BIOPROSPECTING OF NOVEL BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGUS

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CERTIFICATE

This is to certify that the thesis entitled " **Bioprospecting of Novel Bioactive Compounds from Endophytic Fungus** " was submitted to Manonmaniam Sundaranar University in partial fulfillment of the requirements for the degree of Doctor of Philosophy by **T. Suresh,** Reg. No:4405**.** This thesis is the original work of the candidate and has not been submitted, in part or in full, for any diploma, degree, associateship or other similar titles in this on any other university. No portion of the thesis is a reproduction from any other source either published or unpublished without acknowledgement.

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DECLARATION

I do hereby declare that the thesis entitled **" Bioprospecting of Novel Bioactive Compounds from Endophytic Fungus** " has been carried out by me under the guidance of **Dr. B. Xavier Innocent** Associate Professor, P. G and Research Department of Zoology, St. Xaviers College, Palayamkottai, Tirunelveli and **Dr. V. Aldous. J. Huxley,** (C0 – guide) Assistant Professor Biotech Research Laboratory, Department of Zoology, Thiru. Vi. Ka Govt. Arts College, Thiruvarur -3, Tamilnadu, South India and this work has not been submitted elsewhere for any other degree, diploma, fellowship or other similar titles.

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CONTENTS

List of Abbreviations

CEs	-	Clavicipitaceous Endophytes
CRI	-	Cancer Research Institute
DNA	-	Deoxyribo Nucleic Acid
DSE	-	Dark Septate Endophytes
Et Br	-	Ethidium Bromide
GC	-	Gas Chromatography
GSH	-	Growth Stimulating Harmone
HMW	-	High Molecular Weight
Hplc	-	High Performance Liquid Chromatography
LC-MS	-	Liquid Chromatography and Mass spectroscopy
LMPA	-	Low Melting Point Agarose
MEA	-	Malt Extract Agar
MIC	-	Minimum Inhibition Concentration
MTCC	-	Microbial Type Culture Collection
NCEs	-	Non Clavicepataceous Endophytes
NMA	-	Normal Melting Agarose
NMR	-	Nuclear Magnetic Resonense
PBS	-	Phosphate Buffered Saline
PDA	-	Potato Dextrose Agar
PLFAs	-	phospholipid fatty acids
PUFAs	-	polyunsaturated fatty acids
ROS	-	Reactive oxygen species
Rpm	-	Rotation Per Minute
SDA	-	Sabouraud Dextrose Agar
TAC	-	Total Antioxidant Capacity
TLC	-	Thin Layer Chromatography
VOCs	-	Volatile Organic Compounds

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Preface

Natural products discovery have played major role in the search for new drugs, and is the most potent source for the discovery of novel bioactive molecules. Natural products are chemical compounds derived from living organisms. The most prominent producers of natural products can be found within different groups of organisms including plants, animals, marine macro-organisms (sponge, corals and algae), and microorganisms (bacteria, actinomycetes, and fungi). The discovery of natural products involves isolation, structural elucidation and establishing the bio-synthetic pathway of the secondary metabolites. Crude natural products have been used directly as drugs which were low cost and important sources of traditional medicines. The role of natural products in discovery of new therapeutic agents can be demonstrated by an analysis of the number and sources of bioactive agents. There are at least 200,000 natural metabolites with various bioactive properties.

Between 1981 and 2006, about 100 anti-cancer agents have been developed, among which 25% of them were natural product derivatives, 18% were natural product mimics, 11% candidates were derived from a natural product pharmacophore and 9% were pure natural products. Actually 47% of total anticancer drugs and 52% of new chemicals introduced into the market are of natural origin. Since the discovery of potent antimicrobial metabolites against Gram-positive bacteria, penicillin from culture of fungus *Penicillium notatum* by Fleming in 1929, the search for new drugs from microbial origin started. Now, more than 20,000 bioactive metabolites of microbial origin have been reported. Fungi are among the most important groups of eukaryotic organisms that are well known for producing many novel metabolites which are directly used as drugs or function as lead structures for synthetic analogs.

Since the first description of symbiosis as ‘the living together of dissimilar organisms’ onwards an array of symbiotic lifestyles has been identified in plants on natural ecosystems with mycorrhizal fungi and/or fungal endophytes. These fungal symbionts can have profound effects on plant ecology, fitness, and evolution, shaping plant communities and manifesting strong effects on the community structure and

diversity of associated organisms. The fossil record indicates that plants have been associated with endophytic and mychorrhizal fungi for more than 400 years and were likely associated when plants colonized land, thus playing a long and important role in driving the evolution of life on land. Endophytic fungi are a rich source of novel organic compounds with interesting biological activities. They represent a relatively unexplored ecological source, and their secondary metabolism is particularly active because of their metabolic interactions with their hosts. Apart from that, lots of fungicidal, herbicidal and antibacterial activities of endophytic fungal secondary metabolites have been reported. Based on this milieu, new efforts are taken to screen the bioactivity of endophytic fungal metabolites especially anticancer properties. This study also analyzed the toxicity profile and suitable solvent for extraction, and lead compound characterization, which are essentially needed for drug discovery.

OBJECTIVES

The present study was indented to explore the following objectives.

- To isolate potent metabolites from endophytic fungus using different solvent systems
- To evaluate the efficacy of metabolites through various Antibacterial, Antifungal, Antiviral and Anticancer assay systems
- To identify and characterize the lead compound of endophytic fungi, which is responsible for bioactivity

Review of Literature

Introduction

"The living together of unlike organisms" is known since de Bary (1879) as symbiosis. In natural ecosystems plants are potential hosts for a broad spectrum of microbes (usually bacteria and fungi) that can live on the surface as epiphytes or colonize plant tissues as endophytes. The term symbiosis (from the Greek: syn "with" and biosis "living") can be applied to different kind of associations. They can be roughly categorized into mutualism, commensalism and parasitism according to benefits for both partners (mutualistic symbiosis), microbial benefit not affecting the host plant (commensalistic symbiosis) or negative impact on host fitness (pathogenic or parasitic interaction) (Rodriguez, *et al.*, 2008). The borders between these categories are, however, not fixed. For example, mycorrhizal interactions, the most known and wide-spread terrestrial plant-fungus symbioses, are usual mutualistic (Li and Liu, 2004). Depending on environmental conditions, however, the fungus can have negative impact on plant development (Davis *et al.*, 2003). Also, genetic factors can switch this interaction (Redman *et al.*, 1999). On the other hand, pathogenic fungi can colonize their hosts without causing any symptoms (Omacini *et al.*, 2001). In addition to this, a large spectrum of endophytic fungi represent a continuum of plant-fungal associations, ranging from positive over neutral to negative interactions (Brundrett, 2004). These fungal endophytes are represented by diverse taxonomic groups (Arnold *et al.*, 2007; Rodriguez *et al.*, 2009). Like the mycorrhizal fungi, they have been recognized to be ancient (Krings *et al.*, 2007), mainly characterized by the production of important secondary metabolites (Strobel, 2004) and have significant effects on plant fitness and ecology (Brundrett, 2004; Rodriguez *et al.*, 2008).

Fungal endophytes

The term endophyte comes from the Greek that denotes a broad spectrum of plant endosymbionts from bacteria to insects colonizing inside any organ of the plant with variable life styles (Schulz and Boyle, 2005). Fungal endophytes are fungi that grow internally in living plant tissues for at least part of their life cycle without causing disease symptoms (Stone *et al.,* 2000). They include a large spectrum from latent pathogens to mutualistic symbionts (Schulz and Boyle, 2005). Although asymptomatic fungal colonization inside plant tissues has been known since the 19th century (Guerin, 1898), the presence of these endophytes was related to a syndrome called toxicosis suffered by cattle fed in pastures of colonized grass (Bacon *et al.,* 1977). The procedures most commonly used for recovering endophytes from plant tissues are based on surface disinfection of apparently healthy plant organ samples to kill epiphytic fungi (Bills, 1996). Fragments of disinfected plant samples are subsequently placed on different culture media. After incubation, when endophytic hyphae emerge from the plant tissue and start growing in the agar medium, the isolation of the fungus can be carried out. Fungi that emerge from these samples can be identified by means of phenotypic (morphological) or genotypic (molecular) characters (Arnold and Lutzoni, 2007; Higgins *et al.,* 2007).

Obligated biotrophs or fungi not growing well in the agar medium have been detected by molecular techniques (Neubert *et al.,* 2006). Sequencing of ribosomal DNA and internal transcribed spacers (ITS) has improved taxonomic studies of sterile isolates obtained in endophyte surveys (Guo *et al.,* 2000; Higgins *et al.,* 2007). Fungal endophytes can be isolated from leaves, stems and/or roots. They seem to be saprophytic and therefore potentially cultivable on agar and, they are highly diverse within the phyla *Ascomycota* and *Basidiomycota* representing distinct functional groups (Arnold and Lutzoni, 2007; Rodriguez *et al.,* 2009; Selosse *et al.,* 2009). These fungi can show positive effects on the plant host like growth promotion and enhancement of resistance and tolerance to biotic and abiotic stresses

(Mandyam and Jumpponen, 2005; Waller *et al.,* 2005; Kavroulakis *et al.,* 2007; Rodriguez *et al.*, 2008).

Diversity of fungal endophytes

Fungal endophytes have been recognized in two major groups. "Clavicipitaceous endophytes (CEs)" (Ascomycota, Clavicipitaceae) colonize shoots and rhizomes of a narrow host range of cool- and warm-season grasses (Poaceae), while "nonclavicepataceous endophytes (NCEs)" have been isolated from shoots and/or roots of almost all sampled plants and are phylogenetically diverse, most of them belonging to Ascomycota (Schulz and Boyle, 2005; Rodriguez *et al.,* 2009). CEs and NCEs have been categorized into four distinct functional groups or classes according to host range, colonized plant tissue, biodiversity, transmission, fitness benefits and colonization pattern (Rodriguez *et al.,* 2009). The CEs (class 1 endophytes) include the fungal teleomorphic genera Epichloe and Balansia corresponding to the anamorphs Neotyphodium and Ephelis, respectively. They form systemic intercellular infections with a hyphal gradient along the plant axis and are therefore primarily transmitted vertically by seeds (Schulz and Boyle, 2005; Kuldau and Bacon, 2008). The NCEs have been divided into three functional groups (class 2, 3 and 4) (Rodriguez *et al.,* 2009). Class 2 endophytes extensively colonize both above-and below-ground tissues, but with limited biodiversity in individual plant hosts. They are transmitted vertically and horizontally and confer non-habitat and habitat-adapted benefits to the plant host. Endophytic fungi in this class belong to a few members of the Agaricomycotina and Pucciniomycotina (Basidiomycota) (Rodriguez *et al.,* 2009) and also to Pezizomycotina (Ascomycota) such as *Phoma sp*. and *Arthrobotrys spp*. (Lopez-Llorca *et al.,* 2006) and *Fusarium culmorum*, *Colletrichum spp.* and *Curvularia protuberata* in roots, rhizomes, stems and leaves (Rodriguez *et al.,* 2008). NCEs class 3 present a localized colonization restricted to shoots, but with high biodiversity in individual tissues. They have been isolated from tropical forest to boreal and Arctic plant populations (Arnold *et al.,* 2003; Higgins *et al.,* 2007). They are horizontally transmitted by wind, rain and insects

(Arnold, 2008; Feldman *et al.,* 2008). The foliage isolates usually correspond among the Ascomycota to the Sordariomycetes, Dothidiomycetes, Pezizomycetes, Leotiomycetes and Eurotiomycetes, while basidiomycetous isolates belonging to Agaricomycotina, Pucciniomycotina and Ustilaginomycotina have been less frequently isolated from foliage than from woody tissues. This could be, however, underestimated because of the low recovering on agar cultures (Arnold *et al.,* 2007; Higgins *et al.,* 2007). Their benefits to the plant seem to be more complex and non-habitat-adapted (Rodriguez *et al.,* 2009). Class 4 endophytic fungi colonize only roots. They have a broad host range and belong to different phylogenetic groups among Ascomycota and among non-mycorrhizal members of the order Sebacinales (Basidiomycota) (Selosse *et al.,* 2009).

Secondary metabolites produced by endophytes

Endophytes may produce new bioactive substances *in vitro* and *in planta* because of a continual metabolic interaction with their hosts and the environment and, this metabolic interaction is important for the symbiosis of both partners (Schulz *et al.,* 2002; Schulz and Boyle, 2005; Suryanarayanan *et al.,* 2009). Endophytic fungi exhibit a higher proportion of new substances with biological activity than soil isolates and, the spectrum varies depending on the conditions like habitat, plant and substrate (Schulz *et al.,* 2002). These secondary metabolites have been isolated and characterized according to their ecological role and their potential in industry and medicine (Strobel and Daisy, 2003).

Fungal endophytes produce diverse metabolites in various chemical substance classes such as cytochalasines, steroids, chinones, phenols, isocoumarins, terpenoids, xanthones, enniatines, tetralones and benzopyranones which show antibacterial, antifungal, antimalarial, antiviral and anticancer activities (Li *et al.,* 2005; Suryanarayanan *et al.,* 2009). Certain endophytes produce a mixture of volatile organic compounds (VOCs) which consist of various alcohols, esters, ketones, acids and lipids with synergistic activities against pathogenic bacteria and fungi

(Strobel, 2006). Secondary metabolites with antagonistic activity towards pathogenic fungi can be also produced by endophytic fungi in axenic cultures which eases their analysis and application (Kim *et al.,* 2007). The discovery of fungal endophytes inside these plants with the capacity of producing the same compounds however shifted the focus of new drug sources from plants to fungi. In this way the substances could be produced by fermentation processes which reduced the production costs (Strobel, 2003; Suryanarayanan *et al.,* 2009). This was the case for taxol, a diterpene initially extracted from yew tree (*Taxus brevifolia*) and subsequently turned out to be also produced by fungal endophytes (Strobel *et al.,* 1996). Being synthesized in both, the host and the fungus, raised the question, if the corresponding genes were horizontally transferred between endophyte and plant (Stierle *et al.,* 1993).

Cancer status and therapeutics

Cancer is one of the most dangerous diseases reported in the world of medical science. The medical term of cancer is malignant neoplasm. It is the class of disease in which a group of cells display uncontrolled growth, beyond the normal limit. The branch of medicine concerned with the study, diagnosis, treatment and prevention of cancer is oncology. According to the report of American Cancer Society 8.7 million people died from cancer in the world during the mid of the year 2008. The survey of Cancer Research Institute (CRI) in Mumbai, emphasis that 4,00,000 Indians die every year from cancer and the disease is in growing proportion and increases 11% annually. The world cancer report, issued by International Agency for Research on cancer tells, that cancer prevalence are set to increase at a global alarm to aware of this epizootics (Li *et al.,* , 2007). Cancer rates could increase by 50 % to 15 million new cases in the year 2020. This will be mainly due to steadily aging populations in both developed and developing countries and also due to current trends in tobacco usage and the growing adoption of unhealthy lifestyles. The report also reveals that cancer has emerged as a major public health problem in developing countries. This shows the harmful growth of this killer disease, so the control of cancer disease is very important. Recent studies on tumor inhibitory compounds of plant origin have

yielded an impressive array of novel structures besides; epidemiological studies suggest that consumption of diets containing fruits and vegetables. Major source of phytochemicals and micronutrients, may reduce the risk of developing cancer (Li *et al.,* 2007)

Natural Products as Anticancer Agents

Progress has also been made in the treatment of some cancers. Certain cancers can be effectively controlled by drug combinations, such as acute lymphocytic leukemia, Hodgkin's disease, some non-Hodgkin's lymphomas and testicular cancer can be controlled by drug combinations. Relative to 1971, the consequences of chemotherapy are managed with greater efficacy through the use of antiemetics and immunostimulants.

In the War on Cancer, and through the history of combating cancer, natural products have played an important role in the development of contemporary cancer chemotherapy. Between 1960 and 1982 the National Cancer Institute screened around 114,000 extracts from an estimated 35,000 plant samples for anticancer activity. They initiated a new natural products program with a new *in vitro* human cancer cell line screen in 1987, and as of December, 1991, 28,800 plant samples had been collected from over 20 countries to screen for anticancer activity (Cragg, 1997) An important aspects of the use of traditional medicinal remedies and plants in the treatment of burns and wound in the potential to improve healing the same time to reduce the financial burden (Chandramohan and Siva Kumar, 2009). Several plants and herbs had been used experimentally to treat skin disorders, including wound injuries, in traditional medicine (Rodriguez *et al.,* 2009) Household uses basil leaf extract *Ocimum basilicum* was highly effective in inhibiting carcinogen-included both the tumor nodely at perinatal level. There are an estimated 70000 plants species that are used for health care purpose (Dahiya and Kumar, 2008). India is a good source of medicinal Plant and has a gold mine of well Practice Knowledge of herbal medicine

As a result of this research, a number of clinically useful and market-approved drugs were developed. A survey showed that among the 87 approved anticancer drugs, 62% are of natural origin or are modeled on natural product parents (Cragg *et al.*,1997). According to Newman and Cragg, (2007) as many as 65 % of formally synthetic hypertension drugs are plant based.

Among those clinically useful drugs include paclitaxel (Taxola) (Chaves *et al.,* 2009) and camptothecin, a natural product precursor for water-soluble derivative. These substances embrace some of the most exciting new chemotherapeutic agents currently available for use in a clinical setting. Although significant progress has been made in cancer chemotherapy, current drugs are ineffective against many common cancers and are often very toxic. Also at the current time, it is apparent that drug-based therapeutic strategies will predominate into the 21st century. For these reasons the discovery of new drugs effective against resistant solid tumors is an important and necessary strategy in improving chemotherapy. Undoubtedly more effort is needed to search for new cancer drugs with the aid of better screening methods from plants and other natural sources.

With regards to the selection and collection of material from natural sources for the discovery of naturally occurring anticancer drugs, various methods may be employed. There is the random method, where complete collection of plants found in a given area is screened. With this method, large numbers of species can be collected in a short period of time. Another strategy is to target plant families which are known to be rich in biologically active compounds. A third and most fascinating approach is an ethno botanical method, where local people's knowledge about the medicinal uses of the indigenous plants is taken into consideration when making plant selections

Secondary metabolites of fungal endophytes

Stierle *et al.,* (1993) researched on taxol and taxane production by *Taxomyces andreanae,* an endophytic fungus of Pacific yew, *Taxus brevifolia*. The hyphomyceteous fungus grown in a semi-synthetic liquid medium produced taxol

and related compounds. Taxol was identified by mass spectrometry, chromatography and reactivity with monoclonal antibodies specific for taxol. Both [1-14C] acetic acid and L-[U-14C] phenylalanine served as precursors of [14C] taxol in fungal cultures.

Schulz *et al.,* (2002) reviewed on how endophytic fungi serve as a source of novel biologically active secondary metabolites. Accordingly in course of the last 12 years, 6500 endophytic fungi were isolated from herbaceous plants and trees screened them for biological activities and have isolated and determined the structures of the biologically active compounds. The substances isolated were originated from different biosynthetic pathways belonging to diverse structural groups. The potential role of the endophyte and its biologically active metabolites in its association with its host has been investigated. Correlations were found between biological activity and biotope e.g. a higher proportion of the fungal endophytes in contrast to the soil isolates inhibited at least one of the test organisms for antialgal and herbicidal activities. It was seen that the fungal endophytes possess the exoenzymes necessary to colonize their hosts. Certain endophytic interactions associated with roots of the host may be mutualistic improving growth of the host and supplying the mycobiont with enough nourishment to extensively colonize the host's roots. Further plant defense metabolites are higher in plants infected with endophytes. Hence the interaction fungal endophyte plant host is characterized by a finely tuned equilibrium between fungal virulence and plant defence. Not only must the endophyte has to compete with epiphytes and pathogens but presumably also has to regulate metabolism of the host in their delicately balanced association. The utilization of a biotope such as that of the fungal endophyte is one aspect of intelligent screening and that fungi in different biotopes are still need to be exploited.

Antifungal products are vastly produced by majority of the endophytes. Griseofulvin-producing endophyte was first reported in fungus from *Abies holophylla* and was evaluated in vivo antifungal activity against plant pathogenic fungi. Based on nuclear ribosomal sequence analysis, the fungus was identified and labeled as *Xylaria* sp.. Two antifungal substances, griseofulvin and dechlorogriseofulvin were purified

from liquid cultures of *Xylaria sp*. and identified through mass and NMR spectral analyses. Compared to dechlorogriseofulvin, griseofulvin showed high in vivo and in vitro antifungal activity and effectively controlled the development of rice blast (*Magnaporthe grisea*), rice sheath blight (*Corticium sasaki*), wheat leaf rust (*Puccinia recondita*) and barley powdery mildew (*Blumeria graminis*) (Park *et al.,* 2004).

Duran *et al.,* (2005) did structure elucidation of bioactive compounds isolated from endophytes of *Alstonia scholaris* and *Acmena graveolens*. An endophyte of the genus *Xylaria* was isolated from a stem of *A. scholaris* its mycelia and exudate extracted and the extract assayed for growth inhibition of HeLa cancer cells in vitro. Several known compounds were isolated and identified based on NMR, infrared and mass spectral data. The compounds identified are 19, 20-epoxycytochalasin C; 19, 20-epoxycytochalasin D and xylobovide. Two other compounds, fusaric acid and dehydrofusaric acid were discovered in an endophyte of the Hypocreales family inhabiting the plant *A. graveolens*.

Macia-Vicente *et al.,* (2009) were experimental in producing cytotoxic secondary metabolites from the fermentation broth of the endophytic fungus isolated from the fruits of *Brucea javanica*. In vitro cytotoxic assays were performed using leukemia cell line. LC-MS analysis of the F4 fraction of n-butanol extracts of secondary metabolites revealed bruceocin and canthin–6 one compounds as cytotoxic constituents. These compounds were previously reported in the same host plant. Hence the present study could demonstrate the possibility of the endophytic fungi living symbiotically within the host plant producing cytotoxic secondary metabolites.

Lactones of endophytic origin have antiparasitic activity against Plasmodium falciparum. Three lactones were isolated from the culture medium of the endophytic fungus Xylaria sp. One was identified as (+) -phomalactone. The others were 6-(1-propenyl)-3, 4, 5, 6-tetrahydro-5-hydroxy-4Hpyran- 2-one and 5-hydroxymellein. Compounds 1 and 2 were reported for the first time as constituents

of Xylaria. Also this study was the first report showing the activity of these lactone compounds against a chloroquine-resistant Plasmodium falciparum strain, (Matsumoto *et al.,* 2002).

Different fungal species have been exploited as an alternative source of plant secondary metabolites. Endophytic fungi colonize plants internally without apparent adverse effects and do occur ubiquitously in plants. They are known to produce a number of important secondary metabolites including anticancer, antifungal, antidiabetic and immunosuppressant compounds e.g. paclitaxel, torreyanic acid, cytochalasins etc. have been isolated from endophytic sources. The discovery of Stierle and his co-workers, studies carried out by Strobel and Daisy had raised scope of using the endophytic fungus as a sustainable alternative source of important plant secondary metabolites. However our poor understanding of the evolutionary significance of these organisms and their dynamic interaction with their respective hosts results in failure of exploiting endophytic fungi in diverse arenas (Stadler *et al.,* 2010).

Kusari *et al.,* (2009) isolated, identified and characterized an endophytic fungus, *Aspergillus fumigatus* from *Juniperus communis* and *L. horstmann*, as a novel producer of deoxypodophyllotoxin and performed *in vitro* antimicrobial assay against a panel of pathogenic bacteria. The study concluded the production of deoxypodophyllotoxin (found in the host) by the cultured endophyte which is an enigmatic observation. This demonstrates the horizontal transmission of genes from the host plant to its endophytic counterpart. It would be interesting to further study the deoxypodophyllotoxin production and regulation by the cultured endophyte as well as their scale up process for consistent and dependable production.

Fungal fatty acids

Biochemical changes also play a vital role during the growth of fungi. Maysarah, (2010) made such studies in *Penicillium sps*. Changes in the lipid constituents of *P. atrovenetum* were studied during the growth and development of

this fungus. The total fatty acids increased to a maximum during the log phase and the major fatty acids were Palmitic, Stearic, Oleic and Linoleic acids. Younger mycelium contained a much lower percentage (on the basis of total fatty acids) of Linoleic acid compared to the ungerminated spores. Oleic acid was increasing as well as the remaining fatty acids like Myristic, Palmitoleic, Pentadecanoic acid increased slightly but Palmitic acid remained constant. Ergosterol was the only sterol detected. The following changes in lipids appear to be associated with the development and aging of fungi: (i) the presence of a relatively high content of non saponifiable lipid and ergosterol in the young mycelium and their later decrease with age and (ii) a shift from more unsaturated to less unsaturated fatty acids with age (Etten *et al.,* 1965).

Frisvad *et al.,* (2008) analyzed cellular fatty acid compositions of fungi and that they can be differentiated from one another on this basis. Many fungi were found to possess the same fatty acids but produced different relative concentrations of each. Some fungi differed in both the fatty acids produced and in the relative concentrations of others. Multivariate discriminant analysis demonstrated that all of the species included in the study had significantly different ($P < 0.001$) fatty acid profiles. Significant differences in fatty acid composition were also found at the intraspecific level. Both culture temperature and age affected fatty acid composition in the fungi examined but when these factors were held constant, variance in fatty acid composition was not a problem and fungal fatty acid profiles could be differentiated statistically.

Xu *et al.,* (2008) investigated the mycorrhizal fungal interactions in soil ecosystems. The estimation of their biomass is of importance in order to understand their possible role in soil nutrient processes. He analyzed that the signature fatty acid provides a new and promising tool for the estimation of fungal biomass in soil and roots. In biomass estimation primarily the phospholipid fatty acids (PLFAs) are suitable. Through the use of specific PLFAs it is possible to study interactions between mycorrhizal mycelia and bacteria in soil as well as between AM fungal mycelia and mycelia of saprophytic and parasitic fungi in soil and in roots. AM fungi

in particular store a large proportion of their energy as lipids and by using the signature fatty acids it is possible to determine the relation between membrane and storage lipids which could be an indication of energy storage levels. Various aspects of how the fatty acid signatures can be used for studies related to questions of biomass distribution and nutritional status of mycorrhizal fungi are discussed.

Higgins *et al.,* (2007) discussed the developments in fungal taxonomy. Accordingly both the type of fatty acid present and its relative concentration are useful characteristics for separating taxa. Until recently these techniques were only rarely used in fungal taxonomy. Although fewer different fatty acids are produced by fungi than by bacteria, these analyses are increasingly used for differentiating fungi. Recently Gas Chromatography combined with methods of multivariate statistical analysis has successfully been used to study the fatty acids of numerous and varied filamentous fungi including Oomycetes, Zygomycetes, Basidiomycetes and even sterile mycelia which are useful even at intraspecific level. These production techniques are applicable to the production of other polyunsaturated fatty acids (PUFAs), and will contribute to the improvement of fermentation technology especially in the field of fungal cultivation (Huang *et al.,* 2008).

Analysis and Purification of fungal fatty acids

Purification of 9, 12-cis-hexadecadienoic acid by HPLC and further GC analysis was done in Trichoderma sp. AM076, isolated from a freshwater sample. It was found to accumulate 9, 12-cis-hexadecadienoic acid (16: ω4), was the best carbon source for the conversion of Palmitoleic acid to 16: ω4. methyl 16: ω4 content reached 17.4 mg/g dry mycelia (443 mg/L) when the fungus was grown in a medium that contained 2.0% methyl myristate, 1.5% yeast extract and 2.0% methyl palmitoleate, pH 6.0, for 5 d at 12 °C it shaking (Schulz *et al.,* 2002). The lipid content of a source material/sample is traditionally determined gravimetrically by solvent extractions. The more commonly methods for lipid extraction are Soxhlet method, acid hydrolysis method, Bligh and Dyer methods. These extraction methods

vary in their lipid extraction efficiency. The total lipid by solvent extraction represents the content of crude fat. Crude fat is heterogeneous material consisting of a mixture of triacylglycerols, phospholipids, fatty acids, sterols, waxes and pigments which may also contain non-fat material hence needs to be purified and often fails to accurately estimate the inhibitory effect on various cancer cell lines (Li *et al.*, 2007).

Enormous studies have been made in the development of methods for the analysis of lipids using High Performance Liquid Chromatography (HPLC). In addition, it could be claimed that there is no type of lipid separation for which Thin Layer Chromatography (TLC) was once favoured that cannot now be done by HPLC. The latter offers great versatility in that it can be used in the adsorption, reversed-phase, ion exchange and silver ion modes. It operates at room temperature, so is particularly suited to molecules containing thermally labile functional groups. It is possible to isolate the complex lipids from TLC plates and then re-chromatograph them with more polar solvents (and with silica gel without added binder) to isolate each of the individual phospholipid classes say with comparable resolution to HPLC. A host of bonded-phases, offering varying selectivities in specific analyses, are available commercially and many have yet to be properly explored in lipid applications, (Tomsheck *et al.*, 2010).

Turbidometric growth studies of fungi

A microbroth kinetic model based on turbidity measurements was developed in order to analyze the growth characteristics of three species of filamentous fungi (*Rhizopus microsporus, Aspergillus fumigatus* and *Scedosporium prolificans*) characterized by different growth rates in five nutrient media (antibiotic medium 3, yeast nitrogen base medium, Sabouraud broth, RPMI 1640 alone, and RPMI 1640 with 2% glucose). Among the different growth phases distinguished, the smallest variability in growth rates among the strains of each species was found during the log phase in all nutrient media. *R. microsporus* and *A. fumigatus* grew better in Sabouraud and yeast nitrogen base medium than in RPMI 1640. None of the media provided

optimal growth of *S. prolificans*. The germination of Rhizopus spores and *Aspergillus* and *Scedosporium conidia* commenced after 2 and 5 h of incubation, respectively. In conclusion, the growth curves provide a useful tool to gain insight into the growth characteristics of filamentous fungi in different nutrient media and may help to optimize the methodology for antifungal susceptibility testing (Meletiadis *et al.,* 2001).

Turbidometric growth curves of different filamentous fungi in the presence of increasing concentrations of antifungal drugs were studied. 24 clinical mold isolates, including Rhizopus oryzae, *Aspergillus fumigatus, Aspergillus flavus,* and *Scedosporium prolificans,* were tested against itraconazole, terbinafine, and amphotericin B according to NCCLS guidelines. Exposure to increasing drug concentrations resulted in prolonged lag phases of the turbidimetric growth curves. The lag phases of the growth curves at drug concentrations which resulted in more than 50% growth (for itraconazole and terbinafine) and more than 75% growth (for amphotericin B) after 24 h of incubation for *R. oryzae, 48 h for Aspergillus sp.,* and 72 h for *S. prolificans*. Using this system, itraconazole and terbinafine resistance (presence of >50% growth) as well as amphotericin B resistance (presence of >75% growth) was determined within incubation periods of 5.0 to 7.7 h for *R. oryzae* (for amphotericin B resistance incubation for up to 12 h was required), 8.8 to 11.4 h for *A. fumigatus*, 6.7 to 8.5 h for *A. flavus*, and 13 to 15.6 h for *S. prolificans* while awaiting formal MIC determination by the NCCLS reference method.

Biotic and abiotic elicitors as enhancers for metabolite production

Wali *et al.,* (2006) revealed the ability of fungi to utilize crude glycerol as an alternative to conventional carbon substrates for growth and lipid production. Screening revealed that 40 of the 61 isolates tested had increased biomass yield compared to glucose, when crude glycerol was utilized. 29 of these isolates possessed the ability to completely metabolize 14 gL-1 of glycerol after 7-14 days. The top four candidates belonged to the genera *Galactomyces* and *Mucor*. Overall *Galactomyces*

sp. proved to be better suited for lipid production. In addition to producing biomass with a high lipid content (up to 45 % w/w), *Galactomyces sp.* also exhibited high biomass yields (up to 25 gL-1). The results obtained in this study compare favourably and in some cases exceed, other literature reported values for biomass and lipid production using glycerol. Ahmaed *et al.*, (2006) reported the effects of various process parameters on the production of Gamma Linolenic acid in submerged fermentation. Seven strains belonging to Mucorales were taken for the study. Oleaginous endophytic strain belonging to Western Ghats of Kerala produced 8% GLA using glucose as sole carbon source. Optimal conditions of 30ºC, 200 rpm for 7 days yielded maximum dry biomass and GLA respectively. Also combination of yeast extract, corn steep liquor and baker's yeast in ratio of 1:1:1 serve as better organic nitrogen source for increased GLA and lipid production.

Okane *et al.*, (2008) studied the effect of metal ion concentrations on lipid and Gamma Linolenic acid production in *Cunninghamella sp*. Effects of different concentrations of Magnesium sulphate, Ferric chloride, Zinc sulphate, Copper sulphate and Manganese sulphate were examined. Mg2+, Fe2+ and Zn2+ showed significant effect on lipid accumulation by the fungi. In particular Zn2+ showed 74% increase in GLA content of the fungi. Hence the study suggests that critical concentrations of metal ions for optimal production of lipids are required that might interfere in pathways involving lipid biosynthesis. Tauk *et al.*, (2009) studied lipid formation and Gamma linolenic acid production by *Mucor sp*. and *Rhizopus sp.* grown on vegetable oil. The fungal strains were tested in bioscreen automated system to select the best nutritional source. Submerged cultivation was carried out in media containing sole carbon/nitrogen source. Increased growth of fungi was observed in media containing vegetable oil with higher concentration of lipids. Results revealed higher amount of GLA was obtained with *Mucor circinelloides* in cultures containing sesame oil.

Shentu *et al.*, (2007) have shown in their studies that enhancement of productivity was established with several fold increase that addition of

oligosaccharide (oligomannuronate and oligoguluronate blocks) resulted in a 50% increase in penicillin G yield for *Penicillium chrysogenum.* Environmental abiotic and biotic stress factors have been proved to effect variety of responses in microbes. Elicitors as stress factors induce or enhance the biosynthesis of secondary metabolites added to a biological system. They are classified into various groups based on their nature and origin: physical or chemical, biotic or abiotic. Carbohydrates as biotic elicitors (oligosaccharides, oligomannuronate, oligoguluronate and mannan Oligosaccharides) have also been used widely in small amounts (mg/ml) as elicitor molecules in bacterial and fungal fermentations for overproduction of commercially important secondary metabolites.

Bioactivity assay and Apoptotic studies

Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth of a human breast cancer cell line in culture revealed results that Linoleic acid stimulated MDA-MB-231 cell growth with an optimal effect at a concentration of 0.75 μg/ml, whereas oleic acid produced growth stimulation at 0.25 μg/ml but was inhibitory at higher concentrations. Docosahexaenoic acid exhibited a dose-related inhibition of cell growth at concentrations ranging from 0.5 to 2.5 μg/ml and eicosapentaenoic acid was less effective. Similar inhibitory effects occurred with other saturated fatty acids, Rose and Connolly (1990).

Rodrigues and Hasse (2000) performed antimicrobial assays with the secondary metabolites produced by endophytic fungi from *Spondias mombin* (Anacardiaceae). Few of the isolated endophytes were chosen for preparation of culture broth extracts: *Guignardia sp. Phomopsis sp*. and *Pestalotiopsis guepinii.* Extracts were separated by chromatographic methods and tested for biological activities. The crude extracts were tested against 14 organisms including actinomycetes, Gram-negative and Gram-positive bacteria, yeast and filamentous fungi. All fungal extracts inhibited actinomycete growth. *Guignardia sp.* was active against *Escherichia coli, Staphylococcus aureus, Saccharomyces cerevisiae,*

Geotrichum sp. and *Penicillium canadensis*. Culture extracts of *P. guepinii* were active against *S. cerevisae,* while strains of Phomopsis sp. showed a pronounced antifungal effect against *Cladosporium elatum, Mycotypha sp.* and *S. cerevisae*.

Chromosomal DNA and mitochondrial dysfunctions play a role on mammalian cell death induced by oxidative stress. The major biochemical dysfunction of chromosome is the presence of an ordered cleavage of the DNA backbone which is separated and visualized as an electrophoretic pattern of fragments. Oxidative stress provides chromatin dysfunction such as single strand and double strand DNA fragmentation leading to cell death. More than 1 Mb of giant DNA 200-800 kb or 50-300 kb high molecular weight (HMW) DNA and internucleosomal DNA fragments are produced during apoptosis or necrosis induced by oxidative stress such as glutathione (GSH) depletion in several types of mammalian cells. Reactive oxygen species (ROS) mediated DNA fragmentation is enhanced by polyunsaturated fatty acids including Arachidonic acid or their hydroperoxides leading to necrosis. Mitochondrial dysfunction on decrease of trans-membrane potential accumulation of ROS membrane permeability transition and release of apoptotic factors during apoptosis or necrosis has been implicated. This review refers to the correlation of chromosomal DNA fragmentation and apoptosis or necrosis induced by GSH depletion and the possible mechanisms of oxidative stress-induced cell death (Higuchi, 2004).

Strobel, (2003) evaluated the anticancer potential of 11 plants used in Bangladeshi folk medicine. The extracts were tested for cytotoxicity uses the brine shrimp lethality assay, sea urchin eggs assay, hemolysis assay and MTT assay using tumor cell lines. The extract of *Oroxylum indicum* showed the highest toxicity on all tumor cell lines as well as on the sea urchin eggs. The extract of *Aegle marmelos* exhibited toxicity on all used assays but in a lower potency than *Oroxylum indicum*. The study concludes that only the extracts of *Oroxylum indicum, Moringa oleifera* and *Aegles marmelos* could be considered as potential sources of anticancer compounds among the tested plant extracts. Further studies are necessary for

chemical characterization of the active principles and more extensive biological evaluations.

Discovery of Oleic acid as the major component of olive oil that is responsible for a healthy Mediterranean diet was mentioned and the prevention of breast cancer by Oleic acid was examined. One study indicated that the protection of olive oil against breast cancer may be due to Oleic acid components rather than to the acid itself. Oleic acid is a monounsaturated fatty acid with a symmetrically placed double bond. Its IUPAC name is cis-9-octadecenoic acid its lipid shorthand name is 18:1 cis-9 and the CAS registry number is 2027-47-6 (Shu *et al.,* 2005).

Gabler *et al.,* (2010) demonstrated the cytotoxic activity of *Muscodor albus* crude extracts mixed with different solvents viz. hexane, chloroform, ethylacetate, 80 % ethanol and water extracts prepared from roots and stems of *Muscodor albus*. The cytotoxic activity was tested on Vero (V) cells using the MTT assay. Viability of cell cultures was evaluated in presence and absence of the extracts. Different and random combinations of plant part (stem, root) solvent were checked where they found the chloroform-root extract exhibited the most effective cytotoxic activity at 00 µg/ l (70.3 %) and the hexane-root and water-stem extracts of *Muscodor albus* were not cytotoxic at 00 µg/ l. Arnold *et al.,* (2007) studied on the biological activities of extracts from endophytic fungi isolated from *Garcinia* plants. Sixty five crude extracts from 51 selected endophytic fungi were tested for various bioactivities. 80% of the fungal extracts showed antimycobacterial (76.9%), antimalarial (14.1%), antiviral (16.7%), antioxidant (22.2%), antiproliferation (11.1% against NCI-H187 and 12.7% against KB cells) and cytotoxicity to Vero cells (40.0%). Molecular methods based on internal transcribed spacer rRNA sequence analysis was used to identify 15 bioactive isolates as *Aspergillus sp, Botryosphaeria sp, Curvularia sp, Fusicoccum sp, Guignardia sp, Muscodor sp, Penicillium sp, Pestalotiopsis sp and Phomopsis sp*. These results again prove that endophytes are potential sources of various bioactive natural products.

Aly *et al.,* (2008) produced bioactive metabolites from the endophytic fungus *Ampelomyces sp*. isolated from the medicinal plant *Urospermum picroides*. Extracts of cultures of Ampelomyces sp. exhibited considerable cytotoxic activity when tested in vitro against L5178Y cells. Chromatographic separation yielded 14 natural products including pyrone and sulfated anthraquinones that were identified based on H1 and 13C NMR and mass spectral studies. Few compounds exhibited strongest cytotoxic activity against L5178Y cells while few showed antimicrobial activity against the Gram-positive pathogens, *Staphylococcus aureus, S. epidermidis* and *Enterococcus faecalis*. The study further indicates the production of bioactive natural products by the endophyte in their host under in-situ conditions.

Li *et al.,* (2007) have found that the *C. militaris* extract can inhibit growth of MCF-7 human breast cancer cells in a dose and time-dependent manner. In addition to the apoptotic genes the levels of the methyltransferase gene DNMT1 and DNMT3a transcripts were also suppressed in MCF-7 cells incubated with the *C. militaris* extract. Methylation in some tumor-suppressor genes may potentially lead to regained expression of these genes and subsequent inhibition of cancer cell growth and its extract inhibits human breast cancer cell growth through an apoptosis cascade by inducing pro-apoptotic and suppressing antiapoptotic marker gene expression. *C. militaris* extract reduced DNA methylation through the suppression of methyltransferase transcripts leading to the recovery of tumor-suppressor genes and eventually inhibiting tumor cell growth.

Mercier and Jimenez, (2009) did preliminary screening of endophytic fungi from medicinal plants in India for antimicrobial and antitumor activity. 16 endophytic fungal isolates tested were found to exhibit antitumor activity in the yeast cell-based assay. The screening of antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, yeast and fungi was carried out on isopropanol extracts prepared from 121 isolates of endophytic fungi isolated from medicinal plants in India that includes *H. indicus*. Sensitivity was found to vary among the microorganisms. *Bacillus subtilis, Saccharomyces cerevisiae* and *Alternaria sp*. were susceptible to all

endophytic fungi extracts. None was found effective against *Salmonella typhimurium*. 16 endophytic fungal isolates tested were also found to exhibit antitumor activity in the yeast cell-based assay.

Anticancer drugs from fungal endophytes

Endophytic fungi are capable of living in host plants without causing any symptoms (Petrini *et al.,* 1992). To date, endophytic fungi have been separated into four classes based on host range, type of tissue(s) colonized, colonization in planta, diversity in planta, transmission and fitness benefits (Rodriguez *et al.,* 2009). Moreover, some endophytic fungi may produce secondary metabolites with potential for antimicrobial or anticancer property (Xu *et al.,* 2008). The endophytic fungus *Taxomyces andreanae* produced taxol with anticancer activity (Stierle *et al.,* 1993). It is also exemplified by isolation of anticancer drugs such as Taxol (paclitaxel) (Stierle *et al.,* 1993; Strobel *et al.,* 1996), camptothecin (Puri *et al.,* 2005), and podophyllotoxin (Kusari *et al.,* 2008) from endophytic fungal strains.

Fungal endophytes derived active compounds

Many plants and algae have been reported as hosts of fungal endophytes (Davis *et al.,* 2003). Among the host plants, the medicinal herbs are one of the important groups of hosts for endophytic fungi (Huang *et al.,* 2008; Li *et al.,* 2007; Li *et al.,* 2004; Xu *et al.,* 2008). Previous reports have demonstrated that fungal endophytes from medicinal herbs show efficacy as pharmaceutical and agricultural compounds, especially from Chinese herbs (Kusari *et al.,* 2008; Li *et al.,* 2004; Li *et al.,* 2007; Shentu *et al.,* 2007). Recently, certain isolates of endophytic fungi from Chinese herbs have been used as biocontrol agents for agricultural crops (Redman *et al.,* 1999; Schulz *et al.,* 2002; Backman and Sikora, 2008; Mejia *et al.,* 2008; Macia-Vicente *et al.,* 2009).

Other reports showed that certain endophytic fungi produced more than twelve metabolites similar to those produced by host plants with therapeutic function, including alkaloids, steroids, terpenoids, isocoumarin derivatives, quinines,

phenylpropanoids, phenylpropanoids and ligans, peptides, phenol and phenolic acid, aliphatic compounds and chlorinated metabolites (Li and Liu, 2004; Shu *et al.,* 2005; Strobel, 2003; Wang *et al.*, 2007).

Chapter – I

The isolation and preliminary bioactivity screening (antibacterial and antifungal) of bioactive metabolites of endophytic fungus

Introduction

Fungal endophytes colonize internal plant tissues without causing disease symptoms or overt tissue damage in their hosts (Musa *et al.,* 2011). This group comprises mycorrhizal fungi together with the non-mycorrhizal fungi, of which the latter occur in above-ground plant tissues and in roots (Brundrett, 2004). Numerous nonmycorrhizal endophytes, usually *Ascomycota*, have been isolated from gymnosperms to angiosperm monocots and eudicots in a wide diversity of habitats (Raviraja 2005). According to their plant hosts, their taxonomy and their ecological functions the non-mycorrhizal endophytes were grouped into two major groups such as Ascomycota, Clavicipitaceae (Petrini 1996). These nonclavicipitaceous endophytes being a highly diverse group with broad plant host range (Rodriguez *et al.,* 2009) and also colonize in some grasses (Kuldau and Bacon, 2008).

In some interactions, these fungi can exert positive effects on the plant based on the delivery of particular compounds, such as auxins (Vadassery *et al.,* 2008), polyketides, terpenoids (e.g. taxol) and derivatives of indol (Strobel *et al.,* 2004; Zhang *et al.,* 2006), or by the induction of plant defense mechanisms (Waller et al., 2005; 2006). In return, the niche inside plant tissues protects the microorganisms and can even supply their demand for nutrients (Mandyam and Jumpponen 2005). Non-clavicipitaceous endophytes include fungi that colonize specifically plant roots (Rodriguez *et al.,* 2009; Selosse *et al.,* 2009) including the heterogeneous complex of dark septate endophytes (DSE) which are conidial or sterile inhabitants of diverse plant species from arctic to temperate habitats. DSE produce melanized septate hyphae, microsclerotia in the roots and can influence plant growth both positively and negatively (Mandyam and Jumpponen 2005). Much information is available for

nonmycorrhizal endophytes in roots of forest trees and shrubs, in alpine and subalpine plants and in terrestrial orchids (Joshee *et al.,* 2009). However, little is known about the bioactivity of such edophytic fungal metabolites, which is very much needed in drug development.

The current chapter was aimed to isolate endophytic fungus from diversified tomato plants and the isolates were characterized to determine their taxonomic position and colonization pattern. This study also concentrated the preliminary bioactivity screening such as antibacterial and antifungal properties of endophytic fungal metabolites.

Materials and methods

Isolation of Endophytic fungus

Four to six-month old tomato plants without disease symptoms were collected from Plant tissue culture unit, Department of Biotechnology, Malankara Catholic College, Mariagiri, Kaliakkavilai. The tomato plants were kept in semi-opened plastic greenhouses for few days and were transplanted directly to soil after growth in seedbeds. Then the plants were sampled in their total root system. Initially, the roots were intensively washed with tap water, disinfected with 70% ethanol for one minute and random samples were directly transferred to 5% of a commercial sodium hypochlorite solution (2.5% NaOCl) for five (secondary roots) or ten minutes (primary roots). After washing three times with sterile distilled water, thirty fragments of approximately 1-2 cm were cut per each disinfected root system and the effectiveness of surface sterilization was verified on potato dextrose agar (PDA; Hi-media, Mumbai) using the imprint technique (Swofford, 2000). Subsequently, each root fragment were subdivided into three pieces and each one was transferred on PDA, sabouraud dextrose agar (SDA; Hi- media, Mumbai) and 2% malt extract agar (MEA; Hi- media, Mumbai) supplemented with 0.5 mg ml-1 chloramphenicol (Merk, India) and incubated at 25^{o}C. Each fragment was checked weekly for eight weeks for mycelia emerging from the cut ends. Emergent mycelia were subcultured to new PDA plates and incubated in darkness at 25^{o}C to recover pure fungal colonies. Pure isolates were maintained on PDA covered with sterile oil at 4^{o}C.

Characterization of endophytic fungus

After recovering on agar, isolates showed limited growth were removed, because they are not suitable for morphological characterization and analysis of colonization patterns. According to the morphological features of hyphae and spores, the remaining 6 isolates (EF01and EF06) with dark mycelia were selected for further analyses. Hyphal characteristics, conidiogenous cells and type of spores or conidia

were studied by light microscopy (Labomed, Italy) at magnifications of 200 and 400x. Identification of putative endophytes was carried out based on these characters at least to genus level (Ellis 1976).

Growth in different media

Pure colonies of the selected endophytes were grown on different culture media such as PDA (Merck, India), corn meal agar (Sigma-Aldrich, Germany), SDA (Hi Media, Mumbai) and 1.5 % water agar (Hi Media, Mumbai) at 22^{o} C or 25 ^{o}C. The fungus was grown in different pH (5.0, 5.5, 6.0, 6.5 & 7.0). Growth rate of the colonies was measured.

Isolation of secondary metabolites from endophytic fungus

The selected endophytes grown on PDA media was dried in room temperature and about 250 g of finely powdered material was refluxed three times in a 1 liter capacity round bottom flask in a water bath for about 6 h using 500 ml of appropriate solvents (methanol). The extracts were filtered and concentrated to recover the excess solvents in another distillation system. The concentrated extract (about 100ml) was again filtered through a Whatman no. 1 filter paper fitted with a Buchner funnel using suction pressure. Finally it was reduced to thick oily natured crude extract in a rotary vacuum evaporator (JSGW-Buchi type) at 40^{o}C, collected in air–tight plastic vials and stored in the refrigerator for further activity studies.

Antibacterial activity

The agar plate diffusion assay was used to evaluate the antimicrobial activity against four different bacterial strains such as *Bacillus subtilis* (MTCC 441), *Micrococcus luteus* (MTCC 1541), *Escherichia coli* (MTCC 443) and *Klebsiella pneumoniae* (MTCC 109). A 100 µl of bacterial liquid culture, in an exponential growth phase, was spread onto the surface of Muller Hinton agar plate. Immediately, 100 µl of crude extract of the fungal isolates were loaded onto the well. The culture was incubated at 30^{o}C for 18 h and the zone of inhibition was measured. Standard

discs of Tetracycline (10 µg) and Chloramphenicol (10 µg) was used as positive control for antibacterial activity.

TLC Screening

About 1 mg of each extract was used for chromatography. A solvent system of 4:1 CHCl3: MeOH was prepared and placed in a tank and the lid was replaced. The plant extract and the fungi extracts were spotted, each one on separated origin on the plate. Chromatographic separations were carried out using silica gel (Merck, type 60), precoated silica gel. The plates was placed carefully into the tank and covered with the lid. After development, the plate was removed and the solvent front was marked with pencil and allowed to dry. TLC plates were viewed under UV light at 254 nm for fluorescence quenching spots and at 366nm for fluorescent spots. Ceric sulphate solution was used as spraying reagent to detect the spots.

Screening of extracellular enzymes

The isolated strains were tested for their ability to produce extracellular enzymes that degrade starch, proteins and olive oil, for a screening of enzymes amylase, protease and lipase production, respectively. The production of all enzymes was determined by the observation of color intensity and diameter of degrading halos formed in the solid cultivation media.

Amylase

The endophytic microorganisms were inoculated on Petri dishes containing YM medium and incubated at 30 °C for 24 h. The production of amylase was determined using starch medium agar with 0.5 % soluble starch (Bastos, 2005). After 48 h, 10 mL of iodine solution (30 % iodine) was applied in each plate, and the halos around the colonies were measured. All assays were performed in triplicate.

Lipase

The medium used for the determination of lipase production contained the following compounds (per liter): peptone, 3.0 g.L-1; K2HPO4, 2 g.L-1; MgSO4, 1 g.L-1, rhodamine B, 0.01 g.L-1; yeast extract, 2 g.L-1; agar, 18 g.L-1 and 20 g.L-1 olive oil (Lin *et al.,* , 1995). In order to obtain colonial growth, Inoculated plates were

incubated at 30 °C for 48 h. The presence of orange fluorescent halos around colonies under UV rays observation indicated the presence of positive lipase-production. All assays were performed in triplicate.

Protease

Protease activity was determined by inoculating the microbial strain in a culture medium containing triptone, 46.43 g.L-1; yeast extract, 2.79 g.L-1; skim milk powder, 23.21 g.L-1 and agar, 18.57 g.L-1 adapted from (Tang *et al.*, 2008). After 48 h at 30 °C, the halos around the colonies were measured. All assays were performed in triplicate.

Results

Isolation and Identification of endophytic fungi

37 fungal isolates were initially obtained from 20 root fragments of tomato plants. Among the 37 isolates, 31 do not show growth in culture after recovering from oil and were therefore not further characterized. After the isolation, a total of 6 different isolates were recovered from 20 disinfected tomato roots. The number of fungal isolates recovered from roots was 12 (60%). 6 isolates could be roughly categorized according to their morphological characteristics.

Characterization of fungal endophytes

The characterization of 4 different endophytes were displayed in the table: . these isolated produces to types of mycelium types. one type mycelium is branched, septate; hyaline, pale brown or grayish white. Conidiomata is acervular, separate or confluent, composed of hyaline to dark brown, thin or thick walled texture angularis; dehiscence irregular. Setae are sparse. Conidiogenous cells are enteroblastic to phialidic, hyaline. Conidia are fusiform, apices obtuse 9 – 24 X 3-4.5 µm. The textures of second type mycelium are spongy, cottony and flocculate. The colour of the colony is bright white at young stage and light pale orange at mature stage. Conidigenous cells are lining the base of the conidia. The conidia are septate, 5 celled, cylindrical to fusiform, 24.0 × 9 µm and versi coloured. The upper two median cells of conidia are darker than the lower median cell, bearing appendages. The upper two cells are dark brown and the lower cells are pale brown. Based on the morphological characteristics of fungal colony and conidia, four species were successfully identified as *Aspergillus flavus* (EF01), *Aspergillus sp*. (EF02), *Curvularia lunata* (EF03) and *Cladosporium sp*. (EF04), Two species remained unknown (Plate I).

Table 1. The morphological description of isolated endophytic fungi

Sl. No	Code	Description	Species
1.	EF01	Colonies white or silvery white, reverse green to orange brown or reddish brown with thin or thick walled texture angularis. Conidial heads columnar in size. Vesicles globose to ovate, metulae fertile over entire vesicle, conidial heads splitting over age. Conidia smooth, irregular. Setae are sparse globose, 2-3cm in diameter. Conidia are fusiform	*Aspergillus flavus*
2.	EF02	The colony was pulvinate in appearance, green at the centre, with light yellow radial rays and white color edges. The diameter of the colony was 1.05 cm/day. The conidiophores were 500-530 cm in length and 7.5 cm in width. The conidial heads were columnar, compact, about 15 cm in length and 13 cm in breadth from the line of the phyllade heads. The vesicles were flask shaped aseptate in nature.	*Aspergillus sp.*
3.	EF03	Colonies brownish white, with sparse aerial mycelium and small dense felty patches, elsewhere reverse white to grey, conidial masses salmon pink. Some cultures have abundant greyish white aerial mycelium with poor sporulation and no distinct acervuli.	*Curvularia lunata*
4.	EF04	The centre of the colony was green in color with radial rays from the centre and white edged margins. The diameter of the colony was 0.5 cm/day. The diameter of the spore was 6.2 cm, the phyllade was 1.0 cm in length. The color of the colony turns to powdery green on maturity.	*Cladosporium sp*.
5.	EF05	Full brown and whitish mass	unknown
6.	EF06	Green mass with thick edge	unknown

Growth in different mycological media

The radial growth of the fungal isolate was studied on different solid media like PDA, CMA, SDA and water agar. Among the various media, maximal growth was observed on PDA medium. The fungus was grown in different pH (5.0, 5.5, 6.0, 6.5 & 7.0) of PDA Broth medium for 21 days at 30^{o}C and the dry weight of mycelial growth was studied. Maximum dry weight of the mycelium EF01 was obtained at pH 6.0 when compared with the growth on other pH's of the medium (table. 2).

Table 2. Growth of endosymbionts in different mycological media

Endosymbionts	PDA					CMA					SDA					Water agar				
	5	5.5	6	6.5	7	5	5.5	6	6.5	7	5	5.5	6	6.5	7	5	5.5	6	6.5	7
EF01	+	+	+++	+	+	-	+	+	+	+	-	+	+	+	+	-	-	+	+	+
EF02	+	+	++	+	+	-	-	+	-	-	-	-	+	+	-	-	-	+	-	-
EF03	-	+	+	+	+	-	-	+	+	-	-	-	+	+	-	-	-	+	-	-
EF04	-	+	+	+	+	-	-	+	+	+	-	+	+	+	+	-	-	-	-	-
EF05	-	-	+	+	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-
EF06	-	-	+	+	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-

(- - No growth, + - minimum, ++ - moderate, +++ - maximum)

Antibacterial activity

Preliminary antibacterial screening was carried out on the crude extracts of the six endophytic fungi species isolated from tomato plants against the four standard human bacterial pathogens. The antibacterial effect of the fungi extracts is shown in Table 3. The inhibition zone produced by EF01 values were highly variable, ranging between 14-37 mm. The extracts from *Cladosporium sp. Aspergillus sp.* and two of the unknown species were effective (I. Z. D. > 20 mm) against all the bacterial strains.

Table 3. Antimicrobial potential of endophytic fungi with potential pathogens (in mm)

Endosymbionts	*B. subtilis*	*M. luteus*	*E. coli*	*K. pneumoniae*
EF01	37	30	28	16
EF02	32	26	24	16
EF03	14	12	14	08
EF04	20	20	18	14
EF05	18	16	14	14
EF06	21	18	18	18

Phytochemical screening

TLC separation of crude extracts using the solvent system, CHCl3: MeOH in a 4:1 ratio visualized under UV365nm and detected with ceric sulphate spray reagent, revealed the presence of many spots in all samples except sample 1 and 7 prepared from two of the unknown fungi. The obtained pattern might be attributed to the presence of lipophilic, sterols and/or triterpenes compounds in addition to different classes of compounds of intermediate polarity. The pattern of spots in the plant crude extracts resembles that is shown by the endophytic fungi extracts, thus suggesting the presence of similar chemical constituents in the two sources (plate – 2).

Screening of extracellular enzymes

The results obtained suggest that the microorganisms have the ability to use starch and lipids as energy sources, while the production of protease enzymes was not detected, as shown in Table 4.

Table. 4. Enzymatic index of endophytic fungal strains

Endosymbionts	Amylase	Lipase	Protease
EF01	27.0	25.0	0.0
EF02	24.0	22.0	0.0
EF03	20.0	18.0	0.0
EF04	18.0	18.0	0.0
EF05	00.0	18.0	0.0
EF06	00.0	16.0	0.0

Discussion

During this study, a survey has been conducted for the diversity of endophytic fungi associated with tomato plant. A total of six fungal endophytes were isolated and cultured in the laboratory. Morphological investigations, using both macroscopic and microscopic features, have resulted in the identification of four fungal species: *Aspergillus flavus*, *Aspergillus* sp., *Curvularia lunata* and *Cladosporium sp*. Three fungal species, although subjected to the same morphological investigations, remains unidentified. Overall, these preliminary results suggest that tomato hosts are the suitable host for many endophytic fungi. This study represents the first attempt to isolate and identify endophytes from tomato plant Indian scenario and thus it provides a research base for further investigations. Among that the culture media, maximal growth was observed in PDA medium with pH 6.0 when compared with the growth on other pH's of the medium. The antibacterial effect of the fungi extracts, the EF01 extracts produced highly variable zones ranging between 16-37 mm. The extracts from *Cladosporium sp. Aspergillus sp.* and two of the unknown species were also effective against all the bacterial strains.

The yield of bioactive compounds can sometimes be substantially increased by the optimization of physical (temperature, salinity, pH and light) and chemical factors (media components, precursors, and inhibitors) for the growth of microbes (Thakur *et al.*, , 2009; Miao *et al.*, 2006; Kumara and Rawal, 2008; Zain *et al.*, 2009; Gautam *et al.*, 2011; Bhattacharyya and Jha, 2011). Among the tested media, maximum mycelial dry weight (74 mg/25 mL) was recorded in potato dextrose broth medium, followed by malt extract medium (55 mg/25 mL) and czapek's dox broth (51 mg/ mL) whereas sabouroud's broth and nutrient broth showed 28 mg/mL and 30 mg/mL respectively. Similarly, maximum bioactive metabolite was produced in potato dextrose broth (23 mm against *Klebsiella sps*) followed by malt extract broth (17 mm against *Staphylococcus aureus*) and czapek's dox broth (19 mm against *Klebsiella sps*) (Gogoi *et al.*, 2008; Ritchie *et al.*, 2009; Jain and Pundir, 2011).

Minimum production of bioactive metabolite was observed in sabourod's broth (5 mm against *Klebsiella sps*) and nutrient broth (7 mm against *Vibrio cholera*). Perrone *et al.,* (2008) reported the potato dextrose broth medium as the best medium for the maximum growth of *Drechslera hawaiiensis*, the foliar blight pathogen of *Marsilea minuta*. Similarly the marine derived fungus *Arthrinium c.f. saccharicola* was investigated by Miao *et al.,* (2006) and suggested that the culture medium had an effect on mycelial growth and metabolite profile.

Production of bioactive metabolites by the strain was high in the modified yeast extract-malt extract-dextrose broth as compared to other tested media (Kiranmayi *et al.,* 2011). Zain *et al.,* (2009) reported that the growth and secondary metabolites production of *Aspergillus terreus*, *Penicillium janthinellum* and *Penicillium duclauxii* were significantly affected by the type of the growth medium and further yeast extract showed the best mycelial growth and secondary metabolite production. Whereas in the present study potato dextrose showed the best mycelial growth and bioactive compound production and Sabourod's medium showed lowest values. Similar results were reported in *Aspergillus* strain where maximum dry weight (71 mg/25 mL) was recorded in potato dextrose broth medium and maximum zone of inhibition (25 mm) against *Bacillus subtilis* (Bhattacharyya and Jha, 2011).

The effects of different carbon sources on biomass and bioactive metabolite production by *Aspergillus terreus*. Among the various carbon sources tested, sucrose was the best carbon source for both biomass (78 mg/25mL) and bioactive metabolite production (24 mm against *Escherichia coli*). Moderate growth and bioactive metabolite production was observed in glucose supplemented media. Starch was the least utilized carbon compound by the isolate and even the bioactive production was very low. Bhattacharyya and Jha. (2011) reported that the *Aspergillus sp*. grew on all the carbon sources and tested against bacterial pathogen *Bacillus subtilis*, and the maximum growth and bioactivity of the strain was noted when the sucrose was used as a sole carbon source. The results are in good agreement with Bandara *et al.,* (2006). Similar results were obtained in the present study where maximum biomass and bioactive metabolite production (zone of inhibition, 24 mm) was obtained in

sucrose supplemented media. Healy *et al.,* (2004) have described the importance of various nitrogen sources in maximizing the growth rate of the fungal strain and the antibiotic production. He also reported the nitrogen source may influence the antibiotic production in *S. rimosus*. Atta *et al.,* (2011) presented that the optimal antimicrobial activity was obtained with sodium nitrate in the culture medium of *Streptomyces albidoflavus*. Whereas in the present study medium supplemented with yeast extract showed maximum growth and bioactive metabolite production.

Bandara *et al.,* (2006) pointed out that most of the microorganisms have the ability to synthesis antimicrobial compounds at pH ranging from 5.5 to 8.5. The maximum bioactive metabolite production in potato dextrose broth at pH 6.0 was reported by Gogoi *et al.,* (2008). Gogoi *et al.,* (2008) also investigated the influence of pH on the growth and production of bioactive metabolite by an endophyte *Hypocrea sp.* isolated from *Dillenia indica* Linn in North-East, India. *C.gloeosporioides* isolates grew well at pH 5 while pH 6 was found preferred for the sporulation (Liu *et al.,* 2008). In the present study maximum growth as well as increased antimicrobial metabolites was obtained at pH 6 suggesting the acidophilic characteristics of the isolate. Similar results have been reported for several *Aspergillus sp*. The growth and antibacterial activity of *Aspergillus sps* was influenced by pH of the medium.

To date references containing the screening of enzymes from endophytic microorganisms in Petri dishes are still scarce, but former studies used these strains to degrade xylan and mannan, as reported by Liu *et al.,* (2008). It is assumed that during the incubation period, the bacteria and fungi tested released enzymes (amylases, proteases and oxidases) that actively degraded components of the culture medium. Comparative analysis of the production of extracellular enzymes detected variability among isolates of Baru, which can be very useful information for the identification of isolates.

This chapter describes the use of fungal endophytes (EF01 and EF02) isolated from the tomato plants and demonstrates a partial use of these microorganisms in biotechnological processes and their potential as source of bioactive compounds.

Further studies are still needed to identify the compounds related to the anticancer activity. Interestingly, this appears to be one of the first works in Indian scenario achieved by a tomato endophytic microorganism.

Chapter – II

Biotoxicity and anticancer screening of chosen endophytic fungal metabolites

Introduction

Endophytic microorganisms are a significant reservoir of novel bioactive secondary metabolites including anti-microbial, anti-insect, anti-cancer, antidiabetic and immunosuppressant compounds with their great potential applications in agriculture, medicine and food industry (Zhao *et al.*, 2011). These bioactive compounds could be mainly classified as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols and lactones (Yu *et al.*, 2010; Zhou *et al.*, , 2010). World health problems caused by drug resistant bacteria and fungi are increasing. Many pathogenic microorganisms have developed resistance due to the misusage or long-term usage of the same class of antibiotics. An intensive search for newer and more effective antibiotics to deal with these problems is now underway. The isolation of novel secondary metabolites from the endophytes is a progressive field in research (Huang *et al.*, 2008). Endophytes, which occupy a unique biotope with global estimation up to one million species, are a great choice to avoid replication in the study of natural products to assist in solving not only plant diseases, but also human and animal health problems (Gimenez *et al.*, 2007). Since the endophytes can be found in nearly all living plant species, a scientific basis in plant selection is necessary for the study of endophytes in order to isolate microorganisms with pharmaceutical potential (Tong *et al.*, 2011). Approximately 4,000 secondary metabolites of fungal origin have been described to possess biological activities (Zhao *et al.*, 2011). The number of secondary metabolites produced by fungal endophytes is larger than that of any other endophytic microorganisms (Zhang *et al.*, 2006). In the present study, the endophytic

fungi were isolated from living tomato plants and the fungal extracts were screened for their biotoxicity and anticancer properties.

Materials and methods

The methanolic extracts (ES01 and ES02) which were isolated in the earlier chapter were used in this study.

Biotoxicity studies

Brine shrimp cytotoxicity

About 0.1 g of *Artemia salina* cysts was aerated in 1lit capacity glass cylinder (jar) containing filtered seawater. The air stone was placed in the bottom of the jar to ensure complete hydration of the cysts. After 24 h, the newly hatched free-swimming pink-colored nauplii were harvested from the bottom outlet. As the cyst capsules floated on the surface, this collection method ensured pure harvest of nauplii. The freshly hatched free-swimming nauplii were used for the bioassay. The assay system was prepared with 2 ml of filtered seawater containing chosen concentration of methanolic extracts of ES01 and ES02 in cavity blocks (Embryo cup). Parallel vehicle control (using 2 % methanol) and negative control wells also kept. In each, 20 nauplii were transferred and the setup was allowed to remain for 24 h, under constant illumination. After 24 h, the dead nauplii were counted with a hand lens. Based on the percent mortality, the LD_{50} of the test compound was determined using probit scale.

Larvicidal activity

As the larval stages of mosquitoes (*Culex* sp.) were more accessible for control, the early second and final fourth instar larvae were chosen for the experiments. The susceptibility or resistance of the mosquito larvae to the selected concentration of the extracts was studied by adopting standard bioassay protocols. Observations were made after 24 h of treatment for larvicidal activity.

Ichthyotoxicity assay

Fingerlings (1.5-2.0cm) of *Oreochromis mossambicus* were used for evaluating the ichthyotoxic potential. Five Fingerlings were introduced in each experiment and control. Glass bowls containing 1000 ml seawater dissolved with chosen concentration of the extracts were used. Immediate reflex changes and mortality were observed continuously for six hours at 1 h interval for the next 12 h. After 24 h of exposure, the numbers of dead and live fishes were counted. The acute toxicological reflexes were observed and recorded. The mortality percentage was converted into probit scale to determine the LD_{50} values.

Cell lines and its preparation for experiment

HeLa Cell line (ATCC CCL-2) were obtained from American Type Culture Collection (Manassas, VA), maintained in continuous exponential growth by twice-a-week passage in RPMI 1640 medium supplemented with 8.25% fortified bovine calf serum (Hi Media) and penicillin (100 IU/ml)- streptomycin (100 g/ml), and incubated in the presence or absence of drugs at 37°C in a humidified atmosphere containing 5% CO_2. SH-SY5Y Neuroblastoma Cell lines were obtained from Amala Cancer Centre and similarly maintained in RPMI 1640 medium .These cell lines were maintained in the Animal tissue culture and Biomedicine lab, Biogene tech laboratory, Hyderabad.

MTT assay

MTT assay is a standard colorimetric assay (an assay which measures changes in colour) for measuring cellular proliferation (Cell growth). It is used to determine cytotoxicity of potential medicinal agents and other toxic materials. Yellow MTT (3-4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple Formosan in the mitochondria of living cells. A solubilization solution (usually either dimethyl sulfoxide or a solution of the detergent sodium

dodecyl sulphate in dilute hydrochloric acid) is added to dissolve the insoluble purple Formosan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wave length (usually between 500 and 600 nm) by a spectrophotometer.

This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion is directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent is causing death of cells can be deduced, through the production of a dose response curve.

On the first day of the experiment, one T-25 flask of SH-SY5Y Neuroblastoma Cell lines was trypsinized and 5 ml of complete media was added to trypsinized cells. Further the cells were centrifuged in a sterile 15 ml falcon tube at 500 rpm in the swinging bucked rotor (400 x g) for 5 min. The media was removed and cells were resuspended to 1.0 ml with complete media. The cells per ml were counted. The cells diluted to 75,000 cells per ml with complete media. 100 µl of cells were added (7500 total cells) into each well and incubated overnight. On the second day, cells were incubated overnight with methanolic extracts of ES01 and ES02 at the dose of 10 mg/ml each, in a different experimental setups. On the third day of the experiment, 20 µl of 5 mg/ml MTT was added to each well. One set of wells with MTT was incubated but no cells as the control group. The plates were incubated for 0, 12, 24, 36, 48, 60, 72 hours at 37^{o}C in culture hood. The media was removed carefully and 150 µl MTT solvent was added. The cells were agitated on orbital shaker for 15 min and the absorbance at 590 nm was read with a reference filter of 620 nm.

Hydrogen Peroxide Scavenging assay

The ability of the extracts to scavenge hydrogen peroxide was determined. Hydrogen peroxide solution (40 mM) was prepared in phosphate buffer (pH 7.4).

The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extracts (1 mg ml^{-1}) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows:

$$\% \text{ Scavenged } (H_2O_2) = [(Ao - A1)/Ao] \times 100$$

where Ao was the absorbance of the control and A1 was the absorbance in the presence of the sample of extract and standard.

DPPH Radical scavenging assay

10-100 µg of extracts were added to 295 µl DPPH solution (4,5 mg DPPH (HIMEDIA) in 100 ml methanol) in each well of 96 well plates. The absorbance at 517 nm was then monitored at 15 seconds interval from 0 to 5 min. Methanol was used as the blank solution. Ascorbic acid as a positive control representing 100% radical scavenging activity in each experiment.

COMET assay (Single cell eletrophoresis)

1% (500 mg per 50ml Phosphate buffered Saline (PBS)) and 0.5% Low Melting Point Agarose (LMPA) (250 mg per 50 ml PBS) and 1.0% Normal Melting Agarose (NMA) (500 mg per 50 ml in Milli Q water) were prepared. The Agarose (LMPA and NMA) were boiled to get the agarose solidified. The microscopic slides were diped in NMA up to one-third the frosted area. To the agarose coated slide, 75 µL of LMPA (0.5%; 37ºC) priorly mixed with 10,000 lymphocytes was added. The agarose on the slides were allowed to solidify in the room temperature for 20 minutes. Third agarose layer (80 µL LMPA) was added to the slide and coverslip was placed. The slides were incubated in lysing solution at 4ºC for 2 hours. The slides were placed side by side on the horizontal gel box near one end, sliding them as close together as possible. The buffer was filled in the reservoirs with freshly made pH>13

Electrophoresis Buffer until the liquid level completely covers the slides. Then the slides were let to incubate in the alkaline buffer for 20 minutes to allow for unwinding of the DNA and the expression of alkali-labile damage.The power supply was turned on to 24 volts (0.74V/cm) with the current of 300 milliamperes. The slides were let for 15 minutes for electrophoresis and removed from the setup. Further the slides were stained stained with 80µL 1X Ethidium Bromide for 5 min and then dipped in chilled distilled water to remove excess stain. For visualization of DNA damage, observations were made of EtBr-stained DNA using a 40x objective on a fluorescent microscope.

Trypan blue exclusion cell viability assay

Trypan blue is a vital stain that is used to stain dead cells blue. It is diazodye. Live cells or tissues with intact cell membranes will not be colored. Since cells are very selective in the compounds that pass through the membrane, in a viable cell trypan blue are not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a microscope. Trypan blue is commonly used in microscopy (for cell counting) and in laboratory mice for assessment of tissue viability. The method cannot distinguish between necrotic and apoptotic cells. It is also useful to observe hyphea of fungi and stramenopiles. Trypan blue is derived from, tolidine that is, any of several isomeric bases, $C_{14}H_{16}N_2$, derived from toluene. Trypan blue is so-called because it can kill trypanosomes, the parasites that cause sleeping sickness. The dye is also known as diamine blue and Niagarablue.

Trypan blue is a dye that is used to determine the viability of a cell. Living cells exclude the die; whereas dead cells will take up the blue dye. The blue stain is easily visible and cells can be counted using a light microscope. The cells culture (treated with ES01 and ES02) was diluted to 100 cells/ml with 1x Phosphate buffered Saline (PBS) solution. 5 ml cell suspension was taken with equal volume of trypan blue solution. 50 microliter of cell trypan blue mixture was taken into micropippet

and placed in the edge of Haemocytometer. The solution was allowed to run along the grooves with parallel gentle shaking of haemocytometer slide. The slide was placed under 10X objective lens in a Phase contrast microscope (Labomed) and cells were counted from each chamber. The number of stained cells and the total viable cells were counted from the haemocytometer and recorded.

DNA Fragmentation assay (Triton X100 lysis method)

$4X10^6$ HeLa cells (ATCC CCL-2) were incubated with 2 mg of dried fungal extract from each sample was incubated separately in 1 ml eppendrof tube for 10 minutes. The incubated cells were further collected in 1.5 ml eppendorf tube and centrifuged at 3000 rpm to collect the cells. The supernatant was discarded. The cell pellet was then suspended with 0.5 ml PBS. 55 microliter of Triton X100 lysis buffer was added in the cell mixture at 4^0C. The tubes were centrifuged at 4^0C for 30 minutes and the supernatant was transferred to 1.5 ml eppendorf tubes. 1:1 mixture of Phenol: Chloroform along with one tenth quantity of sodium acetate solution. The tubes were agitated gently for 10 minutes and centrifuged again at 3000 rpm. The two fold quantity of ice cold with the pellet along with one tenth equivalent of Sodium acetate buffer. The above step was repeated for 3 times and finally centrifuged at 5000 rpm. The pellet was re-suspended in 30 micro liter of deionized water with RNAse solution (0.4ml water + 5µl of RNase).

Agarose Gel Electrophoresis

The lysed ingredients of DNA fragmentation assay were added in 250mL conical flask and stirred well. Then the conical flask was kept in microwave oven for one minute. 1µL of ethidium bromide (10mg/ml) was added with the solution and mixed thoroughly. The gel was poured at the gel setup without bubbles at 60 ^{0}C and the combs were inserted. The setup was kept undisturbed for 30 minutes to allow the gel to solidify.

Running the gel

0.5X of TBE buffer was poured into the gel tank until the gel is submerged for 2 mm depth. The Samples from Triton x100 lysis reaction was mixed with 5 µL of loading buffer and each sample was loaded into adjacent wells along with Hind III lambda DNA digest marker. The gel was initially run at 5V for 5min before increasing to 100V. The gel was allowed to run until the tracking dye reaches the end of the gel. Further the gel was taken from the setup and documented with BioRAD Gel documentation system.

Results

Biotoxicity studies

Brineshrimp cytotoxicity assay

The results of *Artemia* cytotoxicity bioassay are depicted in Fig. 1.b. The secondary metabolites of the ES01 exhibited high toxicity against *Artemia* nauplii followed by ES02 (table -5). The LC_{50} values of ES02 and ES01 are 0.40%, and 0.32% respectively. The toxicity profile of these two secondary metabolites indicated that they were moderately toxic. Temperature had significantly influenced the toxicity of these endosymbiontic metabolites. The toxicity profiles of endosymbiotic extracts considerably decreased at $20^0 \pm 2^0$C. At this temperature, the toxicity of ES02 was reduced as it resulted in 60% mortality at 1.0 %; while it resulted in 95.00% mortality at the same concentration at $30^0 \pm 2^0$C. Similarly ES01 showed 67.0% mortality at 1% while it produced 100% mortality at $30^0 \pm 2^0$C.

Table: 5. *Artemia* cytotoxicity profile of endosymbiontic metabolites at 30 and 20^0C

	Concentration	Mortality (%)	
		30 $\pm 2^0$C	20 $\pm 2^0$C
ES01	0.2%	20.2±3.6	0
	0.4%	60.0±7.0	20.0±1.2
	0.6%	90.6±3.2	40.0±2.1
	1.0%	100±0.0	67.2±0.8
ES02	0.2%	10.2±2.6	0
	0.4%	50.0±7.0	20.0±3.4
	0.6%	67.2±0.8	40.0±5.6
	1.0%	95.0±2.5	60.0±7.3

Mean ±SD n-10 experiments

Larvicidal effect

The results of the mortality profile of second instar larvae by endosybiotic secondary metabolites showed in the figure. The results indicated that the second instar larvae were most susceptible than the fourth instar larvae (Table -6). The secondary metabolites of ES02 had more potent larvicidal activity followed by

ES01. Larvicidal potential of both metabolites were more or less same kind of activity and they produced 100% mortality at 10% level. Notably the fourth instar larvae were completely resistant at the concentration that produced 100% mortality for the second instar larvae

Table 6. Larvicidal profile of endosybiotic secondary metabolites on second and fourth instar larvae of *Culex sp*.

MSMs	Concentration (Mg/ml)	Mortality (%)	
		Second instar larvae	Fourth instar larvae
ES01	1 6	100±0.0 60.2±2.6	10.2±2.71 0
ES02	10 6	100±0.0 61.2±3.0	0 20.2±1.72

Mean ± SD, n=10 experiments

Ichthyotoxicity

Ichthyotoxicity profiles of endosybiotic secondary metabolites are presented in Table. 7. The extract of ES02 was toxic up to 4 mg/ml where as 2 mg/ml was less toxic and did not influence mortality within 6 h. ES01 was less toxic and as 20.0% mortality was recorded only at 2 mg/ml.

Table 7. Ichthyotoxicity profile of endosymbionts to *Oreochromis mossambicus* fingerlings

Species	*Concentration*	*Mortality (%)*	*Time of death (h)*
ES01	4	100	6
	2	20.0±2.89	6
	1	0	-
ESO2	4	100	2
	2	60.4±3.0	6
	1	0	-

Mean±SD n=10

All the endosymbiotic extracts exhibited more or less same sort of behavioral changes in *O. mossambicus* (Table -7). Initially the fishes exhibited erratic movements and then inclined towards one side. Later, they rapidly went for surface respiration followed by settling at bottom or rapid swimming activity with non-directional bursts, which culminated in dwelling at bottom and mortality.

Table 8: General behavioral changes observed in *Oreochromis mossambicus* exposed to ichthyotoxic endosymbiotic extracts

Stages	***Behavioural changes***
State I: Initial signs	a. Increase in ventilatory frequency Erratic /rapid movements
Stage II: Secondary signs	a. lined towards one side b. Loss of swimming activity
State III: Advanced signs	a. Surface respiration b. Lined to bottom c. Art of sporadic uncontrollable swimming with non directional bursts

MTT assay

The result of MTT assay after the administration of ES01 and ES02 extracts were shown in plate 2. It was noted that the both extracts has killing effect over the tested cell lines. Maximum cytotoxicity was observed in ES02 extract over SH-SY5Y Neuroblastoma Cell lines (78%) at 48 hrs incubation. The different concentration of endosymbiotic extracts (ESEs) were utilized in the present study. Among these 800 µl showed better response after 48hours of incubation. The moderate cytotoxic responses were noted in the ES01 (58 and 48%) extract administered SH-SY5Y Neuroblastoma Cell lines. The same trend was noted in the results of other cell lines (table- 9).

Table: 9 The cytotoxicity potential of choosen extracts over SH-SY5Y Neuroblastoma Cell lines and He la cell lines at different concentration .

Cell lines	extracts	Concentration of crude extract (µ/ml)	Percentage Cytotoxicity %	
			24hrs	48hrs
SH-SY5Y Neuroblastoma Cell lines	ES01	400	22	43
		800	49.16	78.40
	ES02	400	Nil	23
		800	28.36	58.04
	Toxol	400	Nil	23
		800	28.36	58.04
He la cell lines	ES01	400	18.14	32.18
		800	29.54	48.20
	ES02	400	34.8	58
		800	58.96	78.12
	Toxol	400	Nil	18.22
		800	24..36	30.64

Trypan blue exclusion cell viability assay

Trypan blue exclusion cell viability assay for ESEs were shown in table 10 and diagramatically presented in Fig 1. Based on the result the methanolic extracts of ES01 shows decreasing trend in terms of viability. But in the other etract showed the major variation than the control in 24 hours, 48 hours and 72 hours of incubation. It indirectly indicated that over trypane blue inclusion in ES01 administered group produced more cell death (Cancer cell death) .

Table: 10 : Hydrogen Peroxide Scavenging Activity

Incubation time	ES01 (A1)	ES02 (A2)	Taxol (A3)
10	21.4±1.14	22.0±0.70	0±0
20	22.0±1.58	25.2±0.83	2.0±0.70
30	33.4±2.4	27.2±0.83	6.4±1.40
40	42.2±1.92	35.2±1.30	11.6±0.89
50	56.0±1.58	35.2±1.30	12.6±1.14
60	56.2±1.78	36.2±1.30	14.8±0.83
70	71.0±1.58	44.0±1.0	16.8±0.83
80	73.8±1.48	46.4±1.67	17.2±0.83
90	96.4±1.14	56.4±1.40	17.6±0.54
100	97.8±1.30	66.8±0.85	20.6±1.14

Fig 1: Hydrogen Peroxide Scavenging Activity

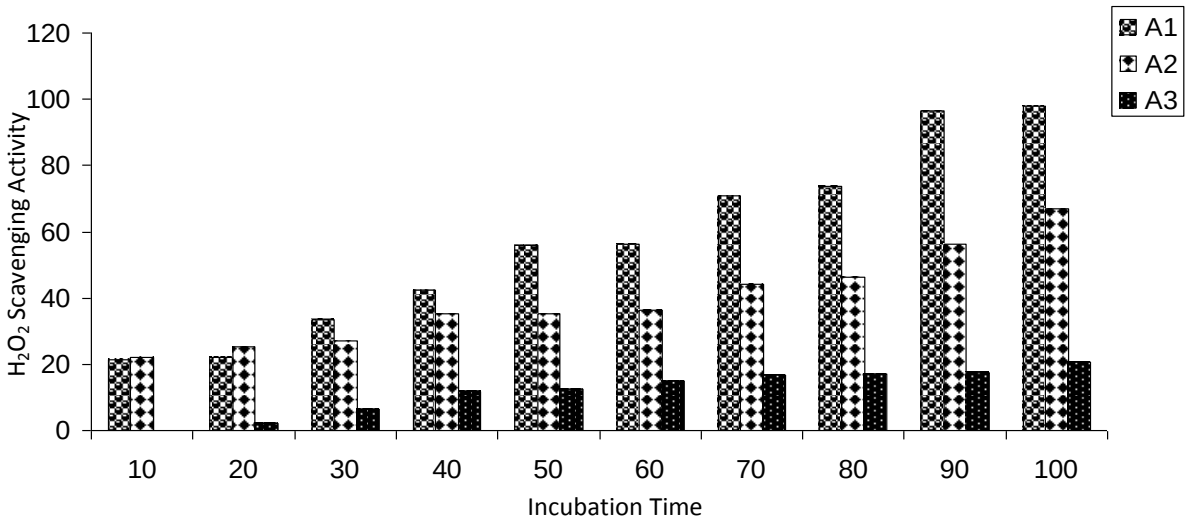


Table 11. DPPh radical scavenging activity of Endosymbionts

Incubation time	ES01 (A1)	ES02 (A2)	Taxol (A3)	Control
10	82.8±0.83	84.6±1.81	43.8±1.78	47.4±1.51
20	92±1.58	53.4±0.91	86.0±1.58	56.6±1.14
30	86±1.0	93.7±1.48	94.8±0.83	54.6±0.54
40	85.8±0.83	85.0±1.58	47.9±0.90	49.4±0.54
50	87.2±1.30	95.44±1.63	77.1±1.47	56.4±0.54
60	91.6±1.14	62.56±0.94	93.2±1.30	57.4±0.54
70	83.6±1.67	92.46±1.53	67.1±1.37	56.6±0.54
80	84.1±0.74	91.2±1.30	67.6±1.31	56.8±0.44
90	82.2±1.92	74.02±0.69	37.4±1.14	45.6±0.54
100	96.56±1.12	64.96±0.65	6.6±1.14	638±1.09

Fig 2: DPPh radical scavenging activity of Endosymbionts

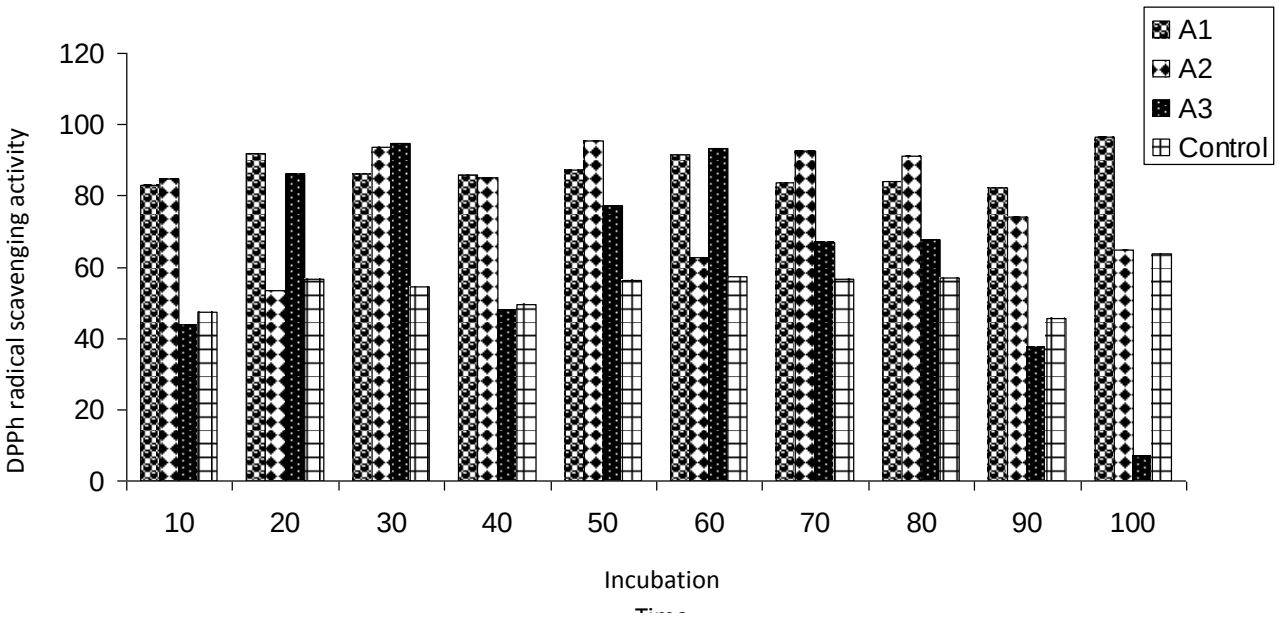
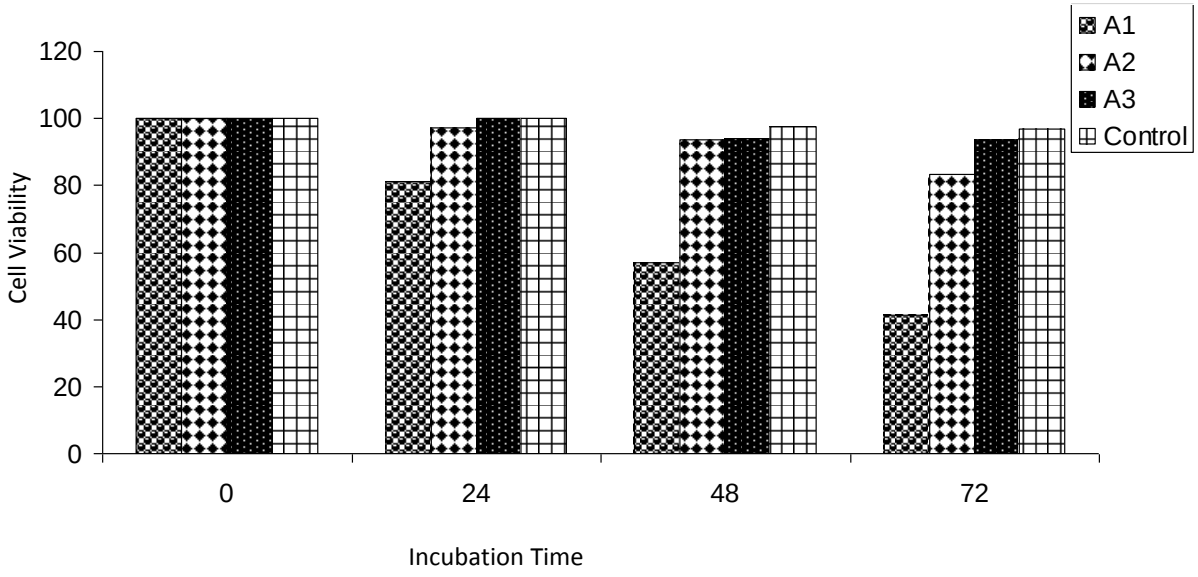


Table 12: Trypan blue Cell Viability of Endosymbionts

Incubation time	ES01 (A1)	ES02 (A2)	Taxol (A3)	Control
0	100±0	100±0	100±0	100±0
24	81.3±0.93	97.2±0.83	100±0	100±0
48	57.0±1.58	93.8±0.83	94.0±1	97.4±0.54
72	41.46±1.45	83.2±1.09	93.6±1.14	96.8±0.83

Fig. 3: Trypane Cell Viability of Endosymbionts



Hydrogen peroxide scavenging assay

The results of H2O2 scavenging activity is shown in fig 1 .The results clearly displayed that the plant extracts ES01 (A1) produced high percentage of Hydrogen peroxide scavenging activity. (Values) It was followed by ES02 (A2) and Taxol (A3). It was also noted that that the concentration of extracts play an important role in Hydrogen peroxide scavenging activity. Here Hydrogen peroxide scavenging activity is directly proportional to the concentration (Table - 10).

DPPH radical Scavenging activity

The results of DPPH radical Scavenging activity are depicted in fig 3 and table 11. The results clearly indicated that the extract of ES01 (A1) induced more DPPH scavenging profile than the other groups in all concentrations. Interesingly in higher concentration (90 and 100 µg) The taxol (A3) extract showed less activity than ascorbic acid (as control) But the other extracts ES02 showed consistent scavenging activity. It was also noted that the ES02 produced high activity in low concentrations (Fig.2).

COMET Assay for DNA fragmentation

The results of COMET assay displayed in plate 4. ES01 (A1) extract has showed perfect cell without any damage of DNA particle. But in control huge DNA degradation was noted. Like that in other plant extracts administered groups also produced some DNA damage (less than the control)

Discussion

The present study was carried to evaluate the anticancer activity of endophytic fungal extracts ES01 and ES02 against taxol (isolated from fungus *Pestalotiopsis pauciseta Sacc*). The cell lines such as He- La cells and SK-Mel -2 cell lines were used to determine the anticancer study. The various assays systems such as *In vito* Cytotoxicity, Apoptosis assay, Comet assay, DPPH assay, telomerase assay, Caspase assay and DNA fragmentation assay were performed. The results showed all the both extracts have cytotoxicity over the cancer cell lines, but maximum cytotoxicity was observed in ES02 extracts. A common consequence of most abiotic and biotic stresses is increased production of ROS. These ROS may lead to the unspecific oxidation of proteins and membrane lipids or may cause DNA injury. The control of oxidant levels is achieved by antioxidative systems. These defense systems are composed of metabolites such as ascorbate, glutathione, tocopherol, etc. (Rodriguez *et al.,* 2004). Mutualistic associations of endophytes also enhance host plant defense responses against various biotic and abiotic stresses (Redman *et al.,* 2002; Rubini *et al.,* 2005). Thus, the tested total antioxidant capacity (TAC) of four different mangrove plants and their predominant endophytic fungus using a suite of *in vitro* assay systems, such as the β-carotene-linoleate model system, iron chelating capacity, reducing power, and hydroxyl radical/hydrogen peroxide/DPPH radical scavenging. DPPH is usually used as a substrate to evaluate activity of antioxidants.

Increased endophytic fungal biomasses observed in other studies have been associated with increased host tolerance of a range of stresses (Baltruschat *et al.,* 2008). Hydroxyl radicals are the major ROS causing lipid peroxidation and enormous biological damage (Hernández *et al.,* 1995; Yang *et al.,* 2004). Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups, and also by forming hydroxyl radicals that cause many of its toxic effects (Miller *et al.,* 1993). The better performance in hydrogen peroxide assays of fungal endophyte extracts than the mangrove plants extracts again indicts

that endophytic fungi may promote ROS resistance in mangrove plants. The sample extracts of mangrove plants and endophytic fungi hindered the extent of β-carotene bleaching by neutralizing linoleate-free radicals and other free radicals formed in the system (Jayaprakasha *et al.*, 2001). Thus, DPPH, hydroxyl and hydrogen peroxide model system antioxidant assays show that when the host fails to produce antioxidants adequately, the associated endophytic fungi in roots and leaves have enhanced antioxidant potential that may benefit the host. We also found that total phenol contents of endophytic *Aspergillus flavus* were significantly higher than in the free living fungus. Thus production of novel antioxidant metabolites by endophytic *A. flavus* (Zheng *et al.,* 2008) and expression of antioxidant genes (Jitesh *et al.,* 2006) are enhanced under stress conditions. Our results are congruent with earlier observations on endophyte-facilitated salt tolerance in agricultural plants like barley (Baltruschat *et al.,* 2008), tomato (Mittova *et al.,* 2004) and wheat (Marshall *et al.,* 1999).

Successful transfer of *A. flavus* to tobacco plants suggests the fungus may be effectively used in other agriculturally relevant plants. Total biomass of tobacco plantlets increased with *A. flavus* infection; a similar effect occurs upon infection of tobacco and Arabidopsis with the endophyte *Piriformospora indica*. The stimulation of host growth by endophytic fungi is attributable to a fungal phytohormone effect (Nassar *et al.,* 2005). Thus, endophytic fungi with broad host ranges can confer effective tolerance of ROS under abiotic stress conditions, such as high salinity (Rodriguez *et al.,* 2009). Transfer of effective endophytes of mangroves to agricultural plants will probably not only facilitate stress tolerance but also controls yield loss.

In a similar research, Huang *et al.,* (2001) reported that 14.5% of the endophytic fungi from *Taxus mairei, Cephalataxus fortunei* and *Torreya grandis* showed antitumour activity. And in further studies, they isolated some antitumour-active compounds such as taxol and brefeldin A from these active isolates (Wang *et al.,* 2000, 2002). Recently, Stadler *et al.,* (2010) also reported that 17.0% of the endophytic fungi from 81 Thai medicinal plant species exhibited anticancer

activity. These reports and our results strongly support the view that the endophytic fungi of traditional medicinal plants are promising sources of natural anticancer-active compounds (Stierle *et al.*, 1993; Strobel *et al.*,1997; Strobel and Daisy 2003).

The antitumour-active isolates were identified and belonged to 12 taxa, of which some were most commonly isolated endophytic genera such as *Alternaria* and *Pestalotiopsis*, which have also been reported as producers of the anticancer compounds taxol and brefeldin A (Strobel *et al.*, 1996). In contrast, some were rare endophytic genera, e.g. Hainesia, Marssonina and Torula. These may have potential practical value. Among these active isolates, Alternaria was the dominant genus, about 33.3% isolates belonged to it. Moreover, it was noted that different isolates in this genus exhibited different strength of antitumour activity. The diversity of these isolates and their active metabolites is worth further research.

Chapter – III

Isolation and identification of the bioactive active principles from the fungal metabolites

Introduction

Endophytes include an assemblage of microorganisms with different life history strategies: those that, following an endophytic growth phase, grow saprophytically on dead or senescing tissue, avirulent microorganisms, incidentals, but also latent pathogens and virulent pathogens at early stages of infection (Suriyanarayanan *et al.*, 2009). Each plant species may be host to a number of endophytes (Strobel, 2003). Their biological diversity, especially in temperate and tropical rainforests, is large and noticeable. Scientific studies have investigated the anticancer activity of several anthracenedione derivatives, which was separated from the secondary metabolites of the endophytic fungus *Halorosellinia sp.* and *Guignardia sp* (Zhang *et al.*, 2010). To date, endophytes have been most extensively studied for their ability to produce antibacterial, antiviral, anticancer, antioxidants, antidiabetic and immunosuppressive compounds. Developing new anticancer drugs with a higher potency and specificity against cancer cells has therefore become an important goal in biomedical research and concern for the medical fraternity. Moreover that, the chemical ecology of these bioactive metabolites are still exempted/ neglected.

To overcome this lacuna, the present work initiated for the identification of chemical constituents on the endophytic fungus ES02, obtained from a tomato roots. The methanol extract of a fermentation broth of the fungus exhibited antimicrobial and anticancer activity.

Materials and methods

Experimental Procedures

The fungal strain, ES02, was isolated and used in earlier chapter as used in this study. The fungal strain was cultivated in 50 L potato glucose liquid medium (15 g of glucose and 30 g of sea salt in 1 L of potato infusion, in 1 L Erlenmeyer flasks, each containing 300 mL of culture broth) at 25 °C without shaking for 4 weeks.

Extraction and Isolation

The fungal cultures were filtered through cheesecloth, and the filtrate was extracted with EtOAc (3 × 5 L, 12 h each). The organic extracts were concentrated in vacuum to yield an oily residue (20.2 g), was fractionated and purified repeatedly by TLC on silica gel to yield four major compounds: compound 1, compound 2, compound 3 and compound 4, as well as a minor negligible overlapped fraction. The Compound 1 was the compound of choice for further study where it showed a highest activity 89%, while the others were less. The further purification of compound 1 was followed by using UV-2401PC visible spectrophotometer (Shimadzu, Kyoto, Japan).

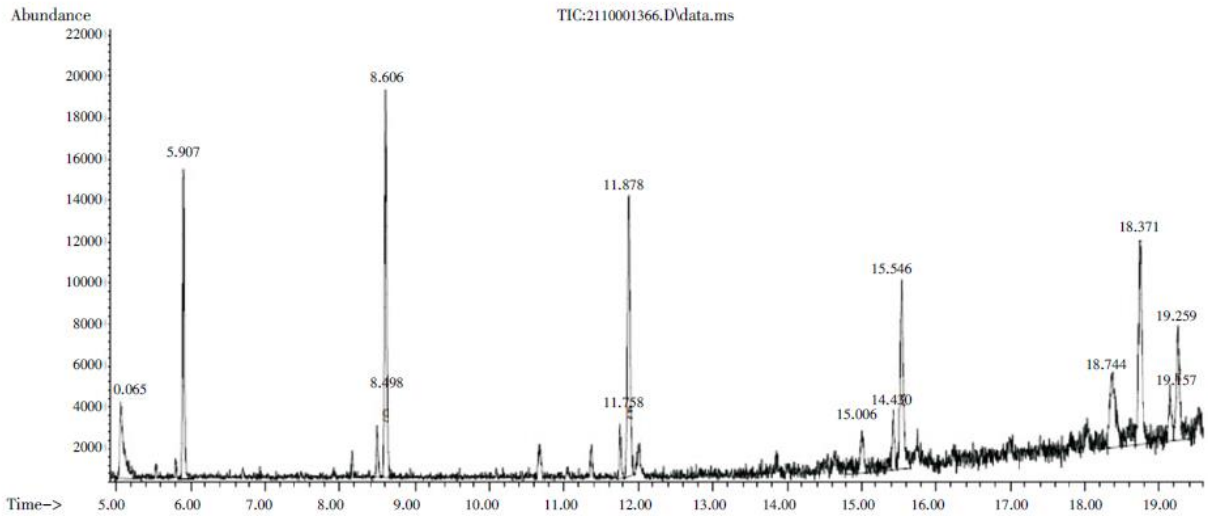
Gas chromatography-mass spectroscopy (GC-MS) analysis

The crude extracts obtained from the endophytic fungal culture broths were concentrated in a rotary evaporator (RE-300, Stuart, UK) at 50°C. The samples were then lyophilized in a lyophilizer (Scanvac, Denmark). 50 µg of the sample was dissolved in 1 ml 50% acetonitrile (HPLC grade) in water (HPLC grade). The sample was then filtered through 0.2 um filter paper (GH polypropylene 0.2 um pore size, 47 mm diameter filter paper) and kept for sonication for 30 min to get a clear solution. The samples were then transferred to UPLC vials (GC-MS certified 12x32 mm clear presilt combo, Waters, India) and kept under refrigeration at 10°C till the

GC-MS analysis. The crude extract exhibiting activity was subjected to GCMS equipped with Agilent 5975 inert XL MSD to find out the active principle of the extracts.

Results

Agar plug paper chromatography of the endophytic fungal metabolites showed presence of UV-visible components in the crude extracts of the endophytic fungal metabolites (ES02)(Plate-5). Thin layer chromatography of the endophytic fungal extracts was seen to form a blue-violet colour in the zone where they were spotted into the TLC plate after treatment with iodine vapours. Faint blue zones were also observed in all endophytic fungal extracts. The extracts were more or less static and exhibited only a negligible movement in TLC chromatogram in relation to the developing solvent front. The GC-MS of the crude endophytic metabolite extracts showed more or less uniform peaks and confirms the presence of aurantioclavine, austdiol, oleic acid, jasmonic acid-ethyl ester, diaportin acid and walleminone. Abscissic acid was detected only in EF02.



Discussion

The presence of a visible yellow coloured spot in the chromatogram may be due to the presence of griseofulvin or related metabolites (Belofsky *et al.*, , 1998). The technique of agar plug TLC is reported to be effective in generating metabolite profiles of fungi (Frisvad *et al.,* 1989). Therefore, prior to more accurate TLC screening of the metabolites, the agar plug technique provided us with an idea of the metabolite production by the endophytic fungi. The production of growth promoting substances or hormones are very crucial in the successful establishment of a symbiotic life cycle within the host plant tissues. Uptake of genes from competing plant pathogens or the plant host itself during the evolutionary course of the symbiosis have generated a much wider range of metabolic diversity in case of fungal endophytes (Strobel, 2003).

A single endophytic fungal isolate like EF02, which was identified to be *Aspergillus sps*, was shown to produce a combination of these metabolites. Thus, it is suggested that the production or absence of Bhagobaty and Joshi (2011) a particular metabolite in the crude extracts of fungal fermentation broths as such, must not be used as specific biochemical markers to ascertain the exact taxonomic identity of the endophytic isolates. It was decided that, more holistic view and tried to analyze the relatedness of the five endophytic fungal species based upon their total metabolic profiles so obtained, using the GC-MS data and the available database. The metabolite production profiles generated for each of the endophytic fungal isolates was used to create a matrix based on the presence (1) or absence (0) of a particular metabolite. The endophytic fungal isolate which was tentatively screened for metabolites using GC-MS and the available database revealed the isolates as potential microbial cell factories for production of a wide range of biomolecules. Most of them were shown to produce mycotoxins in addition to growth promoters / plant hormones. This finding is of relevance, since it leads us to believe that majority of fungi establishing an endophytic relation inside the host plant tissues may be latent

athogens, which have somehow managed to find a perfect balance between their symbiotic and pathogenic roles in the host plant.

Summary

Natural products discovery have played major role in the search for new drugs, and is the most potent source for the discovery of novel bioactive molecules. Natural products are chemical compounds derived from living organisms. The most prominent producers of natural products can be found within different groups of organisms including plants, animals, marine macro-organisms (sponge, corals and algae), and microorganisms (bacteria, actinomycetes, and fungi). The discovery of natural products involves isolation, structural elucidation and establishing the bio-synthetic pathway of the secondary metabolites. Crude natural products have been used directly as drugs which were low cost and important sources of traditional medicines. The role of natural products in discovery of new therapeutic agents can be demonstrated by an analysis of the number and sources of bioactive agents. There are at least 200,000 natural metabolites with various bioactive properties.

Between 1981-2006, about 100 anti-cancer agents have been developed, 25% of them were natural product derivatives, 18% were natural product mimics, 11% candidates were derived from a natural product pharmacophore, and 9% were pure natural products. Actually 47% of total anticancer drugs and 52% of new chemicals introduced into the market are of natural origin. Since, the discovery of potent antibiotic against Gram-positive bacteria, penicillin from culture of fungus *Penicillium notatum* by Fleming in 1929, the search for new drugs from microbial origin started. More than 20,000 bioactive metabolites are of microbial origin have been reported. Fungi are among the most important groups of eukaryotic organisms that are well known for producing many novel metabolites which are directly used as drugs or function as lead structures for synthetic.

Since the first description of symbiosis as ‘the living together of dissimilar organisms’, onwards an array of symbiotic lifestyles has been identified in plants on natural ecosystems with mycorrhizal fungi and/or fungal endophytes. These fungal

symbionts can have profound effects on plant ecology, fitness, and evolution, shaping plant communities and manifesting strong effects on the community structure and diversity of associated organisms. The fossil record indicates that plants have been associated with endophytic and mychorrhizal fungi for more than 400 yrs and were likely associated when plants colonized land, thus playing a long and important role in driving the evolution of life on land. Endophytic fungi are a rich source of novel organic compounds with interesting biological activities. They represent a relatively unexplored ecological source, and their secondary metabolism is particularly active because of their metabolic interactions with their hosts. Apart from that, lots of fungicidal, herbicidal and antibacterial activities of endophytic fungal secondary metabolites have been reported. Based on this milieu, new efforts are taken to screen the bioactivity of endophytic fungal metabolites especially anticancer properties. This study also analyzed the toxicity profile and suitable solvent for extraction, and lead compound characterization, which was essentially needed for drug discovery.

The present study is divided into three chapters. The isolation and preliminary bioactivity screening (antibacterial, antifungal and antiviral) of bioactive metabolites of endosymbionts are being dealt with in chapter I. Various toxicological and anticancer screening of chosen fungal metabolites, are explicated in the second part of the work. Chapter III deals the isolation and identification of the bioactive active principles from the fungal metabolites.

In the chapter I, two endophytic funguses (EF01and EF02) were isolated from selected plants roots and stem by using standard procedures. After incubation (24^0C) for 7 days, hyphal tips of developing fungi were aseptically removed and placed on potato dextrose agar (PDA). Different solvent systems such as eathanol, methanol, and benzene and petroleum ether were used to extract the metabolites from endophytic fungus to perform various bioactivity screening. The antibacterial activity of extracts were carried out against potent bacterial pathogens (MTCC, Chandigarh). The methanolic extracts of ES02 exhibited broad spectrum antibacterial activity (70%) in both gram positive and gram negative bacteria than the other groups.

To determine the toxicity of Methanolic extracts, the cytotoxicity tests such as Brine shrimp lethality bioassay, larvicidal bioassay and Ichthyo toxicity bioassay were performed, using adequate methodology. The results of the brine shrimp cytotoxicity bioassay, the extracts of ES02 exhibited high toxicity against *Artemia nauplii* than the other extract ES01. The results of larvicidal effects indicated that the second instar larvae were more susceptible than the fourth instar larvae. The methanol extracts ES02 1nd ES01 were more or less of the same larvicidal potential. They produced 100% mortality at 10% level. Ichthiotoxicity profiles of fungal extracts displayed high toxicity. The ES02 was extremely toxic and killed all the finger lings of (*Oreochromis mossambicus*) within a short exposure span of 40 minutes at 2% level, although ES01 was produced 49 minutes at 3% level of toxicity.

In the second chapter, these two extracts such as methanolic ES01 and ES02 extracts were subjected for apoptosis regulating efficiency screening. The cell lines such as He- La cells, SK-Mel -2 and Mouse Lymphoma (DLA) cell lines were purchased from ATCC were used as a model. The various assays systems such as *In vito* Cytotoxicity, Apoptosis assay, Comet assay, DPPH assay, telomerase assay, Caspase assay, DNA fragmentation assay and Mitosenser assay were performed using appropriate protocols. The Trypan blue and MTT assay were adopted to determine the cytotoxicity of ES01 and ES02 extracts. The results showed all the both extracts has cytotoxicity over the cancer cell lines, but maximum cytotoxicity was observed in ES02 extracts *H. indicus* administered DLA cell lines (96% in Trypan blue and 78% in MTT) at 48 hrs incubation. The moderate cytotoxic responses were noted in the ES01 extracts administered DLA cell lines. The same trend were noted in the results of other cell lines.

In chapter III, Agar plug paper chromatography of the endophytic fungal metabolites showed presence of UV-visible components in the crude extracts of the endophytic fungal metabolites (ES02). Thin layer chromatography of the endophytic fungal extracts was seen to form a blue-violet colour in the zone where they were spotted into the TLC plate after treatment with iodine vapours. Faint blue zones were

also observed in all endophytic fungal extracts. The extracts were more or less static and exhibited only a negligible movement in TLC chromatogram in relation to the developing solvent front. The GC-MS of the crude endophytic metabolite extracts showed more or less uniform peaks and confirms the presence of aurantioclavine, austdiol, oleic acid, jasmonic acid-ethyl ester, diaportin acid and walleminone. Abscissic acid was detected only in MS02.

This thesis is concluding with the following facts that, the fungal endosymbionts isolated from plant barks are having vast bioactive potential. The methanolic extracts of these endosybionts are proven their antibacterial activity, antifungal activity and antiviral the against the tested organisms. Likewise these extracts are proven their anticancer potential by various bioassays. So, the endosymbiont ES02 may be used as antibacterial anti cancer drugs after further evaluations. The lead components which are present in ES01 may responsible for this anti microbial and anticancer property.

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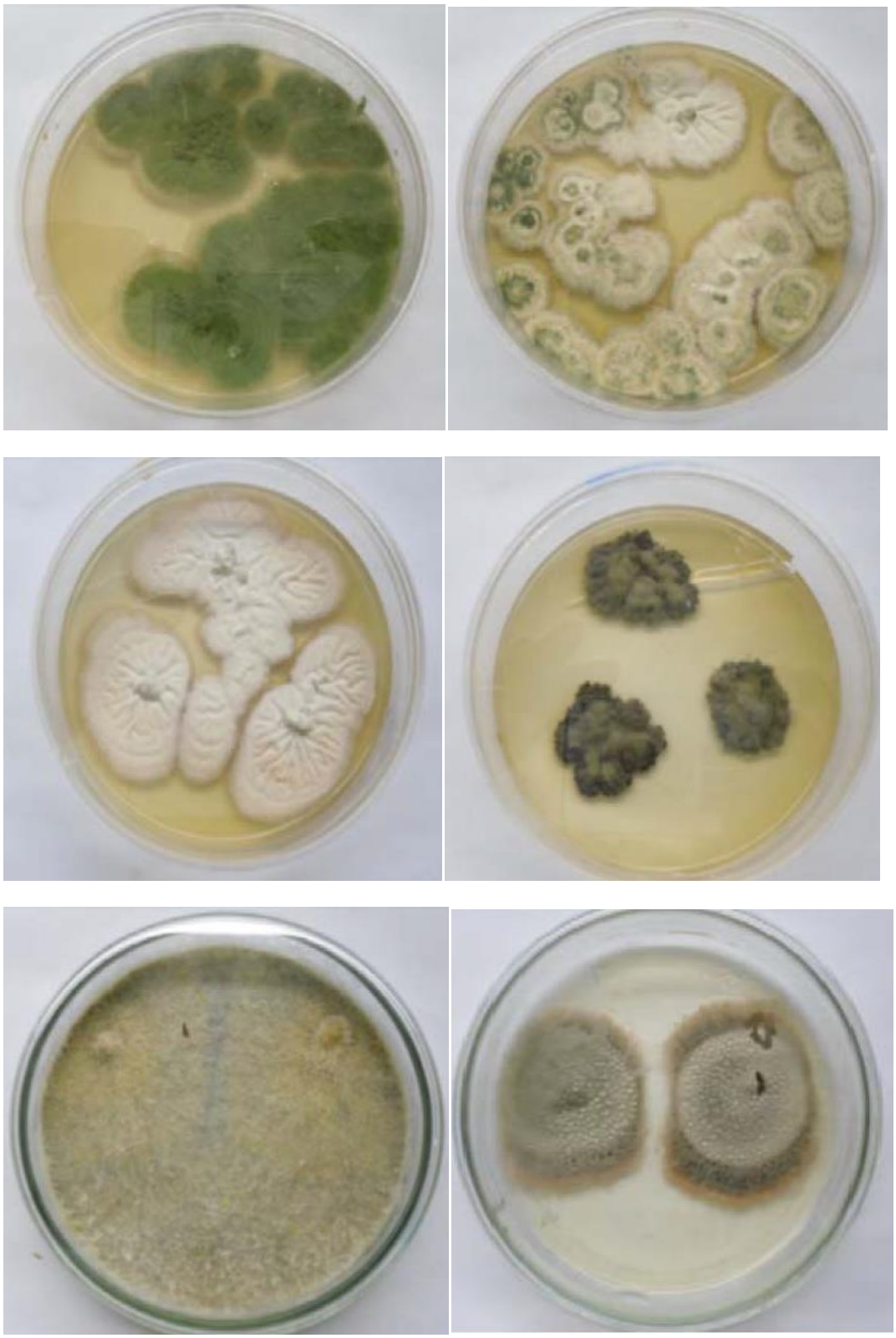
1 The endophytic fungi isolated from tomato plants



A. *Apergillus flavus* B. *Apergillus sp*. C. *Cuvularia lunata* D. *Cladosprium. sp*.
E & F unidentified fungi species (sterile mycelia)

Plate - 2

Thin layer chromatogram under UV254 of endophyte and plant crude extracts Fungi crude extracts in order 1=Unknown; 2= Cladosporium sp.; 3= Curvularia lunata; 4= Aspergillus flavus;5= Aspergillus sp.; 6 & 7 unknown and C= EtOAc plant crude extract. Solvent system: CHCl3: MeOH (4:1)

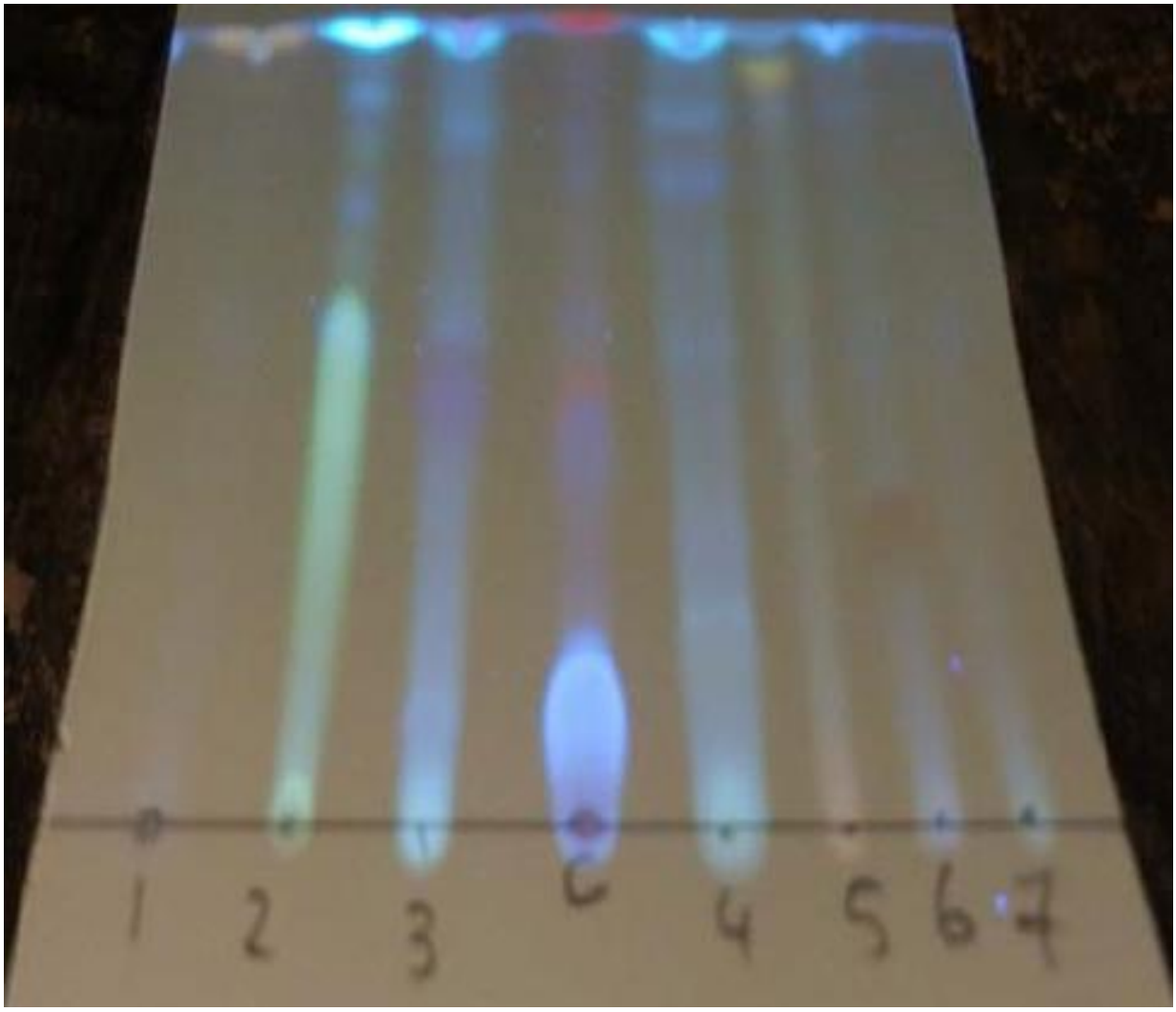
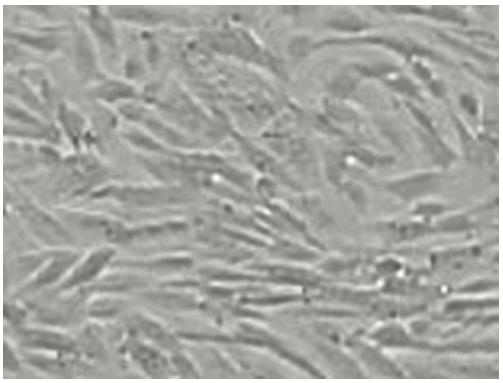
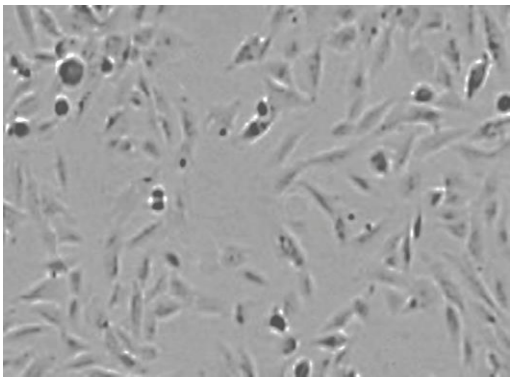


Plate:3

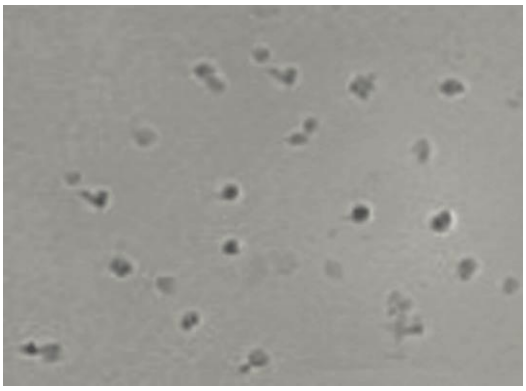
A. Cytotoxicity assay in SH-SY5Y Neuroblastoma Cells Control



B.Cytotoxicity assay in SH-SY5Y Neuroblastoma Cells ES01 treated



C: Cytotoxicity assay in SH-SY5Y Neuroblastoma Cells 3. ES02 treated



D: Cytotoxicity assay in SH-SY5Y Neuroblastoma Cells Toxol Treated

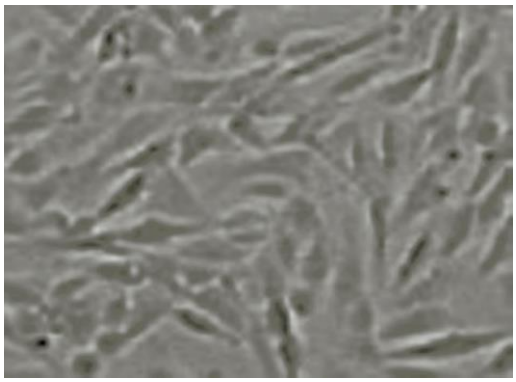


Plate 4 DNA Fragmentation assay

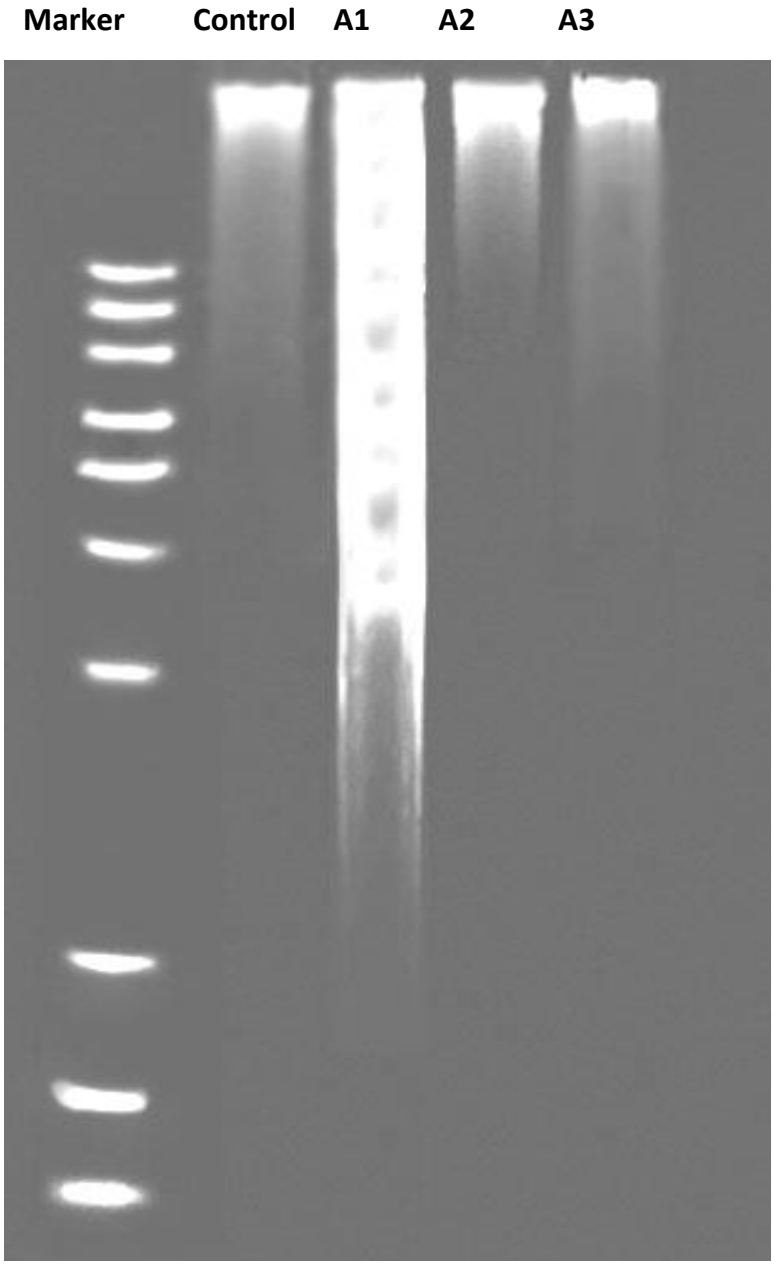


Plate : 5 UV Spectrum Analysis of EF02