Isolation and screening of Vinblastine from Endophytic Fungi

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in
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Submitted
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CANDIDATE'S DECLARATION

I hereby declared that work being presented in the thesis entitled **'Isolation and screening of vinblastine from Endophytic fungi'** in the partial fulfilment of requirements for the awards of degree of Masters of Science in Biotechnology, Department of Biotechnology, Thapar university, Patiala is my own laboratory work during period of **January 2015 to June 2015** under the conception and supervision of Dr. Sanjai Saxena, Professor, Department of Biotechnology, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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This is to certify that the above statement made by candidate is correct and true to the best of my knowledge.

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CERTIFICATE

This is to certify that thesis entitled, "**Isolation and screening of vinblastine from Endophytic fungi**" submitted by **Ms. Geetali Handa (Roll no: 301301004)** in partial fulfilment of the requirements for the award degree of Master of Science Degree in Biotechnology at Thapar University, Patiala is a bonafied work carried out under the esteemed supervision and conception of Dr. Sanjai Saxena and that no part of thesis has been submitted in the award of any other degree.

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Executive Summary

Vinblastine is a chemotherapeutic drug often used for the treatment of bladder cancer. Vinblastine belongs to the group called vinca alkaloids, obtained from plants *Cathranthus roseus* (family- Apocyanaceae), species occurring in West Indies and Madagascar and also cultivated in some parts of India. The mechanism of action of vinblastine is inhibiting the assembly of microtubule by binding to tubulin. This results in arrest in the division of cells.

Endophytic fungi are ubiquitous organisms found in plants, residing intercellular, at least for a portion of their lives without causing apparent symptoms of infection. Most of the plants are known to harbor endophytes. They are chemical synthesizer inside plants which are able to produce bioactive substances with low toxicity toward higher organisms. Endophytes have the ability to produce a variety of secondary metabolites, providing researchers with numerous leads for compounds of pharmaceutical significance and possible development as new drugs. In this study, 20 endophytic fungi have been isolated from plants collected from various biodiversity hotspot in India used for screening of vinblastine.

The endophytic fungi processing the potential to produce vinblastine were initially grown on Czapek Dox Medium. The culture filtrate was screened for their potential to produce vinblastine extracellularly. Subsequently secondary screening was carried out using Phytochemical approach. Out of 20 culture screened, two cultures viz #82CRSTNEY (1.0mg), #6CRSTBRT (5.0mg), exhibited appreciable Vinblastine production. #42CRSTBRT produces appreciable amount of vinblastine (9.9mg).The anticancer potential of the fungal isolate #42CRSTBRT were tested by cytotoxicity assay on A549 cell line by MTT reduction assay. Microscopic identification showed that #42CRSTBRT belongs to *Fusarium sp.* Further for molecular identification, genomic DNA of the #42CRSTBRT isolate was amplified using universal ITS primers. They showed an amplicon in size between 550-600bp. Further characterization is reasonable along with the molecular taxonomy of the endophytic isolates to develop them as a novel source of vinblastine production.

1. INTRODUCTION

Cancer is generally defined as uncontrolled division of cells leading to abnormal growth (defined as cancer) or when they invade the adjoining parts of the body (metastasis).Cancer are generally based on where the tumor is located or where it first started growing in the body. The induction of carcinogenesis is dependent on external and internal factors. The external factors responsible for carcinogenesis are tobacco, obesity, exposure to hazardous chemical, infectious, micro organism while the internal factors include mutations, hormonal and immune disturbances.

The different modalities of cancer treatment are surgery, radiotherapy and chemotherapy. Chemotherapy is the use of drugs or chemicals which inhibit or kill the cancer cell or stop their multiplication cycle. Chemotherapeutic agents may be delivered intramuscularly, intravenously or orally depending upon the type and stage of cancer progression. In recent studies it has been considered that about an estimated 12.7 million new cases of cancer were noticed with approximately 7.6 million deaths only in 2008. Globally, in future by 2030, there may be chances that this number will increase to 22 million. Some studies observed that drugs that were made recently for cancer treatments show nonspecific toxicity to the proliferating normal cells may possess side effects, may not be effective against any type of cancers.

Plants have been an excellent source of chemotherapeutic agent. Recent study revealed that around 47% of total anticancer drugs and 52% of new therapeutic chemicals were introduced into the market by the natural origin (Chin *et al.*, 2006, Newman & Cragg, 2007). Plant-derived compounds have played significant role in the development of clinically useful anticancer drugs. Vinblastine, vincristine, camptothecin (CPT) derivatives, topotecan and irinotecan, etoposide, derived from epipodophyllotoxin, and taxol are some of the medically useful anticancer drugs. Plant-based natural drugs are produced under specific environmental conditions, stress or nutrient availability. It is estimated that One-kilogram paclitaxel is produced after extraction from 10,000 kg bark (Sohn and Okos, 1998). Random collection and cutting down of medicinal plants for extraction of products of interest has led to the extinction of certain number of species making them critically endangered.

Bioactive compounds are useful to provide support and relief in all aspects of the diseased conditions in humans. Degrading environmental continuously, loss in biodiversity, land and water spoilage are upcoming problems that human kind is facing. Endophytes, reside in the tissue of living host, are comparatively understudied and possible source of

2. REVIEW OF LITERATURE

2.1 Vinca alkaloids

Vinca alkaloids are the subset of a class of organic compounds consists of carbon, hydrogen, nitrogen and oxygen and it's derived from plants named alkaloid. The oldest assembly of plant alkaloids are used for treatment of cancers were the vinca alkaloids (Brogan, 2010). They had been used to treat diabetes, high blood pressure and the drugs have even been used as disinfectants. However, the vinca alkaloids are so important for being cancer fighters. There are four major vinca alkaloids used in clinical application- Vinblastine (VBL), vinorelbine (VRL), vincristine and vindesine (VDS). In 2008, research was reported that there was also a new imitation vinca alkaloid, vinflunine that is accepted in Europe for medicinal treatment (Bennouna *et al*., 2008 and Schutz *et al*., 2011).

2.1.1 Vinblastine

Vinblastine was first isolated by Robert Noble and Charles Thomas Beer at the University of Western Ontario from the *Catharanthus roseus* (Owellen *et al*., 1997). Vinblastine was first discovered when it was crushed into a tea. Tea consumption reduced the number of white blood corpuscles. A tea was made by the plant was an ethnic medication for diabetes in earlier times, therefore, it was hypothesized that it might be vinblastine which was effective against cancer of white blood cells such as lymphoma (Thimmaiah and Sethi, 1985). Recently, vinblastine is used for the treatment of large variety of neoplasm and also suggested for the treatment of acute leukemia, Hodgkin's disease and breast and testicular cancer (Miura *et al*., 1987). Guo and Kunming, 1998 reported that vinblastine is produced from *Alternia sp*. isolated from *Cathranthus roseus.*

2.1.2 *Cathranthus roseus*

Catharanthus roseus (L.) which is a significant medicinal plant of the family *Apocynaceae* is used for the treatment of many of the fatal diseases. *C. roseus* which is commonly known as the Madagascar periwinkle and extensively cultivated in north India and its native of Madagascar. the *Catharanthus* alkaloids comprises a group of about 130 terpenoids indole alkaloids (TIAs) (Heijden *et al*., 2004). It was noticed that roots of a plant accumulate ajmalicine and serpentine, important components of medicines and are used for maintaining the high blood pressure and other types of the cardio-vascular diseases. 5

2.1.3 Applications of Vinblastine

2.1.3.1 Anti-cancer agent

It has been reported that for anticancer therapy dynamic mitotic-spindle microtubules are most successful targets. Microtubule-targeted drugs work primarily by increasing or decreasing the cellular microtubule mass. At lower concentrations, microtubule-targeted drugs can suppress microtubule without changing microtubule mass, leads mitotic block and apoptosis. Some microtubule-targeted drugs can act as vascular-targeting agents, swiftly depolymerizing microtubules of newly formed vasculature to cut down the blood supply to tumours (Jordan *et al*., 2004).

2.1.3.2 Antioxidant

Catharanthus roseus (L.) G. Don, a medicinal plant, has an important place in pharmaceutical industry. This plant consist of about 130 terpenoids indole alkaloids (TIA) and, vincristine and vinblastine, are common anticancer drugs. The effect of chromium (Cr) on enzymatic and non-enzymatic antioxidant components on vincristine and vinblastine was studied. These varieties were analyzed under 0, 10, 50, and 100 μM Cr level in order to investigate the plant's protective mechanisms against Cr induced oxidative stress. Vincristine and vinblastine contents were increased under Cr stress condition; this indicates the good antioxidant potential under Cr stress (Rai *et al*., 2014).

2.1.3.3 Anti diabetic activity

The present data evaluate the antidiabetic activity of Vinca rosea, whole plant extracts in alloxan induced diabetic rats for 14 days. At higher dose (500 mg/kg), plant extract exhibited considerable anti-hyperglycemic activity as compared with low doses (300 mg/kg) of extract. The plant extracts showed improvement in body weight and lipid profile as well as regeneration of -cells of pancreas in diabetic rats. Histopathological facts strengthen the healing of pancreas as a possible outcome of their antidiabetic activity by vinca rosea extracts (Ahmed *et al*., 2010).

2.1.3.4 Antibacterial activity

Interpretation of an ethyl methane sulphonate-induced dwarf mutant of *Catharanthus roseus* revealed that the mutant exhibited marked variation in morphometric parameters. In vitro antibacterial activity aqueous and alcoholic leaf extracts was consider against medically 6

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essential bacteria. The mutant extract showed better antibacterial activity against all the tested bacteria except *Bacillus subtilis* (Verma and Singh, 2010).

2.2 Natural sources of Vinblastine

Nature has always kept its basket full of most wonderful gifts for human beings. Nature is a power house of novel bioactive compounds. Natural products are those which are obtained from living organisms like plants, microorganisms (bacteria, fungi) and marine sources etc. throughout the ages, natural products are being employed in the treatment of many kinds of disorders. During the years 1981-2006, approximately 100 anti-cancer agents have been developed, 25% of them were originated from natural products, 9% were the pure natural products, 18% were analogues of natural products, 11% were obtained from a natural product pharmacophore.

Natural Source	Source organism	Reference
Fungi	*Phellinus durrissimus*	Sharma, 1995
	Alternaria sp	Guo and Kunming, 1998
	Fusarium oxysporum	Zhang *et al.*, 2000
	Ganoderma lucidum	Ajith *et al*., 2007
	Pleurotus florida	Ajith *et al*., 2007
	Pleurotus pulmonaris	Ajith *et al*., 2007
	Phellinus rimosus	Ajith *et al.*, 2007
	Fusarium solani	Zhou *et al.*, 2009
Plant	*Vinca rosea*	Johnson *et al.*, 1960
	Catharanthus roseus	Schmeller *et al.*, 1998
		Kumar *et al.*, 2013
	Vinca minor	Yin *et al*., 2011
Yeast	*Saccharomyces cerevisiae*	Brown *et al.*, 2015

Table 1: Natural Sources of Vinblastine

The isolation of the compounds belonging to class vinca alkaloids, Vinblastine and Vincristine, from *Catharanthus roseus* introduced a new era of anticancer drugs. Vinblastine is isolated from various sources ranging from plants to Fungi (Table 1). They were the first anticancer agents in clinical use for the treatment of cancer (Cragg and Newman, 2005). Vinblastine and Vincristine are being used in combination with other anticancer agents for the treatment leukemias, lymphomas, breast and lung cancers, and Kaposi‟s sarcoma (Cragg and Newman, 2005). Most of the synthetic drugs not only kill tumor cells, but also normal cells (Cragg *et al*., 2009). Anticancer drugs derived from the natural sources have proven to be effective and less toxic for cancer therapy (Ma and Wang, 2009; Ravelo *et al*., 2004)

The exploration of secondary metabolites of endophytic fungi has marked its era after the discovery of taxol producing enophytic fungi. Many anticancer agents have been isolated from endophytic fungi. Camptothecin obtained from the endophytic fungus *Fusarium solani* isolated from *Camptotheca acuminata* (Kusari *et al*., 2009) is being used in the treatment of skin diseases (Guo *et al.*, 2008). Ergoflavin, isolated from an endophytic fungi growing in leaves of medicinal plant *Mimusops elengi,* is considered as a novel anticancer agent (Deshmukh *et al.,* 2009). Cochliodinol, an anticancer agent has been obtained from endophytic fungus *Chaetomium* species isolated from stem of *Salvia officinalis* (Debbab *et al.,* 2009). Cytoskyrins, a potential anticancer agent was found to be produced from endophytic fungus *Curvularia lunata* isolated from *Niphates olemda* (Jadulco *et al*., 2002).

2.3 Endophytic fungi

Endophyte alludes to the parasites, yeast and microbes which attack or live inside the tissues of plants without creating any illness or damage to them. They likewise promote development of the host plant and the arrangement of auxiliary metabolites identified with plant defence (Chandra *et al.,* 2010). Endophytes have been found in all parts of plants including xylem and phloem (Petrini, 1986). Endophyte living in the plant host includes persistent metabolic association between the organism and host. Over a long period, endophytic fungi were reported to be possible sources of pharmaceutical lead molecules, and to produce many bioactive secondary metabolites having antimicrobial, anticancer, and antiviral activity .

Endophytes are ubiquitous in nature with rich biodiversity, which have been reported in every plant species examined till date. It is notable that, about 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 revealed that many different types of alkaloids, terpenoids, flavonoids and steroids are produced by endophytic fungi and these metabolites of endophytic 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.

Recent data proved that host plant served as selection system for microbes having secondary metabolites with lower toxicity rate (Strobel, 2003).There are some reports that revealed microbial endophytes has a potential property to mimic the bioactive compounds as produced by the plant itself thus making them a capable source of novel compound. Moreover, biotechnological techniques were used for different microorganism which provide an alternatives for establishing an inexhaustible, reduced time consumption, cost-effective renewable resource of high-rate natural compound (Priti *et al.*, 2009). From medicinal plants or some other plants isolation of endophytic fungi may produce biologically active compounds which is utilized on a large commercial scale because they can be easily cultured in laboratories and instead of harvesting the plants fermentation is used thus affecting the environmental biodiversity

Name of Drug	**Source organism**	**Bioactivity**
Paclitaxel	*Taxus andreanae*	Breast , lung, Prostate cancer
Camptothecin	*Entrophospora infrequens*	Antineoplastic agent
Vincristine	*Fusarium oxysporum*	lymphoma and leukemia
Podophyllotoxin	*Diphylleia sinensis*	Anticancer, antibacterial, immunostimulation
Phenylpropanoids	*Penicillium brasilianum*	anticancer, antioxidant, antimicrobial
Ergoflavin	*Rhinocladiella* sp.	anticancer
Ambuic acid	*Pestalotiopsis microspora*	Antifungal agent
Radicicol	*Chaetomium chiversii*	Cytotoxic activity
Aegiceras corniculatum	*Emericella sp.*	Anti-viral
Javanicin	*Chloridium sp.*	Antibacterial

Table 2: Bioactive compounds isolated from endophytic fungi

The discovery of taxol producing fungi raised the significance of endophytes and their secondary 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, either used alone or in combination with other chemotherapeutic agents for the treatment of a various types of cancers (Croom, 1995). Earlier, it was isolated from the bark of the Pacific yew tree, *Taxus brevifolia* (Wani *et al.*, 1971), because of the limited supply of the drugs alternative sources of these drugs were explored by researchers. 1n 1993, paclitaxel was first time isolated from endophytic fungi isolated from *Taxus brevifolia* (Stierle *et al.*, 1993; Strobel *et al.*, 1993). 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 (Table 2).

2.4 Liquid culture technique for secondary metabolites

Fermentation is the technique of biological conversion of complex substrates into basic compounds by a variety of microorganisms such as bacteria and fungi. Throughout this metabolic breakdown, they likewise release several other compounds apart from the natural products of fermentation; these other 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 referred as "bioactive compounds" since they attain biological activity. Submerged fermentation promotes free flowing liquid substrates, such as molasses and broths. The bioactive compounds are concealed into the fermentation broth. Different media were used for fermentation and it was noted that extracts with higher activity were consistently obtained when the fungi were allowed to grown on nutrient-poor Czapek Dox medium. Nutrient limitations are a distinctive form of stress in fungi, as in other organisms, and the transduction of these stress signals induces a protective reaction to allow survival in a antagonistic environment (Demain, 1986).

2.5 Liquid- liquid extraction

Liquid-liquid extraction involves the separation of substance between two immiscible liquid phases. The two liquid phases are usually, but not all the time, an aqueous solution layer and organic solvent. Most extraction procedure in organic laboratory is carried out by separating funnel. The effectiveness of extraction will rely upon the solubility of the particular compound in the two solvents. The ratio of solubilities is called the Distribution Coefficient (KD). If a compound has a low KD for a given extraction, it is better to opt for a different organic solvent in which the compound is more soluble in order to do liquid-liquid extraction. If this is not reasonable, performing multiple extractions can amplify the amount of compound extracted. Even though water is approximately one of the liquids in the liquid-liquid extraction process, the selection of organic solvent is quite broad. To improvise the extraction of organic compounds from aqueous mixtures, it is applicable to saturate the aqueous phase with salts such as sodium sulphate and calcium chloride.

2.6 Biochemical screening of Endophytic fungi

Biochemical screening is done to measure the presence of alkaloids in a given compound. As it has been reported that vinblastine is a subset of vinca alkaloids and it has a potent property of alkaloids. To detect the presence of alkaloids in a compounds two biochemical assay has been performed. Hager's test is detected by the formation of yellow color ppt. A small amount of sample when mixed with Hager’s regent then the formation of ppt leads to the identification of alkaloids in a particular sample. Similarly, when few drops of sample is mixed with Wagner’s reagent then a yellowish or reddish brown ppt indicates the presence of alkaloids (Kodangala *et al*., 2010).

2.7 Qualitative analysis of endophytic fungi

2.7.1 Thin Layer Chromatography method

Thin Layer Chromatography (TLC) is an easy, rapid, and economical method that gives a fast respond as to how many components are in a mixture. Similar to other chromatographic methods TLC is also based on the principle of separation on the basis of partition coefficient. The separation depends on the relative affinity of compounds towards stationary and mobile phase. The compounds which is under the influence of mobile phase (driven by capillary action) move over the surface of stationary phase. Once separation occurs individual components are visualized as spots at respective level of travel on the plate. Some of the applications of Thin layer chromatography is to verify purity of given samples, identification of compounds like acids, alcohols, proteins, alkaloids, amines, antibiotics etc, to purify samples i.e. for purification process and to keep a confirm on the performance of other separation processes (Cone *et al*., 1963).

It was reported that purity of fungal compound was determined by using solvent system as (a) chloroform: methanol (8:2) (b) chloroform: - methanol (9:1) and (c) ethyl acetate: acetonitrile (8:2) on TLC. Later, the plates were sprayed with ceric ammonium sulphate reagent to produce a brilliant violet color spots. Purity of compound was done by silica gel column chromatography (Kumar *et al.*, 2013).

2.7.2 High-**performance liquid chromatography method**

Chromatography is an analytical practice based on the partition of molecules due to differences in their structure or in composition. High-performance liquid chromatography (HPLC) is a type of liquid chromatography which is used to separate and quantify compounds that have been dissolved in solution. HPLC is also used to conclude the amount of a particular compound in a solution. HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. Stationary phase may be comprised of micron-sized porous particles; a high-pressure pump is necessary to move the mobile phase through the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. Sooner or later, each component elutes from the column as a peak on the data display. Detection of the eluting components is significant, and the method used for detection is dependent upon the detector used. The response of the detector to each component is displayed on a recorder or computer screen and is called as a chromatogram. To collect, store and analyze the chromatographic data, other data-processing equipment are frequently used (Gupta *et al*., 2005).

Purity check of vinblastine and vincristine was checked by HPLC utilizing C18 symmetry column (Waters). Sample for analysis (40 mg) was taken in 40 ml acetonitrile, injected in HPLC section and gradient check was performed by utilizing 5%–95% acetonitrile in water with 0.01% trifluoroacetic acid at flow rate of 0.5 ml/min. A dual wavelength recorder was set at 220 nm and 254 nm and was utilized to detect the compound eluting it from column (Kumar *et al.*, 2013).
In some reported data, for the analysis of compound and RP-18e reversed-phase Chromolith Performance HPLC column were used. During study there is a constant flow rate of 1.2 mL/min. Optimized composition as 21:79 (v/v) acetonitrile–0
.1M phosphate buffer containing 0.5% glacial acetic acid and ph 3.5 maintained at flow rate of 1.2 mL/min,and detection wavelength is at 254nm (Gupta *et al*., 2005).

2.8 Cytotoxicity Assay

Cell lines are populations of pure tumor cells devoid of admixed stromal or inflammatory cells. Normal lung epithelial cell are precious tools for studying the multistage pathogenesis of lung cancers. Two types of normal culture models are presented for studies- primary cultured cells and immortalized cell lines. The major benefit of primary cell models is that it is more close to

the lung tissue origin and thus more is like the lung cell physiology. However, the inter-individual inconsistency, the limited resource and more essentially, the finite life span that does not allow lasting genetic manipulations, make the primary cell model less enviable. The foremost advantage of immortalized non-malignant epithelial lines is the cell lines can be genetically modified in an isogenic system to thoroughly study the genetic modification 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 suitable, modern assays have been optimized for the use of micro titerplates (96-well). This efficiency allows many samples to be analyzed swiftly and simultaneously. Tetrazolium salts (Smith, 1951) are decreased 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 former developed in 1972b DJ Giard through the elimination 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 constantly propagated in vitro for more than 3 years (more than 1,000 cell generations). These cells have a human karyotype and emerge to have been derived from a single parent cell. 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 predictable for cells believed to be responsible for pulmonary surfactant synthesis. The A549 cell line should allow *in vitro* analysis of human surfactant synthesis and secretion and probably provide a source of human surfactant for therapeutic intervention in pulmonary disease states characterized by surfactant deficiency (Lieber *et al*., 1976). XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) has been proposed to substitute 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 concern should be taken when closely related or morphologically alike 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).

3. AIM OF STUDY

1. Isolation, screening and identification of vinblastine producing endophytic fungi from *Catharanthus roseus*.
2. Biochemical and analytical analysis of vinblastine from endophytic fungi.

2.1.3 Applications of Vinblastine

2.1.3.1 Anti-cancer agent

It has been reported that for anticancer therapy dynamic mitotic-spindle microtubules are most successful targets. Microtubule-targeted drugs work primarily by increasing or decreasing the cellular microtubule mass. At lower concentrations, microtubule-targeted drugs can suppress microtubule without changing microtubule mass, leads mitotic block and apoptosis. Some microtubule-targeted drugs can act as vascular-targeting agents, swiftly depolymerizing microtubules of newly formed vasculature to cut down the blood supply to tumours (Jordan *et al*., 2004).

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4. MATERIALS AND METHODS

4.1 Maintenance and sub-culturing of endophytic fungi

The cultures were procured from already maintained repository in laboratory. The maintenance of the cultures involved preparation of Potato dextrose agar (PDA) plates, sub-culturing of cultures and long term preservation.

4.1.1 Preparation of PDA (Potato dextrose agar) plates

39g of PDA was dissolved in 1000 ml double distilled water, stirred to mix properly and was transferred into 250 ml Erlenmeyer flasks and autoclaved at 121°C at 15 psi for 15 min. Aseptically, 25 ml of sterilized PDA was poured into pre-sterilized 90mm glass petri-plates and allowed to solidify at room temperature. The plates were stored in incubator at the temperature 26 ± 2°C until further use.

4.1.2 Sub culturing of Endophytic fungi

A total of 20 endophytic fungi isolated from *Catharanthus roseus* were aseptically sub cultured on PDA plates and incubated at 26°C with 12 h light/dark period for 7-10 days till profuse fungal growth was observed. The loop full of pure culture was aseptically inoculated on to PDA slants containing 10% glycerol for long term preservation at 26 ±2 ºC.

4.2 Production of culture filtrate

The 20 endophytic isolates were subjected to submerged fermentation in Czepak Dox broth (CZD) medium. 5mm mycelial discs of 7 day old culture were inoculated into 100 ml presterilized

CZD broth in Erlenmeyer flasks under aseptic conditions and were incubated in shaker-incubator at 120 rpm, 26°C for 7-10 days. After the incubation period is over, the fungal mycelium was separated from broth through filtration using whattman filter paper followed by centrifugation at 10,000 rpm for 10 min to get cell free culture filtrate. The cell free filtrate was then lyophilized till further use.

4.3 Liquid-Liquid Extraction

The extraction of bioactive residue is the first step in the utilization of various phyto chemicals in the preparation of various neutraceuticals, pharmaceuticals and cosmetic products. Liquid–liquid extraction of the lyophilized culture filtrate was adopted for isolation of the bioactive

fraction.The culture filtrate was extracted using ethyl acetate in the ratio of 1:3 as the solvent system. The organic layer so obtained was pooled and the extraction procedure was repeated thrice.Subsequently, the obtained organic layer was dehydrated using anhydrous sodium sulphate and evaporated to dryness to get crude fraction. The fraction so obtained was weighed, reconstituted in methanol and stored at −20ºC until further use.

4.4 Primary Screening for Vinblastine producing endophytic fungi

The ethyl acetate fractions isolated from different endophytic were then screened for the presence of Vinblastine by adopting specific biochemical assays of vinca alkaloids.

4.4.1 Wagner's Test

It is a specific test to detect the presence of alkaloids (Kodangala *et al*., 2010). Wagner's reagent was prepared by dissolving 1.27 g of iodine and 2 g of potassium iodide in 100 ml distilled water. To the 100 µl of ethyl acetate fraction of each endophytic fungi, 4-5 drops of wagner's reagent was added and observed for the presence of reddish-brown precipitate formation. Vinblastine and methanol was used as positive and negative control respectively.

4.4.2 Hager's Test

Briefly, to the 100 µl of ethyl acetate fraction, 4-5 drops of Hager's reagent (saturated solution of picric acid) was added and observed for the presence of yellow precipitate formation (Kodangala *et al*., 2010). Vinblastine and methanol was used as positive and negative control respectively.

4.5 Thin Layer chromatography (TLC)

The crude ethyl acetate fraction of the positive isolates were subjected to preparative thin layer chromatography (TLC). For the preparation of TLC plates, 20 x 15 x 5 mm glass plates were properly washed and coated with silica gel (Merck) of 0.5 mm thickness. The coated plates were activated by incubating at 100 ºC for 3 h prior to use. The sample and positive control (Vinblastine, Sigma, 1 mg/ml) were spotted on to activated TLC plate just 1 cm above the subordinate edge of plate with the help of capillary tube and allowed to air dry. Simultaneously, the TLC chamber was saturated with different solvent systems (Binary and tertiary) consisting of mixture of solvents of different polarities and ratios for 20 min. The TLC plate was kept in saturated TLC chamber in such a way that the applied spot is above from the solvent level and the plate was allowed to develop. When the solvent front reaches upto the desired level, the TLC

plate was taken out and allowed to air dry. The Chromatogram was developed by keeping the TLC plate in iodine chamber. Vinblastine was used as standard for the comparison of Rf value and alkaloidal spectrum. Retention factor (Rf) value of each band was obtained as the ratio of distance move by solute to that of solvent front

$$\text{Retention Factor } (R_f) = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent front}}$$

4.6 High Performance Liquid Chromatography (HPLC)

The purity and concentration of crude ethyl acetate fraction of selected positive isolate was determined by HPLC (Perkin Elmer- 200 series pump).40 µg of ethyl acetate fraction was dissolved in methanol, injected into the HPLC column and isocratic elution was carried out. Trifluoroacetic acid (0.01%) and acetonitrile in the ratio of 70:30 was used as mobile phase with a flow rate of 1ml/min. To estimate the concentration of fungal Vinblastine, different dilutions of standard vinblastine (Stock- 1 mg/ml, Sigma Aldrich) ranging from 0.01-0.05mg/ml were prepared in HPLC grade Methanol. 20 µl of each dilution was injected into C18 (5 µm) reverse phase discovery column (Sigma Aldrich) and analysed. The data of the peak area vs. concentration of the standard Vinblastine obtained were used to estimate the quantity of fungal vinblastine in crude EA extract.

4.7 Anti-cancer activity of fungal Vinblastine by MTT reduction assay

The crude Ethyl acetate extract of selected endophytic fungi was then tested for its cytotoxicity potential using MTT reduction assay (Mosmann, 1983). A549 lung cancer cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. The cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin and 1ml/L tylosin in humified incubator (5%CO2) at 37 ºC. The adherent cells from were detached by trypsnization from 25cm2 flask and the viable cells were counted by using 0.5% trypan blue dye exclusion method and cells were seeded at a density of ~2 × 104 cell per well in a 96-well microtitre plate. The Microtitre plate was incubated at 37 ºC, 5% CO2 for 24 h. After the incubation is over, the media was removed from each well by aspiration and 40 µg of ethyl acetate extract was added to the respective test wells. The plate was incubated for 72 h at 37 ºC, 5% CO2. After the culmination of incubation period, 20 µL of MTT reagent (5 mg/mL) was added to each well and

again incubated for 4 h. Subsequently, DMSO was added in place of media to dissolve purple colored formazan crystals. The viability of cells was determined by measuring the absorbance at 570nm using Biotek throughput reader, Power wave 340. Vinblastine (Sigma, 1mg/ml)) was used as positive control.

	1	2	3	4	5	6	7	8	9	10	11	12
A	M	M	M	MC	MC	MC	PC	PC	PC	B	B	B
B	S1	S2	S3	S4	S5	S6	S7	S8	B	B	B	B
C	S1	S2	S3	S4	S5	S6	S7	S8	B	B	B	B
D	S1	S2	S3	S4	S5	S6	S7	S8	B	B	B	B
E	B	B	B	B	B	B	B	B	B	B	B	B
F	S1	S2	S3	S4	S5	S6	S7	S8	B	B	B	B
G	S1	S2	S3	S4	S5	S6	S7	S8	B	B	B	B
H	S1	S2	S3	S4	S5	S6	S7	S8	B	B	B	B

Fig no. 1: Showing template for cell cyto-toxicity activity
S1 to S8 refers to different concentrations of ethyl aceate extract of #42CRSTBRT
M refers to the only media + MTT but no cells
MC refers to cells + MTT

The %age cell viability was obtained by the following formula

$$\frac{OD_{Control} - OD_{test}}{OD_{Control}} * 100$$

4.8 Identification of potential endophytic fungi

The selected positive isolate was identified by Classical and Molecular taxonomy tools.

4.8.1 Morphotaxonomy

The potential isolate was visualized under the microscope to identify the culture on the basis of morphological and microscopic characteristics. The culture was grown over different media such as Potato dextrose agar (PDA), Synthetischer nahrstoffarmer agar (SNA), Cathranthus leaf agar (CLA), Cathranthus paste agar for 4–6 weeks at 26 °C. Morphological features such as colony growth rate, color, appearance were critically observed and noted. For Microscopic examination, 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 was placed and teased properly with the needles and 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.

4.8.2 Molecular identification of endophytic fungi

4.8.2.1 Genomic DNA isolation

The genomic DNA isolation of fungal isolate was carried out from 3-4 days old fungal culture

grown on PDA plates using the Wizard® Genomic DNA purification kit (Promega, USA). Briefly, 0.5 -1 g of fungal mycelia was grounded to very fine powder in mortar and pestle using liquid nitrogen. 660-750 µl of the Nuclei Lysis buffer was added to it and again crushed. The contents were transferred to a 1.5ml micro centrifuge tube and vortexed followed by incubation at 65 °C in water bath for 15 min. After the incubation is over, the microcentrifuge tubes were centrifuged at 12,000 rpm for 5 min to remove cell debris. Further 5µl of RNase was added to each tube and incubated at 37°C for 15 min followed by addition of 200 µl of protein precipitation solution. After this, the micro centrifuge tubes were centrifuged at 12,000 rpm for 3 min to remove contaminating protein layer. The aqueous phase containing DNA was transferred to isopropanol containing micro-centrifuge tube and centrifuged at 13,000 rpm for 3 min. The DNA pellet was rinsed with 70% ethanol followed by centrifugation at 13,000 rpm for 1 min. Then pellet was air dried and dissolved in 50 µl of DNA Rehydration buffer (Tris EDTA buffer (pH=8)). The qualitative estimation of the DNA isolated was done by agarose gel electrophoresis.

4.8.2.2 Agarose Gel Electrophoresis

Agarose gel (0.8%) was prepared in 1X TAE (Tris Acetate EDTA) buffer 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 mixed with the 6X loading dye were loaded into wells and allowed to run at 50V for 1h. The DNA fragments were visualized under UV transilluminator. 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 260 nm and 280 nm to determine the concentration and purity of the sample. 1 OD is equivalent to 50µg/ml DNA sample. The concentration of the DNA sample was calculated using following formula

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

The purity of the DNA sample was determined by taking the ratio of absorbance at 260 nm and at 280 nm. 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.

4.8.2.3 PCR amplification, sequencing and Phylogenetic identification

Polymerase chain reaction is a sensitive technique devised by Kary Mullis which is used to amplify number of copies of specific region of DNA. For the identification of fungi, Internal transcribed spacer (ITS) rDNA region is universally accepted. ITS1-5.8S-ITS2 region was amplified by employing primers ITS1 (5ˇTCCGTAGGTGAACCTGCGG3ˇ) and ITS4 (5ˇTCCTCCGCT TATTGATATGC3ˇ) as described by White *et al*., 1990. The primers were

synthesized by Xcelris Labs Ltd., Ahmadabad, Gujarat.

S.no	Reagents	Stock concentration	Quantity	Final concentration/ 25µl
1.	Autoclaved double distilled water	_	15µl	_
2.	Taq buffer	10 X	2.5µl	1X
4.	dNTPs	2.5mM	2.0µl	0.2mM
5.	Forward Primer (ITS1)	10µM	2.0µl	0.8 µM
6.	Reverse Primer (ITS4)	10µM	2.0µl	0.8 µM
6.	Taq DNA Polymerase	5U/µl	1.0 µl	2.5 U
7.	Template DNA	25 ng/µl	1.5 µl	37.5ng

Table 3: Different reagents used during PCR reactions.

Amplification was performed in 25µl reaction mixture (Table 3) containing- 1.5µl of extracted fungal genomic DNA, 0.8µM of each primer (ITS1 and ITS4), 0.2mM of dNTP

(Bangalore GeNei), 2.5U 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 96 ºC for 5 min followed by 39 cycles of 95 ºC for 45 sec, 58 ºC for 60 sec, 72 ºC for 45 sec followed by final extension at 72 ºC for 7 min. The PCR amplicons were resolved by using agarose gel electrophoresis (1.5 % agarose gel dissolved in 1X TAE buffer) at 50 V for 1.30 h. 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). The purified amplicon was sent for direct sequencing to Xcelris Labs Ltd., Ahmadabad, Gujarat.

The obtained chromatograms were manually edited and final sequence was subjected to BLAST analysis to ascertain the positional homology with closely related micro -organism and identification of the potential isolate.

RESULTS:

5.1 Re-culturing

20 Endophytic fungi used in the present study were sub cultured regularly on PDA plates and maintained at 26˚C. For their long term preservation, all the isolates were transferred on to PDA slants containing 10% glycerol and maintained at 26˚C. The endophytic fungi in the present study were isolated from medicinally important plant *Catharanthus roseus* belonging to family *Apocynaceae* collected from the Western Ghats of India. Out of 20 endophytic fungi, 6 isolates were isolated from leaf and 14 from stem of *Catharanthus roseus* (Figure 2, Table 4). Plants have a long history of use in the treatment of cancer. Over 3000 plant species have been reported for the treatment of cancer (Hartwell, 1982). The present study explores the endophytic fungi

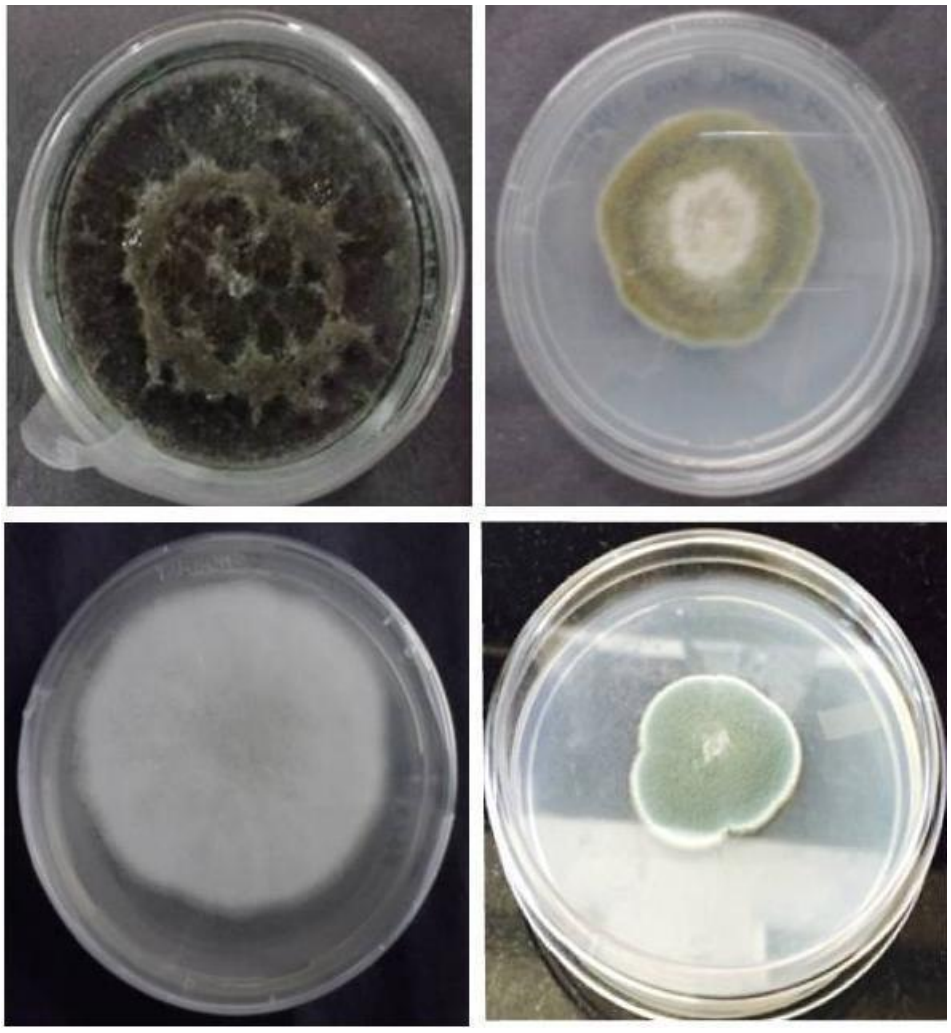


Figure 2 Pure cultures of different endophytic fungi on PDA plates

S. No	Culture Code	Identification	Plant part	Place of sampling	Vinblastine Production	
					Wagner's Test	Haeger's Test
1.	#16CRLBRT	*Fusarium* sp.	Leaf	BRT wildlife sanctuary, Karnataka	-	-
2.	#37CRLBRT	*Xylaria* sp.	Leaf	BRT wildlife sanctuary, Karnataka	-	-
3.	#6CRSTBRT	Unidentified	Stem	BRT wildlife sanctuary, Karnataka	++	++
4.	#15CRSTBRT	*Fusarium* sp.	Stem	BRT wildlife sanctuary, Karnataka	-	-
5.	#12CRSTBRT	*Alternaria* sp.	Stem	BRT wildlife sanctuary, Karnataka	-	-
6.	#17CRSTBRT	*Fusarium* sp.	Stem	BRT wildlife sanctuary, Karnataka	-	-
7.	#17(B)CRSTBRT	*Fusarium* sp.	Stem	BRT wildlife sanctuary, Karnataka	-	-
8.	#21CRSTBRT	Unidentified	Stem	BRT wildlife sanctuary, Karnataka	+	+
9.	#37CRSTBRT	*Xylaria* sp.	Stem	BRT wildlife sanctuary, Karnataka	-	-
10.	#42CRSTBRT	*Fusarium* sp.	Stem	BRT wildlife sanctuary, Karnataka	+++	+++
11.	#43CRSTBRT	Unidentified	Stem	BRT wildlife sanctuary, Karnataka	-	-
12.	#39CRLNEY	Unidentified	Leaf	Neyyar, Kerala	-	-
13.	#10CRSTNEY	Unidentified	Stem	Neyyar, Kerala	-	-
14.	#22CRSTNEY	*Xylaria* sp.	Stem	Neyyar, Kerala	-	-
15.	#39CRSTNEY	*Penicillium* sp.	Stem	Neyyar, Kerala	-	-
16.	#82CRSTNEY	*Alternaria* sp.	Stem	Neyyar, Kerala	+	+
17.	#52CRLYEL	Unidentified	Leaf	Yelandur, Karnataka	-	-
18.	#59CRLYEL	Unidentified	Leaf	Yelandur, Karnataka	-	-
19.	#75CRLYEL	Unidentified	Leaf	Yelandur, Karnataka	-	-
20.	#137CRSTYEL	*Schizophylum* sp.	Stem	Yelandur, Karnataka	-	-

(+++) indicates very good intensity of precipitation, (++) average precipitation, (+) poor precipitation, (-) no precipitation

Table 4:Endophytic isolates used for screening of Vinblastine production

isolated from medicinally important plant, specifically reported for the anticancer bioactive compounds, for the possible development of novel bioactive commodities that can be a potential candidate for the development of anticancer agents.

5.2 Production of culture filtrates and Solvent extraction

The isolates under present study were subjected to secondary metabolites production in liquid medium. Biomass production was considered as a parameter to deduce the growth rate of fungal cultures. #17CRSTBRT was showing the highest and #137CRSTYEL was showing the lowest

S.no	Culture code	Biomass Weight (g)	Volume (ml)	pH
1.	#16CRLBRT	3.78	16	7.01
2.	#37CRLBRT	4.90	12	7.40
3.	#6CRSTBRT	3.75	11	7.35
4.	#15CRSTBRT	3.79	13	5.65
5.	#12CRSTBRT	2.32	15	5.62
6.	#17CRSTBRT	1.80	10	7.02
7.	#17(B)CRSTBRT	2.66	16	7.14
8.	#21CRSTBRT	8.88	13	8.30
9.	#37CRSTBRT	4.32	17	7.01
10.	#42CRSTBRT	5.06	15	7.39
11.	#43CRSTBRT	6.75	13	5.62
12.	#39CRLNEY	3.57	15	8.01
13.	#10CRSTNEY	7.41	11	7.35
14.	#22CRSTNEY	7.54	12	7.31
15.	#39CRSTNEY	6.23	15	8.81
16.	#82CRSTNEY	8.75	12	8.32
17.	#52CRLYEL	2.17	20	7.34
18.	#59CRLYEL	4.09	10	8.05
19.	#75CRLYEL	3.91	11	7.49
20.	#137CRSTYEL	3.92	17	5.42

Table 5: Biomass production of different cultures under study

biomass production. Biomass production of some of fungal cultures ranging from highest to lowest is listed in Table 5. The pH of culture filtrates of most of the isolates were found to be alkaline, the pH range was 5.62 – 8.32. The culture filtrate of #16CRLPAL, #52CRLYEL was found to be acidic i.e. pH- 5.62 followed by #82CRSTNEY with pH- 5.65.

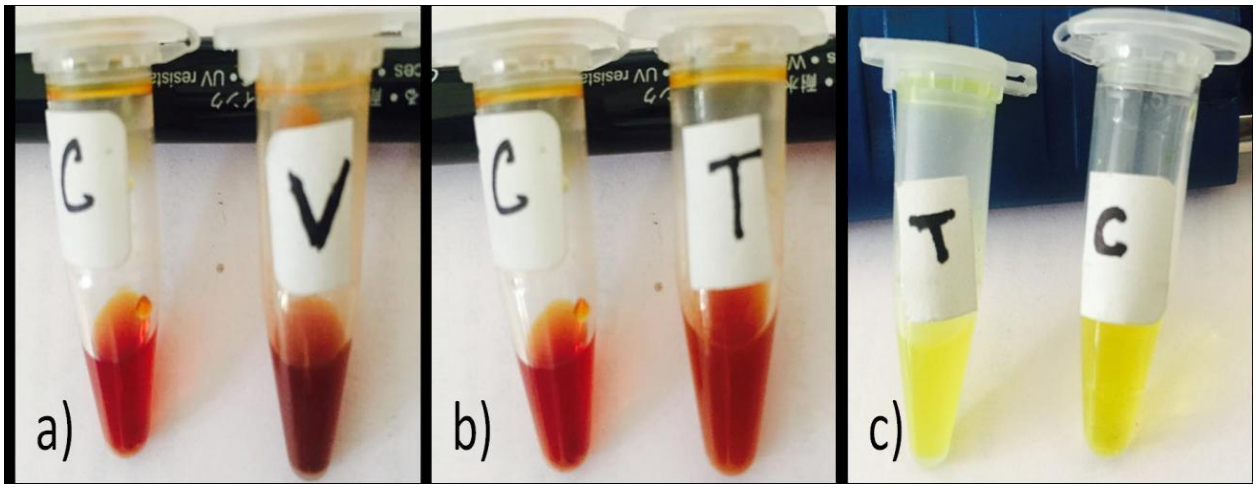
The cell free filtrates of all 20 fungal cultures were subjected to liquid-liquid extraction by ethyl acetate. The bioactive residue was reconstituted in methanol. The maximum yield of the bioactive residue was obtained from #42CRSTBRT (9.9 mg) and was found to be lowest in the case of #82CRSTYEL (1.0 mg) (Table 6). Ethyl acetate extracts of all fungal cultures were subjected to biochemical screening assays.

Culture Code	Yield of crude bioactive residue (mg.)
#42CRSTBRT	9.90
#6CRSTBRT	5.00
#17(B)CRSTBRT	2.10
#21CRSTBRT	2.00
#82CRSTYEL	1.00

Table 6: Yield of bioactive residue of different cultures under study

5.3 Primary screening for Vinblastine producing endophytic fungi

Two biochemical tests, specific for detection of Vinca alkaloids, viz. Wager's test and Hager's test were used for Vinblastine detection in ethyl acetate extracts of all endophytic fungi. Only Figure 3 showing Biochemical tests of #42CRSTBRT a)-b) Reddish brown precipitate formation in Wager's test c) Yellow precipitation in Hager's Test. C- Control (Methanol), V- Vinblastine (Positive control), T- Test (Ethyl acetate extract of #42CRSTBRT)Figure 4: TLC analysis of crude fungal vinblastine along with standard Vinblastine 264 isolates viz. #6CRSTBRT, #21CRSTBRT, #42CRSTBRT and #82CRSTNEY were found to be positive for vinblastine production. In Wager's and Hager's test, the reddish brown and yellow precipitate formation respectively confirms the presence of alkaloidal compounds in sample (Figure 3). Depending upon the intensity of precipitate, #42CRSTBRT was found to be potent producer of Vinblastine followed by #6CRSTBRT, #21 CRSTBRT and #82CRSTBRT. Hence, #42CRSTBRT was selected for further testing.



5.4 Thin Layer chromatography (TLC)

The crude ethyl acetate extract was separated on to different combinations of solvent system as listed in Table 7. The best separation was achieved in Ethyl acetate: Methanol in the ratio of 45:55 which separated the crude fraction into three separate bands of Rf value 0.81, 0.77 and 0.54. The second band was found to have same Rf value to that of standard Vinblastine (Figure 4)

S. No	Solvent system	Ratio	Inference
1.	Ethyl acetate : Hexane	80 : 20	No separation
2.	Ethyl acetate : Methanol	50 : 50	Single band
3.	Hexane: Methanol	50 : 50	No separation
4.	Chloroform : Methanol	80 : 20	No separation
5.	Ethyl acetate : Methanol	45: 55	Three bands

Table 7: showing different solvent systems used for fractionation of crude ethyl acetate extract of #42CRSTBRT

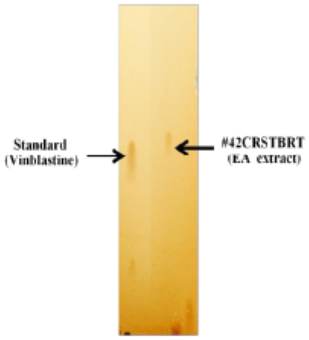
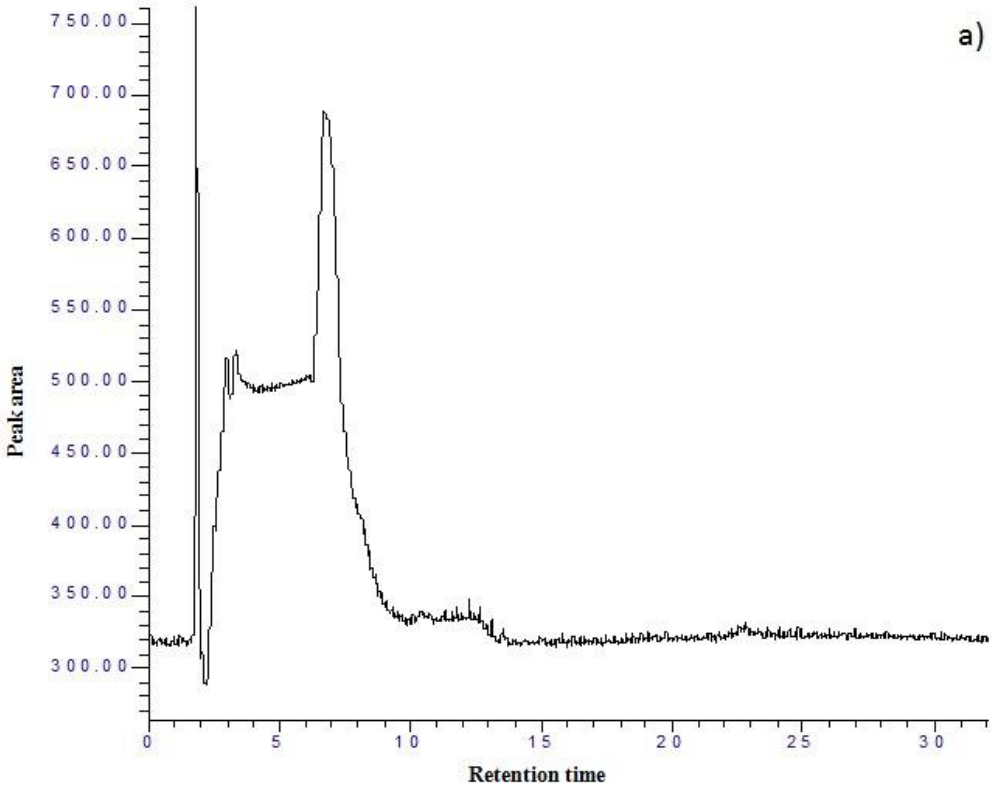


Figure 4: TLC analysis of crude fungal vinblastine along with standard Vinblastine

5.5 High Performance Liquid Chromatography (HPLC)

The concentration of crude fungal Vinblastine was estimated from peak area vs concentration of standard vinblastine which was found to be 9.9 mg. The crude Ethyl acetate extract of #42CRSTBRT showed a peak at retention time of 6.5 min which was at same retention time as that of standard Vinblastine (Figure 5)



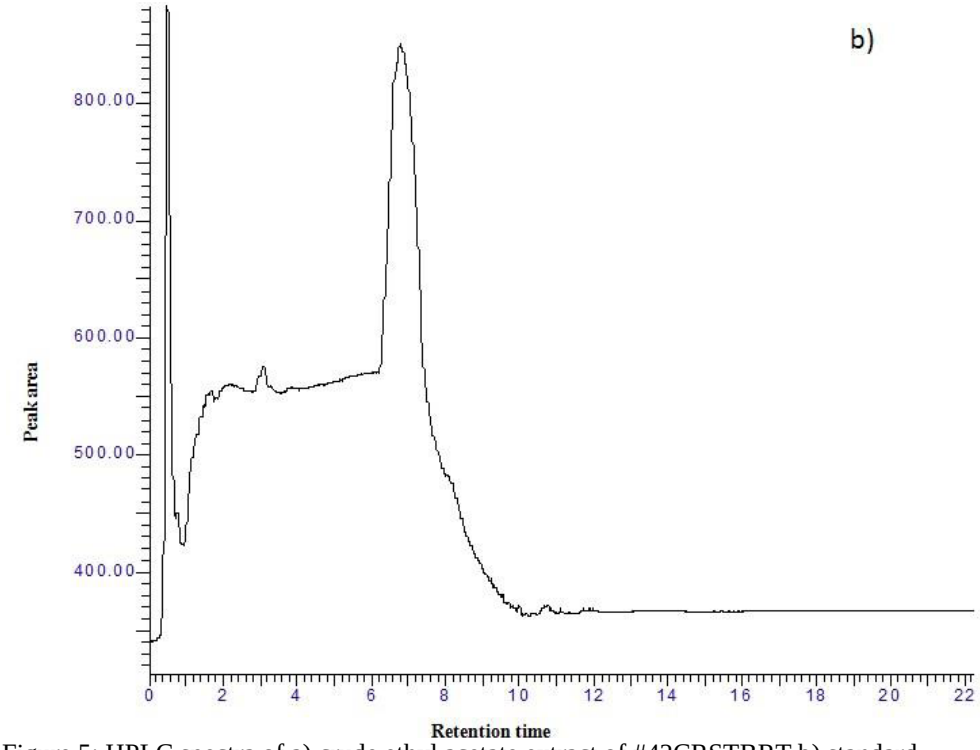


Figure 5: HPLC spectra of a) crude ethyl acetate extract of #42CRSTBRT b) standard Vinblastine

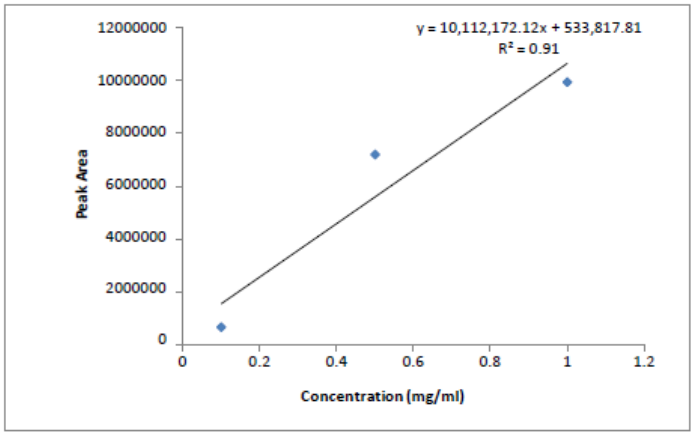


Figure 6: Standard curve of Vinblastine

From the graph the concentration of fungal vinblastine was found to be 0.045mg with retention time 6.71 min and peak area 993404.98µV.s. The retention time of fungal vinblastine was found to be identical to that of pure Vinblastine (Sigma Aldrich).

Conc (mg/mL)	Area of peak (µV.s)	Retention time
0.1	655101.08	6.72
0.5	7191784.97	6.77
1	9934042.78	6.72

Table 8: Peak area and retention time of different concentrations of Vinblastine

5.6 Anticancer activity of Fungal Vinblastine

The crude ethyl acetate extract was further tested for cytotoxicity on A549 cell line by MTT reduction assay. A549 cells are adeno-carcinomic epithelial cells which grow as monolayer as shown in Figure 8. Different concentrations of the crude ethyl acetate extract of #42CRSTBRT ranging from 5µg to 100µg/ml was tested for cytotoxicity assessment on A549 lung cancer cell line. Pure Vinblastine was used as positive control and exhibited 86% cytotoxicity.

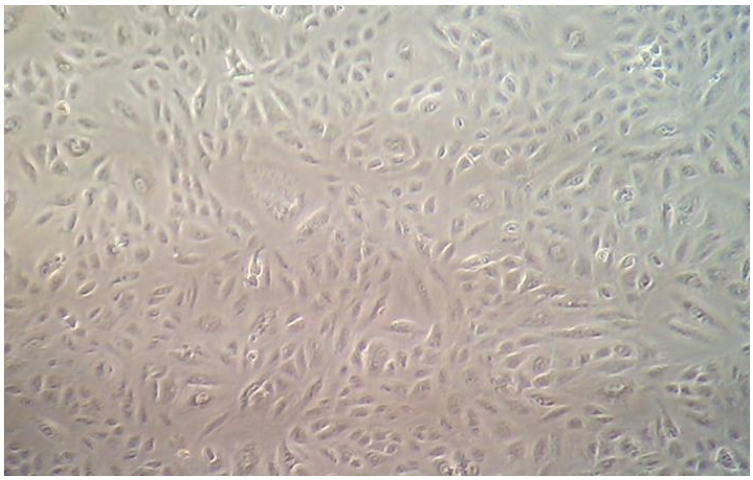


Figure 7: showing A549 epithelial monolayer cells

Pure Vinblastine was used as positive control and exhibited 86% cytotoxicity. The yield ethyl acetate fraction of #42CRSTBRT (1mg/ml) was re-constituted in methanol for the determination of the MIC. The different concentrations prepared for MIC assessment along with their antitumor activity is listed in Table 9, Figure 8

Effective Concentration (µg/ml)	% age cytotoxicity
100	76.4
75	39.1
50	23.2
25	15.0
20	14.1
15	13.7
10	10.7
5	6.5

Table 9: Concentrations and % age cytotoxicity of different concentrations used for determination of MIC

As the concentration of test fraction was increasing, the % age cytotoxicity was also increasing. The highest concentration i.e. 100 µg/well found to possess 76% cytotoxicity. The minimum inhibitory concentration was 5 µg/well as it was showing 7% 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 ethyl acetate fraction of #42CRSTBRT containing vinblastine needs to be purified and characterized for further studies

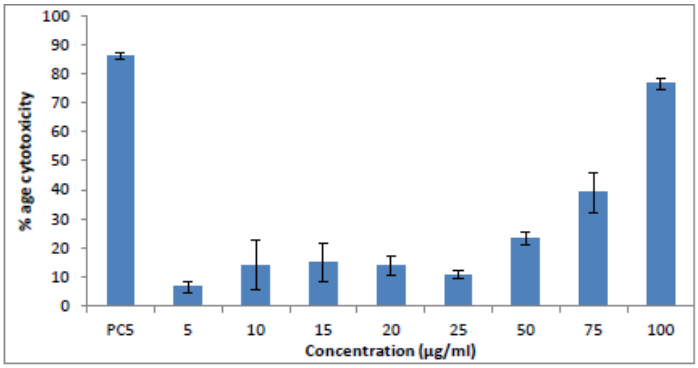


Figure 8: Percentage (%) cytotoxicity of different concentrations of #42CRSTBRT. Vinblastine was used as positive control.

5.7 Identification of Endophytic fungi

5.7.1 Morphotaxonomy

The colony growth rate, appearance etc over different medium are listed in Table 10, Figure 9. Over CPA, Hyphae hyaline, coenocytic, thick and branched. Short and stout phailides are present

over which macroconidia develops. Macroconidia 4-6 celled (Mostly 4 celled), fusiform to ellipsoidal in shape. Over CLA, macroconidia are slightly curved, apical cell conical and basal cell blunt (4-celled). Over SNA

Macroconidia 4-6 celled (mostly 4 celled), apical cell blunt shaped and basal cell extended foot like. Over PDA, Macroconidia 4 celled, apical cell hooked and basal cell blunt in shape.

Colony Characteristics		PDA	CPA	CLA	SNA
Color	Front	White	White	White	White
	Back	Peach	White	White	White
Appearance		Downy	Floccose at the centre and downy at the periphery	Floccose at the centre and downy at the periphery	Floccose at the centre and downy at the periphery
Growth rate		Moderate	Moderate	Moderate	Moderate
Margins		Wavy	Rough	Rough	Rough
Diameter		80mm	72mm	78mm	75mm

Table 10: Colony morphology of *Fusarium* sp., #42CRSTBRT

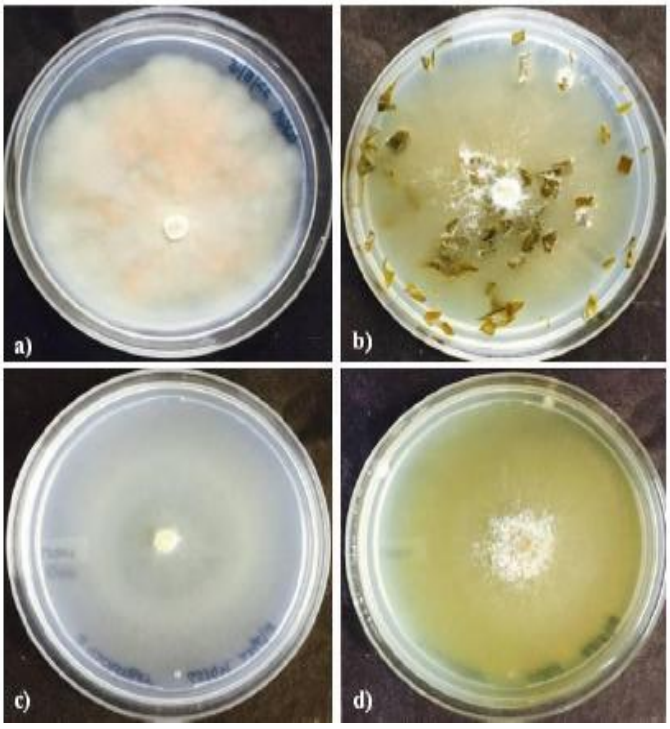


Figure 9: Colony morphology of *Fusarium* sp. (#42CRSBRT) over different media a) Over PDA initially white later turning to peach colored b) Over CLA white colored c) over SNA d) over CPA

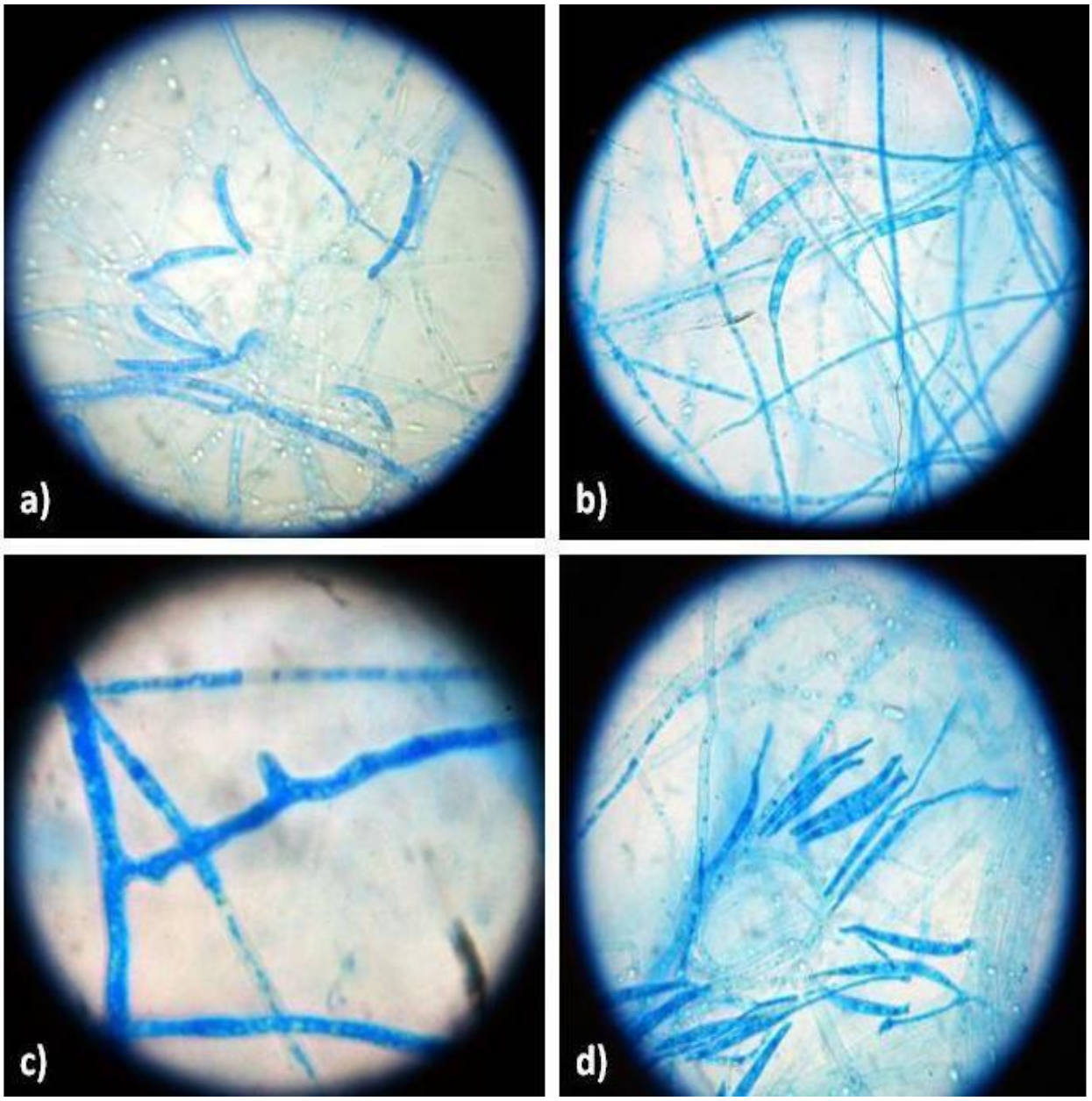


Figure 10: Microscopic features of #42CRSTBRT a,b,d: Sickle shaped macroconidia over various CLA ,CPA and SNA respectively. c) short and stout phailide borne over hyphae.

5.8.2 Molecular identification

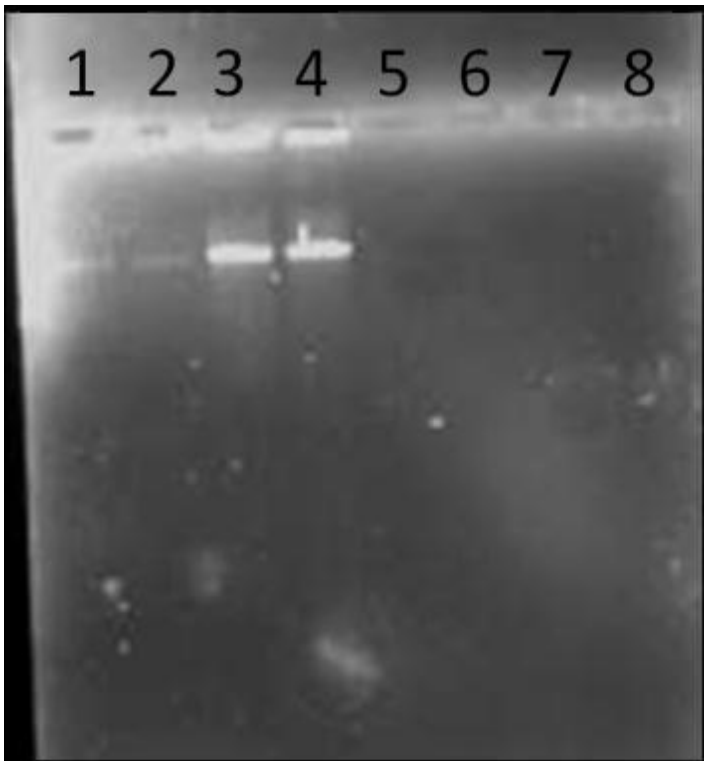
5.8.2.1 Genomic DNA isolation and PCR amplification

The Concentration of genomic DNA (Figure 12a) of #42CRSTBRT was estimated by taking the absorbance at 260 nm and the amount was 50ng/µl by using formula

Concentration (µg/ml) = O.D 260nm x 50 µg/ml x Dilution factor

The PCR amplicon was resolved on 1.5% agarose gel in order to check the size on the basis of the mobility and comparison with the 1000 bp ladder. The size of the amplicon was found to be

approximately 550 bp to 600 bp (Figure 11b). This size can be easily compared to the ITS region, which was amplified in order to characterize the fungi at molecular level.



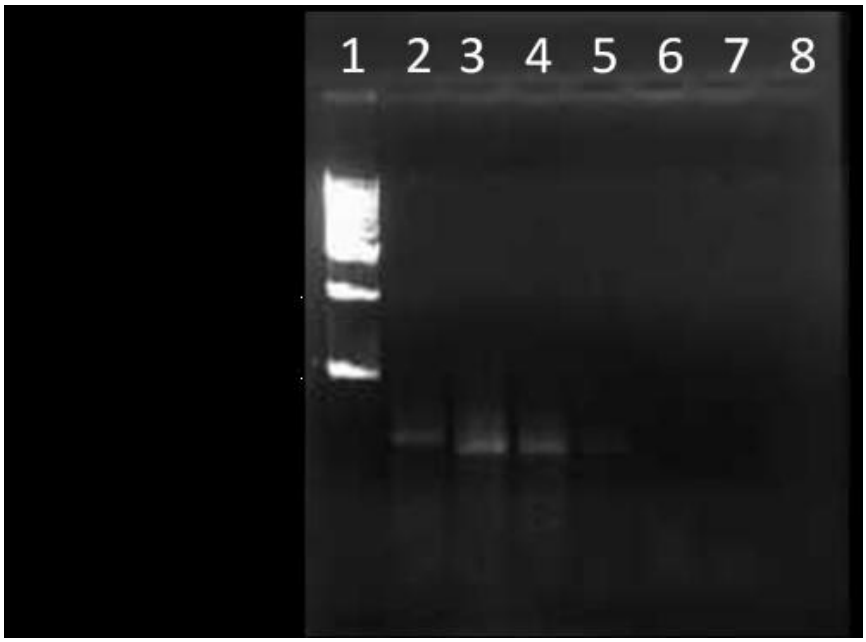


Figure 11 a) Genomic DNA isolation of #42CRSBRT; Lane 1-4: Genomic DNA of

#42JRSTBRT b) PCR amplicon of ITS region of #42CRSTBRT, Lane1- 1000bp DNA ladder Lane 2: ITS amplicon of #42CRSBRT

5.8.2.2 Sequencing and BLAST analysis

The PCR amplicon of ITS region of #42CRSBRT was sent for sequencing and final sequence was subjected to homology analysis by subjecting the final consensus sequence to similarity search by using BLAST. The BLAST analysis showed 96% sequence similarity with *Fusarium equiseti*, *Fusarium incarnatum* and other species of *Fusarium* genus (Table 11).

S.No	Name of Species	Accession Number	Query Coverage	Sequence Identity
1.	*Fusarium equiseti* JH04	JX205167	97%	96%
2.	*Fusarium equiseti* KA	JQ690085	98%	96%
3.	*Fusarium equiseti* T34	FJ459976	97%	96%
4.	*Fusarium sp.* NRRL45996	GQ505760	97%	96%
5.	*Fusarium sp.* NRRL22244	GQ505685	97%	96%
6.	*Fusarium sp.* NRRL13379	GQ505690	97%	96%
7.	*Fusarium equiseti* HO2-7655	EU595566	97%	96%
8.	*Fusarium incarnatum* NJM01770	AY633745	97%	96%
9.	*Fusarium equiseti*	AB425996	97%	96%
10.	*Fusarium oxysporum* A2S3D1	KJ767070	98%	96%
11.	*Fusarium equiseti* NRRL29134	GQ505694	97%	96%
12.	*Fusarium chlyamdosporum*	EU520242	97%	96%
13.	*Fusarium laceratum* NRRL20423	GU505682	97%	96%
15.	*Fusarium oxysporum* FOC156	JN400714	97%	96%

Table 11 BLAST analysis of #42CRSTBRT

For the proper speciation of *Fusarium* sp., MLST approach is to be implemented. Hence, On the basis of morphological as well as molecular taxonomy, the potential isolate #42CRSTBRT was identified as *Fusarium sp.*

6. CONCLUSION

The present study portrays that endophytic organisms are the small scale living organisms which can be turned out to be better source of vinblastine production with effective anticancerous properties. Out of 20 endophytic fungi from medicinal plants of Indian pharmacopeia #42CRSTBRT which has been isolated from *C.roseus* displayed most extreme potential of vinblastine production by both the assays (qualitative and quantitative assays) #42CRSTBRT showed the significant level of cytotoxicity on A549 cell line. Further taxonomic identification of #42CRSTBRT was carried out using microscopic and molecular methods. #42CRSTBRT was identified as *Fusarium sp.*

Further, studies on characterization along with phylogenetic placement of the endophytic isolates would encourage the possibilities for therapeutic employment of the compound as an anticancer drug.

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APPENDIX

MEDIA

1. Czapek Dox Broth

Sucrose – 30 g

$NaNO_3$ - 3 g

K_2HPO_4 - 1 g

KCL - 0.5 g

$MgSO_4.7H_20$- 0.5 g

$FeSO_4.7H_2O$ – 0.01 g

Distilled water – 1 L

Final pH (at 26 ˚C) – 7.0±0.2

Autoclave at 121 ˚C for 15 min

2. Potato Dextrose Agar

Potatoes – 200 g

Dextrose – 20 g

Agar – 15 g

Final pH (at 26 ˚C) – 5.6±0.2

Autoclave at 121 ˚C for 15 min

3. Synthetischer Nahrstoffarmer Agar

KNO_3 – 1 g

K_2HPO_4 – 1 g

KCL – 0.5 g

$MgSO_4.7H_2O$ – 0.5 g

Glucose – 0.2 g

Saccharose – 0.2 g

Agar – 20 g

Distilled water – 1 L

Final pH (at 26 ˚C) – 7.0±0.2

Autoclave at 121 ˚C for 15 min

4. Water Agar

Agar - 20 g

Distilled water – 1 L

Autoclave at 121 ˚C for 15 min

BUFFERS

1. 50X TAE

Tris base - 242 g

Glacical acetic acid - 57.1 ml

0.5 M EDTA - 10 ml

Distilled water – 1 L

2. 1 X TE

Tris -HCL (pH 8.0) - 10 ml

EDTA - 0.1 mM

Distilled water – 100 ml

3. 0.8% Agarose

Agar - 0.8 g

TAE - 100 ml