VINCA ALKALOIDS FROM ENDOPHYTIC FUNGI: ISOLATION, PURIFICATION, CHARACTERIZATION AND BIOASSAYS

Thesis submitted to University of Pune

For the degree of

DOCTOR OF PHILOSOPHY

IN BIOTECHNOLOGY

BY

ASHUTOSH KUMAR

UNDER THE GUIDANCE OF

DR. ABSAR AHMAD

DIVISION OF BIOCHEMICAL SCIENCES

NATIONAL CHEMICAL LABORATORY

PUNE - 411 008,

INDIA

FEBRUARY 2011

Affectionately Dedicated

To

My Beloved Mother

Research is to see what everybody else has seen and to think what nobody else has thought.

-Albert-Szent-Gyorgi (1893-1986) U. S. Biochemist

ACKNOWLEDGEMENT

*The work presented in this thesis would not have been possible without sincere support of my **mother**.*

First and foremost, I would like to extend my sincere gratitude to my research guide Dr. Absar Ahmad for introducing me to this exciting field of science and for his dedicated help, advice, inspiration, encouragement and continuous support, throughout my Ph.D. His enthusiasm, integral view on research and his mission for providing high-quality work, has made a deep impression on me. During our course of interaction during the last six years, I have learnt extensively from him, including how to raise new possibilities, how to regard an old question from a new perspective, how to approach a problem by systematic thinking, data-driven decision making and exploiting serendipity. Every time, I meet him, I can feel "the fire in the belly" which he always talks about. I owe him lots of gratitude for having me shown this way of research. His constant guidance, cooperation, motivation and support have always kept me going ahead. I owe a lot of gratitude to him for always being there for me and I feel privileged to be associated with a person like him during my life. I am really glad to be associated with a person like Dr. Absar Ahmad in my life.

My special words of thanks should also go to my research co-guide late Dr. M.I.Khan for his continuous support, guidance, cooperation, encouragement and for facilitating all the requirements, going out of his way.

I would like to thank our collaborator Dr. P.R.Rajmohanan, Dr. H.B.Borate and Dr. (Mrs). Shanta Kumari for their help during the course of investigation.

I am thankful to, Dr. Vidya Gupta, Dr. C. G. Suresh and Dr. Dhiman Sarkar for their help and cooperation during my tenure as research scholar.

My heartfelt thanks to fellow labmates, Dr.Sreekanth, Asad, Shadab ,Ravi, Imran, Prasad, Sana, Vidyut,Vidya, Ankita,Rusha, Purva and Rasika for always standing by my side and sharing a great relationship as compassionate friends. I will always cherish the warmth shown by them.

I would like to express my deep felt gratitude to my friends Dr.Feroz, Dr.Atul, Dr.Vinod , Dr.Sajid, Dr. Sarvesh, Dr.Shivshankar, Avinash, Ansary, Madhurima, Sonali, Upasna, Sampa, Anamika, Satish, Deepak ,Yogesh, Fazal, Amol, Harish, Manisha, Vitthal, Harish,Urvashi, Mansi, Raju and Ishwar from whom I have received unfailing support and encouragement during my research.

A special mention of thanks to my Trainees in NCL-Vinayak, Rakesh, Leena, Sushmita, Anushree, Swati, Rajashree, Venna, Smita, Yogendra, Sheetal, Aparna, Vidhya, Shanawaz, Hasan, Rusha,Swati, Rajshree, Swapnil, Milind, Nirupma, Neha , Shubhashree , Sujeet, Varsha and many more for their constant support and cooperation during my stay.
A special thanks to Sana, Ankita, Varsha, Shubhashree and Neha for their support during the completion of my work.

My special regards to my teachers because of whose teaching at different stages of education has made it possible for me to see this day. Because of their kindness I was able to reach a stage where I could write this thesis.

I must thank Dr.Sushma Gaikwad, Dr.Sumedha Deshmukh for their kind support throughout my tenure at NCL.

I acknowledge Indira Mohandasan, Giri jee and Trehan jee who were always ready to give their timely help whenever required.

I owe my deepest gratitude towards my beloved ***MOTHER*** *for her eternal support and understanding of my goals and aspirations. Her infallible love and support has always been my strength. Her patience and sacrifice will remain my inspiration throughout my life. Without her help, I would not have been able to complete much of what I have done and become who I am. She had taught me another aspect of life, that, "goodness can never be defied and good human beings can never be denied"*

As always it is impossible to mention everybody who had an impact to this work however there are those whose spiritual support is even more important. I feel a deep sense of gratitude for my Father, who formed part of my vision and taught me good things that really matter in life.

I would be grateful to my better half Mudita, for her love and moral support. I am thankful to my cute son Roshan for giving me happiness during the last two years of my studies.

My heartfelt regards goes to my sisters, brothers, nieces and nephews for their love and affection.

I am also very much grateful to all my family members for their constant inspiration and encouragement.

I also thank Ma'am (Mrs. Absar Ahmad) for her love and care, which never made me feel that I am away from my mother.

I gratefully acknowledge ***Council of Scientific and Industrial Research****, for providing me financial support and* ***Director, NCL*** *to allow me to carry out my research in this esteemed laboratory.*

Finally, I am thankful to library staff and administrative staff of NCL for cooperation.

ASHUTOSH KUMAR

CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Vinca alkaloids from endophytic fungi: Isolation, Purification, Characterization and Bioassays",** submitted by Mr. Ashutosh Kumar, for the Degree of *Doctor of Philosophy*, was carried out by the candidate under my supervision at Division of Biochemical Sciences, National Chemical Laboratory, Pune - 411008, Maharashtra, India.

Date: **Dr. Absar Ahmad**

Place: Pune (Research Guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled **"Vinca alkaloids from endophytic fungi: Isolation, Purification, Characterization and Bioassays",** submitted for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at Division of Biochemical Sciences, National Chemical Laboratory, Pune- 411 008, Maharashtra, India, under the supervision of Dr. Absar Ahmad. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Date: **Ashutosh kumar**

Place: Pune (Research Scholar)

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Chapter 1

General Introduction

Introduction:

Plants, microorganisms and animals have provided to mankind a large variety of biologically active compounds which have found diverse applications in health and curing diseases since the very beginning of human civilization. Initially, plants and other natural sources were the main parts of folk medicines practiced by the ancient man in different parts of the world. They gave rise to the traditional system of medicine. From folk medicine and traditional systems of medicine, medicinal and aromatic plants and other natural products were adopted into the modern system of medicine after they had been found as effective drugs through chemical and pharmacological screening. In the initial stages of development of modern medicine, plants and plant products formed an important part of pharmacopoeia. However, because of significant development in synthetic drug chemistry and antibiotics, there was a certain amount of decline in the use of plants in modern medicine and at one time, one would have thought that ultimately chemist will be able to synthesize all the active constituents of plants which are required by the modern medicine. Since modern medicine has not been able to provide cure to some of the diseases like cancer, AIDS, cardiovascular diseases, arthritis, etc the future of mankind is partially dependent for their health care needs on medicinal and aromatic plants growing on different parts of the world.

Natural product chemistry has witnessed over the years, discoveries of novel molecules by organic chemists, working on different products of metabolism from the biological kingdom. Plants and microorganisms have provided to mankind a large variety of biologically active compounds such as terpenoids, steroids, flavonoids, phenols, quinoles alkaloids and peptides which have found diverse applications in health care needs. In addition to food, clothing and shelter, plants have also provided all the medicaments for man and his domestic animals for thousands of years.

Diversity of Bioactive Metabolites

Plants have provided dyes, perfumes, spices, poisons, cosmetics and several well known medicines such as taxol, etc. Microbes are also known to produce a range of low volume and high value drugs such as hallucinogens, ergot alkaloids and taxol. These compounds are often restricted to a narrow set of species within a phylogenetic group. Thus, they are

species-specific and are not directly referable to the essential metabolic functions associated with growth of the concerned species. In other words, these compounds are "secondary" in origin and distinct from primary products of metabolism responsible for the synthesis of the various macromolecules responsible for the normal growth process. Bu'lock (1961) was the first to recognize the diversity of naturally occurring metabolites in the biological systems.[1] Since these molecules were different and more complex than those involved in primary metabolic pathways, he introduced the term secondary metabolites to characterize them. Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of organisms. They possess highly complex structures arising through the action of multiple enzyme system which are regulated by multiple genes. Unlike primary metabolites, absence of secondary metabolites results not in immediate death, but in long-term impairment of the organism's survivability or aesthetics, or perhaps in no significant change at all. Since the early days of mankind, plants with secondary metabolites have been used by humans to treat infections, health disorders and illness. Only during the last 100 years, have natural products been partly replaced by synthetic drugs, for which plant structures were a lead in many instances (e.g. salicylic acid for aspirin). The use of plant drugs for medical treatment is possible since plants have evolved bioactive secondary metabolites that have been selected during evolution as a means against various diseases. On a global scale, medicinal plants are mainly used as crude drugs and extracts. However, with the discovery of endophytic microbes several of the more potent and active substances are isolated from endophytic fungus.

The function or importance of these compounds to the organism is usually of an ecological nature as they are used as defenses against predators, parasites and diseases, for interspecies competition, and to facilitate the reproductive processes (coloring agents, attractive smells, etc). A number of therapeutically useful low volume and high valued life saving drugs such as taxol, and other bioactive molecules have been isolated from plants, microbes, and plant associated microbes and endophytic fungi.

Bioactive metabolites from plants:

The healing powers of plants or their extracts have been used since ancient times for different ailments and have provided valuable drugs such as analgesics (morphine), antitussives (codeine), antihypertensives (reserpine), cardiotonics (digoxin), antineoplastics (vinblastine and taxol) and antimalarials (quinine and artemisinin) etc. In addition to the above drugs, plants have also provided dyes, perfumes, spices, poisons, cosmetics etc. The total number of natural products produced by plants has been estimated at over 500,000 [2]. Plant cells produce two types of metabolites. Primary metabolites are involved directly in growth and metabolism, viz. carbohydrates, lipids and proteins. Secondary metabolites are considered products of primary metabolism and are generally not involved in metabolic activity viz. alkaloids, phenolics, essential oils, terpenes, sterols, flavonoids, lignins, tannins, etc. These secondary metabolites are the major source of pharmaceuticals, food additives, fragrances and pesticides [3,4,5,6]. Seven plant-derived drugs currently used clinically for various types of cancers are taxol from *Taxus* species, vinblastine and vincristine from *Catharanthus roseus*, topotecan and irinotecan from *Camptotheca accuminata*, and etoposide and teniposide from *Podophyllum peltatum*[6].

Paclitaxel (Taxol), a tubulin binding diterpenoid was first isolated from the pacific Yew tree *Taxus brevifolia*[7]. Because of its ability to bind specifically to B-tubulin and its cytotoxicity at lower concentrations, it is being used for the treatment of several classical tumors. It was approved by USDA for clinical use in ovarian cancer and breast cancer. *Comptotheca acuminate*, a medicinal plant specifically distributed in China is rich in the anticancer compound such as topotecan, irinotecan and camptothecin. The drug mayapple, often called Padophyllum is obtained from rhizomes and roots of *Podophyllum peltatum*. Active constitutents of the plants are present in rhizomes in the form of resin called podophyllin/podophyllotoxin[8]. The important compounds of *Podophyllum peltatum* are etoposide and teniposide. *Calophyllum inophyllum*, a woody plant is medicinally important because of the presence of several classes of bioactive compounds such as anti-human immuno-deficiency virus (HIV) dipyranocoumarins[9]. The

inophyllums and (+) calanolide isolated from *Calophyllum inophyllum* showed strong activity against HIV-1.

Artimisinin, an endoperoxide produced by aerial parts of *Artemisia annua* is an effective against sensitive and multidrug resistant strains of *Plasmodium*, the malarial agent with little or no toxicity to human[10]. *Phyllanthus amarus*, is a potential plant for the treatment of hepatitis B by suppressing the growth and replication of the virus[11]. Phyllanthin and hypophyllanthin present in *Phyllanthus amarus* are also reported as hepatoprotective agents and protect hepatocytes against carbon tetrachloride (CCl4) and galactosamine induced cytotoxicity in rats[12]. Leaves and seeds of *Garcinia dulcis* have been used for the treatment of lymphatitis, parotitis and struma[13].

Opium poppy (*Papaver somniferum*) was one of the most important plants used by the ancient man all over the world. Opium contains more than 2 dozen alkaloids. The main alkaloids present in the opium and used in medicine are morphine, codeine, papaverine and thebaine etc. Egyptian Henbane (*Hyoscyamus muticus*) was used by Egyptians more than 400 years ago. It has been adopted in modern medicine only recently. The drug is obtained from leaves and flowering tops of the plant *Hyoscyamus muticus*. It is mainly used as a raw material for the production of hyoscyamine, hyoscine and atropine. Leaves of corkwood tree *Duboisia myoporoides* contain a number of tropane alkaloids. The most important alkaloids are hyoscine and hyoscyamine. Tobacco alkaloids nicotine and nornicotines are also found. More than 500 alkaloids have been reported from *Rauvolfia serpentina*. The most important alkaloids from this plant which are used in medicine are reserpine, rescinnamine and deserpidine[8].

*Panax quinquefolium (*Ginseng) has been used in Chinese medicine for more than 400 years. Ginseng contains a number of chemical compounds. However, the activity is due to a number of saponins termed ginsenosides. The plant *Dioscorea* contains steroidal sapogenin, diosgenin in their roots. Diosgenin itself is not a medicinal product, but it is one of the most important raw materials used for the synthesis of steroidal drugs, which include corticosteroids, sex hormones, oral contraceptives and anabolic steroids. Senna (*Cassia angustifolia*) leaves and pods contain anthraquinone glycosides, the important ones are sennosides A and B which are used in medicine. The husk of the psyllium seed

(*Plantago ovata*) contains colloidal mucilage mainly consisting of xylose, arabinose, galacturonic acid with rhannose and galactose.

Overexploitation of plants, particularly roots, tubers and bark when used for commercial purposes, particularly drugs has endangered 4,000 to 10,000 species of medicinal plants[14]. Pharmaceutical industries are interested in getting plant based drugs from a microbial source in order to get rid of geographical and political barriers as well as environmental conditions. Thus, from a practical point of view, microbial fermentation as a means of producing drugs or bioactive substances has several advantages.

Bioactive metabolites from microbes:

Microbial sources have proved to be goldmines of bioactive metabolites and with the discovery of therapeutically useful compounds starting with penicillin, the search for diverse novel molecules with biological activity is intensified. It is well known, that actinomycetes (Gram positive bacteria) have proved to be the most versatile group of microorganisms as far as synthesis of secondary metabolites are concerned. In recent years there has been a major focus upon the screening of fungal sources for novel secondary metabolites. Pearce (1997) gave a comprehensive review on bioactive fungal metabolites[15].

Penicillin and cephalosporin are the two most important and widely used antibacterial antibiotics produced by fungi. Recent advances in penicillin therapy including the production of semisynthetic derivatives with wider antibacterial spectra have been reviewed extensively in literature. Penalva *et al*., have reviewed the optimization of penicillin biosynthesis in fungi[16]. Penicillin and other related compounds have been reported from other fungi and this topic has been reviewed in detail by Lechevalier[17]. Presently, a large number of useful antibacterial antibiotics have been discovered from prokaryotes, primarily the actinomycetes. Griseofulvin is a clinically important antibiotic produced by fungi and used in the treatment of fungal infections. The properties, biosynthesis, and fermentation of griseofulvin have been reviewed. It has been used as an effective antibiotic against fungal infections, especially dermatomycosis of animals and humans by oral therapy[18].

Anticancer and antitumor activity of diverse mould metabolites with varying degrees of effectiveness in combating the disease and different levels of toxicity to the normal healthy cells have been reported in literature. Novel cell cycle inhibitors termed tryptostatins have been isolated from *Aspergillus flavus*. While compounds inhibiting metastasis have been identified from the fungus *Nattrassia mangferae*. The enzyme farresyltransferase is involved in the process and the inhibition of this enzyme would be a potential target for anticancer activity. A number of fungal metabolites including gliotoxin, andrastins, kurasoins, and fusidinol have exhibited this activity. Kurasoins are products of *Paecilomyces*[19]. While fusidienol has been isolated from *Fusidium griseum* [20].

Fungi as sources for novel antifungal agents have been screened and among the many compounds isolated from fungi the echinocandins and the pneumocandins are highly potent and promising and are currently in clinical trials. The echinocandins are produced by *Aspergillus nibulans* and are lipopeptides which are inhibitory to B-1, 3 glucan synthesis. This compound has shown activity against both candida and pneumocytis carinii, which is the causal agent of pneumonia in immunocompromised (especially AIDS) patients. Schwartz *et al*., 1989 reported the isolation of a new antifungal lipopeptide similar to echinocandin B from a fungus, *Zalerion arboricola* [21].

One of the interesting developments has been the discovery of fungal compounds which are inhibitors of squalene synthesis and are also potent antifungal agents. Zaragozic acids and squalestatins independently discovered by Glaxo and Merck laboratories are both inhibitors of squalene synthetase and also find application in the lowering of serum cholesterol levels. Bills *et al*., 1994 have given an account of the distribution of zaragozic acids using fungi[22]. As a result of screening fungi for hypocholesterolemic agent, very useful and clinically important drugs have been developed. The most important is Lovastatin which was first reported by Merck from *Aspergillus terreus*.

The best known and widely used immunosuppressive compound of fungal origin is cyclosporine, a metabolic product of *Tolypocladivum inflatum.* Orignally discovered as an antifungal agent from this fungus besides *Cylindrocarpon lucidum*. It was subsequently discovered to have excellent immunosuppressive activity and useful for the

treatment for organ transplants. The cyclosporins represent a group of biologically active secondary metabolites from *Tolypocladivum* as well as other genera such as *Beauveria, Fusarium, Paecilomyces and Verticillium.* Mycophenolic acid is another example of a fungal based immunosuppressant. It was initially discovered from *Penicillium brevicompactum* in 1896 and subsequently found in a number of other penicillia. A metabolite with immunosuppressive activity was discovered from *Isaria sinclarii*.

Search for antiviral activity is planned by understanding the biochemical events that predispose the onset of viral infection. For example, during HIV replication, a series of complex events including specific binding with viral RNA and with viral and host protein is involved. The viral protein has a regulatory role in the transport of viral RNA into host cytoplasm and it is envisaged that blocking this interaction could yield antiviral compounds. Scientists at the Bristol Myers Squibb Laboratories identified from *Trichoderma harzianum* compounds designated Harziphilone and Fleephilone which exhibited such inhibitory activities.

Fungal metabolites active against protozoa, nematodes and insects have received widespread attention and interest. Cures have been sought among microbial metabolites for Protozoan infections like amoebiasis due to *Entamoeba histolyitca* and coccidiosis in chick caused by *Eimeria tenella*. Antiamoebin, a peptide isolated from *Emericellopsis synnematicola* and related species has shown high activity against these pathogens and has been demonstrated to be effective against other protozoan parasites like *Trichomonas vaginalis* and *Trypanosoma evansii*. Tabata *et al,* 1995 isolated Fudecalone from a *Penicillium* sp. which was a terpene type metabolite which was inhibitory to *Eimeria tenella*[23].

Nematode trapping fungi and their possible role in the control of plant pathogenic nematodes destructive to crop plants has also received attention. Predaceous fungi like *Arthrobotrys, Dactyella* and *Dactylaria* have been studied mycologically as possible biocontrol agents. From *Arthrobotrys oligospora*, oligosporons have been identified which are nematicidal metabolites.

Insecticidal metabolites have also been widely studied among which the polyoxins and nikkomycins produced by actinomycetes are useful for control of insects by virtue of their ability to inhibit chitin synthesis. It has been observed that fungal scleroctia which

help the fungi to tide over unfavourable conditions in the natural environment elaborate compounds which are insect repellants or insecticidal. A group of compounds known as Aflavines have been identified from sclerotia of several *Aspergillus* species as well as from the ascostroma of *Eupenicillium crustaceum*. Cyclodepsipeptides which are toxic to insects and other invertebrates have been identified from several entomogenous fungi and include Beauvericin from *Beauveria bassiana*[24]. *Alternaria alternata* produces a metabolite termed Tentoxin and this is a phytotoxin with herbicidal activity. Search for fungal metabolites having vasodilator activity and prevention of platelet aggregation has led to the discovery of some interesting compounds. A new alkaloid, amauromine has been isolated from the culture broth of *Amauroascus* with vasodialating activity.

Many fungi produce toxic compounds which adversely affect human and animal and these toxic metabolites are termed mycotoxins. Aflatoxin produced by *Aspergillus flavus* is one of the most stable carcinogenic compounds produced in groundnut and a variety of other food materials. Other examples of mycotoxins include, Zearalenone from *Fusarium graminearum* which causes vulvovaginitis and infertility in cattle and pigs. Trichothecenes are another group of toxin produced by different species of *Fusarium*. Slatramine is a toxin from *Rhizoctonia leguminicola* causing excessive salivation in cattle referred to as "Slobber Syndrome". Islandicin and Luteoskyrin are carcinogenic toxins from *Penicillium islandicum* that cause hepatitis in humans. The epothilones, isolated from *Myxomycetes* (gliding bacteria) have drawn the eyes of many researchers as a potential anti-tumor agent[25].

Ergot consists of dried sclerotia of the fungus *Claviceps purpurea* parasitic on rye. Although, ergot powder was used to hasten childbirth by midwives for thousands of years, its use in modern medicine was first reported by German physician Lonicer in 1582. The first scientific report of ergot as a cytotoxic agent was made by the American physician Starns in 1808. However, the most important alkaloids, which are therapeutically important, are ergometrine, ergometrinine, ergotamine, ergosine, ergocryptine, ergocrystine, etc. In addition, a number of other alkaloids of minor nature are also present. Ergometrine derivatives are mostly used to stop haemorrhage after childbirth. Ergotamine is used against migraine. A derivative of ergometrine is also used

in migraine. Ergotoxine group of alkaloids in equal parts have been used for controlling essential hypertension and other peripheral disorders[8].

Bioactive metabolites from endophytes

An endophyte is a bacterial or fungal microorganism, which reside inside the healthy tissues of the host plant typically causing no damage to them. Endophytic fungi mimic the chemistry of the respective host plant and produce the same bioactive natural products or derivatives as their host plant[26]. Since the discovery of endophytes in Darnel, Germany, in 1904, various investigators have defined endophytes in different ways, which is usually dependent on the perspective from which the endophytes were being isolated and subsequently examined. Bacon and White gave an inclusive and widely accepted definition of endophytes-"microbes that colonize living, internal tissues of plant without causing any immediate, overt negative effects"[27]. While the symptomless nature of endophytic occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the biodiversity of endophytes suggest that they can also be aggressive endophytes or opportunistic pathogens. Fungi and bacteria are the most common microbes existing as endophytes. Between them fungi are the most commonly isolated one. It turns out that the vast majority of plants have not been studied for their endophytes. Thus enormous opportunities exist for recovery of novel fungal forms, taxa and biotypes. It is estimated that there may be as many as 1 million different fungal species, yet only about 100,000 have been described[28]. It seems obvious that endophytes are a rich and reliable source of genetic diversity and novel, undescribed species.

Almost all vascular plant species examined to date were found to harbor endophytic bacteria or fungi[29]. Moreover, the colonization of endophytes in marine algae, mosses and ferns has been recorded. As a matter of fact, endophytes are important components of microbial diversity[30]. Commonly, several to thousands of endophyte species can be isolated from a single plant, and amongst them, at least one species showing specificity. The environmental conditions under which the host is growing, also effect the endophytic population and the endophyte population can be more diversified in tropical areas. Moreover, genotypic diversity has been observed in single endophytic species originating

from conifers, birch and grasses. Accordingly, endophytes are ubiquitous in the plant kingdom with the population being dependent on host species and location.

Some phytopathogens in the environment are of endophyte origins[31]. During the long co-evolution of the phytopathogen and its host plant, an endophytic mutant may result from balanced antagonism and/or gene mutation. Dual cultures of the host calli and endophytes demonstrated that both the endophytes and the host calli excrete metabolites toxic to each other[32]. Further investigation led to the development of a hypothesis that the endophyte–host interaction could be a balanced pathogen–host antagonism. Freeman and Rodriguez[33] found that a naturally occurring nonpathogenic endophytic mutant developed from the mutation of a single locus in the genome of the wild-type *Colletotrichum magna*, a pathogen causing anthracnose in cucurbit plants. This mutant is able to grow systemically inside the host plant without pathogenic symptoms, but retaining wild-type levels of *in vitro* sporulation, spore adhesion, appressoria formation, infection and host specificity. The *Acremonium* (asexual fungi now reclassified in the genus *Neotyphodium* Glenn, Bacon and Hanlin)[34] endophytes, which usually inhabit tall fescue, perennial ryegrass (*Lolium perenne* L.), and many cool-season grasses, are considered mutualistic symbionts of the host grasses. The grass and the endophytic fungus are so intimately associated that they act 'as a whole', much like 'a single organism'. And, indeed, some of these endophytic *Neotyphodium* species can only spread by infecting seeds from the mother plants[35]. Endophytes colonizing inside plant tissues usually get nutrition and protection from the host plant. In return, they confer profoundly enhanced fitness to the host plants by producing certain fungal metabolites. Endophyte infected plants often grow faster than non-infected ones[36]. This effect is at least in part due to the endophytes production of phytohormones such as indole-3-acetic acid (IAA), cytokines, and other plant growth-promoting substances and/or partly owing to the fact that endophytes could enhance the uptake of nutritional elements such as nitrogen and phosphorous[37].

Certain endophytes improve the ecological adaptability of hosts by enhancing their tolerance to environmental stresses and resistance to phytopathogens and/or herbivores including some insects feeding on host plant. Endophyte infected grasses posses an increased tolerance to drought and aluminium toxicity[38]. Furthermore, some endophytes are able to provide the host plant with protection against some nematodes[39], mammals[40]

and insect herbivores[41] as well as bacterial and fungal pathogens. Some endophytes are capable of enhancing the hosts allelopathic effects on other species co-growing nearby, usually being competitor for the nutrition and the space. This could be the reason why some plants with special endophytes are usually competitive enough to become dominant species in successional fields[42].

Biodiversity and Endophytes

Of the myriad of ecosystems on earth, those having the greatest biodiversity seem to be the ones having endophytes with the greatest number and the most biodiverse microorganisms. Tropical and temperate rainforests are the most biologically diverse terrestrial ecosystems on earth. The most threatened of these spots cover only 1.44% of the land's surface, yet they harbor more than 60% of the world's terrestrial biodiversity. As such, one would expect that areas of high plant endemicity also possess specific endophytes that may have evolved with the endemic plant species. Ultimately, biological diversity implies chemical diversity because of the constant chemical innovation that exists in ecosystems where the evolutionary race to survive is most active. Tropical rainforests are a remarkable example of this type of environment. Competition is great, resources are limited, and selection pressure is at its peak. This gives rise to a high probability that rainforests are a source of novel molecular structures and biologically active compounds[43]. Bills *et al*[22] describe a metabolic distinction between tropical and temperate endophytes through statistical data which compares the number of bioactive natural products isolated from endophytes of tropical regions to the number of those isolated from endophytes of temperate origin. Not only did they find that tropical endophytes provide more active natural products than temperate endophytes, but they also noted that a significantly higher number of tropical endophytes produced a larger number of active secondary metabolites than did fungi from other tropical substrata. This observation suggests the importance of the host plant in influencing the general metabolism of endophytic microbes.

Isolation of Endophytes

Plant materials are thoroughly washed and surface sterilized with different surface sterilizing agents such as mercuric chloride ($HgCl_2$), ethanol etc. in order to remove epiphytic microbes and then cut into small pieces and placed on PDA plate. After several days of incubation, hyphal tips of the fungi are removed and transferred to PDA slants and then screened for bioactive metabolites. Isolations of these fungi from surface sterilized tissues were utilized to determine their epiphytic or endophytic nature[44].Because of the slow growth of the endophytic fungi, major difficulties are encountered in isolation and purification of these cultures for extended periods as required in most of the isolations. Several different stains have been used to detect these fungi in grasses. Epidermal peels from leaf sheaths, pith scrapings, stem and seeds have been examined by Sampson[45] used cotton blue or gentian violet followed by Gram's iodine solution. Other investigators used lactophenol cottonblue[46, 47], lactophenol trypan blue[48] and aniline blue[49, 50] for staining mycelium. Clark *et al*. soaked the seeds with aniline blue lactic acid[51]. Enzyme-linked immunosorbent assay (ELISA) was used recently to detect the presence of these fungi in seeds or plant tissues[52, 53]. Saha *et al*., developed a rapid staining method of these endophytic fungi using rose bengal stains[54]. Rose Bengal was an improvement over trypan blue because it was extremely quick and safe. A number of bioactive metabolites have been isolated from endophytic fungi.

Alkaloids from Endophytes

Endophytic fungi produce a variety of clavine and peptide alkaloids and several other biologically active compounds both *in vivo* and *in vitro* similar to those produced by the ergot fungus *Claviceps* sp. In *Claviceps*, ergot alkaloids are confined to sclerotium. In contrast, the alkaloids can be isolated from whole parts of the infected plant. Hardy *et al*., 1986 and Lyons *et al*., found that the presence of alkaloids in infected plants varied in different plant parts (leaf blades, sheaths,flowering culms) at different times of the year and alkaloid concentration were higher in young than old leaves and leaf sheaths than in leaf blades in case of plants infected with endophytes[55-58]. Bacon *et al.,* for the first time demonstrated the endophytic fungus of grass grown in synthetic medium produced precursors of auxins *in vitro*[59]. *In vitro* auxin production by Porter *et al*. [60].Besides, a variety of biologically active compounds viz. simple indoles, lysergic acid amide, prenylated indoles, lolitriem, C_{28} sterols, pyrrolizidine alkaloids, amino acid derivatives are also known from these fungi both *in vitro* and *in vivo* [26].

Antibiotics from Endophytes:

A unique peptide antimycotic, termed cryptocandin, ambuic acid, jesterone, phomopsichalasin have been isolated from endophytic fungus *Cryptosporoipsis quercina Pestalotiopsis microspora, Pestalotiopsis jesteri*, *Phomopsis* sp., respectively[61-63]. Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*, displays antimicrobial activity against bacteria as well as against the fungus *Helminthsporium sativum* [64].

Antiviral compounds from Endophytes:

Two novel fascinating human cytomegalovirus protease inhibitors, cytonic acids A and B have been isolated from the solid-state fermentation of the endophytic fungus *Cytonaema* sp. It is apparent that the potential for the discovery of compounds, from endophytes, having antiviral activity is in its infancy. However, some compounds that have been found are promising[65].

Volatile Antibiotics from Endophytes:

Muscodor albus is a newly described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree)[66]. This fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds (Gary A. Strobel *et al.*, 2001). The ecological implications and potential practical benefits of the "mycofumigation" effects of *M. albus* are very promising. The potential use of mycofumigation to treat soil, seeds, and plants may soon be a reality. In fact, this organism is already on the market for the decontamination of human wastes. Using *M. albus* as a screening tool, it has now been possible to isolate other endophtyic fungi that produce volatile antibiotics[67].

Anticancer drugs from Endophytes:

Recent interest has focused on the study of endophytic and bark associated fungi from medicinal plants. One of the most significant discoveries in this area has been the isolation of an endophytic fungus, *Taxomyces andreanae* from the bark of Pacific yew (*Taxus brevifolia*) which has been shown to produce taxol, the antitumor diterpenoid produced by the *Taxus* plant [68]. The concept has been gaining ground since this discovery that the endophytic fungi associated with medicinal plants may have the potential to acquire the capability to produce the valuable metabolites of medicinal value from the parent plant and such fungi could become independent alternative sources for these metabolites which can be produced by fermentation. This opens a tremendous new area for research and development, particularly for screening the innumerable medicinal plants of indigenous origin for endophytes and evaluating them for their potential to manufacture the valuable compounds of plant origin. An endophytic fungus, *Gliocladium* sp. was found to produce taxol and 10-DABIII (10-deacteylbacctain III). These compounds were purified by TLC and HPLC and characterized using UV spectroscopy, ESI-MS, MS/MS and proton NMR. One litre of the *Gliocladium* sp. yielded 10 µg of Taxol and 65 µg of 10-DAB III[69]. The isolation of Taxol-producing endophyte *Taxomyces andreanae* has provided an alternative approach to obtain a cheaper and more available product *via* microorganism fermentation. After that, Taxol has also been found in a number of different genera of fungal endophytes either associated or not to yews,

such as *Taxodium distichum*[70]; *Wollemia nobilis* [71]*; Phyllosticta spinarum* [72]; *Bartalinia robillardoides* [73]; *Pestalotiopsis terminaliae* [74]; *Botryodiplodia theobromae* [75].

Another important anticancer compound is the alkaloid Camptothecin, a potent anti-neoplastic agent which was firstly isolated from wood of *Camptotheca acuminata.* Kusari *et al* isolated endophytic fungus from *Camptotheca acuminata* that produces Camptothecin and analogues. Similarly, Podophylotoxins were reported from fungus *Phialocephala fortinii* isolated from *Podophyllum peltatum*. Podophylotoxin and analogues are clinically relevant mainly due to their cytotoxicity and antiviral activities and are valued as a precursor to useful anticancer drugs like etoposide, teniposide, and etopophos phosphate[76,77]. "Phenylpropanoids" have attracted much interest for medicinal use as anticancer, antioxidant, antimicrobial, anti-inflammatory, and immunosuppressive properties[78]. Despite the phenylpropanoids belong to the largest group of secondary metabolites produced by plants, reports showed the production of such compounds by endophytes. The endophytic *Penicillium brasilianum*, found in root bark of *Melia azedarach*, promoted the biosynthesis of phenylpropanoid amides[79]. Wagenaar and co-workers reported identification of three novel "cytochalasins", bearing antitumor activity from the endophyte *Rhinocladiella* sp.[80].

Antioxidants from endophytes

Two compounds, pestacin and isopestacin have been obtained from culture fluids of *P. microspora*, an endophyte isolated from combretaceous plant, *Terminalia morobenesis*. Both pestacin and isopestacin display antimicrobial as well as antioxidant activity[81]. Isopestacin was suspected of antioxidant activity based on its structural similarity to the flavanoids. Electron-spin resonance spectroscopy measurements confirm its anti-oxidant activity; the compound is able to scavenge super oxides and hydroxyl free radicals in solution. Pestacin was later described from the same culture fluid occurring naturally as a racemic mixture and also possessing potent antioxidant activity.

Insecticides from Endophytes:

Several endophytes are known to have anti-insect properties. Nodulisporic acids, novel indole diterpenes that exhibit potent insecticidal properties against the larvae of the blowfly, work by activating insect glutamate-gated chloride channels. The first nodulisporic compounds were isolated from an endophyte, a *Nodulisporium* sp., from the plant *Bontia daphnoides*. This discovery has since resulted in an intensive search for more *Nodulisporium* spp. or other producers of more-potent nodulisporic acid analogues[82]. Another endophytic fungus, *Muscodor vitigenus*, isolated from a liana (*Paullina paullinioides*), yields naphthalene as its major product. Naphthalene, the active ingredient in common mothballs, is a widely exploited insect repellant.

Antidiabetic agents from Endophytes

The action of insulin is initiated by its binding to the insulin receptor (IR), a disulfide-bonded heterotetrameric membrane protein. A nonpeptidal fungal metabolite (L-783,281) was isolated from an endophytic fungus (*Pseudomassaria* sp.)[83]. This compound acts as insulin mimetic and, unlike insulin, is not destroyed in the digestive tract and may be given orally.

Immunosuppressive compounds from Endophytes

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients, and in the future they could be used to treat autoimmune diseases such a rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinol A and B[84].

The Microbiology Department at Sandoz Ltd. developed a computer-aided evaluation program to screen and evaluate fungi for bioactivity. The program can recognize and eliminate from study, the common fungi producing known compounds and thereby direct attention to the evaluation of rare samples, which are more likely to produce metabolites with novel bioactivity. This approach resulted in the discovery of the fungus *Tolypocladium inflatum*, from which cyclosporine, a hugely beneficial immunosuppressant, was isolated[85]. This example perfectly depicts the current aim of

many investigators to seek out rare endophytes from interesting and uncommon hosts and environments.

Nanosystems in Cancer

Cancer is the second leading cause of death next to cardiovascular diseases. An uncontrolled growth and multiplication of cells resulting into harmful tumor is called cancer. Cancer is characterized by the ability to invade adjacent tissues and even distant organs. It causes loss of cell differentiation, forms tumors, and interferes with normal body functions resulting in the death of affected persons if diagnosed very late. A tumor, caused by cancer is an abnormal mass of tissue formed as a result of excessive, uncoordinated, autonomous and purposeless proliferation of cells. There are two types of tumors, benign (eg. brain tumor) and malignant (breast cancer) tumor. Cancers are classified according to embryonic origin of the tissue from which the tumor is derived such as carcinomas, sarcoma, lymphomas, and leukemia. The environmental agents that cause cancer are known as carcinogens. It is not exactly known how a normal cell is converted into cancerous cell. The carcinogenic agents are responsible for causing malignant changes in the cell. There are different types of carcinogenic agents such as chemical carcinogens, radiations, biological agents, dietary factors, genetic factors and also cancers due to habits and customs. Cancer is being treated by surgery, radiation therapy, (such as gamma rays, x-rays, cobalt-60 and radium), chemotherapy (such as alkylating agents, anti-metabolites, antitumor antibiotic and plant alkaloids), immunotherapy. Mechanistic aspect of cancer and its therapy has been critically reviewed by Ji Luo *et al.* and Boyle and Costello [86, 87].

Nanomaterials of different chemical compositions, such as liposomes, micelles or inorganic nanoparticles hold tremendous potential as carriers for drugs to target cancer cells. Several anti-cancer drugs including paclitaxel [88, 89], doxorubicin [90], 5-fluorouracil [91] and dexamethasone[92] have been successfully formulated using nanomaterials. Polylactic/glycolic acid (PLGA) and polylactic acid (PLA) based nanoparticles have been formulated to encapsulate chemotherapeutic agent, dexamethasone [92]. The effectiveness of drug delivery systems can be attributed to their small size, reduced drug toxicity,

controlled time release of the drug and modification of drug pharmacokinetics and biological distribution. Too often, chemotherapy fails to cure cancer because some tumor cells develop resistance to multiple anticancer drugs. In most cases, resistance develops when cancer cells begin expressing a protein, known as p-glycoprotein that is capable of pumping anticancer drugs out of a cell as quickly as they cross through the cell's outer membrane. New research shows that nanoparticles may be able to get anticancer drugs into cells without triggering the p-glycoprotein pump [89, 93].

Targeting cancer cells with nanoparticles

Cancer has remained the most dreadful disease till date. A variety of chemotherapeutics are available to deal with a variety of cancers. However, use of chemotherapy to target cancer not only kills cancer but also targets normal dividing cells of the body. Moreover, even the most potent therapeutic is rendered useless owing to its failure to reach the therapeutic target *in vivo*. Therefore, the need is to focus the research on targeted drug delivery which would only deliver the chemothereapeutics to cancerous cells while sparing the normal cells.

Recently researchers have started exploiting receptor mediated endocytosis to achieve a target specific drug delivery where the therapeutics can be targeted to the diseased tissue with the use of strategy of coupling therapeutics to certain molecules which have recognition by specific receptors present on the target tissue. Today with tremendous research being carried out on cancer, it has become evident that most cancers and their metastases over – express certain receptors, which act as potential drug target for cancer therapy. High levels of αhFR (folate receptors) are over expressed on a variety of human cancers like ovarian, breast, brain, lung and colorectal cancers but are restricted in normal tissues[94]. Internalization of folate hFR involves receptor mediated endocytosis. Consequently, several strategies have been developed for the targeted drug delivery or drugs to hFR - positive tumor cells. Covalent attachment of therapeutic agents to hFR targeted monoclonal antibodies have shown significant targeting efficiency in patients with ovarian cancer. Alternatively, folate derivatized anticancer treatments have been successfully applied *in vitro* for hFR-specific delivery. Evidence for overexpression of

transferrin (a plasma glycoprotein) receptor on tumor cells including oral, prostrate and breast cancer cells implicate its role in target- specific delivery.

Lately, a new therapy called localized hyperthermia (also called local thermal therapy or thermotherapy) has shown promise in killing cancer cells. Hyperthermia is the application of concentrated therapeutic heat generated to treat cancer, which implies use of super paramagnetic iron oxide nanoparticles. In hyperthermia, desired body tissue is implanted with super paramagnetic iron oxide nanoparticles which are then super heated to high temperature (45^{o}C) by an external magnetic field (magnetic field hyperthermia). High temperature can damage and kill cancer cells, usually with minimal injury to normal tissues. In normal tissues, blood vessels dilate on heating. Unlike healthy cell, a tumor is a tightly packed group of cells in which circulation restricted and sluggish. When heat is applied to the tumor, vital nutrients and oxygen are cut off from tumor cells resulting in cell death owing to collapse of tumors vasculature. The super paramagnetic iron oxide nanoparticles used for hyperthermia are biodegradable and biocompatible and enhance the sensitivity of magnetic resonance imaging[95]. It has been observed that after intravenous injection, most super paramagnetic iron oxide nanoparticles accumulate in kuffer cells in the liver and in the reticuloendothelial system in the spleen. After being metabolized by cells, the iron is introduced in the normal plasma iron pool and can be incorporated into the hemoglobin of red cells and used in other metabolic processes. Dendrimer-coated super paramagnetic iron oxide nanoparticles (magneto dendrimers) have been used for cellular labeling.While ferum oxide, a dextran coated super paramagnetic iron oxide nanoparticles (FDA approved) have been used as MRI contrast agent for hepatic imaging.

Targeting angiogenesis with nanoparticles

Robust angiogenesis underlies aggressive growth of tumors. Therefore, one of the mechanisms to inhibit angiogenesis is to starve tumor cells. Angiogenesis is regulated through a complex set of mediators and recent evidence shows that integrin αvβ3 and vascular endothelial growth factors (VEGFs) play important regulatory roles. Therefore, selective targeting of αvβ3 integrin and VEGFs is a novel anti-angiogenesis strategy for treating a wide variety of solid tumors. One approach is to coat nanoparticles with

peptides that bind specifically to the αvβ3 integrin and the VEGF receptor[96]. The synthetic peptide bearing Arg-Gly-Asp (RGD) sequence is known to specifically bind to the αvβ3 integrin expressed on endothelial cells in the angiogenic blood vessels, which can potentially inhibit the tumor growth and proliferation. Following hydrophobic modifications, glycol chitosan is capable of forming self-aggregated nanotube and has been used as a carrier for the RGD peptide, labelled with fluoresein isothiocyanate (FITC-GRGDS) [97].

Scientists at National Chemical Laboratory, Pune, India addressed the use of microorganisms such as fungi and plant extracts in the synthesis of nanomaterials, over a range of chemical compositions that includes metals, semi-conductors (quantum dots), alloys, oxides and bio-minerals. This opens up the exciting possibility of developing chemically and physically hard to synthesize inorganic nanomaterial such as oxide nanoparticles[98-101]. These oxide nanoparticles are important in applications such as hypothermia, drug delivery, targeted delivery etc. and are conventionally synthesized under harsh environments like extremes of temperature, pressure and pH. In contrast, biological processes occur under ambient conditions with room temperature, atmospheric pressure and physiological pH. Ansari *et al* synthesized CdS nanoparticles by enzymatic route and then coupled the nanoparticle with lectin[102].

SUPPLY

The productivity of vinblastine and vincristine is very low in plants (0.001-0.0003%) resulting in their extraordinary high price. Vinblastine is a dimeric indole alkaloid and is formed by coupling of vindoline and catharanthine catalysed by horseradish peroxidase[103]. The yield of coupling products, (15' 20'- anhydro vinblastine) was reported very low (0.9%). Vinblastine is converted into vincristine by the oxidation of its methyl group. Most of the key enzymes of the indole alkaloid biosynthetic pathway have been isolated from seedlings and / or cell suspension cultures of the *C. roseus* [104]. The cell cultures do not produce dimeric and monomeric indole alkaloids but catharanthine is produced in considerable amounts. Vincristine, vinblastine and vindoline were reported only in shoot cultures and differentiated tissues but not in roots[105, 106]. Recently a stable, high producing and salt tolerant cell lines of *C. roseus* plant has been developed to achieve industrial production of the alkaloids.

The low yield of these drugs in cell cultures is one of the major limitations, and many strategies have been tried to improve indole alkaloid production in *C.roseus* cell cultures. Particularly the improvement of catharanthine production in *C. roseus* cell cultures is of great interests for pharmacologists and chemists because catharanthine and vindoline can be coupled to form vinblastine in high yield, and vindoline is abundant in plants[107]. Elicitors can also modulate the production of these alkaloids as reported by Moreno *et al.* in 1981[108]. Low vinblastine and vincristine contents in the plants have also encouraged intense research for alternative production methods involving metabolic engineering[105, 109], semisynthesis[110] or even total chemical synthesis[111, 112]. Total synthesis has proved difficult due to a structural complexity of the molecules and complicated reaction steps involving stereo chemical constraints. Various semi-synthetic procedures have been developed for these alkaloids on the basis of chemical [113, 114] or enzymatic [115] coupling of commercially available catharanthine and vindoline. As a means of simpler and economically feasible semi-synthesis of vinblastine and vincristine, a photochemical one pot synthesis has been proposed. The present synthetic procedures and reaction mechanism is partly based on the approach that has been described in details in the photochemical method. Production of vinca alkaloids in plant cell cultures did not lead to

a significant improvement and today it is accepted that biotechnological approaches in plant cell culturing may not provide an instant solution to this problem.

Clinical Pharmacology

Vincristine

Pharmacokinetic studies of vincristine are limited by a lack of sensitive assays for measuring plasma concentrations of vincristine. Plasma clearance is rapid owing to extensive tissue binding and large volume of distribution. Vincristine is thought to have tri-exponential pharmacokinetics with rapid distribution following bolus injection, β phase distribution of 50–155 min, and an elimination half-life of approx 85 h [116-120]. Children appear to have higher plasma clearance than adults. Vincristine is metabolized in the liver by cytochrome P450 3A, and concomitantly administered drugs may either competitively inhibit or induce cytochrome P450 3A clearance of vincristine [121, 122].

Vincristine accumulates in many tissues such as the lung, liver, kidney, bone marrow, intestinal mucosa, pancreas, and spleen. It is largely excluded from the brain, eye, and adipose tissue [123]. Vincristine is excreted as either unchanged drug or as a metabolite in the bile and feces[124]. Vincristine has little renal excretion. The relationship between the plasma pharmacokinetics and antitumor effects of vincristine is not fully defined.

Vincristine is typically administered as a bolus intravenous infusion at 1.4 mg/m^2 in adults (maximum dose of 2 mg), and at 1.5–2.0 mg/m^2 in children (maximum dose of 2.0–2.5 mg). Vincristine may be given up to once weekly, however, the dosing schedule varies based upon the malignancy, response, and other concomitantly administered drugs. Neurotoxicity is the dose-limiting toxicity, and attempts to use continuous infusion instead of bolus injection to reduce neurotoxicity have been inconclusive [125, 126]. The dose of vincristine is reduced in the setting of severe hepatic dysfunction or severe neurotoxicity. Typically patients will receive 50% of the planned dose for moderate hyperbilirubinemia, and 25% for severe hyperbilirubinemia[127].

Vinblastine

Pharmacokinetic studies of patients treated with a bolus injection of vinblastine are consistent with a triexponential pharmacokinetic model, similar to vincristine. Vinblastine is rapidly distributed from the plasma to tissues, primarily to the lung, liver, spleen, and kidneys. The elimination half-life of vinblastine is approx 29 h, with very little drug remaining in the body at 48 h [128, 129]. Similar to the other vinca alkaloids, vinblastine is metabolized by the hepatic cytochrome P450 3A enzyme. This pathway may be impaired in patients with hepatic dysfunction and may be affected by other medications, which either induce or inhibit the activity of cytochrome P450 3A. Vinblastine is largely excreted in the bile and feces, with little renal excretion [129]. The dose of vinblastine in children and adults is typically 6 mg/m^2, with modifications for hepatic dysfunction and hematological tolerance.

Side Effects

Vincristine

Neurotoxicity is the dose-limiting side effect of vincristine. Vincristine-induced neuropathy is a cumulative toxicity; however, some symptoms develop within the first few weeks of treatment. Initial neurotoxic signs and symptoms include symmetrical sensory impairment and parasthesias. Patients may later have loss of deep tendon reflexes, develop gross motor abnormalities such as foot or wrist drop, or experience a decrease in fine motor skills such as writing. Autonomic polyneuropathy is seen in some patients, manifested by constipation, paralytic ileus, bladder dysfunction, and impotency. Many of the symptoms resolve within weeks to months of discontinuation of therapy, however residual neurotoxicity has been documented[125, 126]. The severity of neurotoxicity may be influenced by the dosage and frequency of administration. Doses of vincristine are typically capped at 2 mg or 2.5 mg because of concerns that autonomic neurotoxicity is more affected by the size of a single dose rather than cumulative dose [126, 130, 131]. Vincristine-induced neurotoxicity is greatest in infants and the elderly and may be related to dose calculations. A correlation between neurotoxicity and obstructive liver disease has also been shown, to result in impaired biliary excretion of vincristine [131].

Concomitant administration of radiation therapy or chemotherapeutic agents such as L-aspariginase may also worsen the neurotoxicity associated with vincristine[132]. Of note, severe central nervous system toxicity has been reported in patients who were given high doses or who have a disrupted blood brain barrier. Intrathecal administration of vincristine is almost always fatal, and therefore this must be carefully avoided [133]. In addition to neurotoxicity, patients will experience mild neutropenia and anemia following administration of vincristine. This is readily reversible and does not usually result in treatment delay [134]. The development of alopecia and rash following vincristine is variable depending upon the dose and duration of treatment. Common gastrointestinal side effects include constipation, abdominal cramping, nausea and vomiting. Patients may also complain of urinary symptoms secondary to polyuria, dysuria, or bladder rentention[135].

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Vinblastine

Myelosuppression is the dose-limiting toxicity of vinblastine. Neutropenia is the most common manifestation of myelosuppression, with anemia and thrombocytopenia being less frequent. Neutropenia occurs approximately 4–10 days following administration of the drug, and counts usually recover within 7–21 days of administration. Neurotoxicity is less common with vinblastine than with vincristine, and usually occurs after prolonged administration or in combination regimens[136, 137]. Patients may complain of gastrointestinal side effects such as mucositis and stomatitis; nausea and vomiting may occur but are less common. Mild alopecia is seen and is reversible. There are case reports of acute hypertension and pulmonary edema, but these are infrequent side effects of vinblastine.

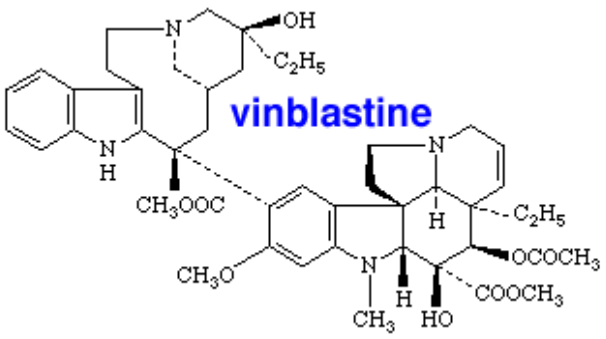
The medicinal plant Catharanthus roseus contains large number of terpenoid indole alkaloid (TIAs) with over 70 compounds isolated and identified. It is one of the most extensively investigated medicinal plants, chiefly due to the presence of two of the most important anti-tumour agents employed in medicine, the bis-indole alkaloids vinblastine and vincristine. It is known that the biosynthesis of vinblastine in C. roseus plants begins with the amino acid tryptophan and the monoterpenoid geraniol, and requires the involvement of at least 35 intermediates, 30 enzymes, 30 biosynthetic and 2 regulatory genes, as well as 7 intra and intercellular compartments[138, 139]. The plant accumulates these metabolites only at low yields. Thus there exists a large demand for these anticancer alkaloids. One tonne of *Catharanthus roseus* leaves yield 50 grams of vincristine sulphate in crude form. On further purification, 40 grams of vincristine is obtained.

***Catharanthus roseus* Linn. G.Donn.**

LOW YIELD IN PROCESS

Vincristine, the antileukaemia drug which is in demand, suffers from the disadvantage of very low yields from the source material, and so is prohibitively expensive. Vinblastine, another anticancer drug from the same plant is present at levels 1000 times higher than vincristine and its cost is one third as that of vincristine. Vinblastine is now being used as the parent drug to obtain, through structural modifications, the prodrug vincristine[109].

Vinblastine

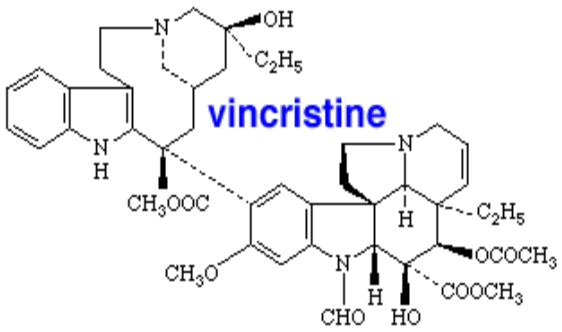


Chemical Data	
Formula	$C_{46}H_{58}N_4O_9$
Mol. mass	810.974 g/mol
Pharmacokinetic data	
Bioavailability	n/a
Metabolism	Hepatic (CYP3A4-mediated)
Half life	24.8 hours (terminal)
Excretion	Biliary and renal

HISTORY

Vinblastine was first isolated by Robert Noble and Charles Thomas Beer from the Madagascar periwinkle plant. Vinblastine's utility as a chemotherapeutic agent was first discovered when it was crushed into a tea. Consumption of the tea led to a decreased number of white blood cells; therefore, it was hypothesized that vinblastine might be effective against cancers of the white blood cells such as lymphoma.

VINCRISTINE



Chemical Data	
Formula	$C_{46}H_{56}N_4O_{10}$
Mol. mass	824.958 g/mol
Pharmacokinetic data	
Protein binding	~75%
Metabolism	Hepatic
Half life	19 to 155 hours
Excretion	Mostly biliary, 10% in urine

History

Having been used as a folk remedy for centuries, studies in the 1950s revealed that C. roseus contained more than 200 alkaloids, many of which are biologically active. While initial studies for its use in diabetes mellitus were disappointing, the discovery that it caused myelosuppression (decreased activity of the bone marrow) led to its study in mice with leukemia, whose lifespan was prolonged by the use of a vinca preparation. Treatment of the ground plant with Skelly-B defattenining agent and an acid benzene extract led to a fraction termed "fraction A". This fraction was further treated with aluminium oxide, chromatography, trichloromethane, benz-dichloromethane and separation by pH to yield vincristine.Vincristine was approved by the United States Food and Drug Administration (FDA) in July 1963 as Oncovin. The drug was initially

discovered by a team lead by Dr. J.G. Armstrong; it was then marketed by Eli Lilly and Company.

Biosynthesis of vinca alkaloids

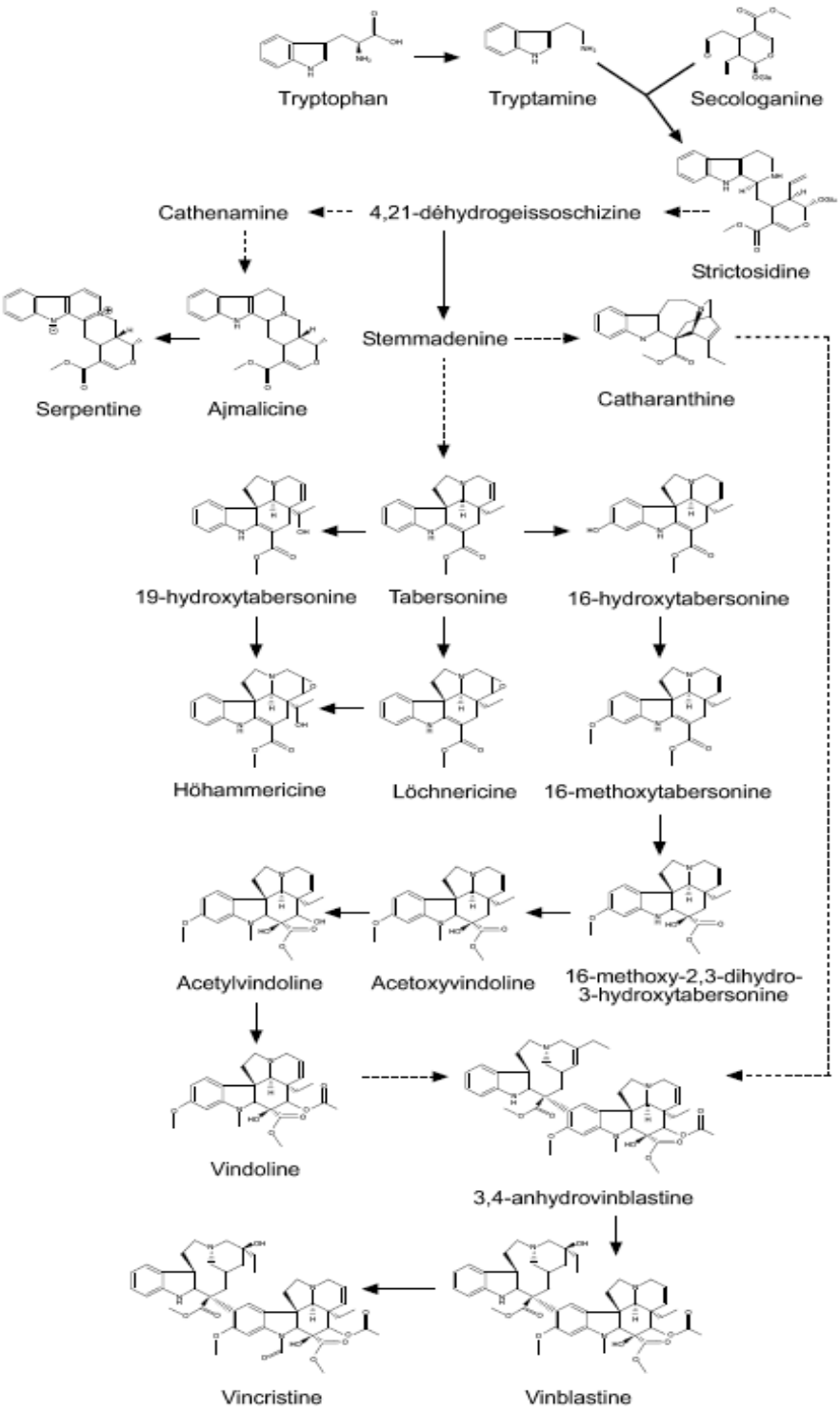
The various *Catharanthus* alkaloids belong to the class of terpenoid indole alkaloids, that is, they consist of two moieties derived from two separate metabolic pathways- The Mevalonate pathway which gives the non tryptophan moiety; and shikimic acid pathway which gives the tryptophan moiety is obtained from tryptophan. The complex structure of these alkaloids usually contains two nitrogen atoms; one is the indole nitrogen (in the tryptophan- derived moiety) and the second is generally two carbons removed from the Beta- position of the indole ring. The non- tryptophan moiety is derived from mevalonic acid and it is a C10-geraniol (monoterpenoid) contribution in the case of these alkaloids. This portion with suitable rearrangements leads to formation of three types of alkaloids- i) Coryanthe- type alkaloids (ii) Iboga- type alkaloids (iii) Aspidosperma- type alkaloids. It is believed that the coryanthe- type monoterpenoid moiety is metabolically most primitive. The reactive form of terpene involves an aldehyde group. The loss of one carbon atom during biogenesis, to give C9 unit is largely common.

Geraniol by a series of conversions forms loganin and then secologanin (a monoterpenoid glucoside). A key intermediate in the biogenesis of the monoterpene indole alkaloids is 3 alpha (S)- strictosidine, formed by the enzymatic condensation of tryptamine and secologanin. The enzyme strictosidine synthase is responsible for this important reaction. Strictosidine then leads to formation of cathenamine (a coryanthe- type of alkaloid); enzyme involved is cathenamine synthase. Cathenamine further gives rise to ajmalicine (enzyme- ajmalicine synthase) and serpentine. Both ajmalicine and serpentine are also coryanthe- type alkaloids. Cathenamine through a series of reactions also leads to formation of catharanthine (iboga- type) and vindoline (aspidosperma- type). Catharanthine and vindoline are monomeric indole alkaloids and occur free in the plant. 3',4'-Anhydrovinblastine is a key intermediate in the coupling of catharanthine and

vindoline and the enzyme involved are peroxidases. It is further converted to vinblastine and vinblastine after oxidation gives vincristine[140, 141].

Biosynthetic pathway of the Vinblastine and vincristine

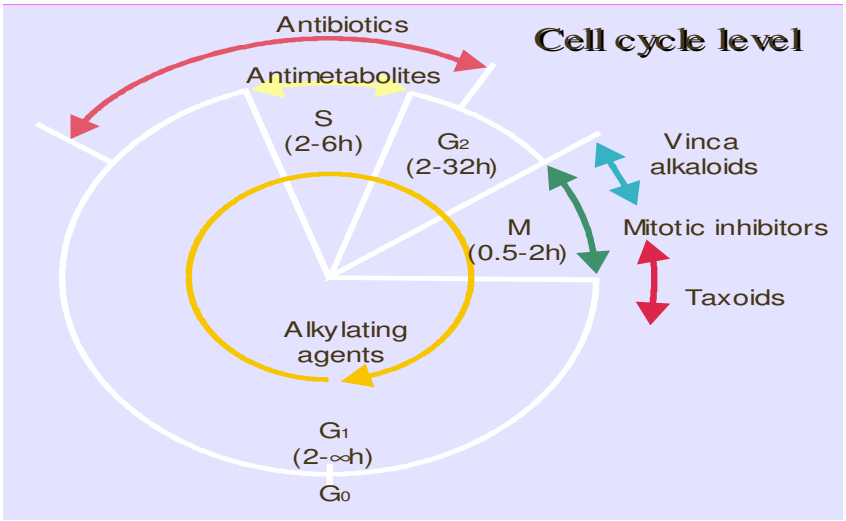
(Tikhomiroff *et al*,2002 and John *et al.,* 2000)



Mode of action of Vinblastine and Vincristine

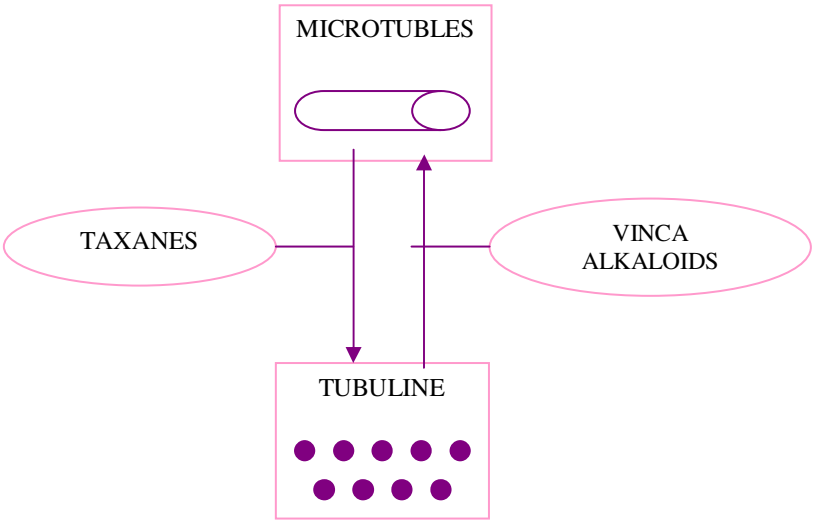
Among the many biochemical effects seen after exposure of cells and tissues to the *Vinca* alkaloids are disruption of microtubules, inhibition of synthesis of proteins and nucleic acids, elevation of oxidized glutathione, alteration of lipid metabolism and the lipid content of membranes, elevation of cyclic adenosine monophosphate (cAMP) and inhibition of calcium-calmodulin regulated cAMP phosphodiesterase. The *Vinca* alkaloids are relatively hydrophobic molecules that partition into lipid bilayers in the uncharged state, altering the structure and function of membranes. Of their diverse effects, their only well-documented direct action is disruption of microtubules, which results from their reversible binding to tubulin (William *et al*, 1994).(The subunit protein of microtubules). Vinca alkaloids are pharmacologically active at most of the concentrations and biochemical effects associated with exposure to the *Vinca* alkaloids is probably secondary to disruption of microtubules, although it is possible that drug-induced changes in lipid bilayers may alter some membrane-dependent processes. At high intracellular concentrations, these compounds induce formation of large crystalline aggregates that are composed of tubulin and drug. Despite their many biochemical actions, the antineoplastic activity of the *Vinca* alkaloids is usually attributed to their ability to disrupt microtubules, causing dissolution of mitotic spindles and metaphase arrest in dividing cells.

However, disruption of microtubules also leads to toxicity in nonmitotic neoplastic cells, and although the *Vinca* alkaloids are classified as mitotic inhibitors, their antineoplastic activity in the clinical treatment of cancer probably arises from perturbation of a variety of microtubule-dependent processes, as well as from disruption of the cell cycle and induction of programmed cell death.



Microtubules are involved in many cellular processes besides mitosis, and exposure to *Vinca* alkaloids give rise to diverse biologic effects, many of which could impair essential functions, both in dividing and in non dividing cells. Morphological changes and cell death after treatment with vinblastine and vincristine have been seen in non dividing normal as well as leukemic lymphocytes and also in cultured leukemic cells during interphase and in G1 and S-phase cells. Chemotaxis in human monocytes and directional migration of cultured tumor cells are inhibited by vinca alkaloids. Microtubules are required for the transport of various metabolites and the movement of organelles, including mitochondria and secretory granules, along neuronal processes. Exposure of nervous tissue to vinca alkaloids inhibits axonal transport, causing neurotoxicity. The vinca alkaloids also inhibit secretory processes, apparently as a result of perturbations in membrane trafficking with disruption of the cytoskeleton. Platelets, which depend on the integrity of the peripheral ring of microtubules for their discoidal shape, become spherical after treatment with vinca alkaloids. These few examples illustrate that the vinca alkaloids exert a variety of potentially cytotoxic effects that are unrelated to mitotic inhibition.

Although the effects of the vinca alkaloids on the organization and function of microtubules have been extensively characterized, establishing the nature and number of vinca alkaloid binding sites on tubulin has been difficult because of methodological problems, However, it appears that each heterodimer of tubulin possesses a single "vinca-specific" site of high intrinsic affinity and an unknown number of nonspecific sites of low affinity. Attempts to compare the tubulin-binding capabilities of different vinca alkaloids are also complicated by differences in assay conditions and methods of analysis of ligand-binding data. Nevertheless, some generalizations can be made.



Vinca alkaloids inhibit polymerization of tubulin & Taxanes induced polymerization.

For example, the relative strength of drug binding to vinca-specific sites on the alpha- and beta-heterodimers of tubulins is vincristine > vindesine > vinblastine. Also, Vincristine and Vinblastine are more potent inhibitors of *in vitro* assembly than vinorelbine. It should also be noted that both the velbanamine and vindoline moieties are required for site-specific binding of the vinca alkaloids to tubulin. (William *et al* Wiley–Liss, New York, 1994.)

Microtubules are dynamic tube-shaped polymers composed of the heterodimeric protein tubulin. Polymerization of tubulin into microtubules occurs by a nucleation-elongation mechanism in which formation of a short microtubule "nucleus" composed of tubulin heterodimers is followed by growth of the microtubule at its ends by the reversible addition of tubulin subunits. However, microtubules do not attain a true equilibrium. GTP, which binds reversibly at an exchangeable site in the tubulin dimer, is irreversibly hydrolyzed to GDP and P_i as (or shortly after) the tubulin polymerizes onto the growing microtubule end this creates polymers with unique non-equilibrium dynamics. At microtubule ends, stochastic transitions occur between phases of relatively slow growth and rapid shortening. The opposite ends of the microtubule differ kinetically, with one end, called the plus end, being more dynamic than the opposite minus end. Regulation of the transitions between growing and shortening at both microtubule ends appears to be due to the stochastic gain and loss of a stabilizing "cap" consisting of a short region of tubulin-GTP or tubulin-GDP-P_i. Loss of the cap is thought to be required for initiation of a shortening phase, and the rate-limiting step has been postulated to be a conformational change in tubulin that is associated with GTP hydrolysis or P_i release[142].

During mitosis, microtubule dynamics are greatly increased as compared with the dynamics during interphase. Spindle microtubules exchange their tubulin with soluble tubulin in the cytoplasmic pool with half-times on the order of ~10-15 s, 20-100-fold faster than during interphase. The rapid dynamics of microtubules during mitosis play an essential role in the formation of the spindle and in movement of the chromosomes. At prometaphase, the plus ends of microtubules rapidly grow out from the centrosomes, probing the cytoplasm by continuous excursions of growing and shortening until they encounter and become attached to the kinetochores of the chromosomes. In addition, rapid treadmilling occurs during mitosis. Growth occurs at the plus ends of microtubules tethered to the kinetochores of the chromosomes, and balanced shortening occurs at the minus ends that are embedded in the centrosomes.

Vinblastine binds to tubulin in intact microtubules with two widely different affinities depending upon whether the tubulin binding site is located at the microtubule ends or is situated along the microtubule surface. The binding sites on the microtubule surface have

low affinity for vinblastine 1-2 sites per molecule of tubulin dimer in microtubules; K_d 0.25-0.3 mM (Jordan, 1986). Binding of vinblastine at high concentrations to these sites *in vitro* depolymerizes the microtubule at both ends by the peeling of protofilaments and leads to formation of tubulin-vinca alkaloid paracrystals in cells (Wilson *et al,* 1994). Suppression of tubulin exchange at microtubule ends, which occurs at low vinblastine concentrations in the absence of significant microtubule depolymerization, appears to be due to the reversible binding of vinblastine to high affinity binding sites located uniquely at one or both microtubule ends (~16 binding sites per microtubule, K_d 1-2 µM (Wilson *et al*, 1982). It was found that vinblastine inhibits tubulin exchange at microtubule ends by 50% when an average of only one or two molecules of vinblastine is bound per microtubule. In addition, vinblastine reduces the rate of tubulin loss from plus ends, kinetically capping this end in the absence of significant microtubule depolymerization (Jordan *et al*, 1990). Video microscopy of individual microtubules, both in living BSC-1 cells and *in vitro* with microtubules assembled from bovine brain tubulin, indicated that vinblastine significantly suppresses dynamic instability at microtubule plus ends at vinblastine concentrations that are below the concentrations required to reduce the microtubule polymer mass (Toso, *et al*,1993).

Fungi are one of the major sources of natural bioactive molecules. Over 4000 bioactive metabolites of fungal origin have been described. In some cases plant-associated fungi are able to make the same bioactive compounds as the host plant itself. One of the best examples is the discovery of gibberellins from *Fusarium fujikuroi* in the early 1930's. The pathways of gibberellin biosynthesis in the fungus and higher plants are identical upto GA12, suggesting the possibility of intergeneric genetic exchange between plant and fungus. This observation led to the prospect that endophytic fungi, associated with *Taxus brevifolia*, may produce Taxol and was confirmed by Strobel *et al* (1993). Thus the success of finding fungal taxol prompted us to isolate endophytic fungi from *Catharanthus roseus* to produce low volume and high valued drugs, vinblastine and vincristine. Potentially, a fungal source would reduce the price of the above life-saving drugs and also save the plant from extinction in some areas.

Vinblastine and vincristine have been produced from leaves of field grown plants by cell and tissue culture, callus culture, cell suspension culture, shoot culture, hairy roots cultures of *Catharanthus roseus* semi synthesis as well as total synthesis. Kharwar *et al.* (2008) isolated several endophytic fungi from *Catharanthus roseus* foun in India but to the best of our knowledge there is so far no report of isolation of these drugs from the above endophytes[143]. Zahng *et al.* [144] and Tung *et al.* [145] discovered that vincristine is produced by *Fusarium oxysporum*, an endophyte of *Catharanthus roseus;* while Guo and Kunming isolated vinblastine from *Alternaria sp*. isolated from *Catharnthus roseus*[146]. However, they have showed the production of vinblastine and vincristine by TLC and HPLC only. Since the quantity of both these drugs from all the sources are extremely low, the supply of vinblastine and vincristine, the wonder drugs to cure cancer, are still limited. Therefore, considering the importance of vinblastine and vincristine (low volume and high valued drugs) and their supply at low cost to the patients and no cost to the environment, a screening program aimed at isolating endophytic fungal strains associated with leaves of *C.roseus* was initiated at Biochemical Sciences Division, NCL. Focus has been laid particularly to identify non-sporulating, slow growing uncommon strains of fungi. So far 52 such cultures have been brought into pure cultures and screened for vinblastine and vincristine production extracellularly. We have identified fungal (endophytic) strains which produce vinblastine and vincristine, purified with TLC, HPLC and characterized by UV-VIS, ESI-MS and ^{1}HNMR. Fungal mediated transformation of vinblastine to vincristine, catharanthine and vindoline to vinblastine and secologonin and tryptophan to vinblastine has been achieved using *Fusarim oxysporum* at room temperature. Vinblastine was used to conjugate with microbially synthesized highly fluorescent gadolinium oxide nanoparticles.

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Chapter 2

Isolation, purification and maintenance of endophytic fungi associated with leaves of *Catharanthus roseus*

Summary:

Endophytic fungi were isolated from the leaves of *Catharanthus roseus* obtained from different areas of Pune. The leaves were cut into small pieces approximately (0.2 cm x 0.2 cm) and surface sterilized with 0.01% mercuric chloride ($HgCl_2$) solution for 90 seconds and washed thoroughly with sterilized distilled water. Residual water on the surface was removed by soaking on sterile blotting paper. Small pieces of leaves were placed on the surface of potato dextrose agar (PDA) poured into Petri dishes. From the fungal population, only the slow growing and unusual fungi were considered for further study. After 10-15 days, fungi were observed growing from the leaves in the plates. Individual hyphal tips of the various fungi were removed from the PDA plates and placed again on PDA and incubated at room temperature for at least 10-15 days. Each fungal culture was checked for purity and transferred to agar slant by hyphal tip as well as single spore isolation method (Strobel *et al*, 1993, 1996 and Ahmad *et al*, 1991, 2009). A total of 52 endophytic fungi were isolated from the leaves of *Catharanthus roseus* plant collected from above locations. Stock cultures were maintained by subculturing at monthly intervals. After growth at pH 7 and temperature 25^{o}C for 7 days, the slants were maintained at 15^{o}C. From actively growing stock cultures, subcultures were made on fresh slants and after 7 days of incubation at pH 7 and temperature 25^{o}C were used as the starting material for fermentation experiments for low volume and high valued vinca (vinblastine and vincristine) alkaloids.

Introduction:

The term endophyte (Greek: endo=within, phyte= plant) has been defined as an organism contained or growing within the substrate plant whether parasitically or not. Endophytes may contribute to their host plant by producing a plethora of substances that provide protection and ultimately survival value to the plant. Endophytes are relatively unstudied and potential sources of novel natural products for exploitation in medicine, agriculture and industries. Consequently, the opportunity to find new and interesting endophytic microorganisms among myriads of plants in different climates and ecosystems is great.

Recent interest has been focused on the study of endophytic and bark associated fungi from medicinal plants. One of the most significant discoveries in this area has been the isolation of an endophytic fungus, *Taxomyces andreanae* from the bark of Pacific yew (*Taxus brevifolia*) which has been shown to produce taxol, the antitumor diterpenoid produced by the *Taxus* plant[1-7]. The concept has been gaining ground since this discovery that the endophytic fungi associated with medicinal plants may have the potential to acquire the capability to produce the valuable metabolites of medicinal value from the parent plant and such fungi could become independent alternative sources for these metabolites which can be produced by fermentation. This opens a tremendous new area for research and development, particularly for screening the innumerable medicinal plants of indigenous origin for endophytes and evaluating them for their potential to manufacture the valuable compounds of plant origin[8-12]. Another important anticancer compound is the alkaloid Camptothecin, a potent anti-neoplastic agent which was firstly isolated from wood of *Camptotheca acuminata.* Kusari *et al* isolated endophytic fungus from *Camptotheca acuminata* that produces Camptothecin and analogues[13-14]. Similarly, Podophyllotoxins were reported from fungus *Phialocephala fortinii* isolated from *Podophyllum peltatum*[15-17]. Podophyllotoxin and analogues are clinically relevant mainly due to their cytotoxicity and antiviral activities and are valued as a precursor to useful anticancer drugs like etoposide, teniposide, and etopophos phosphate[18].

Sreekanth *et al* (2009) isolated endophytic fungi from Indian Yew tree *Taxus baccata*, and then screened for taxol production. Out of the 40 fungal cultures screened, one fungus *Gliocladium* was found to produce taxol and 10-DABIII (10-deacteyl baccatin

III). These compounds were purified by TLC and HPLC and characterized using UV spectroscopy, ESI-MS, MS/MS and proton NMR. One litre of the *Gliocladium sp*. yielded 10 µg of Taxol and 65 µg of 10-DAB III[7]. Husain *et al.* (1993*)* isolated (–) Jasmonic acid from the fungus *Botryodiplodia theobromae* and Ahmad *et al.* (2001) isolated ergot alkaloid and indole alkaloids from the endophytic fungus *Balansia sclerotica* [8-12].We took the advantage of this feature and started the isolation and purification of endophytic fungi from *Catharanthus roseus* plant in order to isolate low volume and high valued vinca alkaloids such as vinblastine and vincristine.

Materials:

Catharanthus roseus plant**,** 0.01% mercuric chloride ($HgCl_2$) solution, PDA medium (Potato: 250 gm, D-glucose: 20 gm, Agar-Agar: 20 gm per lit.), muslin cloth, scissor, sterile distilled water, blotting paper, agar media, Petri plates.

Methods:

Collection of *Catharanthus roseus* plants:

Catharanthus roseus plants were collected from different areas of Pune, to determine the number and species of endophytes present in leaf tissues. After plant selection, leaves were cut with the help of sterile scalpel and placed in sterile plastic bags to store the material at 4^{o}C until isolation procedure was started.

Isolation of endophytic fungi from *Catharanthus roseus*:

Endophytic fungi were isolated from the leaves of *Catharanthus roseus* obtained from different areas of Pune. The leaves were cut into small pieces approximately (0.2 cm x 0.2 cm) and surface sterilized with 0.01% mercuric chloride ($HgCl_2$) solution for 90 seconds and washed thoroughly with sterilized distilled water. Residual water on the surface was removed by soaking on sterile blotting paper. Small pieces of leaves were placed on the surface of potato dextrose agar (PDA) poured into Petri dishes. From the fungal population, only the slow growing and unusual fungi were considered for further study. After 10-15 days, fungi were observed growing from the leaves in the plates. Individual hyphal tips of the various fungi were removed from the PDA plates and placed again on PDA and incubated at room temperature for at least 10-15 days. Each fungal culture was checked for purity and transferred to agar slant by hyphal tips as well as single spore isolation method (Strobel *et al* 1996 and Ahmad *et al* 1991). A total of 52 endophytic fungi were isolated from the leaves of *Catharanthus roseus* plant collected from the above location. Stock cultures were maintained by subculturing at monthly intervals. After growth at pH 7 and 25^{o}C for 7 days the slants were maintained at 15^{o}C. From actively growing stock cultures, subcultures were made on fresh slants and after 7 days incubation at pH 7 and 25^{o}C, were used as the starting material for fermentation experiments for low volume and high valued vinca (vinblastine and vincristine) alkaloids.

Results:

Endophytic fungi from *Catharanthus roseus*:

A total of 52 endophytic fungi were isolated from the leaves of *Catharanthus roseus* plant collected from the above locations (Fig.1 and Fig. 2). We have not observed other microbes such as bacteria and actinomycetes during isolation. Most of the fungi isolated were unusual and slow growing. All the endophytic fungi isolated were brought to pure culture. Stock cultures were maintained by subculturing at monthly intervals. After growth at pH 7 and temperature 25^{o}C for 7 days, the slants were maintained at 15^{o}C. From actively growing stock cultures, subcultures were made on fresh slants and after 7 days incubation at pH 7 and temperature 25^{o}C were used as the starting material for fermentation experiments for isolation of vinca (vinblastine and vincristine) alkaloids.

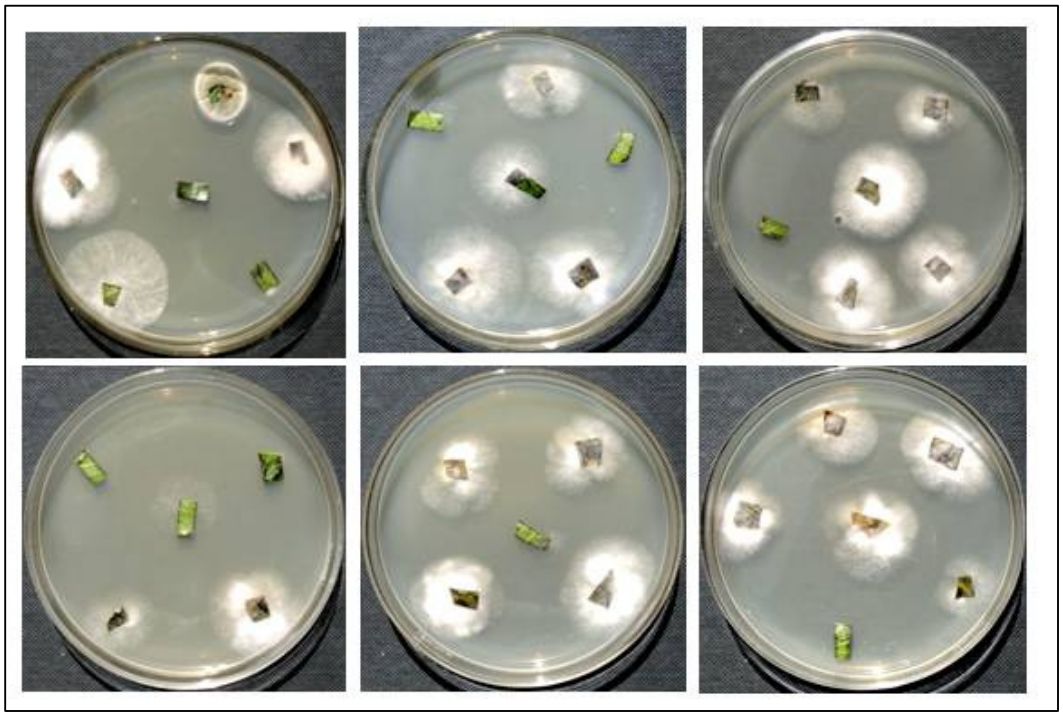
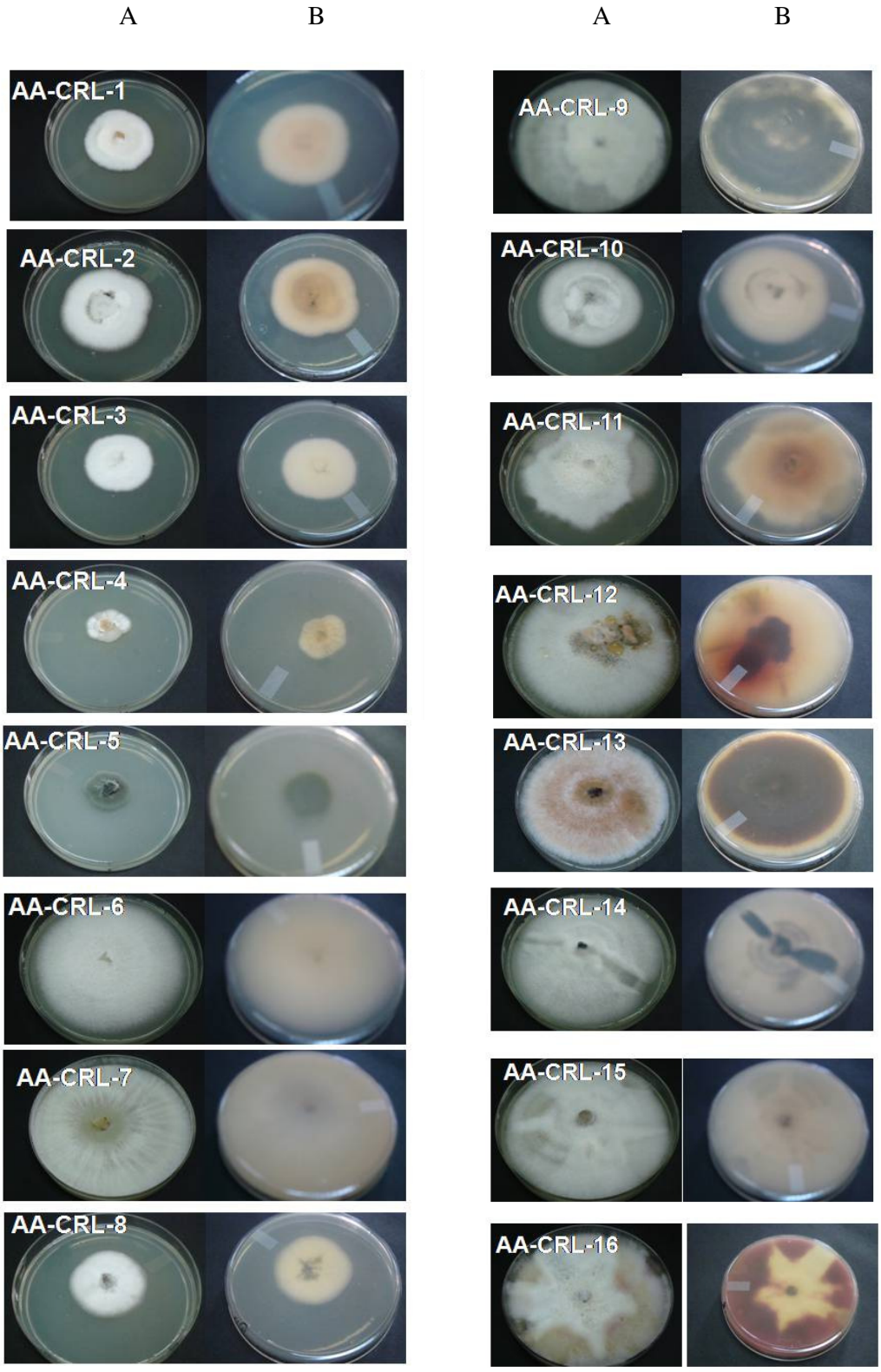


Fig.1- Leaf pieces of *Catharanthus roseus* showing the emergence of endophytic fungi after 10-12 days.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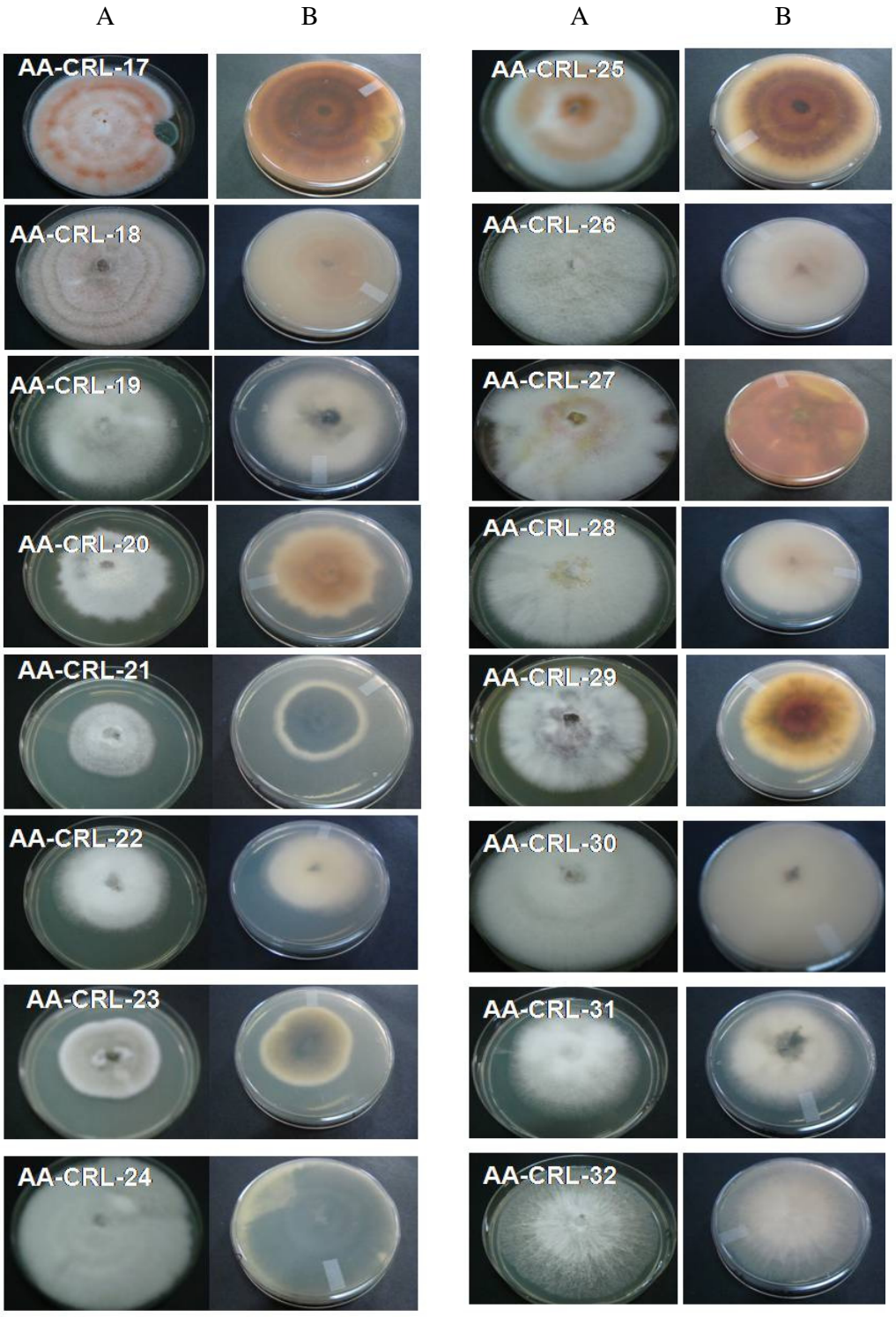
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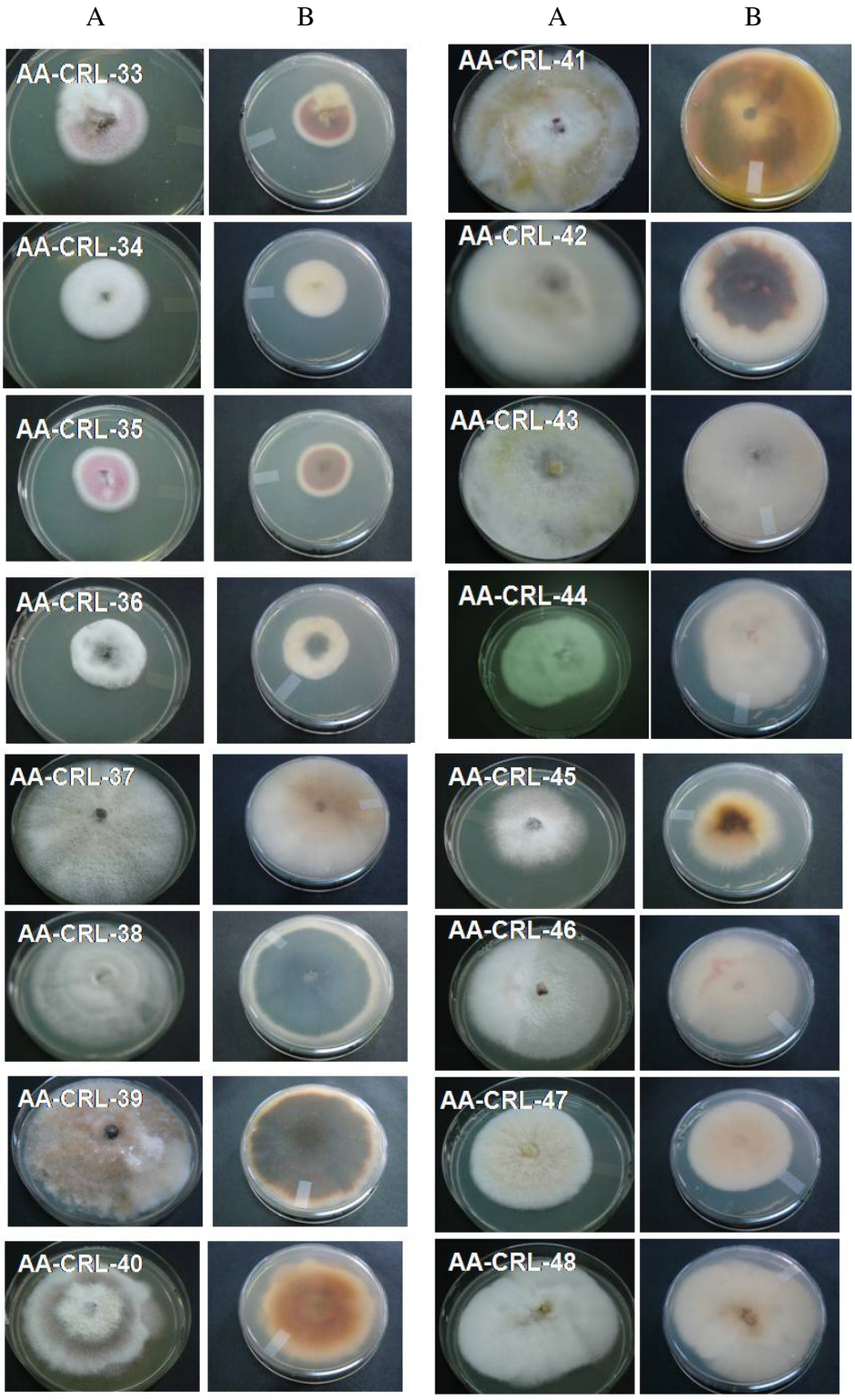
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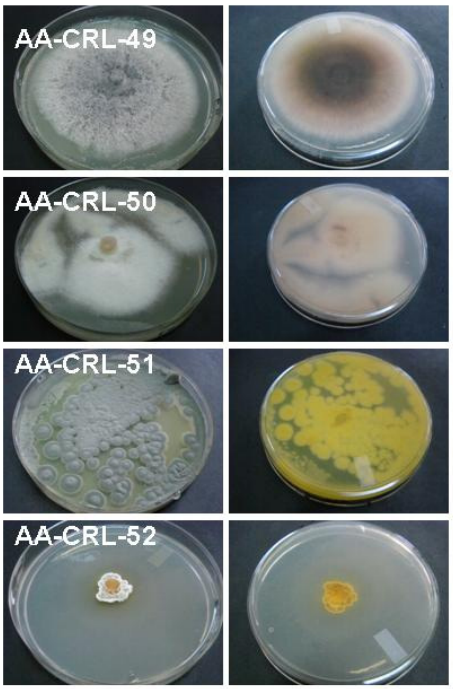

Fig. 2- Colonies of different endophytic fungi isolated from leaves of *Catharanthus roseus* growing on potato dextrose agar medium. Colony shape: front (A) and back (B) side of a PDA plate.

Discussion:

The aim of the present work was to isolate, purify and identify slow growing and unusual endophytic fungi associated with leaves of *Catharanthus roseus* plant. Because of the slow growth of these fungi, major difficulties are encountered in purifying these fungi and in maintaining pure cultures for extended periods required in most of the investigation[19-30]. Isolation of endophytic fungi from the surface of sterilized tissues of excised leaves indicate the endophytic nature of the fungi. Endophytic nature of several *Balansia* sp. has been established[31]. However, some species of *Balansia* namely *Balansia cyperi* and other related genera could not be isolated from their host after surface sterilization. They have been considered as epiphytes. Isolations of endophytic fungi from surface sterilized tissue can be a useful method for distinguishing their endophytic or epiphytic nature [32-39]. The endophytic fungi isolated from *Catharanthus roseus* represent different genera of fungi and screened for vinca alkaloids.

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Chapter 3

Screening of endophytic fungi for production of vinca alkaloids (vinblastine and vincristine)

Summary:

Production of vinblastine and vincristine by 52 endophytic fungi isolated from the leaves of *Catharanthus roseus* plant was studied by a two stage fermentation procedure. These fungi were grown in 500 ml Erlenmeyer flask containing 100 ml MGYP medium. The flasks were inoculated with agar plugs containing mycelium from 7 days old PDA slants. The inoculated flasks were incubated at 25-27^{o}C on a rotary shaker (240 rpm) for 4 days. These cultures were used as seed cultures (First stage). For vinblastine and vincristine production, 10 ml seed cultures were transferred to 500 ml flask containing 100 ml vinca alkaloids-1 (VM-1) medium. The flasks were incubated at 25-27^{o}C on a rotary shaker (240 rpm) for 20 days (second stage). After 20 days of incubation, the culture was harvested and passed through four layers of muslin cloth to separate the mycelia from the culture broth. Both the culture filtrate and mycelia were lyophilized to dryness and extracted with equal volumes of ethyl acetate each time. The extracts were pooled and dried with anhydrous sodium sulphate and concentrated at 40^{o}C *invacuo* to yield crude extract. A small amount of crude extract was dissolved in ethyl acetate and used for screening of vinblastine and vincristine with the help of standard vinblastine and vincristine.

Introduction:

The productivity of vinblastine and vincristine is very low in plants (0.001-0.0003%) resulting in their extraordinary high price[1]. Vinblastine is a dimeric indole alkaloid and is formed by coupling of vindoline and catharanthine catalysed by horse radish peroxidase (Goodbody *et al*, 1988). The yield of coupling product, (15, 20-anhydro vinblastine) was reported very low (0.9%) by Endo *et al*, (1988). Vinblastine is converted into vincristine by the oxidation of its methyl group, Hamad and Nakazawa (1991). Most of the key enzymes of the indole alkaloid biosynthetic pathway have been isolated from seedlings and/or cell suspension cultures of the *C. roseus*. The cell cultures do not produce dimeric and monomeric indole alkaloids but catharanthine is produced in considerable amounts. Vincristine, vinblastine and vindoline were reported only in shoot cultures and differentiated tissues but not in roots. Recently a stable, high producing and salt tolerant cell line of *C. roseus* plant has been developed to achieve industrial production of the alkaloids.

The low yield of these drugs in cell cultures is one of the major limitations, and many strategies have been tried to improve indole alkaloid production in *C. roseu*s cell cultures. Particularly, the improvement of catharanthine production in *C. roseus* cell cultures is of great interest for pharmacologists and chemists because catharanthine and vindoline can be coupled to form vinblastine in high yield, and vindoline is abundant in plants, (Misawa and Goodbody, 1996). Elicitors can also modulate the production of these alkaloids as reported by Moreno *et al* in 1981. Low vinblastine and vincristine contents in the plants have also encouraged intense research for alternative production methods involving cell culture[2, 3], metabolic engineering[4], semisynthesis[5, 6] or even total chemical synthesis[7]. Total synthesis has proved difficult, due to a structural complexity of the molecules and complicated reaction steps involving stereo chemical constraints. Various semi-synthetic procedures have been developed for the production of these alkaloids on the basis of chemical [5, 6] or enzymatic [8] coupling of commercially available catharanthine and vindoline. As a means of simpler and economically feasible semi-synthesis of vinblastine and vincristine, a photochemical one pot synthesis has been proposed. The present synthetic procedures and reaction mechanism is partly based on the approach that has been described in details in the photochemical method. Production of vinca alkaloids

in plant cell cultures did not lead to a significant improvement and today it is accepted that biotechnological approaches in plant cell culturing may not provide an instant solution to this problem. Therefore, considering the importance of vinblastine and vincristine (low volume and high valued drugs) and their supply at low cost to the patients and low cost to the environment, a screening programme aimed at isolating endophytic fungal strains associated with the leaves of *C. roseus* was initiated at biochemical sciences division, National Chemical Laboratory, Pune, India. Focus has been laid particularly to identify non-sporulating, slow growing, uncommon strains of fungi. So far 52 such cultures have been brought into pure cultures and screened for vinblastine and vincristine production in the medium containing glucose, tryptophan, geranium oil, vitamins, etc.

Materials:

Vinca alkaloids-1(VM-1) liquid culture medium, distilled water, MGYP medium, PDA medium, rotary shaker, muslin cloth, chloroform, methanol, silica gel (0.5mm thickness), lyophilizer, ceric ammonium sulphate reagent, vinblastine (Sigma-Aldrich), vincristine (MP Biomedicals), Erlenmeyer flasks (500 ml), liquid nitrogen, ethyl acetate, anhydrous sodium sulphate, rotavapour, TLC silica gel plates, pH meter.

Methods:

Preliminary studies on the production of vinca alkaloids by 52 endophytic fungi were carried out in 5 liquid culture media. Among the media tested, vinca alkaloids production in significant quantities was observed in one medium. Hence, further studies were carried out on the production, isolation and quantification of the alkaloids using this medium. The composition of the medium (per liter H_2O) here after referred as production medium is as follows:

Vinca media (VM)-1:

Glucose: 3%, Succinic acid: 1%, Sodium benzoate: 100 mg, Peptone: 1%, Magnesium sulphate: 3.6 mg, Biotin: 1 mg, Thiamine: 1 mg, Pyridoxal: 1 mg, Calcium pentothenate: 1 mg, Phosphate buffer: 1 ml (pH 6.8), L-Tryptophan: 0.1%, Geranium oil: 0.05%

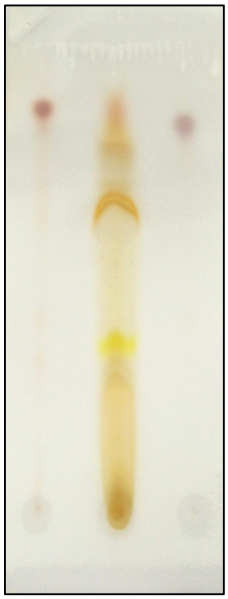
Production of vinca alkaloids by 52 endophytic fungi isolated from the leaves of *Catharanthus roseus* plant were studied by a two stage fermentation procedure. These fungi were grown in 500 ml Erlenmeyer flasks containing 100 ml MGYP medium. The flasks were inoculated with agar plugs containing mycelium from 7 days old PDA slants. The inoculated flasks were incubated at 25-27^{o}C on a rotary shaker (240 rpm) for 4 days. These cultures were used as seed cultures (First stage). For vinca alkaloids production, 10 ml seed cultures were transferred to 500 ml flask containing 100 ml vinca alkaloids-1 (VM-1) medium. The flasks were incubated at 25-27^{o}C on a rotary shaker (240 rpm) for 20 days (Second stage). After 20 days of incubation, the culture was harvested and passed through four layers of muslin cloth to separate the mycelia from the culture broth. The culture filtrates were lyophilized to dryness, and

extracted with equal volumes of ethyl acetate each time[9]. The extracts were pooled and dried with anhydrous sodium sulphate and concentrated at 40°C *invacuo* to yield crude extract. A small amount of crude extract was dissolved in ethyl acetate and subjected to thin layer chromatography (TLC) on silica gel (0.5 mm thickness) using chloroform: methanol (8:2) as a solvent system. The TLC plates were sprayed with ceric ammonium sulphate reagent[10]. Vinca alkaloids spots produced brilliant violet color as well as purple color with above spraying reagent. Alkaloids spectrum of crude extract was identified using reference samples as standards. Standard vinblastine and vincristine were employed as reference for comparison of Rf values.

Results:

Vinca alkaloids from culture filtrate:

Out of 52 endophytic fungi screened for vinca alkaloids, the culture filtrates of AA-CRL-6 and AA-CRL-20 extracted with ethyl acetate yielded a brown residue after the removal of the solvent. The substance was named as crude extract. The crude extract of AA-CRL-6 on TLC on silica gel-G using chloroform: methanol (8:2) solvent system produced five spots, when the plates were sprayed with the above reagent (Fig. 1).



1 2 3

1: Standard vinblastine,
2: Crude extract,
3: Standard vincristine
Detection: Ceric ammonium sulphate reagent

Fig.1: TLC showing vinca alkaloids spectrum of crude extract of culture filtrate of AA-CRL-6 on silica gel using Chloroform:Methanol (8:2) solvent system

The Rf value of the spots (vinca alkaloids) on TLC of the crude extracts using chloroform:methanol (8:2) solvent system when compared with the standard vinblastine, vincristine showed identical Rf values of two spots (Rf value 0.77 and 0.74) with vinblastine and vincristine. The spots were characteristic to vinca alkaloids

(Fig.1). The compounds when chromatographed with standards vinblastine, vincristine showed to be identical with vinblastine and vincristine.

1 2 3

1: Standard vincristine,
2: Crude extract,
3: Standard vinblastine
Detection: Ceric ammonium sulphate reagent

Fig.2: TLC showing vinca alkaloids spectrum of crude extract of culture filtrate of AA-CRL-20 on silica gel G using Chloroform: Methanol (9:1) solvent system.

Similarly, the crude extract of AA-CRL-20 on TLC on silica gel using chloroform: methanol (9:1) solvent system produced six spots, when the plates were sprayed with the above reagent (Fig.2). The Rf value of the spots (vinca alkaloids) on TLC of the crude extracts using chloroform: methanol (9:1) solvent system when compared with the standard vinblastine and vincristine showed identical Rf values of two spots (Rf value 0.69 and 0.60) with vinblastine and vincristine. The spots of vinblastine and vincristine produced by crude extract (AA-CRL-6 and AA-CRL-20) showed nearly identical color with the standards when sprayed with ceric ammonium sulphate reagent. The studies showed that AA-CRL-6 and AA-CRL-20 produced vinca alkaloids in the culture. TLC analysis confirmed the identification of vinca alkaloids as vinblastine and vincristine. In addition to vinblastine and vincristine, three vinca

alkaloids present in the crude extract of AA-CRL-6 and four also present in the crude extract of AA-CRL-20 as evident from TLC could not be identified.

Discussion:

Studies on the isolation of vinca alkaloids produced by endophytic fungi (AA-CRL-6 and AA-CRL-20) of *Catharanthus roseus* show that AA-CRL-6 and AA-CRL-20 produce vinblastine and vincristine *in vitro*. TLC analysis of the extracts from culture filtrate of AA-CRL-6 and AA-CRL-20 confirm this conclusion. In addition to these alkaloids, the fungus AA-CRL-6 also produces three unidentified alkaloids and AA-CRL-20 produces four unidentified alkaloids though in traces. Kharwar *et al.* (2008) isolated several endophytic fungi from *Catharanthus roseus* from India but to the best of my knowledge there is so far no report of isolation of these drugs from the above endophytes[11]. Zahng *et al.* (2000) and Tung *et al.* (2002) discovered that vincristine is produced by *Fusarium oxysporum*[12, 13], an endophyte of *Catharanthus roseus;* while Guo and Kunming (1998) isolated vinblastine from *Alternaria sp*. isolated from *Catharnthus roseus*[14.] However, they have identified the production of vinblastine and vincristine by TLC and HPLC only[15-20]. To the best of our knowledge, this is the first report of isolation of vinblastine and vincristine from endophytic fungi isolated from India. However, these alkaloids are commonly found in *Catharanthus roseus* plant in traces. These observations support present findings. This suggests that the ability to synthesize vinblastine and vincristine derivatives resides not only in the *Catharanthus roseus* plant, *Fusarium oxysporum* and *Alternaria* sp. but also in endophytic fungi of *Catharanthus roseus* isolated by us.

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Chapter 4

Cultural, morphological and molecular characteristics of vinca alkaloids producing endophytic fungi of *Catharanthus roseus*

Summary:

For studying the cultural and morphological characters, the endophytic fungi were grown on PDA medium. Cultural characters such as colour and nature of the growth of the colony were determined by visual observation. Morphological characters of the fungus like mycelia, conidiophores and conidia were microscopically studied (Carl Zeiss Axiovert 25 Inverted Microscope). Mycelia, conidiophores and conidia produced from the fungi in culture were examined under a microscope. The culture that produced vinblastine and vincristine were also identified using Internal Transcribed Spacer (ITS) sequence analysis.

Introduction:

The correct identification of fungi is of great practical importance not only in the industry but also in clinical pathology, biotechnology and environmental studies. The earliest system for fungal classification and identification up to species level relied on morphological characters, mainly those of reproductive structures, spore morphology and the manner in which the spores are produced, host range and secondary metabolites profile. However, this method of classification presents critical limitations such as sterility of fungal cultures that have not developed reproductive structures or morphological similarity among the members of different species [1].

In recent years, molecular biology techniques have provided newer tools for the correct identification of fungi up to species level. In fungi, intra specific genomic variations have also been studied by electrophoretic karyotyping and by the random amplified polymorphic DNA (RAPD) method which takes advantage of the polymerase chain reaction (PCR) technology. PCR has provided accessibility to many genes for study and has also provided an alternative to tedious and cumbersome cloning procedures. In future years, PCR technology can be expected to have significant and greater impact in fungal systematic studies. Application of RAPD using PCR technology for the efficient elimination of duplicate strains in microbial screening for metabolites has been advocated by Fujimori and Okada. Taking the example of *Trichoderma*, in which species are difficult to distinguish on the basis of morphological features alone, these authors showed that the electrophoretic band patterns of the PCR products gave results consistent with the morphological, physiological and ecological data on these strains. The electrophoretic band patterns of 74 strains of *Trichoderma* correlated well with their morphological, cultural properties, metabolite production profiles and ecological data. They concluded that RAPD is the simple, efficient and reliable method for the selection of fungal strains employed in screening for bioactive metabolites.

Ribosomal RNA (rRNA) and ribosomal genes (rDNA) have been studied for their usefulness in fungal systematics. It is accepted that ribosomal genes are well characterized, ubiquitous and easily accessible via PCR technology. In ribosomal DNA sequencing, intergeneric transcribed spacers (ITS) and intergeneric regions

(IGR) are more variable than the coding regions and these variable rDNA regions could offer valuable guidelines for characterizing and differentiating between two closely related species. It is possible that the ITS/IGR ratio could be suitable for designing species specific oligonucleotide probes for fungal identification. Based on DNA sequence analysis or internal transcribed spacers of ribosomal DNA, phylogenetic studies have been carried out with important plant pathogenic fungi such as *Phytophthora*[2] and *Puccinia*[3]. Lee *et al.,* have designed synthetic oligonucleotide probes from ITS sequence data to differentiate between different *Phytophthora* sp. Phylogenetic relationship among *Fusarium solani* and its *formae speciales* is identified on the basis of their morphological and molecular characterization (Lee Y.M). The endophytic fungal complex of stem, leaves and roots of *Catharanthus roseus* were isolated and identified by Kharwar *et al.,* (2008) by morphological and molecular basis. Since vinblastine and vincristine are being produced from the leaves of *Catharanthus roseus* plant, we have isolated 52 endophytic fungi only from the leaves of *Catharanthus roseus* plant. Two positive strains which are producing vinca alkaloids (vinblastine and vincristine) have been identified on the cultural, morphological and molecular basis.

Material:

Catharanthus roseus leaves, PDA , MGYP agar medium, CRLDAB, CRLDA, CMA, V8 juice agar , liquid nitrogen, CTAB buffer, proteinase K, RNase A, chloroform, isoamyl alcohol, ethanol, 0.8% agarose gel, Gel-red, Gel-Doc, ITS-1 and ITS-4 Primers (Bangalore Genei, India), dNTPs (Bangalore Genei, India), Taq Polymerase (Bangalore Genei, India), 1% agarose gel, applied Biosystems Veriti 96-well Thermal Cycler, Carl Zeiss Axiovert 25 inverted microscope, petri plate (10 cm dia.) and B.O.D. incubator.

Methods:

Microorganism:

AA-CRL-6 and AA-CRL-20 were isolated from *Catharanthus roseus* leaves and was maintained on potato dextrose agar (PDA) slants have optimum growth at pH 7.0 and temperature 27^{o}C. Sub culturing was done to maintain the stock cultures, at monthly intervals and preserved at 15^{o}C.

Cultural and morphological characters of the fungi:

For studying the cultural and morphological characters, the fungi were grown on PDA medium. Cultural characters such as color and nature of the growth of the colony were determined by visual observation. Morphological characteristics of the fungus like mycelia, conidiophores and conidia were microscopically studied (Carl Zeiss Axiovert 25 inverted microscope). Mycelia, conidiophores and conidia produced by the fungi in the culture were examined under a microscope[4].

Nutritional studies and factors affecting the growth and sporulaton of the endophytic fungi:

The fungi were grown on natural, semi synthetic and synthetic media. PDA, MGYP, *Catharanthus roseus* leaf decoction agar (CRLDA), *Catharanthus roseus* leaf decoction agar boiled (CRLDAB), Richard's medium were prepared as reported in the literature. Corn meal agar (CMA) and V8 juice agar were prepared as described by the manufacturer (Hi Media India)[5]. For studying the growth of the fungus on solid media, petriplate (10 cm dia.) poured with 25 ml of each medium were used. Each

Petri plate was inoculated in the centre with a mycelial disk (8 cm) cut from a sporulating 10 days old culture growing on PDA. The Petri plates were incubated at 25°C-27°C in B.O.D incubator. Observations on growth pattern and sporulaton were made after 10 days. Sporulaton of the fungus in solid media (PDA, MGYP Agar, Richard's, CRLDAB, CRLDA, CMA and V8 juices) was determined on the basis of number of spores present per field under uniform magnification. Data set comprising of the morphological characters based on previously published description is in Table-1 and Table-2. Information regarding shape, temperature, conidiophores, morphological characters of the colonies, average diameters of the colony are also listed in Table- 1 (AA-CRL-6) and in Table- 2 (AA-CRL-20).

Molecular characterization of fungal strains AA-CRL-6 and AA-CRL-20:

Isolation of genomic DNA:

The genomic DNA of the endophytic fungal strains was extracted according to the method described by Lodhi *et al*., (1994) with slight modifications[5, 6]. For the isolation of DNA, the fungus was grown in 500 ml Erlenmeyer flask containing 100 ml MGYP medium. The growth was initiated by inoculating spores from 7 days old PDA slant. The flasks were incubated on a rotary shaker (200 rpm) for 48 hr at 28°C. The contents were centrifuged at 8000 rpm for 15 min, washed repeatedly to remove the media constituents. 5 g of wet fungal mycelia was freeze-dried and also ground to powder form in liquid nitrogen, followed by addition of 8-10 ml of Cetyl Trimethyl Ammonium Bromide (CTAB) extraction buffer, pH 8 containing 0.2% β-mercaptoethanol. The composition of 2X CTAB extraction buffer was Tris-Base (100 mM; pH 8.0); CTAB (2% w/v); NaCl (1.4 M); EDTA (20 mM); PVP-40 (1-2%); β-mercaptoethanol (0.2-2%). After that 20 µl of proteinase K (20 mg/ml) was added and incubated at 65°C for 1 hr. This was followed by addition of 20 µl RNase A (10 mg/ml) and further incubation at 65°C for 15 min. To the supernatant collected after centrifugation (8000 rpm, 10 min), 10 ml chloroform: isoamylalcohol (24:1) was added. The mixture was shaken for 5 min and centrifuged at 10,000 rpm, 4°C for 15 min. Two volumes of CTAB precipitation buffer (1% CTAB; 50 mM Tris (pH 8.0); 10 mM EDTA), was added to the supernatant and kept at room temperature for 1 hr. The pellet collected after centrifugation was dissolved in 5 ml of chloroform: isoamylalcohol (24:1). Two volumes of absolute alcohol were added to the aqueous

phase to precipitate the DNA. DNA was pelleted and washed with 70% ethanol, air dried and dissolved in 0.5 ml of 0.1 M Tris EDTA buffer pH 8 and stored at 4°C temp. The quality of the isolated DNA was checked on 0.8 % agarose gel stained with Gel-red.

PCR amplification of ITS regions:

ITS regions from the fungal strains were amplified using PCR with a final reaction mixture volume of 15 µl containing 0.4 µl fungal DNA solution (40 ng), 10.5 µl 10X buffer, 4 µl (0.2 mM) dNTPs, 1 µl (1µM) each of the universal eukaryotic primers (forward primer) ITS1- TCCGTAGGTGAACCTGCGG, (reverse primer) ITS4- TCCTCCGCTTATTGATATGC (White *et al*., 1990) and 0.5 U/µl Taq polymerase. Thermocycling parameters were: for ITS region, initial denaturation at 95°C for 3 min, 36 cycles: 95°C for 30 sec, 52°C for 30 sec, 72°C for 1 min; final extension was at 72°C for 3 min. The resulting PCR products were analyzed on 1% agarose gel containing Gel-red [7-10].

DNA sequencing and sequence analysis:

PCR amplicons obtained using ITS1 and ITS4 were sequenced using Sanger's dideoxynucleotide chain termination method (Sanger *et al*., 1977) and was carried out at Chromous Biotech Pvt. Ltd. Bangalore, India. The most identical sequences of strains AA-CRL-6 and AA-CRL-20 were identified from data base of GenBank using nucleotide BLAST algorithm programme (Altuch *et al*., 1997). Multiple sequence alignment was performed for the sequences obtained from BLAST search using Clustal-W algorithm[9]. Phylogenetic analysis was carried out using DAMBE and MEGA4 [10-12]. The above methods were used to analyse the sequence of the fungus AA-CRL-6 and AA-CRL-20. The data generated was converted in to trees using neighbour joining method [11-14].

Results:

Cultural and morphological characters of endophytic fungi:

The fungus AA-CRL-6 grown on PDA medium produced slow growing colonies. Colonies on PDA showed the following characters: snow white, circular, flucculose, compact, reverse pale white, hyphae profusely branched, septate, smooth, hyaline, phialide produced from short lateral hyphae[15]. The strain also produced three types of spores called macroconidia, microconidia and chlamydospores. Macroconidia, fusoid, with prominent foot cell, 3-septate, hyaline, fewer in number than microconidia. Microconidia are produced from short lateral phialide. Microconidia are oval to cylindrical, straight to curved, variable in shape and size, produced abundantly and single celled. Chlamydospores formed after 10-15 days, produced from lateral hyphae, globose to sub globose, hyaline, smooth or rough walled, terminal, form singly (Fig. 1)[16]. Most of the morphological characters of the strain (AA-CRL-6) agree with the known features of *Fusarium oxysporum.*

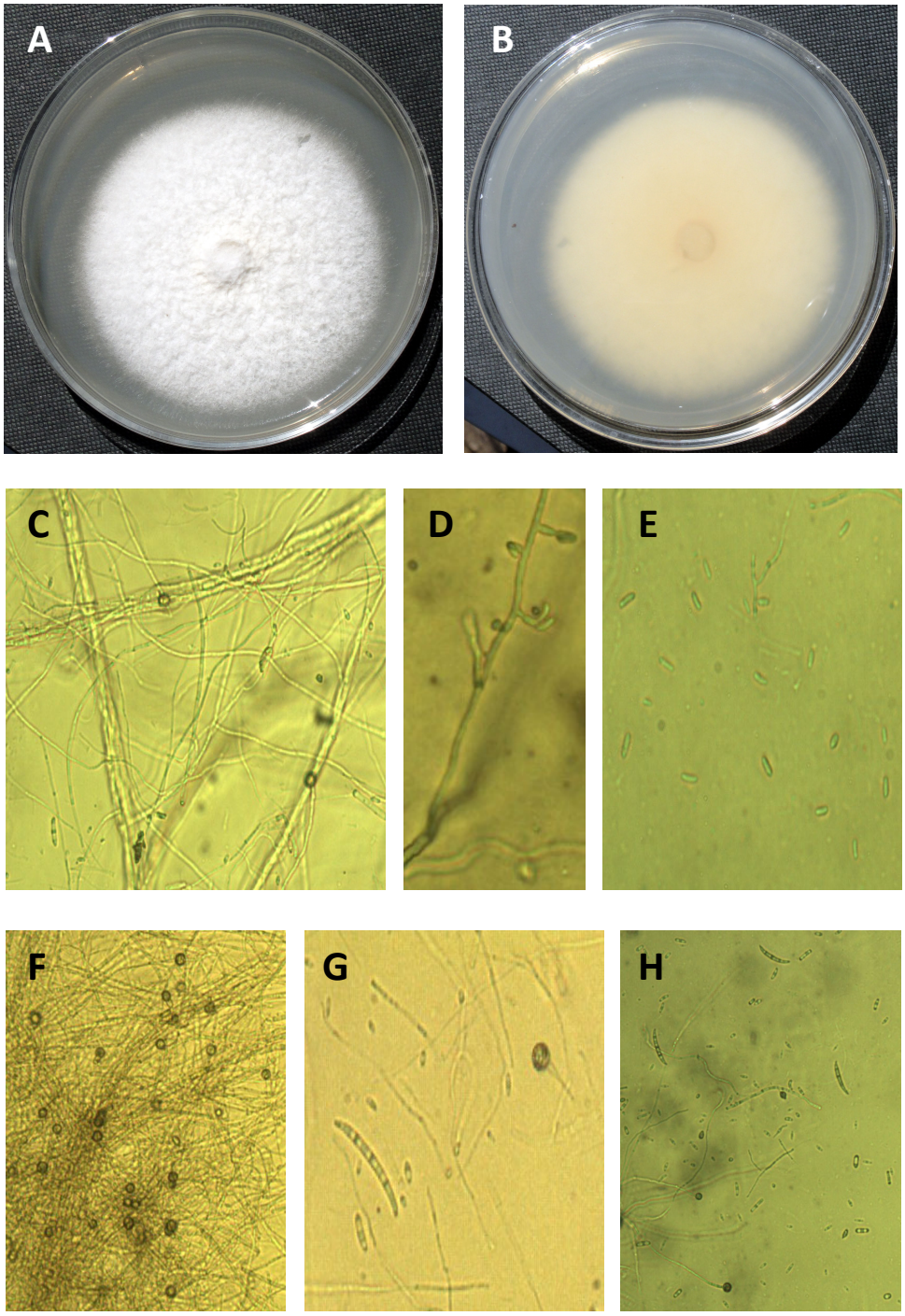

Fig. 1: Morphological features of AA-CRL-6. The fungal strain was grown on PDA for microscopic observation. Colony shape: front (A) and back (B) side of a PDA plate. Mycelium(C), Microconidiophores with microconidia (D), Microconidia (E), Chlamydospores (F) formed on PDA. Macroconidia, microconidia and chlamydospores formed on PDA (G), Macroconidia and microconidia formed on CMA (H).

The other positive strain AA-CRL-20 isolated and grown on PDA medium produced slow growing colonies. Colonies on PDA showed the following characters: white, circular, flucculose, compact and raised, reverse light brown, hyphae profusely branched, septate, smooth, hyaline, phialide arising laterally on hyphae[17]. Produced three types of spores called macroconidia, microconidia and chlamydospores. Macroconidia are produced from lateral phialide, 3-septate, wedge shape, hyaline large in number. Microconidia are produced from lateral long slender phialide accumulated on terminal end of phialides, variable in shape and size, unicellular, oval or kidney shaped, hyaline. Chlamydospores are formed singly or in pairs or chains, subglobose with a smooth or wrinkled wall (Fig. 2)[18]. Most of the morphological characters of the strain (AA-CRL-20) agree with the known features of *Fusarium solani.*

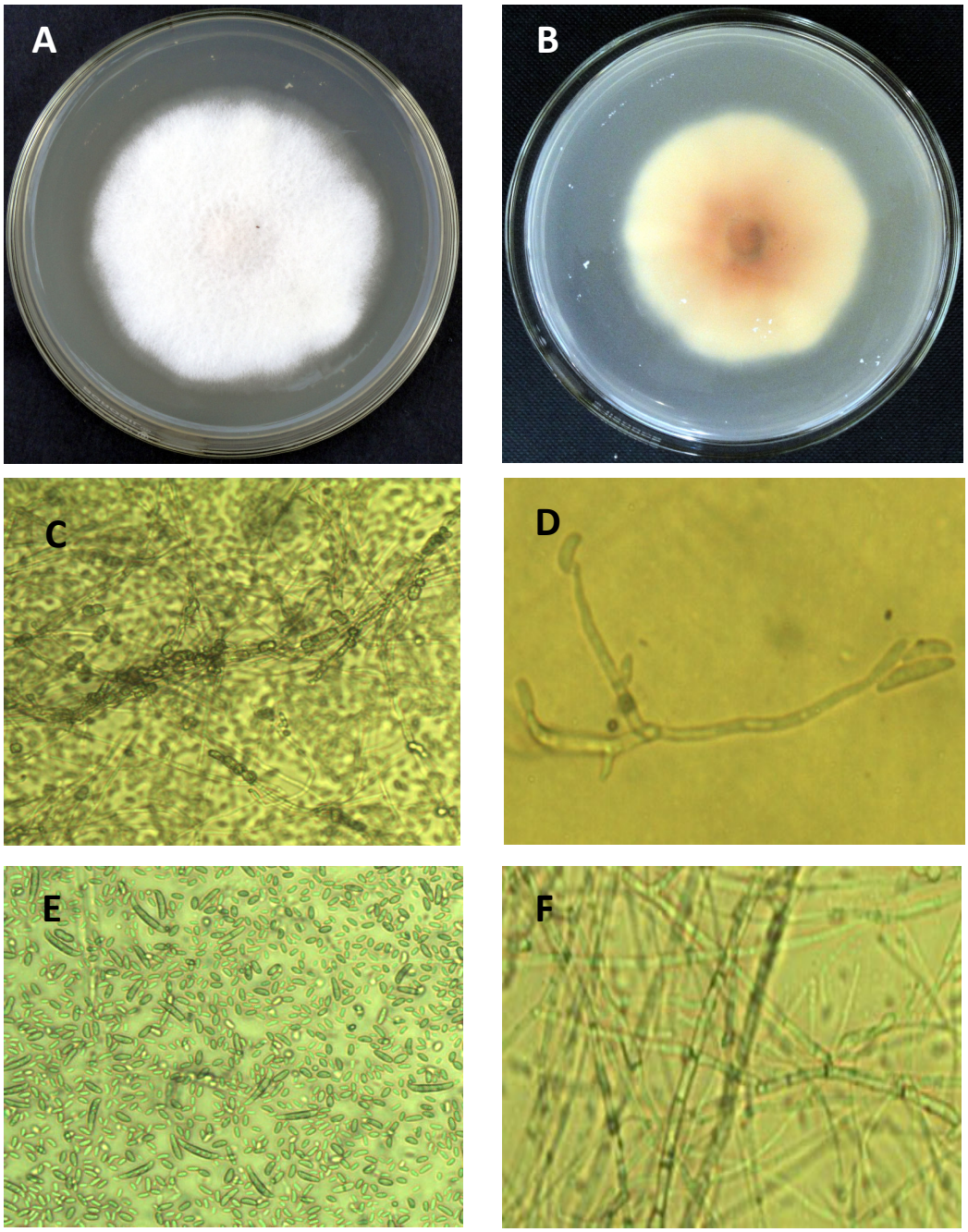

Fig. 2: Morphological features of AA-CRL-20. The fungal strain was grown on PDA for microscopic observation. Colony shape: front (A) and back (B) side of a PDA plate. Mycelium and chlamydospores (C) formed on PDA, Microconidiophores and macro conidiophores along with micro conidia (D), Macroconidia and Microconidia (E) formed on PDA, Mycelium formed on V8 Juice medium (F).

Effect of various nutrient media on the growth and sporulation:

The fungus AA-CRL-6 could not showed significant variation in growth rate when grown on various, natural, semi-synthetic and synthetic media. The results are presented in Table- 1 and Fig.3. Results showed that the fungus can be grown on a variety of media. Among the seven different media tested, it was found that the Richards media was the best for the growth of the fungus followed by MGYP agar and PDA. V8 juice also gave good growth. However, poor growth was recorded in CMA, CRLDAB and CRLDA. The diameter of the fungus on CMA, CRLDAB and CRLDA was very close to PDA and MGYP agar medium. But the mycelial growth is sparse in CMA, CRLDAB and CRLDA. The strain produced macroconidia, microconidia and chlamydospores in PDA, MGYP, CMA, CRLDAB and CRLDA. But on Richards's medium and V8 juice the strain produce only microconidia and chlamydospres but no macroconidia.

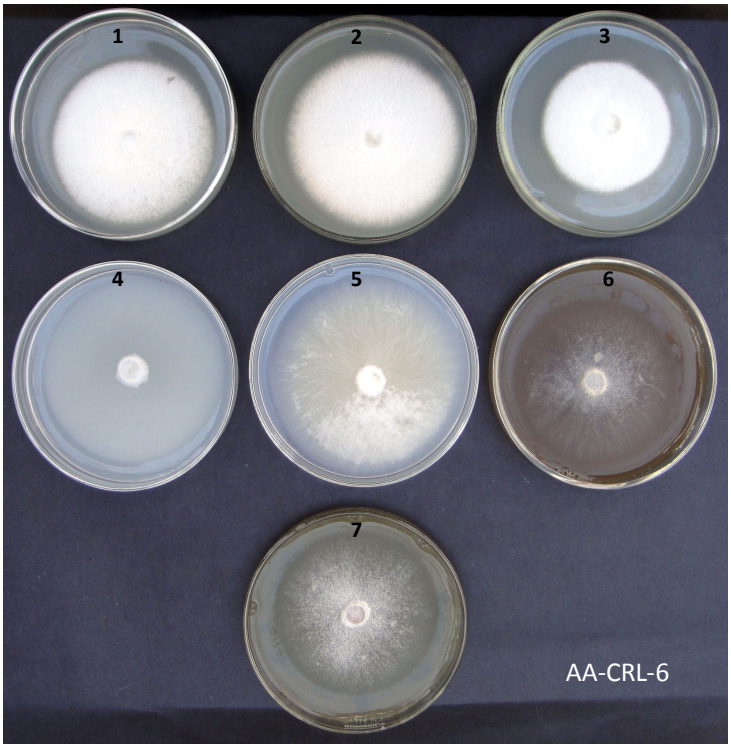

Fig 3: Effect of various nutrient media (1: PDA, 2: MGYP, 3: V-8 juice, 4: CMA, 5: Richard's medium, 6: CRLDAB, 7: CRLDA.) on the growth and sporulation of the fungus AA-CRL-6 after 10 days.

The fungus AA-CRL-20 could not showed significant variation in growth rate when grown on various, natural, semi-synthetic and synthetic media. The results are presented in Table- 2 and Fig. 4. Results showed that the fungus can be grown on a variety of media. Among the seven different media tested, it was found that the Richard's medium was the best for the growth of the fungus followed by PDA and MGYP. V8 juice also gave good growth. However, poor growth was recorded in CMA, CRLDAB and CRLDA media. The diameter of the fungus on CMA, CRLDAB and CRLDA was very close to PDA and MGYP medium. But the mycelial growth was sparse in CMA, CRLDAB and CRLDA. The strain produced macroconidia, micro conidia and chlamydospores in PDA, MGYP, V8 juice and CMA. But on Richards's medium, CRLDAB and CRLDA the strain produce only microconidia and chlamydospres but no macroconidia.

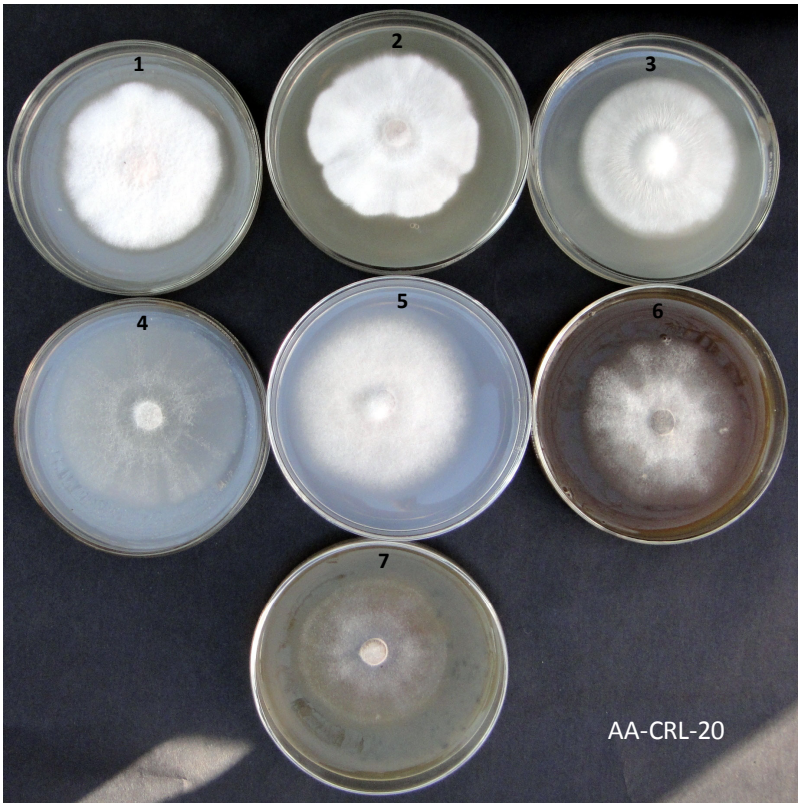

Fig 4: Effect of various nutrient media (1: PDA, 2: MGYP: V8 juice, 4: CMA, 5: Richard's medium, 6: CRLDAB, 7: CRLDA) on the growth and sporulation of the fungus AA-CRL-20 after 10 days.

Morphological Characteristics of Fungus AA-CRL-6:

Characters	PDA	MGYP	V8 Juice	CMA	Richard's Media	CRLDAB	CRLDA
Average diameter (mm) of the colonies after 10 day.	76	80	59	75	82	70	71
Morphological characters of the colony	snow white, circular, flucculose, compact.	white, circular, flocculose, compact and raised.	snow white, circular, compact.	submerged , circular, sparse.	white, circular, not so compact and slightly raised.	ash coloured, circular, not so compact.	ash coloured, circular, not so compact.
Colony color	white in front and pale white on reverse.	white in front and pale white on reverse.	white in front and on reverse.	opaque in front and on reverse.	white in front and pale white on reverse.	ash coloured in front and dark on reverse.	ash coloured in front and dark on reverse.
Macro conidia	few	few	absent	many	absent	few	few
Micro conidia	many	many	few	abundant	few	abundant	abundant
Chalamydospore	abundant	many	many	abundant	few	abundant	abundant
Optimum temperature for growth	25-27^{o}C	25-27^{o}C	25-27^{o}C	25-27^{o}C	25-27^{o}C	25-27^{o}C	25-27^{o}C

Table 1: Growth and morphological characters of the colony of fungus AA-CRL-6

Morphological–– Characteristics of Fungus AA-CRL-20:

Characters	PDA	MGYP	V8 Juice	CMA	Richard's Media	CRLDBA	CRLDA
Average diameter (mm) of the colonies after 10 day.	68	66	65	70	71	63	61
Morphological characters of the colony	white, slightly wavy, compact and raised.	white, wavy, compact.	white, circular and compact.	white, circular, less compact.	snow white, circular and compact.	ash coloured, circular and not so compact.	ash coloured, circular and not so compact.
Colony color	white in front and light brown on reverse.	white in front and pale white on reverse.	white in front and on reverse.	white in front and pale white on reverse.	white in front and light brown on reverse.	ash coloured in front and dark on reverse.	ash coloured in front and dark on reverse.
Macroconidia	few	few	few	many	absent	absent	absent
Microconidia	abundant	abundant	abundant	abundant	abundant	abundant	abundant
Chlamydospore	abundant	abundant	abundant	abundant	abundant	abundant	abundant
Optimum temperature for growth	25-27^{o}C	25-27^{o}C	25-27^{o}C	25-27^{o}C	25-27^{o}C	25-27^{o}C	25-27^{o}C

Table 2: Growth and morphological characters of the colony of fungus AA-CRL-20

Genomic DNA, PCR amplification and Sequence analysis:

The method of Lodhi *et al.,* used for the genomic DNA isolation resulted in obtaining high quality DNA (Fig. 5). Universal eukaryotic primers (ITS1 and ITS4) used for the amplification of ITS regions successfully amplified fungal genomic DNA producing fragments of 0.48 kb of AA-CRL-6 and 0.52 kb of AA-CRL-20 (Fig. 6). Sequence analysis was done using ChromasV 2.0 program and the sequence profile was obtained in FASTA format (Seq. 1- AA-CRL-6, Seq. 2 AA-CRL-20). BLAST analysis of the sequences determined the identical sequences from the data base. Majority of the hits from BLAST search were from *Fusarium oxysporum* for endophytic strain AA-CRL-6 and *Fusarium solani* for endophytic strain of AA-CRL-20. The ITS sequences showed 98% similarity of AA-CRL-6 with *Fusarium oxysporum* and 100% similarity of AA-CRL-20 with *Fusarium solani*. The sequence analysis of AA-CRL-6 determined that the 487 bps fragment consist of a partial ITS1 region at 5' end (1 to 119 bps), 5.8 S ribosomal RNA (120 to 275 bps), ITS2 (276 to 429 bps) and partial 28S ribosomal RNA (430 to 487 bps) at 3' end. The sequence analysis of AA-CRL-20 determined that the 523 bps fragment consist of a partial ITS1 region at 5' end (1 to 135 bps), 5.8 S ribosomal RNA (136 to 293 bps), ITS2 (294 to 465 bps) and partial 28S ribosomal RNA (466 to 523 bps) at 3' end. All the sequences were aligned using CLUSTAL-W programme and the homology between the sequences was determined.

Phylogenetic analysis of the sequences:

The phylogenetic trees were constructed using MEGA 4 software employing neighbour joining method. The analyses were carried out using ITS sequence of fungal isolates AA-CRL-6 and AA-CRL-20 respectively. Fig. 7 and Fig. 8 shows phylogenetic analysis of AA-CRL-6 and AA-CRL-20 fungal isolate respectively. The analysis was done separately for each isolate. Sequence homology alignment showed 98% sequence similarity of AA-CRL-6 strain with *Fusarium oxysporum* and 100% sequence similarity of AA-CRL-20 strain with *Fusarium solani*. The phylogenetic analyses confirmed that fungal strain AA-CRL-6 is closely related with *Fusarium oxysporum*, while isolate AA-CRL-20 is closely related with *Fusarium solani.*

Genomic DNA of endophytic fungus AA-CRL-6 and AA-CRL-20:

A B

A: AA-CRL-20 B: AA-CRL-6

Fig. 5: Genomic DNA of endophytic fungus AA-CRL-6 and AA-CRL-20 on 0.8 % agarose gel.

PCR Amplification and Sequencing of ITS Gene:

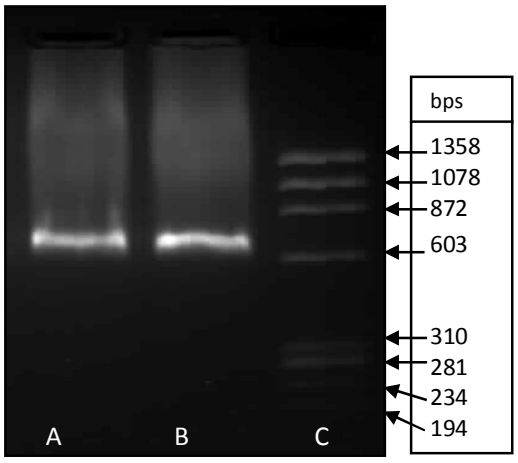

Fig.6: Lane A: 0.52 kb PCR amplicon obtained from AA-CRL-20, Lane B: 0.48 kb of AA-CRL-6 using ITS1 and ITS4 and Lane C low range standard molecular weight marker (Φx174).

ITS1 regions of AA-CRL-6:

>AA-CRL-6_ITS1

ACTTCCACTTGTTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGAGCC

CGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCAT

AAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCATGAAGAA

CGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGATCATCGAAT

CTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGC

GTCATTTCAACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGT

TCCTCAAATTGATTGGCGGTCACGTCGAGCTTCCATGCGTAGTAGTAAAACCC

TCGTTACTGGTAATCGTCGCGGCCACAAAAGTTAAACCCCAACTTCTGAATGT

TGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGATATCAATAAGCGGA

GGAA

Seq.1: Corresponding regions in ITS1 amplified PCR amplicon of AA-CRL-6.

Internal transcribed spacer 1: 1....119 bps
5.8S ribosomal RNA: 120....275 bps
Internal transcribed spacer 2: 276....429 bps
28S ribosomal RNA: 430....487 bps

ITS1 regions of AA-CRL- 20:

>AA-CRL-20_ITS1

TCAACCCTGTGAACATACCTAAACGTTGCCTCGGCGGGAACAGACGGCCCGTG

AAAACGGGCCGCCCCCGCCAGAGGACCCCCTAACTCTGTTTCTATAATGTTTC

TTCTGAGTAAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGC

TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA

ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGG

CGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGT

TGGGGATCGGCGGAGGCCCTCCGTGGGCACACGCCGTCCCCCAAATACAGTGG

CGGTCCCGCCGCAGCTTCCATCGCGTAGTAGCTAACACCTCGCGACTGGAGAG

CGGCGCGGCCACGCCGTAAAACCCCCAACTCTTCTGAAGTTGACCTCGAATCA

GGTAGGAATACCCGCTGAACTTAAGCATATCAAAAGCGGAGGAA

Seq.2: Corresponding regions in ITS1 amplified PCR amplicon of AA-CRL.20

Internal transcribed spacer 1: 1....135 bps
5.8S ribosomal RNA: 136....293 bps
Internal transcribed spacer 2: 294....465 bps
28S ribosomal RNA: 466....523 bps

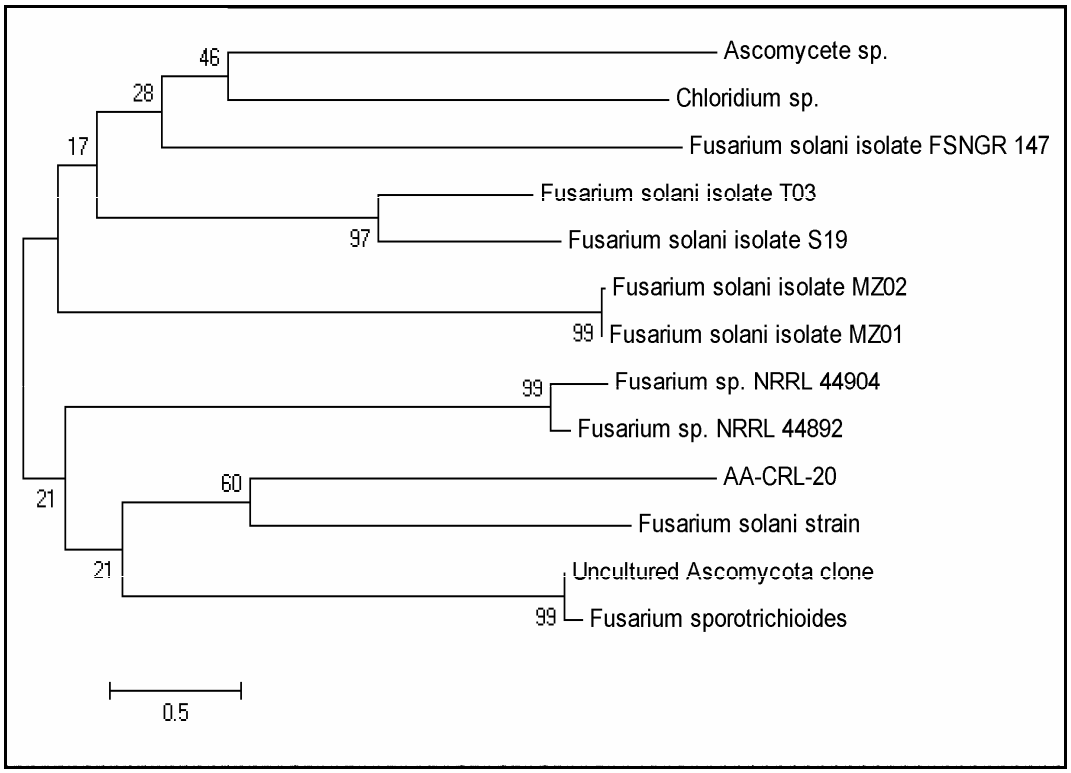

Fig. 7: Phylogenetic relationships of selected members with fungus AA-CRL-20.

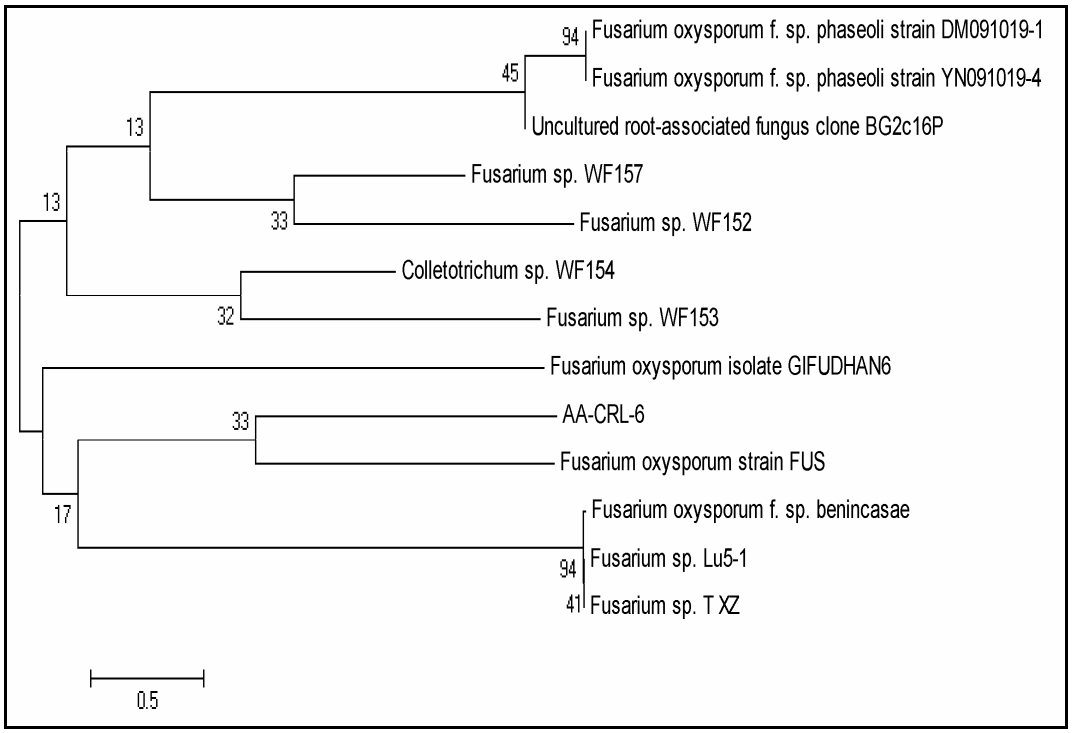

Fig 8: Phylogenetic relationships of selected members with fungus AA-CRL-6.

Discussion:

Morphology of AA-CRL-6 and AA-ARL-20 isolate obtained from *Catharanthus roseus* mostly agree with the description of Nelson et al, 1983. The fungus AA-CRL-6 grown on PDA medium produce slow growing colonies. Colonies on PDA show the following characters: snow white, circular, flucculose, compact, reverse pale white, hyphae profusely branched, septate, smooth, hyaline, phialide produced from short lateral hyphae. The strain also produces three types of spores called macroconidia, microconidia and chlamydospores. Macroconidia, fusoid, with prominent foot cell, 3-septate, hyaline, fewer in number than microconidia. Microconidia, produce from short lateral phialide. Microconidia are oval to cylindrical straight to curved, variable in shape and size produce abundantly and single celled. Chlamydospores form after 10-15 days, produce from lateral hyphae, globose to sub globose, hyaline, smooth or rough walled, terminal, form singly (Fig. 1)

The other positive strain AA-CRL-20 isolated and grown on PDA medium produce slow growing colonies. Colonies on PDA show the following characters: white, circular, flucculose, compact and raised, reverse light brown, hyphae profusely branched, septate, smooth, hyaline, phialide arising laterally on hyphae (ref) produce three types of spores called macroconidia, microconidia and chlamydospores. Macroconidia produce from lateral phialide, 3-septate, wedge shape, hyaline large in number. Microconidia, produced from lateral long slender phialide accumulate on terminal end of phialides, variable in shape and size, unicellular, oval or kidney shaped, hyaline. Chlamydospores form singly or in pairs or in chains, sub-globose with a smooth or wrinkled wall (Fig. 2)

All of the above mentioned characters are identical to those described for the fungus, *Fusarium* sp. of fungal strain AA-CRL-6 and *Fusarium solani* of fungal strain AA-CRL-20. Hence fungus AA-CRL-6 can be referred as *Fusarium* sp. and AA-CRL-20 *Fusarium solani*. Studies on the different media on the growth and sporulation of *Fusarium* sp. and *Fusarium solani* have been studied. Studies indicate that fungi can be grown on media such as natural, synthetic and semi-synthetic.

The ITS sequence analysis and homology alignment of isolate AA-CRL-6 using BLAST and CLUSTAL-W programme respectively revealed highest similarity with genus *Fusarim oxysporum* The phylogenetic studies also indicate that the closest relative of fungus AA-CRL-6 is *Fusarium oxysporum.* Therefore, considering the cultural and morphological characters, we identified the fungus as *Fusarium oxysporum*. The sequence analysis and phylogentic study do support the identification. Similarly, sequence analysis and homology alignment of isolate AA-CRL-20 using BLAST and Clustal-W programme respectively showed highest similarity with the fungus *Fusarium solani*. The phylogenetic studies also conclude that the closest relative of fungus AA-CRL-20 is *Fusarium solani*. Considering the cultural and morphological characters, we identified the fungus as *Fusarium solani*[22]. The sequence analysis and phylogenetic studies do support the identification.

Although *Fusarium* sp and *Fusarium solani* has widespread occurrence in plant and soil across India and different endophytic fungi have been isolated from Indian *Catharanthus roseus*, but no attempts have been made to isolate vinca alkaloids from above endophytic fungi. Zahng *et al.,* (2000) and Tung *et al.,* (2002) discovered that vincristine is produced by *Fusarium oxysporum*, an endophyte of *Catharanthus roseus;* while Guo and Kunming (1998) isolated vinblastine from *Alternaria* sp. isolated from *Catharanthus roseus*. However they have showed the production of vinblastine and vincristine by TLC and HPLC only. The *Fusarium oxysporum* which we isolated produce both vinblastine and vincristine. The isolation of vinblastine and vincristine from culture filtrate of *Fusarium oxysporum* and *Fusarium solani* is the first demonstration of this occurring in fungi isolated from Indian *Catharanthus roseus* plant. Thus suggests the ability to synthesize vinblastine and vincristine resides not only in the fungal strain isolated from *Catharanthus roseus* of above mentioned countries, but also from endophytic fungi of *Catharanthus roseus* growing in India. In conclusion, we have isolated endophytic fungi such as *Fusarium oxysporum* and *Fusarium solani* producing vinblastine and vincristine. Since the quantity of vinblastine and vincristine is higher in *Fusarium oxysporum* as compared to *Fusarium solani*, therefore further work carried out only on *Fusarium oxysporum*.

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Chapter 5

Isolation, purification, characterization and bioassays of vinblastine

Summary:

Endophytic fungi were isolated from the leaves of *Catharanthus roseus* obtained from different areas of Pune and then screened for vinblastine production. Out of the 52 fungal cultures screened, two endophytic fungi were found to produce vinblastine. The vinblastine was purified by TLC and HPLC and characterized using UV-spectroscopy, ESI-MS, MS/MS, and proton NMR. One litre of fungal culture yielded 76 µg of vinblastine. The purified vinblastine from the fungus showed cytotoxicity towards cancer lines HL-60 (leukemia), J774 and THP1.

Introduction:

The vinca alkaloids have been widely used as chemotherapeutic agents for the treatment of both childhood and adult malignancies. The vinca alkaoids were initially isolated from dried leaves of periwinkle plants, *Catharanthus roseus*, formerly called *Vinca rosea.* The vinca alkaloids induce cytotoxicity by binding directly to tubulin and inhibiting microtubule polymerization. This results in impaired microtubule dynamics and mitotic spindle formation, leading to cell death[1]. The vinca alkaloids are currently used in a broad spectrum of malignancies, including both solid tumors and hematological malignancies. The most important alkaloids of *Catharanthus roseus* which have been found to have pharmacological activity and clinical application as anticancer agents are vincaleukoblastine (vinblastine) and vincristine. Vinblastine was first isolated by Robert Noble and Charles Thomas Beer from the Madagascar periwinkle plant[2]. Vinblastine's utility as a chemotherapeutic agent was first discovered when it was crushed into tea. Consumption of the tea led to a decreased number of white blood cells; therefore, it was hypothesized that vinblastine might be effective against cancers of the white blood cells such as lymphoma. The actual content of vinblastine in leaf is very small. Average content of vinblastine is 0.002%[3]. However, in a few exceptional cases alkaloid content upto 0.01% of vinblastine has been reported. Vinblastine sulphate, sold as velbane is used principally for the treatment of Hodgkin's disease, and is also used against lymphosarcoma, choriocarcinoma, neuroblastoma, and carcinoma of breasts, lungs and testes. It is also used against leukemia, especially monocytic variety. Presently the clinical supplies of vinblastine and related drugs are derived from natural sources only. The low yields of this alkaloid in plant, combined with their high market price has encouraged intense research for alternative methods for the production of this alkaloid, such as total synthesis [4, 5] or semi-synthesis [6] of cell and tissue culture [7, 8.] The prospects for production of alkaloids by plant cell culture have been reviewed. However, the most valuable components of dimeric alkaloid, vinblastine have so far only been detected in callus or organ culture[9]. Productivity of vinblastine is very low in plants resulting in their extra ordinarily high price. Efforts to explore biotechnological options for the production of vinblastine *in vitro* have also not been successful yet[10]. Pharmaceutical industries are interested in getting plant based drugs from a microbial source in order to get rid of geographical and political barriers as well as from environmental

conditions. From a practical point of view, microbial fermentation as a means of producing drug or a bioactive substance has several advantages.

Fungi are one of the major sources of natural bioactive molecules. Over 4000 bioactive metabolites of fungal origin have been described. In some cases, plant-associated fungi are able to make the same bioactive compounds as the host plant itself. One of the best examples is the discovery of gibberellins from *Fusarium fujikuroi* in the early 1930's. The pathways of gibberellin biosynthesis in the fungus and higher plants are identical upto GA12, suggesting the possibility of intergeneric genetic exchange between plant and fungus. This observation led to the prospect that endophytic fungi, associated with *Taxus brevifolia*, may produce Taxol and was confirmed by Strobel *et al* (1993). Thus the success of finding fungal taxol prompted us to isolate endophytic fungi from *Catharanthus roseus* to produce low volume and high valued drugs, vinblastine and vincristine. Potentially, a fungal source would reduce the price of the above life-saving drugs and also save the plant from extinction in some areas. We have identified fungal (endophytic) strains which produce vinblastine and vincristine, purified with TLC, HPLC and characterized by UV- VIS, ESI-MS and ^{1}HNMR.

Materials:

Erlenmeyer flasks (500 ml), PDA media, MGYP medium, rotary shaker, vinca alkaloids media (VM-1), muslin cloth, lyophilizer, liquid nitrogen, ethyl acetate, anhydrous sodium sulphate, rotavapour, TLC (0.5 mm thick, 20cm x 20cm) silica plate, chloroform, methanol, ethyl acetate, ceric ammonium sulphate reagent, vinblastine (Sigma-Aldrich), silica gel column, HPLC (Waters), C18 symmetry column, HPLC grade acetonitrile, MilliQ water, trifluoroacetic acid, Shimadzu PC 101 Spectrophotometer, HPLC grade methanol, M/S applied Biosystems APIQSTAR pulsar (ESI-MS) mass spectrophotometer, HPLC Grade acetic acid, Bruker AV 400FT spectrometer, $CDCl_3$ solvent, HL-60, J774 and THP-1 cell lines, 10% DMSO, Centrifuge, trypsin solution, Carl Zeiss Axiovert 25 Inverted Microscope, MTT reagent, isopropanol, Beckman Coulter spectrophotometer, micro titer plates, RPMI-1640, MEM medium and incubator.

Methods:

The fungus, *Fusarium oxysporum* maintained on potato dextrose agar (PDA) slants have optimum growth at pH 7.0 and temperature 27^{o}C. Sub culturing was done to maintain the stock cultures, at monthly intervals and preserved at 15^{o}C. Starting materials for fermentation experiments were taken from an actively growing stock culture, which were subcultured on fresh slants and incubated for 7 days at pH 7.0 and temperature 27^{o}C.

Isolation, purification and characterization of vinblastine from the endophytic fungus *Fusarium oxysporum*:

A two stage fermentation procedure was employed for the isolation of vinblastine by *Fusarium oxysporum*. In the first stage, 500 ml Erlenmeyer flasks containing 100 ml medium (MGYP) were inoculated with 7 days old culture and incubated at 28^{o}C on a rotary shaker (240 rpm) for 4-5 days, which was used as seed culture (I stage). Later, 10 ml seed culture was transferred to 500 ml Erlenmeyer flask containing 100 ml production medium called as vinca media-1 (VM-1) which were incubated at 28^{o}C for 20 days as shake culture (II stage), after which it was harvested and used for further study.

Culture filtrates and mycelia were separated with the help of muslin cloth and then lyophilized. Lyophilized culture filtrate was extracted using ethyl acetate as a solvent system[11]. The organic layer was separated from the aqueous layer using separating funnel. The extraction was repeated thrice and the solvent was dried using anhydrous sodium sulphate and concentrated under vacuum using rotavapour at 40^{o}C in order to get crude extract. A small amount of crude extract was dissolved in ethyl acetate and subjected to thin layer chromatography (TLC) on silica gel-G (0.5 mm thickness) using chloroform: methanol (8:2) as a solvent system. The TLC plates were sprayed with ceric ammonium sulphate reagent. Vinca alkaloids spots produced brilliant violet color as well as purple color with above spraying reagent.

Purification of fungal vinblastine was done by silica gel column chromatography. The crude extract was loaded on silica gel column (60-120 mesh size, 40 cm x 2 cm length width) pre-equilibrated with chloroform and eluted with a gradient of chloroform: methanol (100% chloroform, 9:1, 8:2, 7:3, 1:1 and 3:7 and 100% methanol). Fractions containing compounds with Rf values similar to that of the standard vinblastine were pooled and subjected to preparative TLC on a 0.5 mm thick (20 cm x 20 cm) silica plate and developed in chloroform: methanol (8:2) solvent system. The putative band of fungal vinblastine was scraped and eluted out with methanol [12]. Purity of the isolated compound was checked on TLC in the following solvent systems such as (a) chloroform: methanol (8:2) (b) chloroform: methanol (9:1) and (c) ethyl acetate: acetonitrile (8:2) [12].

HPLC and Spectroscopic analysis:

Purity of fungal vinblastine was determined by HPLC using C18 symmetry column (Waters) [11, 13, 14]. Sample was taken in 40 µl acetonitrile, injected in HPLC column and gradient elution was performed using 5% - 95% acetonitrile with 0.01% trifluoroacetic acid at flow rate of 0.5 ml /min. A dual wavelength recorder set at 220 nm and 254 nm was used to detect the compound eluting from column[13, 15-19]. The absorption maximum of the purified compound was determined by Shimadzu PC 101 spectrophotometer. Sample was dissolved in HPLC grade methanol and spectral data was collected over 200-700 nm range.

ESI-MS and MS-MS analysis:

Molecular mass of the purified compound was determined by M/S applied Biosystems APIQSTAR pulsar (ESI-MS) mass spectrophotometer. Sample for the analysis was dissolved in HPLC grade methanol, water, acetic acid in the ratio of 50:50:0.1. Samples were then analyzed by infusion method (injected into MS) at flow rate of 5 µl/min and at an IS voltage of 3800V in TOF mode. Spectrum from a range of m/z 100-1400 Dalton was obtained [10, 17, 20, 21]. Fragmentation of the desired molecule was obtained by acquiring the product ion spectrum using MS-MS[10] with similar parameters as used for ESI-MS. Molecular ions of the standard vinblastine were also obtained for comparison.

Quantification of vinblastine by HPLC:

A 2 mg/ml concentration of a standard vinblastine solution was prepared in HPLC grade acetonitrile. 50 µl of the above standard solution (2 mg/ml) plus 950 µl of HPLC grade acetonitrile was taken to make the final concentration of 100 µg/ml. 10 µl of this solution was injected into HPLC and analysed. Similarly, in order to quantify vinblastine present in 1 litre of the culture filtrate, the culture filtrate obtained after 20 days was extracted and purified by HPLC. The purified fungal Vinblastine obtained was dissolved in 1 ml of HPLC grade acetonitrile and then 10 µl of this purified solution was injected into HPLC and analysed. The data of the area peak vs. concentration of the standard obtained was used to estimate the quantity of fungal vinblastine in per litre culture filtrate [13, 22].

Nuclear Magnetic Resonance (^{1}H NMR) analysis:

^{1}H NMR analysis was carried out on a Bruker AV 400 FT spectrometer operating at 400 MHz NOCR spectrometer. Sample was dissolved in $CDCl_3$ and scanned overnight**.** NMR spectrum was measured with a spectral width of 8223.68 Hz and an acquisition time of 1.818s and a relaxation delay of 1.0 s were used[9, 10, 23-25].

Cytotoxicity of purified vinblastine against cancerous cell lines:

General maintenance of HL-60, J774 and THP-1 cell lines:

HL-60, J774 and THP-1 cell lines were kept frozen (0.5 ml stock) at 2-5 x 10^6 cells/ ml in RPMI-1640, MEM and MEM (without FCS) respectively containing 10%

DMSO. Frozen stocks were thawed at 37°C and then diluted with fresh pre-warmed medium (RPMI-1640, MEM and MEM) to 10 ml (all supplements). Centrifugation was done for 10 mins at 1000 rpm and supernatant was discarded. Appropriate volume of fresh pre-warmed complete medium (RPMI-1640, MEM and MEM) was used for resuspension of centrifuged solvent and later seeded into culture flasks containing medium (RPMI-1640, MEM and MEM) (25 ml/80cm^2 flask). The approximate seeding ratio from frozen stock is one ampoule to two flasks. Replace medium every 3-4 days as required, so as to grow cells to 90% confluency. 10 ml trypsin solution was added per flask and flasks were left for 2-5 minutes at room temperature. Microscopic observation of rounding up of cells confirms the cell detachment. Approximately 8 ml of the trypsin was decanted off and cells were kept for further 5 minutes. Cells were dislodge the by tapping the flask gently. To each well, 10 µl litre of MTT reagent (5 mg /ml) was added. Then cells were stained for 20 hrs at 37°C. After that, 200 µl acidified isopropanol was added to solubilize the purple formazan crystal formed. Absorbance was measured at 490 nm with a Beckman Coulter spectrophotometer.

Cytotoxicity studies:

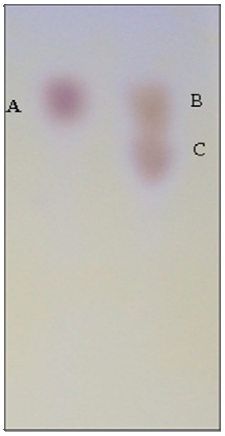
Cytotoxicity of the purified vinblastine, against cancerous cell lines HL-60 (Leukemia), J774 (Murine macrophages) and THP-1 (Human acute monocytic leukemia) was determined by MTT assay. The cell suspension at a concentration of 1x10^{4}cells/ml was added in 96 well microtiter plates. Culture media used for HL-60, J774 and THP-1 were RPMI-1640, MEM and MEM respectively. Plates containing culture media and test compound were incubated for 24 hrs for HL-60, 72 hours for J774 and also 72 hrs for THP-1 at 37°C, 5% v/v CO_2 and 95% humidity. All the samples were in triplicates. To each well, 10 µl of MTT reagent (5 mg/ml) was added and plate was incubated for 1 hr at 37°C. 200 µl of acidified isopropanol was added at the end of this period to stop the reaction and further incubation was done for 4 hrs to solubilize the purple formazan crystals produced. Beckman Coulter spectrophotometer was used to measure absorbance at 490 nm [26].

Results:

Vinblastine from culture filtrates:

The culture filtrates, when extracted with ethyl acetate yielded a brown residue after the removal of the solvent. The substance was named as crude extract. The crude extract, on TLC on silica gel G using chloroform: methanol (8:2) solvent system, produced five clearly differentiated brilliant purple, violet and three yellow colored spots when sprayed with ceric ammonium sulphate reagent (Fig.1, Ch.-3). The Rf value of the spots on TLC of the crude extract using chloroform: methanol (8:2) solvent system when compared with standard vinblastine and vincristine showed identical Rf value of two spots (Rf value 0.77 and 0.70) with vinblastine and vincristine. The spots of vinblastine and vincristine produced by crude extract showed identical color with the standards when sprayed with ceric ammonium sulphate reagent[27]. In addition to vinblastine and vincristine, two vinca alkaloids present in the crude extract as evident from TLC could not be identified.

For the purification of vinblastine from culture filtrate, the crude extract was fractionated on silica gel column with chloroform: methanol (100% chloroform, 9:1, 8:2, 7:3, 1:1 and 3:7 and 100% methanol). Partially purified vinblastine with chromatographic properties similar to that of the standard vinblastine (Fig.1) was obtained. The partially purified vinblastine on prep TLC yielded considerably pure compound. Partially purified vinblastine obtained from preparative TLC showed single dark purple spot that later turned to very dark purple (within Rf value 0.77) when sprayed with ceric ammonium sulphate reagent on TLC as shown in Fig.2[27]. The purity of the fungal vinblastine showed similar chromatographic properties as that of the standard vinblastine in three different solvent systems, a, b and c on TLC.



A**:** Standard vinblastine

B: Partially purified vinblastine

C: Partially purified vincristine

Detection: Ceric ammonium sulphate reagent

Fig.1: TLC of partial fungal vinblastine from culture filtrates along with standard vinblastine on silica gel using chloroform: methanol (8:2) solvent system.

A B

A: Purified fungal vinblastine

B: Standard vinblastine

Detection: Ceric ammonium sulphate reagent

Fig.2: TLC of fungal vinblastine purifed from culture filtrates along with standard vinblastine on silica gel using chloroform: methanol (8:2) solvent system.

HPLC and spectroscopic analysis:

The homogeneity of the purified compound was confirmed by HPLC analysis, which showed a single, symmetrical peak with RT 36.6 mins on C18 symmetry column (Fig.3). Absorbance of the eluting compound showed high intensity at 220 nm and relatively low at 254 nm. The UV absorption analysis showed a peak showing absorption at 220 nm. An absorbance maximum of standard vinblastine was also obtained for comparison (Fig.4).

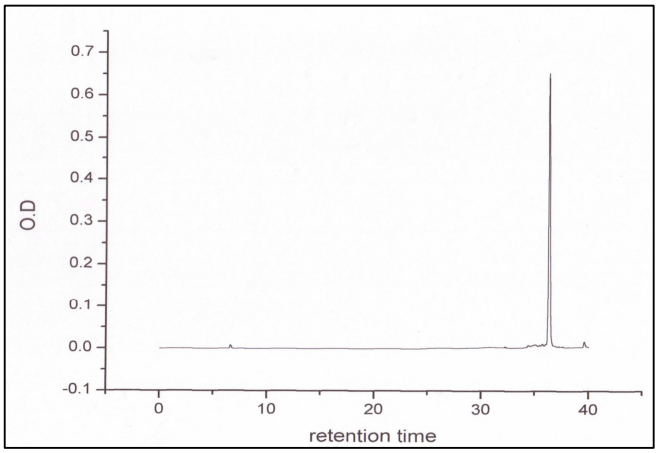


Fig.3: HPLC profile of pure fungal vinblastine with RT of 36.6 min.

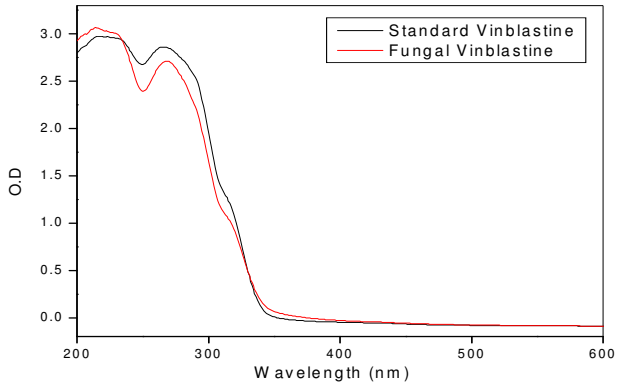


Fig.4: UV absorption spectrum of standard vinblastine and fungal vinblastine.

ESI-MS and MS-MS analysis:

Electrospray ionization mass spectrometry yielded a major ion at 811 (Fig.5).

In MS-MS fragment ion at m/z 355, 522, 542, 733, 751, 793 and 811 were seen.

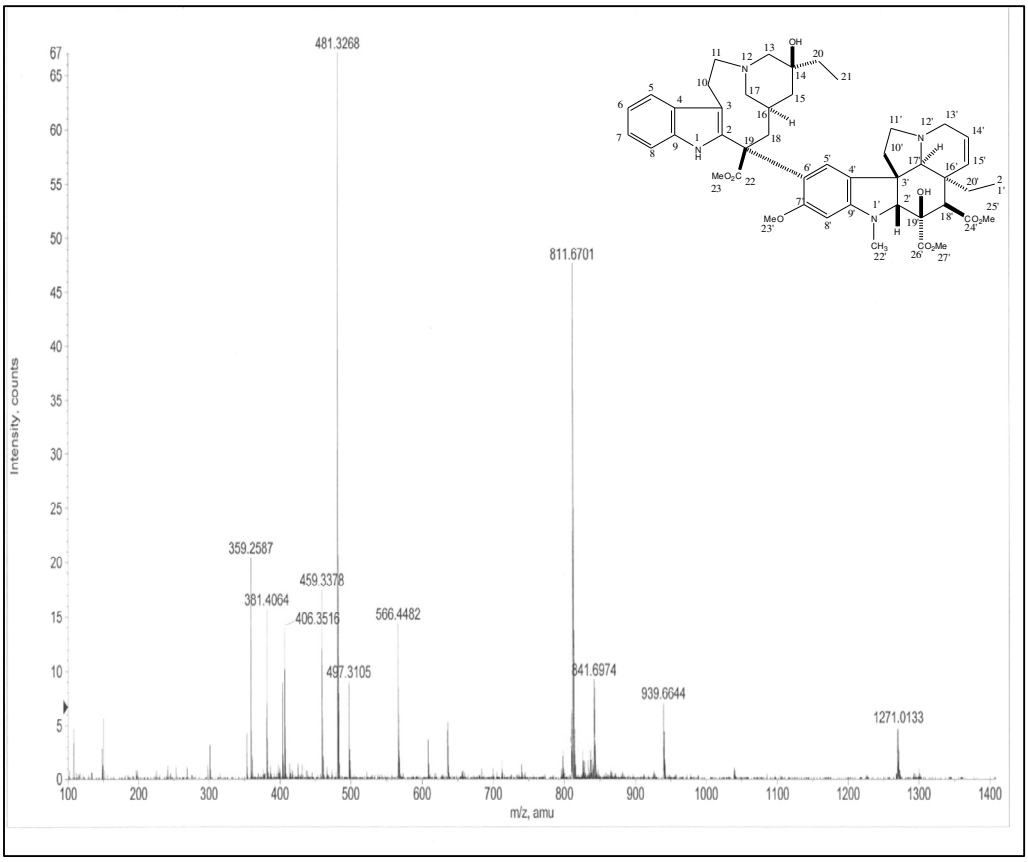


Fig.5: ESI-MS spectrum of m/z 811(M+H) ion of fungal vinblastine.

MS-MS:

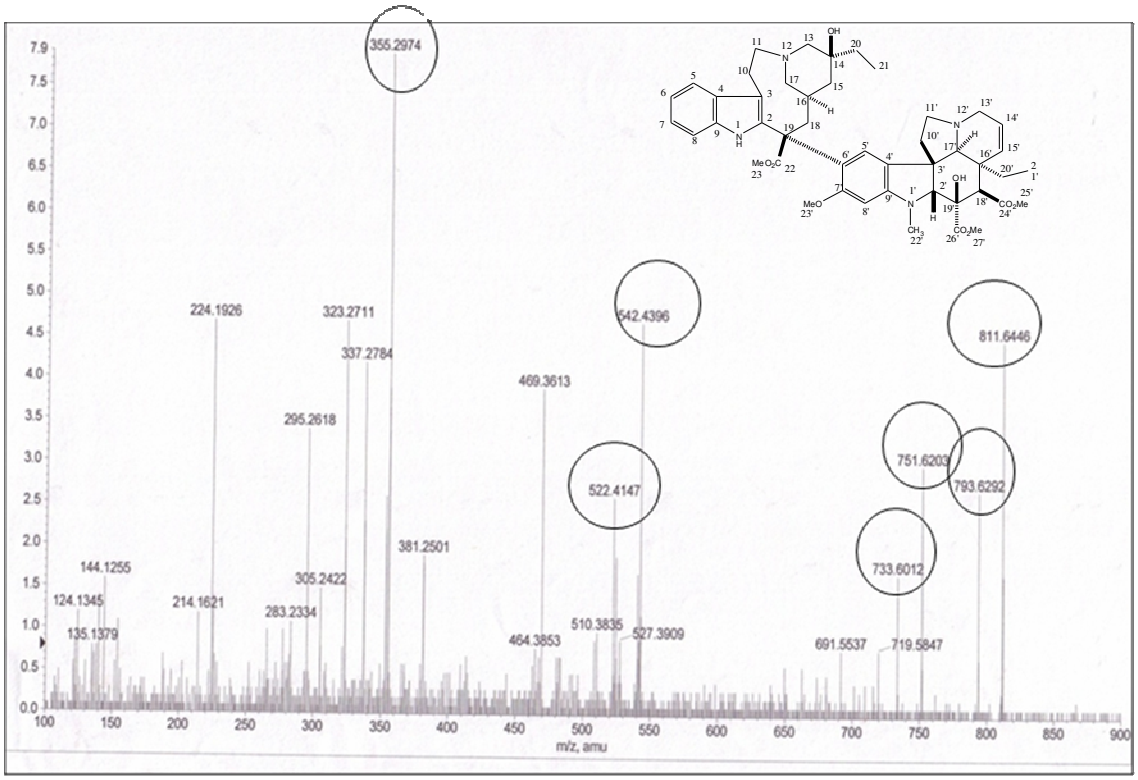


Fig.6: MS-MS spectrum of the purified fungal vinblastine showing product ions 811 m/z (M+H) ion attributing to fragmentation patterns i.e. 355, 522, 542, 733, 751, 793 and 811.

Quantification of vinblastine through HPLC:

The data of area peak vs. vinblastine concentration, obtained in case of the standard sample was used to estimate quantity of fungal vinblastine. The isolation of vinblastine from 1 litre culture filtrate yielded 76 µg.

Proton NMR (^{1}H NMR) analysis:

A comparison of the ^{1}H NMR spectrum of fungal vinblastine and the standard vinblastine sulphate (procured from Sigma Aldrich) is shown in Fig.7. The spectrum obtained for the fungal vinblastine matches with the standard sample. The chemical shifts are summarized in Table (1). These values match with the chemical shifts reported in literature [9, 10, 23-25]. The minor changes in the chemical shifts are likely to be due to the fact that, the standard sample is obtained as the sulphate salt while the fungal vinblastine is extracted as the base.

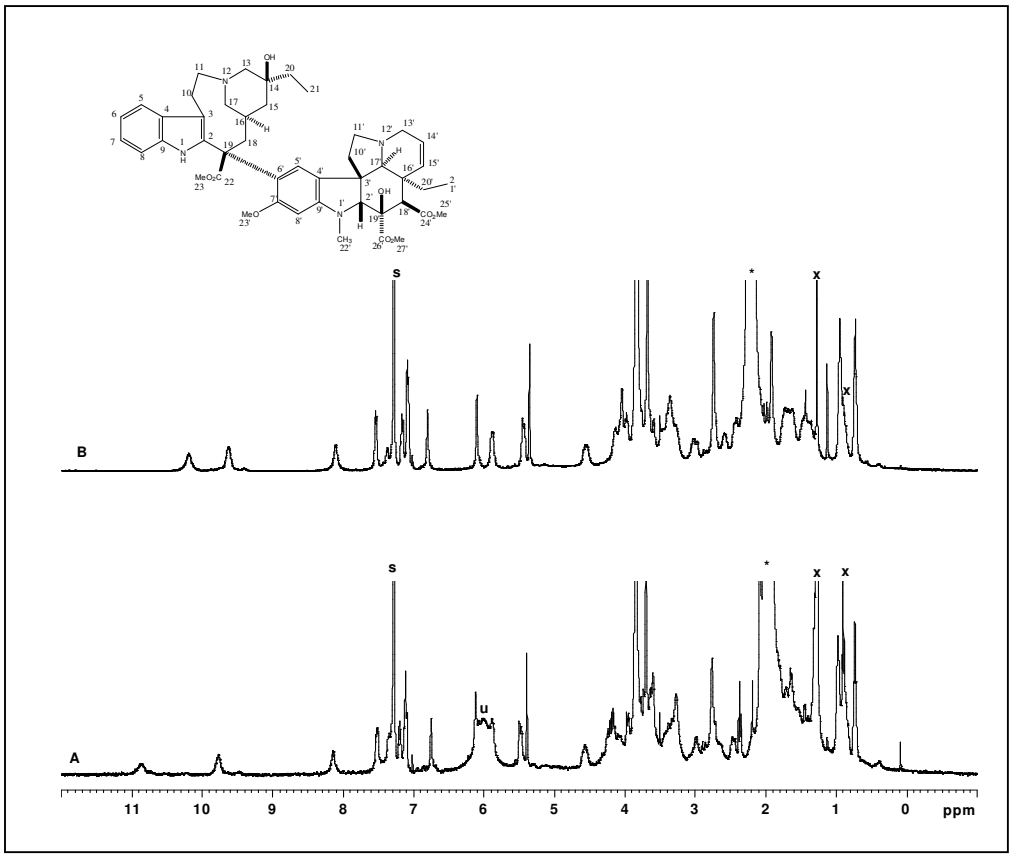
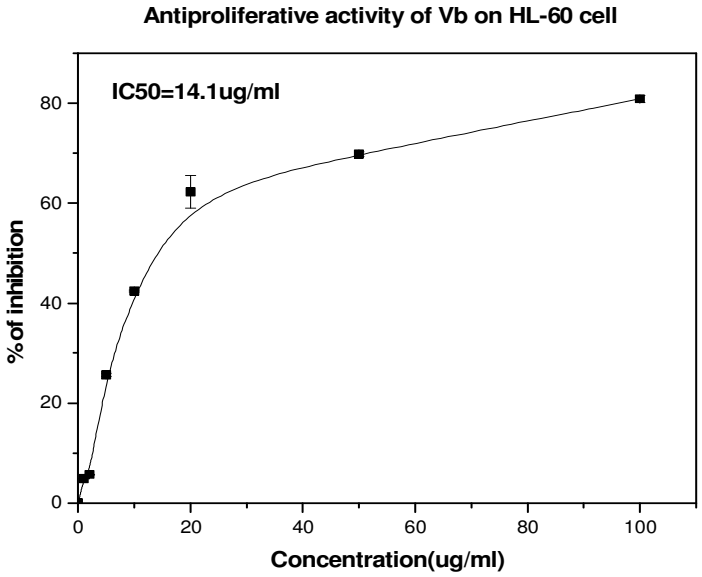


Fig.7: 400 MHz ^{1}H NMR spectra of fungal vinblastine (A) and standard vinblastine sulphate (B). The signals marked with‘s’ are coming from the residual solvent ($CHCl_3$). The signals marked with * and x are due to contamination from trace amounts of water (from the solvent) and n- hexane. A broad signal marked ú’ at ~6 ppm in A is an unidentified impurity.

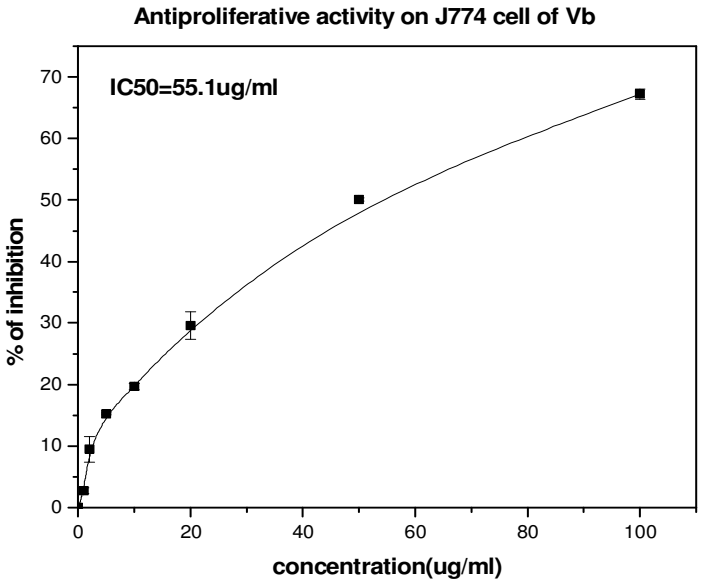
	Standard Vinblastine			**Fungal Vinblastine**	
Sr No.	Chemical Shift δ (in ppm)	**Coupling Constant (in Hz)**		**Chemical Shift δ (in ppm)**	**Coupling Constant (in Hz)**
21'	0.6400 t (3H)	7.41	21'	0.6436 t (3H)	6.70
21	0.8611 t (3H)	7.06	21	0.7928 t (3H)	6.70
16	1.0392 s (1H)		16	1.0383 s (1H)	
15	1.1843 (2H) overlap		15	1.1212 (2H) overlap	
10'b	1.3436 s (1H)over		10'b	1.3489 s (1H)	
25'	1.3436 s (3H)over		25'	1.5559 q (3H)	7
20'b	1.8273 s (1H)		20'b	1.9959 s (1H)	
20'a	1.8977 s (1H)		20'a	1.9959 s (1H)	
20b	1.9388 s (1H)		20b	1.9959 s (1H)	
20a	2.3242 s (1H)		20a	2.2774 t (1H)	7.49
10'a	2.3541 s (1H)		10'a	2.3725 d (1H)	12
13b	2.5062 s (1H)		1'	2.6726 s (3H)	
1'	2.6488 s (3H)		13b	2.7904 obsc (1H)	
18b	2.9433 s (1H)		18b	2.9017 s (1H)	
17b	2.9433 s (1H)		17b	3.1818 s (1H)over	
13a	3.1841 (1H) over		13a	3.1818 s (1H) over	
17'	3.2661 s (1H)		17'	3.1818 s (1H)over	
11'b	3.2661 s (1H)		11'b	3.2699 d (1H)	17.49
17a	3.3128 (1H)over		17a	3.4163 s (1H)	
11a	3.4142 s (1H)		11a	3.4955 (1H) over	
13'b	3.4936 d (1H)	6	13'b	3.5078 (1H) over	
27'	3.5908 s (3H) over		11b	3.5533 d (1H)	2.4
11b	3.5908 s (1H) over		27'	3.6083 s (3H)	
13'a	3.7354 s (1H)over		13'a	3.6394 d (1H)	4
10b	3.7354 s (1H)over		10b	3.6842 (1H) over	
23'	3.7354 s (3H)over		23'	3.7527 (3H)over	
10a	3.7530 (1H) over		10a	3.7527 (1H)over	
23	3.7530 s (3H)over		23	3.7527 (3H)overlap	
2'	3.8879 d (1H)	4	2'	3.7527 (1H) over	
14	3.9542 s (1H)(broad)		14	3.8606 obsc (1H)	
19'	3.9880 s (1H)		19'	4.0998 (1H) over	
11'a	4.0439 s (1H)		11'a	4.1572 (1H) over	
18a	4.4544 s (1H) (broad)		18a	4.4759 s (1H) br	
18'	5.2650 s (1H)		18'	5.3041 s (1H)	
14'	5.3508 dd (1H) (broad)	10, 5	14'	5.3829 dd(1H)	10, 5
15'	5.7915 s (1H)(broad)		15'	5.7867 d(1H)	7.97
5'	6.0139 s (1H)		5'	6.0333 s (1H)	
8'	6.7125 s (1H)		8'	6.6618 s (1H)	
7	6.9872 s(1H)		7	7.0305 t (1H)	8.25
6	7.0062 s (1H)		6	7.1157 t (1H)	7.42
5	7.0815 t (1H)	7.2	5	7.2619 d (1H)	8
8	7.4456 d (1H)	7	8	7.4278 d (1H)	7.52
1	8.0166 s (1H)		1	8.0566 s (1H) br	

Table1: Proton NMR (^{1}HNMR) of standard vinblastine and fungal vinblastine (Chemical shift in ppm and coupling constant in Hz.)

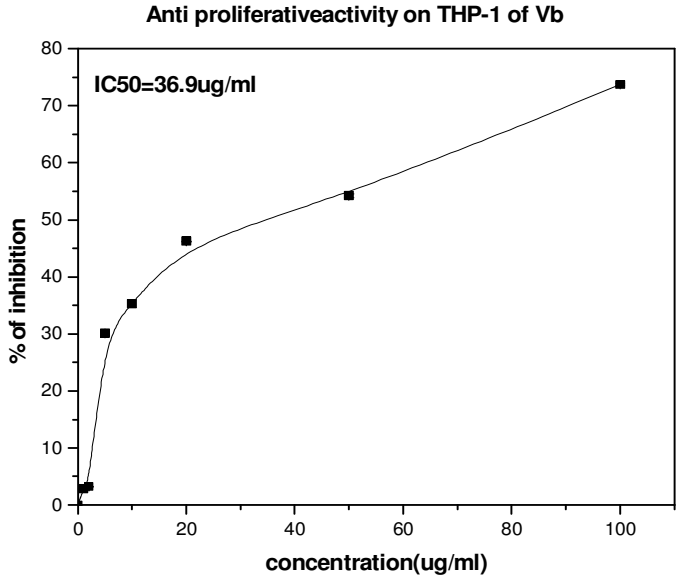
Cytotoxicity of Purified fungal vinblastine against Cancerous cell lines:



Graph 1: Antiproliferative activity on HL-60 of vinblastine (IC_{50}: 17.2 µM)



Graph 2: Antiproliferative activity on J774 of vinblastine (IC_{50}: 67.9 µM).



Graph 3: Antiproliferative activity on THP-1 of vinblastine (IC_{50}: 45.4 µM).

50% cell proliferation inhibitions were observed with 17.2 µM, 67.9 µM and 45.4 µM of fungal vinblastine against HL-60, J774 and THP-1 cell lines respectively.

Discussion:

Studies on the isolation of the vinca alkaloids produced by *Fusarium oxysporum* indicate that the fungus produces vinblastine in the culture filtrate *in vitro*. TLC, HPLC and other analyses of the extracts from culture filtrate confirm this conclusion. In addition to vinblastine and vincristine, this fungus also produces few unidentified vinca alkaloids, although in trace amounts. A number of endophytic fungi have been isolated by Kharwar *et al.* (2008) from *Catharanthus roseus* grown in India. But there is so far no report of vinblastine and vincristine from above endophytic fungi. However, Zahng *et al.* (2000) and Tung *et al.* (2002) discovered that vincristine is produced by *Fusarium oxysporum*, an endophyte of *Catharanthus roseus;* while Guo and Kunming (1998) isolated vinblastine from *Alternaria* sp. isolated from *Catharanthus roseus*. They showed the presence of above drug with the help of TLC and HPLC only. To the best of our knowledge, this is the first report of isolation of vinblastine from *Fusarium oxysporum* isolated from Indian *Catharanthus roseus* plant. The necessary precursor molecule such as tryptophan and geraniol required for the formation of vinca alkaloids were provided in the growth medium as they are essential for vinblastine production as reported earlier from other sources.

The ability of *Fusarium oxysporum* to make vinblastine was confirmed by isolation of compound having chromatographic properties similar to those of standard vinblastine in three solvent systems a, b and c which showed a single dark purple spot on TLC when sprayed with ceric ammonium sulphate reagent. Fungal vinblastine on HPLC C18 symmetry column showed a single symmetrical peak at retention time of 36.6 mins that confirmed its homogeneity. Absorption maximum of the purified fungal vinblastine was found to be at 220 nm as reported earlier [13, 15-19]. In ESI-MS, molecular ions at m/z 811 attributable to $(M+H)^+$ confirmed its molecular weight to be 810 m/z [10,17, 20, 21]. MS-MS showed fragment ions at m/z attributable to structures which are usually seen as vinblastine fragment ions[10]. ^1H NMR spectrum matched with that of standard vinblastine spectrum [9, 10, 23-25]. Vinblastine obtained per liter of culture was estimated to be approximately 76 µg. This is the first report of vinblastine from an endophyte of *Catharanthus roseus* growing in India. Anti-tumor activity of vinblastine was checked against HL-60, J774 and THP-1cell lines, showing IC_{50} 14.1, 55.1 and 36.9 µg/ml fungal vinblastine respectively [28-33]. Vinblastine showed less cytotoxicity in J774 cell lines. However,

in case of HL-60 and THP-1, the cell growth inhibition was significantly higher even at low vinblastine concentration.

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Chapter 6

Isolation, purification, characterization and bioassays of vincristine

Summary:

The endophytic fungi which produced vinblastine also produced vincristine extracellularly from the same medium. The vincristine was purified by TLC and HPLC and characterized using UV-spectroscopy, ESI-MS, MS/MS, and proton NMR. One litre of fungal culture yielded 67 µg of vincristine. The purified vincristine from the fungus showed cytotoxicity towards cancer lines HL-60 (leukemia), J774 and THP1.

Introduction:

The most important alkaloid of *Cathranthus roseus* which has been found to have pharmacological activity and clinical application as an anticancer agent is vincristine. Vincristine sulphate sold as oncovin is used particularly for treatment of acute leukaemia in children including lymphocytic leukemia. It is also used for the treatment of Hodgkin's disease, Wilm's tumor, neuroblastoma, rhabdosarcoma, and reticulum-cell sarcoma[1]. Average content of vincristine is 0.0005% [2]. Presently vincristine is being isolated from the leaves of *Cathtaranthus roseus* plants at a very low concentration (0.0005%). Because this alakaloid is being produced by the plants only at a very low level, *C.roseus* cell cultures have been studied for many years as a potential way to produce this therapeutically important indole alkaoids as well as the monomers vindoline, cathtaranthine. Vincristine is being produced from leaves of field grown plants, cell and tissue culture, callus culture[3], cell suspension culture, shoot culture and hairy root culture of *C.roseus*, semi [4] as well as total synthesis [5, 6]. Attempts to produce vincristine industrially by cell suspension culture have been unsuccessful due to many biological and technological limitations [3]. One of these limitations, is the low yield of this drug in the cell cultures due to lack of some enzymes in the pathway leading to the formation of necessary intermediates. Productivity of vincristine is very low in plants (0.0003%) resulting in their extra ordinarily high price. Production of vinca alkaloids in plant cell cultures did not lead to a significant improvement and today it is accepted that biotechnological approaches in plant cell culturing may not provide an instant solution to this problem. Plant associated fungi are able to make the same bioactive compounds such as isolation of an endophytic fungus, *Taxomyces andreanae* from the bark of pacific yew (*Taxus brevifolia*) which has been shown to produce taxol, the antitumor diterpenoid produced by the *Taxus* plant [7-10]. This concept has been gaining ground since the discovery that the endophytic fungi associated with medicinal plants may have the potential to acquire the capability to produce the valuable metabolites of medicinal value from the parent plant and such fungi could become independent alternative sources for these metabolites which can be produced by fermentation. This opens a tremendous new area for research and development, particularly for screening the innumerable medicinal plants of indigenous origin for endophytes and evaluating them for their potential to manufacture the valuable compounds of plant origin.

Sreekanth et al., 2009 isolated endophytic fungi from Indian Yew tree *Taxus baccata*, and then screened for taxol production. Out of the 40 fungal cultures screened, one fungus *Gliocladium* was found to produce taxol and 10-DABIII (10-deacteyl bacctain III). These compounds were purified by TLC and HPLC and characterized using UV spectroscopy, ESI-MS, MS/MS and proton NMR. After the successful isolation and purification of taxol, we started the isolation and purification of endophytic fungi from *Catharanthus roseus* plant in order to isolate vincristine. We have identified fungal (endophytic) strains which produce vinblastine and vincristine, purified with TLC, HPLC and characterized by UV- VIS, ESI-MS and ^{1}HNMR.

Materials:

PDA media, Erlenmeyer flasks (500 ml), MGYP medium, rotary shaker, vinca alkaloids media (VM-1), muslin cloth, lyophilizer, liquid nitrogen, ethyl acetate, anhydrous sodium sulphate, rotavapour, TLC (0.5 mm thick, 20 cm x 20 cm) silica plate, chloroform, methanol, ethyl acetate, ceric ammonium sulphate reagent, vincristine (MP Biomedicals), silica gel column, HPLC (Waters), C18 symmetry column, HPLC grade acetonitrile, MilliQ water, trifluoroacetic acid, Shimadzu PC 101 Spectrophotometer, HPLC grade methanol, M/S applied Biosystems APIQSTAR pulsar (ESI-MS) mass spectrophotometer, HPLC Grade acetic acid, Bruker AV 400FT spectrometer, $CDCl_3$ solvent, HL-60, J774 and THP-1 cell lines, 10% DMSO, centrifuge, trypsin solution, Carl Zeiss Axiovert 25 Inverted Microscope, MTT reagent, isopropanol, Beckman coulter spectrophotometer, micro titer plates, RPMI-1640, MEM medium and incubator.

Methods:

The fungus, *Fusarium oxysporum* maintained on potato dextrose agar (PDA) slants has optimum growth at pH 7.0 at temperature 27^{o}C. Subculturing was done to maintain the stock cultures, at monthly intervals and preserved at 15^{o}C. Starting materials for fermentation experiments were taken from an actively growing stock culture, which were subcultured on fresh slants and incubated for 4 days at pH 7.0 and temperature 27^{o}C.

Isolation, purification and characterization of vincristine from the endophytic fungus *Fusarium oxysporum*:

A two stage fermentation procedure was employed for the isolation of vincristine from *Fusarium oxysporum*. In the first stage, 500 ml Erlenmeyer flasks containing 100 ml medium (MGYP) were inoculated with 7 days old culture and incubated at 28^0C on a rotary shaker (240 rpm) for 4-5 days, which was used as seed culture (I stage). Later, 10 ml seed culture was transferred to 500 ml Erlenmeyer flask containing 100 ml production medium called as vinca medium-1 (VM-1) which was incubated at 28^oC for 20 days as shake culture (II stage), after which it was harvested and used for further study.

Culture filtrate and mycelia were separated with the help of muslin cloth and then lyophilized. Lyophilized culture filtrate was extracted using ethyl acetate as a solvent system[11]. The organic layer within (ethyl acetate) was separated from the aqueous layer using separating funnel. The extraction was repeated thrice and the solvent was dried using anhydrous sodium sulphate and concentrated under vacuum using rotavapour at 40^oC in order to get crude extract. A small amount of crude extract was dissolved in ethyl acetate and subjected to thin layer chromatography (TLC) on silica gel-G (0.5 mm thickness) using chloroform: methanol (8:2) as a solvent system. The TLC plates were sprayed with ceric ammonium sulphate reagent. Vinca alkaloids spots produced brilliant violet color as well as purple color with above spraying reagent.

Purification of fungal vincristine was done by silica gel column chromatography. The crude extract was loaded on silica gel column (60-120 mesh size, 40 cm x 2 cm length width) pre -equilibrated with chloroform and eluted with a gradient of chloroform: methanol (100% chloroform, 9:1, 8:2, 7:3, 1:1 and 3:7 and 100% methanol). Fractions containing compounds with Rf values similar to that of the standard vincristine were pooled and subjected to preparative TLC on 0.5 mm thick (20cm x 20cm) silica plate and developed in chloroform: methanol (8:2) solvent system. The putative band of fungal vincristine was scraped and eluted out with methanol [12]. Purity of the isolated compound was checked on TLC in the following solvent systems (a) chloroform: methanol (8:2) (b) chloroform: methanol (9:1) and (c) ethyl acetate: acetonitrile (8:2) [12].

HPLC and spectroscopic analysis:

Purity and characteristic of fungal vincristine was determined by HPLC using C18 symmetry column (Waters) [2, 11, 13, 14]. Sample was taken in 40 µl acetonitrile, injected in HPLC column and gradient elution was performed using 5% - 95% acetonitrile with 0.01% trifluoroacetic acid at flow rate of 0.5 ml /min. A dual wavelength recorder set at 220 nm and 254 nm was used to detect the compound eluting from column [13, 15- 19]. The absorption maximum of the purified compound was determined by Shimadzu PC 101 spectrophotometer. Sample was dissolved in HPLC grade methanol and spectral data was collected over 200-700 nm range.

ESI-MS and MS-MS analysis:

Molecular mass of the purified compound was determined by M/S applied Biosystems APIQSTAR pulsar (ESI-MS) mass spectrophotometer. Sample for the analysis was dissolved in HPLC grade methanol, water, acetic acid in the ratio of 50:50:0.1. Samples were then analyzed by infusion method (injected into MS) at flow rate of 5 µl/min and at an IS voltage of 3800V in TOF mode. Spectrum from a range of m/z 100-1000 Dalton was obtained [4,17, 20, 21]. Fragmentation of the desired molecule was obtained by acquiring the product ion spectrum using MS-MS^4 with similar parameters as used for ESI-MS. Molecular ions of the standard vincristine were also obtained for comparison.

Quantification of vincristine by HPLC:

2 mg/ml concentration of a standard vincristine solution was prepared in HPLC grade acetonitrile. 50 µl of the above standard solution (2 mg/ml) plus 950 µl of HPLC grade acetonitrile was taken to make the final concentration of 100 µg/ml. 10 µl of this solution was injected into HPLC and analysed. Similarly, in order to quantify vincristine present in 1 litre of the culture filtrate, the culture filtrate obtained after 20 days was extracted and purified by HPLC. The purified fungal vincristine obtained was dissolved in 1 ml of HPLC grade acetonitrile and then 10 µl of this purified solution was injected into HPLC and analysed. The data of the area peak vs. concentration of the standard obtained was used to estimate the quantity of fungal vincristine in one litre of culture filtrate [13, 22].

Nuclear Magnetic Resonance (^{1}H NMR) analysis:

^{1}H NMR analysis was carried out on a Bruker AV 400 FT spectrometer operating at 500 MHz NOCR spectrometer. Sample was then dissolved in Methanol-d_4 and scanned overnight**.** NMR spectrum was measured with a spectral width 8223.68 Hz and an acquisition time of 1.818s and a relaxation delay of 1.0 s were used [5, 23, 24]**.**

Cytotoxicity of purified vincristine against cancerous cell lines:

General cell maintenance of HL-60, J774 and THP-1cell lines:

HL-60, J774 and THP-1 cell lines were kept frozen (0.5 ml stock) at 2-5 x 10^6 cells/ ml in RPMI-1640, MEM and MEM (without FCS) respectively containing 10% DMSO. Frozen stocks were thawed at 37^0C and then diluted with fresh pre-warmed medium (RPMI-1640, MEM and MEM respectively) to 10ml (all supplements). Centrifugation was done for 10 mins at 1000 rpm and supernatant was discarded. Appropriate volume of fresh pre-warmed complete medium (RPMI-1640, MEM and MEM respectively) was used for resuspension of centrifuged solvent and later seeded into culture flasks containing medium (RPMI-1640, MEM and MEM respectively) (25 ml/80 cm^2 flask). The approximate seeding ratio from frozen stock is one ampoule to two flasks. Replace medium every 3-4 days as required, so as to grow cells to 90% confluency. 10 ml trypsin solution was added per flask and flasks were left for 2-5 minutes at room temperature. Microscopic observation of rounding up of cells confirms the cell detachment. Approximately 8 ml of trypsin was decanted off and cells were kept for further 5 minutes. The cells were dislodged by tapping the flask gently. To each wells, 10 µl litre of MTT reagent (5 mg/ml) was added. Then cells were stained for 20 hrs at 37^0C. After that, 200 µl acidified isopropanol was added to solubilize the purple formazan crystal formed. Absorbance was measured at 490 nm with a Beckman Coulter spectrophotometer.

Cytotoxicity studies:

Cytotoxicity of the purified vincristine, against cancerous cell lines HL-60 (leukemia), J774 (Murine macrophages) and THP-1(Human acute monocytic leukemia) was determined by MTT assay. The cell suspension at a concentration of 1x10^4 cells/ml was added in 96 well microtiter plates culture media used for HL-60, J774 and THP-1

were RPMI-1640, MEM and MEM respectively. Plates containing culture media and test compound were incubated for 24 hours for HL-60, 72 hours for J774 and also 72 hours for THP-1 at 37^0C, 5% v/v CO_2 and 95% humidity. All the samples were in triplicates. To each well, 10 µl of MTT reagent (5 mg/ml) was added and plate was incubated for 1 hour at 37^0C. 200 µl of acidified isopropanol was added at the end of this period, to stop the reaction and further incubation was done for 4 hours to solubilize the purple formazan crystals produced. Beckman Coulter spectrophotometer was used to measure absorbance at 490 nm [25].

Results:

Vincristine from culture filtrate:

The culture filtrates when extracted with ethyl acetate yielded a brown residue after the removal of the solvent. The substance was named as crude extract. The crude extract, on TLC on silica gel G using chloroform:methanol (8:2) solvent system, produced five clearly differentiated brilliant purple ,violet and three yellow coloured spots when sprayed with ceric ammonium sulphate reagent[26] (Fig.1, Ch-III). The Rf value of the spots on TLC of the crude extract using chloroform: methanol (8:2) solvent system when compared with standard vincristine and vinblastine showed identical Rf value of two spots (Rf value 0.74 and 0.77) with vincristine and vinblastine. The spots of vincristine and vinblastine produced by crude extract showed identical colour with the standards when sprayed with ceric ammonium sulphate reagent [26]. In addition to vincristine and vinblastine, two vinca alkaloids present in the crude extract as evident from TLC could not be identified.

For the purification of vincristine from culture filtrate, the crude extract was fractionated on silica gel column pre-equilibrated with chloroform and chloroform: methanol (100% chloroform, 9:1, 8:2, 7:3, 1:1, 3:7, 100% methanol). Partially purified vincristine with chromatographic properties similar to those of the standard vincristine was obtained. The partially purified vincristine on prep TLC yielded considerably pure compound. Partially purified vincristine obtained from preparative TLC showed single dark violet spot (with Rf value 0.74) when sprayed with ceric ammonium sulphate reagent on TLC (Fig.1). The purity of the fungal vincristine showed similar chromatographic properties as those of the standard vincristine in three different solvent systems a, b and c on TLC.

A B

A: Standard vincristine

B: Purified fungal vincristine

Detection: Ceric ammonium sulphate reagent

Fig.1: TLC of fungal vincristine purified from culture filtrate along with standard vincristine on silica gel G using chloroform: methanol (8:2) solvent system.

HPLC and spectroscopic analysis:

The homogeneity of the purified compound was confirmed by HPLC analysis, which showed a single, symmetrical peak with RT of 34.9 min. on C18 symmetry column (Fig.2). Absorbance of the eluting compound showed high intensity at 220 nm and relatively low at 254 nm. The UV absorption analysis showed a peak showing absorption at 220 nm. An absorbance maximum of standard vincristine was also obtained for comparison (Fig.3)

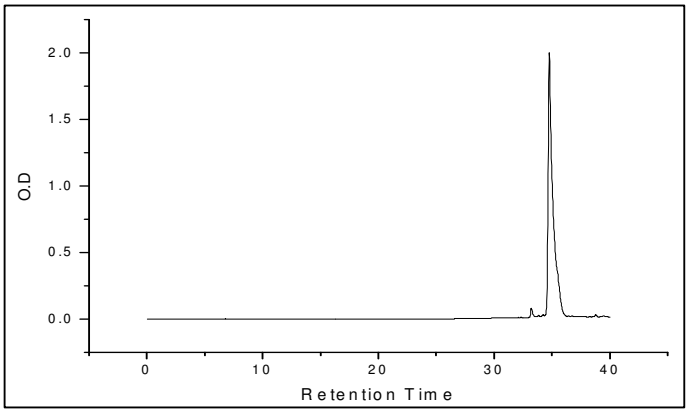


Fig.2: HPLC profile of pure fungal vincristine with RT of 34.9 min.

.

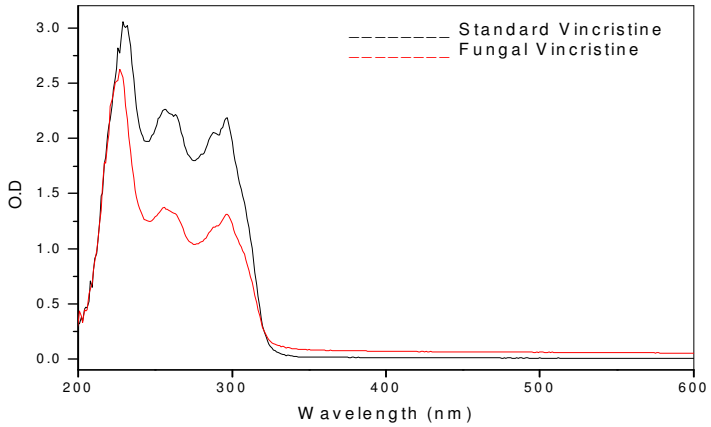


Fig.3: UV absorption spectrum of standard vincristine and fungal vincristine.

ESI-MS and MS-MS:

Electrospray ionization mass spectrometry yielded at major ion at m/z 825 (Fig.4).

In MS-MS fragment ion at m/z 766, 807 and 825 were seen (Fig.5).

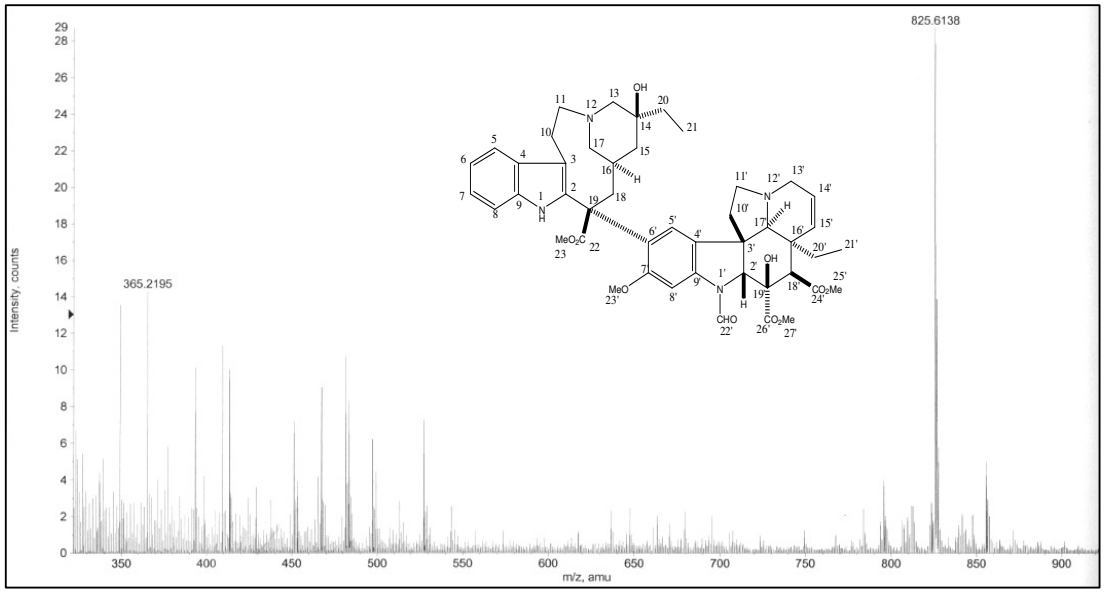


Fig.4: ESI-MS spectrum of m/z 825 (M+H) ions of fungal vincristine.

MS-MS:

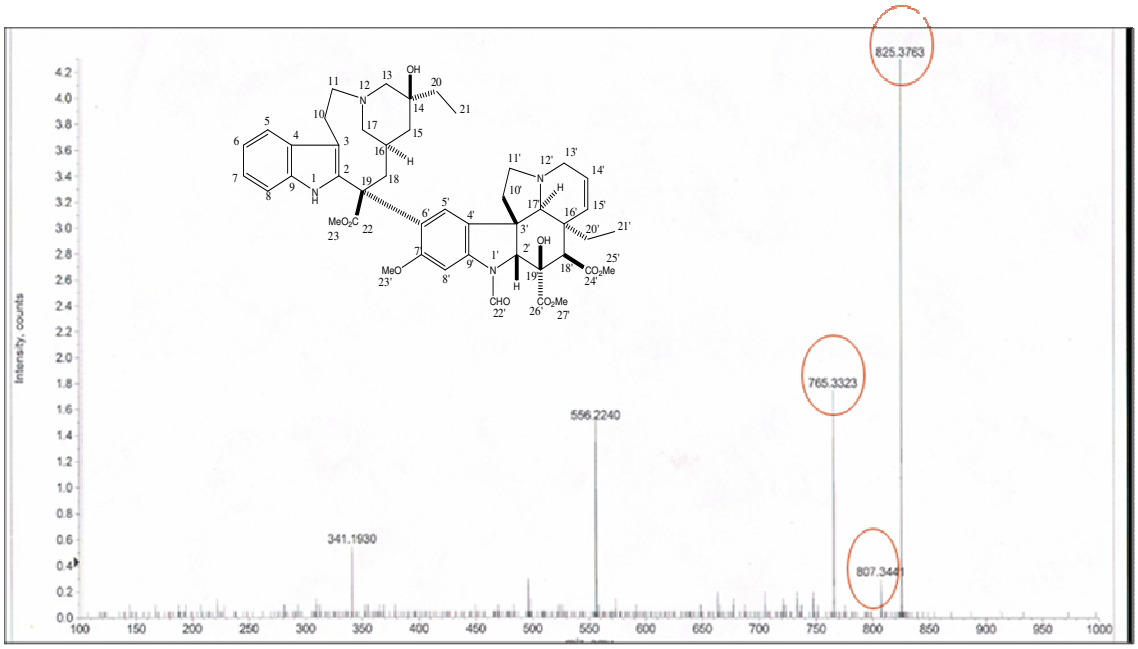


Fig.5: MS-MS spectrum of the purified fungal vincristine showing product ion m/z 825 (M+H) ions attributing to fragmentation patterns i.e. 766, 807 and 825.

Quantification of vincristine through HPLC:

The data of area peak vs. vincristine concentration, obtained in case of the standard sample was used to estimate quantity of fungal vincristine. The isolation of vincristine from 1 litre culture filtrate yielded 67 µg of vincristine.

Proton NMR (^{1}H NMR):

A comparison of the ^{1}H NMR spectrum of fungal vincristine and the standard vincristine sulphate (procured from MP Biotech) is shown in Fig.6. The spectrum obtained for the fungal vincristine matches with the standard sample. The chemical shifts are summarized in Table-1. These values are well matching with the chemical shifts reported in literature [5, 23, 24]. The minor changes in the chemical shifts between

standard and fungal vincristine are likely to be due to the fact that, the standard sample is obtained as the sulphate salt while the fungal vincristine is extracted as the base.

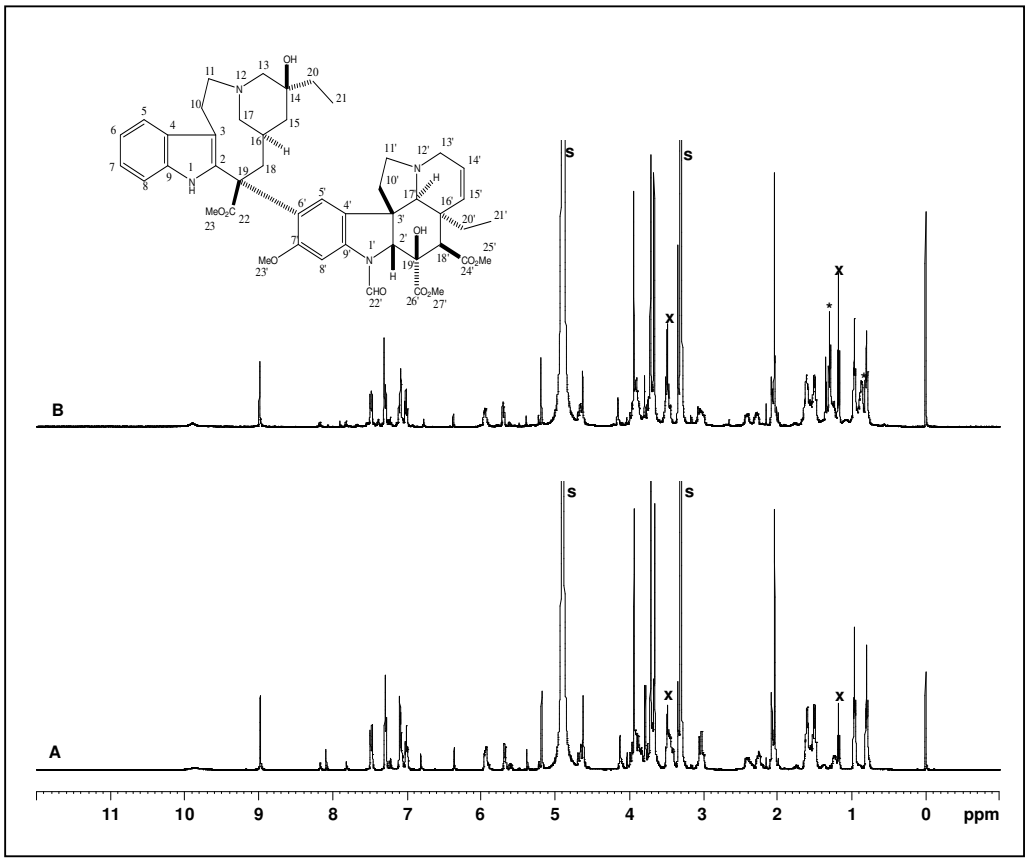


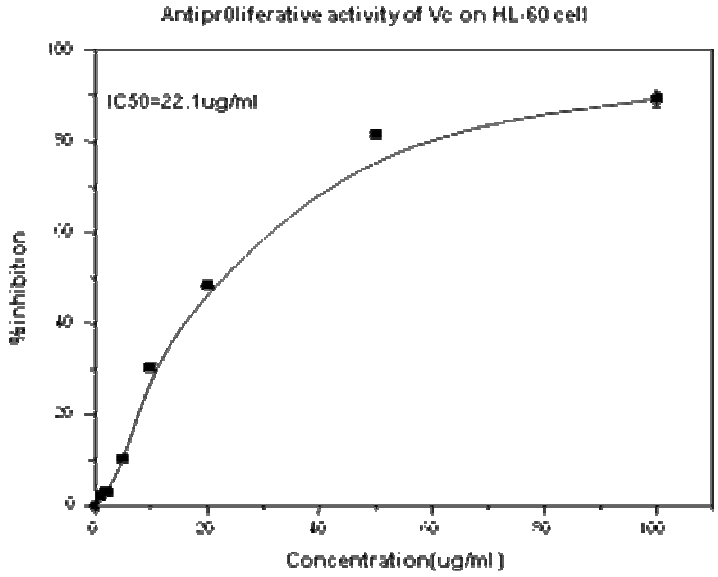
Fig.6: 500 MHz ^{1}H NMR spectra of fungal vincristine (A) and standard vincristine sulphate (B). The signals marked with's' are coming from the residual solvent ($CHCl_3$). The signals marked with * and x are due to contamination from trace amounts of water (from the solvent) and n- hexane.

	Standard Vincristine			Fungal Vincristine	
Sr No.	**Chemical Shift δ (in ppm)**	**Coupling Constant (in Hz)**		**Chemical Shift (in ppm)**	**Coupling Constant (in Hz)**
21’	0.7976 t (3H)	7.15	21’	0.7993 t (3H)	7.4
21	0.9576 t (3H)	7.50	21	0.9591 t (3H)	
15a	1.1765 t (1H)	6.96	15	1.1768 t (2H)	
15b	1.2274 s (1H) (b)		16	1.2338 obsc (1H)	
16	1.5200 (1H) overlapped		20’b	1.2845 s (1H)over	
20a	1.5005 q (1H)		20’a	1.2954 t (1H)over	7.25
20’b	1.5890 (1H)overlapped		20a	1.3431 obsc (1H)	7.04
20’a	1.6091 (1H) overlapped		20b	1.4981 q (1H)	7.20
20b	1.6241 (1H) overlapped		10’b	1.4981 q (1H)	
10’b	1.6358 (1H) overlapped		10’a	1.5532 (1H) over	
25’	2.0373 s (3H)	7.54	25’	1.6068 br (3H)	
10’a	2.0784 m (1H)	14	18b	2.0337 (1H)	
18b	2.2614 d (1H)(b)	14.59	17b	2.0425 s (1H)	
17b	2.4110 d (1H)	6	13b	2.0764 s (1H)	
13b	3.0268 d (1H)		13a	2.2780 (b) (1H)	
13a	3.0268 d (1H)		17’	2.4161 d (1H)	
	3.3052 p (solvent)		11’b	3.0083 d (1H)	
17’	3.3441 s (1H)		11a	3.0566 d (1H)	7.26
11’b	3.4103 s (1H) (broad)		13’ b	3.3443 s (1H)	
23	3.4796 m (3H)		11b	3.4426 m (1H)	
27’	3.6585 s (3H)		23	3.4872 obsc (3H)	
11a	3.6697 (1H) overlapped		27’	3.6650 s (3H)	
13’b	3.6884 (1H) (b)		17a	3.6720 s (1H)	
23’	3.7184 s (3H)	3	23’	3.7168 s (3H)	
11b	3.7874 d (1H)	4.68	10b	3.7454 d (1H)	
17a	3.8320 d (1H)	9.86,		3.3055 p(solvent)	
10b	3.8649 dd (1H)	5.34	10a	3.7931 d (1H)	
10a	3.9064 d(1H)	5.78	14	3.9042 obsc (1H)	

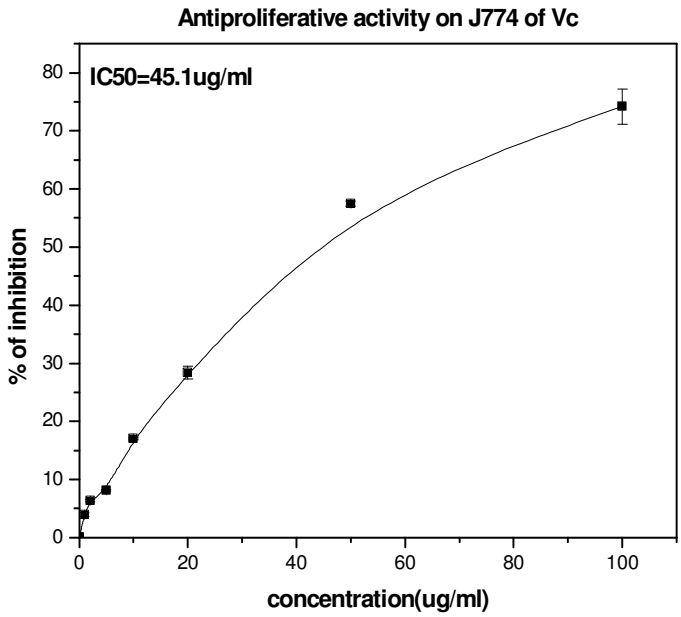
14	3.9340 s (1H)overlapped		19’	3.9385 s (1H)	
19’	3.9340 s (1H)overlapped		13’a	3.9636 (1H) over	14.00
13’a	3.9925 s (1H)		11’a	4.1553 s (1H) br	
11’a	4.1248 s (1H)		2’	4.6298 s (1H) br	15.77
2’	4.6215 s (1H)		18a	4.6626 q(1H) over	
18a	4.6597 q (1H)			4.9261 s(solvent)	
	4.9014 s (solvent)	12.26, 5	18’	5.1890 s (1H)	
18’	5.1827 s (1H)		15’	5.6996 d (1H)	
15’	5.6782 d (1H)		14’	5.9506 dd (1H)	1.64
14’	5.9481 dd (1H)	10.46	6	7.0173 t (1H)	1.64
6	7.0101 t (1H)	10.46,	7	7.0885 s (1H)	
7	7.0721 t (1H)	4.72	5	7.0964 t (1H)	6.99
5	7.0971 (1H) overlapped	7.80	5’	7.2916 s (1H)	
5’	7.2865 s (1H)	7.80	8’	7.3106 s (1H)	
8’	7.3000 s (1H)		8	7.4913 d (1H)	
8	7.4860 d (1H)		22’	8.9956 s (1H)	
22’	8.9854 s (1H)	7.80			

Table- 1: Proton NMR (^{1}H NMR) of standard vincristine and fungal vincristine (Chemical shifts in ppm and coupling constants in Hz).

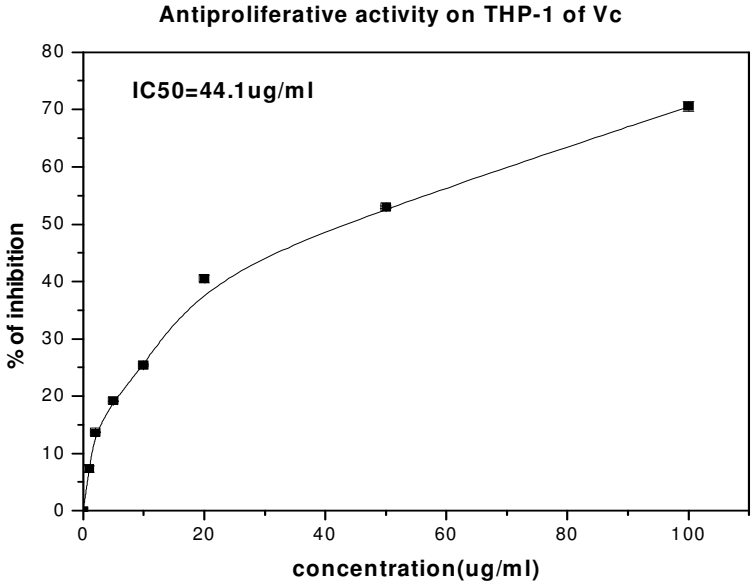
Cytotoxicity studies of purified fungal vincristine:



Graph 1: Antiproliferative activity on HL-60 of vincristine (IC_{50}: 26.7 µM)



Graph 2: Antiproliferative activity on J774 of vincristine (IC_{50}: 54.6 µM)



Graph 3: Antiproliferative activity on THP-1 of vincristine (IC_{50}: 53.4 µM)

50% cell proliferation inhibitions was observed with 26.7 µM, 54.6 µM and 53.4 µM of fungal vincristine against HL-60, J774 and THP-1 cell lines respectively.

Discussion:

Studies on the isolation of the vinca alkaloids produced by *Fusarium oxysporum* indicate that the fungus produces vincristine in the culture filtrate *invitro*. TLC, HPLC and other analyses of the extracts from culture filtrate confirm this conclusion. In addition to vincristine and vinblastine, this fungus also produces few unidentified vinca alkaloids, although in trace amounts. A number of endophytic fungi have been isolated by Kharwar *et al.* (2008) from *Catharanthus roseus* grown in India. But there is so far no report of vinblastine and vincristine from above endophytic fungi. However, Zahng *et al.* (2000) and Tung *et al.* (2002) reported that vincristine is produced by *Fusarium oxysporum*, an endophyte of *Catharanthus roseus* while Guo and Kunming (1998) isolated vinblastine from *Alternaria* sp. isolated from *Catharanthus roseus*. They identified the presence of above drug with the help of TLC and HPLC only. To the best of our knowledge, this is the first report of isolation of vincristine from *Fusarium oxysporum* isolated from Indian *Catharanthus roseus* plant. The necessary precursor molecules such as tryptophan and geraniol required for the formation of vinca alkaloids were provided in the growth medium as they are essential for vincristine production as reported earlier from other sources.

The ability of *Fusarium oxysporum* to make vincristine was confirmed, by the isolation of compound having chromatographic properties similar to those of standard vincristine in three solvent systems a, b and c which showed a single dark violet spot on TLC when sprayed with ceric ammonium sulphate reagent. Fungal vincristine on HPLC C18 symmetry column showed a single symmetrical peak at retention time of 34.9 min. that confirmed its homogeneity. Absorption maximum of the purified fungal vincristine was found to be at 220 nm as reported earlier [13, 15- 19]. In ESI-MS, molecular ion at m/z 825 attributable to m/z $(M+H)^+$ confirmed its molecular weight to be m/z 824[4,17,20,21]. MS-MS showed fragment ions at m/z attributable to structures which as usually seen are vincristine fragment ions[4].

^{1}H NMR spectrum matched with that of standard vincristine spectrum [5, 23, 24]. Vincristine obtained per litre of culture was estimated to be approximately 66 µg. This is the first report of vincristine from an endophyte of *Catharanthus roseus* growing in India. Anti-tumour activity of vincristine was checked against HL-60, J774 and THP-1 cell lines, showing IC_{50} 14.1, 55.1 and 36.9 µg/ml respectively. Vincristine showed less cytotoxicity in J774 cell lines [26-29]. However, in case of HL-

60 and THP-1 the cell growth inhibition was significantly higher even at low vincristine concentration.

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Chapter 7

Fungal mediated transformations

I. **Fungal mediated transformation of vinblastine to vincristine.**

II. **Fungal mediated transformation of catharanthine and vindoline to vinblastine.**

III. **Fungal mediated transformation of L-tryptophan and secologanin to vinblastine.**

Summary:

For the biotransformation of vinblastine to vincristine, the fungus *Fusarium oxysporum* was grown in 500 ml Erlenmeyer flask containing 100 ml MGYP medium and kept on rotary shaker (240 rpm) at 27^{o}C for 96 hr. After 96 hr of fermentation, mycelia were separated from the culture broth by centrifugation (5000 rpm) at 20^{o}C for 20 min and then the mycelia were washed thrice with sterile distilled water under sterile conditions. The harvested mycelial mass (20 gm wet wt.) was then resuspended in 100 ml of sterile distilled water in 500 ml Erlenmeyer flask at pH 6 and the biotransformation was started by adding 2 mg of vinblastine which was pre-dissolved in water under sterile conditions. The biotransformations were routinely monitored by periodic sampling of aliquots (5 ml) which were extracted with ethyl acetate. Extracted solvent was dried and concentrated under vacuo. The crude transformed compounds were analysed by TLC. The transformed compounds were purified by TLC and HPLC. The purified compound had similar chromatographic and spectroscopic properties as those of vincristine. Further characterization with the help of ESI-MS led to the conclusion that the compound obtained is vincristine.

Introduction:

Biotransformation is a useful method for production of novel compounds. It leads to enhancement in the production of the desired compound. Biotransformation overcomes the problems associated with chemical analysis leading to basic information to elucidate the biosynthetic pathway[1]. Whole cell of the fungus or purified enzyme from the fungus have received increasing attention as a method for the conversion of lipids, monoterpenes, diterpenes, steroids, triterpenes alkaloids, lignins and some synthetic chemicals, carrying out stereospecific and stereoselective reactions for the production of the bioactive molecules with some potential for pharmaceutical and food industries[2,3]. Endophytes have received attention as a biocatalyst in the chemical transformation of natural products and drugs, due to their ability to modify chemical structures with high degree of stereospecificity. Although, the potential of these endophytic fungi in the field of biotransformation are still limited. The biotransformation of a tertrahydrofuran lignan, (-) –grandisin, by endophytic fungus *Phomopsis sp* from *Viguiera arenaria* was demonstrated by Verza and coworkers[4]. Other endophytic microbes were studied for the capability to biotransform natural products like taxoid[5], alkaloids[6], curcumin pigment[7], betulinic acid, betulonic acid[8].

Fusarium oxysprum has been discovered as an efficient and selective biocatalyst for the biotransformation of cyclohexanone and cyclopentanone to ε-caprolactone and δ-valerolactone (Baeyer-Villiger reaction) respectively, in quantitative yields[9]. The whole cell of the fungus has been used for the biotransformation of ketones. Another fungus *Trichothecium sp*. was found to be an effective biocatalyst for its enantioselective bioreduction of pro chiral ketone such as acetophenone and its analogous compounds to their corresponding (R)- alcohols with good to excellent enantiomeric excesses[10]. Scientists at National Chemical Laboratory have pioneered the biological synthesis of inorganic nanomaterials of different chemical compositions using endophytic fungi and other microorganisms. This opens up the exciting possibility of developing a rational, fungus based biotransformation approach to the synthesis of chemically and physically hard to synthesize inorganic nanoparticles such as oxides[11-14].

Vinblastine and vincristine have been produced from leaves of field grown plants by cell and tissue culture, callus culture, cell suspension culture, shoot culture, hairy roots cultures of *Catharanthus roseus* semi synthesis as well as total synthesis. Since the quantity of both these drugs from all the sources are extremely low, the supply of vinblastine and vincristine, the wonder drugs to cure cancer, are still limited. Therefore considering the importance of vinblastine and vincristine and their supply, the present endophytic fungus which we isolated from *Catharanthus roseus* was used for biotransformation of vinblastine to vincristine, catharanthine and vindoline to vinblastine and secologonin and tryptophan to vinblastine at room temperature. To the best of our knowledge this is a first report for the biotransformation of above mentioned drugs.

Materials:

Erlenmeyer flask (500 ml), shaker, centrifuge, separating funnel, rotavapour, TLC (0.5 mm thick, 20 cmx20 cm) silica plate, HPLC (waters), C18 symmetry column, MilliQ water, M/S applied Biosystems APIQSTAR pulsar mass spectrophotometer, MGYP medium, ethyl acetate, anhydrous sodium sulphate, chloroform, methanol, vinblastine (Sigma-Aldrich), vincristine (MP Biomedicals), ceric ammonium sulphate reagent, HPLC grade acetonitrile, HPLC grade methanol, acetic acid and Sterile distilled water.

Methods:

The endophytic fungus *Fusarium oxysporum* of *Catharanthus roseus* was grown in MGYP medium containing Malt extract, Glucose, Yeast extract, and Peptone at 25-28^{o}C under shaking condition (200 rpm) for 96 hrs. After 96 hours of fermentation, centrifugation (5000 rpm) at 10°C for 20 min was carried out to separate the mycelia from the culture broth. The mycelia were washed thrice, with sterile distilled water under sterile conditions. The harvested mycelial mass (20 gm wet mycelia) was then resuspended in 100 ml sterile distilled water in 500 ml Erlenmeyer flask at pH 6 and the biotransformation was started by adding 2 mg of vinblastine which was predissolved in water under sterile conditions. The whole mixture was put on shaker at 28^{o}C (200 rpm). The biotransformation was routinely monitored by collecting various fractions (2, 6 and 8 days) during the course of reaction, by separating the fungal mycelia from the aqueous component by filtration.

The filtrate (aqueous component) obtained after separating the mycelial mass was extracted with ethyl acetate as a solvent system[15]. The organic layer was separated from the aqueous layer using separating funnel. The extraction was repeated thrice in order to get the maximum recovery of the compounds. The solvent was dried using anhydrous sodium sulphate and concentrated under vacuum using rotavapour at 40^{o}C in order to get the crude extract. The biotransformation of vinblastine to vincristine was analysed on TLC using chloroform: methanol (8:2) as a solvent system and visualized by ceric ammonium sulphate reagent. The analysis was done by comparison with standard vinblastine and vincristine [16].

Biotransformation of vinblastine to vincristine was also determined by HPLC using C18 symmetry column (Waters). Biotransformed sample (2, 6, and 8 days) was taken

in 10 µl acetonirile injected in HPLC column and gradient elution was performed using 5-95% acetonirile at a flow rate of 0.5 ml/min. A dual wave length recorder set at 220 nm and 254 nm was used to detect the biotransformed vincristine[17-24].

Molecular mass of the biotransformed vincristine was determined by M/S applied Biosystems APIQSTAR pulsar (ESI-MS) mass spectrophotometer. Biotransformed extract was dissolved in HPLC grade methanol, water, acetic acid in the ratio of 50:50:0.1. The above sample was then analyzed by infusion method (injected into MS) at the rate of 5 µl/min and at an IS Voltage of 3800V in TOF mode. Spectrum from a range of m/z 100-900 Dalton was obtained [22- 26].

Results:

The crude extract, on TLC on silica gel-G using chloroform: methanol (8:2) solvent system, produced two clearly differentiated purple and violet colored spots after two days, three spots after six days and one spot after 8 days when sprayed with ceric ammonium sulphate reagent[6]. The Rf Value of the spots (2, 6 and 8 days) on TLC of the crude extract using chloroform: methanol (8:2) solvent system when compared with standards vinblastine and vincristine showed identical Rf values of spots (Rf 0.77 and Rf 0.74) with vinblastine and vincristine and third biotransformed compound (0.60) obtained after six days could not be identified due to lack of a standard. Minimum contaminants were obtained in the crude extracts as we did the reaction in sterile distilled water. Analysis of the crude extract showed dark purple spots on TLC, when sprayed with ceric ammonium sulphate reagent, among which one has similar Rf value as that of standard vinblastine (after 2 days of reaction) which confirms that the transformation of the vinblastine was not complete after two days of reaction. Another biotransformed compound exactly matched with standard vincristine. Further analysis of the crude extract obtained after six days showed dark purple spots on TLC, when sprayed with ceric ammonium sulphate reagent, among which one had similar Rf value as that of standard vinblastine (after 6 days of reaction) which confirmed that the transformation of vinblastine was not complete after six days of reaction. Another biotransformed compound exactly matched with standard vincristine and the third spot could not be identified due to the absence of standard. Analysis of the crude extract obtained after eight days showed dark violet spots on TLC, when sprayed with ceric ammonium sulphate reagent, which has similar Rf value as that of standard vincristine which confirmed the transformation of vinblastine to vincristine. Other two spots, which were detected after six days disappeared after 8 days, confirming that the fungus *Fusarium oxysporum* has converted vinblastine to vincristine at room temperature.

A B C D E

A: Standard vinblastine.

B: Biotransformed vincristine (2 days)

C: Biotransformed vincristine (6 days)

D: Biotransformed vincristine (8 days)

E: Standard Vincristine.

Detection: Ceric ammonium sulphate reagent

Fig.1: TLC showing Standard vinblastine, vincristine along with biotransformed vincristine.

HPLC analysis of biotransformed vincristine:

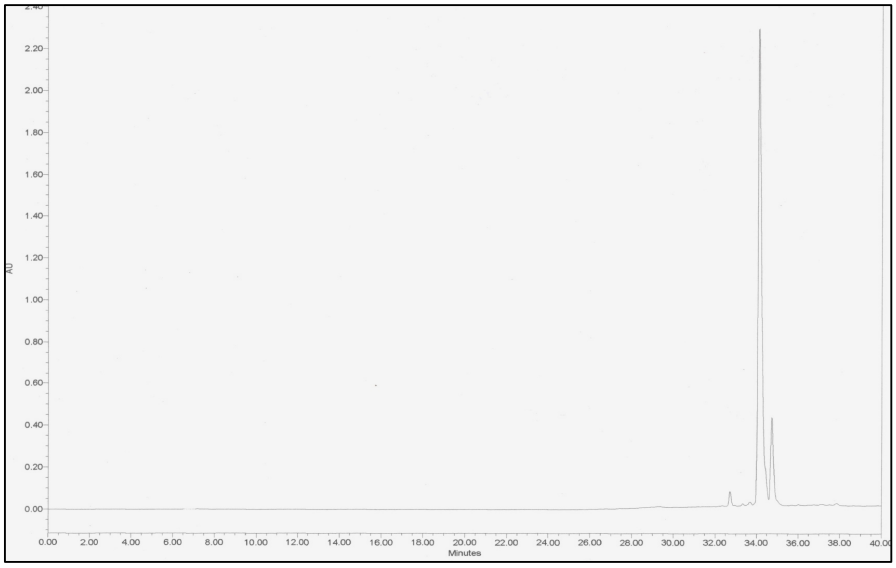


Fig 2: HPLC profile of biotransformed vincristine with retention time of 34.9 min.

Crude extract obtained after eight days on C18 Symmetry column showed peak having equal RT (35.6 min) as that of standard vinblastine and other peak indicating the major compound seen on TLC with RT (34.9 min) as that of standard vincristine.

ESI-MS of biotransformed vincristine (6 days):

ESI-MS yielded ions at 811m/z and 825 m/z which confirmed the presence of vinblastine and vincristine after six days and also confirmed that the fungus *Fusarium oxysporum* converts vinblastine to vincristine at room temperature after eight days [15,16,17,18].

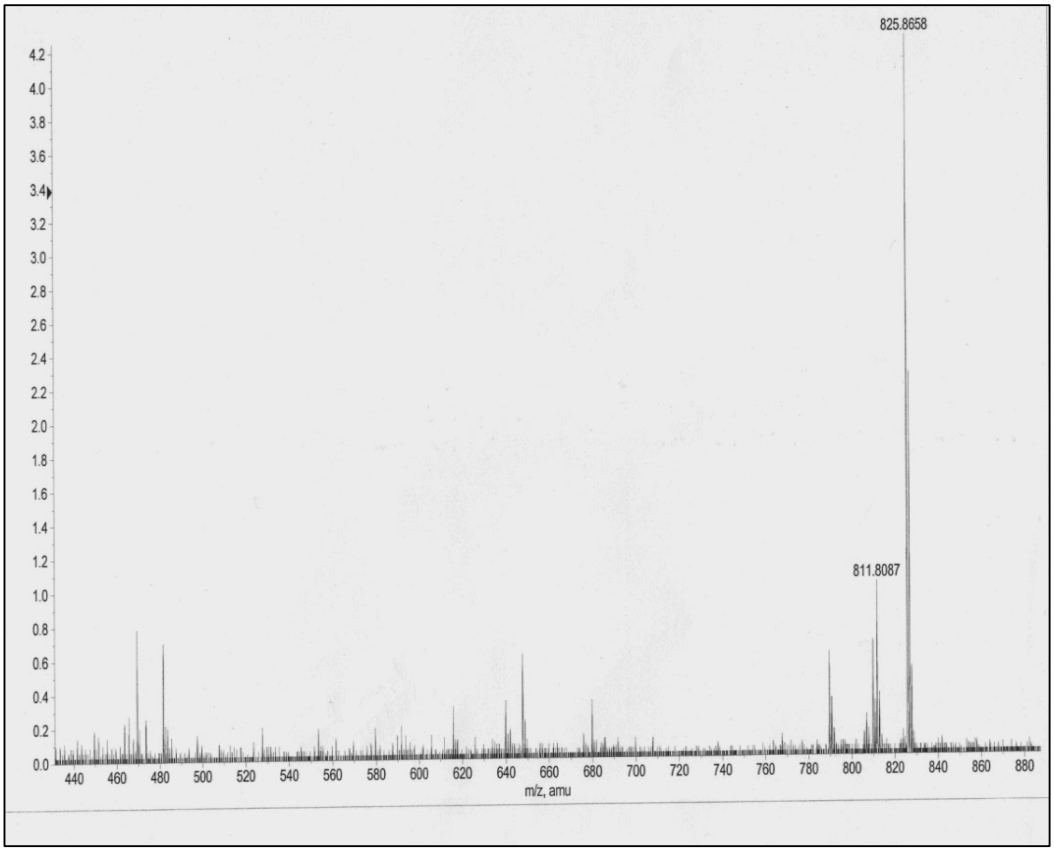


Fig 3: ESI-MS spectrum of m/z 825 (M+H) ions of biotransformed vincristine (after 6 days)

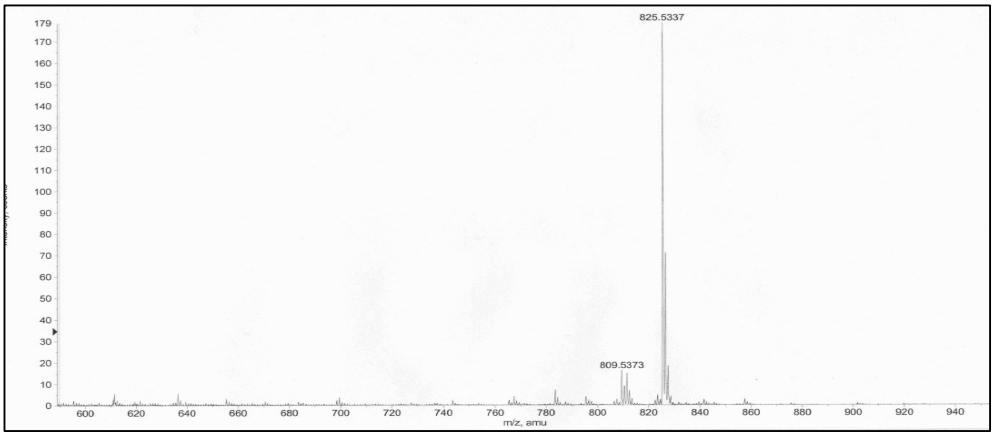


Fig 4: ESI-MS spectrum of m/z 825 (M+H) ions of biotransformed vincristine (after 8 days)

Discussion:

The biotransformation of vinblastine to vincristine at room temperature with the help of fungus *Fusarium oxysporum* was confirmed when ESI-MS analysis showed peak at m/z 825 attributing to the $(M+H)^+$ ion of vincristine[12,14-16]. Studies on the biotransformation of vinblastine to vincristine with the help of fungus *Fusarium oxysporum* indicate that the fungus converts vinblastine to vincristine *in vitro* at room temperature. TLC, HPLC and ESI-MS of crude extract confirm this conclusion. In addition to vincristine, the fungus also produces an unidentified compound after six days which disappeared after eight days. Although biotransformation of vinblastine to vincristine by cell suspension cultures of *Catharanthus roseus* has been reported by Hamada and Nakazawa (1991), but to the best of our knowledge, this is the first report of biotransformation of vinblastine to vincristine from *Fusarium oxysporum* at room temperature.

Summary:

For the biotransformation of catharanthine and vindoline to vinblastine, the fungus *Fusarium oxysporum* was grown and harvested as described above. The harvested mycelial mass (20 gm wet wt.) was then resuspended in 100 ml of sterile distilled water in 500 ml Erlenmeyer flask at pH 6 and the biotransformation was started, by adding 1 mg of vindoline and 1 mg of catharanthine which were predissolved in water under sterile conditions. The biotransformations were routinely monitored by periodic sampling of aliquots (5 ml) which were extracted with ethyl acetate. Extracted solvents were dried and concentrated under vacuo. The crude transformed compound was analysed by TLC. The transformed compound was purified by TLC and HPLC. The purified compound has similar chromatographic and spectroscopic properties as that of vinblastine. However, further characterization with the help of ESI-MS led to the conclusion that the compound obtained is vinblastine.

Materials:

Erlenmeyer flask (500 ml), shaker, centrifuge, separating funnel, rotavapour, TLC (0.5 mm thick, 20 cmx20 cm) silica plate, HPLC (waters), C18 symmetry column, Milli Q water, M/S applied Biosystems APIQSTAR pulsar mass spectrophotometer, MGYP medium, ethyl acetate, anhydrous sodium sulphate, chloroform, methanol, catharanthine (Bioresource Pvt. Ltd), vindoline (Bioresource Pvt. Ltd) ,vinblastine (Sigma-Aldrich), ceric ammonium sulphate reagent, HPLC grade acetonitrile, HPLC grade methanol, acetic acid and sterile distilled water.

Methods:

The endophytic fungus *Fusarium oxysporum* of *Catharanthus roseus* was grown in MGYP medium containing Malt extract, Glucose, Yeast extract, and Peptone at 25-28^{o}C under shaking condition (200 rpm) for 96 hrs. After 96 hrs of fermentation, centrifugation (5000 rpm) at 10°C for 20 min was carried out to separate the mycelia from the culture broth. The mycelia were washed thrice with sterile distilled water under sterile conditions. The harvested mycelial mass (20 gm wet mycelia) was then resuspended in 100 ml sterile distilled water in 500 ml Erlenmeyer flask at pH 6 and the biotransformation was started by adding 1 mg of catharanthine and 1 mg of vindoline which were predissolved in water under sterile conditions. The whole mixture was put on shaker at 28^{o}C (200 rpm). The biotransformation was routinely monitored by collecting various fractions (after 8 days) of the reaction, by separating the fungal mycelia from the aqueous component by filtration.

The filtrate (aqueous component) obtained after separating the mycelial mass was extracted with ethyl acetate as a solvent system[15]. The organic layer was separated from the aqueous layer using separating funnel. The extraction was repeated thrice in order to get the maximum recovery of the compounds. The solvent was dried using anhydrous sodium sulphate and concentrated under vacuum using rotavapour at 40^{o}C in order to get the crude extract. The biotransformation of catharanthine and vindoline to vinblastine was analysed on TLC using chloroform: methanol (8:2) as a solvent system and visualized by ceric ammonium sulphate reagent[16]. The analysis was done with comparison with standard catharanthine, vindoline and vinblastine.

Biotransformation of catharanthine and vindoline to vinblastine was also determined by HPLC using C18 symmetry column. Biotransformed sample (after 8 days) was

taken in 10 µl acetonirile injected in HPLC column and gradient elution was performed using 5- 95% acetonirile at a flow rate of 0.5 per min. A dual wave length recorder set at 220 nm and 254 nm was used to detect the biotransformed vinblastine[17-24]. Molecular mass of the biotransformed vinblastine was determined by M/S applied Biosystems APIQSTAR pulsar (ESI-MS) mass spectrophotometer. Biotransformed extract was dissolved in HPLC grade methanol, water and acetic acid in the ratio of 50:50:0.1. The above sample was then analyzed by infusion method (injected into MS) at the rate of 5 µl/min and at an IS voltage of 3800V in TOF mode. Spectrum from a range of m/z 100-900 Dalton was obtained [22-26].

Results:

The crude extract, on TLC on silica gel-G using chloroform: methanol (8:2) solvent system produced clearly differentiated purple color spot after eight days when sprayed with ceric ammonium sulphate reagent. The Rf value of the spot on TLC of the crude extract using chloroform: methanol (8:2) solvent system when compared with standards vindoline and catharanthine, vinblastine showed identical Rf values of spot (0.84) with vinblastine. Minimum contaminants were obtained in the crude extracts as we did the reaction in sterile distilled water. Analysis of the crude extract showed dark purple spots on TLC, when sprayed with ceric ammonium sulphate reagent, among which one has similar Rf value as that of standard vinblastine, which confirms that the transformation of catharanthine and vindoline to vinblastine was complete after eight days by fungus *Fusarium oxysporum* at room temperature.

A B C D

A: Standard vindoline

B: Standard catharanthine

C: Biotransformed vinblastine

D: Standard vinblastine

Detection: Ceric ammonium sulphate reagent

Fig.1: TLC showing standard vindoline, catharanthine and vinblstine along with biotransformed vinblastine

HPLC profile of biotransformed vinblastine:

Crude extract obtained after eight days on C18 symmetry column showed peak having equal RT (35.6 mins) as that of standard vinblastine.

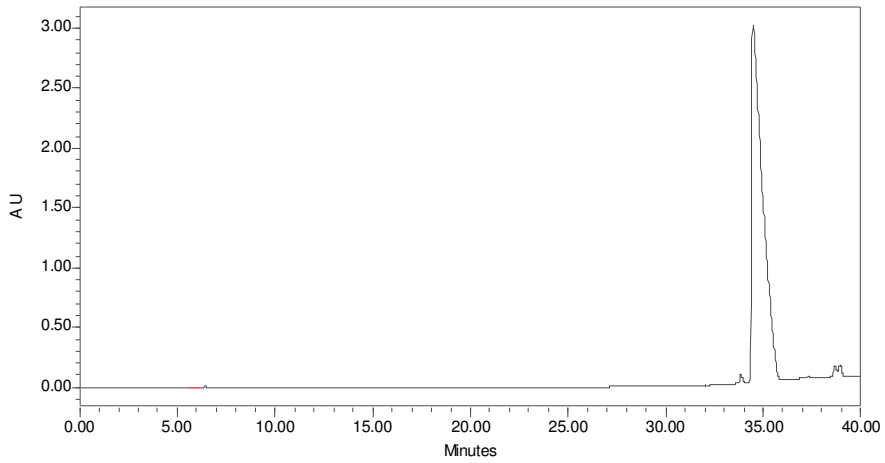


Fig. 2: HPLC profile of biotransformed vinblastine with retention time of 35.6 min.

ESI-MS of biotransformed vinblastine (8 days):

ESI-MS yielded ion at m/z 811 confirmed the presence of vinblastine after 8 days and also confirmed that the fungus *Fusarium oxysporum* converts catharanthine and vindoline to vinblastine at room temperature after 8 days.

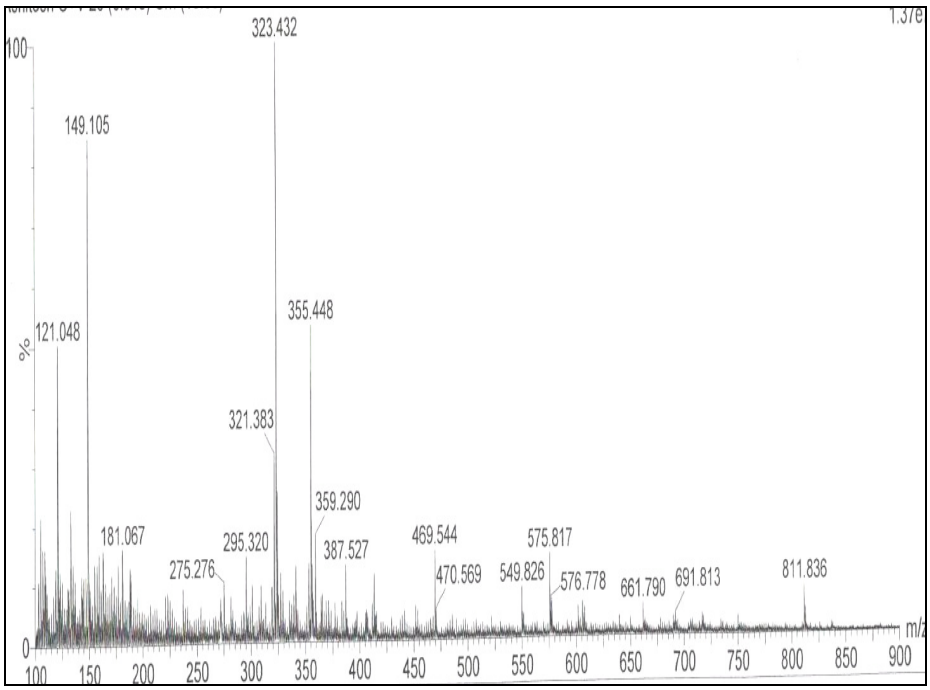


Fig. 3: ESI-MS spectrum of m/z 811 (M+H) ions of biotransformed vinblastine.

Discussion:

The biotransformation of catharanthine and vindoline to vinblastine at room temperature with the help of fungus *Fusarium oxysporum* was confirmed when ESI-MS analysis showed peak at m/z 811 attributing to the $(M+H)^+$ ion of vinblastine. Studies on the biotransformation of catharanthine and vindoline to vinblastine with the help of fungus *Fusarium oxysporum* indicates that the fungus converts catharanthine and vindoline to vinblastine *in vitro* at room temperature. TLC, HPLC and ESI-MS of crude extract confirm this conclusion. Hirata *et al* (1997) reported biomimetic one-pot synthesis of vinblastine: NAD(P)H-mediated vinblastine synthesis from the product of FMN-mediated vindoline-catharanthine coupling under near-ultraviolet light. Coupling of catharanthine and vindoline to form vinblastine has been reported by several groups [27-37]. This is the first report of biotransformation of catharanthine and vindoline to vinblastine from *Fusarium oxysporum* at room temperature.

Summary:

For the biotransformation of L-tryptophan and secologanin to vinblastine, the fungus *Fusarium oxysporum* was grown and harvested as described above. The harvested mycelial mass (20 gm wet wt.) was then resuspended in 100 ml of sterile distilled water in 500 ml Erlenmeyer flask at pH 6 and the biotransformation was started by adding 1 mg of L-tryptophan, predissolved in 1 ml of 1M of NaOH and 1 mg of secologanin which was predissolved in water under sterile conditions. The biotransformations were routinely monitored by periodic sampling of aliquots (5 ml) which were extracted with ethyl acetate. Extracted solvents were dried and concentrated under vacuo. The crude transformed compound was analysed by TLC. The transformed compound was purified by TLC and HPLC. The purified compound had similar chromatographic and spectroscopic properties as that of vinblastine. However, further characterization with the help of ESI-MS led to the conclusion that the compound obtained is vinblastine.

Materials:

Erlenmeyer flask (500 ml), shaker, centrifuge, separating funnel, rota vapour, TLC (0.5 mm thick, 20 cmx20 cm) silica plate, HPLC (waters), C18 symmetry column, MilliQ water, M/S applied Biosystems APIQSTAR pulsar mass spectrophotometer, MGYP medium, ethyl acetate, anhydrous sodium sulphate, chloroform, methanol, L-trypthophan (MP Biomedicals), secologanin (Bioresource Pvt. Ltd.), 1M NaOH, and vinblastine(Sigma-Aldrich), ceric ammonium sulphate reagent, HPLC grade acetonitrile, HPLC grade methanol, acetic acid and sterile distilled water.

Methods:

The endophytic fungus *Fusarium oxysporum* of *Catharanthus roseus* was grown in MGYP medium containing Malt extract, Glucose, Yeast extract, and Peptone at 25-28^{o}C under shaking condition (200 rpm) for 96 hrs. After 96 hrs of fermentation, centrifugation (5000 rpm) at 10°C for 20 min was carried out to separate the mycelia from the culture broth. The mycelia were washed thrice with sterile distilled water under sterile conditions. The harvested mycelial mass (20 gm wet mycelia) was then resuspended in 100 ml sterile distilled water in 500 ml Erlenmeyer flask at pH 6 and the biotransformation was started by adding 1 mg of L-tryptophan, predissolved in 1 ml of 1M NaOH and 1 mg of secologanin which was predissolved in water under sterile conditions. The whole mixture was put on shaker at 28^{o}C (200 rpm). The biotransformation was routinely monitored by collecting various fractions (after 8 days) during the course of reaction, by separating the fungal mycelia from the aqueous component by filtration.

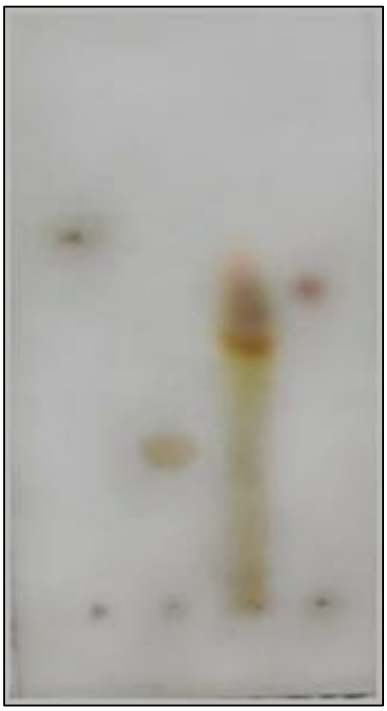
The filtrate (aqueous component) obtained after separating the mycelial mass was extracted with ethyl acetate as a solvent system[15]. The organic layer was separated from the aqueous layer using separating funnel. The extraction was repeated thrice in order to get the maximum recovery of the compounds. The solvent was dried using anhydrous sodium sulphate and concentrated under vacuum using rotavapour at 40^{o}C in order to get the crude extract. The biotransformation of L-tryptophan and secologanin to vinblastine was analysed on TLC using chloroform: methanol (8:2) as a solvent system and visualized by ceric ammonium sulphate reagent[16]. The analysis was done by comparing with standard L-tryptophan and secologanin and vinblastine.

Biotransformation of L-tryptophan and secologanin to vinblastine were also determined by HPLC using C18 Symmetry Column. Biotransformed sample (after 8 days) was taken in 10 µl acetonirile injected in HPLC column and gradient elution performed using 5- 95% acetonirile at a flow rate of 0.5 ml per minute. A dual wave length recorder set at 220 nm and 254 nm was used to detect the biotransformed vinblastine[17-24].

Molecular mass of the biotransformed vinblastine was determined by M/S applied Biosystems APIQSTAR pulsar (ESI-MS) mass spectrophotometer. Biotransformed extract was dissolved in HPLC grade methanol, water, acetic acid in the ratio of 50:50:0.1. The above sample was then analyzed by infusion method (injected into MS) at the rate of 5 µl/min and at an IS voltage of 3800V in TOF mode. Spectrum from a range of m/z 100-900 Dalton was obtained[22-26].

Results:

The crude extract, on TLC on silica gel-G using chloroform: methanol (8:2) solvent system produced clearly differentiated purple color spot after eight days when sprayed with ceric ammonium sulphate reagent. The Rf value of the spot on TLC of the crude extract using chloroform: methanol (8:2) solvent system when compared with standards secologanin, tryptophan and vinblastine showed identical Rf value of spot (0.71) with vinblastine[16]. Minimum contaminants were obtained in the crude extracts as we did the reaction in sterile distilled water. Analysis of the crude extract showed dark purple spots on TLC, when sprayed with ceric ammonium sulphate reagent, among which one had similar Rf value as that of standard, which confirmed that the transformation L-tryptophan and secologanin to vinblastine was complete after 8 days by fungus *Fusarium oxysporum* at room temperature.



A B C D

A: Standard secologanin
B: Standard L-tryptophan
C: Biotransformed vinblastine
D: Standard vinblastine
Detection: Ceric ammonium sulphate reagent

Fig.1: TLC showing standard secologanin, standard L-trytophan and vinblastine along with biotransformed vinblastine

HPLC profile of biotransformed vinblastine:

Crude extract obtained after eight days on C18 symmetry column showed peak having equal RT (35.6 mins) as that of standard vinblastine.

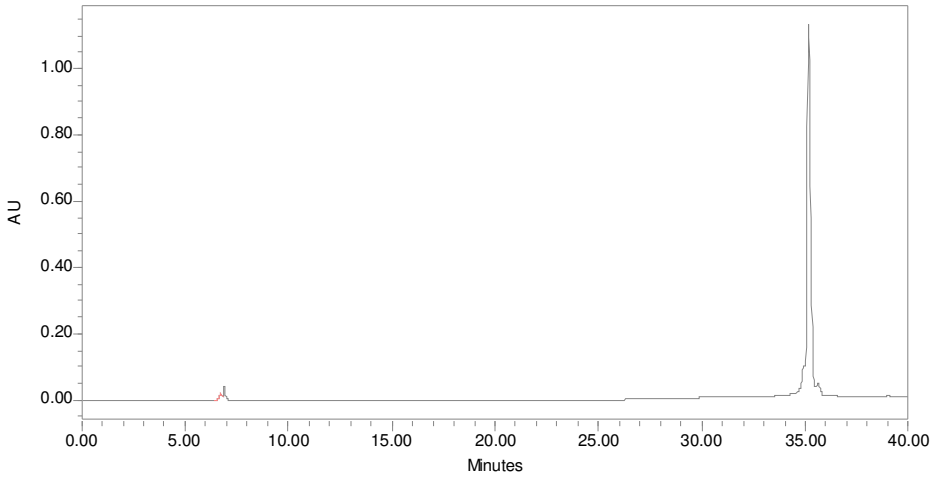


Fig.2: HPLC profile of biotransformed vinblastine with retention time of 35.6 mins.

ESI-MS of biotransformed vinblastine (8 days):

ESI-MS yielded ion at m/z 811 which confirmed the presence of vinblastine after eight days and also confirmed that the fungus *Fusarium oxysporum* converts L-tryptophan and secologanin to vinblastine at room temperature after eight days.

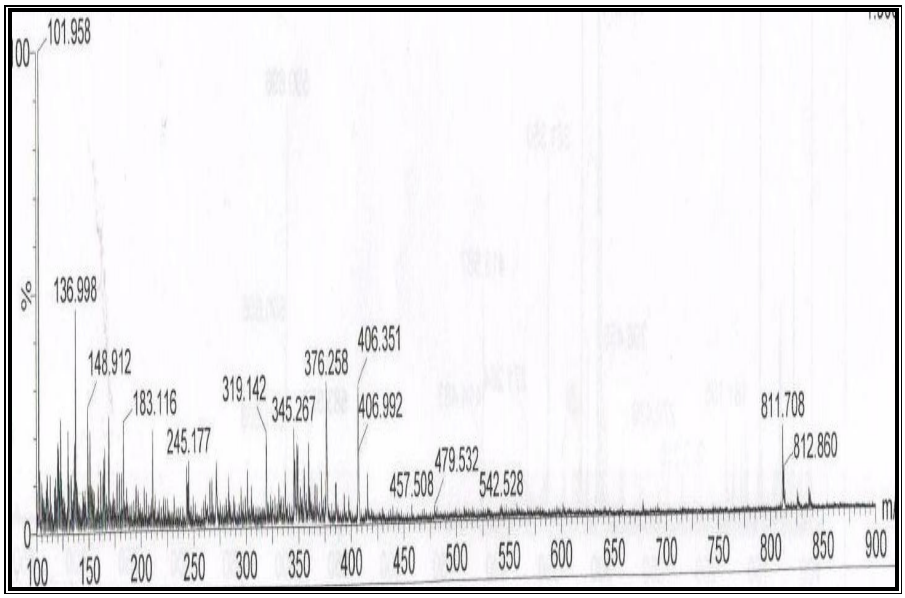


Fig.3: ESI-MS spectrum of m/z 811 (M+H) ions of biotransformed vinblastine.

Discussion:

The biotransformation of L-tryptophan and secologanin to vinblastine at room temperature with the help of fungus *Fusarium oxysporum* was confirmed when ESI-MS analysis showed peak at m/z 811 attributing to the $(M+H)^+$ ion of vinblastine. Studies on the biotransformation of L-tryptophan and secologanin to vinblastine with the help of fungus *Fusarium oxysporum* indicates that the fungus converts L-tryptophan and secologanin to vinblastine *in vitro* at room temperature. TLC, HPLC and ESI-MS of crude extract confirm this conclusion. To the best of our knowledge, this is the first report of biotransformation of L-tryptophan and secologanin to vinblastine from *Fusarium oxysporum* at room temperature.

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CHAPTER 8

Conjugation of nanoparticles with vinca alkaloid

Summary:

Commercially available vinblastine was used to conjugate with gadolinium oxide nanoparticles synthesized using a fungus *Humicola* sp. Biologically synthesized nanoparticles have a native protein coat. The carboxylic group present on these proteins was targeted to couple with secondary amino groups present on vinblastine. 2 mg of vinblastine is dissolved in 1 ml of distilled water and then mixed with 2 mg of gadolinium oxide nanoparticles dissolved in 1 ml of distilled water and then 0.8 ml of formaldehyde (7.5 % v/v) and 0.25 ml sodium acetate was added. The mixture was stirred overnight at room temperature. After stirring, the reaction mixture was concentrated under high vacuum and further purification of vinblastine-gadolinium conjugate was done by HPLC. The conjugate from other chemical contaminants was purified by HPLC using acetonitrile 5%-95% on a C_{18} symmetry column. The compound eluted from the column were detected at 254 and 340 nm (vinblastine-gadolinium conjugate) using a dual wavelength detector.

Introduction:

Nanomaterials of different chemical compostions, such as liposomes, micelles or inorganic nanoparticles hold tremendous potential as carriers for drugs to target cancer cells. Several anti-cancer drugs including paclitaxel[1,2], doxorubicin[3], 5-fluorouracil[4] and dexamethasone[5] have been successfully formulated using nanomaterials. Polylactic/glycolic acid (PLGA) and polylactic acid (PLA) based nanoparticles have been formulated to encapsulate chemotherapeutic agent, dexamethasone[5]. The effectiveness of drug delivery systems can be attributed to their small size, reduced drug toxicity, controlled time release of the drug and modification of drug pharmacokinetics and biological distribution. Too often, chemotherapy fails to cure cancer because some tumor cells develop resistance to multiple anticancer drugs. In most cases, resistance develops when cancer cells begin expressing a protein, known as p-glycoprotein that is capable of pumping anticancer drugs out of a cell as quickly as they cross through the cell's outer membrane. New research shows that nanoparticles may be able to get anticancer drugs into cells without triggering the p-glycoprotein pump[2, 6].

Cancer has remained the most dreadful disease till date. A variety of chemotherapeutics as mentioned above and vinblastine and vincristine are available to deal with a variety of cancers. However, use of chemotherapy to cure cancer not only kills cancer cells but also targets normal dividing cells of the body. Moreover, even the most potent therapy has rendered useless owing to its failure to reach the therapeutic target *in vivo*. Therefore, the need is to focus the research on targeted drug delivery which would only deliver the chemotherapeutics to cancerous cells while sparing the normal cells.

Recently researchers have started exploiting receptor mediated endocytosis to achieve target specific drug delivery where the therapeutics can be targeted to the diseased tissue with the use of strategy of coupling therapeutics to certain molecules which have recognition by specific receptors present on the target tissue. Today with tremendous research being carried out on cancer, it has become evident that most cancers and their metastasis overexpress certain receptors, which act as potential drug targets for cancer therapy. High levels of αhFR (folate receptors) are over expressed on a variety of human cancers like ovarian, breast, brain, lung and colorectal cancers but are restricted in normal tissues. Internalization of folates hFR involves receptor

mediated endocytosis. Consequently, several strategies have been developed for the targeted drug delivery or drugs to hFR - positive tumor cells. Covalent attachment of therapeutic agents to hFR targeted monoclonal antibodies have shown significant targeting efficacy in patients with ovarian cancer. Alternatively, folate derivatized anticancer treatments have been successfully applied *in vitro* for hFR - specific delivery. Evidence for over expression of transferrin (a plasma glycoprotein) receptor on tumor cells including oral, prostrate and breast cancer cells implicates its role in target- specific delivery.

The nanoparticles which our group has synthesized using microbes and plants are capped with natural proteins making them water-dispersible and may bind to integrins or VEGFs (vascular endothelial growth factors). Therefore, targeting integrins and VEGFs is a novel anti-angiogenesis strategy for treating a wide range of solid tumors. Hence, these nanoparticles can be used directly as drugs in the future. Our group has synthesized a range of nanomaterials, of different chemical compositions[7-10] for drug delivery and target drug delivery. In the present study, we have synthesized biocompatible and fluorescent gadolinium oxide vinblastine conjugate.

Materials:

Gadolinium oxide nanoparticles, CH_3COONa (3M), HCHO (7.5% v/v), Vinblastine (Sigma-Aldrich), distilled water, magnetic stirrer, magnetic beads, beakers, HPLC (Waters), C18 symmetry column, HPLC grade acetonitrile, Milli Q water, HPLC grade methanol and Shimadzu PC 101 Spectrophotometer.

Methods:

Conjugation of nanoparticles with Vinblastine:

In order to synthesize highly fluorescent gadolinium oxide nanoparticles-vinblastine conjugate, following studies were carried out. Commercially available vinblastine was used to conjugate with gadolinium oxide nanoparticles synthesized using a fungus *Humicola* sp. Biologically synthesized nanoparticles have a native protein coat. The carboxylic group present on these protein was targeted to couple with secondary amino groups present on vinblastine. 2 mg of vinblastine is dissolved in 1 ml of distilled water and then mixed with 2 mg of gadolinium oxide nanoparticles dissolved in 1 ml of distilled water and then 0.8 ml of formaldehyde (7.5 % v/v) and 0.25 ml sodium acetate was added. The mixture was stirred overnight at room temperature. After stirring, the reaction mixture was concentrated under high vacuum and further purification of vinblastine-gadolinium conjugate was done by HPLC[11-13]. The conjugate from other chemical contaminants was purified by HPLC using acetonitrile 5%-95% on a C_{18} symmetry column[14-21]. The compound eluted from the column were detected at 220 and 340 nm (vinblastine-gadolinium conjugate) using a dual wavelength detector.

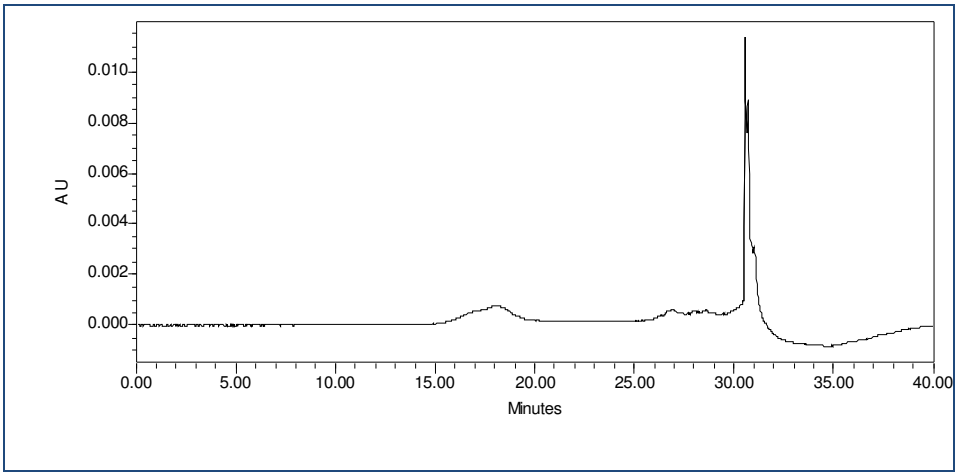
Purification by HPLC:

The conjugation from other chemical contaminants was purified by HPLC using 5-95% actenitrile on a C18 symmetry column. The compounds eluted from the column were detected at 220 nm and 340 nm using a dual wavelength detector.

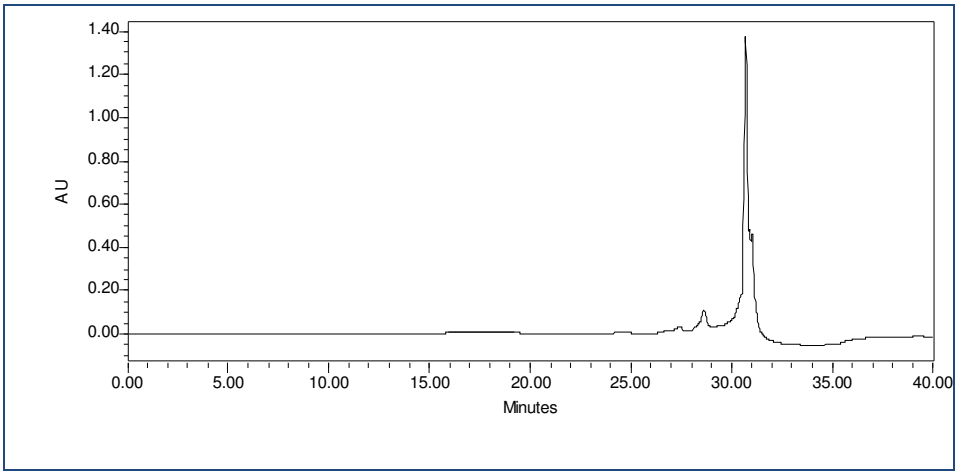
Results:

Gadolinium oxide nanoparticles-vinblastine conjugate:

HPLC profile of gadolinium oxide-vinblastine conjugate showing absorbance at both wavelengths i.e. 340 nm (Fig1) and 220 nm (Fig 2) which are attributing to gadolinium oxide and vinblastine respectively. A single peak at both the wavelengths corresponds to the same retention time confirming the coupling of gadolinium oxide nanoparticles with vinblastine. Other peaks are also coming since there will be unconjugated part of both the entities remain in the solution itself.



HPLC profile of Gadolinium oxide nanoparticles-vinblastine conjugate at 340 nm



HPLC profile of Gadolinium oxide nanoparticles-vinblastine conjugate at 220 nm

Discussion:

Vinblastine was successfully derivatized and then secondary amino group of vinblastine was conjugated with carboxylic group present on gadolinium oxide nanoparticles[11]. However, our studies revealed that biologically synthesized nanoparticles can not only be used for the fluorescence imaging but can be easily coupled with drugs. Ansari *et al*. have synthesized CdS nanoparticles enzymatically and then conjugated with plant lectins[22]. Our group has also synthesized CdS nanoparticles enzymatically and then conjugated with anticancer drug taxol. These observations support our present findings.

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CHAPTER 9

General discussion and conclusions

Discussion:

Recent interest has been focused on the study of endophytic and bark associated fungi from medicinal plants. One of the most significant discoveries in this area has been the isolation of an endophytic fungus, *Taxomyces andreanae* from the bark of Pacific Yew (*Taxus brevifolia*) which has been shown to produce taxol, the antitumor diterpenoid produced by the *Taxus* plant[1-7]. The concept has been gaining ground ever since the discovery that the endophytic fungi associated with medicinal plants may have the potential to acquire the capability to produce valuable metabolites of medicinal value from the parent plant and such fungi could become independent and alternative sources for these metabolites which can be produced by fermentation. This opens a tremendous new area for research and development, particularly for screening the innumerable medicinal plants of indigenous origin for endophytes and evaluating them for their potential to manufacture the valuable compounds of plant origin[8-12]. Another important anticancer compound is the alkaloid camptothecin, a potent anti-neoplastic agent which was first isolated from wood of *Camptotheca acuminata.* Kusari *et al* isolated endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues[13-14]. Similarly, podophylotoxins were reported from fungus *Phialocephala fortinii* isolated from *Podophyllum peltatum*[15-17]. Podophylotoxin and analogues are clinically relevant mainly due to their cytotoxicity and antiviral activities and are valued as a precursor to useful anticancer drugs like etoposide, teniposide, and etopophos phosphate[18].

Shreekant *et al.* (2009) isolated endophytic fungi from Indian Yew tree *Taxus baccata*, and then screened for taxol production. Out of the 40 fungal cultures screened, one fungus *Gliocladium* was found to produce taxol and 10-DABIII (10-deacteyl baccatin III). These compounds were purified by TLC and HPLC and characterized using UV spectroscopy, ESI-MS, MS/MS and proton NMR. One litre of the *Gliocladium* sp. yielded 10 µg of taxol and 65 µg of 10-DAB III[7]. Husain *et al.* (1993) isolated (–) jasmonic acid from the fungus *Botryodiplodia theobromae* and Ahmad *et al*. (2001) ergot alkaloid and indole alkaloids from the endophytic fungus *Balansia sclerotic*[8-12.] We took the advantage of this feature and started the isolation and purification of endophytic fungi from *Catharanthus roseus* plant in order to isolate low volume and high valued vinca alkaloids such as vinblastine and vincristine.

The aim of present work was to isolate, purify and identify slow growing and unusual endophytic fungi associated with leaves of *Catharanthus roseus* plant. Because of the slow growth of these fungi, major difficulties are encountered in purifying these fungi and in maintaining pure cultures for extended periods required in most of the investigation. Isolation of endophytic fungi from surface of sterilized tissues of excised leaves indicates the endophytic nature of the fungi. Endophytic nature of several *Balansia* sp. has been established (Leuchtman and Clay, 1988). However, some species of *Balansia* namely *Balansia cyperi* and other related genera could not be isolated from their host after surface sterilization. They have been considered as epiphytes. Isolations of endophytic fungi from surface sterilized tissues can be a useful method for distinguishing their endophytic or epiphytic nature. The endophytic fungi isolated from *Catharanthus roseus* represent different genera of fungi and screened for vinca alkaloids.

Studies on the isolation of vinca alkaloids produced by endophytic fungi (AA-CRL-6 and AA-CRL-20) of *Catharanthus roseus* show that AA-CRL-6 and AA-CRL-20 produce vinblastine and vincristine *in vitro*[19]. TLC analysis of the extracts from culture filtrate of AA-CRL-6 and AA-CRL-20 confirm this conclusion. In addition to these alkaloids, the fungus AA-CRL-6 also produces three unidentified alkaloids and AA-CRL-20 produces four unidentified alkaloids though in traces. Kharwar *et al* (2008) isolated several endophytic fungi from *Catharanthus roseus* from India but to the best of our knowledge, there are so far no reports of isolation of these drugs from the above endophytes[20]. Zahng *et al* (2000) and Tung *et al* (2002) discovered that vincristine is produced by *Fusarium oxysporum*[21, 22], an endophyte of *Catharanthus roseus;* while Guo and Kunming (1998) isolated vinblastine from *Alternaria* sp. isolated from *Catharanthus roseus*[23.] However they have identified the production of vinblastine and vincristine by TLC and HPLC only[24-28]. To the best of our knowledge this is the first report of isolation of vinblastine and vincristine from endophytic fungi isolated in India. However, these alkaloids are commonly found in *Catharanthus roseus* plant in traces. These observations support present findings. This suggests that the ability to synthesize vinblastine and vincristine derivatives resides not only in the *Catharanthus roseus* plant, *Fusarium oxysporum* and *Alternaria* sp. but also in endophytic fungi of *Catharanthus roseus* isolated by us.

Morphology of AA-CRL-6 and AA-ARL-20 isolate obtained from *Catharanthus roseus* mostly agree with the description of Nelson *et al*, 1983[29]. The fungus AA-CRL-6 growing on PDA medium produce slow growing colonies. Colonies on PDA show the following characters: snow white, circular, flucculose, compact, reverse pale white, hyphae profusely branched, septate, smooth, hyaline, phialide produced from short lateral hyphae [30]. The strain also produces three types of spores called macroconidia, microconidia and chlamydospores. Macroconidia, fusoid, with prominent foot cell, 3-septate, hyaline, fewer in number than microconidia. Microconidia, produce from short lateral phialide. Microconidia are oval to cylindrical straight to curved, variable in shape and size, produce abundantly and single celled. Chlamydospores form after 10-15 days, produce from lateral hyphae, globose to sub globose, hyaline, smooth or rough walled, terminal and form singly [31].

The other positive strain AA-CRL-20 isolated and grown on PDA medium produce slow growing colonies. Colonies on PDA show the following characters: White, circular, flucculose, compact and raised, reverse light brown, hyphae profusely branched, septate, smooth, hyaline, phialide arising laterally on hyphae[32] produce three types of spores called macroconidia, microconidia and chlamydospores. Macroconidia produce from lateral phialide, 3-septate, wedge shape, hyaline large in number. Microconidia, produced from lateral long slender phialide accumulate on terminal end of phialides, variable in shape and size, unicellular, oval or kidney shaped, hyaline. Chlamydospores form singly or in pairs or in chains, sub-globose with a smooth or wrinkled wall [33]. All of the above mentioned characters are identical to those described for the fungus, *Fusarium* sp. of fungal strain AA-CRL-6 and *Fusarim solani* of fungal strain AA-CRL-20. Hence fungus AA-CRL-6 can be referred as *Fusarium* sp. and AA-CRL-20 *Fusarium solani*. Studies on the different media on the growth and sporulation of *Fusarium* sp. and *Fusarium solani* have been studied. Studies indicate that fungi can be grown on media such as natural, synthetic and semi-synthetic.

In sequence analysis using BLAST and CLUSTAL-W both ITS sequences (0.48 kb) show highest similarity with genus *Fusarium oxysporum* The phylogenetic studies indicate that the nearest relative of the fungus AA-CRL-6 is *Fusarium* oxysporum. Considering the cultural and morphological characters, we identified the fungus as *Fusarim oxysporum* [34]. The sequence analysis and phylogenetic analysis do support the identification. Similarly, in sequence analysis using BLAST and CLUSTAL-W

both ITS sequences (0.52 kb) show highest similarity with genus *Fusarium solani*. The phylogenetic studies indicate that the nearest relative of the fungus AA-CRL-20 is *Fusarium solani*. From the cultural and morphological characters, we identified the fungus as *Fusarim solani* [35]. The sequence analysis and phylogenetic analysis do support the identification.

Although *Fusarium oxysprum* and *Fusarium solani* have widespread occurrence in plant soil across India and different endophytic fungi have been isolated from Indian *Catharanthus roseus*, no attempts have been made to isolate vinca alkaloids from above endophytic fungi. The *Fusarium oxysporum* which we isolated produces both vinblastine and vincristine. The isolation of vinblastine and vincristine from culture filtrate of *Fusarium oxysporum* and *Fusarium solani* is the first demonstration of this occurring in fungi isolated from Indian *Catharanthus roseus* plant. Thus, suggests that the ability to synthesize vinblastine and vincristine resides not only in the fungal strain isolated from *Catharanthus roseus* of above mentioned countries, but also from endophytic fungi of *Catharanthus roseus* growing in India. In conclusion, we have isolated endophytic fungi such as *Fusarium oxysporum* and *Fusarium solani* producing vinblastine and vincristine. Since the quantity of vinblastine and vincristine is higher in *Fusarium oxysporum* as compared to *Fusarium solani*, therefore further work was carried out only on *Fusarium oxysporum*.

Studies on the isolation of the vinca alkaloids produced by *Fusarium oxysporum* indicate that the fungus produces vinblastine in the culture filtrate *in vitro*. TLC, HPLC and other analyses of the extracts from culture filtrate confirm this conclusion. In addition to vinblastine and vincristine, this fungus also produces few unidentified vinca alkaloids, although in trace amounts. To the best of our knowledge, this is the first report of isolation of vinblastine from *Fusarium oxysporum* isolated from Indian *Catharanthus roseus* plant. The necessary precursor molecules such as tryptophan and geraniol required for the formation of vinca alkaloids were provided in the growth medium as they are essential for vinblastine production as reported earlier from other sources.

The ability of *Fusarium oxysporum* to make vinblastine was confirmed by the isolation of compound having chromatographic properties similar to those of standard vinblastine in three solvent systems a, b and c which showed a single dark purple spot on TLC when sprayed with ceric ammonium sulphate reagent[19]. Fungal vinblastine on HPLC C18 symmetry column showed a single symmetrical peak at retention time of 36.6 mins that confirmed its homogeneity. Absorption maximum of

the purified fungal vinblastine was found to be at 220 nm as reported earlier [24-27]. In ESI-MS, molecular ions at m/z 811 attributable to $(M+H)^+$ confirmed its molecular weight to be m/z 811[36-39]. MS-MS showed fragment ions at m/z attributable to structures which are usually seen as vinblastine fragment ions[36]. ^{1}H NMR spectrum was nearly matched with that of standard vinblastine spectrum[40-44]. Vinblastine obtained per liter of culture was estimated to be approximately 76 µg. This is the first report of vinblastine from an endophyte of *Catharanthus roseus* growing in India. Anti-tumor activity of vinblastine was checked against HL-60, J774 and THP-1 cell lines, showing IC_{50} 14.1, 55.1 and 36.9 µg/ml fungal vinblastine respectively[45-52]. Vinblastine showed less cytotoxicity in J774 cell lines. However, in case of HL-60 and THP-1, the cell growth inhibition was significantly higher even at low vinblastine concentration.

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The ability of *Fusarium oxysporum* to make vincristine was confirmed by the isolation of compound having chromatographic properties similar to those of standard vincristine in three solvent systems a, b and c which showed a single dark violet spot on TLC when sprayed with ceric ammonium sulphate reagent[19]. Fungal vincristine on HPLC C18 symmetry column showed a single symmetrical peak at retention time of 34.9 mins that confirmed its homogeneity. Absorption maximum of the purified fungal vincristine was found to be at 220 nm as reported earlier[24-27]. In ESI-MS molecular ion at m/z 825 attributable to $(M+H)^+$ m/z confirmed its molecular weight to be m/z 824 [36-39]. MS-MS showed fragment ions at m/z attributable to structures which as usually seen are vincristine fragment ions [36].

^{1}H NMR spectrum matched with that of standard vincristine spectrum [40, 53, 54]. Vincristine obtained per liter of culture was estimated to be approximately 66 µg. This is the first report of vincristine from an endophyte of *Catharanthus roseus*

growing in India. Anti-tumor activity of vincristine was checked against HL-60, J774 and THP-1 cell lines, showing IC_{50} 14.1, 55.1 and 36.9 µg/ml respectively. Vincristine showed less cytotoxicity in J774 cell lines[45, 51, 52, 55-57]. However, in case of HL-60 and THP-1 the cell growth inhibition was significantly higher even at low vincristine concentration.

The biotransformation of vinblastine to vincristine at room temperature with the help of fungus *Fusarium oxysporum* was confirmed when ESI-MS analysis showed peak at m/z 825 attributing to the $(M+H)^+$ ion of vincristine[36-39]. Studies on the biotransformation of vinblastine to vincristine with the help of fungus *Fusarium oxysporum* indicate that the fungus converts vinblastine to vincristine *in vitro* at room temperature. TLC, HPLC and ESI-MS of crude extract confirm this conclusion. In addition to vincristine, the fungus also produces an unidentified compound after six days which disappeared after eight days. To the best of our knowledge, this is the first report of biotransformation of vinblastine to vincristine from *Fusarium oxysporum* at room temperature.

The biotransformation of catharanthine and vindoline to vinblastine at room temperature with the help of fungus *Fusarium oxysporum* was confirmed when ESI-MS analysis showed peak at m/z 811 attributing to the $(M+H)^+$ ion of vinblastine[36-39]. Studies on the biotransformation of catharanthine and vindoline to vinblastine with the help of fungus *Fusarium oxysporum* indicates that the fungus converts catharanthine and vindoline to vinblastine *in vitro* at room temperature. TLC, HPLC and ESI-MS of crude extract confirm this conclusion. To the best of our knowledge, this is the first report of biotransformation of catharanthine and vindoline to vinblastine from *Fusarium oxysporum* at room temperature. The biotransformation of L-tryptophan and secologanin to vinblastine at room temperature with the help of fungus *Fusarium oxysporum* was confirmed when ESI-MS analysis showed peak at m/z 811 attributing to the $(M+H)^+$ ion of vinblastine[36-39]. Studies on the biotransformation of L-tryptophan and secologanin to vinblastine with the help of fungus *Fusarium oxysporum* indicates that the fungus converts L-tryptophan and secologanin to vinblastine *in vitro* at room temperature. TLC, HPLC and ESI-MS of crude extract confirm this conclusion. To the best of our knowledge, this is the first report of biotransformation of L-tryptophan and secologanin to vinblastine from *Fusarium oxysporum* at room temperature.

Vinblastine was successfully derivatized and then secondary amino group of vinblastine was conjugated with carboxylic group present on gadolinium oxide nanoparticles. However, our studies revealed that biologically synthesized nanoparticles can not only be used for the fluorescence imaging but can be easily coupled with drugs. Ansari *et al*. have synthesized CdS nanoparticles enzymatically and then conjugated with plant lectins ( ). Our group has also synthesized CdS nanoparticles enzymatically and then conjugated with anticancer drug taxol. These observations support our present findings.

Conclusion:

During the course of present work, following conclusions have been made:

1. 52 endophytic fungi were isolated and purified from the leaves of *Catharanthus roseus* plant growing in different areas of Pune.
2. These fungi were screened for vinca alkaloid production. Out of 52 endophytic fungi tested, two endophytic fungi AA-CRL-6 and AA-CRL-20 were found to produce vinca alkaloids.
3. Fungal strains AA-CRL-6 and AA-CRL-20 were identified as *Fusarium oxysporum* and *Fusarium solani* respectively based on cultural, molecular, and morphological characterization.
4. Concentration of vinca alkaloids was more in *Fusarium oxysporum* as compared to *Fusarium solani,* therefore further studies were carried out on *Fusarium oxysporum* only.
5. Fungal vinblastine was purified by using column chromatography, preparative TLC and HPLC.
6. Homogeneity of fungal vinblastine was determined by TLC on 3 solvent systems. Fungal vinblastine Rf was found to be 0.77. Vinblastine produced by fungus was quantified to be 76 µg/l. Molecular mass of the fungal vinblastine was determined to be m/z 810 by ESI-MS. MS-MS showed fragments at 355, 522, 542, 733, 751, 793 and 811.
7. Further characterization was done by comparison of standard vinblastine proton spectrum with that of fungal vinblastine proton spectrum and was found to be similar. Cytotoxicity of fungal vinblastine was determined on three different cell lines (HL-60, J774 and THP-1 cell lines). 50% cell proliferation inhibitions were observed with 17.2 µM, 67.9 µM and 45.4 µM of fungal vinblastine against HL-60, J774 and THP-1 cell lines respectively.
8. Fungal vincristine was purified by using column chromatography, preparative TLC and HPLC.
9. Homogeneity of fungal vincristine was determined by TLC on 3 solvent systems. Fungal vincristine Rf was found to be 0.74. Vincristine produced by fungus was quantified to be 67 µg/l. Molecular mass of the fungal vincristine was determined to be m/z 824 by ESI-MS. MS-MS showed fragments at 766, 807 and 825 were seen.
10. Further characterization was done by comparison of standard vincristine proton spectrum with that of fungal vincristine proton spectrum and was

found to be similar. Cytotoxicity of fungal vincristine was determined on three different cell lines (HL-60, J774 and THP-1 cell lines). 50% cell proliferation inhibitions was observed with 26.7 µM, 54.6 µM and 53.4 µM of fungal vinblastine against HL-60, J774 and THP-1 cell lines respectively.

11. Biotransformation of vinblastine to vincristine, catharanthine and vindoline to vinblastine, L-tryptophan and secologanin to vinblastine was done using fungus *Fusarium oxysporum*. Biotransformed vincristine from vinblastine, vinblastine from catharanthine and vindoline and again vinblastine from L-tryptophan and secologanin was purified by HPLC and characterized with the help of ESI-MS.
12. Vinblastine was successfully modified and coupled with microbially synthesized gadolinium oxide nanoparticles. Vinblastine gadolinium oxide conjugate was purified by HPLC.

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ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
°C	Degree Celsius
cm	Centimetre
CMA	Corn Meal Agar
CRLDA	*Catharanthus roseus* leaf decoction agar
CRLDAB	*Catharanthus roseus* leaf decoction agar boiled
CTAB	Cetyl Trimethyl Ammonium Bromide
DAMBE	Data Analysis and Molecular Biology and Evolution
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic acid
ESI-MS	Electospray Ionisation- Mass Spectroscopy
gm	Gram
hr	Hour
HPLC	High performance liquid chromatography
IC50	Half maximal inhibitory concentration
ITS	Internal transcribed spacer
l	liter
M	Molar
MGYP	Malt extract, glucose, yeast extract and peptone
min	Minute
ml	Milliliter
mM	Milli molar
MS-MS	Tandom mass spectrometry
MEGA	Molecular Evolutionary Genetic Analysis
MEM	Minimum Essential Medium
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
nm	Nanometer
OD	Optical density
PDA	Potato Dextrose Agar
PCR	Polymerase Chain Reaction
RPMI 1640 Media	Roswell Park Memorial Institute 1640 Media
RT	Retention Time
RNAase	Ribonuclease
rpm	Revolutions per minute
Taq polymerase	*Thermus aquaticus* polymerase
TLC	Thin Layer Chromatogrphy
UV/Vis	Ultraviolet/ visible spectroscopy
µl	Micro litre
µg	Micro gram