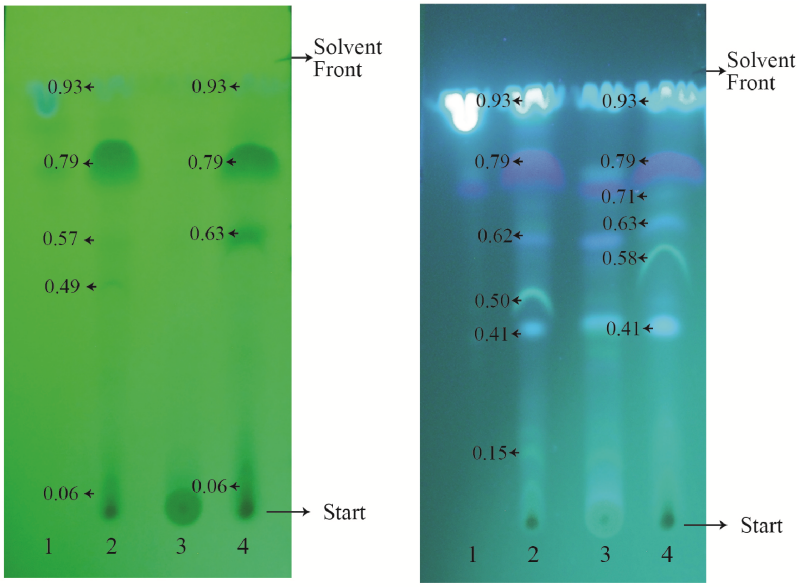


Figure 4.21. Thin layer chromatogram of different solvent extracts of ESEF 6. 1-Mycelia hexane (MH) extract, 2 Mycelia ethyl acetate (MEA) extract, 3-Mycelia methanol (MM) extract, 4- Broth ethyl acetate (BEA) extract observed under UV (A) at 254 nm and (B) at 365 nm.

Table 4.16. Rf values of mycelia ethyl acetate and broth ethyl acetate extracts

MEA Solvent extract		**BEA solvent extract**	
Rf 254 nm	**Rf 365 nm**	**Rf 254 nm**	**Rf 365 nm**
0.06	0.15	0.06	0.41
0.49	0.41	0.63	0.58
0.57	0.50	0.79	0.63
0.79	0.62	0.93	0.71
0.93	0.79		0.79
	0.93		0.93

Presence of major types of metabolites in the solvent extracts was analysed by TLC spraying techniques. The extracts showed positive to fast blue salt (FBS) reagent at an Rf value of 0.8, which revealed the presence of phenolic compounds in precoated silica gel 60 F_{254} (Merck). Anisaldehyde sulphuric acid spray detected a band at Rf 0.45, showing the presence of terpenoids/saponins/carbohydrates/steroids in manually prepared TLC plate. Thin layer chromatogram after reagent spraying is shown in figure 4.22.

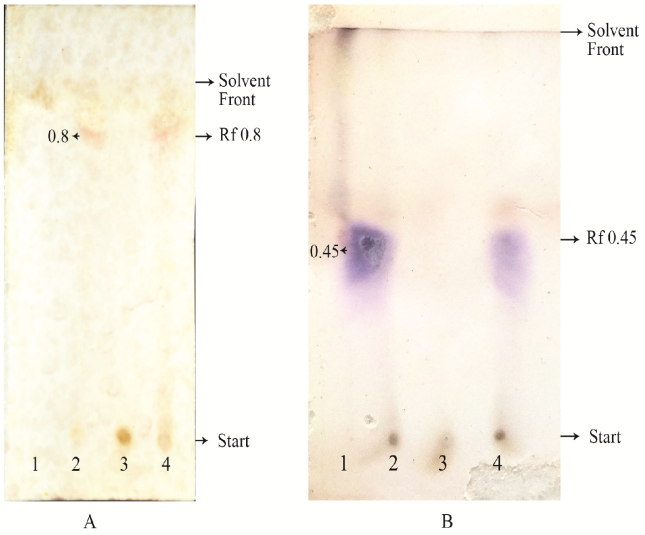


Figure 4.22. Thin layer chromatogram of solvent extracts of ESEF 6 sprayed with FBS and ANS. (A) Sprayed with FBS in precoated silica gel 60 F_{254}, (B) sprayed with anisaldehyde sulphuric acid reagent in manually prepared TLC plate. 1- hexane (MH) extract, 2- Myceliaethyl acetate (MEA) extract, 3-Mycelia methanol (MM) extract, 4- Broth ethyl acetate (BEA) extract observed under visible light.

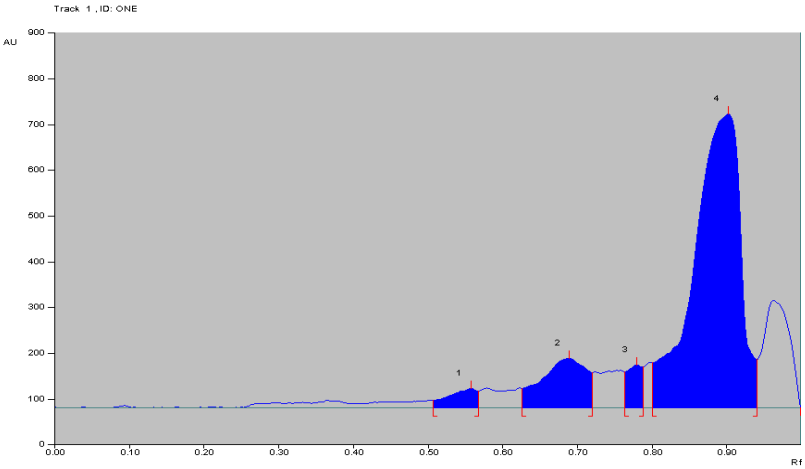
4.3.7. HPTLC profile of solvent extracts of ESEF 6

Qualitative evaluation of MEA and BEA extracts was performed by using high performance thin layer chromatography. The parameters such as number of bands and their corresponding peaks observed under both visible light (550 nm) and UV (at 254 nm and 366 nm) are tabulated (Table 4.17).

Table 4.17. HPTLC profile of MEA and BEA extracts

Sample	Under 550 nm			Under 254 nm			Under 366 nm		
	No. of Band	Rf value	Area (%)	No. of Band	Rf value	Area (%)	No. of Band	Rf value	Area (%)
MEA Extract	4	0.51, 0.63, 0.76, 0.80	3.24, 13.00, 3.76, 80.00	7	-0.06, -0.02, 0.23, 0.38, 0.42, 0.48, 0.59	2.37, 55.73, 4.71, 2.29, 5.37, 10.43, 19.10	5	-0.02, 0.22, 0.31, 0.52, 0.69	73.61, 4.81, 11.82, 7.12, 2.65
BEA Extract	1	0.86	100	4	-0.06, -0.01, 0.05, 0.55	4.26, 61.76, 5.98, 28	2	-0.01, 0.63	89.87, 10.13

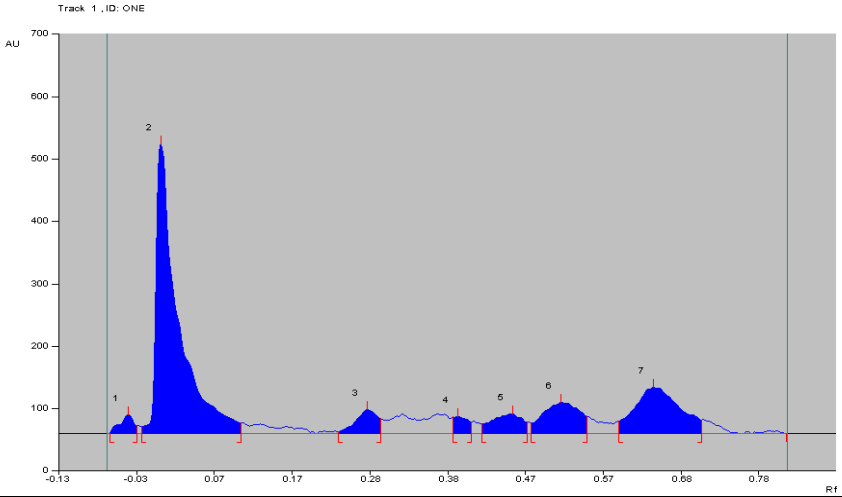
HPTLC profile of MEA extract showed 4 peaks under visible light; the number of peaks, the corresponding Rf and area % is shown in figure 4.23.



Peak	Start Rf	Start height	Maximum Rf	Maximum Height	Maximum %	End Rf	End Height	Area %
1	0.51	15.7	0.56	42.4	4.78	0.57	36.4	3.24
2	0.63	42.6	0.69	108.4	12.23	0.72	77.4	13.0
3	0.76	78.9	0.78	94.0	10.6	0.79	38.9	3.76
4	0.8	98.8	0.9	642	72.4	0.94		80.0

Figure 4.23. HPTLC profile and peak table of MEA extract observed under visible light.

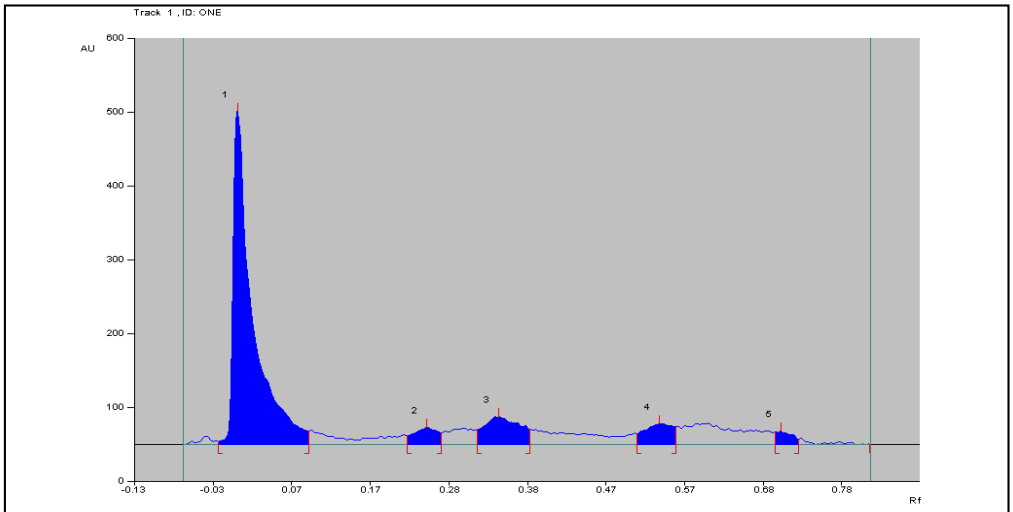
HPTLC profile of MEA extract showed 7 peaks under UV at 254 nm; the number of peaks, the corresponding Rf and area % is shown in figure 4.24.



Peak	Start Rf	Start height	Maximum Rf	Maximum Height	Maximum %	End Rf	End height	Area %
1	-0.06	0.6	-0.04	29.9	4.19	-0.02	12.4	2.37
2	-0.02	10.8	0.01	464.5	64.96	0.11	16.4	55.73
3	0.23	3.2	0.27	38.5	5.39	0.29	23.8	4.71
4	0.38	26.4	0.39	27.0	3.78	0.41	18.5	2.29
5	0.42	15.0	0.46	31.2	4.37	0.48	18.7	5.37
6	0.48	16.9	0.52	49.9	6.98	0.56	27.2	10.43
7	0.59	19.7	0.64	73.9	10.34	0.7	21.6	19.1

Figure 4.24. HPTLC profile and peak table of MEA extract observed under UV at 254 nm.

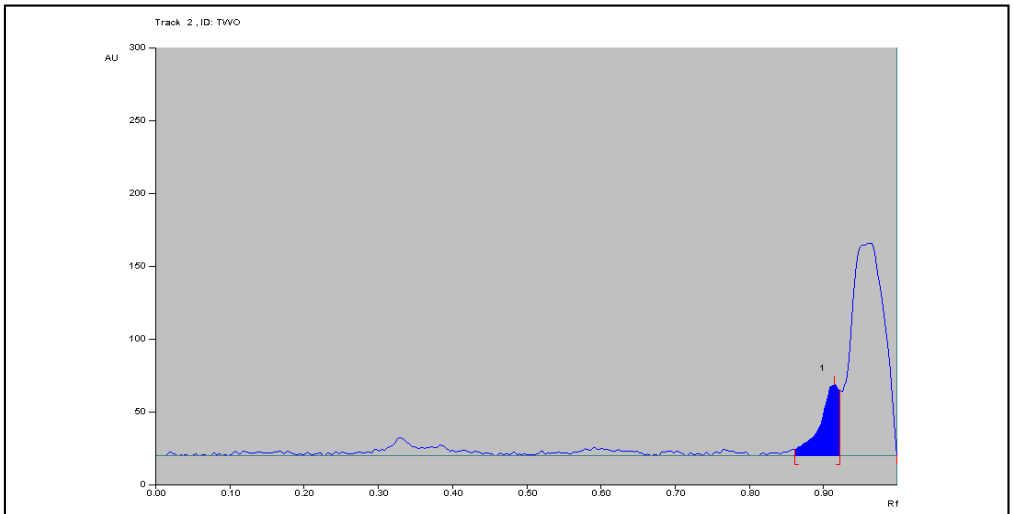
HPTLC profile of MEA extract showed 5 peaks observed under UV at 366 nm; the number of peaks, the corresponding Rf and area % is shown in figure 4.25.



Peak	Start Rf	Start height	Maximum Rf	Maximum Height	Maximum %	End Rf	End height	Area %
1	-0.02	4.3	0.01	451.1	80.78	0.1	18.1	73.61
2	0.22	12.0	0.25	23.3	4.17	0.27	16.2	4.81
3	0.31	20.4	0.34	37.5	6.71	0.38	20.2	11.82
4	0.52	14.3	0.54	28.6	5.12	0.56	24.4	7.12
5	0.69	17.6	0.7	18.0	3.22	0.72	7.7	2.65

Figure 4.25. HPTLC profile and peak table of MEA extract observed under UV at 366 nm.

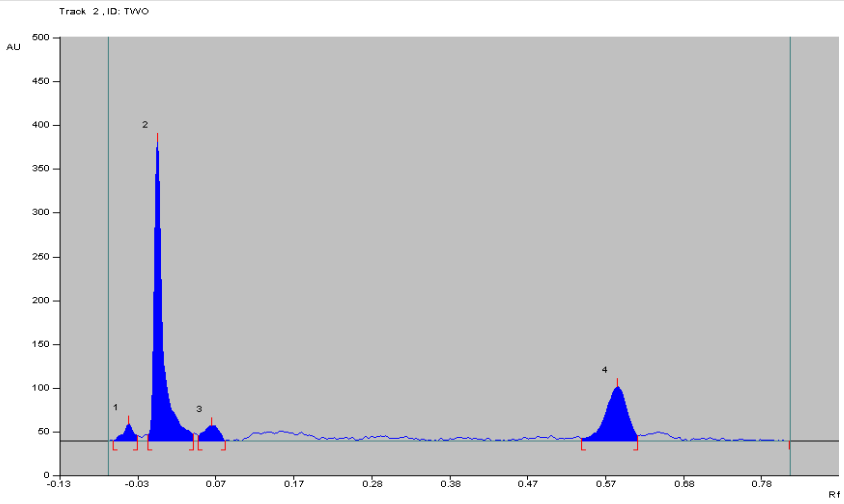
HPTLC profile of BEA extract showed only single peak observed under visible light; the number of peaks, the corresponding Rf and area % is shown in figure 4.26.



Peak	Start Rf	Start height	Maximum Rf	Maximum Height	Maximum %	End Rf	End height	Area %
1	0.86	4.3	0.92	48.7	100.0	0.92	45.0	100.0

Figure 4.26. HPTLC profile and peak table of BEA extract observed under visible light.

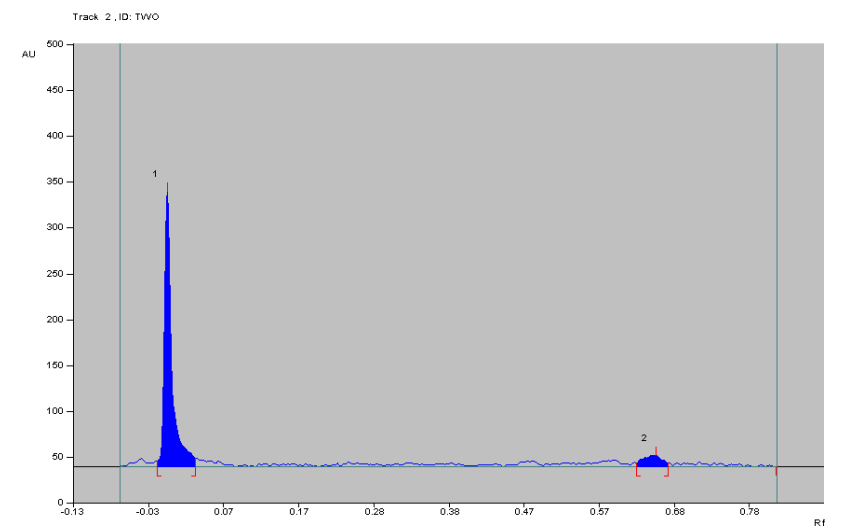
HPTLC profile of BEA extract showed 4 peaks observed under UV at 254 nm; the number of peaks, the corresponding Rf and area % is shown in figure 4.27.



Peak	Start Rf	Start height	Maximum Rf	Maximum Height	Maximum %	End Rf	End height	Area %
1	-0.06	0.5	-0.04	19.4	4.41	-0.03	6.3	4.26
2	-0.01	7.2	0.00	341.8	77.54	0.05	8.3	61.76
3	0.05	6.6	0.07	17.6	4.0	0.09	0.4	5.98
4	0.55	2.6	0.59	61.9	14.05	0.62	6.0	28.0

Figure 4.27. HPTLC profile and peak table of BEA extract observed under UV at 254 nm.

The HPTLC profile of BEA extract showed 2 peaks observed under UV at 366 nm; the number of peaks, the corresponding Rf and area % is shown in figure 4.28.



Peak	Start Rf	Start height	Maximum Rf	Maximum Height	Maximum %	End Rf	End height	Area %
1	-0.01	6.4	0.00	299.6	95.94	0.04	9.6	89.87
2	0.63	3.7	0.65	12.7	4.06	0.67	4.3	10.13

Figure 4.28. HPTLC profile and peak table of BEA extract observed under UV at 366 nm.

4.3.8. Detection of metabolites in MEA and BEA extracts

The post chromatographic derivatization of MEA and BEA extracts was performed by using 2 % ethanolic ferric chloride ($FeCl_3$) and anisaldehyde reagents (ANS) on HPTLC plates for the detection of phenolics and terpenoids/saponins/ carbohydrates/steroids. After derivatization the brown colour band showed the presence of phenolics where as the violet colour band displayed the presence of terpenoids/steroids/saponins/carbohydrates. The bands were also visualized under UV at 254 nm and 366 nm (Figure 4.29 and Figure 4.30).

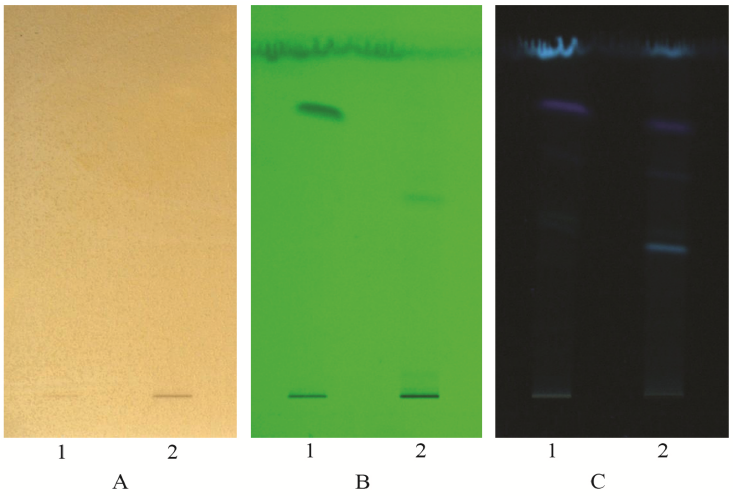


Figure 4.29. Post chromatographic derivatization of MEA and BEA extracts with 2% ethanolic ferric chloride. Mobile phase- chloroform:methanol (7:3) observed under (A) visible light, (B) UV at 254 nm and (C) UV at 366 nm, 1- MEA extract, 2- BEA extract.

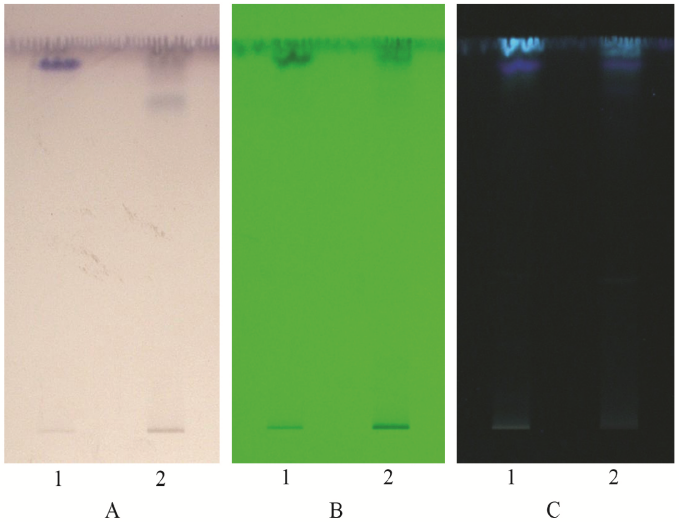


Figure 4.30. Post chromatographic derivatization of MEA and BEA extracts with anisaldehyde sulphuric acid. Mobile phase- chloroform:methanol (9:1) observed under (A) visible light, (B) UV at 254 nm and (C) UV at 366 nm, 1- MEA extract, 2- BEA extract.

4.3.9. Purification of secondary metabolites from ESEF 6 by column chromatography

Column chromatography of MEA and BEA extracts were separately performed on silica gel of 60-120 mesh. The fractions were eluted using chloroform: methanol for both the extracts in the following proportions from 100:0 to 0:100. 45 fractions with 20 ml in each fraction were collected with the flow rate of 2 ml/min. The fractions from MEA extracts were screened for cytotoxic activity by MTT assay on K562 and HCT 116 cell lines, antibacterial activity was screened from the fractions of BEA extracts against the gram positive and gram negative bacteria. Out of the 45 fractions from both the extracts, three fractions on chloroform:methanol (90:10) elution showed the cytotoxic activity while one fraction on chloroform: methanol (90:10) elution displayed antibacterial activity. The active fractions were analyzed on TLC and the bands were scraped out; dissolved in chloroform/ methanol. Further, the active band was scraped out from TLC plates to identify the metabolites. **The TLC scraped active band of the fraction was designated as partially purified active fraction.**

4.3.10. TLC profile of partially purified active fraction from MEA and BEA extracts

The TLC profile of partially purified active fraction from MEA and BEA extracts was developed using chloroform:methanol (7:3). Chromatogram of both the fractions showed a band at Rf value 0.83 under visible light. The presence of the metabolites in the fractions was analyzed by TLC spraying techniques. Anisaldehyde sulphuric acid spray showed a positive response to terpenoids/ saponins/steroids/carbohydrates at the band with Rf 0.83. The chromatogram is shown in figure 4.31.

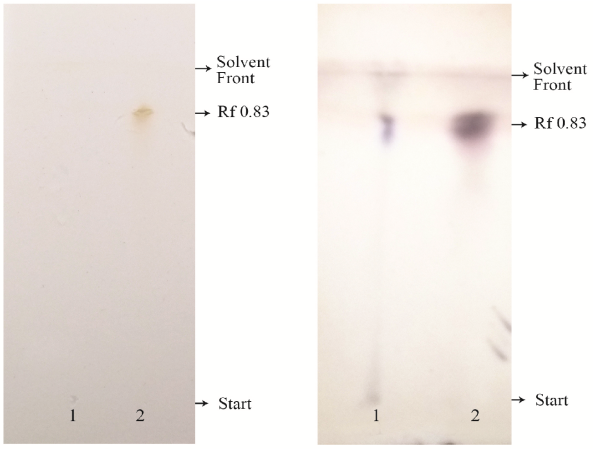


Figure 4.31. Thin layer chromatogram of partially purified active fraction of MEA and BEA extracts. The chromatogram observed under (A) visible light and (B) sprayed with anisaldehyde sulphuric acid, 1- partially purified active fraction of MEA extract, 2- partially purified active fraction of BEA extract.

4.3.11. Detection of metabolites of partially purified active fractions from MEA and BEA extracts

The post chromatographic derivatization of partially purified active fraction from MEA and BEA extracts was performed by using 2 % ethanolic ferric chloride ($FeCl_3$) and anisaldehyde reagents (ANS). The brown colour band showed the presence of phenolics where as the violet colour band displayed the presence of terpenoids/steroids/saponins/carbohydrates. The bands were also visualized under UV at 254 nm and 366 nm (Figure 4.32 and Figure 4.33).

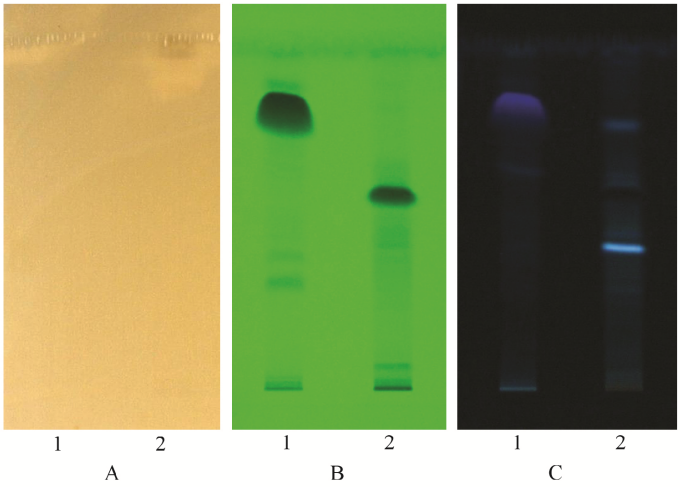


Figure 4.32. Post chromatographic derivatization of partially purified active fraction from MEA and BEA extracts with 2% ethanolic ferric chloride. Mobile phase-Chloroform:methanol (7:3), observed under (A) visible light, (B) UV at 254 nm and (C) UV at 366 nm, 1- MEA extract, 2- BEA extract.

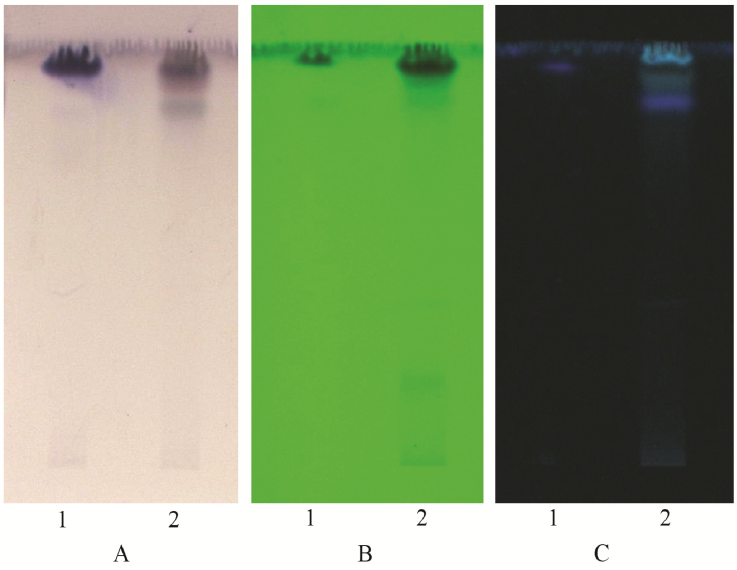
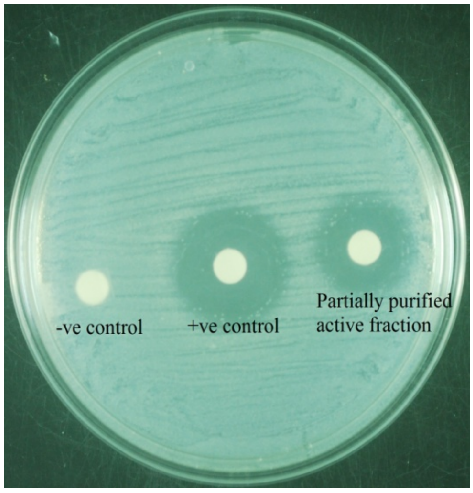


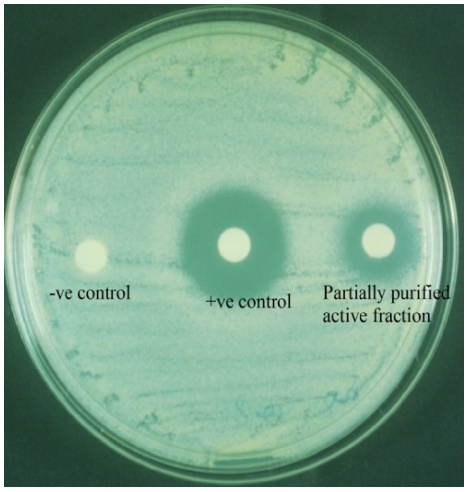
Figure 4.33. Post chromatographic derivatization of partially purified active fraction from MEA and BEA extracts with anisaldehyde sulphuric acid. Mobile phase- chloroform:methanol (9:1), observed under (A) visible light, (B) UV at 254 nm and (C) UV at 366 nm, 1- MEA extract, 2- BEA extract.

4.3.12. Antibacterial activity of partially purified active fraction from BEA extract

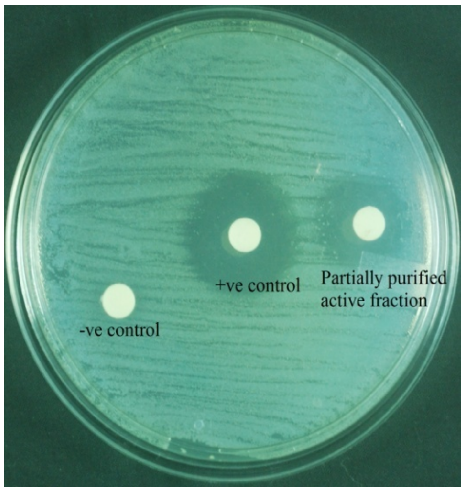
Antibacterial activities of the partially purified active fraction (scrapped out from TLC plates) from BEA extract was tested against the selected gram positive and gram negative bacteria by disc diffusion method (Figure 4.34). Partially purified active fraction showed zone of inhibitions against *K. pneumoniae*, *S. aureus*, *L. monocytogenes* and *E. faecalis*. The zone of inhibition was 18.66±0.57 mm, 13.33±0.57 mm, 14.66±0.57 mm, and 16.66±0.57 mm respectively. Zone of inhibition in mm ± SD is given in table 4.18. The partially purified active fraction from BEA extract showed significant difference in antibacterial activity in comparison with control ($P< 0.05$) (Table 4.18). Analysis of variance in the partially purified fractions exhibited significant variations in antibacterial activity between the microorganisms ($F= 39.00$; $P< 0.001$).



K. pneumoniae



S. aureus



L. monocytogenes

E. faecalis

Figure 4.34. Antibacterial activity of partially purified active fraction from BEA extract. Positive control (Chloramphenicol) - 20µg/Disc, partially purified active fraction- 100µg/Disc and negative control- DMSO.

Table 4.18. Antibacterial activity of partially purified active fraction from BEA extracts

Sl. No.		Diameter of zone of inhibition (mm)			
		K. pneumoniae	***S. aureus***	***L. monocytogenes***	***E. faecalis***
1	Partially purified active fraction	18.66 ± 0.57^{a}	13.33 ± 0.57^{a}	14.66 ± 0.57^{a}	16.66 ± 0.57^{a}
2	+ve control	20^{b}	20.33 ± 0.57^{b}	18 ± 1.73^{b}	20^{b}
3	-ve control	–	–	–	–

Diameter of zone of inhibition in mm ± SD, (n = 3). '-' = No zone of inhibition. Different superscript letter (a-b) indicates significant difference in zone of inhibition between the fraction and the control ($P < 0.05$) (same column).

4.3.13. Cytotoxic activity of the partially purified active fraction from MEA extract

Cytotoxic activity of partially purified active fraction on K562, HCT 116 and L929 cell lines were evaluated by MTT assay. The cells were treated with different concentrations of the partially purified active fraction (0 to 10 µg/ml) for 48 hrs. The percentage of viability with different concentrations is shown in figure 4.35 and the IC_{50} values are tabulated (Table 4.19). The active fraction showed the IC_{50} values 1.04±0.29 µg/ml, 3.65±0.83 µg/ml and 7.71±0.005 µg/ml on K562, HCT 116 and L929 cell lines respectively. Cytotoxic activity of the partially purified fractions was comparable with the standard curcumin. The IC_{50} values of curcumin are 10±1.03 µg/ml, 20±1.25 µg/ml and 10±0.003 µg/ml for K562, HCT 116 and L929 cell lines respectively. Partially purified active fraction from MEA extract showed significant difference in cytotoxic activity on K562, HCT 116 and L929 cell lines ($P< 0.01$) compared to curcumin (Table 4.19). The partially purified fraction showed significantly lower IC_{50} value compared to curcumin and that indicated that the fraction has more cytotoxic activity.

Table 4.19. IC_{50} values of partially purified active fraction from MEA extract on K562, HCT 116 and L929 cell lines

	Cell line (IC_{50} Values in µg/ml)		
	K562	**HCT 116**	**L929**
Partially purified active fraction	1.04 ± 0.29 [a]	3.65 ± 0.83 [a]	7.71 ± 0.005 [a]
Curcumin	10 ± 1.03 [b]	20 ± 1.25 [b]	10 ± 0.003 [b]

Different superscript letters (a-b) indicates the significant difference ($P< 0.01$) between IC_{50} values from partially purified active fraction and curcumin (Same column).

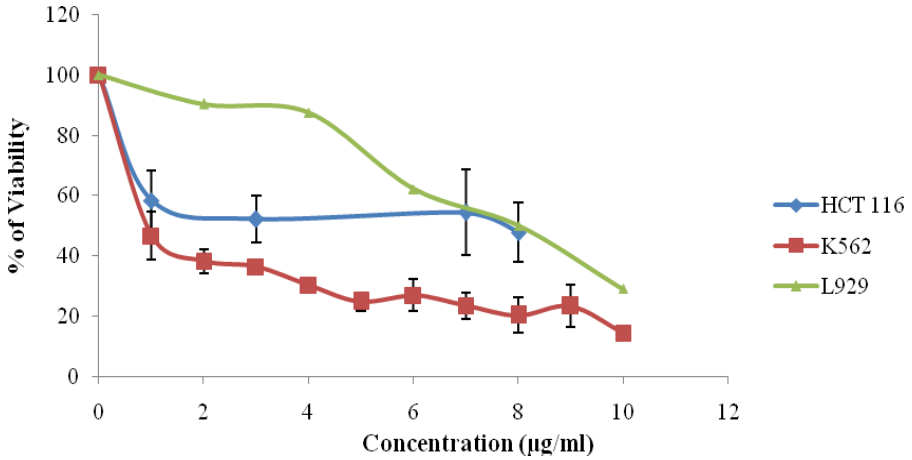


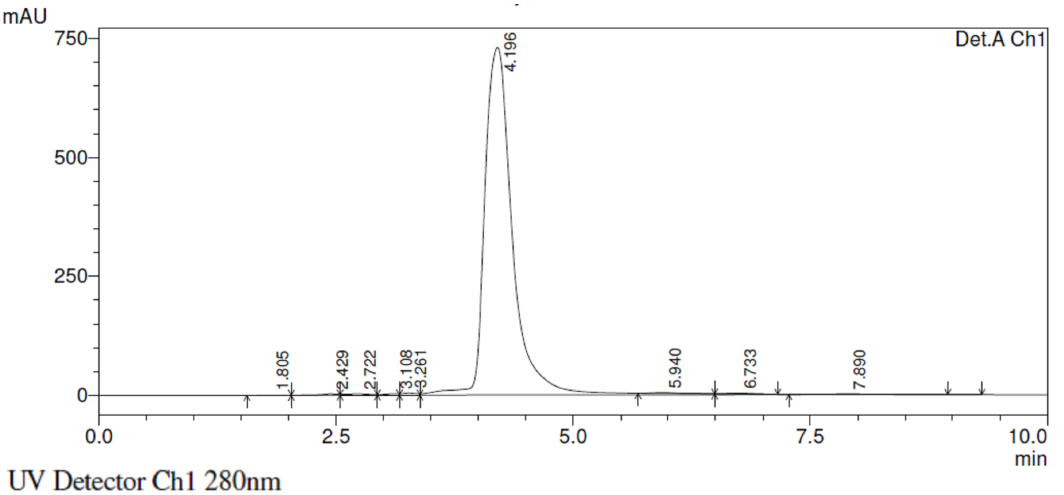
Figure 4.35. Cytotoxic activity of partially purified active fraction on K562, HCT 116 and L929 cell lines. Data represented as mean ± SD (n=3).

4.3.14. HPLC, HR-MS and HR LCMS/MS analysis of partially purified active fractions

Partially purified active fractions from BEA and MEA extracts were further characterized by using HPLC, HR-MS and HR-LCMS/MS analysis.

4.3.14.1. HPLC analysis

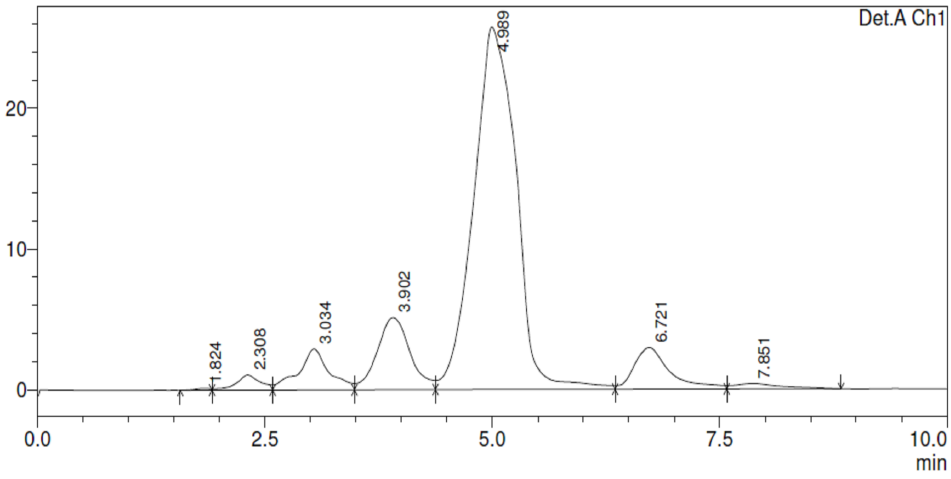
HPLC profile of BEA and MEA partially purified active fractions were generated by using methanol:water (60:40) as mobile phase. BEA and MEA fractions showed a prominent peak with retention time of 4.196 min and 4.989 min respectively. The number of peaks with retention time and area % of BEA and MEA active fractions is exhibit in figure 4.36 and figure 4.37.



UV Detector Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.805	4745	311	0.031	0.042
2	2.429	28518	2430	0.187	0.325
3	2.722	45157	2398	0.296	0.321
4	3.108	27990	2449	0.183	0.328
5	3.261	41685	3998	0.273	0.535
6	4.196	14999928	730557	98.227	97.824
7	5.940	50813	2116	0.333	0.283
8	6.733	32778	1584	0.215	0.212
9	7.890	38999	967	0.255	0.129
Total		15270612	746810	100.000	100.000

Figure 4.36. HPLC profile of partially purified antimicrobial active fraction from BEA extract. HPLC analysis was observed under 280 nm using methanol:water (60:40). The peak table with number of peaks, retention time and area percentage shown; the highest peak in the table was marked.



PeakTable

UV Detector Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.824	1564	127	0.136	0.332
2	2.308	19981	1075	1.738	2.805
3	3.034	68350	2904	5.947	7.576
4	3.902	129266	5120	11.247	13.356
5	4.989	835176	25760	72.664	67.197
6	6.721	81372	2959	7.080	7.718
7	7.851	13656	390	1.188	1.017
Total		1149366	38336	100.000	100.000

Figure 4.37. HPLC profile of partially purified cytotoxic active fraction from MEA extract. HPLC analysis was observed under 280 nm using methanol:water (60:40). The peak table with number of peaks, retention time and area percentage were shown; the highest peak in the table was marked.

4.3.14.2. HR-MS analysis

The HR-MS profile of BEA active fraction has peaks of m/z 217.069, 249.110, 274.999, 277.178, 279.194, 293.173, 295.189, 321.168, 323.184, 325. 199, 339.179, 357.188, and 395.24 with relative abundance of 11, 5, 13, 43, 100, 71, 50, 18, 69, 86, 65, 11 and 5 respectively (Figure 4.38). MEA active fraction showed the base peak with m/z of 453.22 of relative abundance 100. Other peaks with m/z of 153.127, 261.1125, 337.198, and 437.194 with relative abundance of 7, 12, 20 and 15 respectively (Figure 4.39).

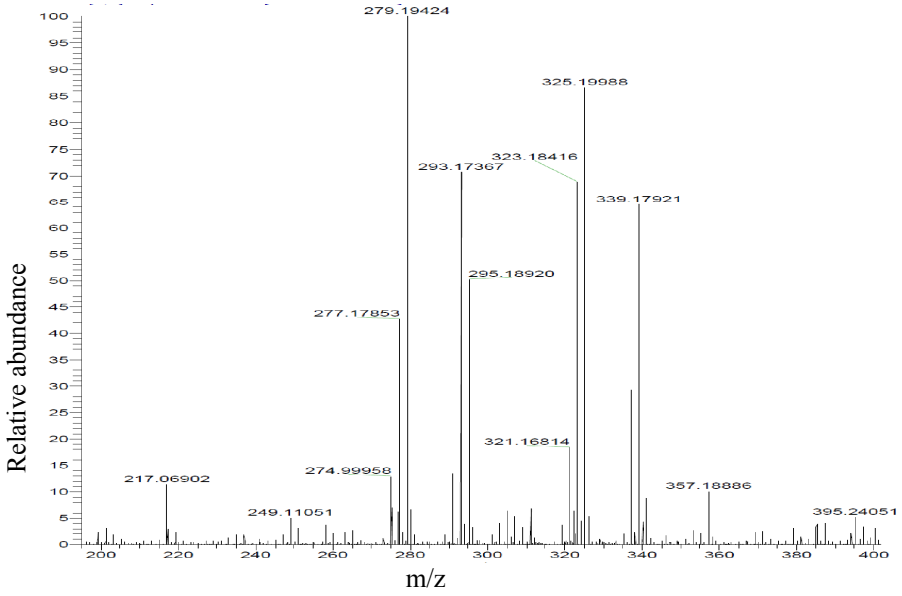


Figure 4.38. HR-MS analysis of partially purified antimicrobial active fraction from BEA extract.

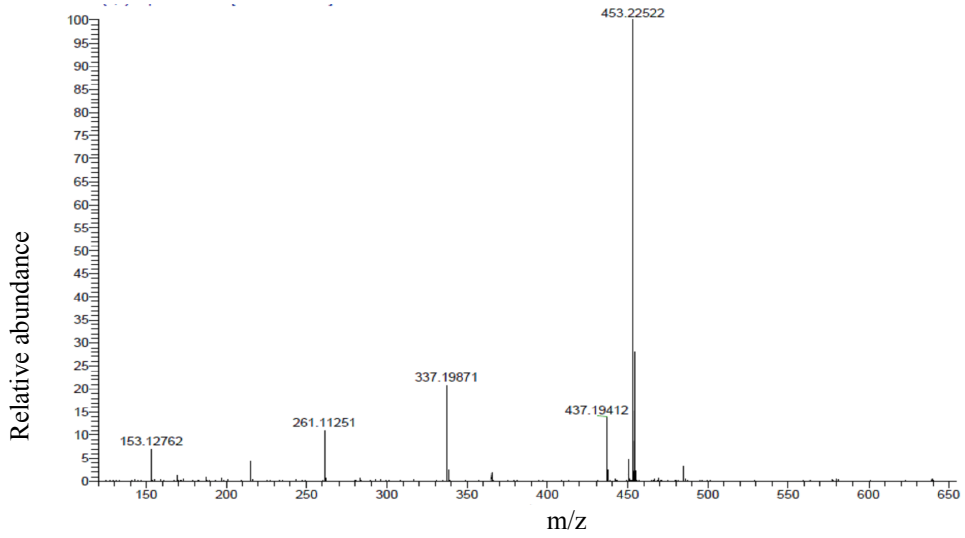


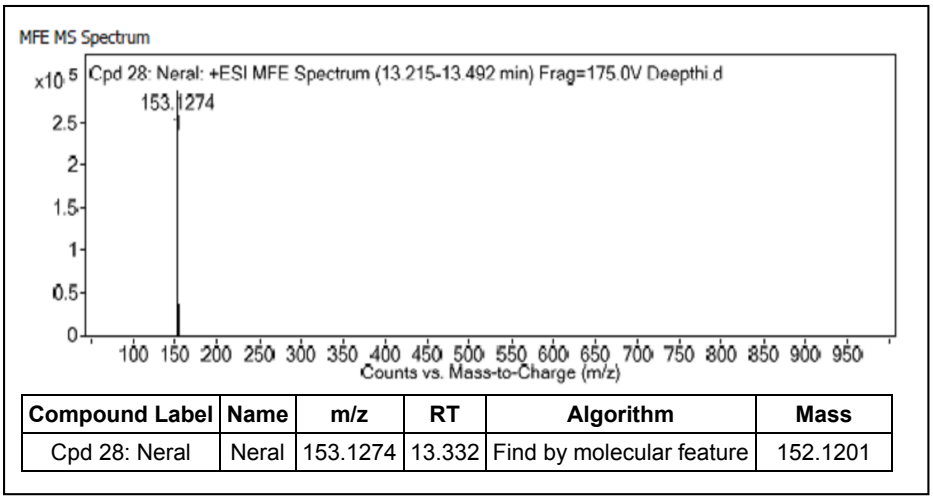
Figure 4.39. HR-MS analysis of partially purified cytotoxic active fraction from MEA extract.

4.3.14.3. HR-LCMS/MS analysis

HR-LCMS/MS analysis was performed for cytotoxic active partially purified MEA fraction. The qualitative compound report of HR-LCMS/MS analysis was included in annexure I. In accordance with HR-MS analysis, four peaks showed same m/z values in HR-LCMS/MS analysis also and the m/z values are 153.1274, 261.1126, 337.197 and 453.342 with corresponding mass 152.12, 260.105, 336.1906 and 452.335. Of these, the 3 compounds with MS/MS analysis and the known

molecular formula are detailed below (Figure 4.40 to Figure 4.42). The structure of these compounds obtained in data base search of HR-LCMS/MS analysis was included in annexure II. The compound with mass of 336.1906 is reported as an uncharacterized compound (Figure 4.43) and the database search showed no similarity with m/z value 437.19412 shown in HR-MS analysis.

1. Neral ((2Z)-3,7-dimethylocta-2, 6-dienal, $C_{10}H_{16}O$, 152.1201)

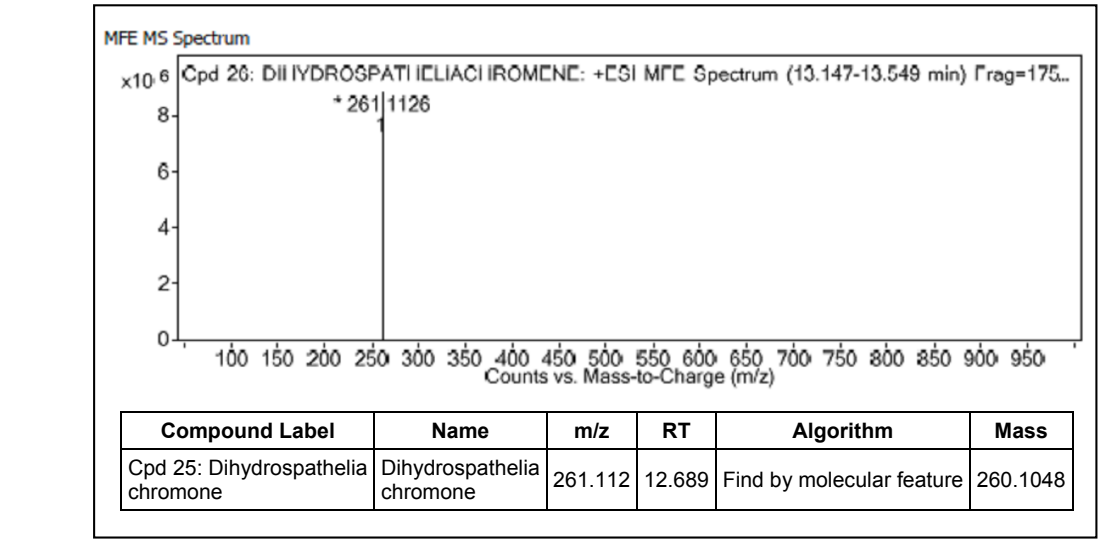


Compound Label	Name	m/z	RT	Algorithm	Mass
Cpd 28: Neral	Neral	153.1274	13.332	Find by molecular feature	152.1201

Figure 4.40. MS/MS profile of neral.

2. Dihydrospatheliachromone

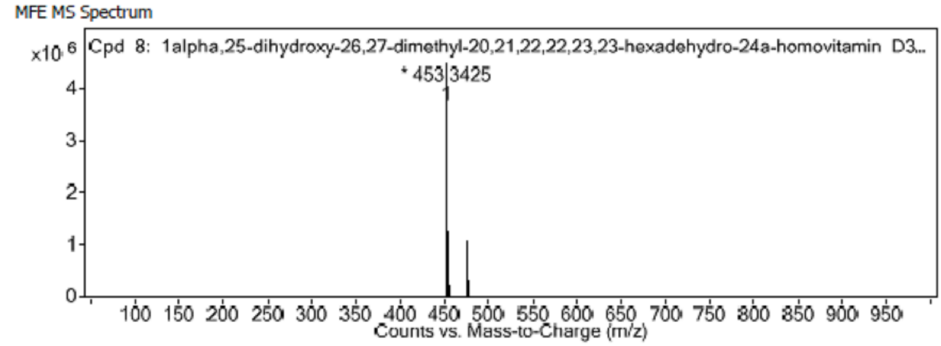
(Isopeucenin, 5-Hydroxy-2,2,8-trimethyl-3,4-dihydropyrano[3,2-g]chromen-6-one, $C_{15}H_{16}O_4$, 260.105)



Compound Label	Name	m/z	RT	Algorithm	Mass
Cpd 25: Dihydrospathelia chromone	Dihydrospathelia chromone	261.112	12.689	Find by molecular feature	260.1048

Figure 4.41. MS/MS profile of dihydrospatheliachromone.

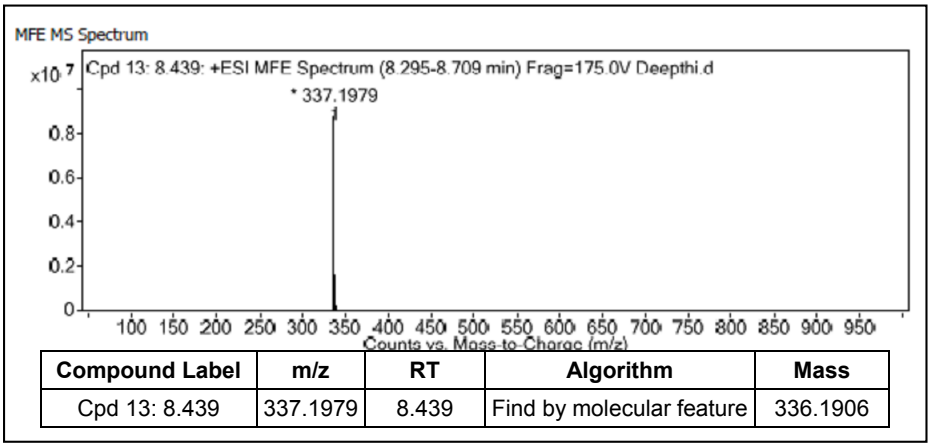
3.1 alpha,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homovitamin D3 ($C_{30}H_{44}O_3$, 452.335)



Compound Label	Name	m/z	RT	Algorithm	Mass
Cpd 8: 1 alpha,25-dihydroxy-26,27-dimethyl-20,21,22,22,23, 23-hexadehydro-24a-homovitamin D3	1 alpha,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homovitamin D3	453.3425	6.279	Find by molecular feature	452.3351

Figure 4.42. MS/MS profile of 1alpha,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homovitamin D3.

4. MS/MS profile and mass of uncharacterized compound



Compound Label	m/z	RT	Algorithm	Mass
Cpd 13: 8.439	337.1979	8.439	Find by molecular feature	336.1906

Figure 4.43. MS/MS profile of uncharacterized compound.

4.4 Evaluation of phytochemical constituents, antibacterial and cytotoxic activities of the host plants, *Elaeocarpus sphaericus* and *Quassia indica*

The leaves of *Elaeocarpus sphaericus* and *Quassia indica* were sequentially extracted with solvents petroleum ether, chloroform, ethyl acetate, methanol and water based on the increasing polarity for 48 hrs of each solvent. Percentage yield of leaves solvent extracts from both the plants is given in table 4.20.

Table 4.20. Percentage yield of leaves extracts of *Elaeocarpus sphaericus* and *Quassia indica*

Solvent extracts	Percentage of yield
ES Petroleum ether	2.14
ES Chloroform	8.69
ES Ethyl acetate	5.8
ES Methanol	3.35
ES Aqueous	2.07
QI Petroleum ether	1.76
QI Chloroform	10.59
QI Ethyl acetate	6.22
QI Methanol	3.90
QI Aqueous	3.20

ES - *Elaeocarpus sphaericus*, QI - *Quassia indica*

4.4.1. Phytochemical screening of leaves extracts from *Elaeocarpus sphaericus* and *Quassia indica*

The preliminary qualitative phytochemical screening of the leaves extracts from *Elaeocarpus sphaericus* showed the presence of major phytoconstituents (Table 4.21). Petroleum ether extract showed the presence of steroids whereas chloroform extract showed the presence of steroids, carbohydrates and saponins. Ethyl acetate extract exhibited the presence of alkaloids, tannins, phenolics, carbohydrates, steroids. The methanol extract showed the presence of alkaloids, flavonoids, phenolics, and terpenoids. Aqueous extracts showed the presence of majority of the phytoconstituents screened.

Preliminary phytochemical screening of the leaves extracts from *Quassia indica* showed the presence of major phytoconstituents (Table 4.22). Petroleum ether extract showed the presence of flavonoids whereas chloroform extract showed the presence of alkaloids, flavonoids, terpenoids, steroids, carbohydrates and saponins. The ethyl acetate extract exhibited the presence of alkaloids, flavonoids, phenolics, and steroids. The methanol extract showed the presence all major constituents except saponins and aqueous extracts have all the major constituents except steroids.

Table 4.21. Phytochemical screening of leaves extracts of *Elaeocarpus sphaericus*

Phytochemical Tests	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Aqueous
Alkaloids	_	_	+	+	+
Flavonoids	_	_	_	+	+
Phenol	_	_	+	+	+
Saponins	_	+	_	_	+
Tannin	_	_	+	_	+
TriTerpenoids	_	_	_	+	+
Glycosides	_	_	_	_	_
Carbohydrates	_	+	+	_	+
Steroids	+	+	+	_	_

'+'= Positive result, '–' = Negative result

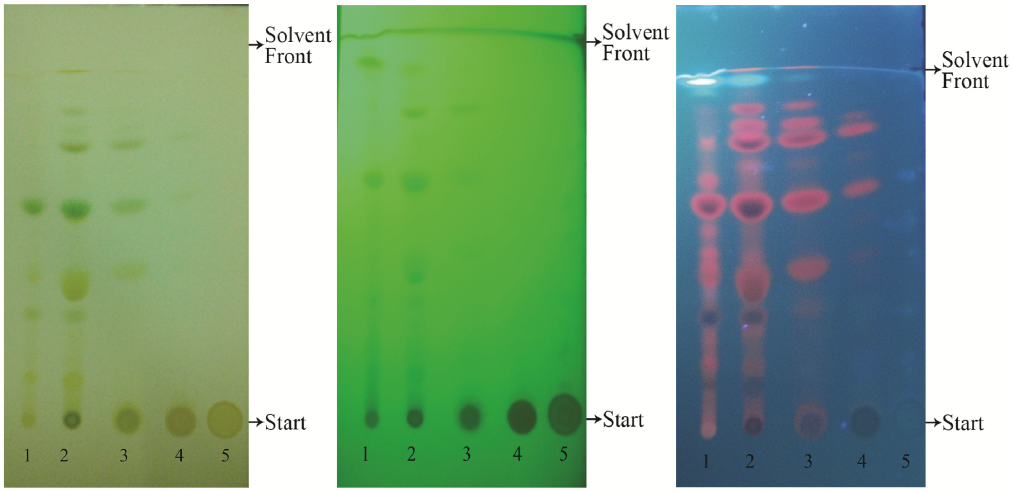
Table 4.22. Phytochemical screening of leaves extracts of *Quassia indica*

	Petroleum ether	Chloroform	Ethyl acetate	Methanol	Aqueous
Alkaloids	_	+	+	+	+
Flavonoids	+	+	+	+	+
Phenol	_	_	+	+	+
Saponins	_	+	_	_	+
Tannin	_	_	_	+	+
Terpenoids	_	+	_	+	+
Glycosides	_	_	_	+	+
Carbohydrates	_	+	_	+	+
Steroids	_	+	+	+	_

'+'= Positive result, '–' = Negative result

4.4.2. Thin layer chromatogram of leaves extracts of *Elaeocarpus sphaericus*

The thin layer chromatographic separation of all the leaves extracts was done by using the petroleum ether:ethyl acetate (8:2) as mobile phase. The profile is shown in figure 4.44. The bands with corresponding Rf values observed under visible light, UV at 254 nm and 366 nm are given in the table 4.23.



A B C

Figure 4.44. TLC profile of leaves extracts of *Elaeocarpus sphaericus.* Petroleum ether: ethyl acetate (8:2) as solvent system viewed under (A) visible light, (B) UV at 254 nm and (C) UV at 365 nm. (1)-Petroleum ether, (2)-Chloroform, (3)- Ethyl acetate, (4)- Methanol, (5)- Aqueous extracts.

Table 4.23. Rf values of leaves extracts of *Elaeocarpus sphaericus*

Extract	Rf Value		
	Visible light	**UV 254 nm**	**UV 365 nm**
Petroleum ether (PE)	0.10, 0.29, 0.39,0.58	0.29,0.40, 0.59,0.91	0.10, 0.15, 0.29, 0.40, 0.53, 0.59, 0.67,0.78,0.87, 0.96
Chloroform (CH)	0.10,0.29, 0.34,0.58, 0.78,0.82, 0.87	0.10, 0.29, 0.36,0.59, 0.78,0.90	0.10,0.15, 0.28, 0.37, 0.58,0.78, 0.82,0.87, 0.96
Ethyl acetate (EA)	0.40,0.59, 0.78	0.41,0.60, 0.80	0.31,0.42, 0.60, 0.78,0.82,0.87, 0.96
Methanol (MM)	0.63, 0.80	–	0.45, 0.63, 0.81
Aqueous (AQ)	–	–	–

'-' = No bands.

4.4.3. Thin layer chromatogram of leaves extracts of *Quassia indica*

Thin layer chromatogram of all the leaves extracts was separated by using the petroleum ether:ethyl acetate (8:2) as mobile phase. The profile is shown in figure 4.45. The bands with corresponding Rf values observed under visible light, UV at 254 nm and 366 nm are given in table 4.24.

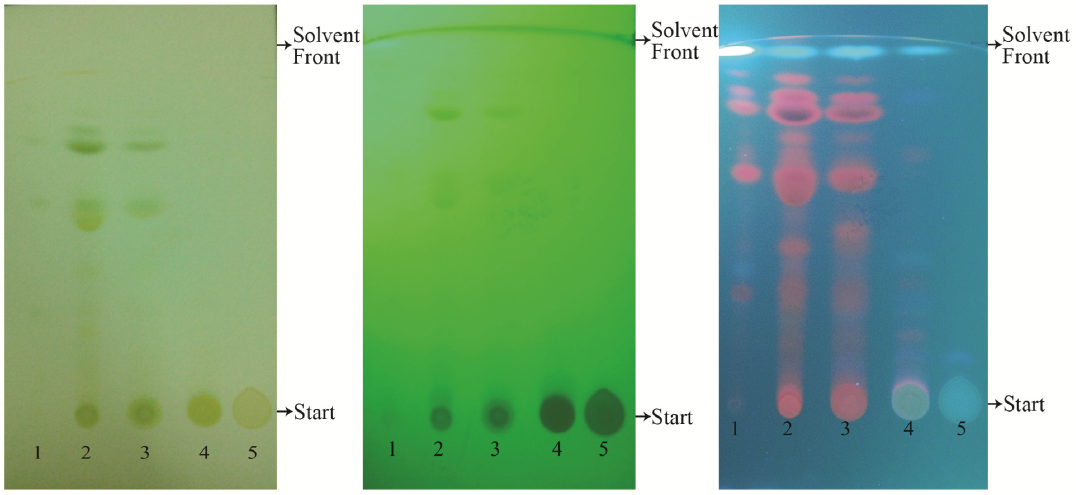


Figure 4.45. TLC profile of leaves extracts of *Quassia indica*. Petroleum ether: ethyl acetate (8:2) as a solvent system viewed under (A) visible light, (B) UV at 254 nm and (C) UV at 365 nm (1)-Petroleum ether, (2)-Chloroform, (3)-Ethyl acetate, (4)-Methanol, (5)-Aqueous extracts.

Table 4.24. Rf values of leaves extracts of *Quassia indica*

Extract	Rf Value		
	Visible Light	**UV 254 nm**	**UV 365 nm**
Petroleum ether (PE)	0.28, 0.61, 0.80	0.92	0.28, 0.33, 0.62, 0.8, 0.96
Chloroform (CH)	0.28, 0.41, 0.55, 0.6, 0.78, 0.83, 0.88	0.55, 0.78, 0.85	0.25, 0.3, 0.41, 0.58,0.81, 0.85, 0.98
Ethyl acetate (EA)	0.52, 0.53, 0.78, 0.82, 0.86	0.58, 0.77	0.31, 0.41, 0.61, 0.78, 0.85, 0.96
Methanol (MM)	–	–	0.16, 0.3, 0.36
Aqueous (AQ)	0.11	–	–

'- ' = No bands

4.4.4. HPTLC profile of *Elaeocarpus sphaericus* and *Quassia indica* leaves extracts

Qualitative evaluation of leaves extracts of *Elaeocarpus sphaericus* and *Quassia indica* were performed by using HPTLC. The profile of both the plant extracts is shown in figure 4.46. The parameters such as the number of bands, corresponding Rf and the area % were tabulated (Table 4.25 and Table 4.26). Chloroform and methanol extract of *Elaeocarpus sphaericus* confirmed the presence of maximum number of compounds (12) under UV at 254nm whereas in the case of *Quassia indica*, the petroleum ether extract showed the presence of maximum number of compounds (12) under UV at 366 nm. The number of peaks, corresponding Rf and the area % of each extract from two plants were included in annexure III and IV.

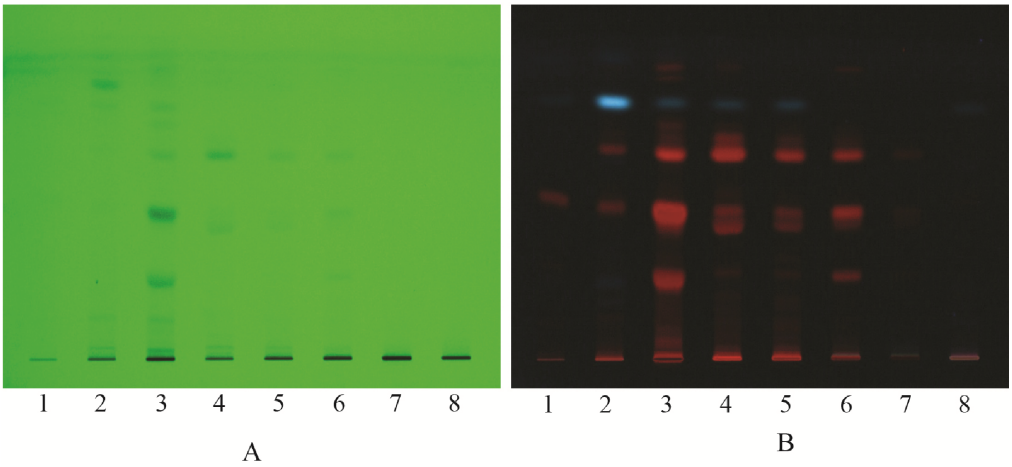


Figure 4.46. HPTLC profile of leaves extracts of *Elaeocarpus sphaericus* and *Quassia indica.* (1)- Petroleum ether, (2)- Chloroform, (3)- Ethyl acetate, (4)- Methanol of *Elaeocarpus sphaericus* and (5)- Petroleum ether, (6)- Chloroform, (7)- Ethyl acetate, (8)- Methanol of *Quassia indica* in petroleum ether: ethyl acetate (8:2) as a mobile phase observed under UV (A) at 254 nm and (B) at 366 nm.

Table 4.25. HPTLC profile of leaves extracts of *Elaeocarpus sphaericus*

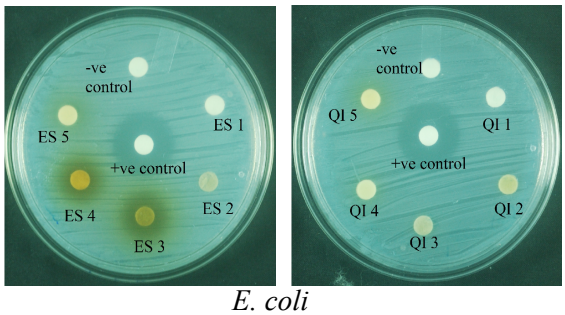
Sample	Under 254 nm			Under 366 nm		
	No. of Band	**Rf value**	**Area (%)**	**No. of Band**	**Rf value**	**Area (%)**
PE Extract	4	0.57,0.91, 1.03,1.12	9.43,24.97, 34.77,30.83	6	0.03,0.11, 0.56,0.89, 1.07,1.13	1.48,8.18, 48.39,16.29, 14.83,10.83
CH Extract	10	0.02,0.08, 0.13,0.24, 0.47,0.74, 0.86,0.93, 1.02,1.14	0.92,0.93, 2.80,11.81, 24.54,7.87, 5.93,12.12, 22.89,10.20	11	0.03,0.10, 0.23,0.44, 0.72,0.86, 0.98,1.04, 1.08,1.14, 1.19	1.33,6.82, 18.37,41.41, 14.09,9.21, 0.80,1.54, 4.00,1.48, 0.85
EA Extract	8	0.01,0.04, 0.48,0.54, 0.74,0.93, 1.01,1.13	30.82,2.88, 7.01,4.88, 17.02,6.82, 12.63,18.28	12	0.01,0.13, 0.20,0.28, 0.36,0.47, 0.54,0.73, 0.87,0.95, 1.08,1.14	24.22,2.25, 1.56,3.26, 2.54,10.5, 10.58,29.36, 5.39,3.15, 4.16,2.95
ME Extract	4	0.00,0.12, 1.02,1.12	62.37,1.30, 18.44,17.88	5	0.00,0.30, 0.52,0.76,1.06	68.76,3.37, 10.25,11.26, 6.36

Table 4.26. HPTLC profile of leaves extracts of *Quassia indica*

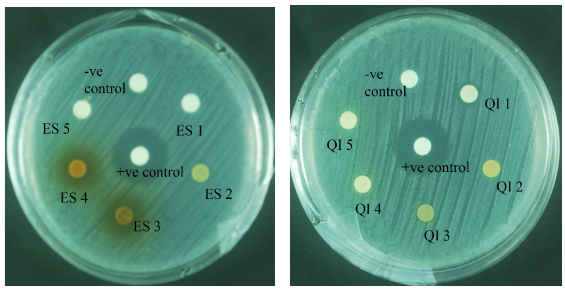
Sample	Under 254 nm			Under 366 nm		
	No. of Band	Rf value	Area (%)	No. of Band	Rf value	Area (%)
PE Extract	12	0.03,0.13, 0.24,0.28, 0.32,0.53, 0.77,0.88, 0.93,1.00, 1.08,1.12	1.76,2.97, 1.21,1.82, 0.81,3.69, 4.31,3.88, 15.73,31.28, 14.9, 17.65	9	0.02,0.12, 0.28,0.50, 0.76,0.88, 0.94,1.07, 1.13	1.53,16.25, 2.01,20.87, 18.11,10.40, 16.40,8.82, 5.60
CH Extract	12	0.04,0.08, 0.14,0.23, 0.30,0.46, 0.55,0.61, 0.74,1.02, 1.08,1.13	3.64,1.07, 2.09,1.45, 2.05,11.31, 4.46,1.65, 32.78,13.07, 10.14,16.29	10	0.01,0.12, 0.30,0.44, 0.54,0.61, 0.73,0.88, 1.06,1.11	4.40,2.25, 2.31,16.59, 10.09,2.73, 50.65,3.45, 3.42,4.10
EA Extract	11	0.00,0.14, 0.18,0.23, 0.27,0.29, 0.49,0.57, 0.76,1.04, 1.13	39.41,1.97, 1.69,1.91, 0.82,7.01, 7.82,4.22, 8.91,15.27, 10.98	9	0.00,0.05, 0.13,0.25, 0.48,0.74, 0.87,1.07, 1.15	30.19,1.81, 1.98,10.64, 26.44,18.97, 4.04, 4.14, 1.80
ME Extract	4	0.00,1.00, 1.07,1.11	61.19,10.53, 7.47,20.80	3	0.00,1.06, 1.14	85.34,8.64, 6.02

4.4.5 Antibacterial activity of leaves extracts of *Elaeocarpus sphaericus* and *Quassia indica*

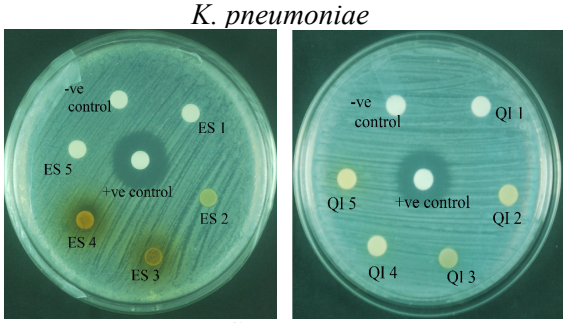
Antibacterial activities of leaves extracts from *Elaeocarpus sphaericus* and *Quassia indica* were tested against the selected gram positive and gram negative bacteria by disc diffusion method. All the solvent extracts from both the plants showed no of zone of inhibition against the gram positive and gram negative organisms tested at 1mg/ml concentrations (Figure 4.47).



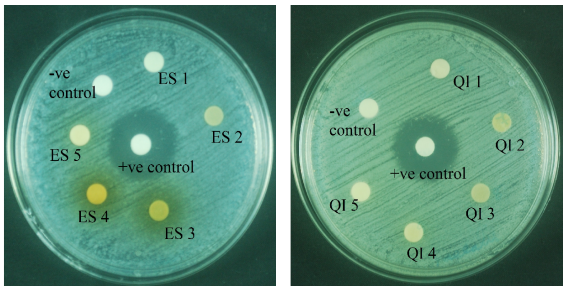
E. coli



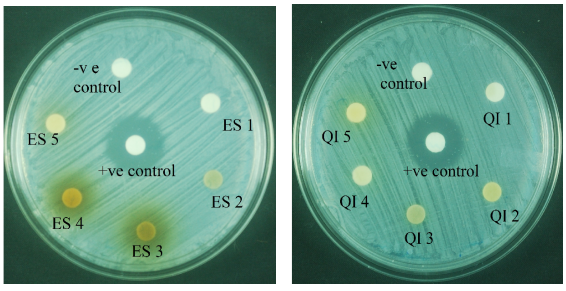
K. pneumoniae



S. aureus



E. faecalis



L. monocytogenes

Figure 4.47. Antibacterial activity of leaves extracts *Elaeocarpus sphaericus* and *Quassia indica*. ES1 and QI1- petroleum ether extracts, ES2 and QI2- chloroform extracts, ES3 and QI3- Ethyl acetate extracts, ES4 and QI4- Methanol extracts, ES5 and QI5-Aqueous extracts. Positive control (Chloramphenicol)-20µg/Disc, leaves extracts- 1mg/Disc and negative control- DMSO.

4.4.6. Cytotoxic activity of leaves extracts of *Quassia indica*

Cytotoxic activity of the leaves extracts of *Elaeocarpus sphaericus* and *Quassia indica* on K562 cell line was evaluated by MTT assay. The cells were treated with different concentrations of leaves extracts from 0 to 300 µg/ml for 48 hrs. The percentage of viability with different concentrations is shown in figure 4.48 and the IC_{50} values are tabulated (Table 4.27). The petroleum ether, chloroform and ethyl acetate extracts of *Quassia indica* exhibited cytotoxic activity and the IC_{50} values are 66±4.82 µg/ml, 33.5±3.81 µg/ml and 26.2±4.07µg/ml respectively. All other extracts from both the plants exhibited no cytotoxic activity up to the given concentrations. The IC_{50} value of curcumin is 12.5±2.17 µg/ml. Analysis of variance showed that there is significant differences in cytotoxic activity of leaves extracts on K562 cell line while comparing with curcumin ($F= 99.18$; $P< 0.001$). Further multiple comparison analysis (Dunnett t- test) revealed that the leaves extracts have significant differences and variations in cytotoxic activity in comparison with control ($P< 0.05$) (Table 4.27). Results showed that the leaves extracts have higher IC_{50} value than curcumin which reveals lower cytotoxic activity.

Table 4.27. IC_{50} values of leaves extracts from *Quassia indica* on K562 cell line

	IC50 values in µg/ml	
Sl. No	**Leaves extracts**	**K562 Cell line**
1	Petroleum ether	66 ± 4.82^{a}
2	Chloroform	33.5 ± 3.81^{b}
3	Ethyl acetate	26.2 ± 4.07^{c}
4	Curcumin	12.5 ± 2.17^{d}

Different superscript letter (a-d) indicates significant difference ($P< 0.05$) between IC_{50} values from different leaves extracts and curcumin (same column).

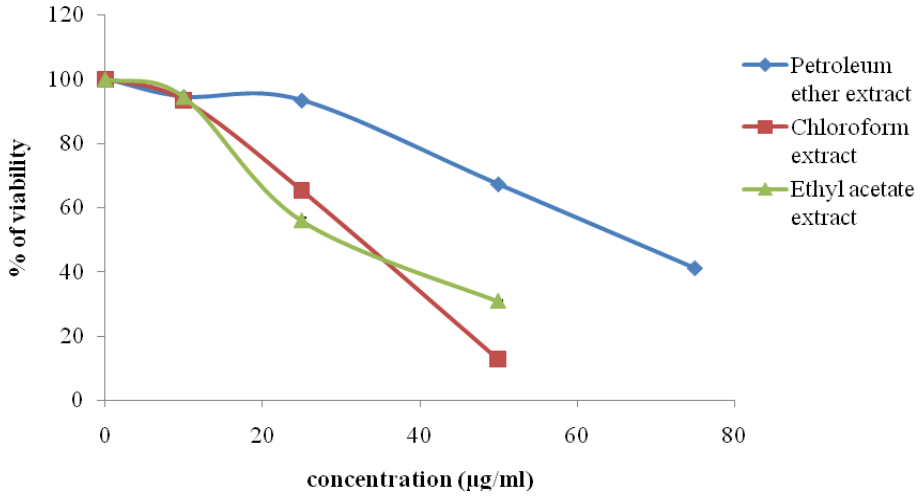


Figure 4.48. Cytotoxic activity of petroleum ether, chloroform and ethyl acetate extracts of *Quassia indica* on K562 cell line.

4.5. Characterization of plant growth regulator indole acetate acid (IAA) from the selected endophytic fungus and the effect of fungus on growth of rice seedlings

All the fungal isolates from *Elaeocarpus sphaericus* and *Quassia indica* were screened for IAA production. Out of the 22 isolates, 3 isolates from *Elaeocarpus sphaericus* and 3 isolates from *Quassia indica* were exhibited the IAA production. The endophytic fungus with highest IAA production selected for further analysis. The fungus was fermented, optimized and IAA was extracted. The isolated IAA was characterized by TLC, HPLC and HR-MS analysis. The fungus was also studied for its effect on growth of rice seedlings.

4.5.1. IAA production by fungal endophytes at different concentrations

The endophytes ESEF 4, ESEF 8 (*Diaporthe* sp.) and ESEF 6 (*Xylaria* sp.) of *Elaeocarpus sphaericus* and QIEF 5, QIEF 6 (*Colletotrichum* sp.) and QIEF 7 (*Fusarium* sp.) of *Quassia indica* were able to produce IAA at varying concentrations of tryptophan supplementation. The amount of IAA produced by endophytic fungi at different concentrations is given in figure 4.49 and table 4.28. Accordingly, the increase in concentration of tryptophan in the media leads to the enhancement of IAA production and the maximum IAA production was exhibited by QIEF 5 in all concentrations (0 to 5 mg/ml) of tryptophan and it ranging from 28.07 µg/ml to 253.69

µg/ml. ANOVA comparison between fungal isolates has significant differences in IAA production at different concentrations of tryptophan (0 to 5 mg/ml) supplementation (F=42.01, F=210.08, F=65.31, F=51.19; $P< 0.001$) (Figure 4.49). Analysis of variance also showed significant difference in IAA production between various concentrations of tryptophan supplementation by the selected endophytic fungi such as ESEF 4, ESEF 6, ESEF 8, QIEF 5, QIEF 6 and QIEF 7 (F= 70.72, F= 70.72, F= 29.26, F=255.75, F=260.96, F=117.29; $P< 0.05$).

Table 4.28. IAA production by endophytic fungi from *Elaeocarpus sphaericus* and *Quassia indica*

Trp. Con. (mg/ml)	IAA production by endophytic fungi (µg/ml)					
	ESEF 4	**ESEF 6**	**ESEF 8**	**QIEF 5**	**QIEF 6**	**QIEF 7**
CDB as control	3.82 ± 0.44	3.82± 0.44	3.82 ± 0.44	3.82 ± 0.44	3.82 ± 0.44	3.82±0.44
0	12.94±1.23	9.04± 4.58	8.34 ± 2.12	28.07 ± 3.64	11.87 ± 1.2	7.51 ± 1.1
1	95.70±9.27	13.99±4.72	56.63±7.14	220.71±13.7	12.09 ± 2.9	72.02±1.5
2	98.47±15.05	17.83±3.31	68.03±16.14	221.24±17.5	37.76 ± 4.3	78.38±8.3
5	114.3±16.31	31.57±7.19	89.82±20.39	253.69±4.39	73.17±4.04	83.86±11

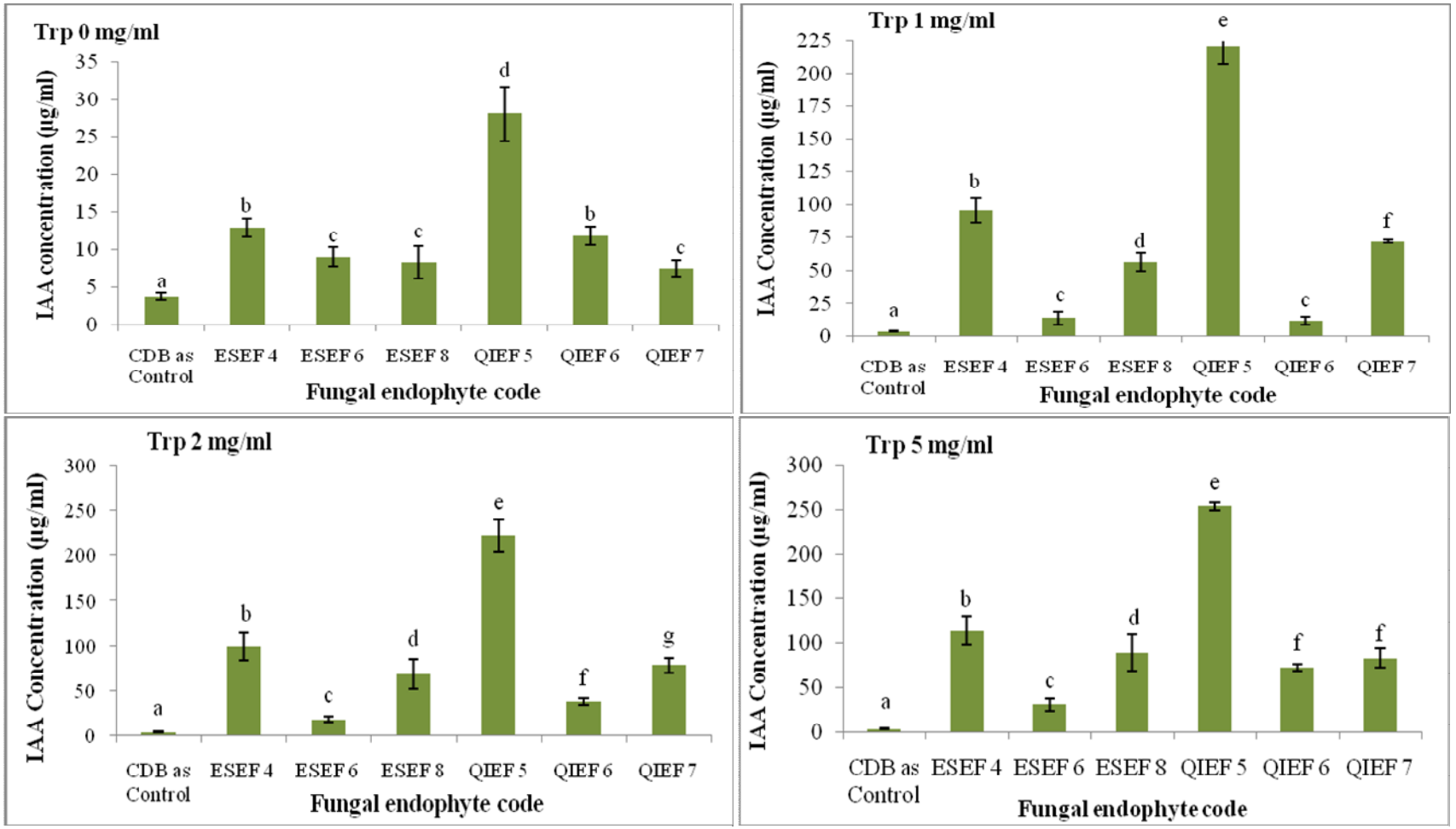


Figure 4.49. IAA production by endophytic fungi from *Elaeocarpus sphaericus* and *Quassia indica* at different con. of trpyptopan. CDB as control (without fungal inoculation), IAA production in µg ± SD, (n=3). Differerent letters on error bars represent mean values have significant differences ($P< 0.01$).

4.5.2. Optimization of QIEF 5 for IAA production

Based on the highest IAA production, QIEF 5 was selected for further analysis. QIEF 5 was fermented and optimized for the production of IAA and it was done in different media with the supplementation of tryptophan (1mg/ml). The effect of different parameters like time, pH and temperature was studied on one factor at a time basis.

4.5.2.1. Effect of different fungal growth media on IAA production

The isolate QIEF 5 was inoculated in czapek dox broth (CDB), potato dextrose broth (PDB) and malt extract broth (MEB) medium with 1mg/ml of tryptophan supplementation and respective IAA production was calculated. Concentration of IAA produced is shown in figure 4.50. The maximum IAA production was seen in PDB as 220.12±19.63 µg/ml followed by CDB as 141.95±5.7 µg/ml and MEB showed least IAA production as 22.95±10.59 µg/ml.

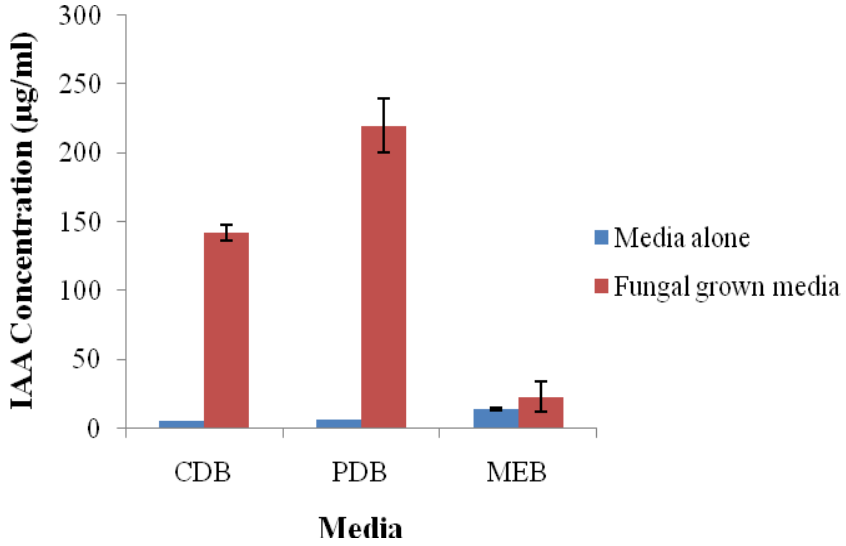


Figure 4.50. Effect of different fungal growth media on IAA production by QIEF 5. CDA- Czapek Dox broth, PDB- Potato dextrose broth, MEB- Malt extract broth, supplemented with 1mg/ml of tryptophan and QIEF 5 was inoculated.

4.5.2.2. Effect of time on IAA production

Endophyte QIEF 5 was inoculated in PDB with 1mg/ml tryptophan and incubated in different time periods. Concentration of IAA produced is given in figure 4.51. The isolate produce IAA in 5 days of incubation and the concentration of IAA was 97.36±15.84 µg/ml. IAA production was higher in 10 days and 15 days of incubation

and the IAA concentration was 220.15±16.32 µg/ml and 222.34±5.04 µg/ml respectively. A gradual increase in IAA production was observed in QIEF 5 with increase in time period up to 15 days.

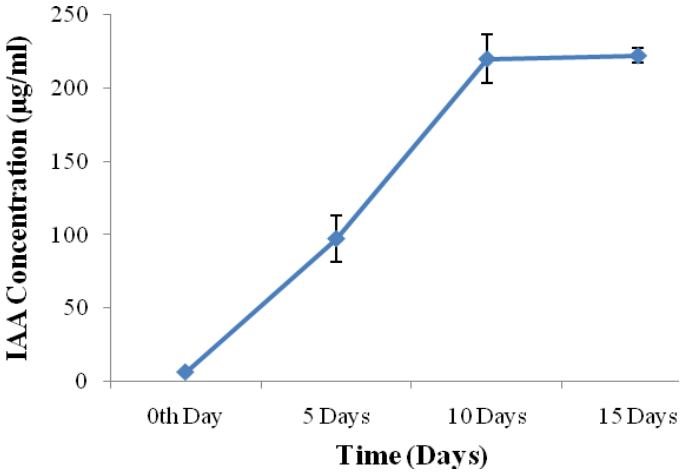


Figure 4.51. Effect of incubation time (Days) on IAA production by QIEF 5. PDB medium was supplimented with 1 mg/ml of tryptophan and QIEF 5 was inoculated.

4.5.2.3. Effect of temperature on IAA production

Endophyte QIEF 5 was inoculated in PDB with 1mg/ml tryptophan and incubated for 10 days at different temperatures (20°C, 27°C, 32°C and 37°C). Concentration of IAA produced is given in figure 4.52. The highest IAA production was 218.37±15.93 µg/ml obtained at 27°C. The endophyte showed less IAA production at remaining temperatures and it was not able to grow at 20°C.

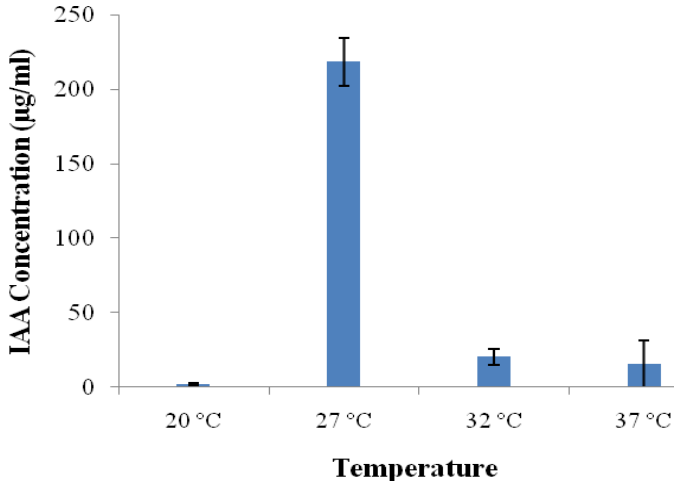


Figure 4.52. Effect of temperature on IAA production by QIEF 5. PDB medium was supplemented with 1mg/ml of tryptophan, QIEF 5 was inoculated and incubated for 10 days.

4.5.2.4. Effect of pH on IAA production

Endophyte QIEF 5 was inoculated in PDB with 1mg/ml tryptophan and incubated for 10 days at room temperature in different pH ranging from 4.5 to 10. Concentration of IAA produced is given in figure 4.53. The maximum IAA production was observed in pH 9.5 with IAA concentration of 203.68±8.3 µg/ml. A decrease in IAA concentration was seen in pH- 10. IAA production was not detected below pH 6.5.

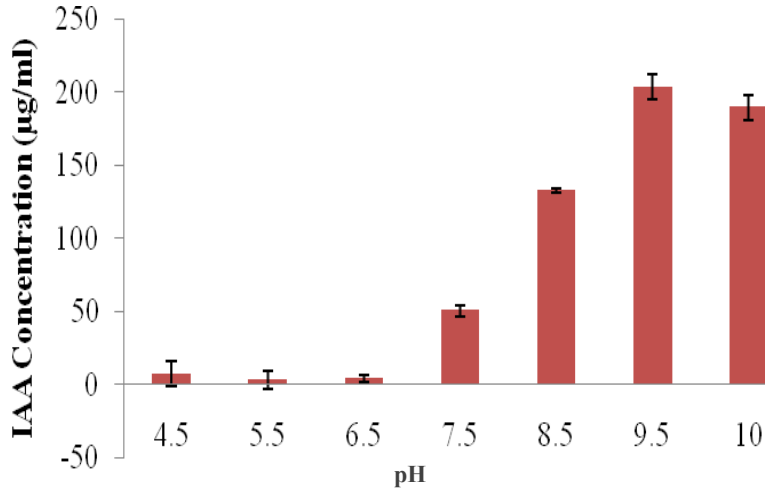


Figure 4.53. Effect of pH on IAA production by QIEF 5. The PDB medium was supplemented with 1mg/ml of tryptophan, QIEF 5 was inoculated and incubated for 10 days.

4.5.3. Fermentation and extraction of endophytic fungus QIEF 5

The fungus, QIEF 5 was grown in above mentioned optimized conditions in PDB medium. After 10 to 15 days of incubation, the broth was acidified to pH 3.0 and extracted with twice the volume of ethyl acetate. The extractive value of ethyl acetate was 0.406±1.2 gm/Litre of broth.

4.5.4. TLC based analyses of the Indole acetic acid from QIEF 5

Thin layer chromatogram of the ethyl acetate extract of QIEF 5 and standard IAA were observed at under UV at 254 nm and visible light sprayed with salkowski reagent (Figure 4.54). Under UV at 254 nm, the presence of the compound was observed in both the standard IAA and QIEF 5 extract with the Rf value 0.769 using chloroform:acetic acid (9.5:0.5) as mobile phase. In visible light both the standard

and QIEF 5 band showed a colour change to pink on spraying it with salkowski reagent showing the same Rf value (Rf- 0.769).

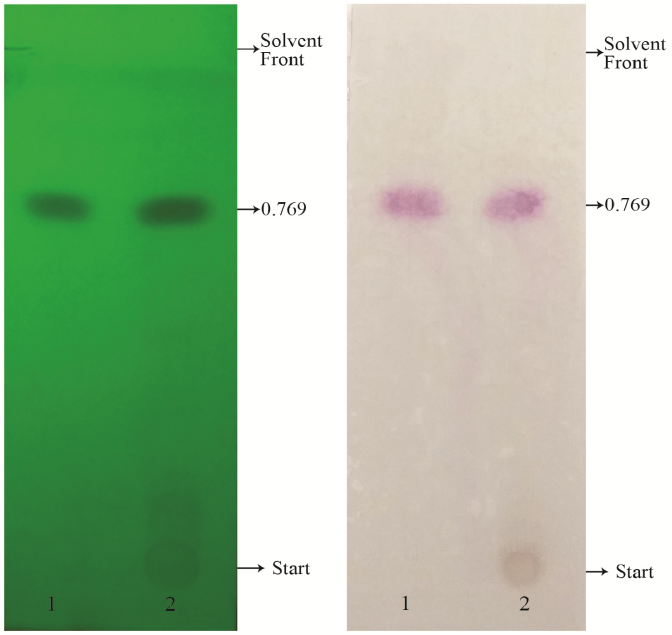
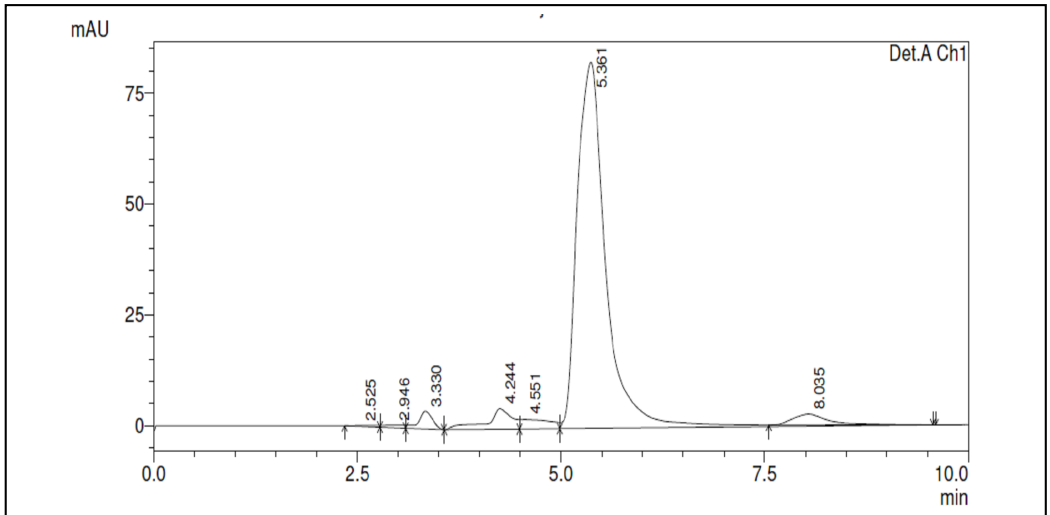


Figure 4.54. TLC based analyses of IAA. (1) Standard IAA and (2) QIEF 5 in chloroform:acetic acid (9.5:0.5) as mobile phase, observed under (A) UV at 254 nm and (B) visible light and sprayed with salkowski reagent.

4.5.5. HPLC and HR-MS profile of standard IAA and QIEF 5

The HPLC profile of standard IAA and QIEF 5 was generated using methanol:water (6:4) with 1 % acetic acid as mobile phase. HPLC of QIEF 5 showed the retention time of 5.24 min, which was almost similar to the retention time (5.361 min) of standard IAA. HPLC profile showing the number of peaks with retention time and area percentage of standard IAA and QIEF 5 is shown in figure 4.55 and figure 4.57. HR-MS profile of QIEF 5 showed the major peaks with m/z as 423.20, 437.25, 453.24 which corresponds to the standard IAA (Figure 4.56 and Figure 4.58).



PeakTable

UV Detector Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.525	5332	193	0.228	0.200
2	2.946	10856	614	0.464	0.635
3	3.330	46447	4043	1.986	4.182
4	4.244	102797	4691	4.395	4.852
5	4.551	54562	2127	2.333	2.200
6	5.361	2043327	82574	87.361	85.400
7	8.035	75625	2448	3.233	2.532
Total		2338944	96691	100.000	100.000

Figure 4.55. HPLC Profile of standard IAA. Observed under UV at 254 nm using methanol: water (6:4) with 1 % acetic acid as mobile phase. The highest peak was marked

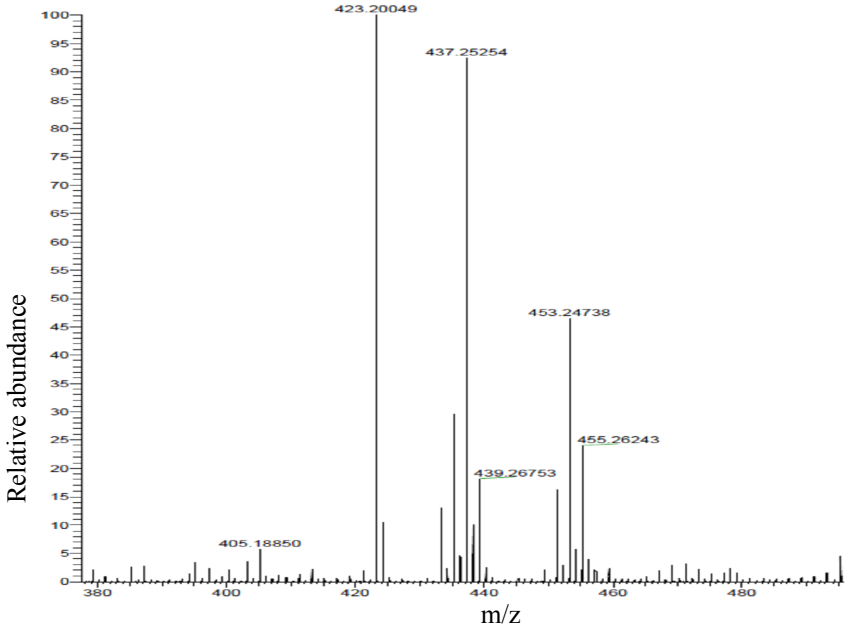
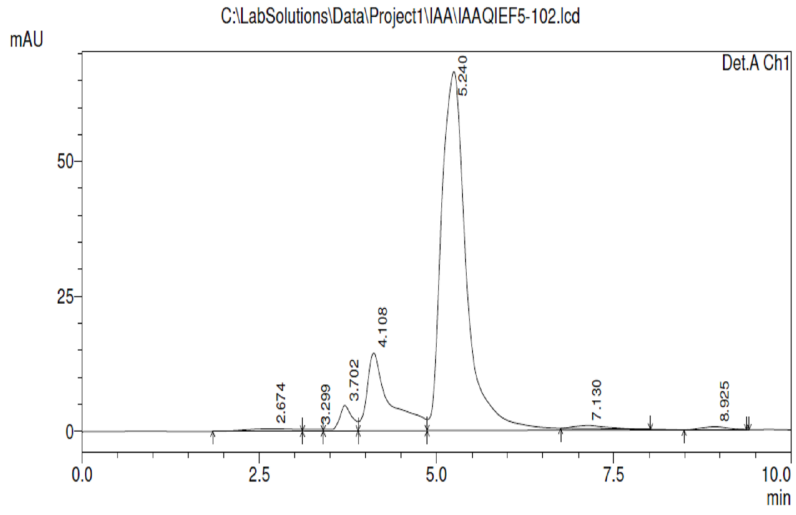


Figure 4.56. HR-MS profile of Standard IAA

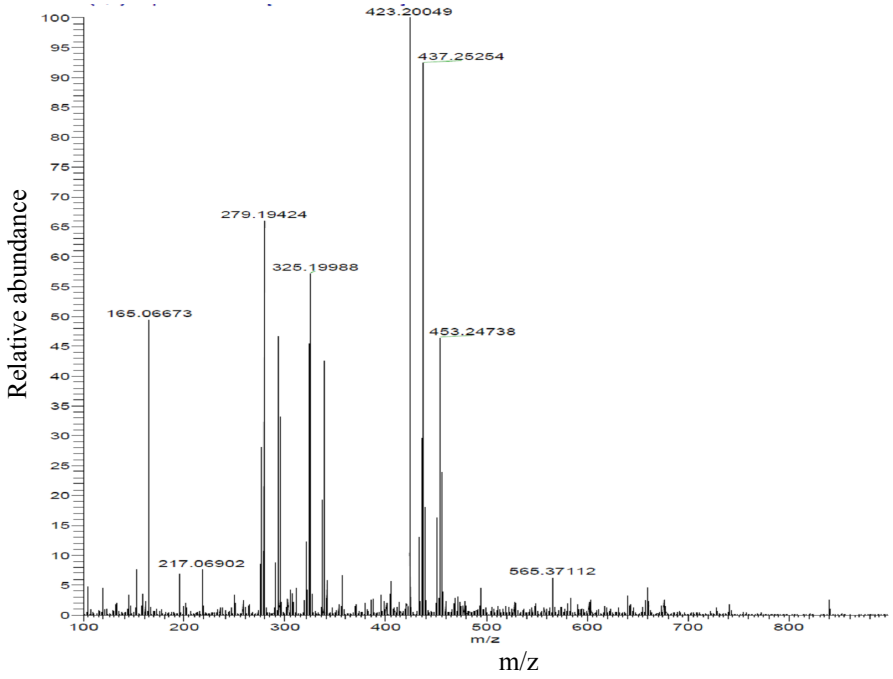


PeakTable

UV Detector Ch1 254nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.674	19372	423	0.933	0.484
2	3.299	4936	292	0.238	0.334
3	3.702	58547	4670	2.821	5.340
4	4.108	324211	14367	15.620	16.428
5	5.240	1636076	66495	78.826	76.035
6	7.130	18354	621	0.884	0.710
7	8.925	14065	585	0.678	0.669
Total		2075562	87453	100.000	100.000

Figure 4.57. HPLC Profile of QIEF 5. Observed under UV at 254 nm using methanol: water (6:4) with 1 % acetic acid as mobile phase. The highest peak was marked.



m/z

Figure 4.58. HR-MS profile of QIEF 5.

4.5.6. Effect of endophytic fungus on promotion of rice seedling growth

Rice seeds were incubated for 24 hrs in to the 10 days fungal grown medium and further germinated in the sterile wet filter paper. Endophytic fungi QIEF 5 had significant effect on the growth of rice seedlings (Figure 4.59). Growth measurements are listed in table 4.29. The shoot length and root length of the rice seedlings were 7.60±0.54 cm and 13.66±0.38 cm which were higher when compared to the control (without fungal inoculation). The shoot length and root length of control seedling was 6.54±0.29 cm and 10.18±0.29 cm respectively. The average weight of the seedlings and the numbers of aerial roots were higher in QIEF 5 compared to control. Fresh weight and dry weight of QIEF 5 seedlings are 70.56±10.84 mg and 10.34±0.27 mg respectively and the control seedlings have 57.28±10.35 mg and 8.50±1.01 mg of fresh and dry weight. Independent t-test for shoot length, root length, number of areal roots and average weight (fresh weight and dry weight) were significantly higher in QIEF 5 ($P< 0.05$) than the control (without fungal inoculation) (Table 4.29).

Figure 4.59. Effect of QIEF 5 incubation on rice seedling growth. A and B: Control- without fungal inoculation. C and D- rice seedlings germinated on filter paper after incubating it with QIEF 5 grown in PDB medium.

Table 4.29. Shoot length, root length and average weight of rice plant seedlings on incubated with QIEF 5

Sample	Shoot length (cm)	Root Length (cm)	No. of aerial roots	Average weight of seedlings (mg)	
				Fresh weight	Dry weight
Control	6.54± 0.29[a]	10.18± 0.29 [a]	4 [a]	57.28 ± 10.35 [a]	8.50 ± 1.01[a]
QIEF 5	7.60± 0.54[b]	13.66± 0.38[b]	8 [b]	70.56 ± 10.84 [b]	10.34 ± 0.27 [b]

Different superscript letter (a and b) indicates significant difference ($P< 0.05$) between control and QIEF 5 (same column).

Chapter 5

DISCUSSION

DISCUSSION

Endophytes are the microorganisms that live in intracellular area of healthy plant tissues at least for one phase of their life cycle without causing obvious symptoms (Kaneko et al., 2010). Mostly, endophytes form a stable relationship with the host plants and they produce many substances with pharmaceutical and agricultural applications (Bernardi-Wenzel et al., 2010; Orlandelli et al., 2012). Plants with good medicinal values are known to be associated with endophytic fungi. With this view in the present study, the medicinal plants *Elaeocarpus sphaericus* and *Quassia indica* were screened for the isolation and identification of endophytic fungi and further isolated the secondary metabolites from endophytic fungi with antibacterial, cytotoxic and plant growth promoting activities.

A total of 203 endophytic fungi were isolated from 122 leaf segments of *Elaeocarpus sphaericus* and 12 morphologically different cultures were selected. Among these, ESEF 12 (*Pestalotia* sp.) was the only fungus identified based on spore characteristics. 10 different isolates were selected from a total of 139 endophytic fungi isolated from 110 leaf segments of *Quassia indica*. From this, QIEF 8 (*Pestalotia* sp.) and QIEF 10 (*Fusarium* sp.) were identified based on spore characteristics. Remaining isolates from both the plants could not be identified due to the lack of spore formation. Diverse endophytic fungi had been isolated from various host plants. The leaves and stems of endemic medicinal plants from Tirumala hills afforded 14 fungal isolates including *Alternaria porri*, *Aspergillus niger*, *Aspergillus flavipes*, *Fusarium oxysporum*, *Nigrospora sphaerica*, *Pestalotiopsis* sp. and some hyphomycetous forms (Anitha et al., 2013). *Fusarium* sp. and *Pestalotiopsis* sp. were comparable in the microscopic characteristics of the identified isolates in the present study. Additionally, 4 genera of endophytic fungi were isolated from different parts of the medicinal plant Makassar fruit (*Brucea javanica* (L.) Merr.). They are *Trichoderma* sp., *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp.; *Fusarium* sp. had the same microscopic characteristics reported in the present observations (Amin et al., 2015). From the red listed endangered medicinal plant *Coscinium fenestratum*, 41 endophytic fungi that belonging to 16

taxa and were identified from 195 samples of stems and leaves. The endophytes include *Phomopsis jacquiniana*, *Alternaria alternate*, *Aspergillus tamarii*, *Aspergillus fumigates*, *Penicillium senticosam*, *Nigrospora oryzae* and sterile mycelia (Goveas et al., 2011).

Molecular techniques are used as the new perspective for the taxonomic identification of endophytic fungi and endophytes that could not be identified based on microscopic characteristics were also subjected to molecular techniques. For instance, ITS sequence of rDNA and the microscopic observations were also used for the identification of *Dothediomycetes* sp., *Alternaria tenuissima*, *Thielavia subthermophila*, *Alternaria* sp., *Nigrospora oryzae*, *Colletotrichum truncatum*, and *Chaetomium* sp. from the leaves and stems of the medicinal plant *Tylophora indica* (Kumar et al., 2011). A total of 33 different taxonomic groups of endophytes from *Calotropis procera* were also identified based on the ITS sequencing of rRNA region. The identified sequences showed full correspondence with their microscopic characteristics (Gherbawy and Gashgiri, 2013). In addition to this, *Colletotrichum gloeosporioides* isolate from tea plant was identified by ITS rDNA sequence which was in agreement with the identity made by microscopic characteristics (Rabha et al., 2016). Endophytic fungi associated with the Amazonian medicinal plant *Carapa guianensis* were identified at genus level by the molecular method ITS PCR and the isolates are *Phomopsis*, *Aspergilllus*, *Beltrania*, *Botryosphaeria*, *Xylaria*, *Diaporthe*, *Endomelanconiopsis*, *Fusarium*, *Guignardia*, *Pestalotiopsis*, *Pilidiella*, *Trichoderma*, and *Colletotrichum* (Ferreira et al., 2015). The current study also uses ITS sequencing and identified all the endophytic fungi of *Elaeocarpus sphaericus* and *Quassia indica*. The genus of the identified endophytic fungi is mentioned in the study. The identified isolates by using molecular techniques are *Xylaria* sp. (ESEF 1, ESEF 2, ESEF 6 and ESEF 7), *Fusarium* sp. (ESEF 3, QIEF 7 and QIEF 10), *Diaporthe* sp. (ESEF 4, ESEF 8 and QIEF 2), *Endomelanconiopsis* sp. (ESEF 5), *Nigrospora* sp. (ESEF 9, ESEF 10 and ESEF 11), *Pestalotiopsis* sp. (ESEF 12 and QIEF 8), *Daldinia* sp. (QIEF 1), *Phyllosticta* sp. (QIEF 3), *Lecanicillium* sp. (QIEF 4), *Colletotrichum* sp. (QIEF 5 and QIEF 6), and *Meyerozyma* sp. (QIEF 9). The molecular characters of the endophytic

fungi ESEF 12, QIEF 8 and QIEF 10 supported the microscopic identification. Inference in bayesian analysis of endophytic fungi from both *Elaeocarpus sphaericus* and *Quassia indica* were studied. Endophytic fungi were positioned into various taxa and the analysis showed the evolutionary relation and the common ancestor of the identified endophytic fungi. Similarly, bayesian analysis identified the endophyte isolate 9143 as *Pestalotiopsis neglecta* (Hoffman et al., 2013).

Colonization frequencies of endophytic fungi of *Elaeocarpus sphaericus* and *Quassia indica* ranged from 3.27 % to 20.49 % and 2.72 % to 20.90 % respectively. The highest colonization frequency in ESEF 4 and QIEF 2 (*Diaporthe* sp.) was observed as 20.49 % and 20.90 % respectively. Colonization of endophytic fungi depends upon the medicinal properties of the plants. The low range of colonization frequency may be due to the secretion of phytochemicals that hold certain antibacterial components (Rajagopal et al., 2010). Previously, Endophytes, *Fusarium oxysporum* and *Chaetomium globosum* associated with leaves of *Calotropis procera* were identified as the two isolates with highest colonization frequency as 2.7 % and 2.9 % (Gherbawy and Gashgiri, 2013). Similarly, *Fusarium* sp. and *Alternaria* sp. related with the leaves of the medicinal herb *Salvia miltiorrhia* were the two dominant fungi with colonization frequencies as 16.13 % and 14.52 % (Lou et al., 2013). The colonization rate of leaves was higher when compared to other plant parts like stem, root and petiole of the host plants (Lakshman and Jayshree, 2013). The medicinal plant *Calotropis procera* exhibited the dominant endophytes *Phaeoramularia calotropidis* and *Guignardia bidwellii* with high colonization frequencies 63.5 % and 21.1 % (Nascimento et al., 2015).

In the present study, diversity and evenness of fungal endophytes from the leaves of the two plants was indexed using shannon diversity index with $H' = 2.248$ and evenness of 0.90 for *Elaeocarpus sphaericus*. *Quassia indica* displayed the diversity index $H' = 1.943$ with an evenness of 0.84. The H' index usually ranges between 1.5 and 3.5. The results pointed out that the two plants had abundant in endophytic fungi and were evenly distributed in the plants. The diversity of endophytic fungi provides the abundant sources of novel and bioactive secondary

metabolites. Comparably, the isolates of woody lianas- *Bauhinia vahlii* showed high diversity in the leaves of the plants from Chilkigarh (Bagchi and Banerjee, 2014). The leaves of the medicinal plants of Malnad region, Karnataka exhibited high diversity index in *Sida acuta* with H'=1.06 and low endophytic diversity index H' = 0.65 in *Achyranthus aspera* (Naik et al., 2014). The different parts of the medicinal plant *Adenocalymma alliaceum* showed high fungal diversity in stem with shannon-weiner index of H' = 2.515 followed by leaf with H' = 2.379 and petiole showed relatively low diversity with H' =2.016 (Kharwar et al., 2011). High species diversity, richness and evenness were noticed in endophytic fungi colonizing the leaves, stem and root of *Angelica sinensis* (Jiang et al., 2013).

Endophytic fungi are capable of producing antimicrobial metabolites. Most of the bioactive natural products from endophytic fungi have the resistance mechanism to overcome the highly drug resistant human and plant pathogen (Yu et al., 2010). In the present work, the ethyl acetate extracts of endophytic fungi ESEF 4 and ESEF 8 (*Diaporthe* sp.), ESEF 5 (*Endomelanconiopsis* sp.), ESEF 6 and ESEF 7 (*Xylaria* sp.), ESEF 9 and ESEF 10 (*Nigrospora* sp.) from *Elaeocarpus sphaericus* showed antibacterial activity against *E. coli*, *K. pneumoniae*, *S. aureus* and *L. monocytogenes* at 100 µg/Disc concentration. Among the isolates, ESEF 6 showed significant antibacterial activity and the zone of inhibitions are 13 mm, 13.66±0.57 mm, 13.66±0.57 mm and 14.33±0.57 mm for *E. coli*, *K. pneumoniae*, *S. aureus* and *L. monocytogenes* respectively. The ethyl acetate extracts of endophytic fungi, QIEF 2 (*Diaporthe* sp.), QIEF 3 (*Phyllosticta* sp.), QIEF 6 (*Colletotrichum* sp.) and QIEF 7 (*Fusarium* sp.) exhibited antibacterial activity against *E. coli*, *K. pneumoniae*, *S. aureus*, *L. monocytogenes* and *E. faecalis* at 100µg/Disc concentration. Of these, QIEF 2 has significant antibacterial activity and the zone of inhibitions is 15.33±0.57 mm for *E. coli*, *K. pneumoniae* and *S. aureus*, 14.33±0.57 mm for *L. monocytogenes*, 16 mm for *E. faecalis*. All the fungal extracts from both the plants showed no antibacterial activity against the gram negative *Pseudomonas aeruginosa*. Generally, the extracts had good antibacterial activity against gram positive bacteria because of the structural differences (Denyer and Maillard, 2002).

Antibacterial activity of endophytic fungi suggested that it could be a potential source of antibacterial compounds (Kaul et al., 2012). Homogeneous endophytic fungi namely *Fusarium* sp., *Pestalotiopsis* sp. and *Colletotrichum* sp. isolated from the two plants *Plumeria acuminate* and *Plumeria obtusifolia* showed antibacterial activities against *S. aureus*, *E. coli*, *S. typhi*, *B. cereus*; but no antibacterial activity was observed against the gram negative *Pseudomonas aeruginosa* (Ramesha and Srinivas, 2014). The Crude extract of *Nigrospora sphaerica* isolated from the leaves of *Indigofera suffruticosa* was also active against the pathogens *E. coli*, *S. aureus*, *K. pneumoniae*, *B. subtilis* and *P. aeruginosa* (Santos et al., 2015). Eight endophytic fungal extracts of the Sri Lankan aquatic plant *Nymphaea nouchali* exhibited the antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus* and *Bacillus cereus* (Dissanayake et al., 2016). In accordance with the present work, the bark of *Cinnamomum mercadoi* holds endophytes like *Colletotrichum* sp., *Fusarium* sp., *Pestalotiopsis* sp., *Penicillium* sp., and sterile mycelia and their extracts showed the antibacterial activity against the selected gram positive and gram negative bacteria (Marcellano et al., 2017).

Endophytic fungi are important and alternative sources of novel anticancer metabolites (Chandra, 2012). In the current investigations, the extracts of endophytic fungi from *Elaeocarpus sphaericus* and *Quassia indica* were screened for the cytotoxic activity against K562 cell line by MTT assay. The endophytes ESEF 6 and ESEF 7 (*Xylaria* sp.), ESEF 9 (*Nigrospora* sp.) and ESEF 12 (*Pestalotiopsis* sp.) from *Elaeocarpus sphaericus* showed cytotoxic activity and the IC_{50} values are 7.80±0.18 µg/ml, 54.5±3.54 µg/ml, 84±2.83 µg/ml and 25.75±3.19 µg/ml respectively. QIEF 1 (*Daldinia* sp.) and QIEF 4 (*Lecanicillium* sp.), the two endophytic fungi identified from *Quassia indica* have the cytotoxic activity with IC_{50} values of 140±3.19 µg/ml and 83.80±1.77 µg/ml. MTT assay is used widely for the in vitro cytotoxic activity, since it describes the relation between the amount of active cells and absorbance at 50 % inhibition (Behera et al., 2003). The lower the IC_{50} values, the higher the potential to inhibit the cell proliferation. Literature showed evidence for similar endophytic fungi (*Xylaria* sp. and *Pestalotiopsis* sp.) isolated from tropical leaves displaying the cytotoxic activity against the human

melanoma cells (MDA-MB-435) and uterine cervical carcinoma cells (KB) (Casella et al., 2013). The commonly found medicinal plant, *Annona muricata*, holds endophytic fungi in their leaves. Of which the ethyl acetate extracts of endophytic fungi *Phomopsis* sp. showed the highest anticancer activity against breast cancer (MCF-7) cell lines with an IC_{50} value of 19.20 µg/ml (Minarni et al., 2017). Recently, the endophytic fungi of two medicinal plants *Enicostemma axillare* and *Ormocarpum cochinchinense* exhibited cytotoxicity against the breast cancer cell lines (Nagarajan and Pandian, 2018).

The endophytic fungus ESEF 6 (*Xylaria* sp.) having significant antibacterial and cytotoxic activities was selected in the study for metabolite isolation. After 21 to 28 days of growth, mycelium was extracted with different solvents and is mentioned as mycelia hexane (MH), mycelia ethyl acetate (MEA) and mycelia methanol (MH) extracts. The spend medium as broth was extracted with ethyl acetate and used as broth ethyl acetate (BEA) extract. BEA extract showed good zone of inhibition against the tested organisms and the zone of inhibitions are 12.66±0.57mm, 15mm, 15mm, 13.33±1.52mm and 13.66±1.15mm respectively for *E. coli*, *S. aureus*, *K. pneumoniae*, *L. monocytogenes* and *E. faecalis*. MH and MEA extract showed cytotoxic activity on K562 and HCT 116 cell lines; non toxic to L929 cell lines up to 10 µg/ml concentrations. The IC_{50} values of MH extract is 8.5±1.32 µg/ml and 5.5±1.5 µg/ml on K562 and HCT 116 cell lines respectively. The IC_{50} values of MEA extract is 3.5±0.08 µg/ml and 2.65±0.70 µg/ml on K562 and HCT 116 cell lines respectively. The cytotoxic activity of MEA extract was comparable with the standard curcumin. A similar sequential extraction method with hexane, ethyl acetate and methanol was used to isolate the metabolites from *Colletotrichum gloeosporioides* with antibacterial activity (Arivudainambi et al., 2011). Ethyl acetate was used for the successful extraction of bioactive compounds from endophytic fungi (Sowphartani and Kathiravan, 2011). Ethyl acetate extracts from *Xylaria* sp. had antibacterial activity against *E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis* and multi drug resistant *S. aureus* (Ramesh et al., 2012). *Xylaria* sp. was also active against human embryonic kidney cells (HEK293) and Jurkat cell lines with IC_{50} values of 2.94 µg/ml and 2.63 µg/ml respectively (Orachaipunlap et al., 2016).

In the present study, MEA and BEA extracts showed phenolic content and antioxidant capacity. BEA extract exhibited high phenolic content and potential antioxidant activity in DPPH assay, reducing power assay and total antioxidant capacity. Phenolics and flavonoids play the major role in antioxidant activity mainly in radical scavenging (Govindappa et al., 2013) and lipid peroxidation (Pawle and Singh, 2014). Antioxidants possess the biological applications like anti-inflammatory, anti-carcinogenic, anti-aging and anti-apoptosis. Free radical scavenging activity by using DPPH is the most common and accurate assay for the evaluation of antioxidants (Yadav et al., 2014) and reducing power assay resulted in the breaking of free radical through the donation of hydrogen atom (Ravindran et al., 2012). Methanolic extract of *Xylaria* sp. isolated from *Ginkgo biloba* displayed significant total phenolic and flavonoid content. Additionally, methanolic extract indicated strong antioxidant activity by DPPH assay and b-carotene–linoleic acid model system (Liu et al., 2007). *Xylaria feejeensis* HMJAU22039 isolated from *Tectona grandis* plant showed appreciable total phenolic content and were highly potent to antioxidant activity by DPPH and nitric oxide scavenging assay (Rebbapragada and Kalyanaraman, 2016). Fungal extract from *Xylaria* sp. of *Mussaenda luteola* showed the presence of phenolics and flavonoids. The extract was also potent to have high antioxidant property by DPPH and reducing power assay (Gunasekaran et al., 2017).

In the present study, both the BEA and MEA extracts were selected for the purification and characterization of the secondary metabolites having antibacterial and cytotoxic activities. The preliminary screening by MTT assay and thin layer chromatography are the methods used for detecting potential metabolites. In the preliminary screening BEA extracts showed the presence of flavonoids and carbohydrates while MEA extract showed the presence of phenolics, carbohydrates and steroids. In earlier, Gunasekaran et al., 2017, reported that the *Xylaria* sp. from the leaves of *Mussaenda luteola* indicated the presence of phenolics and saponins. The soxhlet extracts of petroleum ether, chloroform and ethanol exhibited the presence of sterols, terpenoids, alkaloids, phenols, tannins, flavonoids, and glycosides in qualitative phytochemical screening (Patel and Krishnappa, 2017).

In the present investigation, silica gel column chromatography with silica of mesh size 60-120 for MEA and BEA extracts was done by using chloroform: methanol elution. The fractions on elution with chloroform: methanol (90:10) from MEA extract showed cytotoxic activity while one fraction on elution with chloroform: methanol (90:10) from BEA extract displayed antibacterial activity. The active fractions were analyzed on TLC and the bands were scraped out and dissolved in chloroform/ methanol. Further, the active bands were again analyzed on TLC plates to identify the metabolites. Previously, Wang et al., 2012, reported that the endophytic fungus *Chaetomium globosum* from the medicinal plant, *Curcuma wenyujin* was extracted with ethyl acetate and was subjected to column chromatography by using silica gel of 100-200 mesh for the isolation of bioactive metabolites (Wang et al., 2012). Likewise, the endophyte *Botryospheria obtuse* of the medicinal plant *Bidens pilosa* was extracted with ethyl acetate and silica gel column chromatography was performed with mesh size of 230-400 and by eluting with hexane and ethyl acetate as solvent system (Abdou et al., 2010). *Alternaria* sp. from *Broussonetia papyrifera* was extracted with ethyl acetate and subjected to silica gel column chromatography with 100-200 mesh size for the elution of metabolites having cytotoxic activity (Zhang et al., 2016).

Thin layer chromatography was employed to separate the metabolites based on the polarity of solvents and the spraying reagents were used to detect the secondary metabolites. Thin layer chromatogram of the present study showed 5 different bands in MEA extract observed under UV at 254 nm and 365 nm. Whereas BEA extracts showed 4 bands in UV at 254 nm and 6 bands at 365 nm by using chloroform: methanol (7:3) as mobile phase. The extracts showing positive towards the FBS reagent spraying with Rf 0.8 suggested the presence of phenolics. The extracts showed the band with Rf 0.45 was highlighted by spraying it with anisaldehyde sulphuric acid reagent that may react with terpenoids/saponins/ carbohydrates/steroids. TLC profile of partially purified fractions showed a single band with Rf 0.83 under visible light and it showed the positive response to the anisaldehyde spraying that exhibited the presence of terpenoids/saponins/ carbohydrates/steroids. Previous reports showed that ethyl acetate extracts of

endophytic fungi isolated from *Coleus amboinicus* employed TLC and spraying techniques were employed to detect the chemical groups of the bioactive compounds (Astuti et al., 2014). Different endophytic fungal extracts from *Costus spiralis* identified different classes' of secondary metabolites by using TLC with different spraying reagents (Ascencio et al., 2014). Parptiwi et al., 2018, reported the metabolite analysis of ethyl acetate extracts of endophytic fungi from medicinal plants using TLC and spraying reagents for vanillin and cerium to detect the bioactive compounds. TLC profile of column eluted fraction from the endophytic fungus *Diaporthe* sp. showed a single band of Rf 0.65 with blue fluorescence under UV at 366 nm, identified as the phenolic compound (Patil et al., 2017). Ranjan and Jadeja, 2017, purified the compound from the endophytic fungus *Micrococcus yunnanensis* based on chromatography followed by TLC using combinations of chloroform and methanol as mobile phase and purified compound was detected with Rf 0.26 on thin layer chromatography.

High-performance thin layer chromatography is the extended form of TLC. HPTLC profile of MEA extract detect quantitative analysis of metabolites with high resolution and it showed the presence of 4, 7 and 5 bands under visible light, UV at 254 nm and 366 nm respectively whereas, BEA extracts showed the presence of 1, 4 and 2 bands under visible light, UV at 254 nm and 366 nm respectively. Chromatogram derivatization using MEA and BEA extracts and partially purified active fractions scraped out from TLC plates displayed the violet/blue colour bands exhibiting the presence of saponins/carbohydrates/steroids/terpenoids and the brown colour bands showed the presence of phenolics only in BEA and MEA extracts. Earlier report showed that ethyl acetate extract of *Penicillium* sp. from *Centella asiatica* was analysed the metabolite alkaloids by using HPTLC analysis and visualized the bands with corresponding Rf, an orange/brown coloured band on derivatization displayed the presence of alkaloids (Devi et al., 2012).

In the present investigation, the purified active fraction (scraped out from the TLC plates) of BEA extracts exhibited the antibacterial activity against both the gram positive and gram negative bacteria like *S. aureus*, *K. pneumoniae*, *L.*

monocytogenes and *E. faecalis*. The zone of inhibition was 18.66±0.57 mm, 13.33±0.57 mm, 14.66±0.57 mm, 16.66±0.57 mm respectively. Endophytic fungi are capable of producing antimicrobial substances as secondary metabolites with potential antibacterial activity (Rathnaweera et al., 2015). The metabolites analysed in TLC spraying displayed the presence of carbohydrates/saponins/terpenoids/steroids that may provide the antibacterial activity. Formerly, from the fruiting bodies of the endophytic fungus *Xylaria* sp. 101, one new diterpenoid- xylarenolide and three sesquiterpenoids- xylaranol A, xylaranol B and xylaranic acid were isolated and these compounds have good zone of inhibition against the *E. coli*, *S. aureus*, *Bacillus pumilus* and *Bacillus subtilis* (Li et al., 2010). Helvolic acid, a nortriterperpenoid was isolated and identified from the leaves of *Anoectochilus setaceus*. The compound Helvolic acid was active against the gram positive bacteria *Bacillus subtilis* and the methicillin resistant *S. aureus* (Rathnaweera et al., 2014).

Cytotoxicity of the partially purified active fraction from MEA extract on K562, HCT 116 and L929 cell lines were evaluated by MTT assay. The active fraction showed the IC_{50} values 1.04±0.29 µg/ml and 3.65±0.83 µg/ml on K562 and HCT 116 cell lines respectively. But on L929 cells the fraction showed a moderately higher IC_{50} value 7.71±0.005 µg/ml. The cytotoxic activity of the partially purified fractions was comparable with the standard curcumin and the active fraction was highly potent than Curcumin. *Xylaria* sp. explored as the source of wide range of bioactive secondary metabolites with cytotoxic applications. The bioconstituents analysis by TLC and its spraying showed the presence of a class of metabolites like terpenoids, steroids, carbohydrates or saponins, which may be responsible for the cytotoxic activity. Previously, 7 types of sesquiterpenes known as eremophilane along with the familiar mairetolide F were purified from the endophytic fungus *Xylaria* BCC 21097. From the sesquiterpenes, compounds 2 and 3 revealed the average cytotoxic activities on lung, oral and breast cancer cell lines (Isaka et al., 2010). The endophytic fungus *Xylaria* sp. from the leaves of *Curcuma xanthorrhiza* also possessed three compounds including two new and resacetophenone compounds and was not cytotoxic against murine cancer cell line L5178Y by MTT assay (Hammerschmidt et al., 2015). Similarly from the endophytic fungus *Xylaria*

sp. PSU-H182, five new compounds identified including three dimeric chromaones, one benzamide and cyclohexenone along with four known compounds. The compounds were not much cytotoxically active on breast cancer and uterine cervical carcinoma cell lines (Maha et al., 2016).

The novel polyketides were also isolated from the *Xylaria* sp. NCY2 namely, methyl xylariate C, 1-(xylarenone A) xylariate A, xylarioic acid B, xylariolide A, xylariolide B, xylariolide C, and xylariolide D and an already identified taiwapyrone. The above mentioned compounds were active against the gram positive *S. aureus*, *B. subtilis* and gram negative *E. coli*. These compounds exhibited very low level of cytotoxic activity against the cervical and liver cancer cell lines (Hu et al., 2010). Different types of compounds were purified and identified from various *Xylaria species*. Seven new metabolites including three sesquiterpenoids- xylaritriol, 10-hydroxythujopsene and akotriol, one aliphatic derivative- akoenic acid, one alkaloid-akodionine, and one isocoumarin- akolitserin and 7 known compounds from *Xylaria cubensis* (Fan et al., 2014). From *Xylaria papulis*, four triterpenoids (3-trans-p-coumaroyloxy2α,23-dihydroxyolean-12-en-28-oic acid, chebuloside *I*, arjunolic acid, and taraxerol), papupyrrolal, the pyrrole derivative and flavanoid metabolites myricitrin, quercitrin and myricetin were identified (Cheng et al., 2015) but the cytotoxic activity of the compounds from both *Xylaria cubensis* and *Xylaria papulis* were not reported.

In present investigations, HPLC analysis of the partially purified active fraction (scraped out from TLC plates) of MEA and BEA extracts with antibacterial and cytotoxic activities displayed the retention time of 4.989 min and 4.196 min respectively. Further, the HR-MS profile of partially purified MEA and BEA fractions showed the peaks with corresponding m/z values. The m/z value will be almost similar as the molecular weight of the metabolite present in the active fractions. HR-MS analysis of MEA active fraction showed the peaks with m/z of 153.127, 261.1125, 337.198, and 437.194, 453.22 with relative abundance of 7, 12, 20, 15 and 100 respectively. BEA active fraction showed peaks of m/z 217.069, 249.110, 274.999, 277.178, 279.194, 293.173, 295.189, 321.168, 323.184, 325. 199, 339.179, 357.188 and 395.24 with relative abundance of 11, 5, 13, 43, 100, 71, 50,

18, 69, 86, 65, 11 and 5 respectively. Identical fungus, *Xylaria* sp. isolated from *Vitis labrusca* and the compounds was analyzed in HPLC and displayed, diplosporin with retention time 15.5 min and agistatine derivatives, coriloxin with retention time 6.5 min and 5-carboxy mellein with a retention time 15.7 min (Ibrahim et al., 2014). The compounds cytochalasin D and piliformic acid from two *Xylaria* sp. of Guarana plant was analyzed by chromatographic techniques HPLC (Elias et al., 2018).

HR-LCMS/MS analysis of cytotoxic active partially purified MEA fraction showed four peaks with m/z value similar to HR-MS analysis. The database search of these compounds reported the molecular formula, MS/MS analysis and structure. Of these, the compound with mass 336.1906 is detected as an uncharacterized compound. The 3 detected compounds are neral, dihydrospatheliachromone and a derivative of vitamin D3 (1 alpha, 25-dihydroxy-26, 27-dimethyl-20, 21, 22, 22, 23, 23-hexadehydro-24a-homovitamin D3). The compound neral is also named as citral, lemonal or 3,7-dimethyl-2,6-octadienal. The double bond isomers formed with citral are named as citral A/ geranial or citral B/neral depending upon the E or Z form. Previous reports of Rosecke et al., 2000, identified the compound neral produced by the fungus *Gleophyllum odoratum* isolated from its habitat - sechsenwald forest near to Hamburg. Identical compound was also isolated from the endophytic fungus *Ganoderma lucidum*, a saprophyte on deciduous trees (Ziegenbein et al., 2006). The second detected compound was dihydrospatheliachromone which is also known as isopeucenin. They belong to the group of secondary metabolites called chromenes. An earlier report of peucenin isolation from rhizomes of *Peucedanum ostruthium* was described by Joa et al., 2011. 1alpha,25-dihydroxy-26, 27-dimethyl-20, 21, 22, 22, 23, 23-hexadehydro-24a-homovitamin D3 was the third known compound. It was identified as vitamin D3 derivative. Evidences from literature revealed that fungi like mushrooms are capable of producing D3 (Keegan et al., 2013).

The two medicinal plants selected for endophytic fungi isolation was also evaluated for the phytochemical constituents, antibacterial and cytotoxic activities. *Elaeocarpus sphaericus*, the folk medicinal plant used in Ayurveda, Siddha and Unani. It also possesses good medicinal and biological properties. The second medicinal plant *Quassia indica* is used as analgesic, anti-inflammatory, antifeedent

and antimicrobial agents. The majority of the phytoconstituents of the leaves extracts from *Elaeocarpus sphaericus* showed the presence of steroids in petroleum ether extract while, the presence of steroids, carbohydrates and saponins were seen in chloroform extract. Ethyl acetate extract exhibited the presence of alkaloids, carbohydrates, tannins, steroids and phenolics. The methanol extract showed the presence of alkaloids, flavonoids, phenolics, and terpenoids. Aqueous extracts showed the presence of majority of the phytoconstituents. The second plant exhibited flavonoids in the petroleum ether extract where as the alkaloids, flavonoids, phenolics and steroids were present as the common phytoconstituents in both chloroform and ethyl acetate extracts. Most of the phytoconstituents analyzed were present in methanol and aqueous extracts.

Elaeocarpus ganitrus is the synonym of *Elaeocarpus sphaericus*. Previous research on *Elaeocarpus ganitrus* was done by Pandey et al., 2016. The leaves were extracted with the solvents, petroleum ether, chloroform, methanol, acetone and aqueous extracts and were positive to the phytoconstituents like saponins, terpenoids, alkaloids, steroid, and flavonoids. Comparable results were also obtained in methanolic and acetone extracts with the presence of phytochemicals like steroids, phenols, flavonoids, tannin and glycosides in methanolic and acetone extracts (Dalei and Sahoo, 2016). Phytochemical screening of ethyl acetate extract from the bark of *Elaeocarpus sphaericus* hold the metabolites like tannins, flavonoids, triterpenoids, carbohydrates and glycosides (Talukdar et al., 2016). In accordance with the present study, similar observations were recorded by Anusha and Sudha Bai, 2017. They have successively extracted phytoconstituents from leaves and bark of *Quassia indica* and it contained majority in chloroform, methanol and aqueous extracts but petroleum ether extract had little amount of phytoconstituents. Likewise, Meghna et al., 2018, have reported the presence of flavonoid content in petroleum ether extract of *Quassia indica* leaves. The quantitative analysis of phytoconstituents from both the plants was also screened by using HPTLC analysis and the profile showed the high resolution of bands with the corresponding Rf.

The different leaves extracts- petroleum ether, chloroform, ethyl acetate, methanol and aqueous extracts from both *Elaeocarpus sphaericus* and *Quassia*

indica displayed no antibacterial activity against *E. coli*, *K. pneumoniae*, *S. aureus*, *L. monocytogenes*, and *E. faecalis* tested up to 1 mg/ml concentrations by disc diffusion method. In earlier reports, antibacterial activity of petroleum ether, chloroform, methanol, ethanol and acetone extracts of *Elaeocarpus ganitrus* was done by disc diffusion method. The petroleum ether extract showed no activity against *S. aureus*, *B. subtilis* and *E. coli*. The result was in correlation with the present study whereas methanol, ethanol and acetone extracts showed the antibacterial activity against *B. subtilis*, *S. aureus* and *E. coli* (Pandey et al., 2016). The results in the present study were controversy with the previous reports Rao et al., 2011 and Talukdar et al., 2016. Their results revealed that the aqueous extracts from the leaves of *Elaeocarpus ganitrus* had exhibited the zone of inhibition against *S. aureus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* and ethyl acetate extracts from the bark of *Elaeocarpus ganitrus* showed the maximum inhibition against *E. coli*, *Pseudomonas* and *Klebsiella* sp. Reports of Aiswarya and Pushpalatha, 2017, showed the antibacterial activity of different solvent extracts from the leaves of *Quassia indica* and *Centella asiatica*. Additionally, the methanolic extracts of leaves and bark from *Quassia indica* against *S. aureus* and *E. coli* were displayed a moderate antibacterial activity and comparatively leaf extract had higher antibacterial potential (Anusha and Sudha Bai, 2017).

In the present study, the petroleum ether, chloroform and ethyl acetate extracts from *Quassia indica* on K562 cell line exhibited the cytotoxic activity with IC_{50} values 66±4.82 µg/ml, 33.5±3.81 µg/ml and 26.2±4.07 µg/ml; other leaves extracts were non cytotoxic up to 300µg/ml the concentrations. Whereas, all the solvent extracts from *Elaeocarpus sphaericus* were non cytotoxic to K562 cell line. Reports from earlier studies showed that the aqueous and methanolic extracts of leaf and seed from *Quassia indica* exhibited the cytotoxic activity on leukaemia cell line and the viability was higher in seed methanolic extract (Bhat, 2017).

The present investigation showed the significant variation in antibacterial and cytotoxic activity compared to previous reports. This may be due to the variations in phytochemicals produced in the plant in response to the environmental factors such as temperature, rainfall, soil composition and altitude (Monteiro et al.,

2006). Phytochemicals represent the chemical interface in connection with the plants and environment (Gobbo-Neto and Lopes, 2007). The production of phytochemicals and its metabolism is either directly or indirectly influenced by the environmental factors and the accumulation of phytoconstituents was closely related to the location of the plant (Liu et al., 2015).

Endophytic fungi isolated from *Elaeocarpus sphaericus* and *Quassia indica* were also screened for the production of IAA. It is the most important naturally occurring auxin essential for the growth and development of plants. Auxins help in the embryogenesis, vascular pattern formation, growth of seedlings, flower development and other developmental processes by employing tryptophan as the precursor (Zhao, 2010). The present study also used tryptophan as the precursor for the production of IAA. The ethyl acetate extract of selected isolates produced IAA in the presence and absence of tryptophan; IAA production increase with increase in tryptophan concentrations. Among the isolates, QIEF 5 (*Colletotrichum* sp.) showed the highest IAA production as 253.69±4.39 µg/ml with 5 mg /ml of tryptophan supplement. In 1 mg/ml and 2 mg/ml tryptophan supplement, IAA production was 220.71±13.7 µg/ml and 221.24±17.5 µg/ml respectively. The results are in agreement with Munasinghe et al., 2017. Here the ethyl acetate extract of the fungal isolate *Colletotrichum* sp. isolated from the *Piper nigrum* leaves produced IAA. Fouda et al., 2015, isolated *Penicillium chrysogenum*, *Alternaria alternate* and sterile hyphae associated with the medicinal plant *Asclepias sinaica* which produced IAA at different concentrations of tryptophan (0 to 5 mg/ml). Similarly, Hassan, 2017, isolated bacterial and fungal endophytes from the medicinal plant *Teucrium polium*, that promote the plant growth by producing IAA at different concentrations of tryptophan from 0 to 5 mg/ml. The 22 endophytic bacterial isolates of *Musa* sp. also exhibited the production of IAA in the presence and absence of tryptophan. Among the isolates, *Stenotrophomonas maltophilia* had the maximum IAA production (Ambawade and Pathade, 2013).

The phytohormones produced by the microorganisms are extracellular and are highly influenced by the physical and chemical parameters such as the growth media, incubation time, temperature and pH. *Colletotrichum* sp. (QIEF 5) produced

maximum IAA in PDB media with alkaline pH in 10 days at room temperature. Correspondingly, the physico-chemical parameters of the fungus *Pleurotus ostreatus* have maximum IAA production by 18 days of incubation at 30^{o}C under shaking conditions supplemented with 0.1 % tryptophan (Bose et al., 2013). Moreover, bacterial and fungal isolates of *Teucrium polium* produced IAA in different time period of 2 to 14 days and the IAA production increased with increase in incubation time (Hassan, 2017).

In this work, the presence of IAA in QIEF 5 (*Colletotrichum* sp.) was identified and characterized by TLC, HPLC and HR-MS profile. In TLC, the Rf value of 0.769 corresponds to the standard IAA; HLPC and HR-MS analysis of QIEF 5 had close similarity with the peaks of standard IAA. HPLC profile showed retention time 5.24 min and 5.361 min for QIEF 5 and standard IAA respectively. HR-MS profile of QIEF 5 showed the major peaks of m/z values 423.20, 437.25, 453.24 which correspond to the standard IAA. The results are in accordance with the study of Hoffman et al., 2013, in endohyphal bacterium *Luteibacter* sp. present in the foliar endophytic fungi *Pestalotiopsis* sp. The endophyte-bacterium complex produced IAA and was characterized by TLC and HPLC analysis. The retention time of both the standard and endophyte-bacterium complex IAA was almost similar. *Moringa peregrina* holds the endophytic bacteria *Bacillus subtilis* that produced IAA in 14 days of growth and was characterized by UPLC/MS-MS analysis and compared with the standard IAA (Khan et al., 2016). The culture supernatant of the endophyte *Pleurotus ostreatus* of Jatropha seedcake produced IAA with tryptophan supplementation at 0.1 % which was characterized by TLC and GC-MS analysis. Thin layer chromatogram with an Rf 0.33 and GC-MS with retention time 19.57 min corresponded to the standard IAA (Bose et al., 2013).

Endophytic fungi promote the growth of the plants mainly through the production of phytohormone indole acetic acid (IAA) (Bal et al., 2013). In the present study, the endophyte QIEF 5 (*Colletotrichum* sp.) isolated from *Quassia indica* possess the plant growth promoting indole acetic acid and the fungus isolate influence the enhancement of shoot and root length of rice seedlings. In addition, the

aerial roots and weight of the seedlings inoculated with the fungus was more than the control seedlings. To summarise, QIEF 5 promotes the growth of rice seedlings by the secretion of plant growth regulator IAA. Related reports of Fouda et al., 2015, showed the ability of the fungal endophytes *Alternaria alternata* and the sterile hyphae promote the root growth of the *Zea mays* compared to the control. The effect of endophyte inoculation on varied root growth of the seedlings may be mediated by the IAA production. The bacterial endophytic strain, *Bacillus subtilis* isolated from *Moringa peregrina* raised the growth of tomato seedlings by increasing the root length, shoot length, weight of the seedlings and chlorophyll content (Khan et al., 2016). Fungal endophytes (*Penicillium chrysogenum* and *Penicillium crustosum*) and the bacterial isolates (*Bacillus subtilis* and *Bacillus cereus*) consortiums improve the root growth of maize seedlings, whereas the biomass production was higher in fungal endophytes and its consortium (Hassan, 2017).

Chapter 6

SUMMARY AND CONCLUSIONS

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The medicinal plants *Elaeocarpus sphaericus* and *Quassia indica* were selected for the isolation of endophytic fungi. A total of 203 endophytic fungi were isolated from 122 leaf segments of *Elaeocarpus sphaericus*. Of these, 12 morphologically different endophytic fungi were selected and given unique code of ESEF 1 to ESEF 12. From *Quassia indica*, 139 endophytic fungi were isolated from 110 leaf segments and 10 morphologically different endophytic fungi were selected; fungal isolates were given unique code QIEF 1 to QIEF 10.

Identification of fungi was based on sporulating characteristics and molecular techniques. The morphological observations of each isolates were different from others. Of the selected endophytic fungal isolates from both the plants, *Pestalotiopsis* sp. and *Fusarium* sp. were identified based on the spores produced and remaining isolates could not be identified due to the lack of spore production. Molecular technique- ITS sequencing, identified all the isolates based on the similarity search in BLAST analysis and the sequences were submitted to GenkBank. The genus of the endophytic fungi is mentioned in the study. The identified isolates from *Elaeocarpus sphaericus* are *Xylaria* sp. (ESEF 1, ESEF 2, ESEEF 6 and ESEF 7), *Fusarium* sp. (ESEF 3), *Diaporthe* sp. (ESEF 4 and ESEF 8), *Endomelanconiopsis* sp. (ESEF 5), *Nigrospora* sp. (ESEF 9, ESEF 10 and ESEF 11) and *Pestalotiopsis* sp. (ESEF 12). The identified isolates from *Quassia indica* are *Daldinia* sp. (QIEF 1), *Diaporthe* sp. (QIEF 2), *Phyllosticta* sp. (QIEF 3), *Lecanicillium* sp. (QIEF 4), *Colletotrichum* sp. (QIEF 5 and QIEF 6), *Fusarium* sp. (QIEF 7 and QIEF 10), *Pestalotiopsis* sp. (QIEF 8), and *Meyerozyma* sp. (QIEF 9). Phylogenetic analysis of identified endophytic fungi was positioned into various taxa and it revealed the evolutionary relationship with the existing endophytic fungi. Both *Elaeocarpus sphaericus* and *Quassia indica* have diversity in endophytic fungi. Shannon-weiner diversity index of *Elaeocarpus sphaericus* and *Quassia indica* was 2.248 and 1.943 respectively. Among the isolates, *Diaporthe* sp. (ESEF 4 and QIEF 2) has the highest colonization frequency in both the plants; 20.49 % for *Elaeocarpus sphaericus* and 20.90 % for *Quassia indica* and this fungus may be abundant in these two plants.

All the fungal extracts were screened for antibacterial and cytotoxic activities. The fungal extracts of ESEF 4 to ESEF 8 and ESEF 10, QIEF 2, QIEF 3, QIEF 6 and QIEF 7 possess antibacterial activity. Among these isolates, ESEF 6 (*Xylaria* sp.) and QIEF 2 (*Diaporthe* sp.) exhibited good zone of inhibition. The diameter zone of inhibition of fungal extract of ESEF 6 was 13 mm, 13.66±0.57 mm, 13.66±0.57 mm and 14.33±0.57 mm for *E. coli*, *K. pneumoniae*, *S. aureus* and *L. monocytogenes* respectively; zone of inhibition of the positive control was 19.66±0.57 mm, 19.33±0.57 mm, 20.33±0.57 mm and 17±1 mm for *E. coli*, *K. pneumoniae*, *S. aureus* and *L. monocytogenes* respectively. The zone of inhibition of fungal extract of QIEF 2 was 15.33±0.57 mm for *E. coli*, *K. pneumoniae* and *S. aureus*, 14.33±0.57 mm and 16 mm for *L. monocytogenes* and *E. faecalis* respectively. Positive control showed the diameter zone of inhibition 19 mm, 19.66±0.57 mm, 20.33±0.57 mm, 17.33±2.51 mm and 19.33±0.57 mm for *E. coli*, *K. pneumoniae*, *S. aureus*, *L. monocytogenes* and *E. faecalis* respectively. The fungal extracts of ESEF 6, ESEF 7, ESEF 9, ESEF 12, QIEF 1, and QIEF 4 have cytotoxic activity on K562 cell line. Of these, ESEF 6 (*Xylaria* sp.) has the least IC_{50} value 7.8±0.18 µg/ml. IC_{50} value of curcumin was 12.5±0.08 µg/ml. Based on both antibacterial and cytotoxic activities, ESEF 6 was selected for further isolation and characterization of metabolites produced with antibacterial and cytotoxic activities.

ESEF 6 (*Xylaria* sp.) after 21 to 28 days of growth, mycelium and broth was extracted with different solvents and the extracts were designated as mycelia hexane (MH), mycelia ethyl acetate (MEA), mycelia methanol (MM) and broth ethyl acetate (BEA) extracts. Further, BEA extract possess antibacterial activity against the tested gram positive and gram negative bacteria. The diameter zone of inhibition was 12.66±0.57 mm, 15 mm, 15 mm, 13.33±1.52 mm and 13.66±1.15mm respectively for *E. coli*, *S. aureus*, *K. pneumoniae*, *L. monocytogenes* and *E. faecalis*. Positive control showed the diameter zone of inhibition as 20 mm, 20mm, 20.33±0.57 mm, 18±1.73 and 20 mm for *E. coli*, *S. aureus*, *K. pneumoniae*, *L. monocytogenes* and *E. faecalis* respectively. The partially purified active fraction (after column chromatography followed by scraped out from TLC plates) from BEA extract displayed antibacterial activity against *K. pneumoniae*, *S. aureus*,

L. monocytogenes and *E. faecalis*. The zone of inhibition was 18.66±0.57 mm, 13.33±0.57 mm, 14.66±0.57 mm and 16.66±0.57 mm respectively. Positive control have zone of inhibition as 20 mm, 20.33±0.57 mm, 18±1.73 mm and 20 mm respectively for *K. pneumoniae*, *S. aureus*, *L. monocytogenes* and *E. faecalis*.

In secondary metabolite analysis, BEA extract showed the presence flavonoids and carbohydrates. Besides, the extract and the partially purified active fraction (scraped out from TLC plates) were positive to FBS spraying showing the presence of phenolics and anisaldehyde spraying showed the presence of terpenoids/steroids/saponins/carbohydrates. HPTLC analysis of these extracts displayed the number of spots, Rf and the corresponding area %. Post chromatographic derivatization in HPTLC analysis confirmed the presence of phenolics and terpenoids/steroids/saponins/carbohydrates. HPLC, HR-MS analyses were used to characterize the partially purified active extract fraction of BEA extracts. HPLC analysis showed retention time 4.196 min. HR-MS analysis of BEA active fraction showed peaks of m/z 217.069, 249.110, 274.999, 277.178, 279.194, 293.173, 295.189, 321.168, 323.184, 325. 199, 339.179, 357.188 and 395.24 with relative abundance of 11, 5, 13, 43, 100, 71, 50, 18, 69, 86, 65, 11 and 5 respectively.

MEA extract of ESEF 6 have cytotoxic activity on K562 and HCT 116 cell lines. The IC_{50} values are 3.5±0.08 µg/ml on K562 cell line and 2.65±0.70 µg/ml on HCT 116 cell line. Curcumin showed the IC_{50} value as 12.5±0.08 µg/ml on K562 cell line and 21.83±1.60 on HCT 116 cell line. Partially purified active fraction (after column chromatography followed by TLC scraped out) from MEA extract exhibited cytotoxic activity on K562, HCT 116 and L929 cell lines, IC_{50} values are 1.04±0.29 µg/ml, 3.65±0.83 µg/ml and 7.71±0.005 respectively. Curcumin exhibited the IC_{50} value as 10±1.03 µg/ml, 20±1.25 µg/ml and 10±0.003 µg/ml on K562, HCT 116 and L929 cell lines respectively.

MEA extract showed the presence of phenolics, steroids and carbohydrates. Moreover, the extracts were positive to FBS spraying showed the presence of phenolics and anisaldehyde spraying showed the presence of terpenoids/steroids/

saponins/carbohydrates. HPTLC analysis of these extracts displayed the number of spots, Rf and the corresponding area %. Post chromatographic derivatization in HPTLC analysis confirmed the presence of phenolics and terpenoids/steroids/ saponins/carbohydrates. Further, HPLC, HR-MS and HR-LCMS/MS analysis were used to characterize the partially purified active fractions. HPLC analysis showed retention time 4.989 min. HR-MS analysis showed the peaks with m/z of 153.127, 261.1125, 337.198, and 437.194, 453.22 with relative abundance of 7, 12, 20, 15 and 100 respectively. HR-LCMS/MS analysis followed by data base search detected 3 compounds based on the similar m/z value in HR-MS analysis. The detected compounds are neral, dihydrospatheliachromone and a derivative of homovitamin D3 (1alpha, 25-dihydroxy-26, 27-dimethyl-20, 21, 22, 22, 23, 23-hexadehydro-24a-homovitamin D3).

Both BEA and MEA extracts possess potential antioxidant properties specifically, DPPH radical scavenging activity, reducing power assay and total antioxidant activity by phosphomolybdenum method. The BEA extract possess highest antioxidant activity.

Phytochemical screening of *Elaeocarpus sphaericus* leaves extracts showed the presence of major phytoconstituents. Steroids were present in petroleum ether extract where as steroids, carbohydrates and saponins were present in chloroform extract. Ethyl acetate extract exhibited the presence of alkaloids, tannins, phenolics, carbohydrates and steroids. Majority of the phytoconstituents were present in methanol and aqueous extracts. In *Quassia indica* leaves extracts, flavonoids were present in petroleum ether extract where as alkaloids, flavonoids, terpenoids, steroids, carbohydrates and saponins were present in chloroform extract. Ethyl acetate extract exhibited the presence of alkaloids, flavonoids, phenolics, and steroids. Majority of the phytoconstituents were present in methanol and aqueous extracts. HPTLC analysis of these extracts displayed the number of bands, Rf and the corresponding area %. In *Elaeocarpus sphaericus*, 4 and 6 bands in petroleum ether extract, 10 and 11 bands in chloroform extract, 8 and 12 bands in ethyl acetate extract and 4 and 5 bands in methanol extract were observed under UV at 254 nm and 366 nm respectively. In *Quassia indica*, 12 and 9 bands in petroleum ether

extract, 12 and 10 bands in chloroform extract, 11 and 9 bands in ethyl acetate extract and 4 and 3 bands in methanol extract were observed under UV at 254 nm and 366 nm respectively. Solvent extracts from both the plants have no zone of inhibition against the gram positive and negative organisms tested at 1 mg/ml concentrations. Petroleum ether, chloroform and ethyl acetate extracts from *Quassia indica* exhibited cytotoxic activity on K562 cell line. IC_{50} values are 66±7.08 µg/ml, 33.5±3.54 µg/ml and 26.2±0.71 µg/ml respectively. All other extracts from both the plants exhibited no cytotoxic activity up to 300µg/ml concentrations.

These results suggest that the antibacterial and cytotoxic activities exhibited by the BEA and MEA extracts respectively are solely due to fungal metabolites. While, the plant *Elaeocarpus sphaericus* where in the endophyte ESEF 6 isolated was failed to show the corresponding activities in their leaves extracts. Apart from this, leaves extracts from *Quassia indica* showed cytotoxic activity.

Endophytes from both the plants were evaluated for IAA production. Out of the 22 isolates, *Diaporthe* sp. (ESEF 4 and ESEF 8) and *Xylaria* sp. (ESEF 6) of *Elaeocarpus sphaericus*; *Colletotrichum* sp. (QIEF 5 and QIEF 6) and *Fusarium* sp. (QIEF 7) of *Quassia indica* were able to produce IAA at different concentrations of tryptophan. Highest IAA production was seen in QIEF 5 at different concentrations of tryptophan supplementations. The optimized condition of IAA production from QIEF 5 was in PDB media supplemented with 1mg/ml tryptophan in alkaline condition at room temperature for 10 to 15 days. TLC, HPLC and HR-MS analysis revealed the presence of IAA production from QIEF 5 and which was authenticated by standard IAA as reference.

TLC analysis of IAA from QIEF 5 showed the band at Rf 0.769 with standard IAA as reference and the band showed a colour change to pink on spraying it with salkowski reagent. HPLC analysis of both QIEF 5 and standard IAA showed the peaks with almost same retention time. The retention time of QIEF 5 and standard IAA was 5.24 min and 5.361 min respectively. HR-MS analysis showed the major peaks of mass 423.20, 437.25 and 453.24 which correspond to the standard IAA.

QIEF 5 promotes the growth of rice seedlings. Shoot length and root length of QIEF 5 incubated rice seedlings were 7.60±0.54 cm and 13.66±0.38 cm whereas, the control seedlings (without fungal inoculation) have 6.54±0.29 cm of shoot length and 10.18±0.29 cm of root length. QIEF 5 incubated rice seedlings were higher in growth when compared to the control (without fungal inoculation). Moreover, the average weight of the seedlings and the numbers of aerial roots were higher in QIEF 5 compared to control. Fresh weight and dry weight of QIEF 5 incubated seedlings were 70.56±10.84 mg and 10.34±0.27 mg respectively.

Consequently investigations in the present study revealed that *Xylaria* sp. from *Elaeocarpus sphaericus* produces metabolites with antibacterial, antioxidant and cytotoxic activities. Future studies are needed to isolate and characterize the active principles responsible for the antibacterial, antioxidant and cytotoxic activities and such other favourable activities/applications. Moreover, the studies are needed to prove its efficiency and safety at the next level- animal model studies to eventually become useful for biological applications. *Colletotrichum* sp. from *Quassia indica* produces the plant growth regulator IAA and the fungus influenced the increases in growth of rice seedlings. Thus, the endophytic fungus can be authorized to use in agricultural applications as a biofertilizer. However, biochemical and molecular studies are mandatory in elucidating the role of endophytic fungus on promotion of plant growth. Besides, the beneficial role of the fungus still needs to be investigated under field trials.

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APPENDIX

APPENDIX

Extraction Buffer

CTAB	: 2 %
Tris Cl	: 100 mM
NaCl	: 1.4 M
EDTA	: 20 mM

TE Buffer (pH 8.0)

Tris Cl	: 10 mM
EDTA	: 0.1 mM

Tris acetate EDTA (TAE) buffer

Tris base	: 40 mM
EDTA	: 2 mM
Glacial acetic acid	: 20 mM

Gel loading buffer

Bromophenol blue	: 0.25 %
Glycerol	: 30 %

Stored at 4 °C

Genei marker (100bp)

DNA marker	: 1 µl
6X gel loading buffer	: 3 µl
Sterile distilled water	: 12 µl

Dragendorff reagent (Solution A + solution B (1:1))

Solution A

Basic bismuth nitrite	: 0.85 gm
Glacial acetic acid	: 10 ml
Water	: 40 ml

Solution B

Potassium Iodide	: 8 gm
Water	: 30 ml

Anisaldehyde reagent

Glacial acetic acid	: 10 ml
Anisaldehyde	: 0.5 ml
Methanol	: 85 ml
Con. Sulphuric acid	: 5 ml

Fast blue salt reagent

Fast blue salt B	: 0.5 gm in 100 ml

Spray with FBS reagent follwed by 10 % ethanolic NaOH

Salkowski reagent

Con. Sulphuric acid	: 300 ml
Distilled water	: 500 ml
Ferric chloride ($FeCl_3$)	: 15 ml (0.5M)

McFarland standard (0.5)

1.175 % Barium chloride dihydrate	: 0.05 ml
1 % H_2SO_4	: 9.95 ml

RPMI 1640 medium

RPMI 1640 powder (Himedia)	: 10.4 gm/L
FBS	: 10% v/v
$NaHCO_3$	: 2 gm/L
Streptomycin	: 100 µg/ml
Penicillin	: 100 U/ml

DMEM (Dulbeccos's modified eagle medium)

DMEM powder (Himedia)	: 10 gm/L
FBS	: 10% v/v
$NaHCO_3$	: 3.7 gm/L
Streptomycin	: 100 µg/ml
Penicillin	: 100 U/ml

ANNEXURE

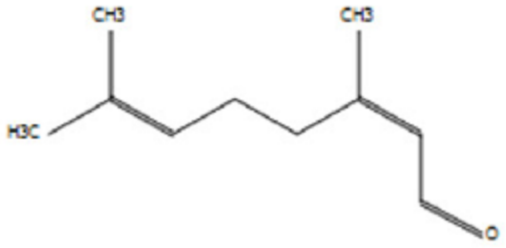
ANNEXURE 1

Compound Table

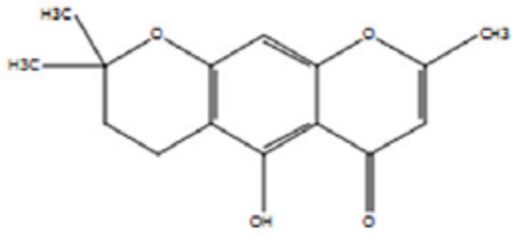
Compound Label	RT	Mass	Name	Formula	MFG Formula	DB Formula	DB Diff (ppm)	Hits (DB)
Cpd 1: p-Hydroxydextroamphetamine	5.318	151.0992	p-Hydroxydextroamphetamine	C9 H13 N O	C9 H13 N O	C9 H13 N O	3.11	8
Cpd 2: methyl 8-[2-(2-formyl vinyl)-3-hydroxy-5-oxo-cyclopentyl]-octanoate	5.378	310.1739	methyl 8-[2-(2-formyl-vinyl)-3-hydroxy-5-oxo-cyclopentyl]-octanoate	C17 H26 O5	C17 H26 O5	C17 H26 O5	13.29	3
Cpd 3: Hydroxysalmeterol	5.511	431.2715	Hydroxysalmeterol	C25 H37 N O5	C25 H37 N O5	C25 H37 N O5	-10.02	1
Cpd 4: 5.512	5.512	414.245						
Cpd 5: Arg Arg Gln	5.726	458.2713	Arg Arg Gln	C17 H34 N10 O5	C17 H34 N10 O5	C17 H34 N10 O5	0.25	4
Cpd 6: Netilmicin	5.727	475.2977	Netilmicin	C21 H41 N5 O7	C21 H41 N5 O7	C21 H41 N5 O7	6.2	2
Cpd 7: 5.898	5.898	519.3238						
Cpd 8: 1alpha,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homovitamin D3 / 1alpha,25-dihy	6.279	452.3351	1alpha,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homovitamin D3 / 1alpha,25-dihy	C30 H44 O3	C30 H44 O3	C30 H44 O3	-13.41	7
Cpd 9: VD 2736	6.514	452.3345	VD 2736	C30 H44 O3	C30 H44 O3	C30 H44 O3	-11.99	10
Cpd 10: Chalcone	7.233	208.087	Chalcone	C15 H12 O	C15 H12 O	C15 H12 O	8.81	4
Cpd 11: 3-O-Methylrimiterol	7.754	237.1387	3-O-Methylrimiterol	C13 H19 N O3	C13 H19 N O3	C13 H19 N O3	-9.44	8
Cpd 12: 8.385	8.385	324.2194						
Cpd 13: 8.439	8.439	336.1906						
Cpd 14: 8.782	8.782	127.9695						
Cpd 15: beta-Erythroidine	8.923	273.136	beta-Erythroidine	C16 H19 N O3	C16 H19 N O3	C16 H19 N O3	1.75	10
Cpd 16: 9.228	9.228	572.2435						
Cpd 17: Dihydrodeoxystreptomycin	9.23	567.2894	Dihydrodeoxystreptomycin	C21 H41 N7 O11	C21 H41 N7 O11	C21 H41 N7 O11	-5.3	1
Cpd 18: 1-Phosphatidyl-1D-myo-inositol 3-phosphate	9.272	470.0243	1-Phosphatidyl-1D-myo-inositol 3-phosphate	C11 H20 O16 P2	C11 H20 O16 P2	C11 H20 O16 P2	-3.41	1
Cpd 19: Protorifamycin I	9.669	639.3081	Protorifamycin I	C35 H45 N O10	C35 H45 N O10	C35 H45 N O10	-5.81	1
Cpd 20: DIHYDROSPATHELIACHROMENE	9.721	260.1046	DIHYDROSPATHELIACHROMENE	C15 H16 O4	C15 H16 O4	C15 H16 O4	1.07	5
Cpd 21: Methyl Jasmonate	10.448	224.1412	Methyl Jasmonate	C13 H20 O3	C13 H20 O3	C13 H20 O3	0.06	7
Cpd 22: 11.650	11.65	116.0627						
Cpd 23: Met His Lys	11.849	414.2042	Met His Lys	C17 H30 N6 O4 S	C17 H30 N6 O4 S	C17 H30 N6 O4 S	1.79	6
Cpd 24: 11.854	11.854	118.0784						
Cpd 25: DIHYDROSPATHELIACHROMENE	12.689	260.1048	DIHYDROSPATHELIACHROMENE	C15 H16 O4	C15 H16 O4	C15 H16 O4	0.39	5
Cpd 26: DIHYDROSPATHELIACHROMENE	13.329	260.1052	DIHYDROSPATHELIACHROMENE	C15 H16 O4	C15 H16 O4	C15 H16 O4	-1.39	5
Cpd 27: Parthenin	13.332	262.1168	Parthenin	C15 H18 O4	C15 H18 O4	C15 H18 O4	14.28	4
Cpd 28: Neral	13.332	152.1201	Neral	C10 H16 O	C10 H16 O	C10 H16 O	0.42	10
Cpd 29: Trp Glu Leu	13.332	446.2079	Trp Glu Leu	C22 H30 N4 O6	C22 H30 N4 O6	C22 H30 N4 O6	19.4	10
Cpd 30: Budesonide	13.333	430.2356	Budesonide	C25 H34 O6	C25 H34 O6	C25 H34 O6	-0.11	10
Cpd 31: 2,4,6,8,10-dodecapentaenal	13.333	174.1068	2,4,6,8,10-dodecapentaenal	C12 H14 O	C12 H14 O	C12 H14 O	-13.61	2
Cpd 32: 2-Butanone, 4-(6-hydroxy-2-naphthalenyl)-	13.334	214.0992	2-Butanone, 4-(6-hydroxy-2-naphthalenyl)-	C14 H14 O2	C14 H14 O2	C14 H14 O2	0.72	5
Cpd 33: LAPACHOL	13.334	242.0944	LAPACHOL	C15 H14 O3	C15 H14 O3	C15 H14 O3	-0.42	6
Cpd 34: Pyridoxamine	13.339	168.0938	Pyridoxamine	C8 H12 N2 O2	C8 H12 N2 O2	C8 H12 N2 O2	-23.19	1
Cpd 35: 13.988	13.988	371.9348						
Cpd 36: 14.471	14.471	677.4708						
Cpd 37: Loxoprofen	14.493	246.1259	Loxoprofen	C15 H18 O3	C15 H18 O3	C15 H18 O3	-1.21	10
Cpd 38: 14.543	14.543	633.4451						
Cpd 39: 14.608	14.608	589.4189						
Cpd 40: 14.672	14.672	545.3928						
Cpd 41: 14.749	14.749	501.3664						
Cpd 42: 4-Hydroxystyrene	14.879	120.0579	4-Hydroxystyrene	C8 H8 O	C8 H8 O	C8 H8 O	-3.4	2
Cpd 43: 14.885	14.885	352.261						
Cpd 44: CEFTIBUTEN	16.922	410.0378	CEFTIBUTEN	C15 H14 N4 O6 S2	C15 H14 N4 O6 S2	C15 H14 N4 O6 S2	-5.65	1
Cpd 45: 4-Chloro-N1-methyl-N1-(4-carboxy-2-hydroxy-2-methylbutyl)-m-benzenedisulfonamide	17.467	414.0349	4-Chloro-N1-methyl-N1-(4-carboxy-2-hydroxy-2-methylbutyl)-m-benzenedisulfonamide	C13 H19 Cl N2 O7 S2	C13 H19 Cl N2 O7 S2	C13 H19 Cl N2 O7 S2	-6.44	1
Cpd 46: N-(4-benzenesulfonamide) arachidonoyl amine	17.738	458.2582	N-(4-benzenesulfonamide) arachidonoyl amine	C26 H38 N2 O3 S	C26 H38 N2 O3 S	C26 H38 N2 O3 S	4.62	4
Cpd 47: 19.065	19.065	470.0974						
Cpd 48: 24,24-difluoro-1alpha,25-dihydroxy-26,27-dimethylvitamin D3 / 24,24-difluoro-1alpha,25-dihydroxy-26,	19.168	480.3379	24,24-difluoro-1alpha,25-dihydroxy-26,27-dimethylvitamin D3 / 24,24-difluoro-1alpha,25-dihydroxy-26,	C29 H46 F2 O3	C29 H46 F2 O3	C29 H46 F2 O3	7.46	3
Cpd 49: 26.756	26.756	112.0999						
Cpd 50: 26.850	26.85	138.9799						

ANNEXURE II

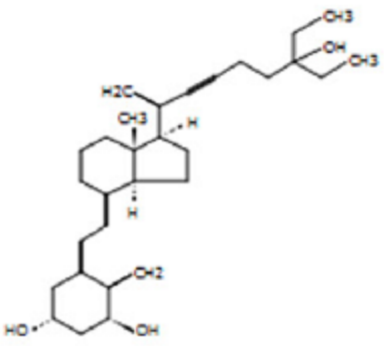
1. Neral ((2Z)-3,7-dimethylocta-2,6-dienal, $C_{10}H_{16}O$, 152.1201)



2. Dihydrospatheliachromone (Isopeucenin, 5-Hydroxy-2,2,8-trimethyl-3,4-dihydropyrano[3,2-g]chromen-6-one, $C_{15}H_{16}O_4$, 260.105)



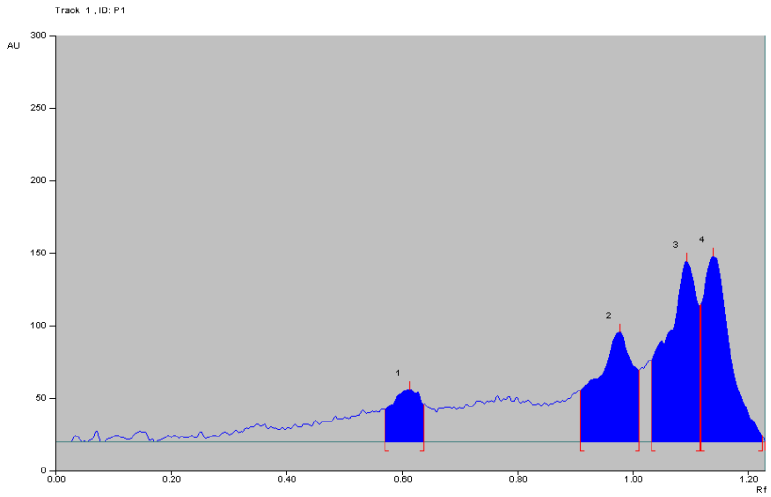
3. 1alpha,25-dihydroxy-26,27-dimethyl-20,21,22,22,23,23-hexadehydro-24a-homovitamin D3 ($C_{30}H_{44}O_3$, 452.335)



ANNEXURE III

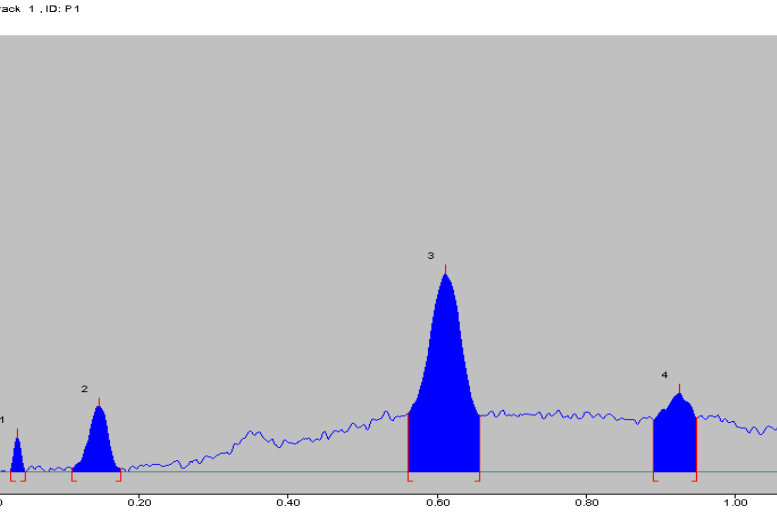
HPTLC profile of leaves extracts of *Elaeocarpus sphaericus*

1). HPTLC profile of petroleum ether extract showed 4 peaks under UV at 254 nm.



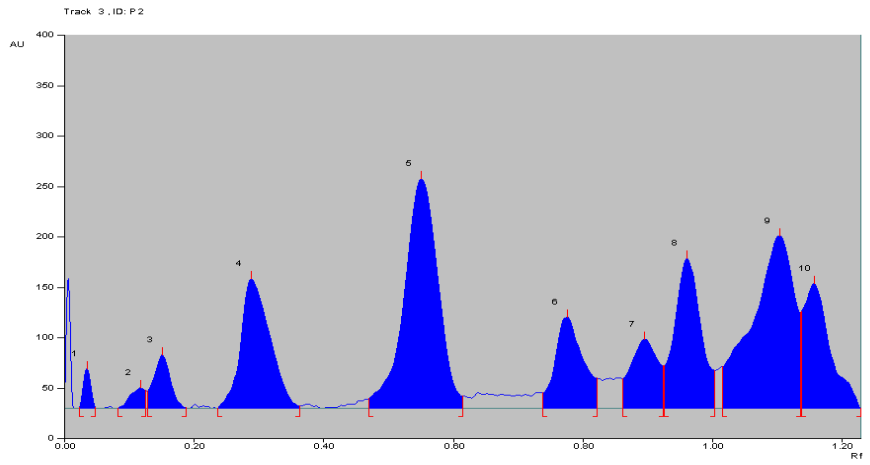
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.57	22.6	0.61	36.2	9.94	0.64	25.9	9.43
2	0.91	35.3	0.98	75.8	20.79	1.01	49.3	24.97
3	1.03	56.1	1.09	124.6	34.2	1.12	94.1	34.77
4	1.12	94.6	1.14	127.8	35.08	1.22	4.1	30.83

2). HPTLC profile of petroleum ether extract showed 6 peaks under UV at 366 nm.



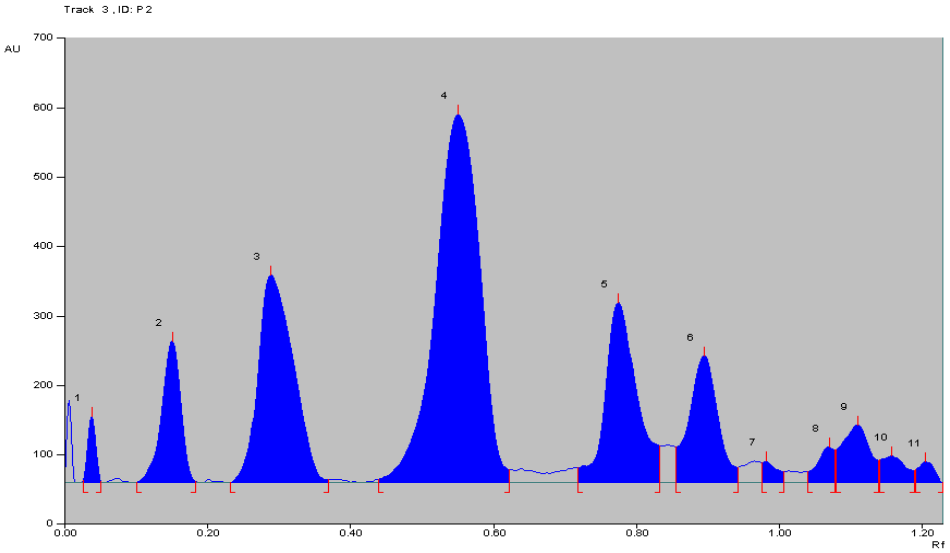
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.03	0.6	0.04	15	6.44	0.05	0.00	1.48
2	0.11	1.4	0.15	28.6	12.28	0.18	1.3	8.18
3	0.56	25.5	0.61	86.5	37.11	0.66	24.7	48.39
4	0.89	22.7	0.93	34.5	14.82	0.95	23.5	16.29
5	1.07	22.8	1.1	37.8	16.21	1.12	27.6	14.83
6	1.13	26.3	1.14	30.6	13.14	1.2	3.2	10.83

3). HPTLC profile of chloroform extract showed 10 peaks under UV at 254 nm.



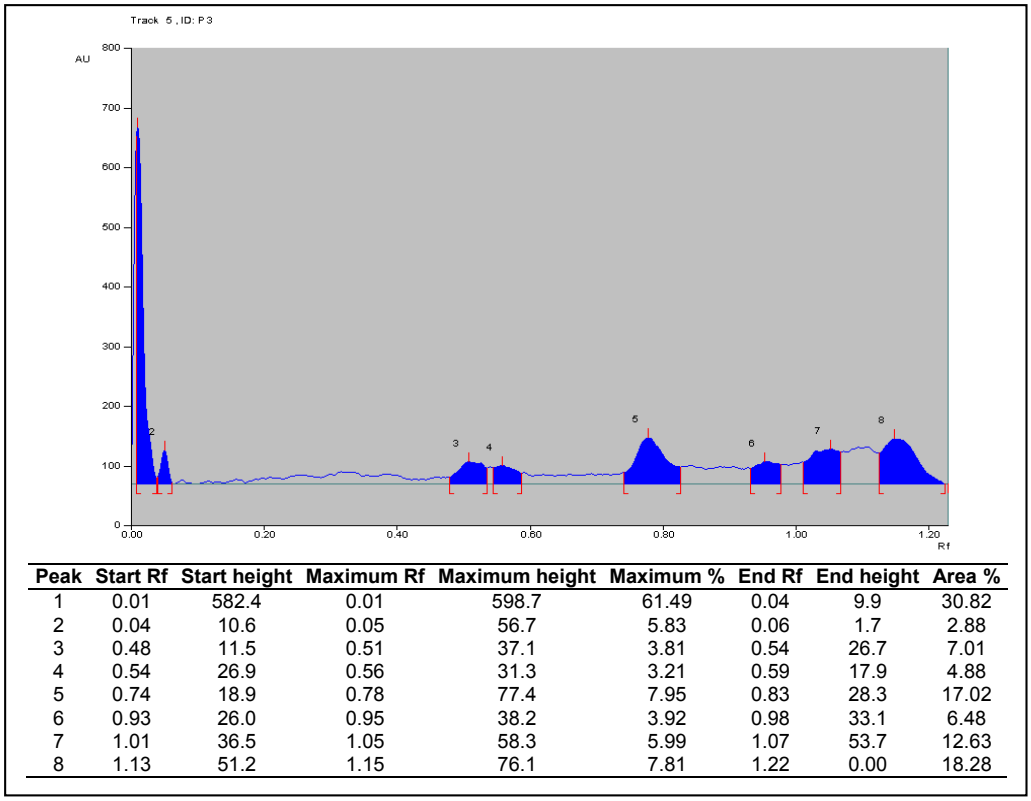
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.02	0.5	0.04	39.0	3.65	0.05	0.8	0.92
2	0.08	0.1	0.12	20.1	1.88	0.12	17.5	0.93
3	0.13	16.7	0.15	53.0	4.95	0.19	0.8	2.80
4	0.24	0.2	0.29	128.3	11.99	0.36	2.4	11.81
5	0.47	10.0	0.55	227.4	21.25	0.62	12.1	24.54
6	0.74	15.5	0.78	90.6	8.46	0.82	30.0	7.87
7	0.86	29.4	0.9	68.4	6.39	0.92	42.4	5.93
8	0.93	42.4	0.96	148.5	13.88	1.0	37.6	12.12
9	1.02	41.3	1.10	171.0	15.98	1.14	35.3	22.89
10	1.14	96.2	1.16	123.9	11.58	1.23	0.00	10.2

4). HPTLC profile of chloroform extract showed 11 peaks under UV at 366 nm.



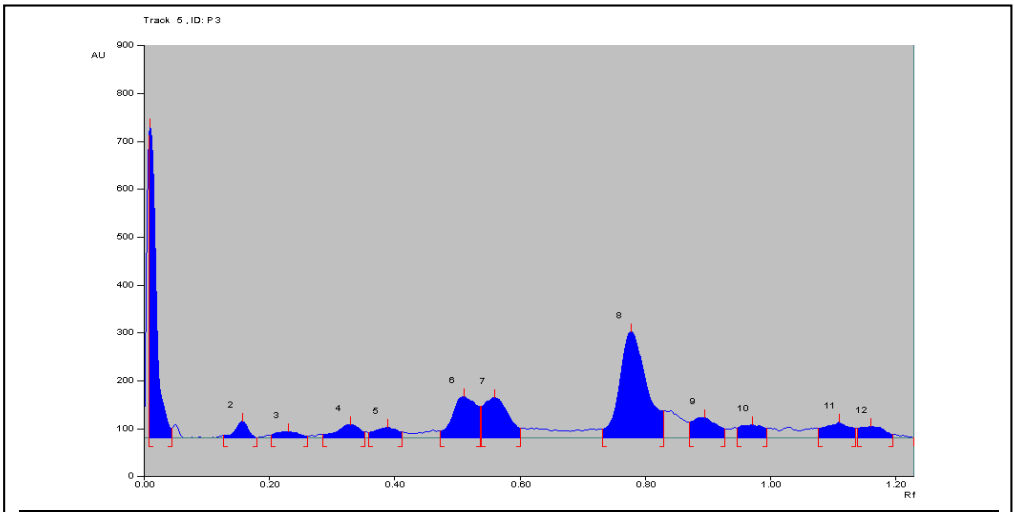
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.03	2.5	0.04	95.2	5.28	0.05	2.0	1.33
2	0.10	0.4	0.15	204.0	11.31	0.18	2.1	6.82
3	0.23	0.1	0.29	299.1	16.58	0.37	3.8	18.37
4	0.44	4.9	0.55	530.3	29.40	0.62	18.0	41.41
5	0.72	21.3	0.78	258.8	14.35	0.83	53.0	14.09
6	0.86	51.7	0.90	182.7	10.13	0.94	21.8	9.21
7	0.98	28.1	0.98	31.1	1.72	1.01	15.2	0.80
8	1.04	15.2	1.07	51.1	2.83	1.08	46.6	1.54
9	1.08	46.7	1.11	83.2	4.61	1.14	31.9	4.0
10	1.14	32.2	1.16	38.5	2.13	1.19	17.0	1.58
11	1.19	17.8	1.20	29.9	1.66	1.23	0.00	0.85

5). HPTLC profile of ethyl acetate extract showed 8 peaks under UV at 254 nm.



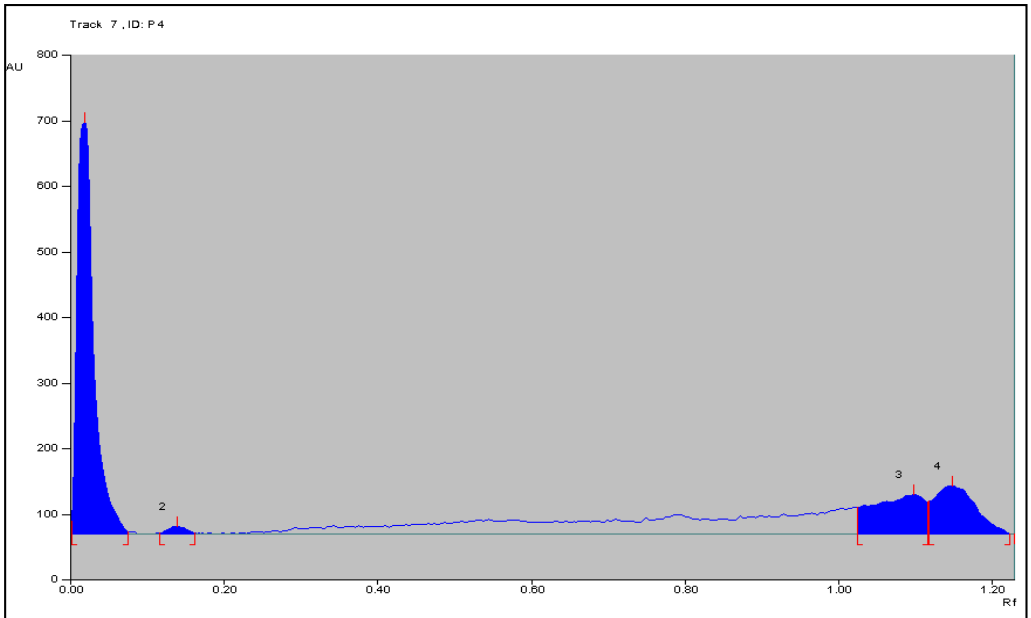
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.01	582.4	0.01	598.7	61.49	0.04	9.9	30.82
2	0.04	10.6	0.05	56.7	5.83	0.06	1.7	2.88
3	0.48	11.5	0.51	37.1	3.81	0.54	26.7	7.01
4	0.54	26.9	0.56	31.3	3.21	0.59	17.9	4.88
5	0.74	18.9	0.78	77.4	7.95	0.83	28.3	17.02
6	0.93	26.0	0.95	38.2	3.92	0.98	33.1	6.48
7	1.01	36.5	1.05	58.3	5.99	1.07	53.7	12.63
8	1.13	51.2	1.15	76.1	7.81	1.22	0.00	18.28

6). HPTLC profile of ethyl acetate extract showed 12 peaks under UV at 366 nm.



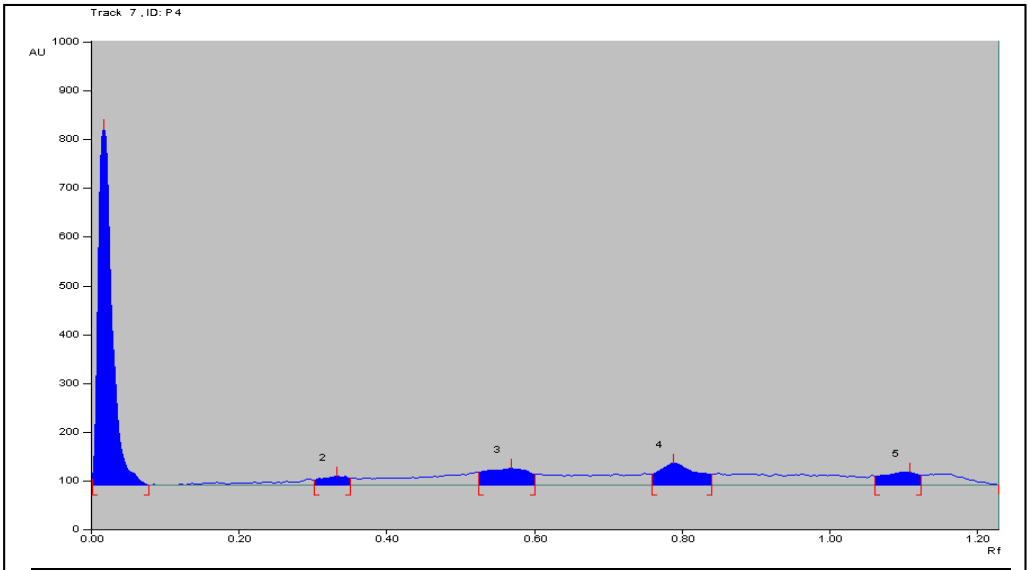
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.01	631.7	0.01	648.8	51.21	0.04	19.8	24.22
2	0.13	4.9	0.16	34.1	2.69	0.18	0.5	2.25
3	0.20	5.8	0.23	12.8	1.01	0.26	3.5	1.56
4	0.28	7.6	0.33	27.8	2.20	0.35	12.6	3.26
5	0.36	11.1	0.39	22.5	1.77	0.41	12.4	2.54
6	0.47	13.8	0.51	86.8	6.85	0.54	66.1	10.59
7	0.54	65.8	0.56	84.5	6.67	0.60	19.7	10.58
8	0.73	18.6	0.78	222.1	17.53	0.83	56.7	29.36
9	0.87	33.6	0.90	42.8	3.38	0.93	18.8	5.39
10	0.95	20.5	0.97	27.6	2.18	0.99	20.6	3.15
11	1.08	19.3	1.11	32.5	2.56	1.14	20.2	4.16
12	1.14	19.9	1.16	24.6	1.94	1.20	7.4	2.95

7). HPTLC profile of methanol extract showed 4 peaks under UV at 254 nm.



Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.00	19.4	0.02	627.8	81.2	0.08	2.9	62.37
2	0.12	1.3	0.14	11.4	1.47	0.16	1.7	1.30
3	1.02	40.4	1.10	60.4	7.76	1.12	49.4	18.44
4	1.12	49.7	1.15	74.0	9.57	1.22	0.3	17.88

8). HPTLC profile of methanol extract showed 5 peaks under UV at 366 nm.

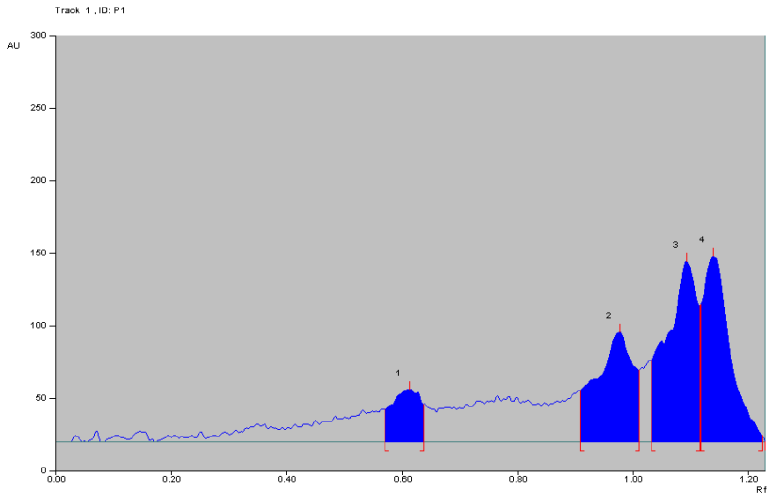


Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.00	12.8	0.02	730.2	85.46	0.08	0.2	68.76
2	0.30	10.8	0.33	18.0	2.10	0.35	14.1	3.37
3	0.52	26.5	0.57	34.5	4.04	0.60	22.7	10.25
4	0.76	22.8	0.79	45.1	5.28	0.84	21.9	11.26
5	1.06	18.2	1.11	26.7	3.12	1.12	19.4	6.36

ANNEXURE III

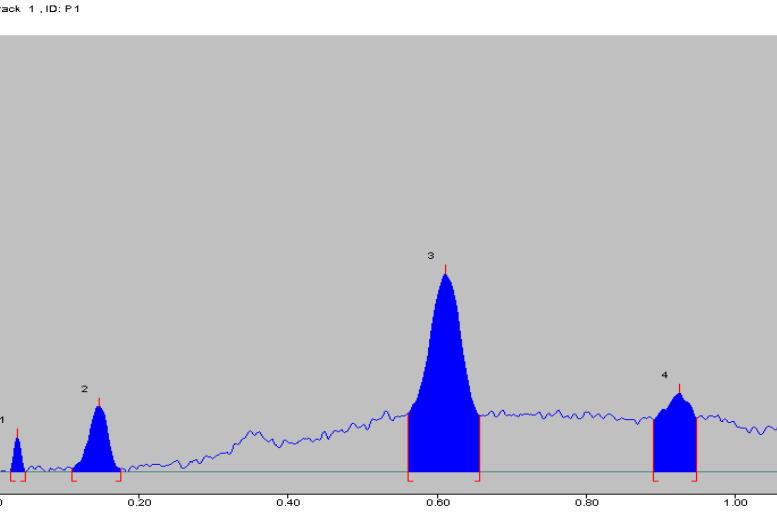
HPTLC profile of leaves extracts of *Elaeocarpus sphaericus*

1). HPTLC profile of petroleum ether extract showed 4 peaks under UV at 254 nm.



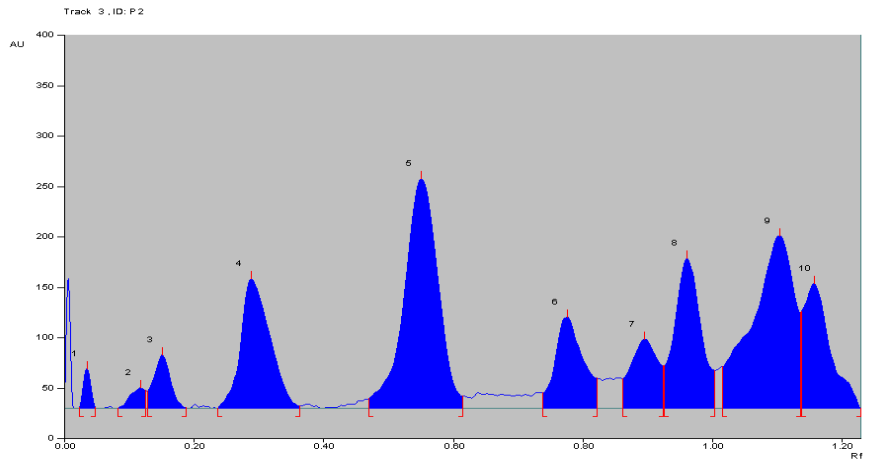
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.57	22.6	0.61	36.2	9.94	0.64	25.9	9.43
2	0.91	35.3	0.98	75.8	20.79	1.01	49.3	24.97
3	1.03	56.1	1.09	124.6	34.2	1.12	94.1	34.77
4	1.12	94.6	1.14	127.8	35.08	1.22	4.1	30.83

2). HPTLC profile of petroleum ether extract showed 6 peaks under UV at 366 nm.



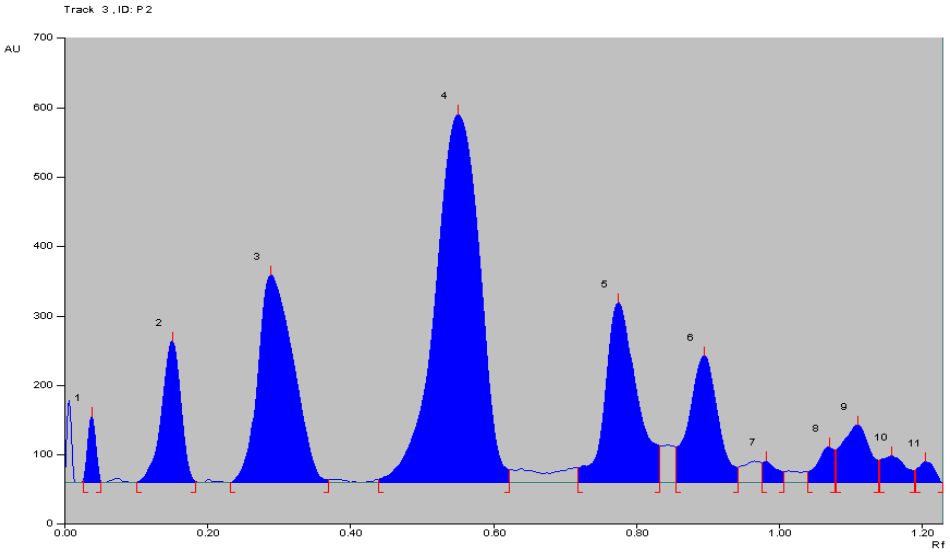
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.03	0.6	0.04	15	6.44	0.05	0.00	1.48
2	0.11	1.4	0.15	28.6	12.28	0.18	1.3	8.18
3	0.56	25.5	0.61	86.5	37.11	0.66	24.7	48.39
4	0.89	22.7	0.93	34.5	14.82	0.95	23.5	16.29
5	1.07	22.8	1.1	37.8	16.21	1.12	27.6	14.83
6	1.13	26.3	1.14	30.6	13.14	1.2	3.2	10.83

3). HPTLC profile of chloroform extract showed 10 peaks under UV at 254 nm.



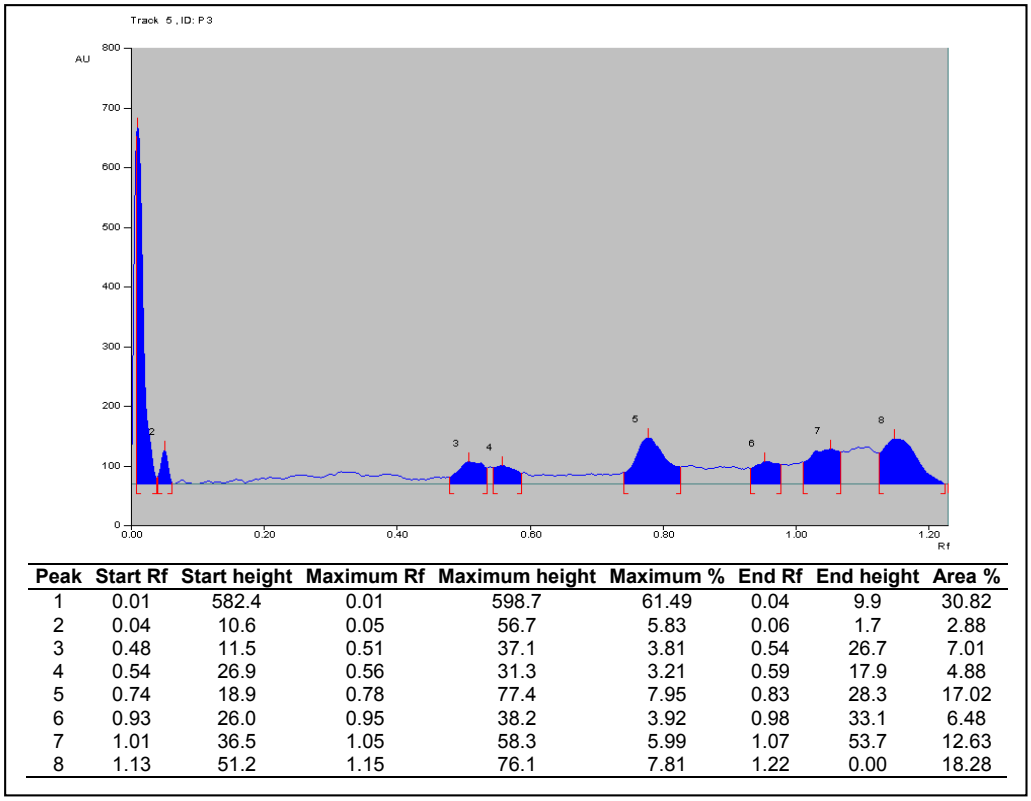
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.02	0.5	0.04	39.0	3.65	0.05	0.8	0.92
2	0.08	0.1	0.12	20.1	1.88	0.12	17.5	0.93
3	0.13	16.7	0.15	53.0	4.95	0.19	0.8	2.80
4	0.24	0.2	0.29	128.3	11.99	0.36	2.4	11.81
5	0.47	10.0	0.55	227.4	21.25	0.62	12.1	24.54
6	0.74	15.5	0.78	90.6	8.46	0.82	30.0	7.87
7	0.86	29.4	0.9	68.4	6.39	0.92	42.4	5.93
8	0.93	42.4	0.96	148.5	13.88	1.0	37.6	12.12
9	1.02	41.3	1.10	171.0	15.98	1.14	35.3	22.89
10	1.14	96.2	1.16	123.9	11.58	1.23	0.00	10.2

4). HPTLC profile of chloroform extract showed 11 peaks under UV at 366 nm.



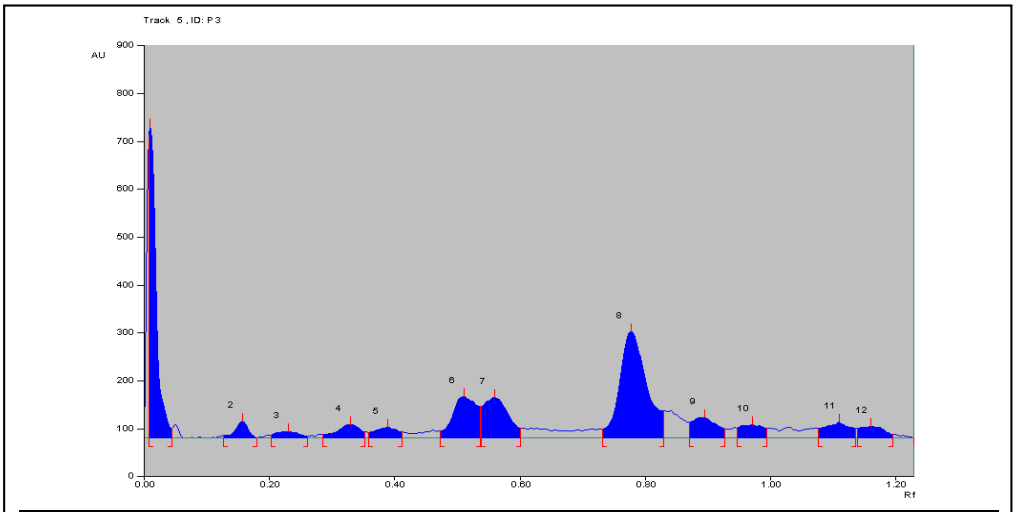
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.03	2.5	0.04	95.2	5.28	0.05	2.0	1.33
2	0.10	0.4	0.15	204.0	11.31	0.18	2.1	6.82
3	0.23	0.1	0.29	299.1	16.58	0.37	3.8	18.37
4	0.44	4.9	0.55	530.3	29.40	0.62	18.0	41.41
5	0.72	21.3	0.78	258.8	14.35	0.83	53.0	14.09
6	0.86	51.7	0.90	182.7	10.13	0.94	21.8	9.21
7	0.98	28.1	0.98	31.1	1.72	1.01	15.2	0.80
8	1.04	15.2	1.07	51.1	2.83	1.08	46.6	1.54
9	1.08	46.7	1.11	83.2	4.61	1.14	31.9	4.0
10	1.14	32.2	1.16	38.5	2.13	1.19	17.0	1.58
11	1.19	17.8	1.20	29.9	1.66	1.23	0.00	0.85

5). HPTLC profile of ethyl acetate extract showed 8 peaks under UV at 254 nm.



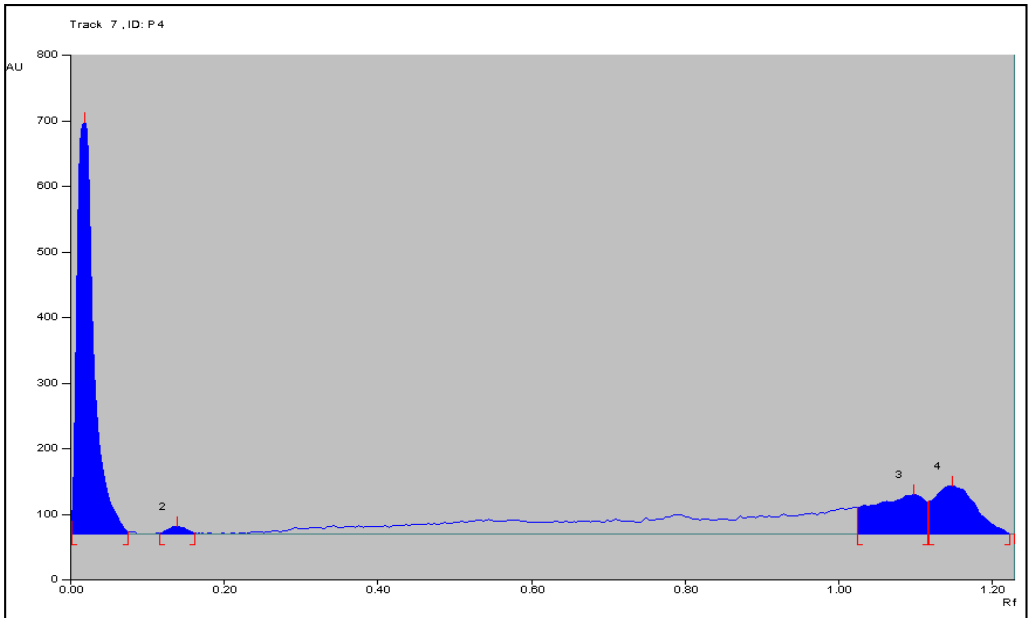
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.01	582.4	0.01	598.7	61.49	0.04	9.9	30.82
2	0.04	10.6	0.05	56.7	5.83	0.06	1.7	2.88
3	0.48	11.5	0.51	37.1	3.81	0.54	26.7	7.01
4	0.54	26.9	0.56	31.3	3.21	0.59	17.9	4.88
5	0.74	18.9	0.78	77.4	7.95	0.83	28.3	17.02
6	0.93	26.0	0.95	38.2	3.92	0.98	33.1	6.48
7	1.01	36.5	1.05	58.3	5.99	1.07	53.7	12.63
8	1.13	51.2	1.15	76.1	7.81	1.22	0.00	18.28

6). HPTLC profile of ethyl acetate extract showed 12 peaks under UV at 366 nm.



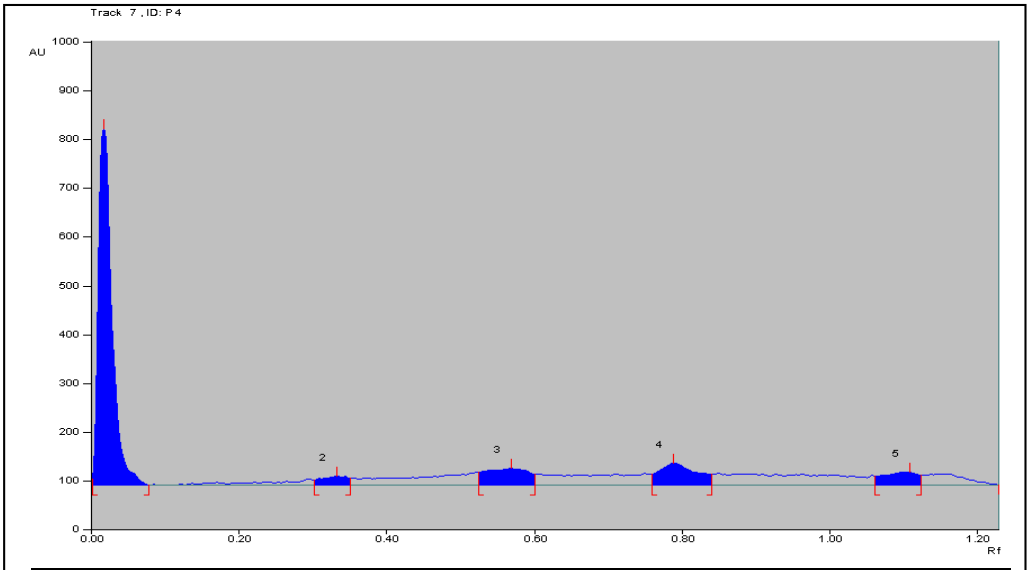
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.01	631.7	0.01	648.8	51.21	0.04	19.8	24.22
2	0.13	4.9	0.16	34.1	2.69	0.18	0.5	2.25
3	0.20	5.8	0.23	12.8	1.01	0.26	3.5	1.56
4	0.28	7.6	0.33	27.8	2.20	0.35	12.6	3.26
5	0.36	11.1	0.39	22.5	1.77	0.41	12.4	2.54
6	0.47	13.8	0.51	86.8	6.85	0.54	66.1	10.59
7	0.54	65.8	0.56	84.5	6.67	0.60	19.7	10.58
8	0.73	18.6	0.78	222.1	17.53	0.83	56.7	29.36
9	0.87	33.6	0.90	42.8	3.38	0.93	18.8	5.39
10	0.95	20.5	0.97	27.6	2.18	0.99	20.6	3.15
11	1.08	19.3	1.11	32.5	2.56	1.14	20.2	4.16
12	1.14	19.9	1.16	24.6	1.94	1.20	7.4	2.95

7). HPTLC profile of methanol extract showed 4 peaks under UV at 254 nm.



Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.00	19.4	0.02	627.8	81.2	0.08	2.9	62.37
2	0.12	1.3	0.14	11.4	1.47	0.16	1.7	1.30
3	1.02	40.4	1.10	60.4	7.76	1.12	49.4	18.44
4	1.12	49.7	1.15	74.0	9.57	1.22	0.3	17.88

8). HPTLC profile of methanol extract showed 5 peaks under UV at 366 nm.

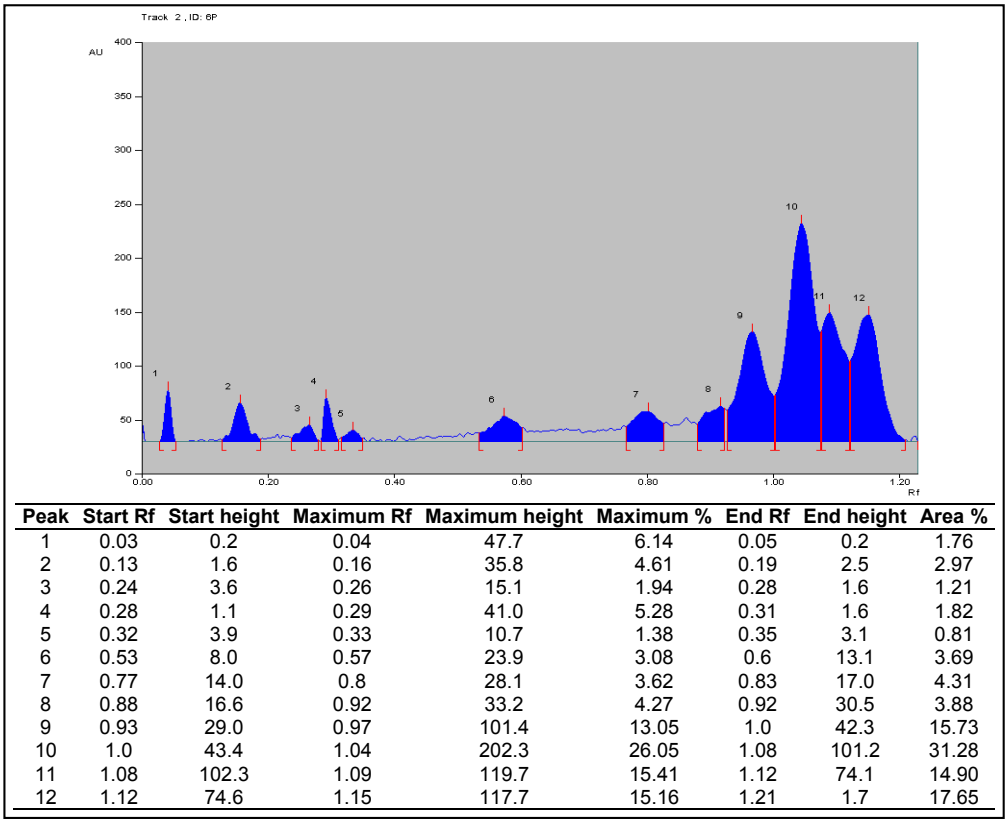


Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.00	12.8	0.02	730.2	85.46	0.08	0.2	68.76
2	0.30	10.8	0.33	18.0	2.10	0.35	14.1	3.37
3	0.52	26.5	0.57	34.5	4.04	0.60	22.7	10.25
4	0.76	22.8	0.79	45.1	5.28	0.84	21.9	11.26
5	1.06	18.2	1.11	26.7	3.12	1.12	19.4	6.36

ANNEXURE IV

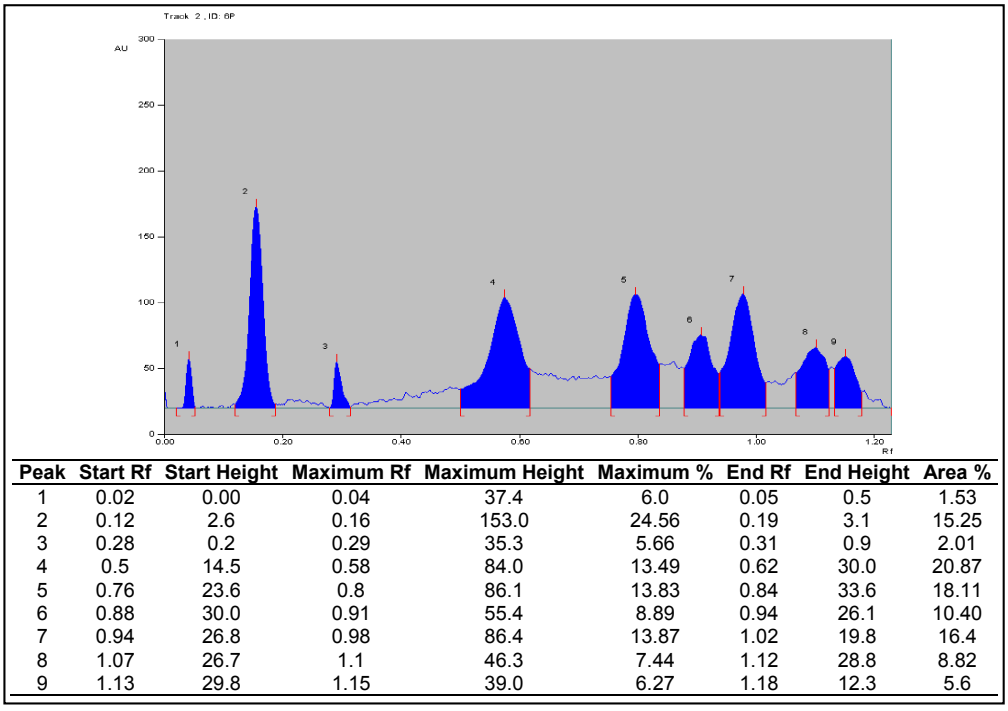
HPTLC profile of leaves extracts of *Quassia indica*

1). HPTLC profile of petroleum ether extract showed 12 peaks under UV at 254 nm.



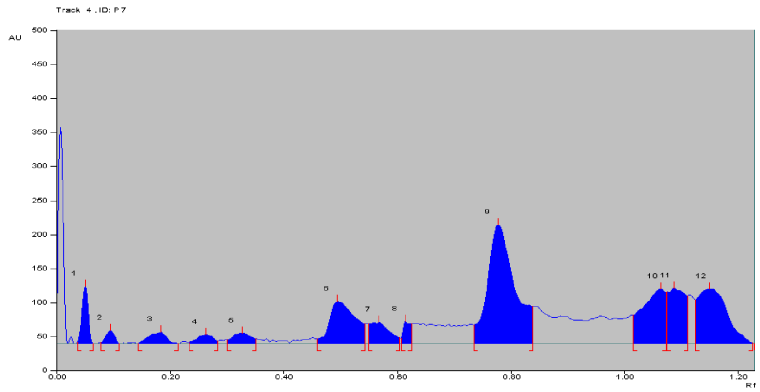
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.03	0.2	0.04	47.7	6.14	0.05	0.2	1.76
2	0.13	1.6	0.16	35.8	4.61	0.19	2.5	2.97
3	0.24	3.6	0.26	15.1	1.94	0.28	1.6	1.21
4	0.28	1.1	0.29	41.0	5.28	0.31	1.6	1.82
5	0.32	3.9	0.33	10.7	1.38	0.35	3.1	0.81
6	0.53	8.0	0.57	23.9	3.08	0.6	13.1	3.69
7	0.77	14.0	0.8	28.1	3.62	0.83	17.0	4.31
8	0.88	16.6	0.92	33.2	4.27	0.92	30.5	3.88
9	0.93	29.0	0.97	101.4	13.05	1.0	42.3	15.73
10	1.0	43.4	1.04	202.3	26.05	1.08	101.2	31.28
11	1.08	102.3	1.09	119.7	15.41	1.12	74.1	14.90
12	1.12	74.6	1.15	117.7	15.16	1.21	1.7	17.65

2). HPTLC profile of petroleum ether extract showed 9 peaks under UV at 366 nm.



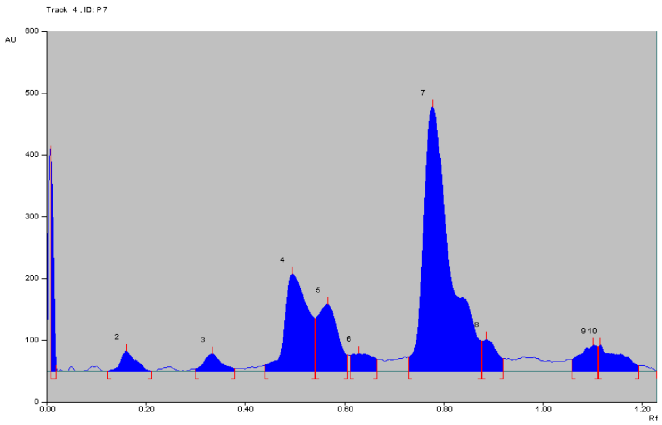
Peak	Start Rf	Start Height	Maximum Rf	Maximum Height	Maximum %	End Rf	End Height	Area %
1	0.02	0.00	0.04	37.4	6.0	0.05	0.5	1.53
2	0.12	2.6	0.16	153.0	24.56	0.19	3.1	15.25
3	0.28	0.2	0.29	35.3	5.66	0.31	0.9	2.01
4	0.5	14.5	0.58	84.0	13.49	0.62	30.0	20.87
5	0.76	23.6	0.8	86.1	13.83	0.84	33.6	18.11
6	0.88	30.0	0.91	55.4	8.89	0.94	26.1	10.40
7	0.94	26.8	0.98	86.4	13.87	1.02	19.8	16.4
8	1.07	26.7	1.1	46.3	7.44	1.12	28.8	8.82
9	1.13	29.8	1.15	39.0	6.27	1.18	12.3	5.6

3). HPTLC profile of chloroform extract showed 12 peaks under UV at 254 nm.



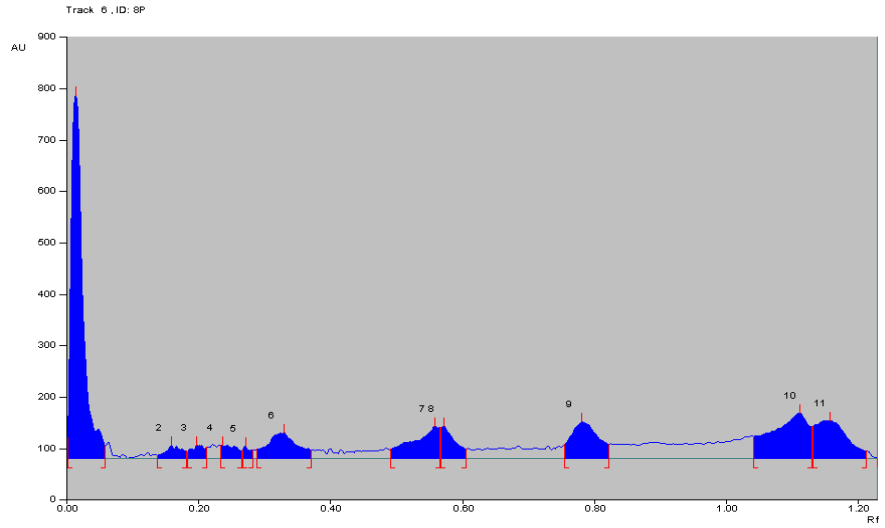
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.04	2.5	0.05	84.0	12.15	0.06	0.2	3.64
2	0.08	1.6	0.09	18.8	2.72	0.11	0.0	1.07
3	0.14	0.1	0.18	17.2	2.49	0.21	0.2	2.09
4	0.23	2.5	0.26	13.2	1.91	0.28	4.1	1.45
5	0.30	5.9	0.33	15.5	2.24	0.35	6.8	2.05
6	0.46	6.8	0.49	61.6	8.91	0.54	29.5	11.31
7	0.55	29.6	0.57	31.0	4.49	0.60	8.7	4.46
8	0.61	7.9	0.62	32.7	4.73	0.62	29.3	1.65
9	0.74	27.8	0.78	174.5	25.25	0.84	54.9	32.78
10	1.02	41.5	1.06	80.3	11.62	1.07	75.6	13.07
11	1.08	75.9	1.09	81.6	11.81	1.11	70.4	10.14
12	1.13	64.4	1.15	80.7	11.68	1.23	0.6	16.29

4). HPTLC profile of chloroform extract showed 10 peaks under UV at 366 nm.



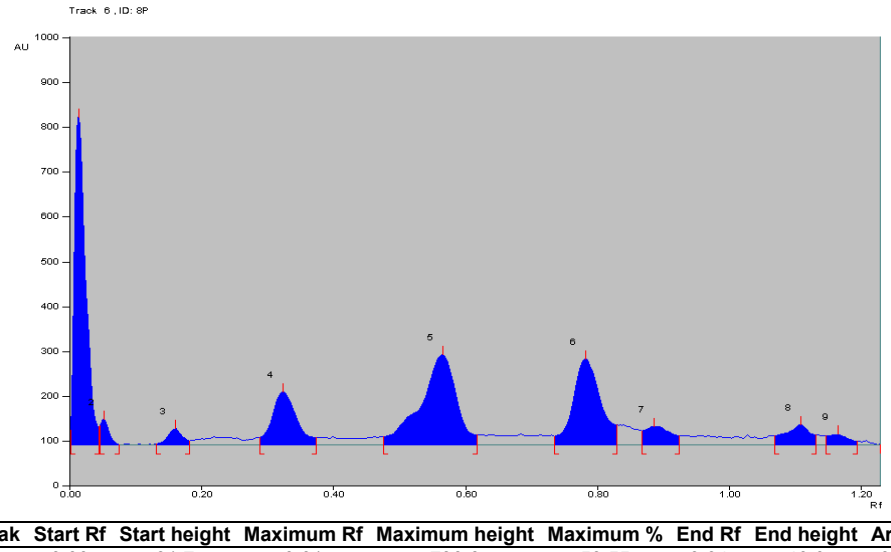
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.01	354.2	0.01	354.2	27.75	0.02	9.3	4.40
2	0.12	0.1	0.16	32.9	2.58	0.21	0.1	2.25
3	0.30	4.2	0.33	28.6	2.24	0.38	4.5	2.31
4	0.44	10.6	0.49	156.8	12.29	0.54	35.8	16.59
5	0.54	85.9	0.57	109.0	8.54	0.61	26.4	10.09
6	0.61	25.4	0.63	29.1	2.28	0.66	20.1	2.73
7	0.73	23.2	0.78	428.3	33.55	0.88	49.1	50.65
8	0.88	49.1	0.89	52.3	4.10	0.92	21.1	3.45
9	1.06	19.6	1.10	42.3	3.32	1.11	40.0	3.42
10	1.11	40.6	1.12	42.3	3.36	1.19	10.1	4.10

5). HPTLC profile of ethyl acetate extract showed 11 peaks under UV at 254 nm.



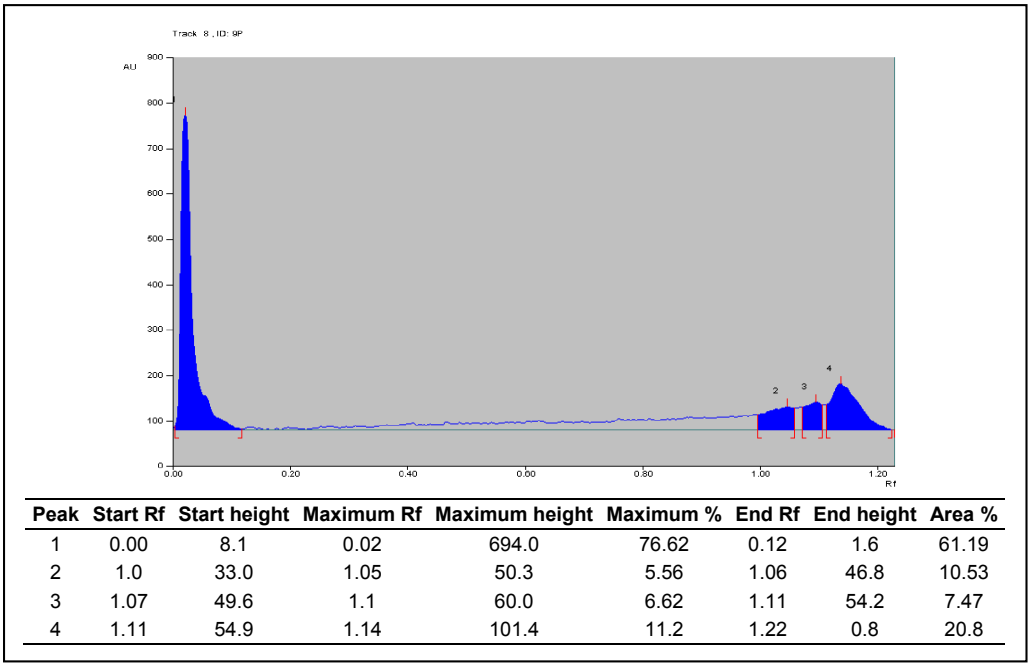
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.00	40.4	0.01	706.7	58.16	0.06	25.4	39.41
2	0.14	6.3	0.16	25.4	2.09	0.18	15.3	1.97
3	0.18	13.4	0.20	25.8	2.13	0.21	21.6	1.69
4	0.23	24.8	0.24	25.3	2.08	0.26	13.7	1.91
5	0.27	16.2	0.27	23.9	1.97	0.28	16.0	0.82
6	0.29	18.0	0.33	50.0	4.12	0.37	16.9	7.01
7	0.49	17.8	0.56	62.7	5.16	0.57	30.4	7.82
8	0.57	60.1	0.57	62.7	5.16	0.61	18.4	4.22
9	0.76	27.0	0.78	70.6	5.81	0.82	28.1	8.91
10	1.04	42.9	1.11	88.6	7.29	1.13	61.3	15.27
11	1.13	62.2	1.16	73.3	6.03	1.21	14.1	10.98

6). HPTLC profile of ethyl acetate extract showed 9 peaks under UV at 366 nm.



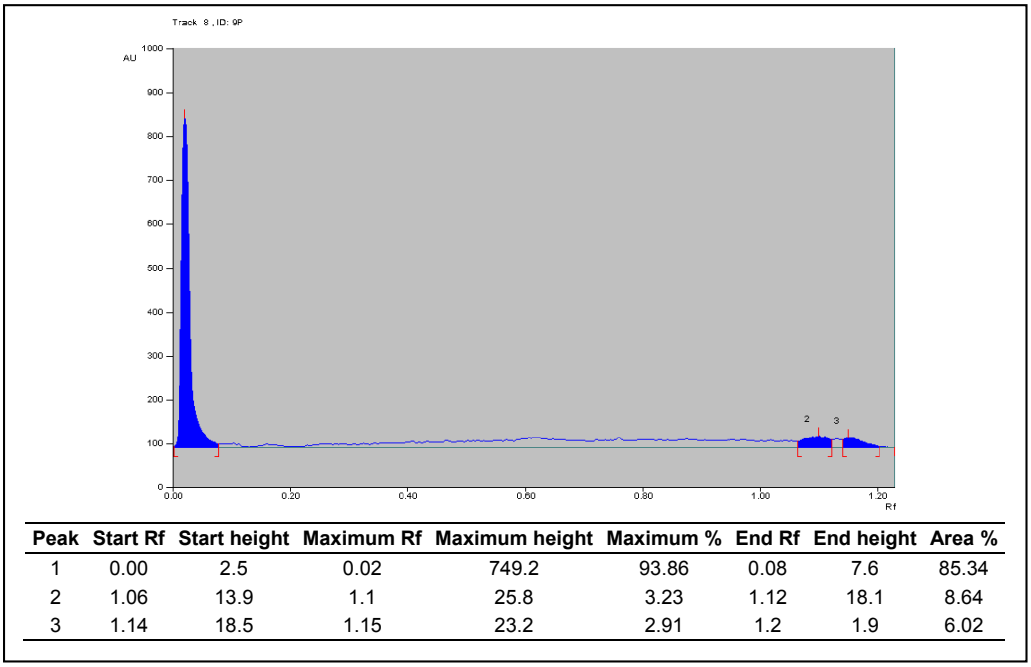
Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.00	31.7	0.01	730.6	50.55	0.04	40.0	30.19
2	0.05	42.7	0.05	57.7	3.99	0.08	0.7	1.81
3	0.13	2.6	0.16	36.2	2.50	0.18	9.1	1.98
4	0.29	16.9	0.32	118.5	8.20	0.37	16.1	10.64
5	0.48	19.0	0.57	200.7	13.89	0.62	22.1	26.44
6	0.74	19.2	0.78	191.4	13.24	0.83	14.5	18.97
7	0.87	31.2	0.89	41.5	2.87	0.92	20.5	4.04
8	1.07	19.7	1.11	45.5	3.15	1.13	21.5	4.14
9	1.15	20.5	1.16	23.4	1.62	1.19	7.8	1.80

7). HPTLC profile of methanol extract showed 4 peaks under UV at 254 nm.



Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.00	8.1	0.02	694.0	76.62	0.12	1.6	61.19
2	1.0	33.0	1.05	50.3	5.56	1.06	46.8	10.53
3	1.07	49.6	1.1	60.0	6.62	1.11	54.2	7.47
4	1.11	54.9	1.14	101.4	11.2	1.22	0.8	20.8

8). HPTLC profile of methanol extract showed 3 peaks under UV at 366 nm.



Peak	Start Rf	Start height	Maximum Rf	Maximum height	Maximum %	End Rf	End height	Area %
1	0.00	2.5	0.02	749.2	93.86	0.08	7.6	85.34
2	1.06	13.9	1.1	25.8	3.23	1.12	18.1	8.64
3	1.14	18.5	1.15	23.2	2.91	1.2	1.9	6.02

PUBLICATION

- **V.C. Deepthi,** Seepana Sumathi, M Faisal and K.K. Elyas. Isolation and identification of endophytic fungi with antimicrobial activities from the leaves of *Elaeocarpus sphaericus* (Gaertn.) K. Schum. and *Myristica fragrans* Houtt. International journal of pharmaceutical sciences and research 9 (7), 2783-2791. Impact factor- 0.59.

CONFERENCE PAPERS

1. Deepthi V C and Elyas K K. Endophytic fungus from the leaves of the medicinal plant *Elaeocarpus sphaericus* (Gaertn.) K. Schum. and their antibacterial activity. International conference on phytomedicine. Bharathiar University, Coimbatore, Kerala, 29th- 31st August 2018. **Best paper** award for the same.

2. Deepthi V C, Elyas K K. Endophytic fungi from the medicinal plants *Elaeocarpus sphaericus* (Gaertn.) K. Schum. and *Myristica fragrans* Houtt.: antimicrobial properties. International conference on emerging synergies in agriculture, food processing engineering and biotechnology, Karunya University, Coimbatore, Kerala, 21st - 23rd February 2018.

3. Deepthi V C, Elyas K K. Isolation and screening of endophytic fungi from the leaves and twigs of two medicinal plants and their within-leaf distributions. 29th Kerala science congress, Marthoma College, Thiruvalla, Kerala, 28th- 30th January 2017.

4. V C Deepthi and K K Elyas. Isolation and identification of endophytic fungi from the leaves of *Elaeocarpus sphaericus* Roxb. and *Myristica fragrans* Houtt. National seminar on recent trends in microbiology, University of Calicut, Kerala, 26th and 27th September 2017.

POSTER

1. Deepthi V C, Faisal Moossa, Elyas K K. Isolation and identification of an endophytic fungus from the leaves of *Elaeocarpus sphaericus* (Gaertn.) K. Schum. Govt. Brennen College, Thalassery, Kerala, 28th- 30th January 2018.

SEQUENCE DEPOSITS

- *Xylaria* sp. isolate ESEF1, ITS, Accession number- MH819666, VC. Deepthi, KK. Elyas
- *Xylaria* sp. isolate ESEF2, ITS, Accession number- MH819667, VC. Deepthi, KK. Elyas
- *Fusarium* sp. isolate ESEF3, ITS, Accession number- MK045725, VC. Deepthi, KK. Elyas
- *Diaporthe* sp. isolate ESEF4, ITS, Accession number- MK050996, VC. Deepthi, KK. Elyas
- *Endomelanconiopsis* sp. isolate ESEF5, ITS, Accession number- MH835348, VC. Deepthi, KK. Elyas
- *Xylaria feejeensis* isolate ESEF6, ITS, Accession number- MH836377, VC. Deepthi, KK. Elyas
- *Xylaria* sp. isolate ESEF7, ITS, Accession number- MK045721, VC. Deepthi, KK. Elyas
- *Diaporthe* sp. isolate ESEF8, ITS, Accession number- MK045723, VC. Deepthi, KK. Elyas
- *Nigrospora* sp. isolate ESEF9, ITS, Accession number- MH910671, VC. Deepthi, KK. Elyas
- *Nigrospora* sp. isolate ESEF10, ITS, Accession number- MH909796, VC. Deepthi, KK. Elyas
- *Nigrospora* sp. isolate ESEF11, ITS, Accession number- MK045718, VC. Deepthi, KK. Elyas
- *Pestalotiopsis* sp. isolate EFEF12, ITS, Accession number- MH915546, VC. Deepthi, KK. Elyas
- *Daldinia eschscholtzii* isolate QIEF1, ITS, Accession number- MH819669, VC. Deepthi, KK. Elyas
- *Diaporthe* sp. isolate QIEF2, ITS, Accession number- MK045724, VC. Deepthi, KK. Elyas
- *Phyllosticta* sp. isolate QIEF3, ITS, Accession number- MH910044, VC. Deepthi, KK. Elyas
- *Lecanicillium* sp. isolate QIEF4, ITS, Accession number- MH910098, VC. Deepthi, KK. Elyas
- *Colletotrichum* sp. isolate QIEF5, ITS, Accession number- MH910090, VC. Deepthi, KK. Elyas
- *Colletotrichum* sp. isolate QIEF6, ITS, Accession number- MK207056, VC. Deepthi, KK. Elyas
- *Fusarium* sp. isolate QIEF7, ITS, Accession number- MK050999, VC. Deepthi, KK. Elyas
- *Pestalotiopsis* sp. isolate QIEF8, ITS, Accession number- MH915547, VC. Deepthi, KK. Elyas
- *Meyerozyma* sp. isolate QIEF9, ITS, Accession number- MK050997, VC. Deepthi, KK. Elyas
- *Fusarium* sp. isolate QIEF10, ITS, Accession number- MK050998, VC. Deepthi, KK. Elyas