Cytotoxic Potential of fungal extracts against A549 cell line and its potential in drug development

A

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in partial fulfilment of the requirement of the degree

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Master of Science

In

Biotechnology

By

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Certificate

This is to certify that the thesis entitled ***"Cytotoxic Potential of fungal extracts against A549 cell line and its potential in drug development "*** being submitted by **Ms Anjali Rani (Roll No-301101004)** in partial fulfillment of the requirements for the award of degree of Master of Science in Biotechnology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of Dr. Sanjai Saxena and Dr. Siddhartha Sharma and that no part of this thesis has been submitted for the award of any other degree.

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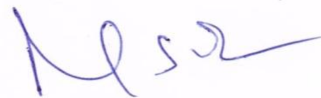

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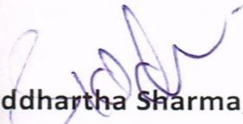

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I hereby declare that the work being presented in the thesis entitled "***Cytotoxic Potential of fungal extracts against A549 cell line and its potential in drug development***" in partial fulfilment of the requirements for the award of degree of Masters in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is my own laboratory work during the period of January 2013 to June 2013, under the conception and supervision of Dr. Sanjai Saxena, Associate Professor and Dr. Siddharth Sharma, Assistant Professor, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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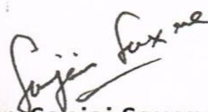

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Abbreviations

S.No	Abbreviation	Full form
1.	IARC	International Agency for Research on Cancer
2.	DNA	De-oxyribonucleic Acid
3.	SCLC	Small Cell Lung Cancer
4.	NSCLC	Non-Small Cell Lung Cancer
5.	DMEM	Dulbecco's modified eagle medium
6.	CZD	Czapek Dox broth
7.	DMSO	Dimethyl sulfoxide
8.	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
9.	FBS	Fetal Bovine Serum
10.	PCR	Polymerase Chain Reaction
11.	MIC	Minimum Inhibitory Concentration
12.	Tris	Tris(hydroxymethyl)aminomethane
13.	EDTA	Ethylenediaminetetraacetic acid
14.	TAE	Tris-acetate-EDTA
15.	ITS	Internal Transcribed Spacer
16.	CZDA	Czapek Dox Agar
17.	dNTP	Deoxyribonucelotide
18.	Pen-Sep	Pencillin and Streptomycin

19.	rpm	Revolutions per minute
20.	µl	Microlitre
21.	µM	Micromoles
22.	V	Volts
23.	ml	Milliliter
24.	psi	Per square inch
25.	EDTA	Ethylenediaminetetraacetic acid
26.	TAE	Tris acetate EDTA
27.	µl	Microliter
28.	mg	Milligram
29.	nm	Nanometer
30.	µM	Micromolar
31.	ng	Nanogram
32.	U	Unit
33.	RNA	Ribonucleic acid
34.	WHO	World health organization
35.	NCI	National Cancer Institute
36.	ATCC	American Type Culture Collection
37.	WCRFI	World Cancer Research Fund International

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Executive summary

The need of new anticancer drugs having high efficiency & less toxicity for lung cancer has increased the exploration of natural sources. The discovery of many important anticancer drugs from endophytic fungi has increased research work on endophytic fungi to explore new anticancer agents.

In the present study, 65 endophytic fungal cultures were subjected to culture filtrate production in synthetic medium, which were then screened for antitumor activity. Primary screening of all the culture filtrates was performed by using Antitumor Potato Disc Assay and 10 fungal cultures possessing significant antitumor activity were selected for further study. Bioactive residues as well as their aqueous fractions were screened for cytotoxicity employing *in vitro* cell culture method on A549 lung cancer cell line. The aqueous fraction of #1048AMSTITYEL was showing significant level of percentage cytotoxicity and it was found to be 96%. Although the % age cytotoxicity of #1048AMSTITYEL was comparable to that of Vinblastine but the MIC of selected culture was very high i.e. 0.5 mg. The IC50 of bioactive residue of #1048AMSTITYEL was found to 17.5 mg/ml. The identification of selected culture was done using molecular and classical tools. #1048AMSTITYEL was tentatively identified as *Lasiodiplodia sp*.

Further purification and characterization of the probable anticancer compound along with identification of the culture upto species level is warranted for therapeutic usage as anticancer drug.

Chapter 1

Introduction

1.Introduction

Cancer has become a growing problem worldwide, the mortality rate due to cancer is increasing every year. Cancer is a term used for disease in which cells divide abruptly and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems (NCI, 2003). It has been considered one of the major cause of death worldwide (about 13% of total deaths) in 2004 (WHO, 2009). Cancer can be of many types depending upon the type of tissue and cell type from which they arise.

As per cancer statistics, India contributes 1 million of the current 5 million deaths in the world, and 2.41 million in developing countries (Thankappan and Thresia, 2007; Pai, 2002) and in 2020, this figure will be projected to 1.5 million (Thankappan and Thresia, 2007; Murray & Lopez, 1996). Lung cancer is the type of cancer that originates in the tissues of the lungs or the cells lining the airways. The main cause of lung cancer is smoking, which contributes about 80-90% of lung cancer cases, other factors include radon gas, asbestos, genetic factors, air pollution and second hand smoke. There are two main types of lung cancer - Small Cell Lung Cancer (SCLC) and Non Small Cell Lung Cancer (NSCLC). These types are diagnosed based on how the cells look under microscope (NCI, 2003). Non Small Lung Cancer has further 3 subtypes: adenocarcinoma, squamous cell carcinoma and large-cell lung carcinoma. Out of these three types, adenocarcinoma contributes around 40% of lung cancer cases.

Common treatments for lung cancer include chemotherapy, palliative care, surgery and radiation therapy. Chemotherapy is the most commonly used treatment in case of lung cancer. Chemotherapy alone or combined with radiation may be used before, after or instead of surgery in treating lung cancer. But Chemotherapy has many side effects like diarrhea, temporary hair loss, weight loss, fatigue depression, nausea, vomiting and anemia. Because of the serious complications due to chemotherapy there is an urgent need of new chemotherapeutic agents that are highly effective and have low toxicity. Lung cancer cell lines have a great contribution in lung cancer research and biomedical studies. Multiple cell lines (estimated at 300–400) have been established from human small cell (SCLC) and non-small cell lung cancer (NSCLC) (Adi *et al.*, 2010). There are many lung cancer cell line that are being used in lung cancer studies e.g. NCI-H1373, NCI-H1395, HCC-827 etc (ATCC). The NSCLC cell line A549 was established in 1976 and has been very widely studied since then (Lieber *et al.*, 1976).

Human beings have always been relied on nature as a resource of potent drugs. Besides direct medicinal application of natural products they can also serve as synthetic or semi-synthetic precursors of novel compounds for medicinal purposes, and also serve as pharmacophores for designing new drugs. Synthetic drugs used for treatment of cancer not only kill tumor cells, but also have marked killing effect on normal cells. On the other hand, drugs from natural sources are more effective and less toxic. Plant produces combination of secondary metabolites having anticancer, antimicrobial, antifungal and antiviral activities etc. Plant based discoveries have helped in isolation of many anticancer drugs like Vincristine, Vinblastine, Paclitaxel, Camptothecin and Taxol. In addition, much of the microbial world and marine world is still unexplored, and there are plenty of bioactive compounds that need to be explored.

Taxol is widely used anticancer drug in clinical field. Taxol, a highly functionalized diterpenoid, is found in each yew (Taxus) species, it was originally isolated from *Taxus brevifolia.* In the early 1990s, a novel taxol-producing endophytic fungus, *Taxomyces andreanae*, was isolated from *Taxus brevifolia* (Strobel *et al.*, 1993). The discovery of Taxol producing endophytic fungi increased the importance of endophytes and shifted natural products exploration from endophytic fungi.

Endophytes are microorganisms (mostly fungi and bacteria) that inhabit plant hosts for all or part of their life cycle. They colonize the internal plant tissues beneath the epidermal cell layers without causing any apparent harm or symptomatic infection to their host, living within the intercellular spaces of the tissues and its seems that they may penetrate the living cells (Strobel, 2003). Endophytic fungi produce various secondary metabolites having anticancer, antifungal, immunosuppressant and antimicrobial activity. As studies have shown that endophytic fungi is group of microorganisms which is poorly investigated till date and the search for bioactive natural products of endophytic fungi, isolated from higher plants, are attracting considerable attention from researchers worldwide, as indicated by the increase of work and publications on therapeutic potential during recent years (Tenguria ***et al.,*** 2011).

Currently there are large number of available bioassay in the development of anticancer drugs for testing their anticancer activity but cytotoxicity assays are very common. Cytotoxicity is an activity that is consistent with anticancer activity against cell lines. Different bioassays are used for screening of anticancer compounds. Antitumor potato disc bioassay is one of them that are developed based on *Agrobacterium tumefaciens* infection and is useful for checking antitumor properties. The rationale behind the use of this bioassay is that tumorigenic mechanism initiated in plant tissues by *Agrobacterium tumefaciens* is in

many ways similar to animals (Srirama *et al*., 2007). *In vitro* cell culture assay plays a key role in the development of new anticancer commodities revealing the cytotoxic potency and differential activity of potential anticancer drug candidate against specific cell lines before *in vivo* studies. The other advantage of use of cell culture for anti-tumor activity is that they are capable of infinite replication and so can provide a limitless source of materials that can be dispersed to laboratories worldwide to allow scientists to directly compare their results from identical study materials (Adi *et al*., 2010).

This study aims to screen endophytic fungi producing secondary metabolites having anti-tumor activity against A549 cell line. A549 cell line has been selected in this study because A549 is lung adenocarcinoma cell line and these cells have been well characterized through their use in a wide variety of molecular studies, such as anti-tumor drug permeability and efficacy analysis, infection assays, respiratory immunotoxicity tests, cell senescence studies, and cytokine expression profiling.

Chapter 2

Review of Literature

2.1 Cancer

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of total deaths) in 2008 (Globocan, 2008; IARC, 2010). Lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year. About 30% of cancer deaths are due to the five leading behavioral and dietary risks- high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use and alcohol consumption. Tobacco use is the most important risk factor for cancer causing 22% of global cancer deaths and 71% of global lung cancer deaths. Deaths from cancer worldwide are projected to continue rising, with an estimated 13.1 million deaths in 2030 (Globocan, 2008; IARC, 2010). Prevention of cancer can be done by avoiding smoking, caloric restriction, exercise, increasing fruits and vegetables in diet, deceasing meat consumption, avoiding direct exposure to sunlight, use of vaccinations, and regular check-ups (Preetha *et al*., 2008).

2.2 Lung cancer

Lung cancer is characterized by uncontrolled growth in tissues of lungs. Lung cancer was the most commonly diagnosed cancer as well as the leading cause of cancer death in males in 2008 globally. Among females, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer death. Lung cancer accounts for 13 % (1.6 million) of the total cases and 18 % (1.4 million) of the deaths in 2008 (Jemal *et al*., 2011).

Most of lung cancer are carcinomas, that originates from epithelial cells. Lung cancer occurs in multiple histologic types as classified by conventional light microscopy. The four major types of lung cancer include squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and small cell undifferentiated carcinoma (Anthony and Jonathan, 2003). Despite extensive research, the mechanisms leading to these different types of lung cancer remain uncertain. Hypotheses have focused on the cells of origin of lung cancers and on the pathways of differentiation of malignant cells. In the initial decades of the smoking-caused epidemic of lung cancer, squamous cell carcinoma was the most frequent type of lung cancer that was observed in the population among smokers, and small cell carcinoma was the next most frequent. In the late 1970's, the first evidence of a shift toward a predominance of adenocarcinoma was noted (Churg, 1994; Vincent *et al*., 1977 and Charloux *et al*., 1997) and now adenocarcinoma of the lung is the most common histologic type of lung cancer (Wingo *et al*., 1999; Travis *et al*., 1995). The decline in lung cancer rates has been more rapid for squamous cell and small cell carcinomas than for adenocarcinoma, which is just beginning to show a lower incidence rate (Wingo *et al*., 1999).

2.2.1 Causes and Treatment of Lung Cancer

Smoking is the leading cause of lung cancer, accounting for 80-90 % of lung cancers. Compared to never smokers, smokers have about a 20-fold increase in lung cancer risk at present. Risk factors of lung cancer in case of non-smokers include second hand smoke, radon, indoor air pollution (combustion of coal and biomass, and cooking fumes in the household), occupational agents (asbestos, arsenic, silica etc.), outdoor pollution, diet, infections of HPV (Human papillomavirus) or HIV (Human immunodeficiency virus) and ionizing radiations etc. (Jonathan *et al.*, 2009). In 1981 published reports from Japan (Hirayama, 1981) and Greece (Trichopoulos *et al.*, 1981) indicated there was increase in lung cancer risk in case of never smoking women married to cigarette smokers. The expert panel concluded that diet having fruits and foods containing carotenoids likely protect against lung cancer (WCRFI, 2007).

Clinical treatments for lung cancer therapy include chemotherapy, radiation therapy, surgery, immunotherapy, and other methods. A histological diagnosis helps in the management of lung cancer. Each case needs a coherent treatment plan which may involve physicians, surgeons, oncologists, radiotherapists, and palliative care services (Brown *et al.*, 1996). Chemotherapy is the use of anticancer drugs given by mouth or injection to destroy cancer cells. Chemotherapy alone or combined with radiation may be used before, after or instead of surgery in treating lung cancer.

In recent years, anti-tumor drugs have been the most frequently used in treatment of cancer (Ma and Wang 2009). The many commercially available anti-tumor drugs can be classified into different types by either or by origin as chemical synthetic drugs (e.g., alkylating agents and antimetabolites) or natural drugs derived from organisms (e.g Taxol, Camptothecin) (Ma and Wang, 2009; DeVita *et al.*, 2008). Most synthetic drugs kill not only tumor cells, but also normal cells, and most of them have severe side effects (Cragg *et al.*, 2009). Antitumor drugs derived from the natural sources have also proven effective and less toxic for cancer therapy (Ma and Wang 2009; Cragg *et al.*, 2009; Ravelo *et al.*, 2004).

2.3. Anticancer agents from natural sources

Nature has always been a potent source of novel bioactive compounds. Natural products are those which are obtained from living organisms like plants, animals, microorganisms (bacteria, fungi) and marine sources (sponges, algae) etc. The discovery of natural compounds has a major role in discovery of the new drugs. Between 1981-2006, about 100

anti-cancer agents have been developed, 25% of them were derived from natural products, 9% were the pure natural products, 18% were mimics of natural products, 11% were obtained from a natural product pharmacophore. Actually, 47% of total anticancer drugs and 52% of new chemicals are of natural origin (Chin *et al.*, 2006; Newman & Cragg 2007). In recent years, large-scale screenings of microorganisms, plants, animals and marine organisms for anti-tumor drugs have been performed to explore new anticancer drugs.

The isolation of the vinca alkaloids, Vinblastine and Vincristine, from *Catharanthus roseus* introduced a new era of anticancer drugs. They were the first anticancer agents in clinical use for the treatment of cancer (Cragg and Newman, 2005). Vinblastine and Vincristine are mainly used in combination with other cancer anticancer drugs in the treatment of variety of cancer, like leukemias, lymphomas, breast and lung cancers, and Kaposi's sarcoma (Cragg and Newman, 2005). Another important anticancer drug, paclitaxel (Taxol) was isolated from the bark of the Pacific Yew, *Taxus brevifolia*. Paclitaxel has high potential to treat ovarian cancer, advanced breast cancer, small and non-small cell lung cancer (Rowinsky *et al.*, 1992). Topotecan and irinotecan are semi-synthetic derivatives of camptothecin and are used for the treatment of ovarian and small cell lung cancers, and colo-rectal cancers, respectively (Creemers *et al.*, 1996; Bertino, 1997).

Cytarabine, the first marine derived anticancer agent was isolated from Caribbean sponge, *Cryptotheca crypta*. Cytarabine is currently used in leukemia and lymphoma. More than 10,000 bioactive molecules have been discovered till date and around 1000 of new molecules are being discovered every year. Marine environment is occupied mainly by invertebrates such as sponges, mollusks, bryozoans, tunicates etc. They produce a large variety natural products including alkaloids, peptides, terpenes, polyketides etc. (Rinehart, 2000).

Antitumor antibiotics are also important chemotherapeutic agents and include members of the anthracycline, bleomycin, actinomycin, mitomycin and aureolic acid families (Cragg and Newman, 1997). Clinically useful agents are the daunomycin and related agents like doxorubicin, idarubicin and epirubicin; the peptolides (exemplified by dactinomycin), the mitosanes (such as mitomycin C) and the glycosylated anthracenone mithramycin. The anthracyclines are among the most used antitumor antibiotics in the clinic and exert antitumor activity mainly by inhibiting topoisomerase II (Binaschi *et al.*, 2000; Patrick, 1997)

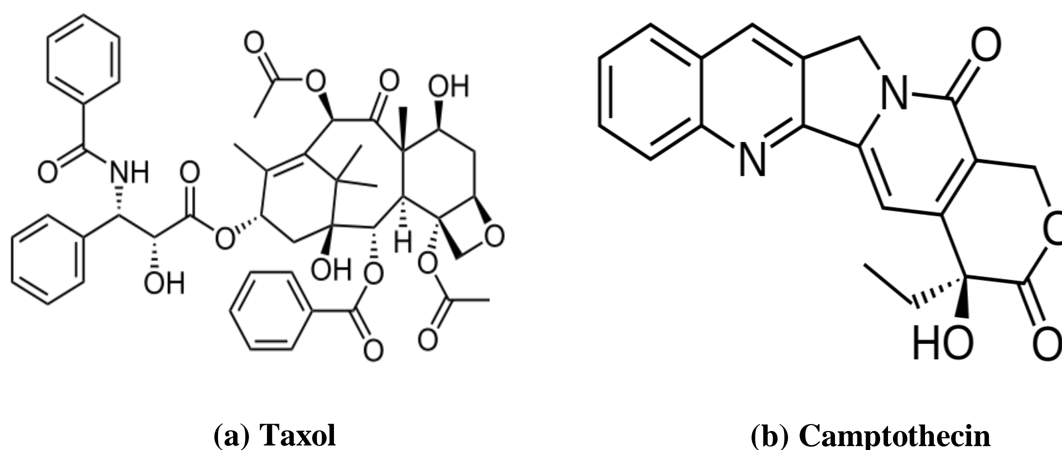
Natural source	Drug name	Source organisms
Plants	Vinca alkaloids (Vinblastine, vincristine)	*Catharanthus roseus*
	Lignans (Etoposide, teniposide)	*Podophyllum* species
	Taxanes (Paclitaxel)	*Taxus* species
	Camptothecins (Topotecan, irinotecan)	*Camptotheca acuminate*
	Cephalotaxanes (Homoharringtonine)	*Cephalotaxus harringtonia*
	Flavones (Flavopiridol)	*Dysoxylum binectariferum*
	Stilbenes (Combretastatin prodrug)	*Combretum caffrum*
Marine organisms	Aplidine	*Aplidium albicans*
	Bengamide analog	*Jaspis* species
	Bryostatin 1	*Bugula neritina*
	Discodermolide	*Discodermia dissoluta*
	Dolastatin 10	*Dolabella auricularia*
	Ecteinascidin 743	*Ecteinascidia turbinata*
	Halichondrin	*Lissodendoryx* species
	Hemiasterlin B analog	*Cymbastella* species
	Isogranulatimide	*Didemnum granulatum*
	Kahalahide F	*Elysia rubefescens*
Microorganisms	Anthracyclins	*Streptomyces species*
	Glycopeptides	*Streptomyces verticillus*
	Peptolides	*Sterptomyces species*
	Mitosanes	*Streptomyces species*
	Rapamycins	*Streptomyces species*
	Staurosporins	*Streptomyces species*
	Epothilones	*Sporangium cellulosum*
	Cryptophycins	*Nostoc species*

Table no. 1: anticancer drugs from natural sources (Schwartsmann *et al.,* 2002)

2.4 Anticancer agents from Endophytic fungi

The investigation of secondary metabolites of endophytic fungi has started after the discovery of taxol producing enophytic fungi. Many anticancer agents have been isolated from endophytic fungi. The alkaloids found in endophytic fungi are also potent anticancer agents. Wagenaar *et al.*, 2000 reported identification of three novel cytochalasins alkaloid from the endophytic genus *Rhinocladiella*, having antitumor activity. Other anticancer alkaloids

include Camptothecin and its derivatives; in China, Camptothecin is used as a drug for treatment of skin diseases (Guo *et al.*, 2008). Camptothecin and its derivatives are important precursors for the synthesis of anticancer drugs, topotecan, and irinotecan (Uma *et al.*, 2008). Camptothecin and two analogues (9-methoxycamptothecin and 10-hydroxycampto-thecin) were also obtained from the endophytic fungus *Fusarium solani* isolated from *Camptotheca acuminata* (Kusari *et al.*, 2009)



(a) Taxol **(b) Camptothecin**

Figure no. 1 Anticancer drugs isolated from endophytic fungi

An important anticancer agent, torreyanic acid which is cytotoxic quinone dimer, was isolated from the endophytic fungus *Pestalotiopsis microspora* associated with a tree *Torreya taxifolia*, that causes cell death by apoptosis and was shown 5 to 10 times more potent cytotoxicity in cell lines (Lee *et al.*, 1996; Puri *et al.*, 2006) identified a novel fungal endophyte (*Trametes hirsuta*) producing lignans “podophyllotoxins”. The lignans are biologically active, and they have potent antioxidant and anticancer properties its derivatives are currently being used in cancer chemotherapy. Also, phenylpro-panoids as anticancer and antioxidant agents, and were reporte to be produced by endophytes (Korkina *et al.,* 2007).

Cytoskyrins, a potential anticancer agent was found to be produced from endophytic fungus *Curvularia lunata* isolated from *Niphates olemda* (Brady *et al.*, 2000; Jadulco *et al.*, 2002). Ergoflavin, isolated from an endophytic fungi growing in leaves of medicinal plant *Mimusops elengi* ; it is a dimeric xanthene which belongs to ergochromes which is considered as a novel anticancer agent (Deshmukh *et al.*, 2009). Secalonic acid D, was isolated from the mangrove endophytic fungus of ergochrome class, are potent anticancer agents showed high cytotoxicity on HL60 and K562 cells (Zhang *et al.*, 2009).

2.5 Endophytic Fungi

Endophytes are microorganisms (bacteria and fungi) living within plant tissues without causing negative effects and therefore they are considered as a novel source of different metabolite having potential for medical, agricultural and industrial exploitation. They colonize the internal plant tissues beneath the epidermal cell layers without causing any apparent harm or symptomatic infection to their host, living within the intercellular spaces of the tissues and its seems that they may penetrate the living cells (Strobel and Daisy, 2003). Over a long period, endophytic fungi were reported to be potential sources of pharmaceutical leads, and to produce many bioactive metabolites having antimicrobial, anticancer, and antiviral activity.

Endophytes are ubiquitous in nature with rich biodiversity, which have been found in every plant species examined to date. It is noteworthy that, of the nearly 3,00,000 plant species that exist on the earth, each individual plant is the host to one or more endophytes (Strobel and Daisy, 2003). Studies have shown that many different types of alkaloids, terpenoids, flavonoids and steroids are produced by endophytic fungi and these metabolites of endophtic fungi have antimicrobial, antiviral, anticancer and antioxidant potential. Most of these compounds produced by endophytic fungi are the same as those produced by the respective host plants, because endophytic fungi can mimic the properties of the host plants in which they reside.

The discovery of taxol producing fungi increased the importance of endophytes and their metabolites. Taxol is an anticancer drug, which is used for treatment of different types of cancers like ovarian and breast cancers, but now it is also used to treat many human tissue-proliferating diseases as well. Paclitaxel, a diterpene compound, has been used alone or in combination with other chemotherapeutic agents for the treatment of a variety of cancers, as well as AIDS-related Kaposi sarcoma (Croom, 1995). Initially it was isolated from the bark of the Pacific yew tree, *Taxus brevifolia* (Wani *et al.*, 1971) but isolation of paclitaxel from plant source requires destruction of trees which is harmful for biodiversity as well. Because of the limited supply of the drugs alternative sources of these drugs were explored by researchers. 1n 1993, pactitaxel was first time isolated from endophytic fungi isolated from *Taxus brevifolia* (Stierle *et al.*, 1993; Strobel *et al.*, 1993). Other methods for paclitaxel production, such as chemical synthesis, tissue and cell cultures of the *Taxus* species are expensive and give low yields (Guenard *et al.*, 1993; Frense, 2007). As the endophytic fungi can mimic the activities of the host plant so exploitation of such medicinal plants for the isolation of endophytic fungi having important biological activities can be done.

Fermentation of endophytic fungi is cost effective method and gives higher yield as compare to other methods.

2.6.1 Liquid culture for secondary metabolites

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi. In the course of this metabolic breakdown, they also release several additional compounds apart from the usual products of fermentation, these additional compounds are called secondary metabolites. Secondary metabolites range from several antibiotics to peptides, enzymes and growth factors (Balakrishnan and Pandey, 1996; Machado *et al*., 2004; Robinson *et al*., 2001). They are also called 'bioactive compounds' since they possess biological activity. Submerged fermentation utilizes free flowing liquid substrates, such as molasses and broths. The bioactive compounds are secreted into the fermentation broth. Different media were used for fermentation and it was found that extracts with greater activity were consistently obtained when the fungi were grown on nutrient-poor Czapek Dox medium. Nutrient limitations are a typical form of stress in fungi, as in other organisms, and the transduction of these stress signals induces a protective response to allow survival in a hostile environment (Demain., 1986).

2.6.2 Solvent extraction

Liquid-liquid extraction involves the distribution of substance between two immiscible liquid phases. The two liquid phases are usually, but not always, an aqueous solution and organic solvent. Most extraction operations in organic laboratory are carried out in separatory funnel. The efficiency of extraction will depend upon the solubility of the compound in the two solvents. The ratio of solubilities is called the distribution coefficient (K_D). If a compound has a low K_D for a given extraction, it is better to search for a different organic solvent in which the compound is more soluble in order to do liquid-liquid extraction. If this is not feasible, doing multiple extractions can increase the amount of compound extracted. Although water is almost always one of the liquids in the liquid-liquid extraction process, the choice of organic solvent is quite wide. To improve the extraction of organic compounds from aqueous mixtures, it is advisable to saturate the aqueous phase with salts such as sodium sulphate and calcium chloride.

2.7 Screening of Endophytic fungi having antitumor activity

The screening of endophytic fungi for their antitumor activity can be done by using Antitumor potato disc assay which is widely used method for prescreening of the different anticancer compounds and extracts (Coker *et al*., 2003).

2.7.1. Antitumor Potato Disc Assay

The inhibition of *A. tumefaciens*-induced tumors (or crown gall) in potato disc tissue is an assay based on antimitotic activity and can detect a broad range of known and novel antitumor effects (McLaughlin and Rogers, 1998). Crown gall is a neoplastic disease plant disease caused by *A. tumefaciens* (Kahl and Schell, 1982; Lippincott and Lippncott, 1975). The validity of this bioassay is predicted on the observation that certain tumorigenic mechanisms are similar in plants and animals (Becker, 1975; Braun, 1972; Karpas, 1982). The bacterium, *A. tumefaciens*, is a gram-negative rod that is the causative agent of Crown Gall Disease. Crown Gall Disease is a disease in which a mass of tissue bulging from stems and roots of woody and herbaceous plants is produced. The tumors produced are histologically similar to those tumors found in humans and animals (Agrios, 1997).

During infection of plant material with *A. tumefaciens,* a tumor-producing plasmid (Ti-plasmid), found in the bacterial DNA, is incorporated into the plant's chromosomal DNA. When plant is wounded it release phenols, etc., which will activate the Ti-plasmid in *A. tumefaciens.* Ti-plasmid causes the plant's cells to multiply rapidly without going through apoptosis, resulting in tumor formation similar in nucleic acid content and histology to human and animal cancers (Agrios, 1997). McLaughlin (1991) concluded that the Crown Gall (potato disc assay) assay could be used as a fairly rapid, in expensive and reliable prescreen for antitumor activity.

2.8 Cytotoxicity Assay

Cell lines offer many advantages over tumors. Cell lines are populations of pure tumor cells without admixed stromal or inflammatory cells. Normal lung epithelial cell are valuable tools for studying the multistage pathogenesis of lung cancers. Two types of normal culture models are available for studies: primary cultured cells and immortalized cell lines. The major advantage of primary cell models is that it is more close to the lung tissue origin and thus more resemble the lung cell physiology. However, the inter-individual variability, the limited resource and more importantly, the finite life span that does not allow long term genetic manipulations, make the primary cell model less desirable. The major advantage of

immortalized non-malignant epithelial lines is the cell lines can be genetically modified in an isogenic system to systematically study the genetic alteration in lung cancer (Adi *et al.*, 2010). In past years, a number of methods have been developed to study cell viability and proliferation in cell culture (Cook and Mitchell, 1989). The most convenient, modern assays have been optimized for the use of micro titerplates (96-well). This miniaturization allows many samples to be analyzed rapidly and simultaneously. Tetrazolium salts (Smith, 1951) are reduced only by metabolically active cells. Thus, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) can be reduced to a blue colored formazan (Mosmann, 1983). The A549 cell line was first developed in 1972b DJ Giard through the removal and culturing of cancerous lung tissue in the explanted tumor of 58-year-old caucasian male. The A549 tumor-cell line, initiated from a human alveolar cell carcinoma, has been continuously propagated in vitro for more than 3 years (more than 1,000 cell generations). These cells have a human karyotype and appear to have been derived from a single parent cell. A549 cells examined by electron microscopy at both early and late passage levels contain multilamellar cytoplasmic inclusion bodies typical of those found in type II alveolar epithelial cells of the lung. At early and late passage levels, the cells synthesize lecithin with a high percentage of disaturated fatty acids utilizing the cytidine diphosphocholine pathway; such a pattern of phospholipid synthesis is expected for cells believed to be responsible for pulmonary surfactant synthesis. The A549 cell line should permit *in vitro* analysis of human surfactant synthesis and secretion and possibly provide a source of human surfactant for therapeutic intervention in pulmonary disease states characterized by surfactant deficiency (Lieber *et al.*, 1976). Tetrazolium dye reduction is dependent on NAD(P)H-dependent oxidoreductase enzymes largely in the cytosolic compartment of the cell. XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) has been proposed to replace MTT, yielding higher sensitivity and a higher dynamic range. The formed formazan dye is water soluble. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate (PMSF) produces a formazan product that has an absorbance maximum at 490-500 nm in phosphate-buffered saline.

2.9 Identification of endophytic fungi

Fungal taxonomy is traditionally based on comparative morphological features (e.g., Lodge *et al.*, 1996; Sette *et al.*, 2006; Crous *et al.*, 2007; Zhang *et al.*, 2008). However, special caution should be taken when closely related or morphologically similar endophytes are being identified, because the morphological characteristics of some fungi are medium dependent

and cultural conditions can substantially affect vegetative and sexual compatibility (Zhang *et al*., 2006; Hyde and Soytong, 2007). Various optimization of growth conditions have been used to promote sporulation of these fungi, such as different culture media, potato dextrose agar (PDA), malt extract agar (MEA), corn meal agar (CMA), potato carrot agar (PCA), and water agar (WA), as well as the inclusion of host tissues in plate cultures (Guo *et al*., 2000).

In contrast, molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels (Sette *et al*., 2006).

Chapter 3

Aim of the study

Aim and objectives of present study include following:

- Screening endophytic fungi for their antitumor activity via antitumor potato disc assay
- Determination of cytotoxic potential of selected fungal isolates against A549 lung cancer cell line
- Identification of endophytic fungi having antitumor activity

Chapter 4

Materials & Methods

4.1 Maintenance of pure cultures of endophytic fungi

The maintenance of the cultures involved Potato dextrose agar (PDA) plates preparation, sub culturing of cultures and long term preservation.

4.1.1 Preparation of PDA (Potato dextrose agar) plates

39g of PDA was weighed and dissolved in 1L double distilled water and stirred to mix properly. After that it was transferred into 250ml Erlenmeyer flasks and autoclaved at 121˚C at 15 psi for 15 min. Aseptically, 25ml of sterilized PDA was poured into pre-sterilized 90mm glass petri plates and allowed to solidify at room temperature.

4.1.2 Sub culturing of Endophytic fungi

65 endophytic fungi isolated from *Aegle marmelos, Cinnamomum malabaricum, Cinnamomum zeylanicium* and *Cinnamomum camphora, Raulwofia serpentina, Camellia sinensis* were aseptically sub cultured on PDA plates and incubated at 26°C for 7-10 days till profuse fungal growth was observed. The loop full of pure culture was transferred aseptically on to PDA slants containing 10% glycerol for long term storage at 26 ±2ºC. Sub culturing of these PDA slants was done after regular intervals of time.

4.2 Production of culture filtrate

The submerged fermentation of 65 endophytic fungi was carried out in Czapek Dox broth (CZD) medium. 5mm mycelial plugs of 4-5 day old culture were inoculated into 100ml pre-sterilized CZD broth in Erlenmeyer flasks under aseptic conditions. The flasks were incubated in shaker-incubator at 120 rpm, 26°C for 7-10 days. After 10 days, the fungal mycelium was separated from broth through filtration using Whatman filter paper followed by centrifugation at 12,000 rpm for 15 min to get cell free culture filtrate. The cell free culture filtrate was then used for primary screening of anti tumor activity.

4.3 Primary Screening:

Primary screening of 65 culture filtrates for anti tumor activity was carried out using anti tumor potato disc assay. But to carry out this test first of all the growth curve of *A. tumefaciens* was deduced.

4.3.1 Growth kinetics of *Agrobacterium tumefaciens*

For the Growth kinetics of the *Agrobacterium tumefaciens,* the broth was inoculated with 1%

inoculum of overnight grown culture of *A. tumefaciens* and then incubated at 28˚C at 120 rpm. Aseptically 2ml of culture was taken after 0h, 12h, 24h, 36h and absorbance was noted at 600nm. Readings were taken in triplicate to reduce error. Growth curve of *Agrobacterium tumefaciens* was obtained by plotting graph of absorbance versus time.

4.3.2 Anti-tumor Potato Disc assay

This assay was carried out as described by Coker, 2003 with slight modifications. *Agrobacterium tumefaciens* was grown on Luria Bertani broth at 28˚C for 48h. Red skin potatoes (*Solanum tuberosum*) were washed under running tap water followed by surface sterilization with 1% sodium hypochlorite for 10 min. Further the potatoes were immersed in 70 % ethanol for 10 min followed by washing with sterilized water. Potato discs of size 10mm were scooped out with the help of sterilized cork borer. Then these potato discs were transferred on to 2% water agar plates. The discs were inoculated with 50µl of total reaction inoculum. Vinblastine was used as positive control and considered to exhibit 100% anti tumor activity. Three negative controls i.e. Czapek Dox broth, 1% DMSO and only A*grobacterium tumefaciens* were used. The composition and volume of final reaction mixture to be inoculated on to potato discs are shown in Table no.2.

	Positive control	Test filtrates	Negative control 1	Negative control 2	Negative control 3
A. tumefaciens broth	25µl	25µl	25µl	25µl	25µl
Vinblastine	19µl	-	-	-	-
CZD broth	-	-	19µl	-	-
DMSO	-	-	-	19µl	-
Test filtrates	-	19µl	-	-	-
Autoclaved Distilled water	6µl	6µl	6µl	6µl	25µl

Table no.2 showing reaction mixture composition and volume of inoculum of Anti-tumor potato disc assay.

After inoculation, petri dishes were sealed with parafilm & incubated at 28˚C for 21 days in dark. On 21st day, the discs were stained with Lugol's reagent (I_2KI: 5% I_2 + 10% KI in distilled water). Lugol's reagent stains the starch in the potato tissue and produce dark blue

to brown color, but tumors do not take up the stain. After 21 days, the potato discs were viewed under Nikon stereo zoom microscope and photographs of tumors protruding on the potato discs were taken.

4.4 Solvent Extraction

The Extraction of bioactive residue is the first step in the utilization of various phytochemicals in the preparation of neutraceuticals, pharmaceuticals and cosmetic products. Bioactive residue was purified from the culture filtrate by liquid-liquid extraction. The extraction of secondary metabolites was carried out by using gradient of different solvents with increasing polarity (Hexane, Dichloromethane, ethyl acetate). Organic solvents in the ratio of 1:1 (v/v) were added to the culture filtrate and shaken vigorously. The aqueous phase was dried by adding anhydrous sodium sulphate. The organic phase was evaporated to dryness at room temperature. The residue obtained was weighed and reconstituted in 1% DMSO and then stock solution of 1mg/ml was prepared and stored at -20°C till further use.

4.5 Secondary Screening

After solvent extraction of selected fungal cultures by using different solvents, their bioactive residue was screened using MTT assay according to previously described method (Mosmann, 1983) on A549 cell line for their antitumor activity.

4.5.1 A549 cell growth

The Human alveolar adenocarcinoma (A549) cell line was grown in a humified atmosphere (5% CO_2) at 37ºC in Dulbecco's Modified minimum essential medium containing 10% FBS and 1% Pen-Sep (Antibiotic).

4.5.2 MTT Assay

For MTT assay, the adherent cells were detached by trypsinization from $25cm^2$ flask on the first day. The viable cells were counted by using 0.5% Trypan Blue dye exclusion method and cell count was adjusted to 2×10^5cells/ml. The cells were seeded using a concentration of 2×10^5cells/ml (100µl/well) in triplicate wells in a 96-well microtitre plate (Figure no.2). The microtitre plate was incubated at 37ºC, 5% CO_2 for 24h. After 24h incubation, the medium of each well was removed by aspiration and replaced with experimental bioactive residue (50µg/well) and incubated for a period of 24h. After this interval, 0.05mg of MTT was added to each well and the plate was incubated for 4h in CO_2 incubator. Subsequently after 4h, the

media was removed from each well and DMSO was added to all wells to dissolve the formazan crystals. Cell viability was determined by measuring the absorbance at 570nm using Biotek throughput reader, Power wave 340. DMSO was used as negative control and considered to have 100% cell viability. Vinblastine was used as positive control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C4	T2	T5	T8	T10	T13	B	B	B	B	B
B	C1	C4	T3	T5	T8	T11	T13	B	B	B	B	B
C	C1	C4	T3	T6	T8	T11	B	B	B	B	B	B
D	C2	T1	T3	T6	T9	T11	B	B	B	B	B	B
E	C2	T1	T4	T6	T9	T12	B	B	B	B	B	B
F	C2	T1	T4	T7	T9	T12	B	B	B	B	B	B
G	C3	T2	T4	T7	T10	T12	B	B	B	B	B	B
H	C3	T2	T5	T7	T10	T13	B	B	B	B	B	B

Fig no. 3: Showing template for MIC
T1 to T13 refers to different concentrations of bioactive residue
C1 refers to the only cells + MTT and no compound
C2 refers to positive control i.e. Cells treated with DMSO + MTT
C3 refers to DMEM Media + MTT and no cells
C4 refers to cells treated with Vinblastine (positive control)
B refers to empty wells .

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C4	S2	S5	S8	S10	S13	S16	S18	S21	S24	S26
B	C1	C4	S3	S5	S8	S11	S13	S16	S19	S21	S24	S27
C	C1	C4	S3	S6	S8	S11	S14	S16	S19	S22	S24	S27
D	C2	S1	S3	S6	S9	S11	S14	S17	S19	S22	S25	S27
E	C2	S1	S4	S6	S9	S12	S14	S17	S20	S22	S25	S28
F	C2	S1	S4	S7	S9	S12	S15	S17	S20	S23	S25	S28
G	C3	S2	S4	S7	S10	S12	S15	S18	S20	S23	S26	S28
H	C3	S2	S5	S7	S10	S13	S15	S18	S21	S23	S26	S29

Fig no. 2: Showing template for cell cytotoxicity activity
S1 to S10 refers to culture filtrates of different endophytic fungi
S11 to S20 refers to ethyl acetate extracted bioactive residue
S21 to S29 refers to aqueous fraction of different endophytic fingi
C1 refers to the only cells + MTT and no compound
C2 refers to positive control i.e. Cells treated with DMSO + MTT
C3 refers to DMEM Media + MTT and no cells
C4 refers to cells treated with Vinblastine (poistive control)

Minimum inhibitory concentration (MIC) of best selected culture was determined by preparing different concentrations of test compound following MTT assay as described above (Figure no.3)

4.6. Identification of the endophytic fungi possessing anti-tumor activity

Endophytic fungi possessing cytotoxicity was identified by using Classical as well as molecular tools.

4.6.1 Morphotaxonomy

The endophytic fungal culture which was showing positive results was examined under the microscope to characterize the culture on the basis of their microscopic and morphological characters. The culture was grown on Potato dextrose Agar (PDA) plates. Briefly, the glass slide was cleaned with alcohol and air dried. A drop of water was put on to glass slide, upon which the mycelial mass taken with the help of fine needle was placed and teased properly. It was then stained with Lactophenol cotton blue (Hi Media). The slide was covered with 18 x 10 mm cover slip avoiding the formation of air bubble and mounted with DPX. The slide was observed at 10X, 40X and 100X using Nikon binocular microscope. The fungi were identified based upon their spore structure and other morphological characteristics.

4.6.2 DNA isolation

The fungal genomic DNA was isolated from 3-4 days old fungal culture grown on PDA plates. 0.5-1g of wet mycelium was crushed to very fine powder by using liquid nitrogen in pestle and mortar. 660-750µl of the extraction buffer was added to it and again crushed. The contents were transferred to a 1.5ml micro centrifuge tube and 10µl of β-mercaptoethanol and 4µl of Proteinase K was added to each tube and vortexed followed by incubation at 65°C in water bath for 1h (intermittent mixing after every 15 min). After incubation, the micro centrifuge tubes were centrifuged at 10,000 rpm for 15 min to remove cell debris. Further 6µl of RNase was added to each tube and incubated at 37°C for 30 min. Equal volume of Phenol: Chloroform (1:1) was added to each tube to precipitate the contaminating protein contents and mixed properly for 15 min followed by centrifugation at 12,000 rpm for 10 min (This step was repeated three times). Transfer the aqueous layer containing DNA to the fresh micro centrifuge tube carefully and add 20µl of 3M sodium acetate and contents of each micro centrifuge was topped up with absolute ethanol and incubated at 4°C overnight. On the next day, the micro centrifuge tubes were centrifuged at 12,000 rpm for 10 minutes; the pellet was washed with 70% ethanol followed by centrifugation at 12,000 rpm for 5 min. Then pellet was air dried and dissolved in 50µl of Tris EDTA buffer (pH=8). The qualitative estimation of the DNA isolated was done by agarose gel electrophoresis.

4.6.3 Agarose gel electrophoresis

0.8% agarose gel was prepared in 1X Tris Acetate EDTA and 0.5µg/ml of ethidium bromide (EtBr) was added and then gel was casted in the electrophoretic apparatus. The gel was allowed to solidify and the comb was carefully removed. The running buffer (1X TAE) was poured into the electrophoretic tank so that the gel is fully immersed into the buffer. The DNA samples were mixed with the 5X loading buffer. The samples were loaded into wells and allowed to run at 50V for 1h. The gel was observed under UV transilluminator for the presence of DNA. Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software.

Quantitative Estimation of the Genomic DNA was done by spectrophotometric analysis of the sample. The absorbance of the sample was taken at 260nm, to determine the concentration of the sample. 1 OD is equivalent to 50µg/ml DNA sample. The concentration of the DNA sample was calculated using following formula.

$$\text{Concentration } (\mu g/ml) = O.D_{260nm} \times 50\mu g/ml \times \text{Dilution factor}$$

The purity of the DNA sample was determined by taking the ratio of absorbance at 260nm and at 280nm. If the ratio is less than 1.6, then there is RNA contamination, if the ratio lies between 1.6-1.8, then DNA sample is pure. If the ratio is more than 1.8, the DNA might be contaminated with protein.

4.6.4 PCR Amplification

The polymerase chain reaction (PCR) is a sensitive technique which is used to amplify number of copies of specific region of DNA. ITS1 + 5.8S + ITS2 rDNA region was amplified using ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCT TATTGATATGC3') primers as described by White *et al.*, 1990. Primers were synthesized by Integrated DNA Technologies (IDT).

S.No	Reagents	Stock concentration	Quantity	Final concentration in 25µl
1.	Autoclaved distilled water	_	15µl	_
2.	Taq buffer	10X	2.5µl	1X
3.	dNTPs	2.5mM	2.0µl	0.2mM
4.	Primers	10µM	2.0µl	0.8µM
5.	Taq DNA Polymerase	3U/µl	0.5µl	1U
6.	Template DNA	25ng/µl	1.0µl	25ng

Table no.3 Different reagents used during PCR reactions.

Amplification was carried out in 25µl reaction mixture (Table no.3) containing- 1µl of extracted fungal DNA, 10µM of each primer (ITS1 and ITS4), 2.5mM of dNTP (Bangalore GeNei), 25mM $MgCl_2$ (Bangalore GeNei), 1.5U of Taq DNA Polymerase (Bangalore GeNei) in 10X Taq buffer (Bangalore GeNei) in a Thermocycler (My Cycler, Bio-Rad Laboratories, Inc). The PCR cyclic conditions consisted of initial denaturation at 96ºC for 5 min followed by 39 cycles of 95ºC for 45 sec, 60ºC for 45 sec, 72ºC for 45 sec followed by final extension at 72ºC for 5 min (Table no.4, Fig 4). The PCR products were resolved by using agarose gel electrophoresis (1.5 % agarose gel dissolved in 1X TAE buffer) at 50V for 1.30h. Gel imaging was performed under UV light in Bio-Rad Gel documentation System using Quantity-1-D analysis software. An approximate 550-600bp PCR product was purified by using the Wizard® SV Gel and PCR clean up system kit (Promega, USA).

Step	Temperature	Time
STEP I : Initial Denaturation	96°C	5 min
STEP II : Denaturation	95°C	45 sec
STEP III : Annealing	60°C	45 sec
STEP IV: Extension	72°C	45 sec
STEP V	Step II to Step IV repeated 39 times	
STEP VI : Final extension	72°C	5min
STEP VII: Store	4°C	∞

Table No.4: Temperature profile of PCR reaction

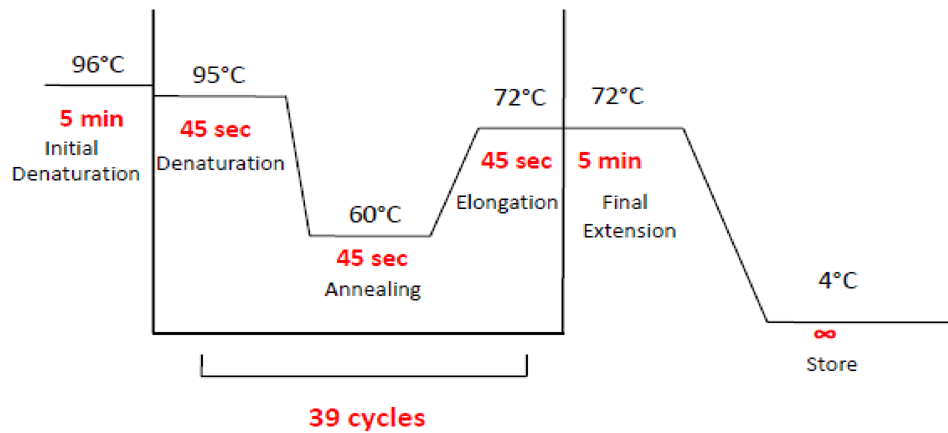


Figure no. 4 showing ray diagram of temperature profile of PCR reaction

Chapter 5

Results & Discussion

5.1 Re-culturing

65 Endophytic fungi used in this study were sub cultured regularly on PDA plates and maintained at 28˚C. For their long term preservation, all the fungal isolates were sub cultured on PDA slants containing 10% glycerol and maintained at 28˚C. The endophytic fungi in the present study were isolated from medicinally important plants belonging to families Rutaceae (*Aegle marmelos*), Lauraceae (*Cinnamomum malabaricum, Cinnamomum zeylanicium, Cinnamomum camphora*), Piperaceae (*Piper nigrum*) Apocynaceae (*Raulwofia serpentina*) *and* Theaceae (*Camellia sinensis*) collected from the biodiversity hot spots of India. From ancient times, medicinal plants have always been a prime source of novel bioactive compounds, so in the present study the endophytic fungi isolated from these medicinally important plants were exploited for production of novel bioactive commodities that can be a potential candidate for the development of anticancer drugs.

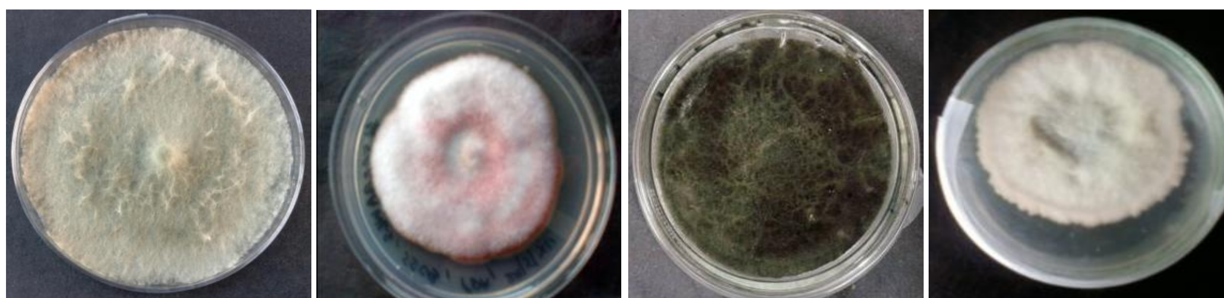


Figure no.5 showing pure endophytic cultures on PDA plates

Out of 65 endophytic fungi, 30 were isolated from *Aegle marmelos*- 15 were from stem, 8 from stem internal tissue, 6 from leaves, 1 was from bark, 21 endophytic fungi were isolated from *Cinnamomum malabaricum*- 5 were from bark, 8 from leaf, 3 from stem, 5 from stem internal tissue, 3 endophytic fungi were isolated from stem internal tissue of *Cinnamomum camphora*, 1 was isolated from stem of *Camellia sinensis*, 6 endophytic fungi were isolated from *Cinnamomum zeylanicium*- 3 were from bark, 2 from stem, 3 from stem internal tissue, 1 was isolated from leaf of *Piper nigrum* , 4 were isolated from *Raulwofia serpentine*- 2 were from leaf and 2 from bark.

5.2. Production of culture filtrates

65 endophytic fungi under study were subjected to secondary metabolites production in liquid medium. Biomass production of some of fungal cultures ranging from highest to lowest is listed in Table no.5.

Biomass production was considered as a parameter to deduce the growth rate of fungal cultures. #59AMSTWLS was showing the highest and #9AMLBRT was showing the

lowest biomass production. The culture filtrates obtained after the centrifugation were further screened using antitumor potato disc assay.

Culture Code	Biomass weight (g)
#59AMSTWLS	1.05 g
#1013AMSTITYEL	0.92 g
#9(b)AMSTYEL	0.38 g
#9AMLBRT	0.19 g

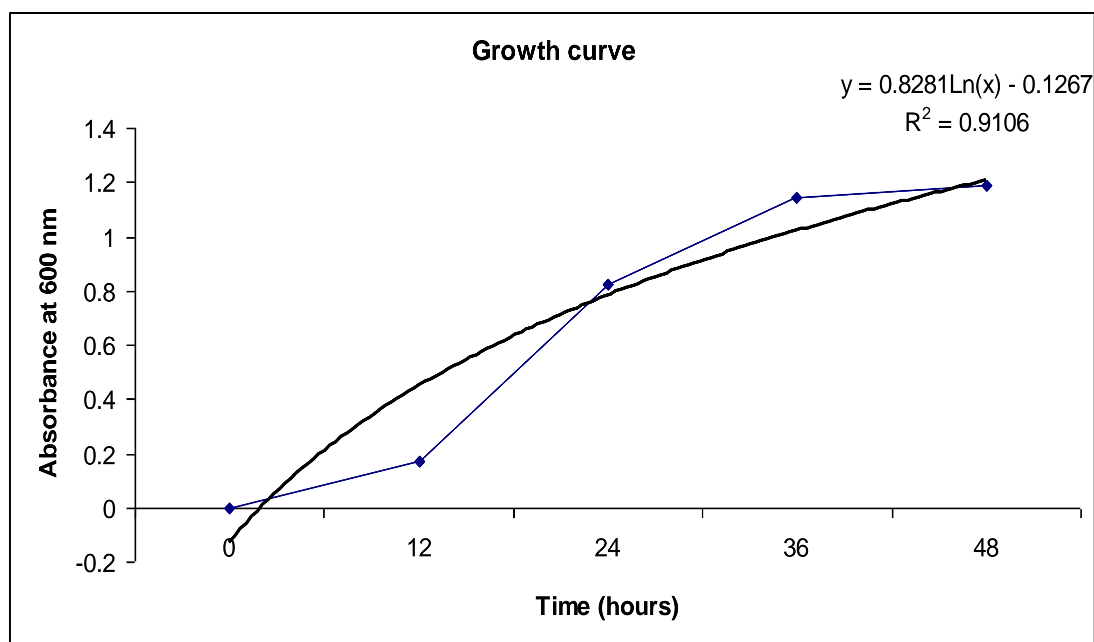
Table no.5 showing the biomass production in culture filtrate production

5.3. Primary screening

All culture filtrates were screened using the antitumor potato disc assay. This assay requires the metabolically active culture of *Agrobacterium tumefaciens* to induce the tumor formation so growth curve analysis of *Agrobacterium tumefaciens* was performed.

5.3.1 Growth kinetics of *Agrobacterium tumefaciens*

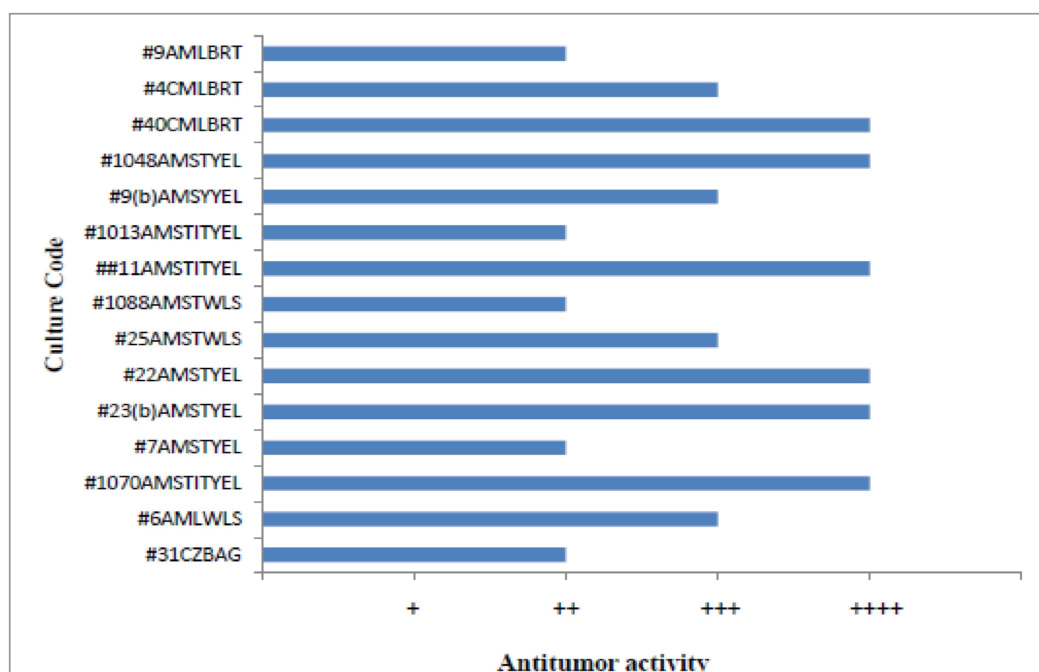
The growth curve revealed that the culture of *A. tumefaciens* comes in its active phase after 14-16 hours. So the culture after 14-16 hours was used in antitumor potato disc assay. The plot of absorbance versus time is shown in Graph No.1.



Graph no. 1 showing Growth curve of *Agrobacterium tumefaciens*

5.3.2 Antitumor potato disc assay

All endophytic fungal cultures under study were subjected to antitumor potato disc assay for their antitumor activity. It was observed that positive control, Vinblastine, showed 100% antitumor activity at concentration of 0.5mg/ml. All the three negative controls (CZD broth, DMSO, only *A. tumefaciens*) showed significant tumor formation confirming that the CZD broth in which the fermentation of endophytic fungal cultures were carried out has no effect on tumor formation. Similarly 1% DMSO, which was used to reconstitute the bioactive residue after solvent extraction, was not showing antitumor activity as well. Selection of potential endophytic fungi was done through comparison of antitumor activity of test filtrates with the positive and negative controls. Only culture filtrates having antitumor activity were able to inhibit the tumor formation by *Agrobacterium tumefaciens*. Data observed in primary screening given in Table no.6. Out of 65 cultures 6 endophytic fungi- #4CMLBRT, ##11AMSTITYEL, #1070AMSTYEL, #1048AMSTYEL, #22AMSTYEL and #23(b) AMSTYEL were found to have highly significant tumor inhibition activity and considered to have 100 % antitumor activity (indicated with ++++ sign), 4 endophytic fungi- #31CZBAG, #6AMLWLS, #25AMSTWLS, #9(b) AMSTYEL were considered to possess 80% antitumor activity (indicated with +++ sign), 5 endophytic fungi were found to have 40-50 % antitumor activity (indicated with ++ sign), 15 cultures were showing 20-30% antitumor activity (indicated with +) (Graph no.2).



Graph no.2 showing antitumor activity of cultures on potato disc assay

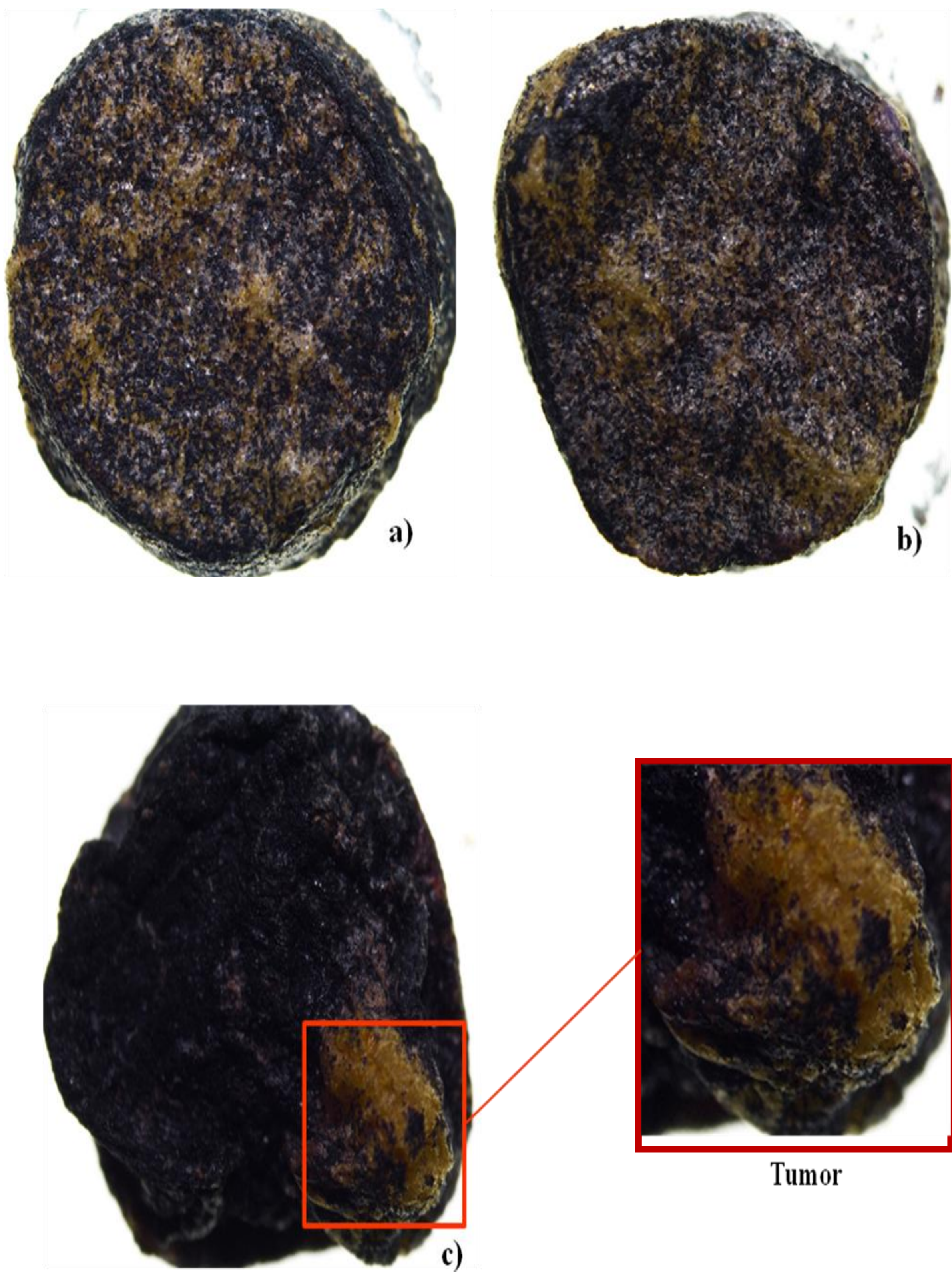


Figure no.6 Tumor formation on potato discs a) Negative control 1 (CZD broth) b) Negative control 2 (with *A .tumefaciens* only) c) Negative control 3 (1% DMSO)

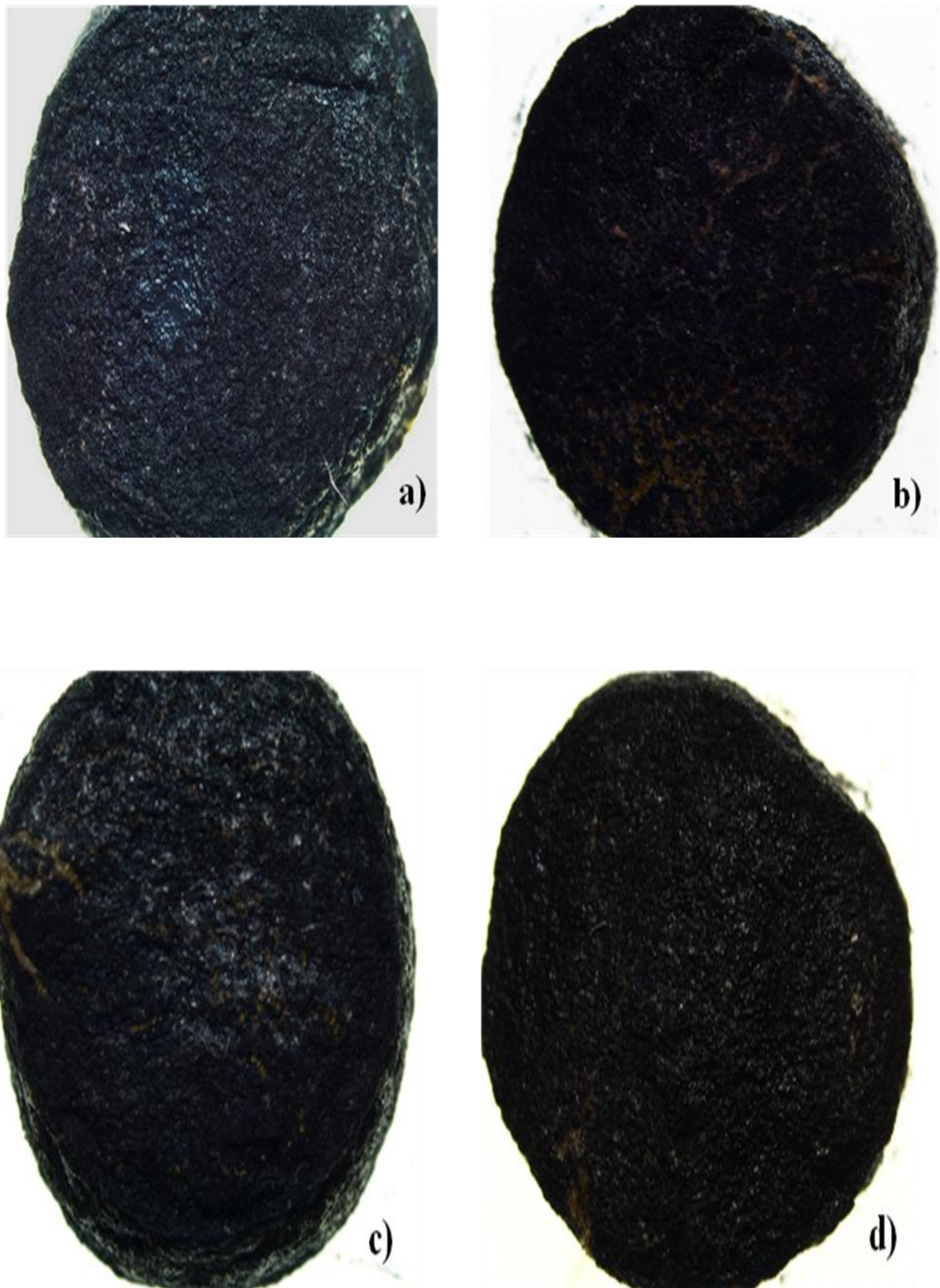


Figure no.7 Antitumor activity of test culture filtrates on potato discs a) Positive control, Vinblastine (0.5mg/ml) b) #1070AMSTITYEL c) #1048AMSTITYEL d) #9(b) AMSTYEL

In rest of 35 cultures, tumor formation was observed on potato disc and considered to have no antitumor activity. From primary screening it was observed that the 10 cultures were showing above 80% antitumor activity and they were selected for further study.

5.4 Solvent Extraction

After primary screening, the cell free broths of selected 10 fungal cultures were subjected to liquid-liquid extraction by ethyl acetate. The bioactive residue was reconstituted in 1% DMSO. The maximum yield of the bioactive residue was obtained from #22AMSTYEL (4.3mg) and lowest in the case of #9(b) AMSTYEL (0.6mg). Ethyl acetate extracts as well as aqueous phase of 10 fungal cultures were again screened on potato discs for antitumor activity.

Out of 10 selected cultures, ethyl acetate extracts of 4 cultures- #23(b) AMSTYEL, #22AMSTYEL, #1070AMSTYEL, #1048AMSTYEL were found to possess tumor inhibition ability and significant antitumor activity was also observed in aqueous phase of two cultures e.g. #1048AMSTYEL, #31CZBAG. So, it was considered that the aqueous phase of culture filtrates contains such bioactive compound which was not extracted. Above 5 cultures, which were showing again good results on potato disc, were then extracted with Hexane, Dichloromethane and ethyl acetate and screened for cytotoxicity on A549 cell line.

5.5 Secondary screening by MTT assay:

Based on the primary screening by potato disc assay for antitumor activity, 10 selected fungal cultures were further screened for their cytotoxic activity on A549 cells (non small cell lung cancer cell line) by MTT assay**.** A549 cells are adenocarcinomic epithelial cells which grow as monolayer as shown in Figure no 8.

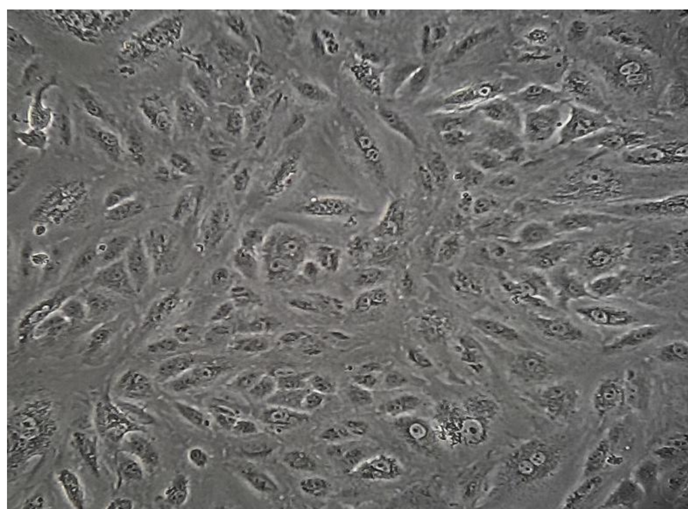


Figure no 8: showing A549 epithelial monolayer cells

S.No.	Culture code	Host plant	Plant part	Sampling location	Antitumor activity
1	#1069AMSTYEL	*Aegle marmelos*	Stem	Yelundur,Karnataka	+
2	#7AMSTYEL	*Aegle marmelos*	Stem	Yelundur,Karnataka	++
3	#22AMSTYEL	*Aegle marmelos*	Stem	Yelundur,Karnataka	++++
4	#1003AMSTYEL	*Aegle marmelos*	Stem	Yelundur,Karnataka	+
5	#23AMSTYEL	*Aegle marmelos*	Stem	Yelundur,Karnataka	+
6	#1032AMSTYEL	*Aegle marmelos*	Stem	Yelundur,Karnataka	+
7	#23(b)AMSTYEL	*Aegle marmelos*	Stem	Yelundur,Karnataka	++++
8	#9(b)AMSTYEL	*Aegle marmelos*	Stem	Yelundur,Karnataka	+++
9	#1048AMSTYEL	*Aegle marmelos*	Stem	Yelundur,Karnataka	++++
10	#1013AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelundur,Karnataka	++
11	#1010AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelundur,Karnataka	-
12	#1058AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelundur,Karnataka	+
13	#1070AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelundur,Karnataka	++++
14	#1007AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	-
15	#1016AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	-
16	#9AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	++
17	#1006AMLBRT	*Aegle marmelos*	Leaf	BRT wildlife sanctuary	-
18	#59AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	+
19	#28AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	+
20	#33AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	-
21	#25AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	+++
22	#20AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	-
23	#37(b)AMSTWLS	*Aegle marmelos*	Stem	Wayanad, Kerala	-

24	#1104AMSTITWLS	*Aegle marmelos*	Stem internal tissue	Wayanad, Kerala	-
25	#1088AMSTITWLS	*Aegle marmelos*	Stem internal tissue	Wayanad, Kerala	++
26	#42AMSTITWLS	*Aegle marmelos*	Stem internal tissue	Wayanad, Kerala	-
27	#61AMLWLS	*Aegle marmelos*	Leaf	Wayanad, Kerala	-
28	#6AMLWLS	*Aegle marmelos*	Leaf	Wayanad, Kerala	+++
29	#11AMBAWLS	*Aegle marmelos*	Bark	Wayanad, Kerala	-
30	##11AMSTITYEL	*Aegle marmelos*	Stem internal tissue	Yelundur,Karnataka	++++
31	#18CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	-
32	#2CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	-
33	#14CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	-
34	#12CMBANEY	*Cinnamomum malabaricum*	Bark	Neyyar, Kerala	+
35	#12CMBABRT	*Cinnamomum malabaricum*	Bark	BRT wildlife sanctuary	-
36	#1CCSTITD	*Cinnamomum camphora*	Stem internal tissue	Darjeeling, west Bengal	-
37	#2CCSTITD	*Cinnamomum camphora*	Stem internal tissue	Darjeeling, west Bengal	-
38	#36CCSTITD	*Cinnamomum camphora*	Stem internal tissue	Darjeeling, west Bengal	-
39	#2CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar, Kerala	-
40	#37CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar, Kerala	-
41	#1CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar, Kerala	+
42	#31CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar, Kerala	+
43	#29CMLNEY	*Cinnamomum malabaricum*	Leaf	Neyyar, Kerala	+
44	#4CMLBRT	*Cinnamomum malabaricum*	Leaf	BRT wildlife sanctuary	++++
45	#40CMLBRT	*Cinnamomum malabaricum*	Leaf	BRT wildlife sanctuary	++
46	#27CMLBRT	*Cinnamomum malabaricum*	Leaf	BRT wildlife sanctuary	-

47	#44CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-
48	#4CMSTNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-
49	#1622CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar, Kerala	-
50	#54(b)CMSTITNEY	*Cinnamomum malabaricum*	Stem	Neyyar, Kerala	-
51	#96CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar, Kerala	+
52	#21CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar, Kerala	-
53	#79CMSTITNEY	*Cinnamomum malabaricum*	Stem internal tissue	Neyyar, Kerala	-
54	#1CMSTITBRT	*Cinnamomum malabaricum*	Stem internal tissue	BRT wildlife sanctuary	-
55	#4CSSTOT	*Cimellia sinensis*	Stem	Ooty,Tamil nadu	-
56	#31CZBAG	*Cinnamomum zeylnicum*	Bark	Guwahati. Assam	+++
57	#4CZSTSTE	*Cinnamomum zeylnicum*	Stem	Assam	-
58	#2164CZSTITG	*Cinnamomum zeylnicum*	Stem internal tissue	Guwahati. Assam	-
59	#2131CZSTITG	*Cinnamomum zeylnicum*	Stem internal tissue	Guwahati, Assam	+
60	#2106CZSTITG	*Cinnamomum zeylnicum*	Stem internal tissue	Guwahati, Assam	-
61	#2PNLNEY	*Piper nigrum*	Leaf	Neyyar, Kerala	+
62	#1RSBANEY	*Rauwolfia serpentina*	Bark	Neyyar, Kerala	-
63	#16RSBANEY	*Rauwolfia serpentina*	Bark	Neyyar, Kerala	-
64	#15RSLBRT	*Rauwolfia serpentina*	Leaf	BRT wildlife sanctuary	-
65	#16RSLBRT	*Rauwolfia serpentina*	Leaf	BRT wildlife sanctuary	-

*++++ Excellent activity, +++ very good activity, ++ good activity, + low activity, - no activity

Table no.6 Primary screening of endophytic fungal isolates for antitumor activity by antitumor potato disc assay

The culture filtrate, ethyl acetate extracts and aqueous fraction of selected 10 fungal cultures were subjected to MTT assay. Vinblastine, a commercially available anticancer drug, was used as positive control and as expected it showed 100% cytotoxicity towards the cancerous cells after 4h of MTT addition.

1% DMSO which was used as negative control was not showing any cytotoxic effect on A549 cells and cells were found to be viable (Figure no.9).

Out of the 10 selected cultures, culture filtrates and ethyl acetate extracted bioactive residue of 5 cultures- #1048AMSTYEL, #23(b)AMSTEYL, #22AMSTYEL, 1070AMSTITYEL, #31CZBAG were showing mild to moderate antitumor activity by MTT reduction assay but their aqueous fraction were found to possess the significant cytotoxicity.

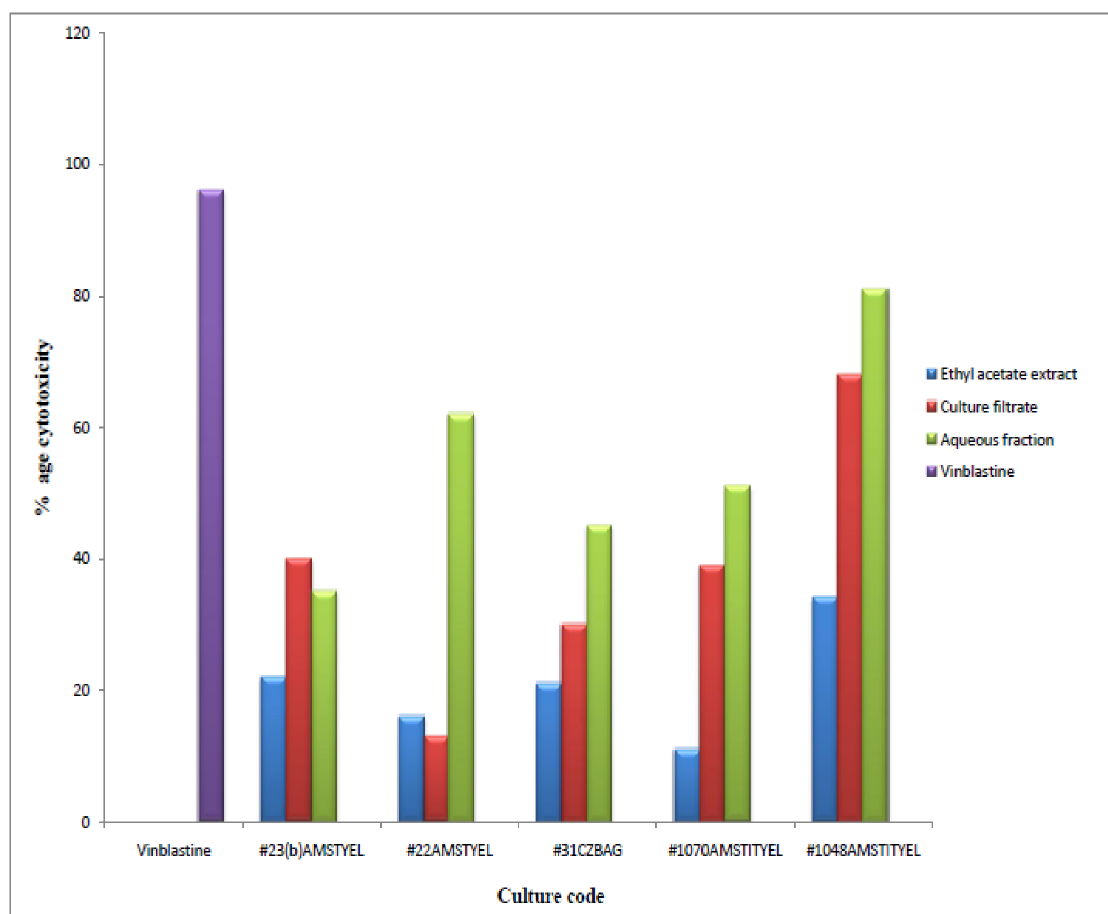
This obtained result was concordant to result observed in potato disc assay of ethyl acetate and aqueous fraction of selected 10 cultures. The comparison of cytotoxic activity of 5 cultures on A549 cells is represented in Graph no. 3, Table no.7.

Culture Code	**% age Cytotoxicity**		
	Culture filtrate	**Ethyl acetate extract**	**Aqueous fraction**
#1048AMSTITYEL	68	34	81
#23(b) AMSTYEL	40	22	35
#22AMSTYEL	13	16	62
#31CZBAG	30	21	45
#1070AMSTITYEL	39	11	51

Table no.7 showing comparison of % age cytotoxicity of 5 fungal cultures

The ethyl acetate extracted bioactive residue was not showing significant level of cytotoxicity, this may be due to the compound with anticancer properties was not extracted with ethyl acetate. So, the selected 5 cultures were extracted with solvents of different polarities i.e. Hexane (non-polar), Dichloromethane (slightly polar), ethyl acetate (slightly polar) these fractions as well as left aqueous fraction were again screened for cytotoxic effect on A549 cells by MTT reduction assay. No antitumor activity found in either of the extracted

bioactive residues of selected 5 cultures but the aqueous fraction of #1048AMSTITYEL was showing highly anti tumor activity. The probable reason of antitumor activity in aqueous

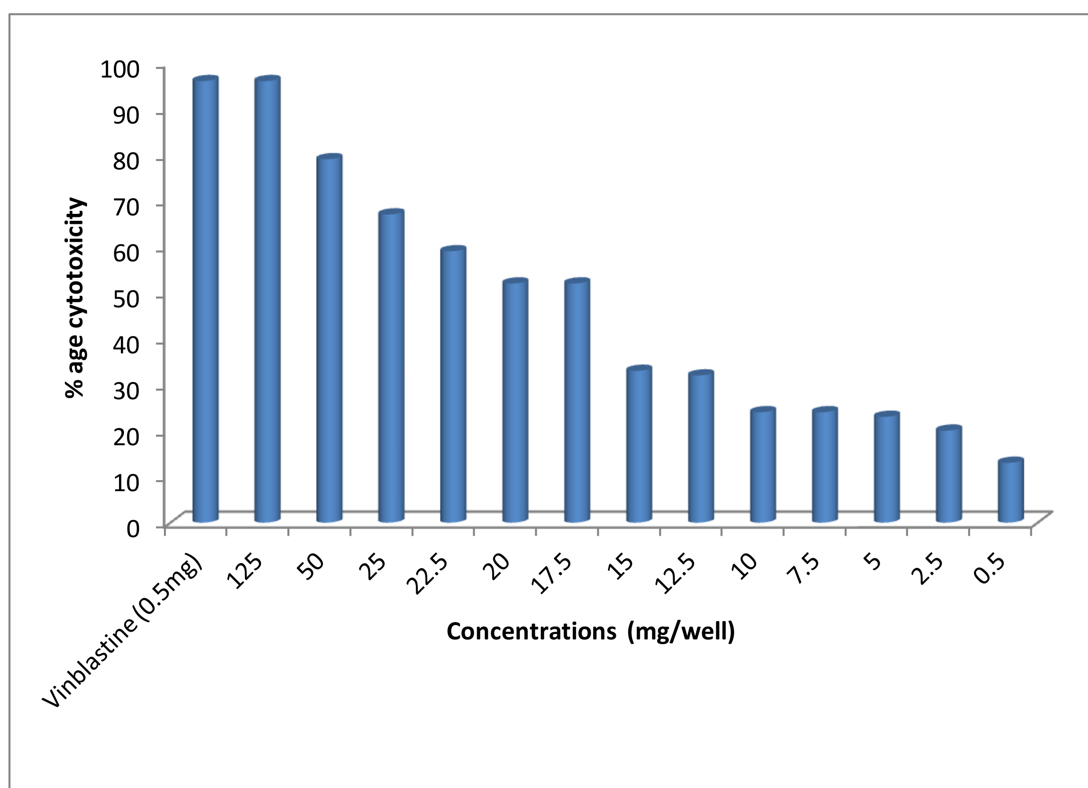


Graph no.3 showing % age cytotoxicity of selected 5 cultures on A549 cell line by MTT assay

fraction may be due to the presence of such bioactive compound that was not extracted in either of the organic solvents used. #1048AMSTITYEL was found to be potent culture exhibiting antitumor activity. The yield of air dried aqueous fraction of #1048AMSTITYEL was 250mg, which was re-constituted in 1% DMSO for the determination of the MIC. The different concentrations prepared for MIC assessment along with their antitumor activity is listed in Table No. 8, Graph no.4.

S.No	Concentration (mg/ml)	Effective concentration in each well (mg/Well)	% Cytotoxicity
1.	250	125	96
2.	100	50	79
3.	50	25	67
4.	45	22.5	59
5.	40	20	52
6.	35	17.5	52
7.	30	15	33
8.	25	12.5	32
9.	20	10	24
10.	15	7.5	24
11.	10	5	23
12.	5	2.5	20
13.	1	0.5	13

Table no.8 showing concentrations and % age cytotoxicity of different concentrations used for determination of MIC



Graph no.4 showing % age cytotoxicity of different concentrations of #1048AMSTITYEL. Vinblastine was used as positive control

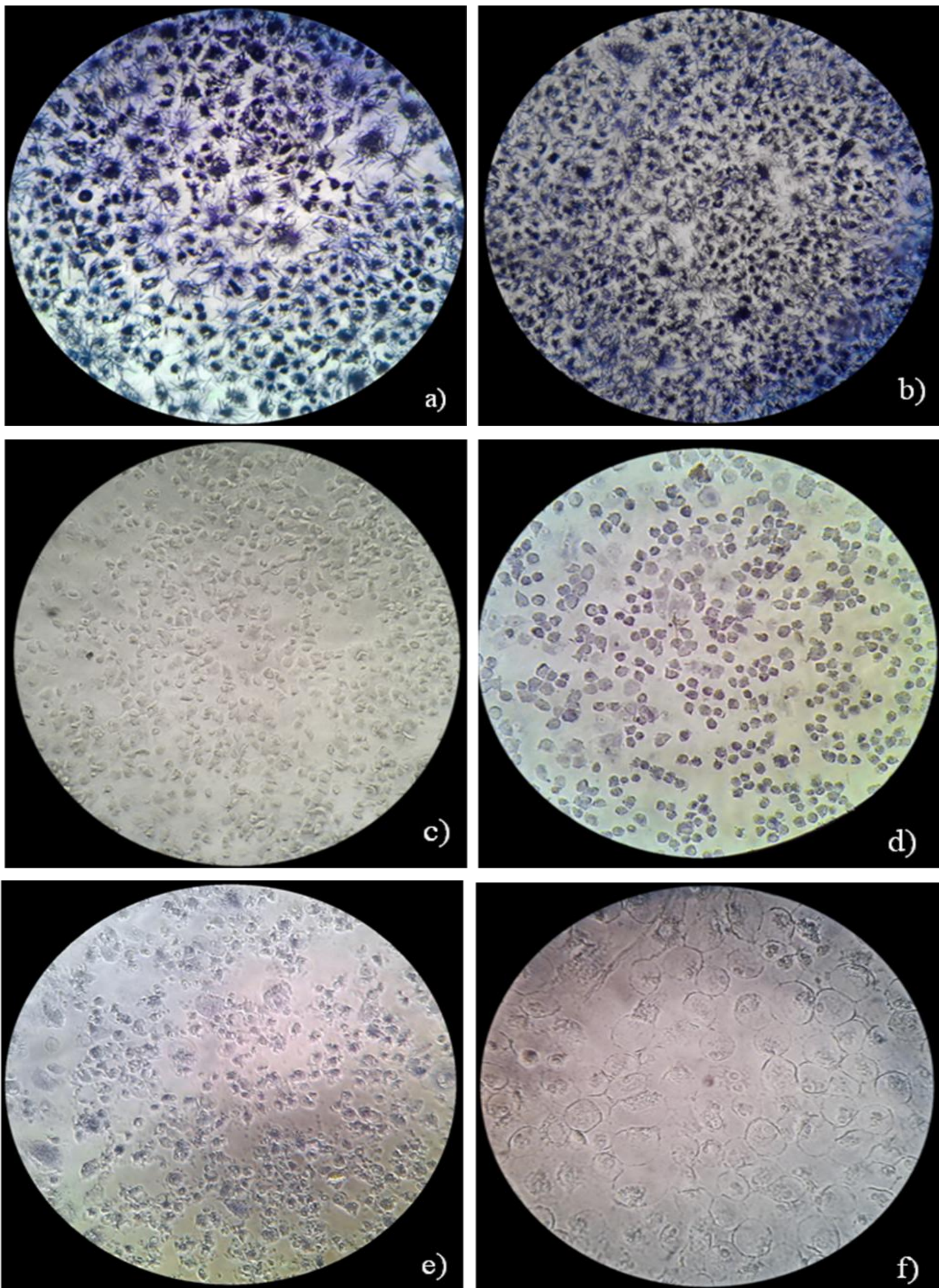


Figure no.9 Images of MTT reduction assay at 20X magnification showing the cell morphology of a) untreated cells and MTT b) cells treated with 1 % DMSO c) cells treated with 0.5mg/ml of Vinblastine d) treated with 50mg/ml e) 100mg/ml f) 250mg/ml of aqueous fraction of #1048AMSTITYEL

As the concentration of test aqueous fraction was increasing, the % age cytotoxicity was also increasing. The highest concentration i.e. 50 mg/well found to possess 96% cytotoxicity which was found comparable to % age cytotoxicity obtained with Vinblastine (0.25mg). The minimum inhibitory concentration was 0.5mg/well as it was showing 13% of cytotoxicity. The concentration below this value was also tested but no cytotoxic activity observed and all the cells were viable forming a purple colored formazan product on MTT addition. The IC_{50} i.e. the concentration which showed 50% cytotoxic activity, was deduced from graph and found to be 17.5mg. The aqueous fraction of #1048AMSTITYEL containing possible anticancer compound needs to be purified and characterized for further studies.

5.6 Identification of Endophytic fungi

5.6.1 Morphotaxonomy

The fungal culture (#1048AMSTITYEL) showing antitumor activity was grown on PDA, so that it can be characterized on the basis of their morphology and microscopic characteristics.

1.On PDA

Colony flocosse, cottony, grayish green color from front side and dark grayish green color from back side, fast growing, Diameter of colony is 90mm after 7 days, non elevated with aerial mycelium. Hyphae is brown colored, septate, long, thin, slender, branched.

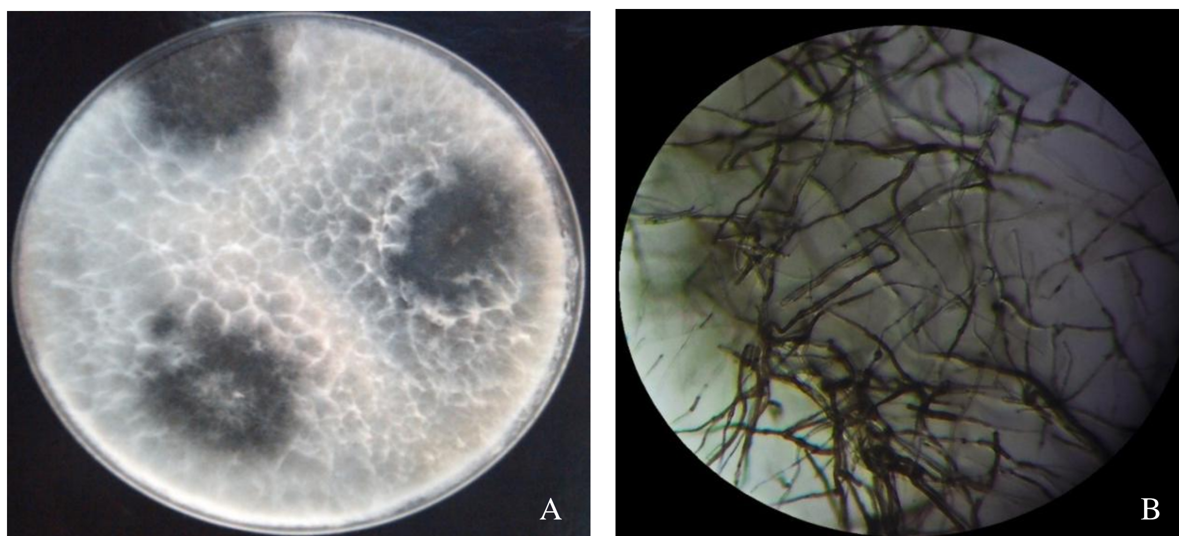


Figure no. 10 showing A) colony of 1048AMSTITYEL on PDA B) Microscopic view of mycelium (on PDA)

Based on the morphological characters, #1048AMSTITYEL is tentatively identified as ***Lasiodiplodia sp.***

5.6.2 Genomic DNA isolation and PCR amplification

The genomic DNA of 1048AMSTITYEL showing significant cytotoxicity was done. (Figure no.11). The Qualitative estimation of DNA was done by agarose gel eleactrophoresis. No smear or RNA bands were seen. The purity and concentration of genomic DNA was quantified by taking absorbance at 260nm and 280nm. The A_{260}/A_{280} was 1.6 confirming that the extracted genomic DNA was pure and free of RNA or protein contamination. The concentration of genomic DNA was found to be 0.321µg/ml.

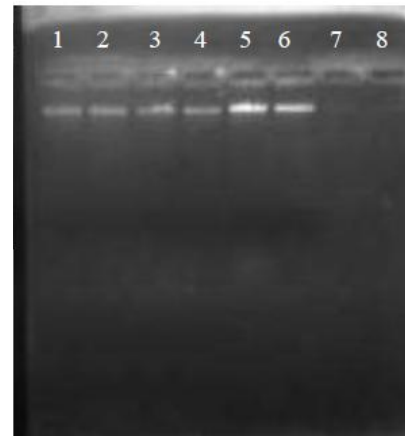


Figure no.11- showing genomic DNA of #1048AMSTITYEL

The ITS1, 5.8S, ITS2 region of the fungi was amplified by using ITS1 and ITS4 primer pair. The amplified product was resolved on to 1.5% agarose gel and the size of the amplicon was found to be approximately 550bp (Figure no.12).The size of the amplicon was deciphered by comparing its mobility in the gel with 500bp ladder.

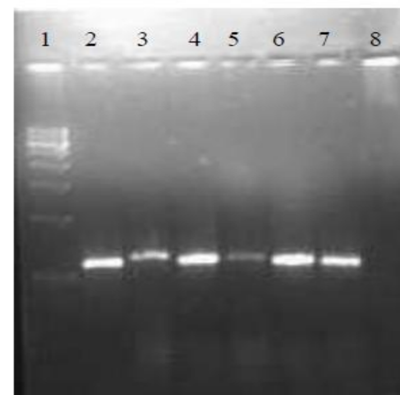


Figure no.12- showing PCR amplicon of ITS region of #1048AMSTITYEL.
Lane1: 500bp ladder
Lane 2-7: 1048AMSTITYEL amplicon

Further taxomonic identification of the endophtyic fungi must be carried out by processing the samples for sequencing and establishing the phylogenetic relationship and hence characterizing the fungi upto species level.

Chapter 6

Conclusion

Conclusion

In the present study, out of 65 endophytic fungal cultures screened, only 10 fungal cultures were showing significant antitumor activity in primary screening by antitumor potato disc assay. Hexane, Dichloromethane, ethyl acetate extracted bioactive residue as well as aqueous fractions of these cultures were screened for cytotoxicity on A549 cell line. Aqueous fraction of #1048AMSTITYEL showed the significant level of cytotoxicity on A549 cell line. Further taxonomic identification of #1048AMSTITYEL was carried out using classical and molecular methods. #1048 AMSTITYEL was tentatively identified as *Lasiodiplodia sp.*

Further purification and characterization of the compound having the cytotoxic activity along with the phylogenetic placement of the potential isolate would open up the possibilities for therapeutic employment of the compound as an anticancer drug.

Chapter 7

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