"Isolation, purification and characterization of secondary metabolites from endophytic fungi of *Phyllanthus* sp."

A Thesis

Submitted to the

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In

Biotechnology

By

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Under the guidance of

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CERTIFICATE

This is to certify that the work discussed in the thesis entitled **"Isolation, purification and characterization of secondary metabolites from endophytic fungi of *Phyllanthus* sp."** by **Taware Ravindra Vitthalrao**, submitted for the degree of Doctor of Philosophy in Biotechnology was carried out under my supervision at the Biochemical Sciences Division of the National Chemical Laboratory, Pune, India. Such materials which have been obtained by other sources have been duly acknowledged in this thesis. To the best of my knowledge, the present work or any part thereof has not been submitted to any other University for the award of any other degree or diploma.

Date:

Place:

Dr. ABSAR AHMAD
(Research Guide)

DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Isolation, purification and characterization of secondary metabolites from endophytic fungi of *Phyllanthus* sp."** submitted to University of Pune for the degree of **Doctor of Philosophy in Biotechnology**, was carried out by me under the guidance of **Dr. Absar Ahmad** and has not formed the basis for the award of any degree, diploma, associate-ship, fellowship, titles in this or any other University or other institute of higher learning. I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Date: Signature of the Candidate

Place: **Taware Ravindra Vitthalrao**

Dedicated to my Beloved
Family and Friends

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ABBREVIATIONS

µg	Microgram
µL	Micro litre
BSA	Bovine serum albumin
CMA	Corn meal agar
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Electron Spray Ionization Mass spectrophotometery
FBS	Foetal bovine serum
g/ gm	Grams
HPLC	High Performance Liquid Chromatography
IC50	50 % inhibitory Concentration
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ITS	Internal Trascription spacer regions
Kb/Kbp	Kilo base pairs
LB(A)	Luria Bertani (Agar)
LC-MS	Liquid chromatography Mass spectrophotometery
mg	Milli gram
MIC	Minimum Inhibitory Concentration
min(s)	Minute(s)
mL	Milli liter
mM	Milli molar

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ng	Nanogram
nm	Nano meter
nM	Nano molar
OD	Optical density
OMA	Oat meal agar
PBS	Phosphate buffered saline
PDA	Potato dextrose agar
SMQ	Sterile Milli Q water
TAE	Tris acetic EDTA buffer
TE	Tris EDTA buffer
TLC	Thin layer chromatography
UV	Ultra violet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Chapter 1

General introduction

Introduction:

Endophytic fungi are microorganisms which spend the whole or part of their lifecycle residing symbiotically within the healthy tissues of higher host plants, inter-and/or intra-cellularly, mimic the chemistry of their respective hosts and interestingly, produce the same bioactive natural products or drugs and derivatives as their hosts; meanwhile causing no damage or disease to them.(94)

A majority of plant species examined for endophytes have proved to be richly loaded with them and it is estimated that over a million fungal endophytes exist in nature. (73) This abundant resource of novel microorganisms holds its roots deep in the history of evolution as evidence of plant associated microbes has been discovered in fossilized tissues of plant roots, stems and leaves, thus indicating that endophytic association is nearly as old as the time higher plants first appeared on the face of earth millions of years ago.(95) Literature suggests that endophytes are related to some phytopathogens found in the environment then and traced back to similar origins. (15) These microorganisms actively penetrated plant tissues through openings and wounds and also proactively by using hydrolytic enzymes like pectinase and cellulase to rupture plant cell walls.(120)

During the long course of co-evolution, the endophytes, by gradual genetic variation adapted themselves to comfortably survive within their hosts and also be an important component of the host plant's micro-ecosystems. (81, 94, 120) This involved the uptake of some plant DNA segments into their own genomes while insertion of their own DNA segments into the host genomes as well; a process known as biotransformation. Overtime, this association between the endophytes and host plants became mutualistic, with both the partners benefiting from each other. The endophytes derive nutrition, habitat and protection from the host and synthesize some bioactive compounds which help the host in resisting external biotic and abiotic stresses, providing immunity to diseases, pathogens, insects, etc. and ensuring fitness and survival of the host.(26, 81) In an attempt to mimic the chemistry of their hosts, endophytes also acquired the capability of biosynthesizing some "phytochemicals" similar to those of their host plants.(87, 120)

The possibility that plant hormone gibberellin can be produced not only from the plant but also using the endophytic fungi associated with it created an instant spark of

interest within researchers, which was further ignited when in 1993, paclitaxel (taxol), the wonder drug for cancer was produced from the endophytic fungi *Taxomyces andreanae* isolated from the bark of Pacific Yew plant *Taxus brevifolia.* (16, 87) Owing to the huge developments in the fields of genetic engineering, microbial fermentation technology, etc. the past two decades have seen a major increase in the number of researchers working hard to further explore the endophytic fungal diversity, and better understand the relationships between endophytic fungi and their host plants, in an attempt to employ endophytes to obtain valuable compounds of plant origin without exploiting plant parts and to improve the productivity of the ones already being derived by optimizing fermentation conditions to reap benefits of abundant renewable supply.(29, 126)

Eversince, endophytic fungi have been used to fabricate a number of valuable plant based bioactive compounds with antimicrobial, insecticidal, antiparasitic, cytotoxic, immuno suppressive and anticancer activities. These can be classified into alkaloids, peptides, terpenoids, steroids, flavonoids, quinones, lignans, phenols, lactones, etc. and include secondary metabolites like podophyllotoxin, camptothecine, vinblastine, vincristine, hypericin, diosgenin, toosendanin , α-irone, β-irone, huperzine, etc.(14, 46, 49, 56, 108, 113, 120, 122, 125, 127) Thus, endophytes hold tremendous promise as an alternative eco-friendly source for efficiently producing valuable bioactive compounds in the future with varied applications in both the research and applied fields of medicine, food industry, agriculture, pest management, etc.

Isolation of Endophytes

In order to remove epiphytic microbes, the plant materials are thoroughly washed and surface sterilized with different surface sterilizing agents such as mercuric chloride ($HgCl_2$), ethanol etc. These are later cut into small pieces and placed on a PDA (potato dextrose agar) plate. After significant period of incubation ranging upto several days, the hyphal tips of the fungi are removed and transferred to PDA slants and screened for bioactive secondary metabolite production. To determine their epiphytic or endophytic nature, isolations of these fungi from surface sterilized tissues were used. In most of the procedures, difficulties are mainly encountered in isolation and purification of these cultures for extended periods , owing to the slow growth rate of endophytes.(4) Many different stains have been explored detect these fungi in

plants. Sampson used cotton blue or gentian violet followed by Gram's iodine solution to examine the presence of endophytic fungi in epidermal peels from stem, seeds, leaf sheaths, pith scrapings, etc.(83) Lactophenol cottonblue, lactophenol-trypan blue and aniline blue were used by other researchers for staining mycelium.(6, 18, 27, 35) Clark *et al*. used aniline blue-lactic acid to soak the seeds.(17) Recently, Enzyme-linked immunosorbent assay (ELISA)was used for the detection of endophytes in seeds and plant tissue systems.(45, 67) A rapid staining method for endophytes using rose bengal stains was developed by Saha *et al.* (82) This method was an improvement over using trypan blue as it was rapid and safe. Eversince, a vast number of bioactive secondary metabolites have been isolated, purified and completely characterized from plant associated endophytic fungal strains.

Bioactive metabolites from endophytic fungi:

[A] Anticancer drugs from endophytes:

(i) Paclitaxel (Taxol):

Paclitaxel and some of its derivatives represent the first major group of anticancer agents which are produced by endophytes. Paclitaxel is a highly functionalized diterpenoid and is found yew (*Taxus*) species worldwide.(109) Its mode of action is to preclude tubulin molecules from depolymerizing during the processes of cell division making it the world's best anti-cancer drug.(107) Apart from cancer, taxol is also used in the treatment of other human tissue proliferating diseases. Presence of paclitaxel in yew species (1993) prompted the study of its endophytes worldwide, with a view to find an alternate source for drug production. However, it was only after several years of hard work that paclitaxel isolation was possible from other yew species around the globe, with endophyte *T. andreanae* isolated from *T. brevifolia* being used for the production of paclitaxel. (87) Eversince, many other plants and their endophytes have been successfully explored for paclitaxel and other bioactive secondary metabolite production. (63, 77, 90, 101)

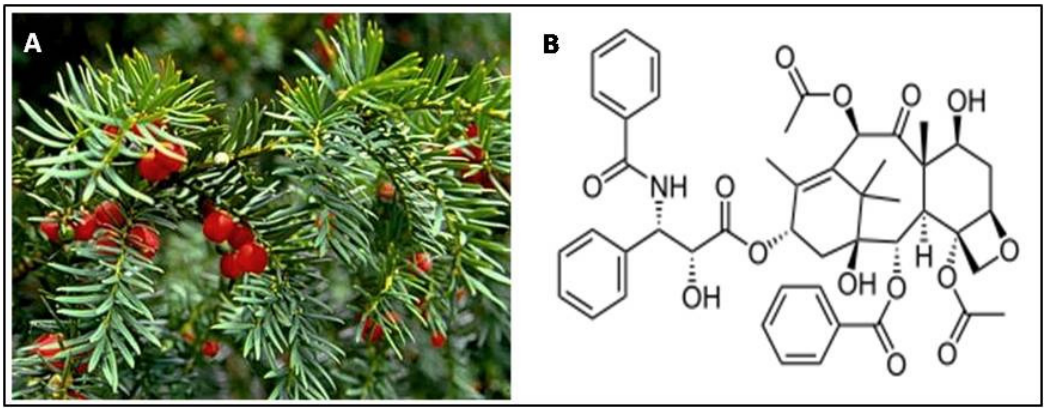

Fig.1 Endophytic fungus isolated from *Taxus brevifolia* (A) produces paclitaxel (B).

India has a huge reserve of *Taxus baccata* species which encouraged our group to explore it for possible production of paclitaxel. A screening programme was initiated and forty slow-growing, few nonsporulating, and uncommon endophytic fungi were isolated from *Taxus* bark, stem, and leaves (needles) and brought to pure culture state. Out of these, one endophytic fungus *Gliocladium* sp. was found to produce paclitaxel and its precursor 10-deacetyl baccatin III (10-DABIII) extracellularly. (86)

Some of the endophytic fungi producing paclitaxel are:

Host plant	**Paclitaxel-producing endophytic fungus**
Taxus baccata	*Botryodiplodia theobromae, Fusarium lateritium, Monochaetia* sp.*, Pestalotia bicilia, Gliocladium* sp.
Taxus wallachiana	*Pestalotiopsis microspora.*
Taxus cuspidata	*Alternaria* sp.*, Botrytis* sp., *Pestalotiopsis microspora.*
Taxus chinensis	*Alternaria alternata, Botrytis* sp., *Fusarium mairei, Metarhizium anisopliae, Mucor rouxianus, Papulaspora* sp.
Taxus brevifolia	*Taxomyces andreanae*
Taxus media	*Cladosporium cladosporioides*
Taxus celebica	*Fusarium solani*
Taxus sumatrana	*Pithomyces* sp.

(ii) Camptothecin:

Camptothecin and its analogue hydroxycamptothecin are regarded as the two most effective antineoplastic agents. Camptothecin is a pentacyclic quinoline alkaloid and

was first isolated from the wood of *Camptotheca acuminate* (Nyssaceae) by Wall *et al.* in 1966. (105) The mode of action of camptothecin is by inhibiting the intranuclear enzyme topoisomerase-1, which is required in DNA replication and transcription during molecular activities.(40) Excessive cropping of the wild trees *Camptotheca acuminata* and *Nothapodytes nimmoniana* (Icacinaceae) found in Indian and China, for the production of camptothecin and related compounds has made it crucial to find an alternate source to obtain these compounds and prevent further exploitation of natural resources. With this view, various researchers attempted to isolate endophytes from these plants and finally, many endophytes like *Entrophospora infrequens, Neurospora* sp., *Fusarium solani,* etc., producing camptothecin and its analogues were successfully isolated from the above mentioned tree species.(50, 76, 79)

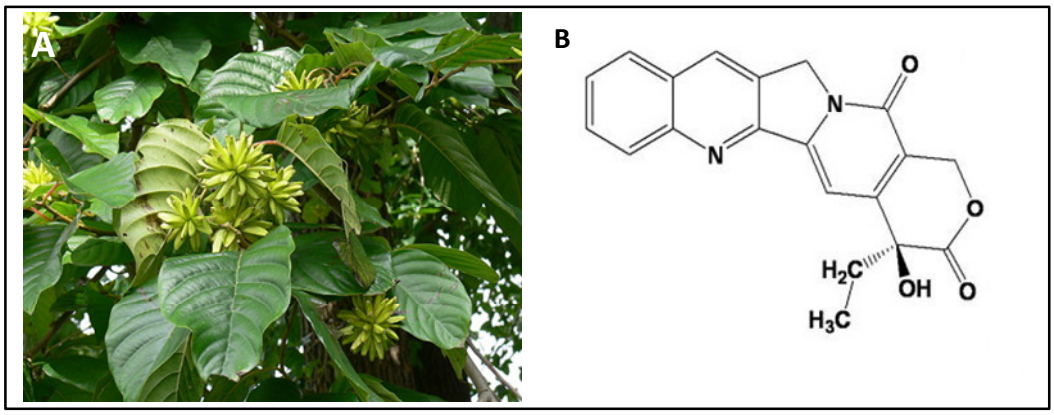

Fig.2 Endophytic fungus isolated from *Camptotheca accuminata* (A) produces camptothecin (B).

Some of the endophytic fungi producing camptothecin are:

Host plant	Camptothecin-producing endophytic fungus
Nothapodytes foetida	*Entrophospora infrequens, Neurospora* sp.
Camptotheca acuminata	*Fusarium solani*
Apodytes dimidiata	*Fusarium solani*

(iii) Podophyllotoxin:

Podophyllotoxin is an excellent anticancer, antiviral, antioxidant, antibacterial, immunostimulation and anti-rheumatic compound mainly isolated from *Podophyllum* (*Sinopodophyllum*) plants. It is an aryltetralin lignan and is used as a precursor for

chemical synthesis of the anticancer drugs like etoposide, teniposide and etopophose phosphate. Other genera in which this compound occurs include *Diphylleia*, *Dysosma* and *Sabina* (*Juniperus).* Yet, supply of this compound proves to be insufficient although the plants used for its synthesis are now being declared endangered due to excessive exploitation. Thus, increasing demand and lack of resources make it very important to find an alternate source for drug production and this is where the use of endophytes steps in. Researchers have now successfully isolated podophyllotoxin-producing endophytic fungi like *Sinopodophyllum hexandrum*, *Diphylleia sinensis, Dysosma veitchii, Alternaria* sp, *Sabina vulgaris, Phialocephala fortinii, Trametes hirsuta, Fusarium oxysporum,* etc. from the above mentioned plant species and attempts are now being targeted towards increasing production of this novel compound and its derivatives. (13, 24, 32, 48, 57, 115, 118)

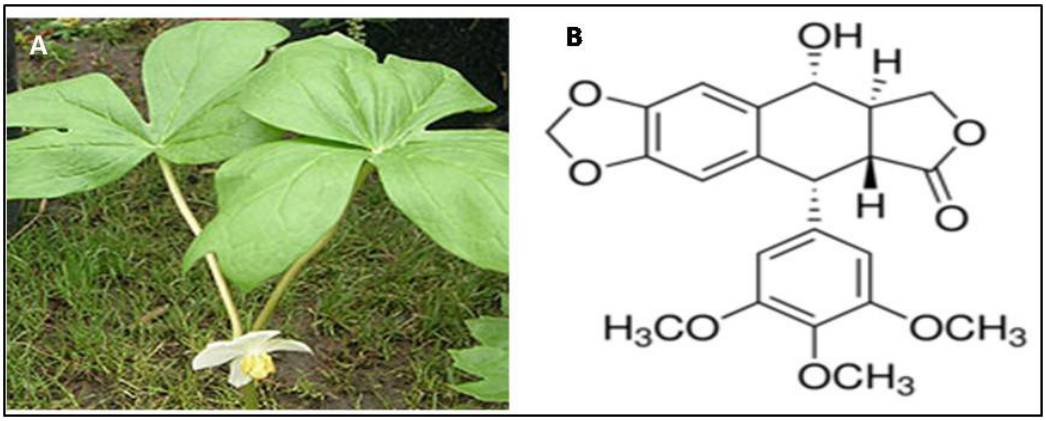

Fig.3 Endophytic fungus isolated from *Podophyllum hexandrum* (A) produces podophyllotoxin (B).

Some of the endophytic fungi producing podophyllotoxin are:

Host plant	**Podophyllum-producing endophytic fungus**
Sinopodophyllum hexandrum (*Podophyllum hexandrum*)	*Trametes hirsuta, Penicillium* sp., *Alternaria* sp., *Alternaria neesex.*
Sinopodophyllum peltatum	*Phialocephala fortinii.*
Sabina recurva (*Juniperus recurva*)	*Fusarium oxysporum.*
Dysosma veitchii	*Monilia* sp., *Penicillium* sp.

(iv) Vinca alkaloids - Vinblastine & Vincristine:

Vinblastine and vincristine are two of the most popular anti-cancer drugs. They are being produced from leaves of field grown plant *Catharnthus roseus* by cell and tissue culture, callus culture, cell suspension culture, shoot culture, hairy roots cultures, semi synthesis as well as total synthesis.

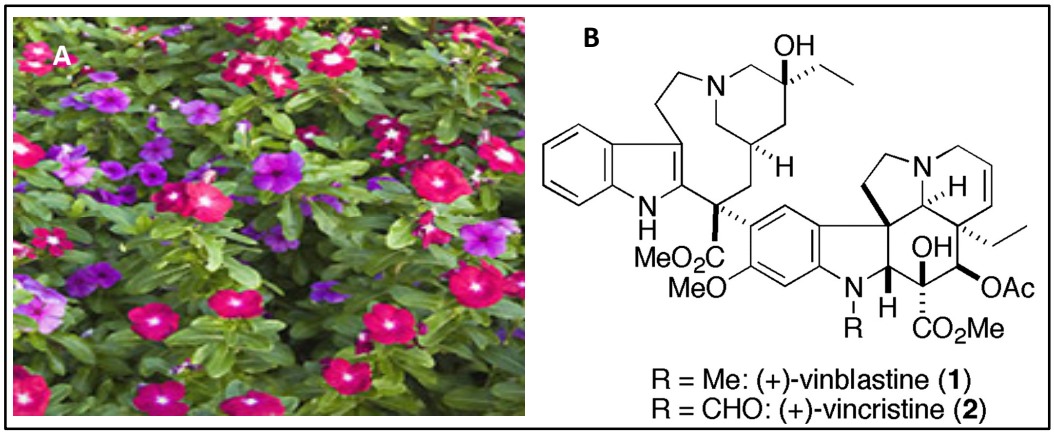

Fig.4 Endophytic fungi isolated from *Catharanthus roseus (*A) produces Vinblastine and Vincristine (B).

It has also been shown that vinblastine and vincristine can respectively be isolated from the endophytes *Alternaria sp*. and *Fusarium oxysporum*, isolated from *Catharnthus roseus* plant.(31, 116, 123) Since the quantity of both these drugs from all the sources are extremely low, the supply of these 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 by our group. We were able to successfully isolate, purify and identify an endophytic fungal strain producing these drugs extracellularly in appreciable amounts.

[B] Antimicrobial compounds from endophytes:

Metabolites with anti-biotic properties are low-molecular-weight natural organic compounds synthesized by microorganisms which are active at low concentrations against other microorganisms. Now, endophytes are known to produce secondary metabolites as a resistance mechanism in response to stress induced by invading

pathogens. These secondary metabolites produced by endophytes belong to a diverse number of structural classes which include alkaloids, peptides, steroids, phenols, flavonoids, quinines, terpenoids, diterpenoids, etc.(39, 44, 94) The increasing levels of drug resistance exhibited by plant and human pathogens has become a global problem and has led researchers into exploring endophytes for production of novel antimicrobial metabolites which can be used not only as drugs but also for other purposes like food-preservation, against food-borne diseases and food-spoilage, etc.

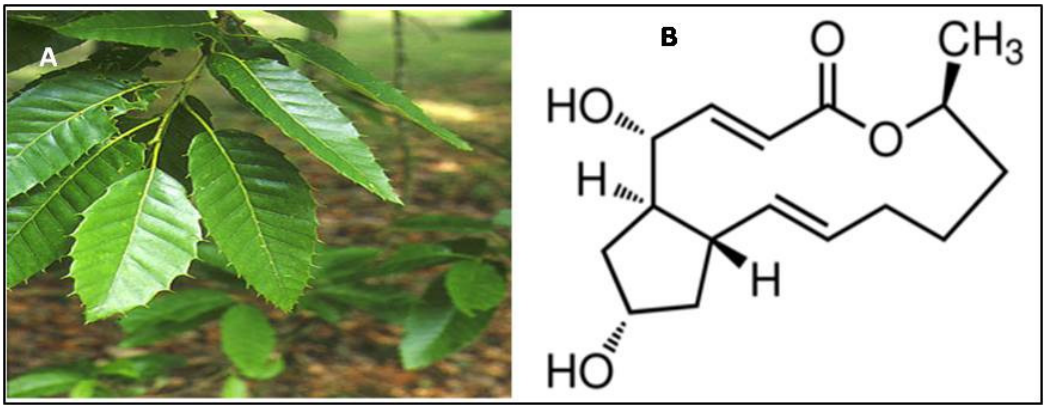

Fig.5 Endophytic fungus *Cladosporium* sp. isolated from *Quercus variabilis* (A) produces brefaldin- A (B).

Eversince, a number of endophytic fungi have been isolated from different plant species worldwide which produce novel anti-microbial compounds; some of which are tabulated below:(1, 53, 74, 104, 106)

Endophytic fungi	**Anti-microbial Compounds Synthesized**
Xylaria sp.	Sordaricin, 7-amino-4-methylcoumarin, Griseofulvin, 2-hexyl-3-methyl-butanodioic acid, Cytochalasin D.
C. globosum	Chaetomugilin A, Chaetomugilin D.
Pezicula sp.	(−)-mycorrhizin A, (+)- cryptosporiopsin .
Pestalotiopsis adusta	Pestalachloride A, Pestalachloride B.
Cladosporium sp.	Brefeldin-A.
Phomopsis cassiae	ethyl 2,4-dihydroxy- 5,6-dimethylbenzoate, phomopsilactone.
Penicillium janthinellum	Citrinin.
Aspergillus fumigatus	Asperfumoid, Fumigaclavine C, Fumitremorgin C, Physcion, Helvolic acid.
Verticillium sp.	2,6-Dihydroxy-2-methyl-7- (prop-1E-enyl)-1-benzofuran-3(2H)-one.
Pestalotiopsis theae	Pestalotheol C (anti-HIV).

[C] Antioxidants from endophytes:

Anti-oxidant activity bearing compounds are highly effective against damage caused by oxygen-derived free radicals and reactive oxygen species (ROS) and contribute towards a range of pathological effects like cellular degeneration, membrane degradation, DNA damages, carcinogenesis, etc. These attributes make antioxidants very promising for effective treatment against a wide spectrum of ROS related illnesses like atherosclerosis, hypertension, ageing, rheumatoid arthritis, neurodegenerative diseases like Alzheimer's and Parkinson's, diabetes mellitus, cardiovascular disease, several types of cancers, etc.

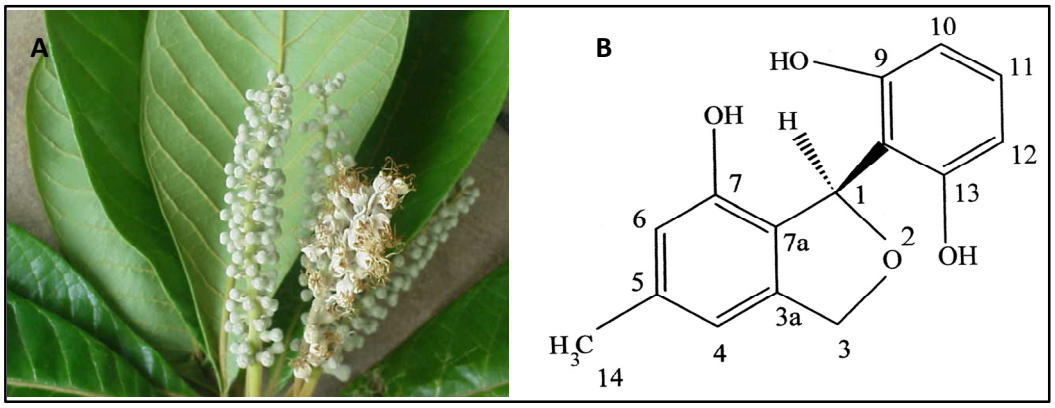

Fig.6 Endophytic fungus *Pestalotiopsis microspora* isolated from *Terminalia morobensis* (A) produces pestacin (B).

Antioxidants are naturally found in medicinal plants, vegetables, fruits and tubers. There are several reports where metabolites from endophytes are used as potentail source of novel natural antioxidants; some of which are given below:(34, 92)

Plant name	**Isolated endophytic fungi**	**Anti-oxidants synthesized**
Terminalia morobensis	*Pestalotiopsis microspora*	Pestacin, Isopestacin, 1,3-dihydro isobenzofurans.
Trachelospermum jasminoides	*Cephalosporium* sp.	Graphislactone A

[D] Ergot alkaloids from endophytes:

Ergot-alkaloids are confined to the sclerotium of the fungus while alkaloids on the other hand can be isolated from whole parts of the plant symbiotic with endophytes. Although, ergot powder obtained from dried sclerotia of the fungus *Claviceps purpurea* parasitic on rye, 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.

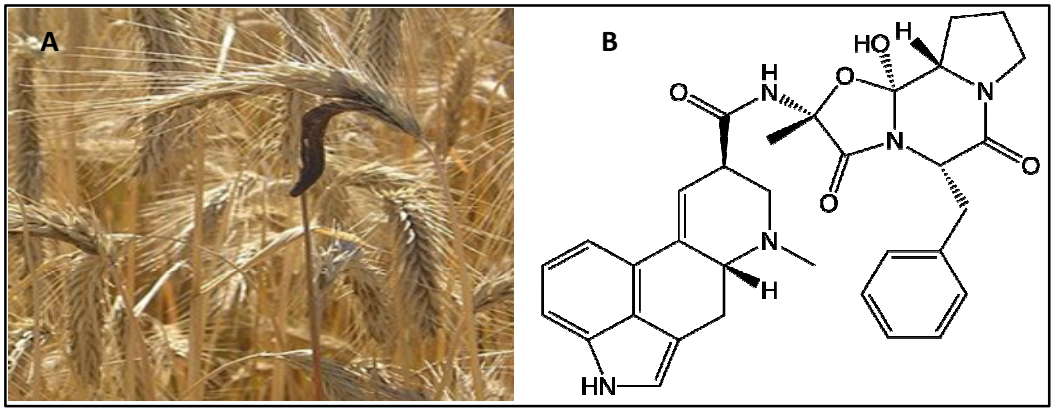

Fig.7 Ergot alkaloids from sclerotium (A) of *Claviceps* (ergot fungus) *purpurea* produces ergotamine (B)

The first scientific report of ergot as a cytotoxic agent was made by the American physician Starns in 1808. Ergometrine derivatives are also used to stop haemorrhage after childbirth.(4, 41) Ergotamine and its derivatives are used against migraine. Ergotoxine group of alkaloids in equal parts have been used for controlling essential hypertension and other peripheral disorders. These ergot alkaloids are now also being synthesized using the endophytic fungi associated with host plants:(7, 8, 33, 58, 75, 94, 117)

Endophytic fungi	Ergot alkaloids synthesized
Claviceps purpurea	Ergometrine, Ergometrinine, Ergotamine, Ergosine, Ergocryptine, Ergocrystine.
Balansia claviceps	Chanoclavine , Ergonovine, Ergonovinine.
Balansia epichloe'	Agroclavine, Chanoclavine, Elymoclavine, Ergonovine, Ergonovinine, Isochanoclavine- I, Panniclavine, 6,7-Secoagroclavine.

Balansia henningsiana	Chanoclavine, Dihydroelymoclavine, Ergonovine, Ergonovinine.
Balansia strangulans	Chanoclavine, 6,7-Secoagroclavine.
Balansia sclerotica	Lysergic acid, Isolysergic acid amide (ILAD)

[E] Antiviral compounds from endophytes:

Cases of drug resistance in viruses have become a common phenomena and researchers worldwide are trying to find a way to combat this situation. In such a case, drugs isolated from endophytic fungi may come to rescue as these belong to a new and improved class of therapeutic agents. Recently, Guo *et.al* isolated two novel human cytomegalovirus protease inhibitors, cytonic acids A and B via solid-state fermentation of the endophytic fungus *Cytonaema* sp. (30) Thus, although in its infancy, the potential of endophytic fungi to produce novel anti-viral compounds cannot be sidetracked and has tremendous scope for discovery, and treatment of various viral diseases like AIDS, poliomyelitis, chicken pox, swine flu, mumps, etc.

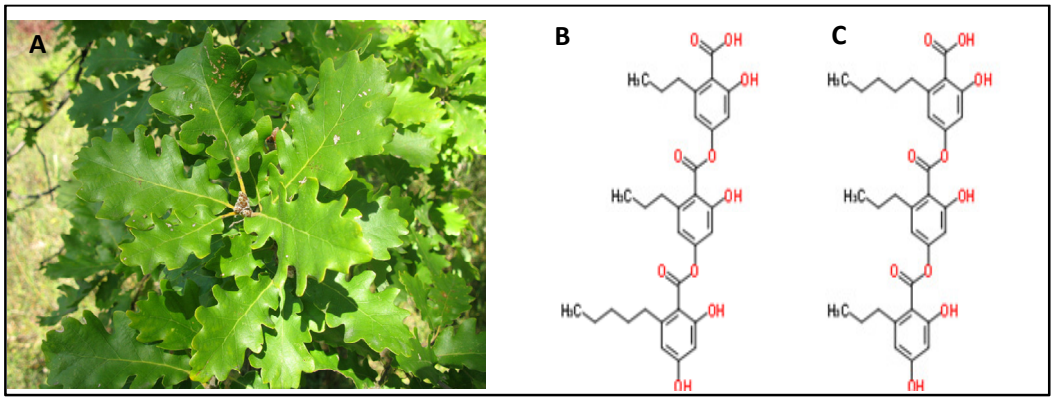

Fig.8 Endophytic fungus *Cytonaema* sp. isolated from *Qurcus sp* (A) produces cytonic acid A and B (B and C).

[F] Antibiotics from endophytes:

A sudden surge in the number of people worldwide facing health problems due to cancers, parasitic protozoans, drug-resistant bacteria, life threatening viruses, and fungi have caused a red-alert. Recurring problems with diseases in patients who have undergone organ transplants and are immunocompromised, emergence of new diseases like AIDS and severe acute respiratory syndrome, presence of naturally

resistant organisms, and development of resistance in pathogenic microorganisms like species of *Staphylococcus*, *Mycobacterium*, *Streptococcus,* etc. call for an immediate development in the variety of drugs being produced and advent of newer and stronger derivatives to tackle these utmost serious issues.

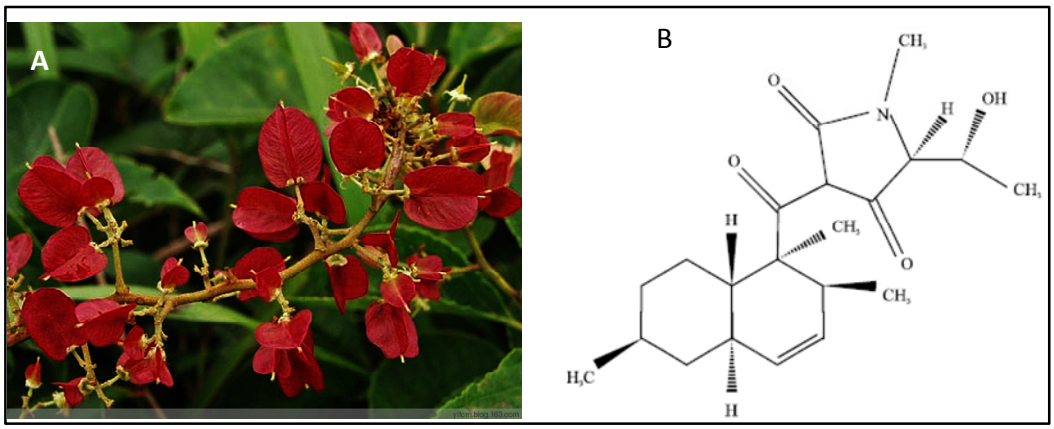

Fig. 9 Endophytic fungus *Cryptosporiopsis quercina* isolated from *Tripterygium wilfordii* (A) produces cryptocin (B).

Antibiotics, i.e. the drugs used in the treatment of most of the above illnesses are low-molecular-weight organic natural compounds synthesized by microorganisms, and which are active at low concentration against other microorganisms. Endophytes have recently proven to be a goldmine for antibiotic production and novel, more powerful drugs and derivatives are now being synthesized using endophytes, a way which is eco-friendly, non-toxic and produces compounds with negligible side-effects to human, plant and animal health. (53–55, 89)

Some of the endophytic fungi producing antibiotics are:

Host plant	**Antibiotic-producing endophytic fungus**	**Antibiotics synthesized**
Tripterigeum wilfordii	*Cryptosporiopsis quercina*	Cryptocandin, Cryptocin
Torreya taxifolia (endangered)	*Pestalotiopsis microspora*	Pestaloside, β glucoside, Pestalopyrone, Hydroxypestalopyrone, Pestalotiopsins A and B, 2-α-hydroxydimeninol, Humulane.
Selaginella pallescens	*Fusarium* sp.	Pentaketide antifungal agent-CR377.

Artemisia mongolica	*Colletotrichum gloeosporioides*	Colletotric acid.
Artemisia annua	*Colletotrichum* sp.	Artemisinin
Mangifera indica (mango), *Psidium guajava* (guava).	*Pestalotiopsis sp.*, *Monochaetia* sp.	Ambuic acid
Fragraea bodenii	*Pestalotiopsis jesteri*	Jesterone, hydroxy-jesterone.

[G] Volatile Antibiotics from endophytes:

An endophytic fungus *Muscodor albus recently* isolated from cinnamon tree has found to be effective against inhibiting or eliminating certain fungi and bacteria by producing a mixture of volatile compounds.(112) A majority of compounds produced by this xylariaceaous (non-spore producing) fungus have been identified by gas chromatography and mass spectrometry, synthesized or obtained separately, and finally made into an artificial mixture which interestingly mimicked the antibiotic effects of the volatile compounds which the fungus produces. This go to say that fungus alone, via a simple and eco-friendly way is producing complex volatile anti-biotics which otherwise takes in a lot of our efforts, time and money to fabricate. As soil fumigation utilizing methyl bromide will soon be illegal in several countries worldwide, mycofumigation effects of *M. albus* and similar fungi to treat soil, seeds, fruits, vegetables, cereals, bulbs, tubers and plants would have potential practical benefits and be safe to the environment and associated flora and fauna. (66, 88, 111)

Some of the endophytic fungi producing volatile antibiotics are:

Host plant	**Volatile antibiotic producing endophytic fungus**
Cinnamomum zeylanicum (cinnamon tree)	*Muscodor albus*
Ananas ananassoides (wild pineapple)	*Muscodor crispans*
Erythophelum chlorostachys (ironwood)	*Muscodor roseus*
Grevillea pteridifolia (fern-leafed grevellia)	*Muscodor roseus*

[H] Insecticidal compounds from endophytes:

The market for bioinsecticides as a part of the insecticide field is gaining popularity owing to their eco-friendly non-hazardous nature, targeting efficacy and negligible toxicity to the soil and nearby water bodies. As the world is becoming aware of the ecological damage done by synthetic insecticides and pesticides, bioinsecticides are being explored as a selective, safe and powerful alternative. Several endophytic fungi have shown to have anti-insect properties. As a resistance mechanism, endophytes produce mild toxic chemicals and secondary metabolites so as to keep away grazing animals, pathogens, insects and ensure fitness and survival of the host. Nodulisporic acids are novel indole diterpenes which exhibit potent insecticidal properties against blowfly larvae. These compounds work by activating the insect's glutamate-gated chloride channels and were first derived from the endophyte *Nodulisporium* sp. isolated from the plant *Bontia daphnoides*. Eversince, intensive research has been carried out to produce more potent analogues of nodulisporic acid and explore more fungal species which produce them and other important insect repellants.(25, 68)

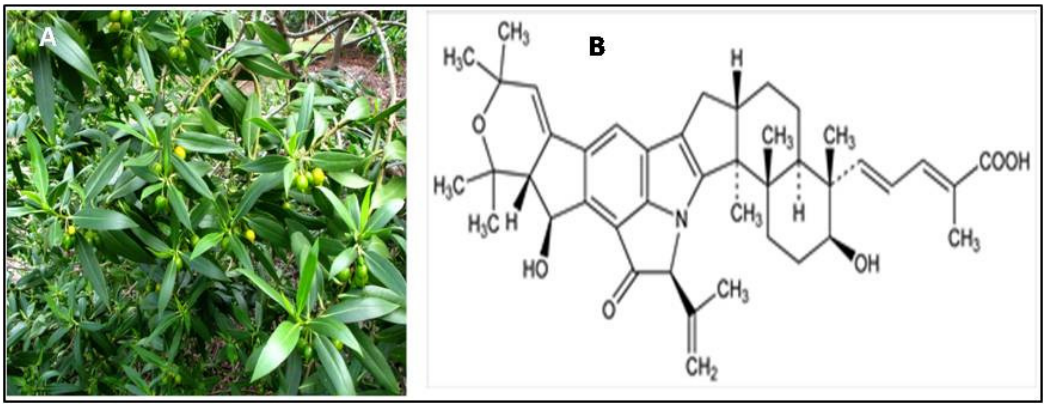

Fig.10 Endophytic fungus *Nodulisporium* sp. isolated from *Bontia daphnoides* (A) produces nodulisporic acid (B).

Some of the endophytic fungi producing insecticides are:

Host plant	**Insecticide producing endophytic fungus**	**Pest species affected**
Bontia daphnoides	*Nodulisporium* sp.	*Aedes aegypti* (mosquito larvae), *Lucilia sericata* (blowfly larvae).
Paullina paullinioides	*Muscodor vitigenus.*	*Cephus cinctus* (wheat stem sawfly).

Festuca arundinacea	*Acremonium coenophialum*	*Diuraphis noxia, Sipha flava, Rhopalosiphum padi, Schizaphis graminum, Acheta domesticus* (house cricket), *Sitobion fragariae* (strawberry aphid), *Tribolium castaneum* (flour beetle).
Lolium perenne	*Acremonium lolii*	*Chaetocnema pulicaria* (corn flea beetle), *Listronotus bonariensis* (argentine stem weevil), *Sphenophorus* sp. (billbug), *Oncopeltus fasciatus* (milkweed bug).

[I] Immunosuppressive compounds from endophytes:

In transplant patients, immunosuppressive compounds like cyclosporin A and FK506 are being used to prevent allograft rejection. In future, these approved immunosuppressive agents may also be used to treat autoimmune diseases such as insulin-dependent diabetes, rheumatoid arthritis, etc. However, although beneficial, these drugs do produce some undesirable side effects. Thus, efforts are now being focused towards employing endophytic fungi for the production of safe and improved immunosuppressive agents.

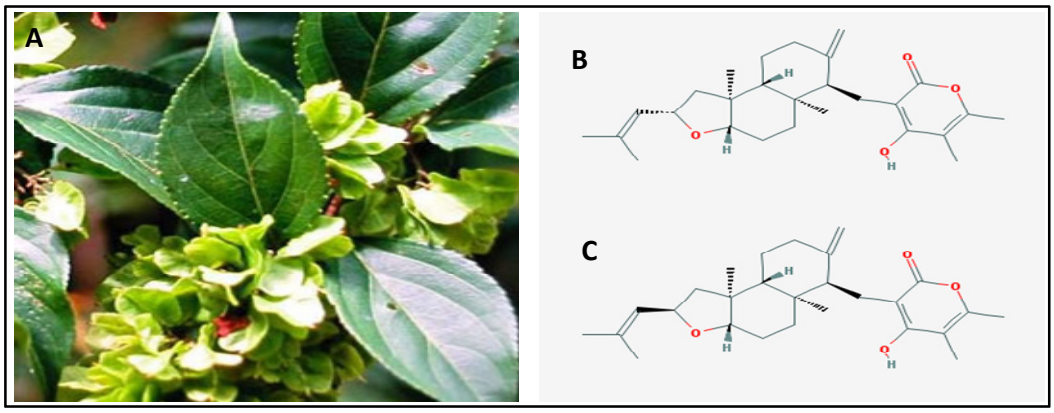

Fig. 11 Endophytic fungus *Fusarium subglutinans* isolated from *Tripterygium wilfordii* (A) produces subglutinol A and B (B and C).

Plants with reported biological activities are selected and screened for a range of endophytic fungi, out of which the ones producing the desired compound are further analyzed. Applying the same logic, researchers recently have discovered two novel immunosuppressive compounds subglutinol A and B, from the endophytic fungus

Fusarium subglutinans isolated from the perennial twining vine *Tripterygium wilfordii*. This plant is being used in traditional Chinese medicine for over two thousand years now, and is popular among chemists as a rich source of cytotoxic diterpene lactones such as tripdiolide and insecticidal alkaloids like wilfordine. The compounds subglutinol A and B which were isolated from the above plant lack toxicity and thus are being studied in greater detail to be developed as fully fledged immunosuppressants.(52) Similarly, endophytes isolated from plants around the globe are being explored as a novel and dynamic alternative to plant based bioactive compounds, which will be cheap, abundant and will produce no side-effects.

[J] Antidiabetic compounds from endophytes:

Autoimmune disorders like insulin-dependent diabetes or *diabetes mellitus* are difficult to cure mainly because of the degradation of the peptidal drug insulin once inside the digestive tract. Thus, endophytic fungus *Pseudomassaria* sp. collected from an African rainforest was employed for the production of a nonpeptidal fungal metabolite which acts as an insulin mimetic compound, and unlike insulin, this compound is not degraded in the digestive system.(119) Moreover, it can be orally administered, sparing the patients of painful syringe shots on a daily basis. This discovery has prompted researchers to further extend the horizons of employing endophytes for the production of various life-saving drugs and other valuable compounds.

Plants which can be targeted for the isolation of antidiabetic compound-producing endophytic fungi

Aegle marmelos ***Cassia fistula***

Syzygium cumini ***Trigonella foenum***

Cinnamomum tamala ***Polyalthia longifolia***

[K] Possible plant based bioactive metabolites which can be targeted from endophytes:

The use of endophytes is not limited to the variety of compounds mentioned so far. Infact, looking at the manner in which these studies are progressing, that day is not far away when endophytes will become independent alternative sources of innumerable metabolites, sparing the parent plants and becoming an inexhaustible source of valuable compounds being produced by simple fermentation processes. Medicinal and aromatic plants of indigenous origin would be screened for their endophytes and evaluated for their true potential to manufacture several life-saving compounds required on a day-to-day basis by plants, animals and humans.

Endophytes will find applications in the synthesis of compounds required in the treatment of almost all problems faced by mankind including malaria, filaria, tuberculosis, kala-azar, arthritis, hypertension, depression, atherosclerosis, memory degeneration, various allergies, respiratory disorders, gastric ulcers, kidney problems, regulation of menses, GIT (gastro-intestinal tract) dysfunction, osteoporosis,

posorosis, Parkinsonism, etc. and will provide a cheap reliable source of safe, eco-friendly drugs.

[L] Biotransformation process using endophytes:

The production of a range of novel compounds by the method of biotransformation proves to be extremely useful. Bioactive molecules with applications in pharmaceuticals and food industries like lipids, steroids, lignins, alkaloids, triterpines, etc. are being synthesized by using the whole cell of the fungus or enzyme purified from it. In the past, biotransformation processes have been carried out by a range of microorganisms, but using endophytic fungi for the same is a relatively recent process. Various drugs like taxol, vinblastine, vincristine, camptothecin, podophyllotoxin, etc. are isolated and purified from the culture filtrate by a two-stage fermentation procedure which lasts for about 2-3 weeks. These drugs can be isolated in a very short span of time if the precursors of the above drugs are incubated with endophytic fungi. For example, if the precursor of taxol i.e. phenylisoserine + 10 deacetylbaccatin is incubated with the endophytic fungus isolated from *Taxus baccata*, taxol is produced in a few days as compared to the several days it takes when the two-stage fermentation process is used.

Recently, endophytic fungi are being applied as a biocatalyst in order to transform the precursor for the production of compound of interest. Microbial transformation of 10-deacetly-7-epitaxol and 1β-hydroxybaccatin I by endophytic fungi isolated from the inner bark of *Taxus yunnanensis* has been reported by Zhang *et al*.(121) Shibuya *et al* have also reported the transformation of Cinchona alkaloids into 1-N-oxide derivatives by endophytic *Xylaria* sp. isolated from *Chinchona pubescens*.(84) The biotransformation of a tertrahydrofuran lignan, (-) –grandisin, by endophytic fungus *Phomopsis* sp. from *Viguiera arenaria* was demonstrated by Verza *et al*.(103) Endophytic fungi have also recently been reported to transform natural products like taxoid, alkaloids, curcumin pigment, betulinic acid, betulonic acid, thioridazine, propranolol, etc.(85)(10)(12) The use of endophytic fungi as a biocatalyst is advantageous because they mimic the chemistry of their respective host plants and make very complex bioactive molecules or drugs because they secrete multi-enzymes and co-factors extracellularly and convert the precursors into desired drugs. It is well known that biological transformations are enantio-selective and produce chiral compounds from racemic mixtures.

Our group has discovered a fungus which is an efficient and selective biocatalyst for the biotransformation of cyclohexanone and cyclopentanone to -caprolactone and valerolactone (Baeyer-Villiger reaction) respectively, in quantitative yields .(59) The whole cells of the fungus have 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.(60) Our group in the past has also extracellularly synthesized a range of inorganic nanomaterials of different shapes, sizes and chemical compositions by fungal biotransformation. (2, 3, 9, 78) From the discussion and examples cited above, it is evident that endophytic fungi are promising and potent resources for diverse secondary metabolite production of high value.

Future prospects:

The discovery of the true potential of endophytes has been one of the major breakthroughs in science in the 21st century. Endophytes have very soon found a wide variety of applications in agriculture, medicine, therapeutics, food processing and preservation, etc.(91, 102, 124) However, synthetically derived compounds, although most produce toxicity and side effects are still in huge demand and thus, studies on endophytes need to be ventured deeper still so as to increase their overall output and obtain many more valuable compounds of plant origin which may then benefit the society at a larger scale.

Exponential advances in engineering and science in the past two decades have further established techniques like microbial fermentation, metabolic technology, microscopy, chromatography, spectroscopy, etc. and have helped researchers dwell further into various unexplored regions of possibilities. Through gene manipulation, mutation and other recombinant DNA techniques, the best quality endophytic fungi samples can be chosen and their productivity increased by tampering with the relevant functional genes in their biosynthetic pathways and colonizing them for optimal production of bioactive compounds. Desired features may be attributed to these compounds and stronger derivatives may be obtained.

Microbial fermentation is a very simple and inexpensive process, occurs within a very short period of time, where the parameters can easily be controlled for the best growth and breeding conditions. Moreover, the efficacy of the fungal culture medium can be

enhanced by using special anti-inhibitory enzymes, feeding precursors, addition of biotic and abiotic elicitors confirmed through metabolic studies, etc. All these developments will lead to an unlimited supply of cheap, completely safe and naturally derived medicines, flavor and aromatic compounds, wound healers, ointments, antiseptics, etc. which will be easily available to all.(39) The entire process will be in complete synchronization with the environment with a view to conserve and preserve the balance of eco-system by preserving the trees which sustain it.

Trichothecene mycotoxins:

A contamination free environment to live in and healthy safe food and water are the basic requirements of each and every human being as well as animals. However, it is easier said than done and mankind has to combat innumerable disease causing micoorganisms almost on an everyday basis in order to stay healthy and survive. The sudden surge in human population in the past few years brought with it unemployment, poverty, low standards of living, exploitation of natural resources, deforestation, climate changes, etc. and providing clean habitat and nourishment for such a vast number of people has become a global problem.

One of the major reasons for outbreaks of diseases in humans and farm animals is due to the spoilage of cereal crops, fruits, vegetables and other agricultural products by mycotoxin contamination produced by a range of imperfect plant pathogenic fungi which infect the produce during cultivation.(62, 70) Mycotoxins (e.g. - aflatoxins, trichothecenes) are secondary metabolites produced by the fungus which provide it with certain ecological advantages to better adapt and grow inside their plant hosts. Apart from mycotoxins, fungal secondary metabolites also include plant growth regulators (e.g. - gibberellins), medicinal compounds (e.g. - penicillin), pigments (e.g. - carotenoids), etc.(47) Mycotoxins accumulate in infected crop plants and upon ingestion, lead to the development of severe diseases (mycotoxicoses) in humans and animals.(98) Manifestation of these toxins may cause anemia, cardiovascular lesions, gastrointestinal disorders, hypotension, immunological problems , lymphoid necrosis, etc.(72, 99) These toxins also adversely affect the plant, causing dwarfism, inhibition of root development and elongation, chlorosis, etc.(20, 64) Thus, it has become extremely important to understand the pathogenicity of these fungi and regulate their toxin biosynthesis so as to put an end to their menace worldwide.

One of the major classes of mycotoxins are trichothecenes, which are known to cause significant impact on cereal and grain crops each year amounting to decay, diseases, product refusal and economic losses. (62, 70) Trichothecenes are made up of a family of over hundreds of toxins with a common tricyclic 12,13 epoxytrichothec-9-ene (EPT) core structure.(28) Trichothecine producing fungi belong to the order Hypocreales, include genera *Fusarium*, *Trichothecium*, *Trichoderma*, *Myrothecium*, *Stachybotrys*, *Spicellum*, *Cephalosporium*, etc., which are well adapted for colonization in a wide array of temperature, pH, moisture and nutrient availability in areas throughout the entire globe. (19)

For example, some species of genus *Fusarium* cause **ear** rots in grains like wheat, barley, maize, oats, etc. (22, 62) *Trichoderma* species are found in soil and associated with diseases of grapes and mushrooms.(21) *Trichothecium* species are found in soil too and other decaying organic matter. Those of genus *Myrothecium* are pathogens of tomato and muskmelon. (36, 43) *Stachybotrys* are indoor environmental contaminants and are the cause of damp building-related illnesses. (71)

Now, trichothecenes are very minute, amphipathic molecules which can passively move across cell membranes. (65, 110) Thus, these can inhibit eukaryotic protein synthesis by preventing the formation of peptide bond at the peptidyl transferase center of 60S ribosomal subunit, thereby affecting the polypeptide chain initiation, elongation as well as termination.(51, 99) Trichothecenes have also been shown to inhibit mitochondrial protein synthesis by interacting with the sulfhydryl groups of proteins.(61, 69, 114) As trichothecenes are easily absorbed by the gastrointestinal and integumentary systems, their rapid proliferation and activity leads to the generation and accumulation of free radicals which eventually produce hazardous levels of oxidative stress and cause rejection of feed, vomiting, dizziness, weakness, hemorrhagic lesions, skin dermatitis and various immunological malfunctions.(80, 93, 110) The level of toxicity increases with increasing oxygenation of EPT i.e. the tricyclic core structure common to all the trichothecene class members. (11)

Classification of trichothecenes is as well based on the substitution pattern of their EPT. Broadly, these have been differentiated into four groups i.e. Type A, B, C and D of which Type A, B and C consist of trichothecenes with simple structure, while Type D consists of the ones with macrocyclic structures.(98, 100) Type A, B and C

are differentiated based on the substitution exhibited at the C-8 positions of their chemical structures. Trichothecenes of Type A include compounds which have either a hydroxyl group, an ester function, or no oxygen substitution at their C-8 position. Those of Type B have a keto (carbonyl) function at C-8 while Type C trichothecenes possess a C-7/C-8 epoxide. Type D trichothecenes on the other hand have an additional ring linking the C-4 and C-15 position and are thus termed macrocyclic. Based upon the reactivity of their functional groups and conformational differences, simple trichothecenes have also been designated into three groups.(97) Group I contains trichothecenes with substitutions only in ring C (from figure), Group II represents those with substitutions in ring A while Group III contains trichothecenes with a keto at C-8 position. However, study reveals that small structural changes can result in large changes in toxicity and it is noted on several occasions that the degree of toxicity of trichothecenes does not necessarily correlate with their chemical classification. In many instances, Type B trichothecenes have found to be relatively more toxic than those of Type A, technically when the exact opposite is expected owing to their respective chemical structures.(23, 42, 71)

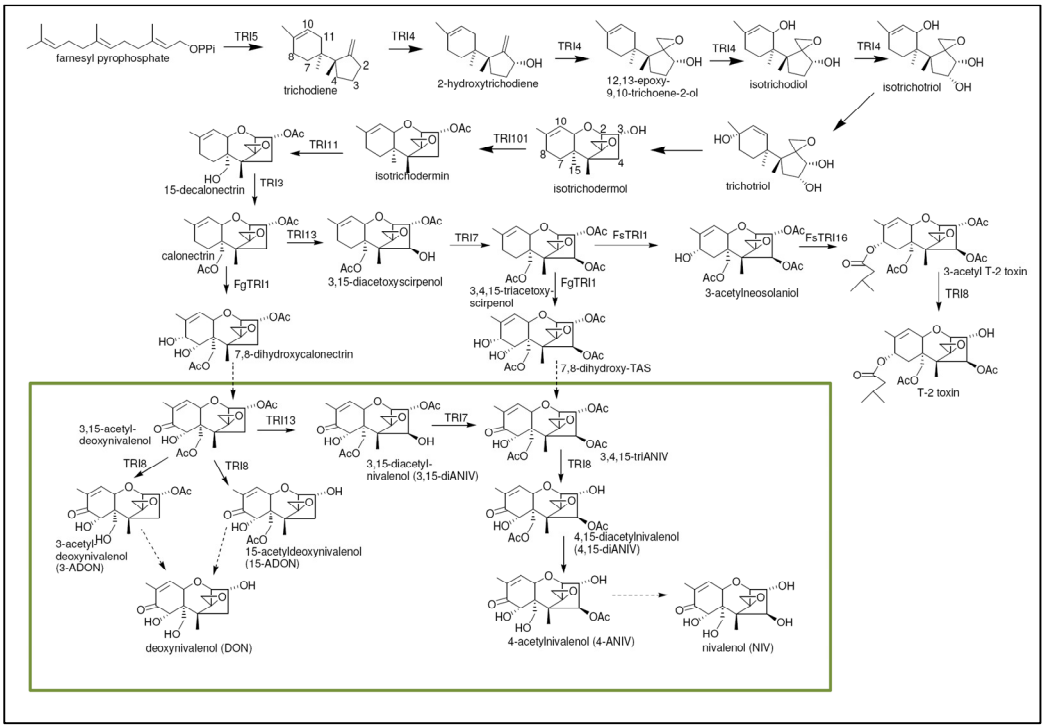

Fig. 12 Proposed trichothecene biosynthetic pathway in *Fusarium* sp.

The biosynthesis of trichothecenes typically begins with the cyclization of farnesyl pyrophosphate, which is a primary metabolic intermediate, to form trichodiene. The Tri5 (terpene cyclase trichodiene synthase) that catalyzes this reaction and the gene which encodes it (*TRI5*) we first found in a Type A trichothecene toxin producing strain of *Fusarium* species.(37, 38) Homologs of this encoding gene have been found in *Trichothecium roseum,* the trichothecene from which clinically valuable compounds like trichothecin and trichothecinol-A have been isolated.(96) Trichothecin lacks a C-3 oxygen function while trichothecinol-A has a C-3 hydroxyl group. (5, 19) The genes in fungi, which are responsible for the biosynthesis of secondary metabolites, are typically always located next to one another in a gene cluster, thus making it very difficult to know exactly which gene controls this oxygenation process or whether the C-3 hydroxyl group is added before cyclization can commence.

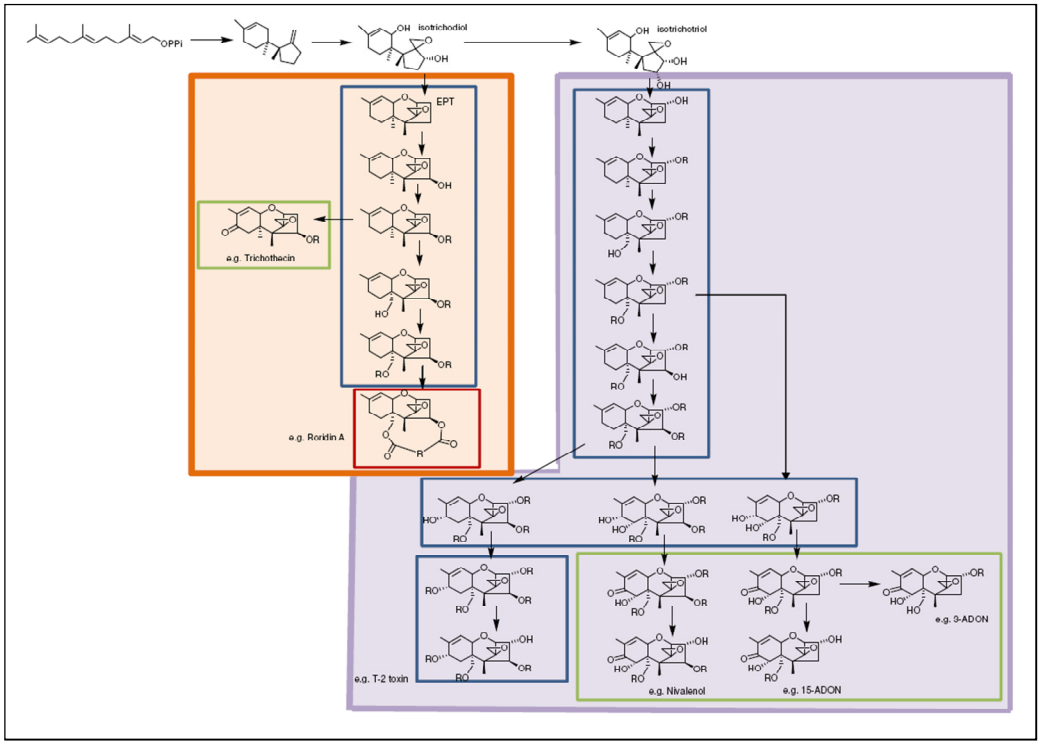

Fig.13 Proposed trichothecene biosynthetic pathways illustrating the divergence into the d-type (from isotrichodiol) (orange box) and the t-type (from isotrichotriol) (violet box) trichothecenes. Blue boxes indicate Type A trichothecenes; green boxes indicate Type B trichothecenes; red box indicates Type D trichothecene.

Paracelsus, a renowned 15th century German-Swiss physician, botanist, alchemist and astrologer mentioned in one of his most historic quotes that "Poison is in everything, and nothing is without poison. Only the dose permits something not to be poisonous" or more commonly "The dose makes the poison". That is to say that substances considered toxic are harmless or even medicinal at low doses, and conversely, an ordinarily harmless substance can be deadly if over-consumed. True to this very day, the same idea applies in the case of trichothecenes and the toxicity they cause. Thus, phylogenetic and evolutionary studies are being conducted upon these fungi , using a combination of chemical and genetical analysis techniques to understand their biosynthesis mechanisms, so that control strategies may be developed to reduce the crop diseases caused by this family of fungi and prevent mycotoxin contamination of grains, building materials, air-conditioning units, etc. associated with these diseases.

Present Research: How and Why??

Our group at NCL Pune is already working on low volume and high valued drugs such as Taxol and Vinca alkaloids from endophytic fungi. We have isolated several endophytic fungi from *Taxus baccata* plant and identified a fungal strain which produces Taxol (paclitaxel) and 10-DAB extracellularly. Similarly, we have isolated endophytic fungi from *Catharanthus* (*Vinca*) *roseus* for the production of vinblastine and vincristine and identified a fungal strain which produces both these drugs extracellularly. Endophytic fungi from different medicinal plants are known to produce the same bioactive compounds as their host plant. Strobel *et. al* and other groups from all over the world have isolated a range of plant based drugs such as taxol, vinca alkaloids, camptothecin and podophylotoxin from the endophytic fungi isolated from *Taxus baccata, Catharanthus* (*Vinca*) *roseus, Camptotheca accumunata* and *Podophyllum* sp. respectively.

As part of our ongoing research for plant based drugs using endophytic fungi, we started the isolation and screening of endophytic fungi from medicinal herb, *Phyllanthus* sp. for the production of various beneficial bioactive secondary metabolites and identified a fungal strain *Trichothecium* sp. which during the present investigation produced only antifungal secondary metabolites against *Aspergillus niger*. These compounds were identified as sesquiterpenoid trichothecene mycotoxins and completely characterized as Trichothecin and Trichothecinol-A. The medicinal herb *Phyllanthus* sp. was used as it is a known astringent, deobstruent, stomachic,

diuretic, febrifugal, antiseptic, employed in dropsy and diseases of urinogenital system; its leaves are expectorant, diaphoretic; seeds are carminative, laxative, astringent to the bowels, tonic to the liver, and used as a remedy for bronchitis, earache, griping, opthalmia and ascites. Fresh roots and leaves of this plant have also been reported to be potent remedy for jaundice. All these features made *Phyllanthus* sp. a very worthy candidate for our study.

During the present investigation, we focused only upon the above two compounds due to their remarkable structural similarities to trichothecene mycotoxins and studied their anti-fungal activity against a range of plant pathogenic fungi, saprophytic fungi and yeast. Also, their apoptotic, anti-proliferative (cytotoxic), and anti-metastatic activities were studies. In future, we will try to isolate other plant based medicinal compounds like phyllanthin, hypophyllanthin, coumarin, etc. based on our previous research experience in this field.

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Chapter 2

Isolation, purification, screening and identification of endophytic fungi from *Phyllanthus* sp. which produces bioactive secondary metabolites.

[A] Isolation, purification and maintenance of endophytic fungi associated with *Phyllanthus* sp.:

Summary:

Endophytic fungi are symbionts residing within plant species which are now being explored as an alternate source of valuable compounds of plant origin. Endophytic fungi were isolated from the leaves, stem and roots of *Phyllanthus* sp. obtained from Pune, India. Each fungal culture was checked for purity and transferred to agar slants by hyphal tip as well as single spore isolation method. From the fungal population, only the slow growing and unusual fungi were considered for further study. Stock cultures were maintained by subculturing at monthly intervals. After growing at a pH of 7 and 25 ^{o}C for 7 days, the slants were maintained at 15 ^{o}C. From an actively growing stock culture, sub-cultures were made on fresh slants and after 7 days incubation at pH 7 and 25 ^{o}C, they were used as the starting material for fermentation experiments.

Introduction:

Endophytic fungi reside in a symbiotic fashion inside their host plants, mimic their chemistry and interestingly, produce the same bioactive natural products and derivatives as their host plants; meanwhile causing no damage to the host.(24, 38–40, 42, 49) One such major example can be seen as in the case of Taxol, An anti-cancer drug which was initially isolated from the bark of Pacific yew plant *Taxus brevifolia* and later also from the endophytic fungus *Taxomyces andreanae* isolated from its bark, thus opening up a new era of research and development.(35) A range of biologically active compounds such as ergot alkaloids, antibiotics, anti-viral compounds, volatile compounds, anti-oxidants, insecticides, anti-diabetic agents, immunosuppressive compounds, etc. have been isolated from endophytic fungi. Endophytic fungi are currently being screened as an alternate source of expensive and non-abundant valuable compounds of plant origin such as taxol, vinblastine, vincristine, camptothecin, podophyllotoxin, etc. in order to make them readily available in the market.(12, 25, 27, 34, 45, 47) All the above drugs are isolated and purified from the culture filtrate by a two stage fermentation procedeure which lasts for about 2-3 weeks. These drugs can be isolated in a very short span of time if the precursors of the above drugs are incubated with endophytic fungi. For example, if

the precursor of Taxol i.e. phenyl-isocerine + 10 deacetly baccatin is incubated with the endophytic fungus isolated from *Taxus baccata*, Taxol is produced in a few days as compared to the several days it takes when the two-stage fermentation process is used. (32, 33)

Fungal endophytes have a wide spread occurrence in higher plants, grasses and cereals in India, but no attempts have been made to isolate and purify these endophytic fungi from *Phyllanthus* sp. for the isolation of bioligcally active metabolites. Tropical species of the genus *Phyllanthus* (Euphorbiaceae) commonly known as 'Bhuiamlki' in India have long been used for the treatment of liver, kidney and bladder problems, intestinal parasites and diabetes.(26) It is considered as an astringent, deobstruent, stomachic, diuretic, febrifugal and is also employed in dropsy and diseases of urinogenital system and has antiseptic properties too.(9) Different parts of the plant are also ethnobotanically reported to have various therapeutic activities e.g. leaves as expectorant, diaphoretic and useful instrangury and sweats; the seeds as carminative, laxative, astringent to the bowels, tonic to the liver, diuretic, useful in bronchitis, earache, griping, opthalmia and ascites.(16) The fresh roots and leaves have been reported to be potent remedy for jaundice.(31) Besides, *Phyllanthus fraternus* and *Phyllanthus maderaspatensis* are also reported to show hepatoprotective activity against carbon tetrachloride induced mitochondrial dysfunction and acetaminophen-induced hepatotoxicity, respectively.(3, 23) The aerial parts of *Phyllanthus niruri* have been reported to contain alkaloids, flavanoids, phenols, coumarins, tannins, terpeniods and lignans. Since the quantity of all these bioactive molecules from all the sources are very low, the supply of all above mentioned compounds to cure liver and other diseases are still limited.

Plant belonging to *Phyllanthus* sp.

Therefore, considering the importance of all above mentioned plant based low volume and high valued drugs and their supply, a screening programme aimed at isolating fungal strains associated with stem and leaves of above mentioned medicinal plant was initiated at our division. Focus has been laid particularly to identify non-sporulating, slow growing and uncommon strains of fungi. So far, 30 such cultures have been brought into pure culture and preliminary laboratory scale experiments to grow and screen the mycelial biomass as well as culture filtrate of the different strains isolated from different parts for possible occurrence of bioactive molecules and related compounds have been initiated.

In this study, we focused on the screening of endophytic fungi for the production of bioactive metabolites as well as other related compounds and to identify the fungi based on their cultural, morphological and molecular (rDNA ribotyping) characteristics.

Materials:

Phyllanthus sp. plant, 0.01% mercuric chloride (HgCl2) 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 *Phyllanthus* sp. plants:

Phyllanthus sp. plants were collected from different areas of Pune, to determine the number and species of endophytes present in leaf, stem and root tissues. After plant selection, the tissues were cut with the help of sterile scalpel and placed in sterile plastic bags to store the material at 4^0C until isolation procedure was started.

Isolation of endophytic fungi from *Phyllanthus* sp.

Endophytic fungi were isolated from the leaves, stem and roots of *Phyllanthus* sp. obtained from Pune, India(1, 6, 14). The samples were cut in to small pieces of approximately 0.5 cm X 0.5 cm and surface sterilized with 0.01% mercuric chloride ($HgCl_2$) solution for 1 min and washed thoroughly with sterile distilled water. Residual water on the sample surface was removed by soaking on sterile blotting paper. Small pieces of leaves, stem and roots were placed on the surface of PDA (Potato Dextrose Agar) poured into Petri dishes. After 10-15 days, fungi were

observed growing from the fragments of the leaves and stem on the plates. Individual hyphal tips of 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 slants by hyphal tips as well as single spore isolation method.(1, 36) From the fungal population, only the slow growing and unusual fungi were considered for further study. Stock cultures were maintained by subculturing at monthly intervals. After growing at a pH of 7 and 25 ^{o}C for 7 days, the slants were maintained at 15 ^{o}C. From an actively growing stock culture, sub-cultures were made on fresh slants and after 7 days incubation at pH 7 and 25 ^{o}C, they were used as the starting material for fermentation experiments.

Results:

Endophytic fungi from *Phyllanthus* sp.:

A total of 30 endophytic fungi were isolated from the leaves, stem and root tissues of *Phyllanthus* plants 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^{0}C for 7 days, the slants were maintained at 15^{0}C. From actively growing stock cultures, subcultures were made on fresh slants and after 7 days incubation at pH 7 and temperature 25^{0}C were used as the starting material for fermentation experiments for isolation of bioactive secondary metabolites.

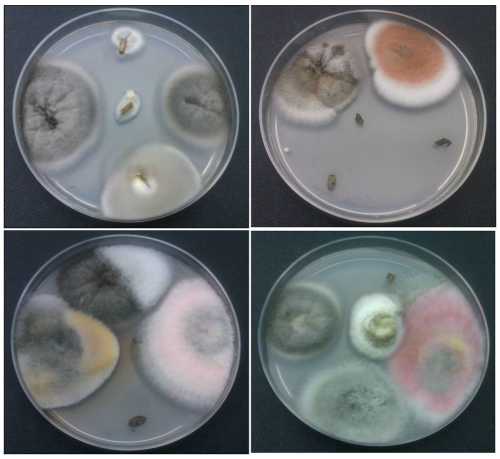
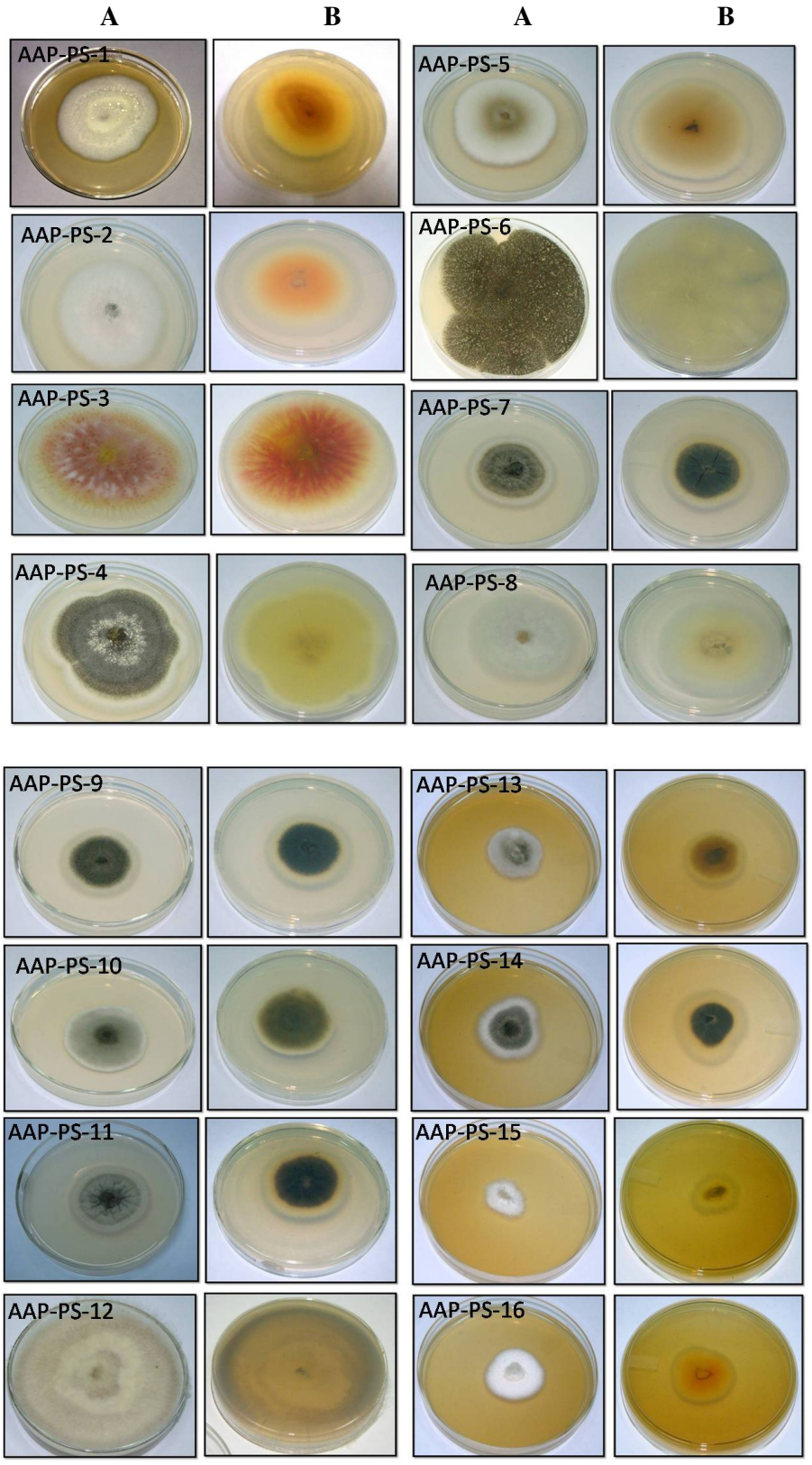


Fig.1- Leaf, stem and root pieces of *Phyllanthus* sp. showing the emergence of endophytic fungi after 10-12 days


A
B
A
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AAP-PS-1
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AAP-PS-3
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AAP-PS-4
AAP-PS-8
AAP-PS-9
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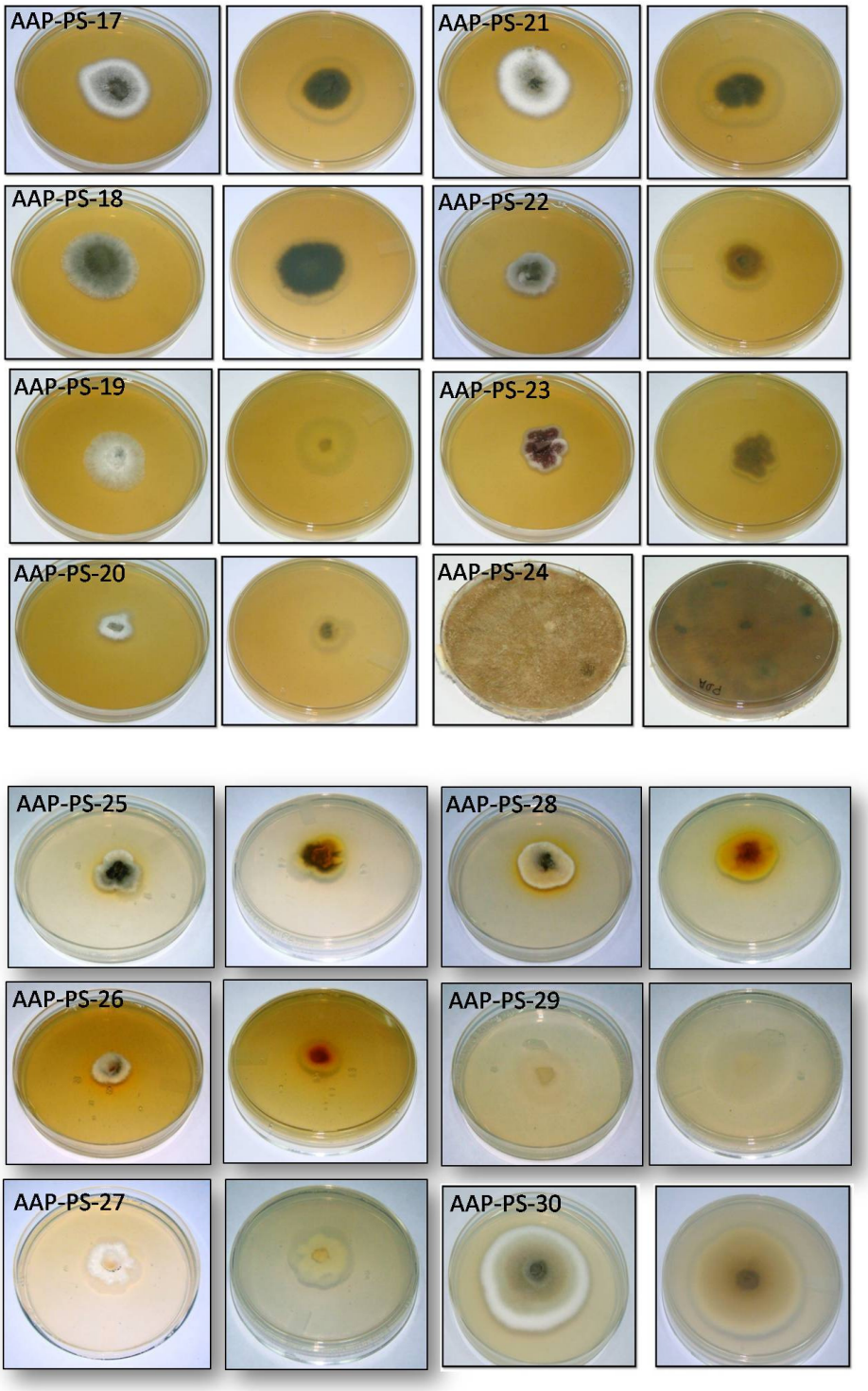


Fig. 2- Colonies of different endophytic fungi isolated from the leaves, stem and root tissues of *Phyllanthus* sp. growing on potato dextrose agar medium. Colony shape: front (A) and back (B) side of a PDA plate.

Discussion:

The aim of the present work was to isolate, purify and identify slow growing and unusual endophytic fungi associated with the leaves, stem and root tissues of *Phyllanthus* sp. 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 the surface of sterilized tissues of excised leaves indicate the endophytic nature of the fungi. Endophytic nature of several *Balansia* sp. has been established. 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. The endophytic fungi isolated from *Phyllanthus* sp. represent different genera of fungi and were screened for bioactive secondary metabolites. (1)

[B] Screening of endophytic fungi for production of bioactive secondary metabolites and other related compounds:

Summary:

Production of bioactive secondary metabolites by the thirty endophytic fungi isolated from different plant parts of *Phyllanthus* sp. was studied by two stage of fermentation procedure. In the first stage these fungi were grown in MGYP medium as submerged culture on a shaker (200 rpm) and in the second stage they were grown in modified S7 medium as stationary culture. After 30 days of incubation, the culture was harvested and passed through four layers of muslin cloth to separate the mycelial mat from the culture filtrate. Both the culture filtrate & mycelia were lyophilized to dryness and extracted thrice with equal volumes of ethyl acetate each time. The extracts were pooled and dried with anhydrous sodium sulphate and concentrated at 40 ^{0}C *in vacuo* to yield crude extract. Antifungal activity against *Aspergilus niger* was used as marker assay for detection of bioactive molecules. The crude extracts derived from fermentation of all endophytic cultures were subjected to above mentioned bioassay. One culture was found to show good activity against *Aspergilus niger* and was selected for further studies.

Introduction:

Endophytes are microorganisms which reside symbiotically with the tissues of higher plants, intra-and/or intercelullarly, causing no damage to their host plants. These microorganisms have a tendency to mimic the chemistry of their respective hosts and produce the same bioactive natural products and derivatives as their hosts. Thus, endophytes have proved to be a rich source of plant derived drugs and other valuable compounds. (19, 43) This symbiotic or mutuality behavior expressed between the endophytes and host plants leads to fitness benefits to both the partners, where the endophytes derive nourishment and protection from the host while the host plant becomes more adept to survive due to various substances produced by the endophytes, which once isolated, purified and characterized, may find huge applications in medicine, agriculture and industry. (17, 41, 42)

Out of the estimated three lack or more plant species playing host to one or more endophytes, very few endophytes have been studied, mainly because ecosystems with

great biodiversity like the temperate and tropical rainforests are still under-explored and thus, several researchers are now focusing their attention and efforts to unearth the true potential of these novel microorganisms with a view to produce plant based bioactive compounds and natural metabolites without utilizing plant parts or harming them in any way. (37, 40) This will prove to be very beneficial to the ecosystem as deforestration would be prevented and global warming and other related serious concerns would be under check.

The process of 'biotransformation' i.e. the synthesis of plant based bioactive compounds by the endophyte associated with the plant, is considered to be an evolutionary mechanism, where these microorganisms, in order to better adapt themselves to their host's conditions, incorporated genetic information from their hosts and as a resistance mechanism began producing mild toxic chemicals and secondary metabolites so as to keep away grazing animals, insects and pathogenic invasion and ensure fitness and survival of the host. Antibiotics are secondary metabolites may be explained as low-molecular-weight natural compounds made by microorganisms and exhibit antibiotic activity at low concentrations against other microorganisms.(7, 22, 37)

A broad range of drugs and bioactive compounds including alkaloids, flavonoids, peptides, quinones, steroids, terpenoids, etc. are now being derived through endophytes and are finding varied applications as antibiotics, antiparasitics, antioxidants, anticancer agents, immunosuppressive and antimicrobial compounds, etc. (11, 43) Many of these anitifungal and antibacterial compounds, apart from being used as drugs, may also be used as food preservatives and prevent food spoilage and food-borne diseases which is a serious issue worldwide. As cases of drug resistance in viruses and pathogenic bacteria are alarmingly on a rise, new and improved therapeutic agents and derivatives are required for effective treatments of diseases in human, plants, and animals. Endophytes can prove to be a promising source for eco-friendly, safe, newer drugs and derivatives and once their fabrication protocol is optimized, bioactive natural compounds can take over synthetic products which exhibit toxicity and produce side-effects. (37, 40, 48)

Materials:

Bioactive secondary metabolite's modified liquid culture medium (**S7)**, distilled water, MGYP medium (composition per lit. dextrose 10g, peptone mycological 5g, yeast extract 3g, malt extract 3g) PDA medium (composition per lit. potato 250g, dextrose 20g, agar 20g), rotary shaker, muslin cloth, chloroform, methanol, silica gel (0.5mm thickness), lyophilizer, 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 bioactive secondary metabolites by 30 endophytic fungi were carried out in 5 liquid culture media. Among the several media tested, secondary metabolites production in significant quantities was observed in only one medium. Hence, further studies were carried out on the production, isolation and quantification of the metabolites using this medium. The composition of the modified S7 medium (per liter H_2O) here after referred as production medium is as follows:

Glucose 1g, sucrose 3g, fructose 6g, soya peptone 1g, zinc sulphate 2.5g, manganese chloride 5mg, magnesium sulphate 3.6mg, cupric nitrate 1mg, calcium nitrate 6.8mg, ferric chloride2mg, biotin 1mg, thiamine 1mg, pyridoxal 1mg, calcium pentothenate 1mg, l-phenylealanine 25mg.

Fermentation, extraction and bioassay:

Production of above compounds by the thirty endophytic fungi isolated from different plant parts of *Phyllanthus* sp. was studied by two stage of fermentation procedure.(35) In the first stage, these fungi were grown in submerged culture and in the second stage they were grown as stationary culture. These fungi were grown in 500 mL Erlenmeyer flasks containing 100 mL Potato Dextrose Broth (PDB) medium. The flasks were inoculated with agar plugs, containing mycelium from 7 days old slants. The inoculated flasks were incubated at 25-27 ^{o}C on a rotary shaker (240 rpm) for 5 days. These cultures were used as seed cultures (First stage). For production of above compounds, 10 ml seed cultures were transferred to 500 mL flasks containing 100 mL modified S7 medium. The flasks were incubated at 25-27 ^{o}C for 21 days as stationary culture (Second stage). After 30 days of incubation, the culture was harvested and

passed through four layers of muslin cloth to separate the mycelial mat from the culture filtrate. Both the culture filtrate & mycelia were lyophilized to dryness and extracted thrice with equal volumes of ethyl acetate each time. The extracts were pooled and dried with anhydrous sodium sulphate and concentrated at 40 ^{0}C *in vacuo* to yield crude extract.

A small amount of crude extract was dissolved in DMSO (dimethyl sulfooxide) and subjected to antifungal activity against *Aspergillus niger* by disc- diffusion method.(10) The culture showing antifungal activity after 48 hrs was selected for further studies.

Results:

Bioactive secondary metabolites from culture filtrate:

Out of 30 endophytic fungi screened for bioactive secondary metabolites, the culture filtrates of AAP-PS-1 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 AAP-PS-1 on prep TLC on silica gel-G using hexane: ethyl acetate (2:1) solvent system produced eight bands when the plates were exposed to iodine vapours. All the bands were cut and eluted with ethyl acetate and tested for their biological (antifungal) activity against *Aspergillus niger*. Out of the 8 bands produced, band numbers 2 and 5 showed strong antifungal activity and thus were further processed for their purification and complete characterization.

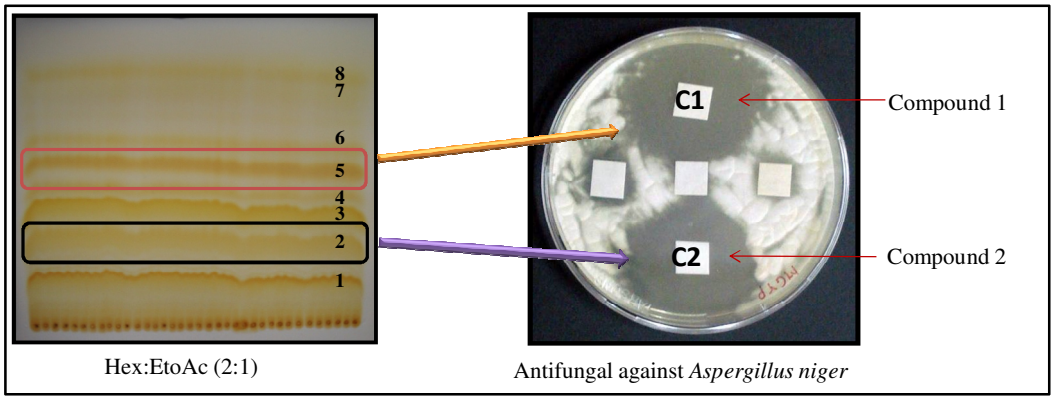


Fig 3. Prep. TLC showing bioactive secondary metabolite's spectrum of crude extract of culture filtrate of AAP-PS-1 on silica gel-G using hexane: ethyl acetate (2:1) solvent system. Detection: Iodine vapors.

Discussion:

Plant based drugs from a microbial source like endophytic fungi will be of immediate interest to pharmaceutical industries as these will help in getting rid of the several geographical and political barriers associated with the transportation of plants as well as from the various environmental conditions which can hamper the quality and production of the desired compounds. Microbial fermentation i.e. microbial production *via* fermentation, or microbial transformation has several advantages over using parts of the plants for the production of drugs and bioactive substances as this can easily be carried out cost-effectively in tank fermenters, providing unlimited renewable supply of drugs and negating the requirement of plant parts. Moreover, different stronger derivatives of the drugs can be obtained by altering the culture conditions. Also, the microbial extraction procedures are very easy and require less solvent in order to purify the drugs.

In the present era of global warming and the ecosystem hanging from a loose thread, this discovery comes as a boon as all the drugs for which plants were earlier being exploited can now be synthesized from the endophytic fungi associated with the plants itself, thus sparing them from extinction in most places. This proves to be true for a very wide extent of plant associated endophytic fungi and has opened up a new area for research and development wherein the drugs could be made available to all at low costs and help in recovering the balance of nature.

[C] Cultural, morphological and molecular characteristics of bioactive secondary metabolites produced from endophytic fungi of *Phyllanthus* sp.:

Summary:

For studying the cultural and morphological characters, the fungus was grown on PDA. 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 Ziess Axiovert 25 Inverted microscope and Nikon Eclipse E200). Mycelia, conidiophores and conidia produced from the fungus in culture were examined under a microscope.

The fungus showed slight variation in growth rate when grown on various nutrient media. Results showed that the fungus can be grown on a variety of natural as well as semi-synthetic media. Among the four different media tested, it was found that V-8 Juice Agar was the best for the growth of the fungus followed by PDA, OMA media and CMA. Excellent sporulation was recorded in PDA and OMA media. Good sporulation was observed in CMA medium and V-8 Juice medium.

Genomic DNA of the fungal culture was extracted by using the Salting out method with some modifications. The quality of the DNA which was isolated was checked on 0.7 % agarose gel stained with ethidium bromide. ITS regions from the fungal strain were amplified using PCR with a Robocycler GRADIENT 96, Stratagene, USA. The primers used were (forward primer) **ITS1-TCCGTAGGTGAACCTGCGG** and (reverse) **ITS4- TCCTCCGCTTATTGATATGC.** The resulting PCR products were analyzed on 1 % agarose gel containing ethidium bromide. Extraction of the fragment DNA was carried out by the protocol as described in Axygen™ GEL elution kit, Biosciences, USA. The gel-eluted fragment was ligated into pGEM-T vector (Promega, USA.). Competent E. coli XL 1 Blue cells were used for transformation. Positive colonies were picked after screening by blue/white selection (disruption of lac Z gene). The alkaline lysis method of Sambrook *et al.* was improvised upon so that samples are processed conveniently for Plasmid DNA extraction. Purified plasmid DNA was given for sequencing. The complete internal transcribed spacer (ITS) sequence of isolate was searched for homology with representative genebank nucleotide sequences using online NCBI blast tool. The internal transcribed spacer (ITS) sequence analysis alongwith morphological and cultural studies confirm that the isolate belongs to genus *Trichothecium.*

Introduction:

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. For proper identification of many fungi, the morphology of conidiophores and the manner in which the conidia are differentiated are required to be studied in detail. Slides prepared from fully sporulated cultures often fail to reveal the developmental stages of conidiophore and conidial morphogenesis. The correct identification of fungi is of great practical importance not only in the industry but also in clinical pathology, biotechnology and environmental studies. (8)

Attempts to find suitable biochemical tools for differentiating taxonomic diversity among the fungi have resulted in different degrees of success. Indole secondary metabolites were considered for differentiating *Penicillium* sp. such as *P. camemberti* and *P.discolor* supplementary to morphological features. (20) Cellular fatty acid analysis of hundreds of fungi belonging to different classes of Oomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and Sterile mycelial forms revealed that many fungi produce the same fatty acid but produce different relative concentrations of each. Both culture temperature and age affected fatty acid compositions, but when these factors were held constant, variance in fatty acid composition was not a problem and fungal fatty acid profiles could be differentiated statistically.

In recent years, molecular biology techniques have provided newer tools for the correct identification of fungi up to species level. These have helped in establishing evolutionary relationships between different taxa in the biological tree and have facilitated their application in fungal systematics, where a new perspective to the phylogenetic relationships among fungal taxa has been brought forward. 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 which has provided accessibility to many genes for study and has also provided an alternative to

tedious and cumbersome cloning procedures. In near future, fungal systematic studies are bound to experience a significant boost due to PCR technology. 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, thus concluding that RAPD is the most 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. Phylogenetic studies of well known plant pathogenic fungi such as *Phytophthora* and *Puccinia* have been carried out based on their DNA sequence analysis or ITS of ribosomal DNA. 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.(18, 46) Kharwar *et al.* isolated and identified the endophytic fungal complex of stem, leaves and roots of *Catharanthus roseus* based on morphological and molecular analysis. (15)

The fact that *Phyllanthus* sp. is being used for the production of several bioactive secondary metabolites led us into further exploring its potential and finally leading to the isolation of 30 new endophytic fungi from different parts of the plant. Out of several fungal cultures screened, one strain (AAP-PS-1) was found to produce

Trichothecin and Trichothecinol-A, which are important secondary metabolites. Thus, further work was carried out on the same and the fungus was identified to be *Trichothecium* sp. based upon its cultural, morphological and molecular studies.

Materials:

Phyllanthus sp. leaves and stem, PDA , MGYP agar medium, CMA, V8 juice agar, liquid nitrogen, CTAB buffer, Proteinase K, RNase A, chloroform, isoamyl alcohol, ethanol, 0.8% agarose gel, Gel-Doc, ITS-1 and ITS-4 Primers (Bangalore Genei, India), dNTPs (Bangalore Genei, India), Taq Polymerase (Bangalore Genei, India), Applied Biosystems Veriti 96- well Thermal Cycler, Carl Zeiss Axiovert 25 inverted microscope, petri plate (10 cm dia.) and B.O.D. incubator. (Add/remove whatever necessary)

Methods:

Microorganism:

AAP-PS-1 was isolated from *Phyllanthus* sp. leaves and was maintained on potato dextrose agar (PDA) slants have optimum growth at pH 7.0 and temperature 27^0C. Sub culturing was done to maintain the stock cultures, at monthly intervals and preserved at 15^0C.

Cultural and morphological characters of the fungus:

For studying the cultural and morphological characters, the fungus was grown on PDA (potato dextrose agar). 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 Ziess Axiovert 25 Inverted microscope and Nikon Eclipse E200). Mycelia, conidiophores and conidia produced from the fungus in culture were examined under a microscope.

Nutritional studies and factors affecting the growth and sporulation of the fungus:

The fungus was grown on the following natural and semi synthetic media: Potato Dextrose Agar (PDA), Oat Meal Agar (OMA), Corn Meal Agar (CMA) and V-8 Juice Agar prepared as described by the manufacturer (Himedia). For studying the growth

of the fungus on solid media, Petriplate (10 cm dia) poured with 25 mL each medium were used. Each Petriplate was inoculated in the centre with a mycelial disc (5 mm) cut from a sporulating 7 days old culture growing on PDA. The Petriplates were incubated at 25-27 ^{0}C in B.O.D incubator. Observations on growth pattern and sporulation were made after 5 days. Sporulation of the fungus in solid media (PDA, CMA, OMA, and V-8 Juice) was determined on the basis of number of spores present per field under uniform magnification and categorized in the following grades as: Good and Excellent. Data comprising of the morphological characters based on previously published description is in TABLE: I. Information regarding shape, temperature, conidiophore, morphological characters of the colony and average diameters of the colony are also listed in Table I.

Molecular characterization of fungal strain AAP-PS-1.

Isolation of fungal genomic DNA

Genomic DNA of the fungal culture was extracted by using the 'Salting out method' with some modification(21). 5 g fungal mycelia was ground into powder using liquid nitrogen, suspended in 5 mL SET buffer (75 mM NaCl, 25 mM EDTA (pH 8.0) and 20 mM Tris (pH 7.5) and placed in the sonicator for 5 minutes with pulse of 5 seconds for disruption of cells. 100 µL lysozyme (final conc. 1mg/ mL) was added to the above solution and incubated at 37 ^{0}C for 60 min. 140 µL of proteinase K (final conc. 0.5 mg/ mL) and 600 µL of 10 % SDS were added and incubated for 2 h at 55 ^{0}C with occasional mixing. 2 mL of 5 M NaCl (final conc. 1.25 M) was added and the mixture was cooled to 37 ^{0}C. 5 mL chloroform and isoamyl alcohol (12 : 1) was added and mixed for about 30 min at room temperature and then the mixture was centrifuged for 20 min at 4,500 g. The supernatant was transferred into a fresh tube and 0.6 volume of chilled ethanol (99 %) was added to precipitate the DNA. The DNA was pelleted and washed twice with 70 % ethanol, air dried and dissolved in 100-200 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at 55 ^{0}C. The quality of the DNA which was isolated was checked on 0.7 % agarose gel stained with ethidium bromide.

Quantification of DNA

Absorbance ratio 260nm / 280nm - 1 $O.D_{260}$ corresponds to 50µg/µl.

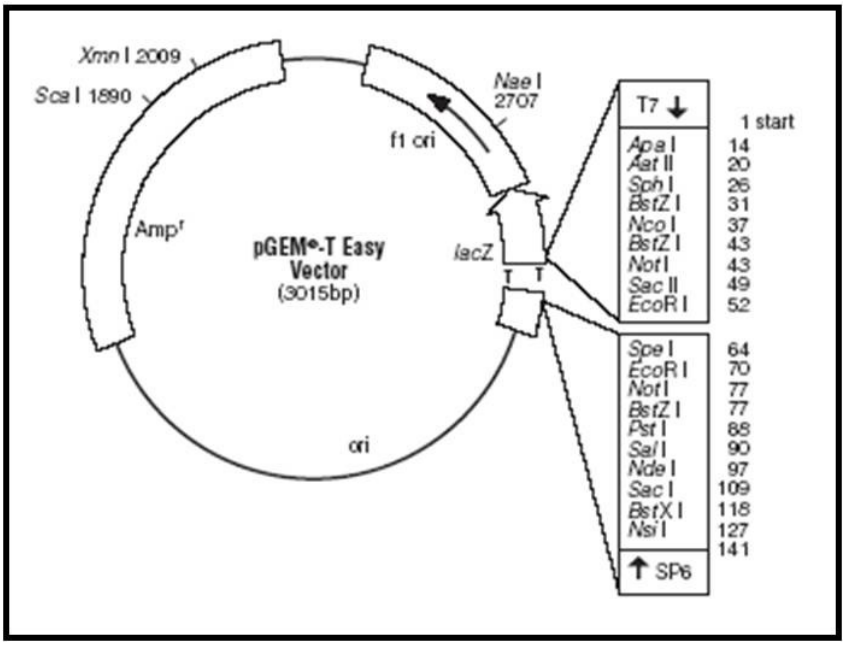
PCR amplification of ITS regions

ITS regions from the fungal strain were amplified using PCR with a Robocycler GRADIENT 96, Stratagene, USA with a final reaction mixture volume of 15 µL containing 0.4 µL fungal DNA solution (40ng), 1.5 µL 10X buffer (Bangalore Gene, India), 4 µL (0.2 mM) dNTPs (Sigma), 1 µL (1 µM) each of the universal eukaryotic primer (forward) **ITS1-TCCGTAGGTGAACCTGCGG**, (reverse) **ITS4-TCCTCCGCTTATTGATATGC**, and 0.5 U/µL Taq polymerase (Bangalore Gene, India). Thermocycling parameters were: for ITS region, initial denaturation at 95 ^{0}C for 3 min, 36 cycles: 95 ^{0}C for 30 sec, 52 ^{0}C for 30 sec, 72 ^{0}C for 1 min; final extension was at 72 ^{0}C for 3 min. The resulting PCR products were analyzed on 1 % agarose gel containing ethidium bromide.

Extraction and purification of DNA from agarose gels

Extraction of the fragment DNA was carried out by the protocol as described in the Axygen^{TM} GEL elution kit, Biosciences, USA. The fragment of interest was excised from the gel and weighed. 100 µg of gel piece was transferred to a 1.5 mL microcentrifuge tube and 300 µL Buffer DE-A was added. The tube was incubated at 70 ^{0}C for 5 to 10 min with intermittent mixing until the gel slice completely dissolved. The gel mixture was cooled down to room temperature and 150 µL Buffer DE-B was added. The solubilized gel was transferred into Axyprep column and centrifuged at 12,000 rpm for 1 min. Supernatant was discarded and the column was washed with 500 µL of wash buffer 1 (provided by Axygen) and centrifuged at 12,000 rpm for 30 seconds. The supernatant was discarded and 700 µL of wash buffer 2 was added and spun at 12,000 rpm for 30 seconds. This step was repeated again;, this ensures the complete removal of salt. Axyprep column was then transferred into a fresh 1.5 mL microfuge tube and DNA was eluted by adding 25-30 µL of elution buffer. After adding the eluent, the tube was left at room temperature for 1 min and centrifuged at 12,000 rpm for 1 min. The filterate containing the DNA was stored at -20 ^{0}C.

Ligation using pGEM®-T Easy Vectors:



pGEM® -T vector map

The gel-eluted fragment was ligated into pGEM-T vector (Promega, USA.)

PGEM-T® vector (50ng/µl)	0.5 µl
Amplicon DNA fragment (20ng/µl)	4.0 µl
Ligase buffer (2X)	5.0µl
T_4 DNA ligase	0.5µl

The ligation mixture was kept at 16ºC overnight and was used for transformation.

Preparation of competent cells using $CaCl_2$ for transformation:

Materials:

Overnight grown culture of E.coli XL 1 Blue.

Sterile LB medium

100mM $CaCl_2$

Method:

- A single colony of E.coli XL 1 –Blue was inoculated in 5 ml of LB medium and grown overnight at 37^0C in incubator shaker at 200 rpm.

- About 500 µl of the overnight grown culture was added to 50 ml of Fresh L B medium and grown for 2 hours at 37^0 C in incubator shaker at 200 rpm.
- Cells were harvested by centrifugation at 5,000 rpm for 10 minutes at 4^0 C.
- The cell pellet was washed with 20 ml ice-cold 100 mM $CaCl_2$ and the pellet was resuspended in 10ml of 100 mM $CaCl_2$ and kept on ice for 30 min.
- The pellet was subsequently centrifuged at 5000 rpm for 10min at 4^oC and was resuspended in 1ml of 100mM $CaCl_2$.
- It was then dispensed in 200 µl aliquots to Eppendorf tubes and kept at 4° C.

Bacterial Transformation:

- Competent E. coli XL 1 Blue cells were used for transformation(29).
- 3µl of the ligated product is mixed with 200µL fresh competent cells and kept on ice for 30 min.
- The cells were then given heat shock at 42^oC for 2 min.
- To each tube, 800 µl of LB broth was added and further incubated at 37^oC for 1h.
- The cells were pelleted by centrifugation at 4000 rpm for 10 min and resuspended in 200 µl of LB broth and spread on LB- agar –amp^{100}, IPTG, X-gal plates.
- The plates were incubated overnight at 37^o C.

Screening for recombinants:

Positive colonies were picked after screening by blue/white selection (disruption of lac Z gene). Each isolated colony was grown in 5ml LB- agar supplemented with ampicillin, incubated overnight and plasmid extraction was done.

Plasmid extraction:

The alkaline lysis method was improvised upon so that samples are processed conveniently for plasmid DNA extraction, with yields of 5-30 µg per 1.5 ml culture depending on the host strain and the plasmid vector(28). This method is recommended for *E.coli* XL 1 blue.

Reagents:

Solution I: 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50 mM Glucose.

Solution II: 0.2 N NaOH, 1 % SDS (freshly prepared).

Solution III: 3.0 M Potassium acetate (pH 4.8).

RNase A to a final concentration of 20 µg / ml.

Chloroform: Isoamyl alcohol (24:1)

70% ethanol

3 M sodium-acetate, pH 5.2.

Method:

- The bacterial cultures were grown overnight at 37°C in LB (Luria Bertani) broth, with 100µg/ml ampicillin.
- About 3 ml of the culture was pelleted for 10 min at 5,000 rpm in a micro-centrifuge.
- The bacterial pellet was resuspended in 200 µL of Solution I by pipetting up and down. Solution II (300 µl) was added and mixed by inversion till the solution becomes clear and incubated.
- The above solution was neutralized by adding 300 µl of Solution III, mixed well and incubated on ice for 5 min.
- The cell debris was removed by centrifuging for 10 min at 12,000 rpm at room temperature.
- The supernatant was transferred to a clean tube; RNase A to a final concentration of 20 µg mL^{-1} (Sambrook et al. 1989) was added and incubated at 37°C for 20 min.
- To the above solution equal volume of chloroform was added, mixed for 30 sec and centrifuged for 10 min at 12,000 rpm.
- The upper aqueous layer was transferred to a clean tube. 0.1 volume of 3 M sodium-acetate and 0.6 volume of Isopropanol was added mixed and kept for precipitation at room temperature for 5 mins.
- It was centrifuged for 10 min at 12,000 RPM. The pellet was washed with 70% ethanol and dried under vacuum for 3 min.
- The dried pellet was dissolved in 40 µl of deionized water and stored at 4°C.
- The plasmid was checked by electrophoresis.

Digestion of the Plasmid DNA:

To release the insert from the vector the Plasmid was digested with EcoR1.

SMQ	14.0 µl
Plasmid DNA	3.0µl
Buffer 3	2.0µl
EcoR1 (NEB, UK)	1.0µl

The mixture was kept at 37º C for 3 hours and run on 1% Agarose gel with 100bp ladder (Bangalore Genei, India).

DNA sequencing and sequence analysis

The cloned fragments were sequenced using Sanger's dideoxy method using ABI Prism Big Dye Terminator Cycle sequencing kit from ABI systems (30). The most identical sequences of the strain **AAP-PS-1** were identified from NR database of Genbank using BLAST algorithm (2). Sequences showing high sequence identity were manually picked for further analysis. Multiple sequence alignment was performed for the sequences obtained from BLAST search using ClustalW algorithm (44).

Results:

Cultural and morphological characters of the fungus:

The fungus AAP-PS-1 isolated and grown on PDA medium produced slow growing colonies. Colonies on PDA were initially white, and with time turned pale rose, flocculose, circular, compact, margin smooth, hyphae branched septate, smooth, and hyaline. Conidophores were long, slender, simple, septate, bearing conidia apically, singly or held together in groups. Conidia were hyaline, two celled, ovoid or ellipsoid. Based on cultural and morphological characters, the strain (AAP-PS-1) was identified as *Trichothecium* sp (4).

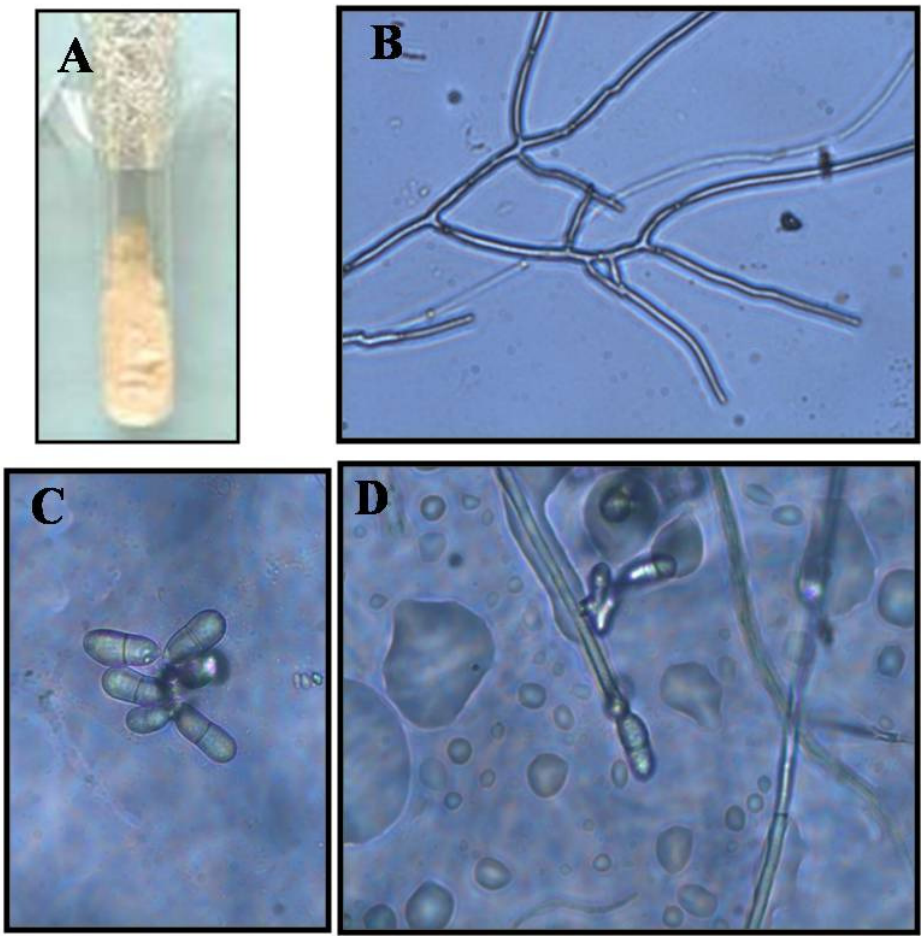


Fig 4. Morphological features of *Trichothecium* sp. (A) Endophytic fungus *Trichothecium* sp. growing on PDA slant after 10 days. (B) Microgaph of mycelium of isolate *Trichothecium* sp. (C). Micrograph of conidia of isolate *Trichothecium* sp. (D). Micrograph of conidiophore bearing conidium of isolate *Trichothecium* sp.

Effect of various nutrient media on growth and sporulation:

The fungus showed slight variation in growth rate when grown on various nutrient media. The results are presented in TABLE 1. Results showed that the fungus can be grown on a variety of natural as well as semi-synthetic media (fig.5). Among the four different media tested, it was found that V-8 Juice Agar was the best for the growth of the fungus followed by PDA, OMA media and CMA also gave good growth. Excellent sporulation was recorded in PDA, OMA media whereas, good sporulation was observed in CMA medium and V-8 Juice medium.

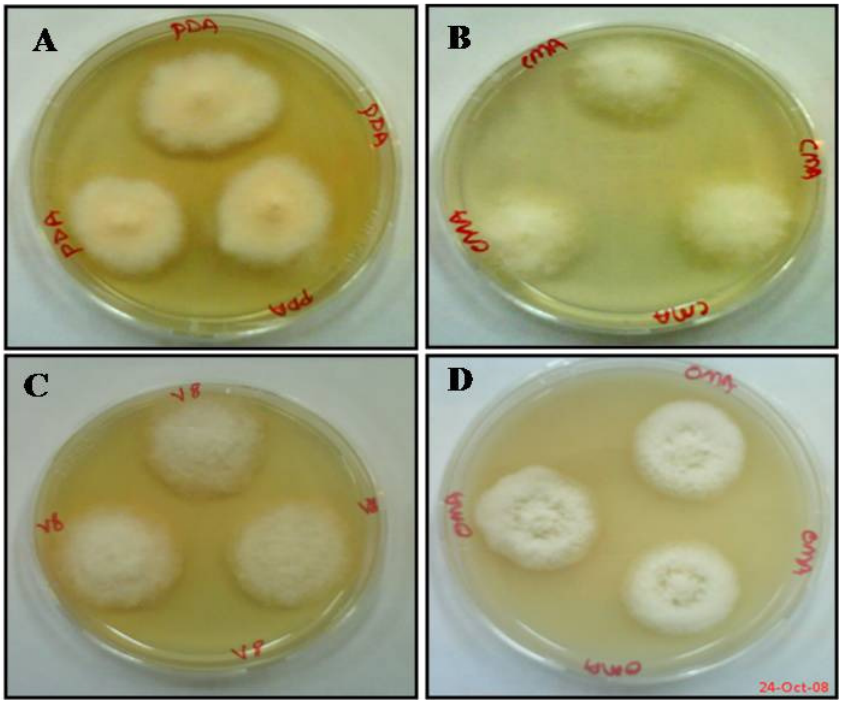


Fig 5. Effect of various nutrient media (A). PDA. (B). CMA. (C) V-8 Juice Agar. (D) OMA) on the growth and the sporulation of the fungus AAP-PS-1 after 5 days.

Morphological characters of the fungus *Trichothecium* sp.

Characters	**PDA**	**V-8 Juice**	**OMA**	**CMA**
Average dia. (mm) of the colonies after 5 day.	32 mm	34 mm	32 mm	29 mms
Morphological characters of the colony	Circular, smooth, flocculose and dense	Circular, smooth, flocculose and dense	Circular, smooth, flocculose and dense	Circular, smooth, flocculose and dense
Colony color	Initially white, and with time turned pale rose.	White in front and pale yellow on reverse	White in front and reverse	White in front and reverse
Conidiophores	Conidophores long, slender, simple, septate, bearing conidia epically, singly or held together in groups.	Conidophores long, slender, simple, septate, bearing conidia epically, singly or held together in groups.	Conidophores long, slender, simple, septate, bearing conidia epically, singly or held together in groups.	Conidophores long, slender, simple, septate, bearing conidia epically, singly or held together in groups.
Conidia	+++	+++	+++	+++
Conidia shape	Hyaline, two celled, ovoid or ellipsoid	Hyaline, two celled, ovoid or ellipsoid	Hyaline, two celled, ovoid or ellipsoid	Hyaline, two celled, ovoid or ellipsoid
Optimum temperature for growth	25- 27^0C	25- 27^0C	25- 27^0C	25- 27^0C

Table I: Growth and morphological characters of the colony of fungus AAP-PS-1.
+++: Excellent. Excellent: More than 100 spore.

DNA extraction from fungal isolate:

Genomic DNA was extracted using salting out method and checked on 0.8% agarose (fig.6). The gDNA was diluted 200 times and quantified on spectrophotometer. The A_{260}nm / A_{280}nm ratio obtained was 1.632 and the concentration of dsDNA was 1200 µg/ml.

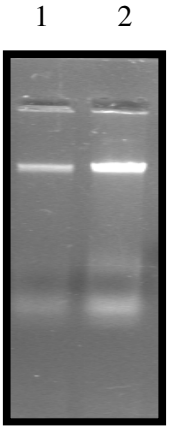


Fig 6: gDNA of endophytic fungus AAP-PS-1on 0.8 % agarose gel.

A good quantity of DNA was obtained by this method and on observation under UV transilluminator showed intact bands of the genomic DNA

PCR amplification by designed primers:

PCR amplification of gDNA (lane1 and 2) using primers ITS1 and ITS4 (fig.7) and lane M standard Molecular weight marker. Lane 1 and 2: 0.7kb PCR amplicon obtained from AAP-PS-1 using primer ITS 1 and ITS 4.

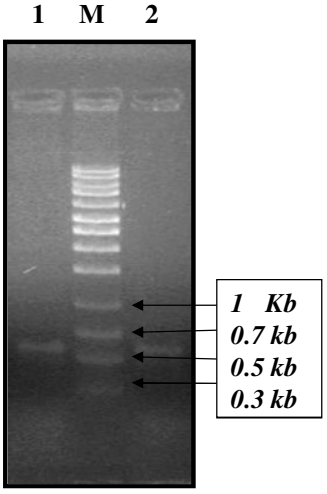


Fig 7: PCR amplification of 'ITS' region fungal gDNA

These bands were cut and eluted as described in Axygen™ GEL elution kit, Biosciences, USA, and dissolved in autoclaved SMQ. This amplified product was cloned in pGEM-T vector.

Transformation (blue/white screening):

The purified 650 bp fragment of gene was ligated in Promega pGEM®-T Easy Vector system. The ligated product was transformed *E.coli* XL 1 Blue competent cells and plated on LB amp plates with Xgal, IPTG (fig. 8)

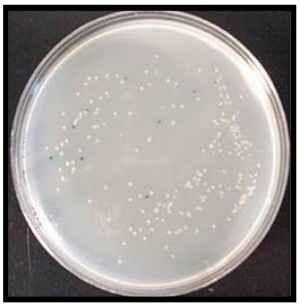


Fig 8: Blue-white screening of recombinant cells.

The plates were incubated overnight at 37 °C. Colonies were observed and transformants were selected by Blue-white screening. The recombinant white colonies were inoculated in 5 ml of LB amp broth and incubated overnight at 37 °C.

Plasmid DNA extraction:

The Plasmid DNA isolation of the colonies was done by alkaline lysis method. 3 µl isolated Plasmid DNA was electrophoresed on 1 % agarose gel (fig. 9).

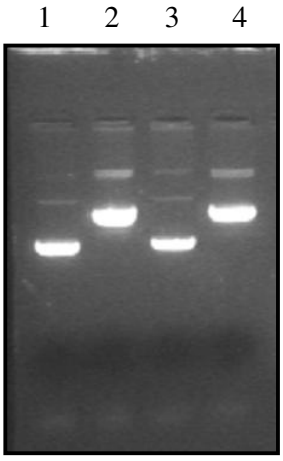


Fig 9: Lane 1-4 Plasmid DNA

The gel was checked under UV transilluminator and three distinct bands of nicked, linear and supercoiled plasmid DNA were observed, where supercoiled migrates ahead towards cathode followed by linear and then nicked.

Sequence analysis of g DNA Fragments:

ITS nucleotide sequence

```
1   ACTAGTGATT  TCCGTAGGTG  AACCTGCGGA  GGGATCATTA  TAGAGTTAAC
51  AAAACAACTC  CCAACCCTTT  GTGAACCTTA  CCTACCGTTG  CTTCGGCGGA
101 CCGCCCCGGG  CGCTGCGTGC  CCCGGACCCA  AGGCGCCCGC  CGGGGACCAC
151 ACGAACCCTG  TTTAACAAAC  ATGTGTATCC  TCTGAGCGAG  CCGAAAGGCA
201 ACAAAACAAA  TCAAAACTTT  CAACAACGGA  TCTCTTGGTT  CTGGCATCGA
251 TGAAGAACGC  AGCGAAATGC  GATAAGTAAT  GTGAATTGCA  GAATTCAGTG
301 AATCATCGAA  TCTTTGAACG  CACATTGCGC  CCGCCAGTAT  TCTGGCGGGC
351 ATGCCTGTCC  GAGCGTCATT  TCAACCCTCG  GGCCCCCCCC  TTTTCCCCTC
401 GCGGGGGAGG  GGGCGGGCCC  GGCGTTGGGG  CCCAGGCGTC  CTCCAAGGGC
451 GCCTGTCCCC  GAAACCCAGT  GGCGGCCTCG  CCGCTGCCTC  CTCCGCGTAG
501 TAGCACAAAC  CTCGCGGGCG  GAAGGCGGCG  CGGCCACGCC  GTAAAACCCC
551 AAACTTTTAC  CAAGGTTGAC  CTCGGATCAG  GTAGGAATAC  CCGCTGAACT
601 TAAGCATATC  AATAAGCGGA  GGAAATC
```

The sequence consists of partial 18S, complete ITS 1, 5.8S, ITS 2 and partial 28S sequence and shows 99% identity with *Trichothecium roseum* 18S rDNA sequence.

- 1 - 39 18S ribosomal DNA partial sequence
- 40 - 223 Internal transcribed spacer 1 complete ribosomal DNA sequence
- 224 - 371 5.8S complete ribosomal DNA sequence
- 372 - 564 Internal transcribed spacer 2 complete ribosomal DNA sequence
- 565 - 627 28S ribosomal DNA partial sequence

Alignment of the ITS nucleotides with known *Trichothecium roseum* 18s r DNA sequences in GenBank Databases:

```
AAP-PS-1                -------------------------------------------------ACTAGTGATT
Trichothecium_roseum     AGGGCCGGAAAGTTCTCCAAACTCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTC
                                                                          *  *

AAP-PS-1                TCCGTAGGTGAACCTGCGGAGGGATCATTATAGAGTTAACAAAACAACTCCCAACCCTTT
Trichothecium_roseum    TCCGTTGGTGAACCAGCGGAGGGATCATTATAGAGTTAACAAAACAACTCCCAACCCTTT
                        ***** ******** **********************************************

AAP-PS-1                GTGAACCTTACCTACCGTTGCTTCGGCGGACCGCCCCGGGCGCTGCGTGCCCCGGACCCA
Trichothecium_roseum    GTGAACCTTACCTACCGTTGCTTCGGCGGACCGCCCCGGGCGCTGCGTGCCCCGGACCCA
                        ************************************************************

AAP-PS-1                AGGCGCCCGCCGGGGACCACACGAACCCTGTTTAACAAACATGTGTATCCTCTGAGCGAG
Trichothecium_roseum    AGGCGCCCGCCGGGGACCACACGAACCCTGTTTAACAAACATGTGTATCCTCTGAGCGAG
                        ************************************************************

AAP-PS-1                CCGAAAGGCAACAAAACAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
Trichothecium_roseum    CCGAAAGGCAACAAAACAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
                        ************************************************************

AAP-PS-1                TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA
Trichothecium_roseum    TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA
                        ************************************************************

AAP-PS-1                TCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATT
Trichothecium_roseum    TCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATT
                        ************************************************************

AAP-PS-1                TCAACCCTCGGGCCCCCCCCCTTTTCCCCTCGCGGGGGAGGGGGCGGGCCCGGCGTTGGGG
Trichothecium_roseum    TCAACCCTCGGGCCCCCCCCCTTTTCCCCTCGCGGGGGAGGGGGCGGGCCCGGCGTTGGGG
                        ************************************************************

AAP-PS-1                CCCAGGCGTCCTCCAAGGGCGCCTGTCCCCGAAACCCAGTGGCGGCCTCGCCGCTGCCTC
Trichothecium_roseum    CCCAGGCGTCCTCCAAGGGCGCCTGTCCCCGAAACCCAGTGGCGGCCTCGCCGCTGCCTC
                        ************************************************************

AAP-PS-1                CTCCGCGTAGTAGCACAAACCTCGCGGGCGGAAGGCGGCGCGGCCACGCCGTAAAACCCC
Trichothecium_roseum    CTCCGCGTAGTAGCACAAACCTCGCGGGCGGAAGGCGGCGCGGCCACGCCGTAAAACCCC
                        ************************************************************


AAP-PS-1                AAACTTTTACCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATC
Trichothecium_roseum    AAACTTTTACCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATC
                        ************************************************************

AAP-PS-1                AATAAGCGGAGGAAATC
Trichothecium_roseum    AATAAGCGGAGGAAAAG
                        ***************
```

Discussion:

The morphology of AAP-PS-1 isolate obtained from *Phyllanthus* sp. mostly agree with the description of Barnett and other researchers. The isolate grew well on a variety of natural as well as semi-synthetic media. Among the four different media tested, it was found that V-8 Juice Agar was the best for the growth of the fungus followed by PDA, OMA media and CMA also gave good growth. Excellent sporulation was recorded in PDA, OMA media whereas, good sporulation was observed in CMA medium and V-8 Juice medium. The isolate grew well at room temperature (26–28 ^{o}C). The method employed for the genomic DNA isolation resulted in obtaining high quality DNA. Universal eukaryotic primers (ITS 1 & 4) were used for the amplification of ITS regions and 18S rRNA successfully amplified fungal genomic DNA producing fragments of 0.7 kb. All the clones obtained showed the respective inserts when subjected to restriction digestion and analyzed on agarose gel. Sequence analysis was done using Chromos V 2.0 program and the sequence profile was obtained in FASTA format. BLAST analysis of the sequences determined the identical sequences from the database. Majority of the hits from the BLAST search were from a group of fungus belonging to genus Trichothecium. The sequence was aligned with ClustalW and the relatedness between the sequences was determined. The sequence was submitted in NCBI gene bank and is retrievable with accession number: JN603460.The internal transcribed spacer (ITS) sequence analysis, alongwith morphological and cultural studies confirms that isolate belongs to genus *Trichothecium.* Endophytic fungi isolated from different plants also grew well on natural and semi-synthetic media. These results thus support present findings.

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Chapter 3

Isolation, purification and characterization of Trichothecin produced by endophytic fungus *Trichothecium* sp.

Summary

Endophytic fungi were isolated from the leaves of *Phyllanthus* sp. plant obtained from different areas of Pune and then screened for trichothecin production. Out of 30 fungal cultures screened, only one AAP-PS-1 was found to produce trichothecin extracellularly in appreciable amounts. Homogeneity of the purified trichothecin was determined by TLC on three different solvent systems. RP-HPLC analysis performed on a Waters model using a C_{18} symmetry pack column with a flow rate of 0.5 ml/min, and the eluting compounds were detected by a dual mode wavelength detector set at 220nm and 240nm. The 1D and 2D NMR (^{1}H, COSY, NOESY, TOCSY, ^{13}C, DEPT, ^{13}C-^{1}H HMBC, ^{13}C-^{1}H HSQC), ESI-MS, HRMS, IR and UV-Vis show conclusively that the isolated compound was Trichothecin. One liter of *Trichothecium* sp. yielded 4.25 mg of Trichothecin.

Introduction

Trichothecenes are biologically active sesquiterpenoid mycotoxins (secondary metabolites) produced by various genera of fungi like *Fusarium*, *Trichothecium*, *Trichoderma*, *Myrothecium*, *Stachybotrys*, *Spicellum*, *Cephalosporium*, etc., and are potent cytotoxic eukaryotic translation inhibitors. These are found worldwide in the environment, food stuffs like grains, vegetables, fruits, juices and alcoholic beverages and pose a great threat to human and animal health. Research by the UN Food and Agriculture Organization (FAO) showed that about 25% of world food production is contaminated by at least one trichothecene mycotoxin. Trichothecenes have a diverse role in plant and animal cells, which is not limited only to the inhibition of protein synthesis. Toxic manifestation of these compounds leads to growth retardation, anaemia, hemorrhagic lesions, vomiting, immune dysfunctions and reproductive disorders.(12, 16)

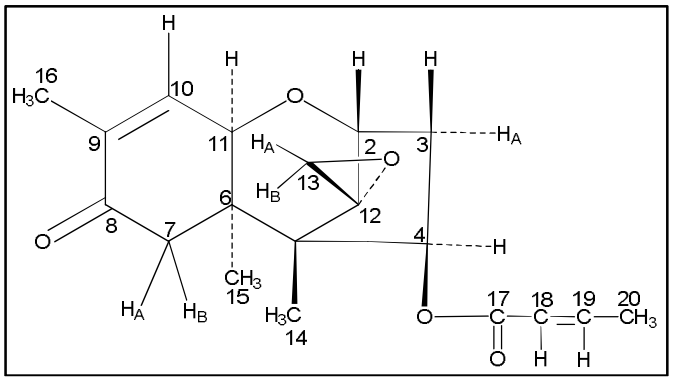


Fig 1. Trichothecin **(Mw: 332, $C_{19}H_{24}O_5$)**

All trichothecenes share a 12,13-epoxytrichothec-9-ene or the EPT core and classification is based on substitution pattern of specific side groups. Trichothecin (TCN) is a member of this group of compounds and was isolated as a stable anti-fungal agent from the cytotoxic organic culture extract of terrestrial filamentous fungus *Trichothecium roseum*, as species of the genus *Trichothecium* have been a rich source of secondary metabolites with reports of about 30 different secondary metabolites from *Trichothecium roseum* alone.(2, 4, 6–8, 10, 13–15, 17) TCN bears a high similarity to the chemical structure of trichothecenes, with added presence of a ketone functional group at C-8 and lack of C-3 oxygen function. Infact, the name 'trichothecenes' was derived from the compound TCN as it was the first naturally occurring trichothecene ever isolated. (17)

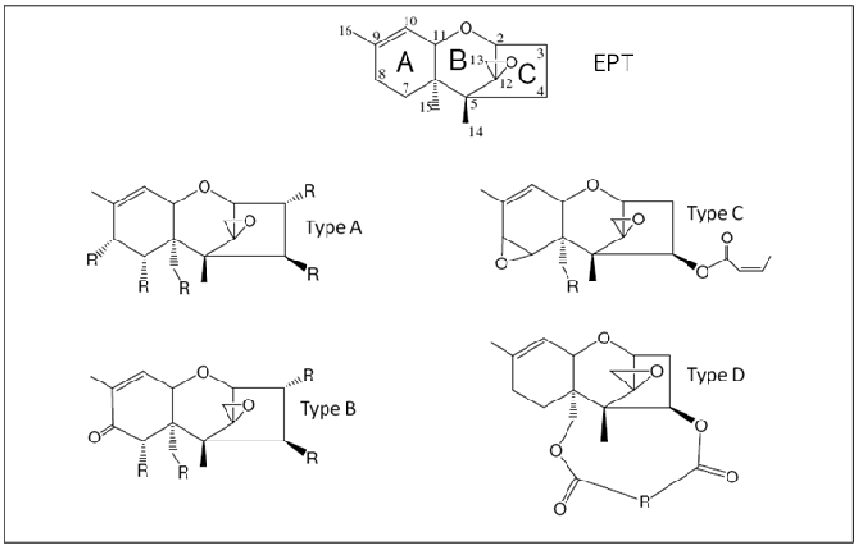


Fig 2. Common tricyclic 12,13-epoxytrichothec-9-ene (EPT) core structure of trichothecenes. R groups may be H, OH, OAcyl, or variations in the macrolide chain.

TCN is extremely stable and its activity is unaffected even after repeated boiling and hour-long autoclaving at 20 lb./sq. inch pressure.(6)**(13)** This remarkable thermostability and neutral reaction of TCN make it an excellent choice for exploring anti-biotic production in soil. Reports suggest that TCN accelerates the bond-break phase of growth in plants. Minute doses of TCN stimulates growth by slightly increasing the rate of bond-break, enough to allow increased elongation but insufficient to cause molecular disorganization. At higher concentrations, the disorganization of the molecular make-up of the wall leads to swelling and bursting of

the hyphal tips in fungi; and in plants, it leads to reduction of overall height and dwarfism.

In animal system, TCN is found to be one of the most potent anti-fungal members which produces the very least of adverse effects on mammalian cells.(1) TCN causes disruption of mitochondrial membrane morphology leading to homosomal condensation and inter-nucleosomal fragmentation, thus affecting the intrinsic pathway of apoptosis. (11) The resultant loss of mitochondrial membrane potential leads to release of apoptogenic factors like cytochrome c (cyt c) from intermembrane space into the cytosol and promotes expression and activation of caspase-9 and caspase-3 respectively. This leads to excessive generation of ROS (reactive oxygen species) and attenuation of cell viability, alongwith affecting apoptosis and apoptotic cascade reactions in HepG2 cells selected from liver, as these cells are always in the process of rapidly dividing and exhibit active ongoing protein synthesis which makes them susceptible to trichothecene infection and abundant toxins can be metabolized. (5)

Table .1 Classification of representative Trichothecenes

Sr. No.	**Trichothecene**	**Simple or Macrocyclic**	**Type [1]**	**Group [2]**	**Type [3]**
1	Trichodermol	S	A	I	d
2	Trichodermin	S	A	I	d
3	4,15-Diacetoxyscirpenol (DAS)	S	A		t
4	Neosolaniol	S	A	II	t
5	T-2 toxin	S	A	II	t
6	Isotrichodermol	S	A	I	t
7	Calonectrin	S	A		t
8	7,8-Dihydroxy calonectrin	S	A	II	t
9	Harzianum A	S	A		d
10	Nivalenol (NIV)	S	B	III	t

11	Deoxynivalenol (DON)	S	B	III	t
12	Fusarenon-X	S	B	III	t
13	Trichothecin	S	B	III	d
14	Trichothecinol A	S	B	III	t
15	Crotocin	S	C		d
16	Satratoxin H	M	D		d
17	Roridin A	M	D		d
18	Baccharin	M	D		d
19	Verrucarin A	M	D		d

[1] Based on presence of C-8 keto group (Type B), C-7, C-8 epoxy (Type C), ring connecting C-4 and C-15 (Type D); [2] Based on substitutions in the A and C ring; [3] Based on presence (t-type) or absence (d-type) of C-3 oxygen function.

In present work, we have isolated an endophytic fungus *Trichothecium* sp. from the medicinal herb *Phyllanthus* sp. and used it for the isolation of TCN. The compound was purified, and completely characterized by standard techniques and a variety of ID and 2D NMR Spectroscopy. Its anti-fungal, anti-cancer (anti-proliferative), anti-metastatic and apoptotic studies all tested positive for great potential of this compound to be developed as a drug for the respective fields, making treatment cheap, eco-friendly, safer and minimizing side-effects.

Materials

Fermentation (Modified S7) medium: per liter composition

Glucose 1gm, Sucrose 3gm, Fructose 6gm, Soya peptone 1gm, Zinc sulphate 2.5gm Manganese chloride 5mg, Magnesium sulphate 3.6mg, Cupric nitrate 1mg, Calcium nitrate 6.8mg, Ferric chloride 2mg, Biotin 1mg, Thiamine 1mg, Pyridoxal 1mg, L-phenylalanine 25mg, Calcium pentothenate 1mg, , Phosphate buffer 1ml (pH 6.8)

MGYP broth: per liter composition

Malt extract 3gm, Glucose 10gm, Yeast extract 3gm, Peptone 5gm.

Solvents used:

Acetonitrile, Methanol, Ethyl acetate, Hexane, Chloroform. All the solvents used were from Qualigens ExceleR grade.

Methods

The fungus *Trichothecium* sp. maintained on PDA slants has optimum growth at pH 7.0 and temp. 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 7 days at pH 7.0 and temp. 27^{o}C.

Isolation and purification of trichothecin:

Fermentation:

Production of trichothecin was studied by a two stage fermentation procedure. In the first stage, the fungal strain was grown in 500ml Erlenmeyer flasks containing 100ml MGYP medium. The flasks were inoculated with agar plugs containing mycelium from 7 days old culture. The inoculated flasks were incubated at 25-27^{0} C on a rotary shaker (240 rpm) for 4-5 days, which was used as seed culture (Ist stage). Later, 10ml seed culture was transferred to 500ml Erlenmeyer flask containing 100ml of fermentation (production) medium called as modified S7 medium and were incubated at 25-27^{0}C as a stationary for 30 days (IInd stage). After which it was harvested and used for further study.

Lyophilization:

Culture filtrate and mycelium were separated with the help of muslin cloth and then lyophilized and stored at 4^{0}C for further processing.

Extraction:

Culture filtrate was extracted with equal volumes of ethyl acetate each time. The organic layer was separated from the aqueous layer using separating funnel. The extraction was repeated thrice and solvent was dried using anhydrous sodium sulphate and concentrated under vacuum using Rotavapor at 40^{0}C in order to get crude extract.

Purification:

Preparative TLC:

A small amount of (0.5 ml) crude extract was dissolved in ethyl acetate and subjected to Thin Layer Chromatography (Prep TLC) on Silica gel G (0.75mm thickness) using hexane:ethyl acetate 2:1 as solvent system. The developed plates were exposed to iodine vapours. The bands are then cut, eluted with ethyl acetate and tested for their

biological activity. The bands showing activity were further processed for their purification.

HPLC analysis:

Small amount of crude (EToAc extract) was dissolved in 1ml of HPLC grade acetonitrile and 10 µl of this solution was further processed to purify active compound by reverse phase HPLC. HPLC analysis performed on a Waters model using a C_{18} symmetry pack column with a flow rate of 0.5 ml/min, and the eluting compound was detected by a dual mode wavelength detector set at 220nm and 240nm. HPLC grade solvents were prefiltered using a Millipore system and analysis was performed. The column was first equilibrated with three column volumes of 100% acetonitrile and eluted with a gradient of 5-95% acetonitrile.

Quantification of trichothecin by HPLC:

Stock solution of different concentrations (1mg/ml, 3mg/ml, 5mg/ml and 10mg/ml) of a purified trichothecin was prepared in HPLC grade acetonitrile. 10 µl of each stock solution was injected in HPLC to generate a standard graph of peak area v/s concentration. Then the known volume of (10 µl) crude dissolved in HPLC grade acetonitrile containing unknown concentration of trichothecin was analysed by HPLC and the peak area of trichothecin containing peak was compared to standard graph to calculate its concentration.

Characterization

UV-Visible analysis:

UV-visible spectroscopy measurements were carried out on Jasco dual beam spectrophotometer (model V- 570) operated at resolution of 1nm.

ESI-MS and HRMS analysis:

Molecular mass of the purified compound was determined by M/S Applied Biosystems API QSTAR pulsar (ESI-MS) mass spectrometer. Samples for the analysis were dissolved in HPLC grade methanol. Samples were then analyzed by infusion method / (injected into MS) at a flow rate of 5 µl/min and at a IS voltage of 3800 V in TOF mode. Spectrum from a range of m/z 200 to 500 Daltons was

obtained. HRMS analysis was carried out using mass spectrometry instrument (model – Autoconcept) by direct injection probe with resolving power of 6000.

FTIR:

FTIR spectroscopy measurements of purified compound taken in KBr pellet were carried out using a Perkin-Elmer spectrum one instrument. Spectrometer was used in the diffuse reflectance mode of resolution 2 cm^{-1}. To obtain good signal to noise ratio, 128 scans of the film were taken in the range of 450-4000 cm^{-1}.

NMR spectra:

All the NMR measurements were carried out on Bruker AV 500 spectrometer operating at 500 MHz and 125 MHz, respectively for ^{1}H and ^{13}C. Approximately 10 mg of the compound was dissolved in $CDCl_3$ in a standard 5mm NMR tube and the ^{1}H, COSY, NOESY, TOCSY, ^{13}C DEPT, ^{13}C-^{1}H HSQC and ^{13}C-^{1}H HMBC experiments were performed on a standard 5mm BBFO probe at ambient temperature (~ 25^{o}C). Chemical shifts in the ^{1}H spectra were referenced to residual $CHCl_3$ peak (7.26 ppm), while ^{13}C spectra were referenced to $CDCl_3$ (77.0 ppm). Approximately 1600 and 800 transients were collected for ^{13}C CPD and DEPT135 spectra of trichothecin. Gradient spectroscopic techniques were employed for all the 2D experiments. 256 experiments (t1 increments) of 8, 8 and 32 scans were used respectively for COSY, NOESY and TOCSY measurements of Trichothecin. COSY and the HMBC spectra were collected in a magnitude mode while a phase sensitive (States-TPPI) mode was used for HSQC and NOESY measurements. A mixing time of 1 sec was employed for NOESY. The number of scans used for each t1 increment for other 2D experiment for trichothecin were as follows: 24 (^{13}C HSQC), 32 (^{13}C HMBC). The ^{13}C HMBC data was optimized for a long range coupling constant of 7 Hz. A pulse sequence employing a double low pass filter was found to give better results for ^{13}C HMBC due to spread in $^{1}J_{C-H}$ values (160 -135 Hz). The HMBC spectra were acquired without proton decoupling during detection. The 90^{o} pulse lengths for ^{1}H and ^{13}C were 13.5 and 10 respectively. Appropriate window functions viz. sine squared bell with no phase shift for all magnitude mode and phase shifted (ssb = 2) sine squared bell for phase sensitive mode were used for data processing. In general, a 1Kx1K data matrix size was used for the 2D experiments.

Results

Production and purification of trichothecin

The culture filtrate of endophytic fungal strain *Trichothecium* sp extracted with ethyl acetate yielded a brown residue after removal of the solvent. The crude extract of the fungus on preparative TLC on silica gel G using hexane: ethyl acetate (2:1) as solvent system produced 8 bands when the plates were exposed to iodine vapours. All the bands were cut and eluted with ethyl acetate and tested for their biological (antifungal) activity against fungus *Aspergillus niger*. Out of 8 bands produced, band no. 2 and 5 showed strong antifungal activity and were further processed for their purification and complete characterization. The 5th fraction obtained from preperative TLC was further processed for its purification by reverse phase HPLC.

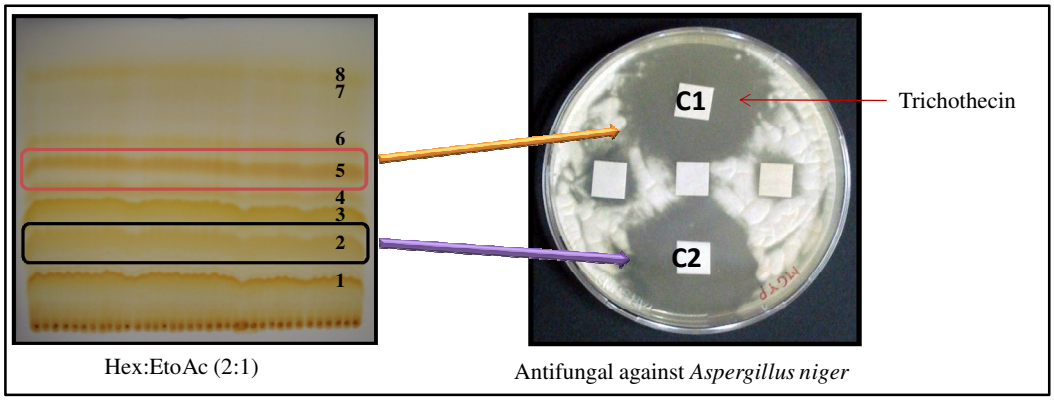


Fig 3. Antifungal activity of crude extract (Band 5) against *Aspergillus niger*.

RP-HPLC of fraction 5 was carried out using Waters separation module. Compound was loaded on analytical C_{18} (4.6 X 250 mm) symmetry pack column with a flow rate of 0.5 ml/min. 40 minutes gradient of milli Q water – acetonitrile (95-5%) were used as a mobile phase. The progress of HPLC was monitored at 240 nm. Each peak was collected separately and tested for antifungal activity. The peak eluted at RT 45 min only showed antifungal activity and processed for its complete characteriszation. The homogeneity of the purified compound was confirmed by HPLC analysis which showed a single symmetrical peak at RT 39 minute on C18 symmetry coloumn.

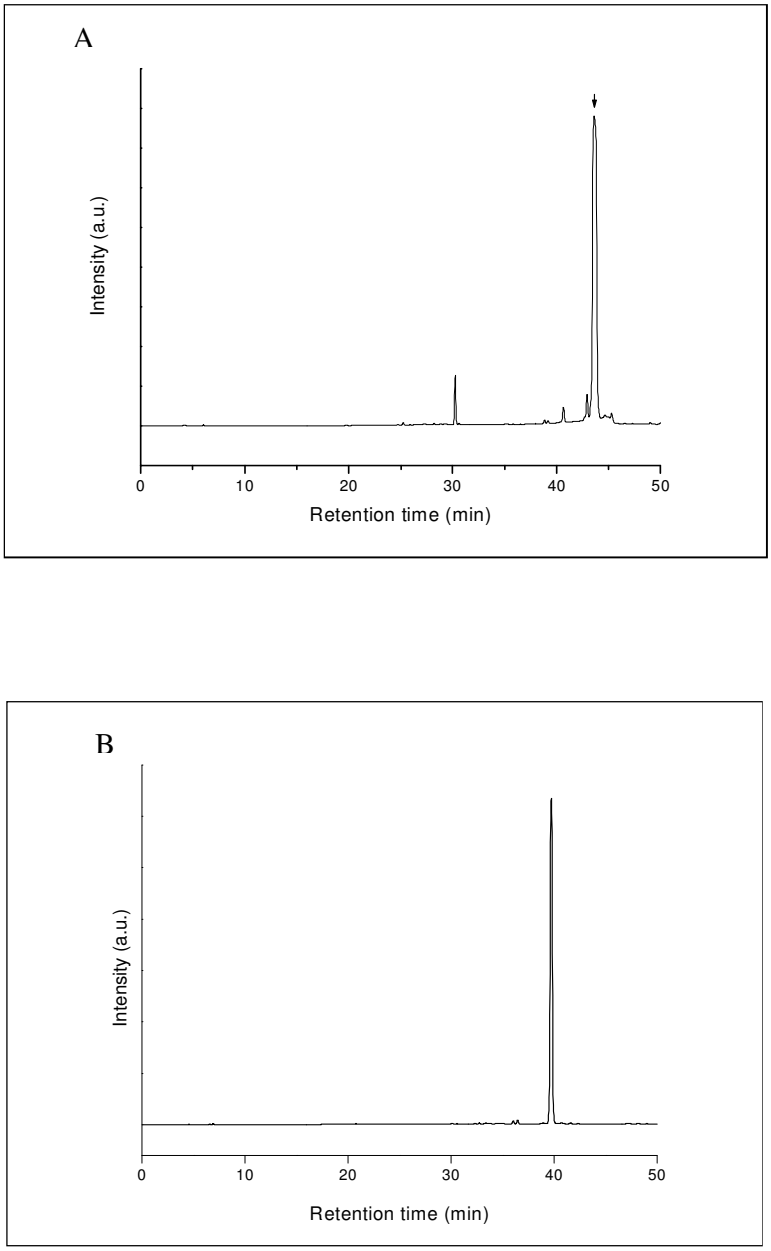


Fig 4. A) RP-HPLC profile of ethyl acetate extract. Peak containing antifungal activity indicated by arrow eluted ar retention time of 45 minute. B) RP-HPLC profile of purified copmound which gave antifungal activity shows peak at retention time of 39 minutes.

Characterization of Trichothecin

UV-Vis spectroscopy

UV-visible spectroscopy measurements of trichothecin were carried out on Jasco dual beam spectrophotometer (model V-570) operated at resolution of 1nm. The UV absorption analysis showed a peak showing absorption maxima at 238 nm in ethanol. This is similar to the reported value of Trichothecin. (3)

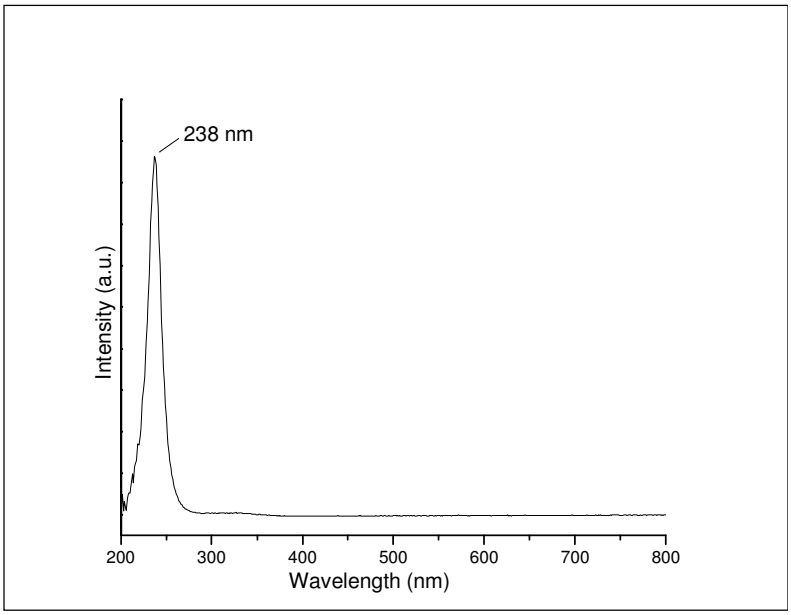


Fig 5. UV-visible spectrum of trichothecin in ethanol

FTIR analysis

FTIR spectroscopy measurements of purified trichothecin taken in KBr pellet were carried out using a Perkin-Elmer spectrum one instrument. Spectrometer was used in the diffuse reflectance mode of resolution 2 cm^{-1}. To obtain good signal to noise ratio, 120 scans of the film were taken in the range of 500-3500 cm^{-1}. Trichothecin shows characteristic 2978 cm^{-1} (C-H stretching), 2923 cm^{-1} (C-H stretching), 2851 cm^{-1} (C-H stretching), 1711 cm^{-1} (C=O stretching), 1671 cm^{-1} (C=C stretching), 1645 cm^{-1} (-C=C- stretching) and 1072 cm^{-1} (C-O stretching). This is similar to the reported value of Trichothecin. (3)

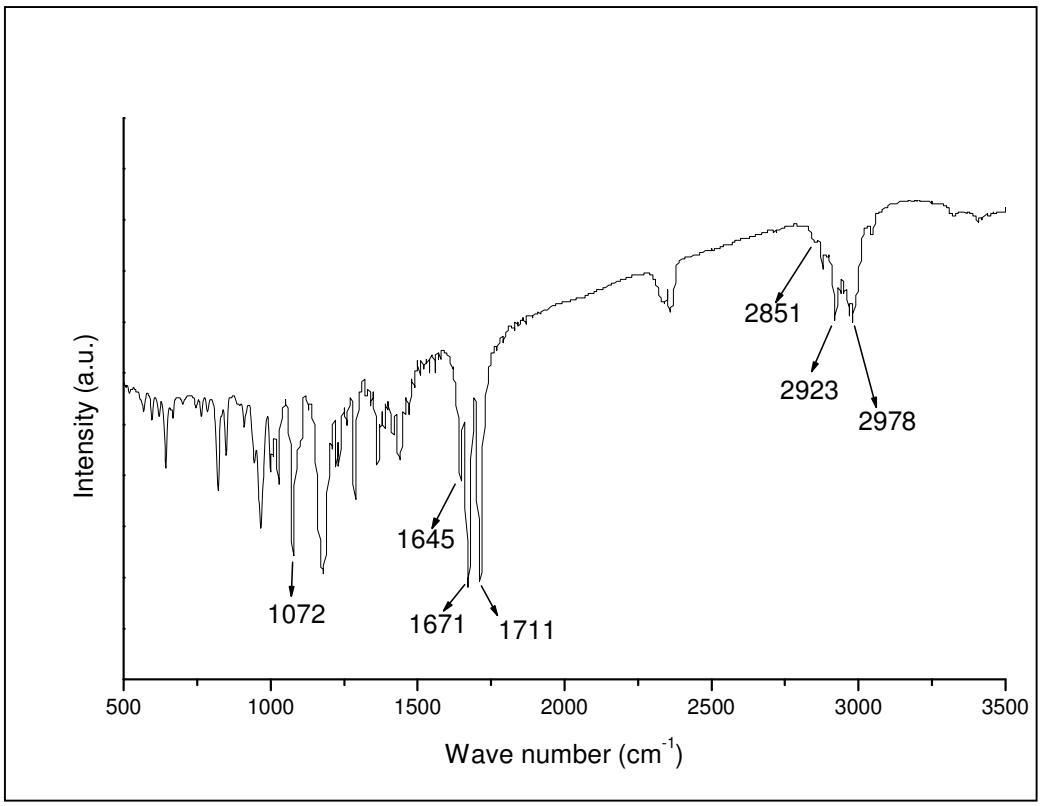


Fig 6. FTIR spectrum of purified trichothecin.

ESI-MS and HRMS analysis

Molecular mass of the trichothecin was determined by M/S Applied Biosystems API QSTAR pulsar (ESI-MS) mass spectrometer. Samples for the analysis were dissolved in HPLC grade methanol and analysed by infusion method / (injected into MS) at a flow rate of 5 µl/min and at an IS voltage of 3800 V in TOF mode. Spectrum from a range of m/z 200 to 500 Daltons was obtained. The spectrum showed molecular ion at *m/z* 333.19 and at *m/z* 355.14 attributing to M+H and M+Na ions of trichothecin respectively. HRMS calculated for $C_{19}H_{24}O_5$: 332.1624, found: 332.1629. This is similar to the reported value of Trichothecin. (3)

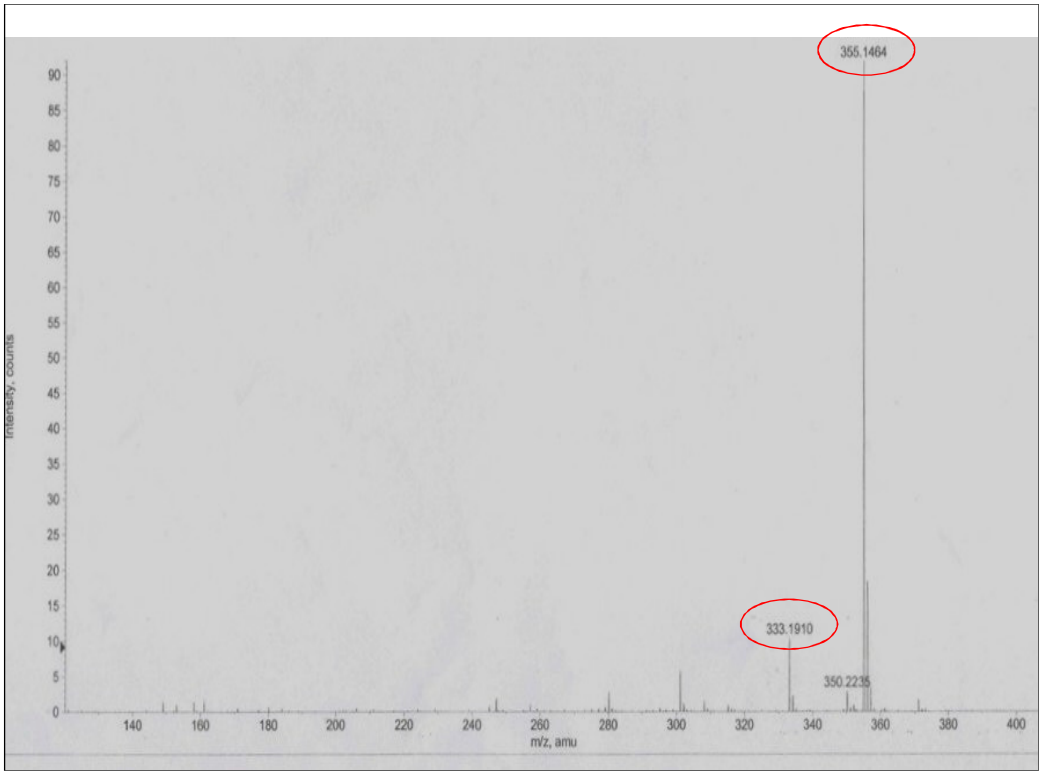


Fig 7. ESI-MS spectrum of purified trichothecin.

Quantification of Trichothecin through HPLC:

The data of area peak vs Trichothecin concentration obtained in case of standard sample was used to estimate the quantity of purified Trichothecin. The isolation of the Trichothecin from 1 litre culture filtrate yielded 4.2 mg of Trichothecin.

NMR analysis

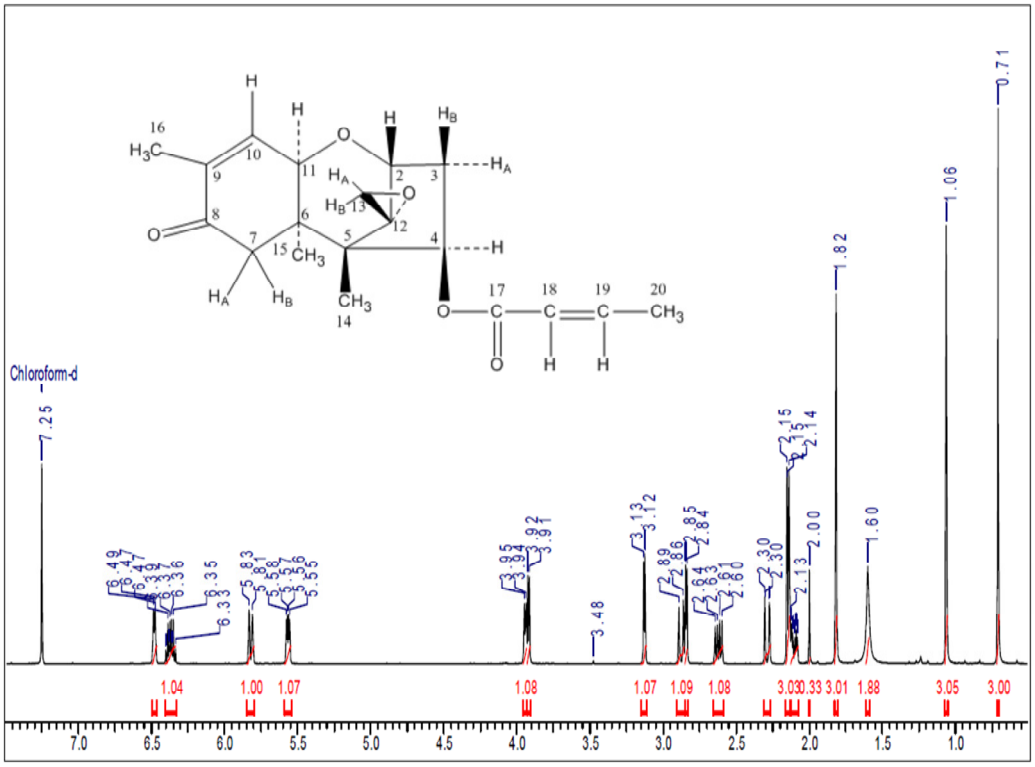


Fig 8. ^{1}H nuclear magnetic resonance (NMR) spectrum of trichothecin.

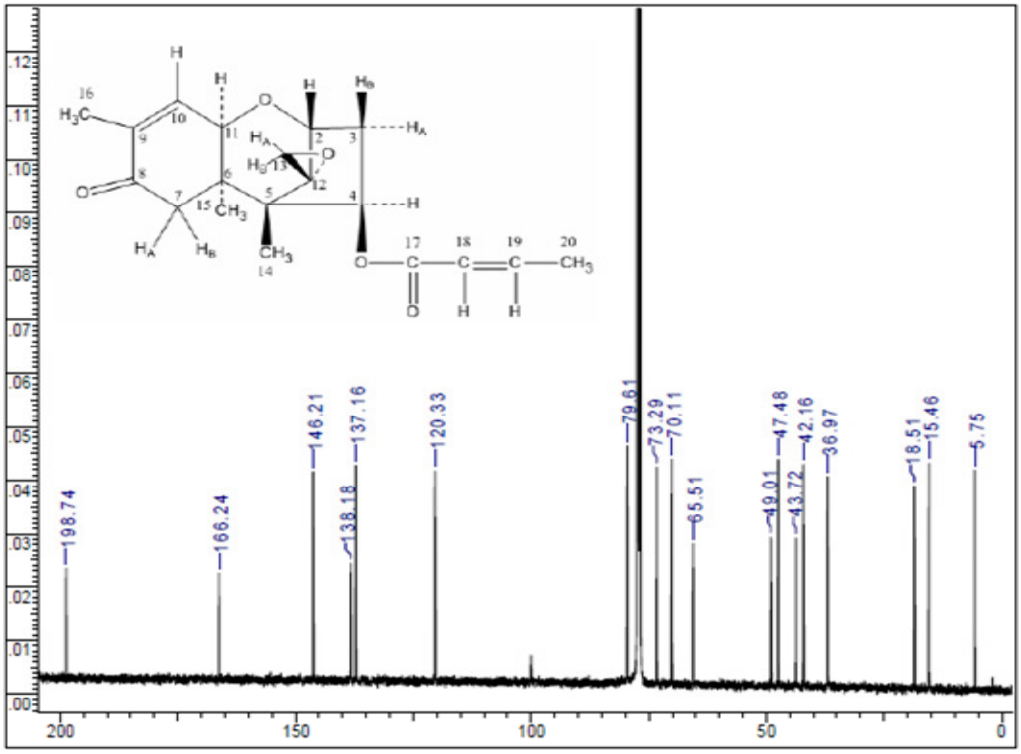


Fig 9. 125 MHz Proton deoupled ^{13}C nuclear magnetic resonance (NMR) spectrum of trichothecin.

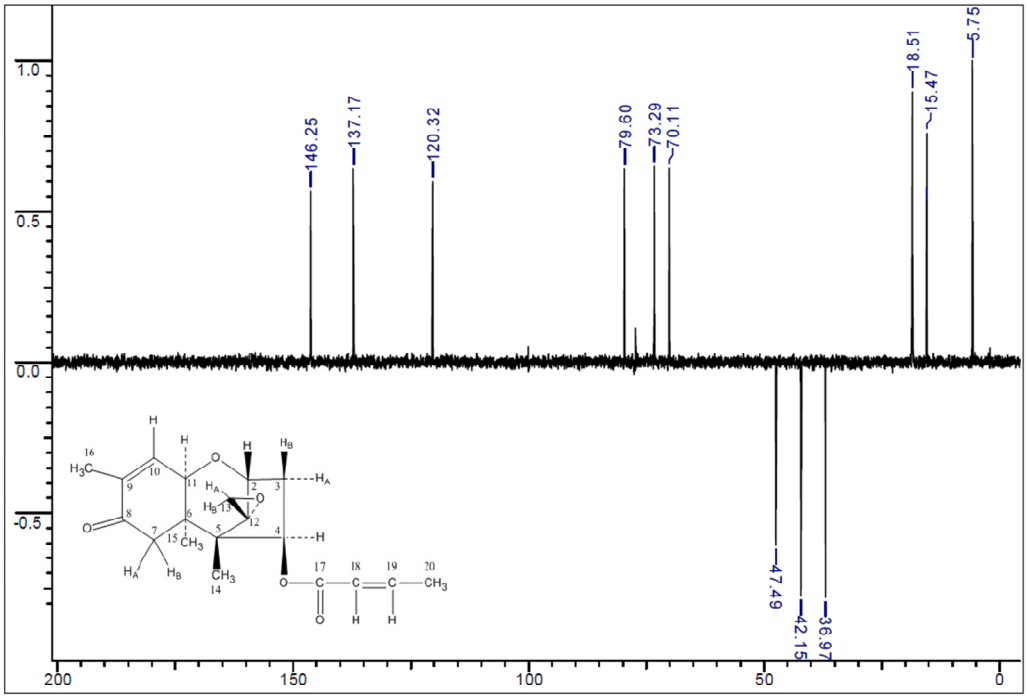


Fig 10. ^{13}C DEPT 135 spectrum of trichothecin, where the CH_2 carbons appear with a negative phase.

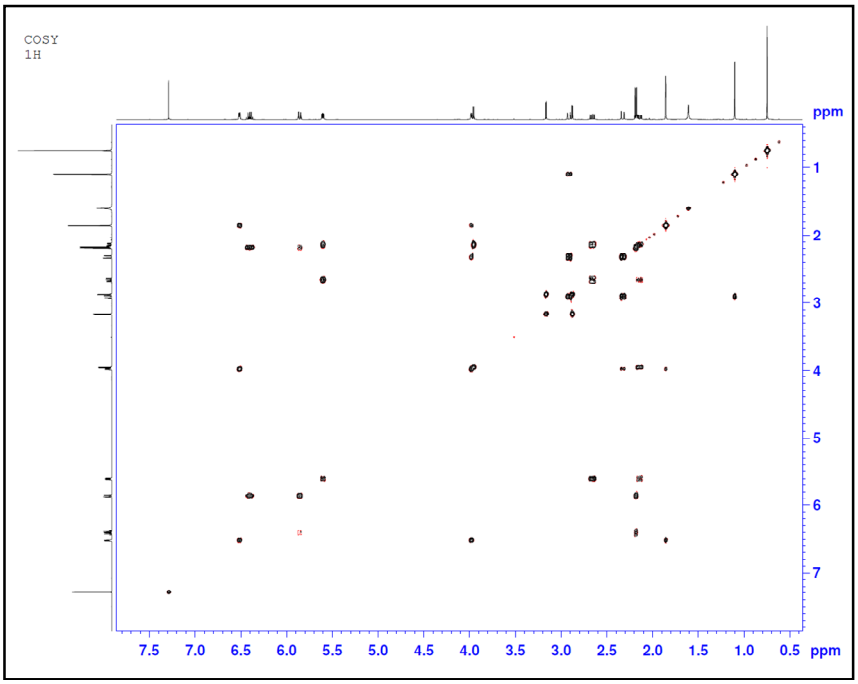


Fig 11. 500 MHz COSY spectrum of trichothecin in 99.8% deuterated $CDCl_3$.

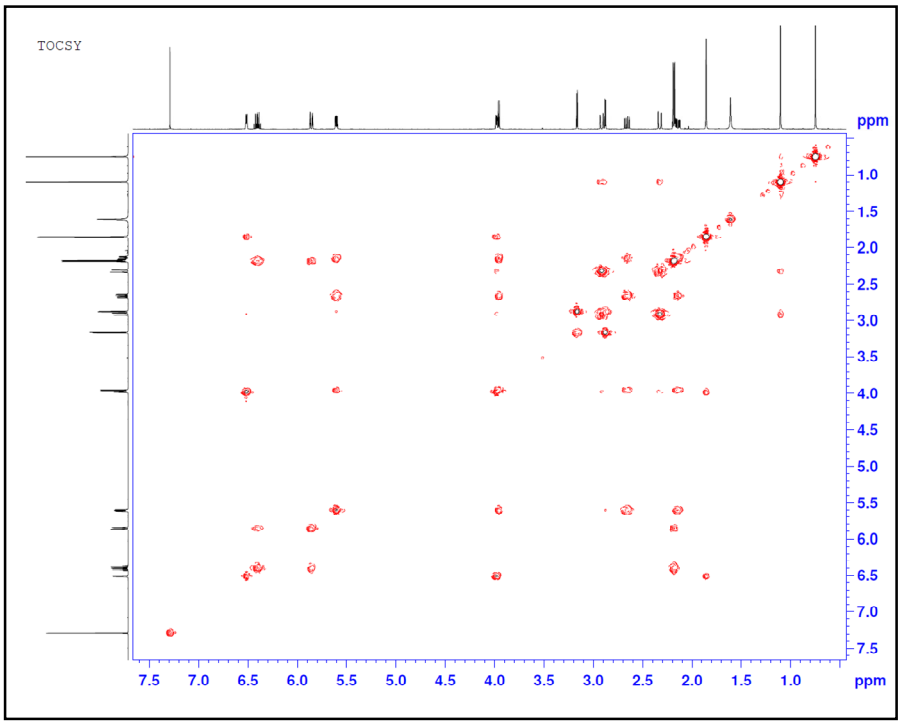
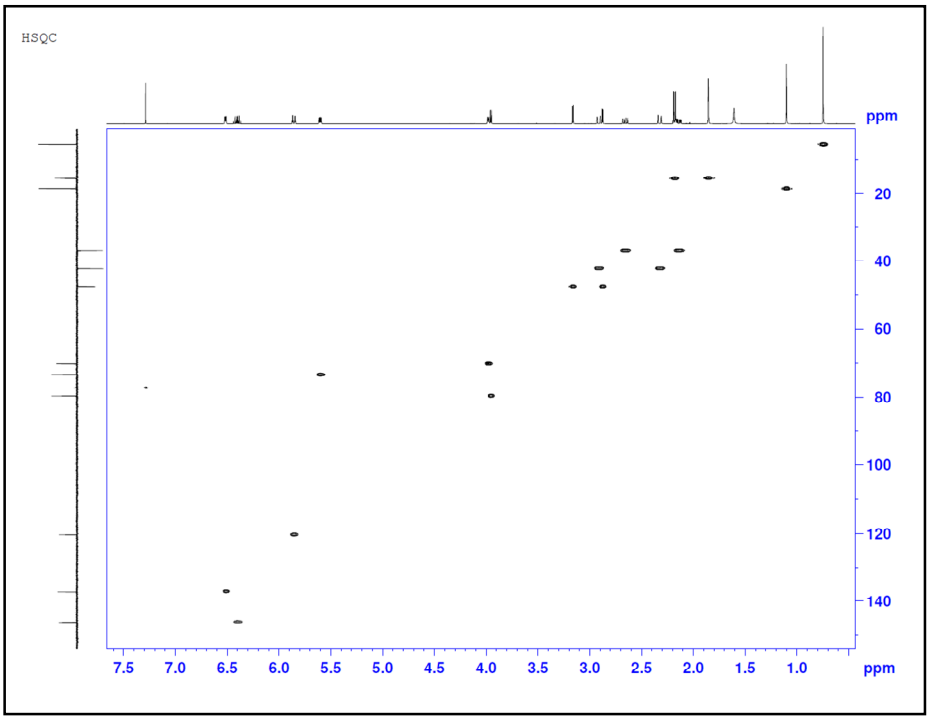
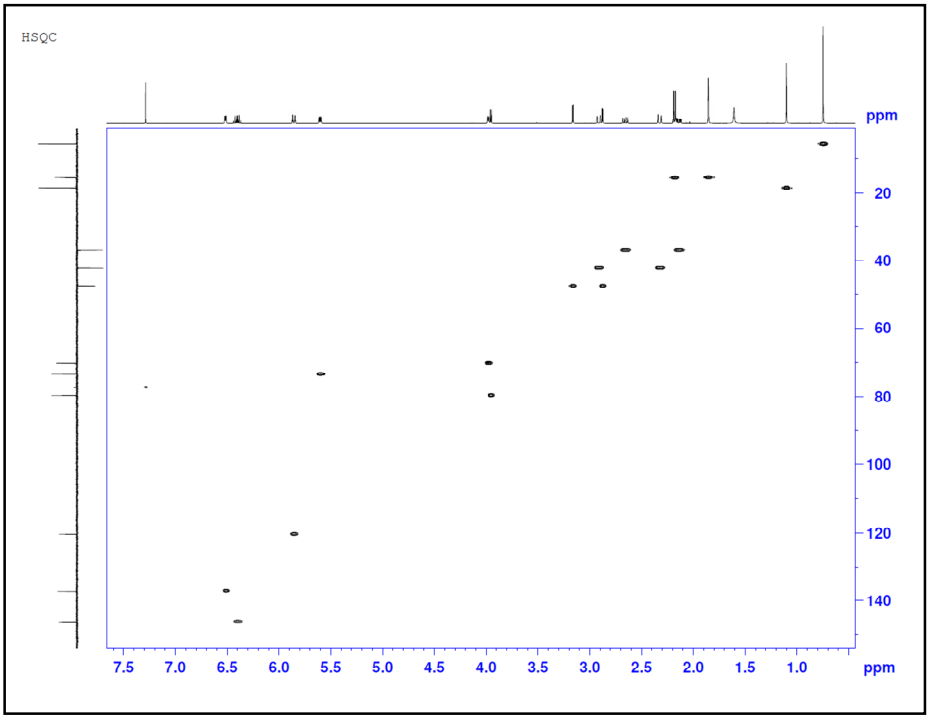


Fig 12. 500 MHz TOCSY correlations of trichothecin in 99.8% deuterated $CDCl_3$.

A



B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20
40
60
80
100
120
140
7.5
7.0
6.5
6.0
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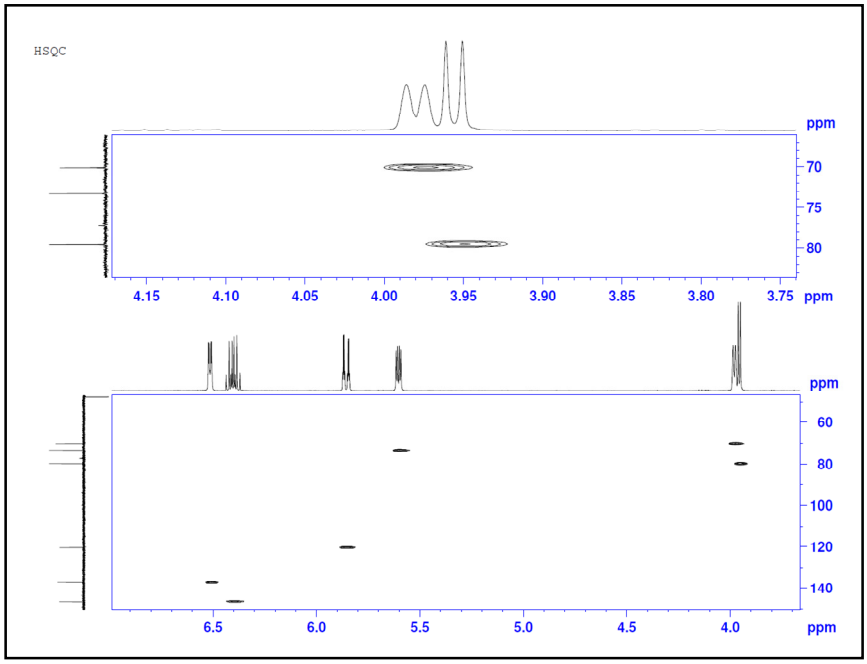
ppm

C


HSQC
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75
80
4.15
4.10
4.05
4.00
3.95
3.90
3.85
3.80
3.75
ppm
ppm
60
80
100
120
140
6.5
6.0
5.5
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4.5
4.0
ppm

D

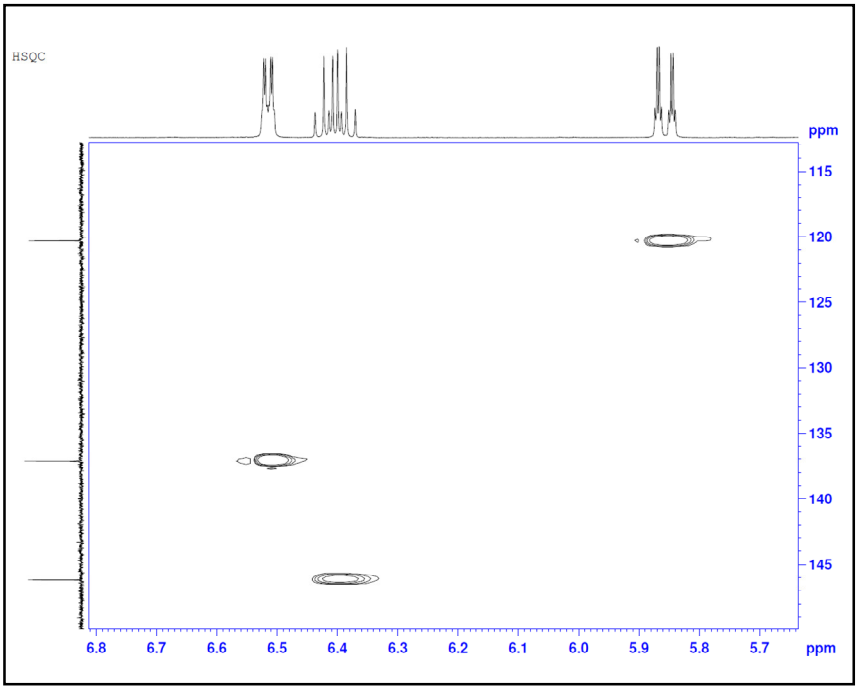
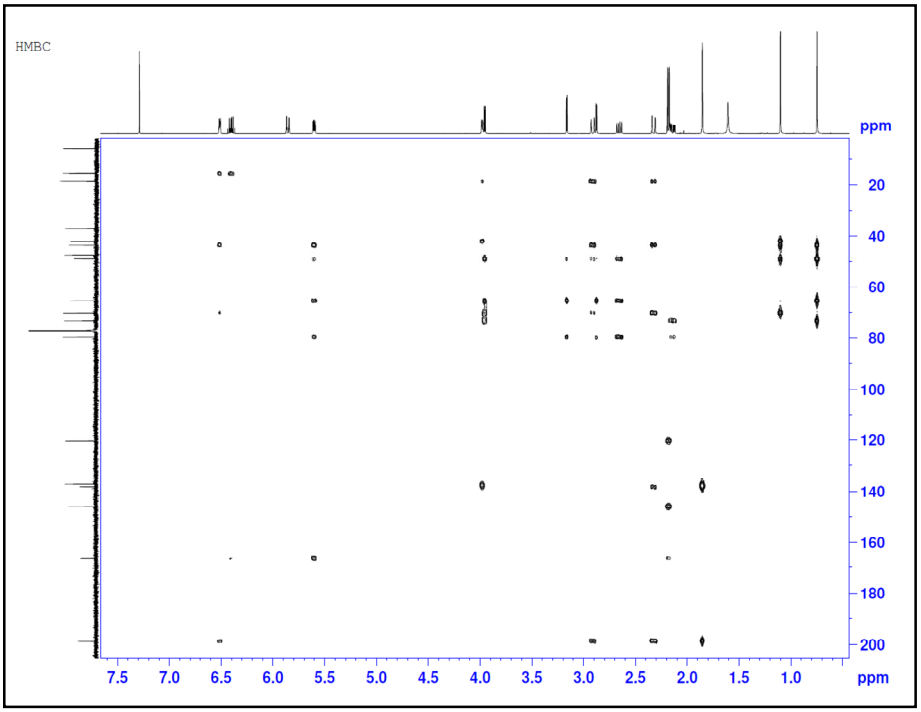
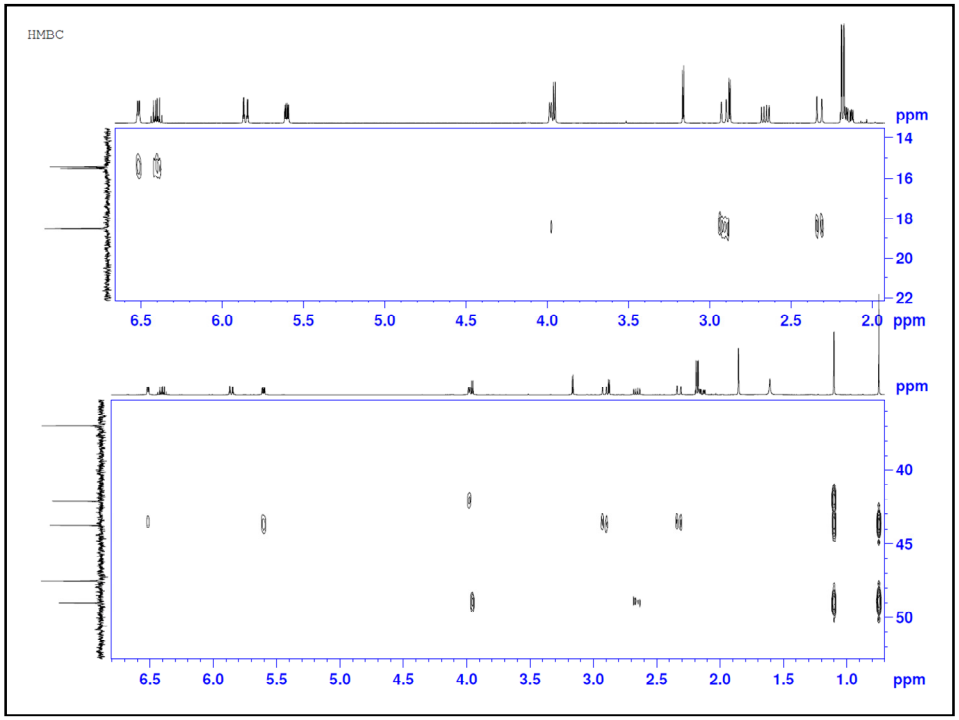


Fig 13. ^{1}H-^{13}C HSQC spectrum of trichothecin: (A) complete ^{1}H-^{13}C HSQC spectrum, (B) (C) and (D) expanded ^{1}H-^{13}C HSQC spectrum.

A



B



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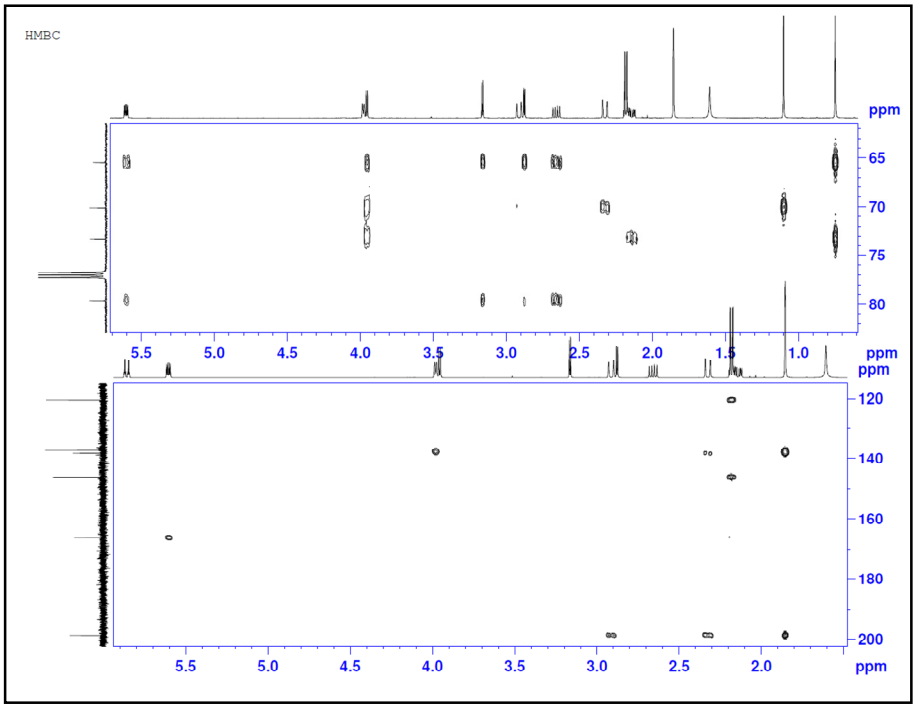

Fig 14. ^{1}H-^{13}C HMBC correlations of trichothecin: Spectrum (A) shows complete while (B) and (C) show expanded ^{1}H -^{13}C HMBC correlations.

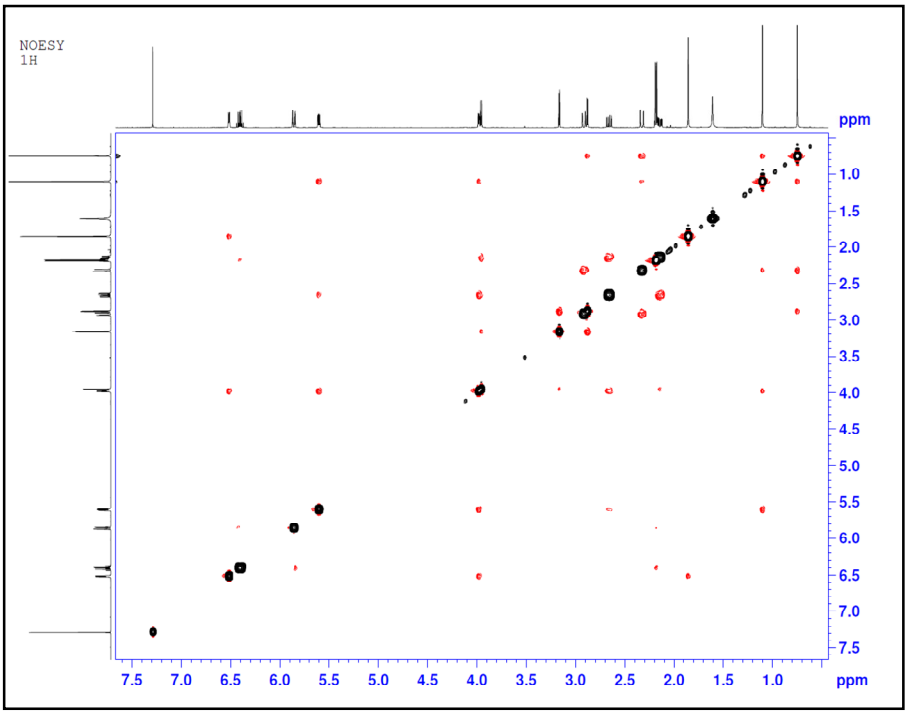

Fig 15. 500 MHz NOESY spectrum of trichothecin in 99.8% deuterated $CDCl_3$.

Structural characterization of trichothecin was carried out using a combination of various 1D/2D ^{1}H and ^{13}C NMR techniques. The ^{1}H spectrum presented in Fig 8. clearly indicated the presence of three olefinic protons in the region 5.6 to 6.6 δ. The splitting patterns and the coupling constants observed clearly showed the presence of methyl group with a vicinal coupling (J~7Hz) as well as long range coupling (J~2Hz) with these olefinic protons. Besides, the coupling constant of ~11 Hz between two of the olefinic protons (at ~5.84 δ and 6.38δ) also suggested the presence of a *cis* arrangement for them. Presence of at least four methyl groups can also be inferred from the aliphatic region of the spectrum, one of which is a doublet (J~7Hz) at ~2.16δ, probably belongs to the methyl group attached to an olefineic carbon. Appearance of proton signals in the region ~4δ is attributable to protons attached to oxygen. More insight to the structure details has been obtained from the ^{13}C NMR (^{1}H decoupled and DEPT135 spectra) which showed 4 methyls, 3 methylenes, 6 methines and 6 quaternary carbons (Fig.9,10). The proton decoupled ^{13}C NMR spectrum and the DEPT (Distortionless Enhancement by Polarization Transfer) spectra of this compound are shown in Fig.9 and 10, respectively. These two spectra are required to differentiate the types of carbons present in the molecule. DEPT spectrum will show only the carbons with attached protons. In DEPT 135 spectrum as given in the Fig.10

shows a negative phase for CH_2 carbons and positive phase for CH and CH3 carbons. The signals that are absent in the DEPT spectrum in comparison with the ^{1}H decoupled spectrum are the quaternary carbons. Various types of carbons present in trichothecin could be thus easily identified.

Three of the methine carbons appeared in the olefinic region (100-150 δ) and two the quaternary carbons in the carbonyl region at 199 δ and 166 δ which are attributable to a keto and an ester carbonyl, respectively.

COSY (COrrelation SpectroscopY) is the simplest 2D NMR experiment and used for indentifying the protons that are scalar coupled to each other. The coupled spins are manifested as off diagonal signals (cross peaks) involving the spin system. The spins those are not involved in any J coupling will have only the diagonal signal. The COSY spectrum of the sample helps to identify the various coupling partners of different protons. Fig. 11 shows COSY spectrum of trichothecin and the various coupled protons are identified. The coupling constant obtained for the 1D proton spectrum is given in Table-1. Total Correlation Spectroscopy (TOCSY) is a two dimension correlation experiment widely used for identification of various spin systems present in a molecule as it shows correlations between all the coupled spins. TOCSY spectrum of trichothecin is given in Fig-12.

Structural characterization by NMR was further achieved by the assignment of various carbons and protons present in the molecule by hetero nuclear correlation experiments, ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Correlation) and ^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation). HSQC experiment correlates chemical shifts of protons with the chemical shift of heteronuclei (^{13}C) directly attached to it. The 2D HMBC experiment allows to obtain a 2D heteronuclear Chemical Shift correlation between long-range coupled proton and heteronuclei (^{13}C). This experiment gives information about protons that are near to (usually separated by two or three bonds) different carbons and provide very useful information about molecular structure, since the long range proton-carbon correlations can include quaternary carbons, in addition to protonated carbons. The ^{1}H-^{13}C HSQC spectrum (Fig-13) clearly brought out that all the methylene carbons present in the molecule have attached inequivalent protons, which is likely to be due to diastereotropic nature of them and hence show strong germinal couplings. The ^{13}C-^{1}H HSQC spectrum of –trichothecin is given in Figs -13 A,B,C and D while the HMBC spectra are depicted in Fig14 A,B and C

Table-1 1H,13C chemical shifts and 1H coupling constant of trichothecin

$\delta_{C,\ mult.}$	δ_H [a] (δ)	Numbering of Atom, coupling constant
--	--	1
79.8 (CH)	3.95d	C2, H2 ($J_{2,3a}$=5.2)
36.98 (CH2)	2.13, ddd, 2.65 (H3Bdd,	C3, H3a, $J_{3a,3b}$=15.73,$J_{3a,2}$=5.09, $J_{3a,4}$=3.68) H3b $J_{3b,3a}$=15.7,$J_{3b,4}$=7.86
73.3 (CH)	5.6 dd	C4, H4,($J_{4,3a}$=7.9,$J_{4,3b}$=3.7)
49 (q)	--	C5
43.5(q)	--	C6
42.16 (CH2)	2.91dd 2.33 dq	C7, H7a, $J_{7a,7b}$= 15.30, $J_{7a,11}$=1.54) H7b $J_{7b,7a}$=15.30,$J_{7b,15}$=1.13)
198.7 (q)	--	C8
138.1 (q)	--	C9
137.2 (CH)	6.53 dq	C10, H10($J_{10,11}$=5.85,$J_{10,16}$=1.56)
70.1 (CH)	3.98 d	C11, H11;($J_{11,10}$=5.8)
65.5 (q)	--	C12
47.49 (CH2)	2.87 dd, 3.14dd	C13, H13a, H13b $J_{13a,13b}$=4.01, $J_{13b,13a}$=4.01)
5.74 (CH_3)	0.74 s	C14, H14
18.5 (CH3)	1.09 s	C15, Me15
15.48 (CH3)	1.86 bq	C16, Me16 ($J_{16,10}$=1.5,$J_{16,11}$=0.7)
166.2(q)	--	C17
120 (CH)	5.85 dq	C18, H18($J_{18,19}$=11.5,$J_{18,20}$=1.8)
146 (CH)	6.40 dd	C19, H19 ($J_{19,18}$=11.4, $J_{19,20}$=7.4)
15.54 (CH3)	2.17 dq	C20, Me20 ($J_{20,19}$=7.4, $J_{20,18}$=1.8)

[a] s – singlet, d – doublet, dd – doublet of doublet, dq – doublet of quadruplet.

[b] w – weaker interactions

Table 2 Homo nuclear correlations of trichothecin

Sr No	δ_H [a] (δ) , assignment	Observed COSY Cross peaks to protons at δ_H	Observed TOCSY Cross peaks to protons at δ_H	Observed NOESY Cross peaks to protons at δ_H
2	3.95d, H2	2.13	2.13, 5.6, 2.65, 2.13	2.13, 3.16
3	2.13, H3a	2.65, 3.95	5.6	3.98, 5.6
	2.65, H3b	2.13, 5.6,	3.95	3.95, 5.6, 2.13,
4	5.6 H4	2.13, 2.65	2.13,2.65, 2.87(w), 3.95	0.74, 1.1, 2.13,2.65, 3.98
7	2.91 H7a	1.09, 2.33	1.09, 2.33	0.74
	2.33 H7b	1.09, 2.91	1.1, 2.87, 2.91, 3.98	0.74, 1.1, 2.91
10	6.53 H10	1.85, 3.98,	1.85,3.98	1.85, 3.98
11	3.98 H11	1.85, 2.33, 6.52	1.85, 2.33(w)	2.65, 5.6, 6.5
13	2.87 H13a,	3.16,	3.16, 2.91,5.6(w),	0.74,3.16, 3.95,
	3.16, H13b	2.87	2.87 2.91,5.6(w),	0.74, 2.87,3.95,
14	0.74 , Me14	--	--	1.1, 2.33, 2.87
15	1.09 ,Me1 5	2.91	2.33, 2.91	0.74,2.33, 5.6, 3.98
16	1.86 , H16	3.98, 6.5	3.98, 6.5	6.5
18	5.85,H18	2.17	2.17, 6.40	2.17, 6.4
19	2.17 , H19	5.85, 6.40	5.85, 6.40	5.85
20	6.40 , H20	5.85, 2.17	5.85, 2.17	5.85

Table 3 Hetero nuclear correlations observed for trichothecin

Sr No	$\delta_{C,\ mult.}$	Observed HSQC Correlations	Observed HMBC Correlations to protons
2	79.8 (C2)	3.95	5.6, 3.16, 2.87, 2.65
3	36.98 (C3)	2.13, 2.65	--
4	73.3 (C4)	5.6	3.95, 2.13, 0.74,
5	49 (q) C5	--	5.6, 3.95, 3.16,2.65, 1.1
6	43.5,C6	--	0.74, 1.1, 2.33, 2.9, 5.6, 6.5
7	42.16 ,C7	2.91, 2.33	--
8	198.7 (C8)	--	6.5, 2.91, 2.33, 1.85
9	138.1 (C9)	--	2.33,1.85
10	137.2 (C10)	6.53,	3.98,1.85
11	70.1 (C11)	3.98	6.5, 3.95, 2.91,2.33 1.1,
12	65.5 (C12)	--	5.6, 3.95, 3.16,2.87, 2.65, 0.74
13	47.49 (C13)	2.87, 3.16	3.95
14	5.74 (C14)	0.74, s	--
15	18.5 (C15)	1.09, s	2.33, 2.91, 3.98
16	15.48 (C16)	1.856,	6.5,
17	166.2(C17)	--	5.6, 2.17
18	120 (C18)	5.85,	2.17
19	146 (C19)	6.40,	2.17
20	15.54 (C20)	2.17,	6.40

Another very important NMR tool for structural characterization is the experiments based on Nuclear Overhauser Enhancement (nOe) spectroscopy. These experiments can be performed in 1D as well as 2D mode. Here, we have employed a 2D nOe experiment, NOESY for characterization. In the NOESY (Nuclear Overhauser Enhancement Spectroscopy) spectrum, the protons those are close in space and hence have NOE will show cross peak between them. Since NOE is inversely proportional to the sixth power of the distance between the spins, the cross peak intensity give information about inter nuclear distances. The spins that are close will show stronger NOE and stronger cross peaks whereas with spins that are more than ~5Å apart will

not show any cross peak. The cross peak intensity diminishes rapidly as inter nuclear distances increases. 2D NOESY spectrum of the sample is shown in Fig.15 and the observed nOe conectivities are summarized in Table 2 and schematically shown in Fig.-16.

From the structural investigations we observed that the product isolated is Trichothecin, NMR spectra of which has already been reported. The stereochemistry of the isolated product aslo found to be similar to the reported data.

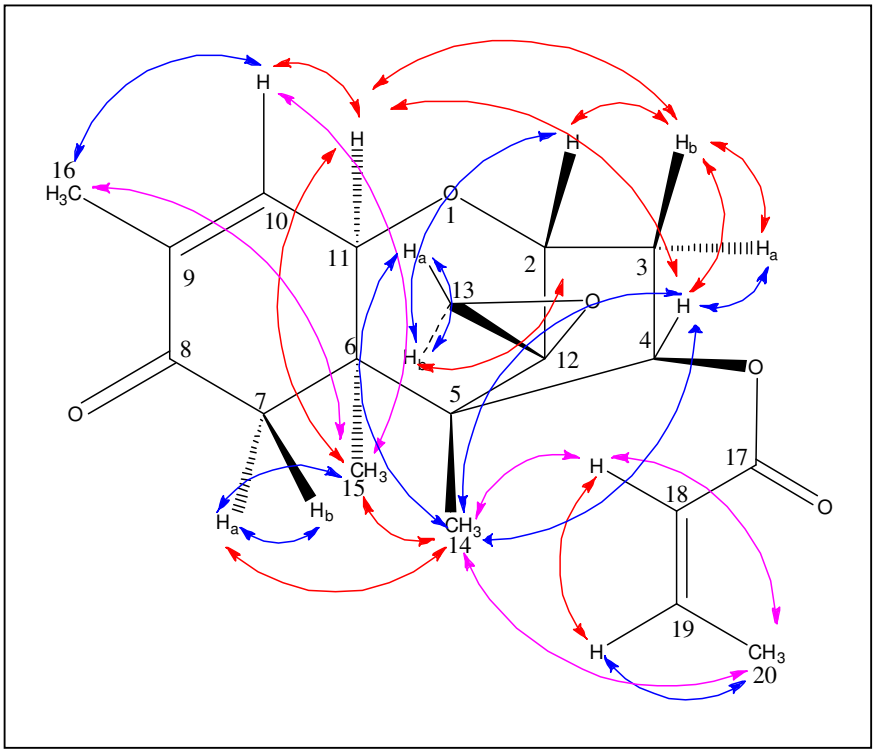

Fig.16. NOE correlations of Trichothecin. Red colour indicates strong interactions, while blue and pink colour indicates medium and weak interactions respectively.

Discussion

Studies on the isolation of the trichothecin produced by *Trichothecium* sp. indicate that the fungus produces trichothecenes (trichothecin) in the culture broth *in vitro*. Trichothecin was isolated and its antifungal activity against *Aspergillus niger* was estimated. HPLC and other analyses of the extracts from culture broth confirm this conclusion. Trichothecin obtained per liter of culture was estimated to be approximately 4.2 mg per liter. Putative trichothecin on HPLC C18 symmetry column showed a single symmetrical peak at retention time of 39 min that confirmed its homogeneity. Absorption maximum of the purified trichothecin was found to be at 238 nm in ethanol. FTIR spectrum of purified trichothecin in KBr pellet shows characteristic stretchings as follows; 2978 cm^{-1} (C-H stretching), 2923 cm^{-1} (C-H stretching), 2851 cm^{-1} (C-H stretching), 1711 cm^{-1} (C=O stretching), 1671 cm^{-1} (C=C stretching), 1645 cm^{-1} (-C=C- stretching) and 1072 cm^{-1} (C-O stretching). In ESI-MS, molecular ions at *m/z* 333 attributing to the (M+H) and at *m/z* 355 attributable to (M+Na) confirmed its molecular weight to be 332. HRMS calculated for $C_{19}H_{24}O_5$: 332.1624, found: 332.1629 which confirmed trichothecin. ^{1}H, C^{13}, COSY, NOESY, TOCSY, HSQC and HMBC NMR spectra were found to match with those reported for trichothecin.(3, 6, 9)

This is the first report of isolation of appreciable amount of trichothecin from endophytic fungus *Trichothecium* sp. isolated from medicinal herb *Phyllanthus* sp. However, trichothecin are commonly found in *Trichothecium roseum* and plant infected by this fungus from various parts of the world as well as India. These observations support the present findings. This suggest that the ability to synthesize trichothecin resides not only in *Trichothecium roseum* and other infected plant from various part of the globe but also from *Trichothecium* sp. isolated by us from *Phyllanthus* plant. This compound previously has been characterized by various standard techniques such as ^{1}H nmr and ^{13}C nmr. Since this compound is reported to have some very important anticancer activities, its complete characterization is must. During the present investigation we completely characterized this compound by variety of 1D and 2D NMR spectroscopy.

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Chapter 4

Isolation, purification and characterization of Trichothecinol-A produced by endophytic fungus *Trichothecium* sp.

Summary

Endophytic fungi were isolated from the leaves of *Phyllanthus* sp. plant obtained from different areas of Pune and then screened for trichothecinol-A production. Out of 30 fungal cultures screened, only one AAP-PS-1 was found to produce trichothecinol-A extracellularly in appreciable amounts. Homogeneity of the purified trichothecinol-A was determined by TLC on three different solvent systems. RP-HPLC analyses performed on a Waters model using a C_{18} symmetry pack column with a flow rate of 0.5 ml/min and the eluting compounds were detected by a dual mode wavelength detector set at 220nm and 240nm.The 1D and 2D NMR (^{1}H, COSY, NOESY, TOCSY, ^{13}C, DEPT, ^{13}C-^{1}H HMBC, ^{13}C-^{1}H HSQC), ESI-MS, HRMS, IR and UV-Vis show conclusively that the isolated compound was trichothecinol –A (31). One liter of *Trichothecium* sp. yielded 4.37mg of Trichothecinol-A.

Introduction

Trichothecenes are a vast group of structurally and chemically related mycotoxin producing fungi which have a strong impact on the health and well-being of humans, plants and farm animals. These include imperfect fungi of various species of *Fusarium*, *Trichothecium*, *Trichoderma*, *Myrothecium*, *Cephalosporium*, *Stachybotrys*, etc. Mycotoxins are complex sesquiterpene secondary metabolites produced by fungi belonging to trichothecenes, which help them to better adapt to their plant host's internal environment and provide them with certain ecological benefits. The mycotoxins are highly stable even during the milling, processing and cooking of food stuffs and thus it is very difficult to get rid of them. (3, 6)

It is these trichothecenes which are responsible for the toxicity and spoilage of grains, fruits, vegetables, tubers and other vegetative products worldwide. Apart from mycotoxicoses, i.e. diseases caused due to consumption of food over-accumulated with trichothecene mycotoxins, these fungi are also responsible for various health problems due to damp indoor environments, skin diseases, the poisoning in a variety of mushroom species, shortening of roots, rotting of stalks and overall dwarfism in plants, etc. Several incidents of emesis in humans and animals due to consumption of cereals infected with trichothecenes have been recorded in early 19th century.(5, 15) Lacks of people succumbed to death in Soviet Union due to reported alimentary toxic

aleukia, a disease caused by consumption of loads of T-2 toxin present in food grains infested with *Fusarium* species.(10) Related outbreaks of such magnitude have also been reported in China and Japan.(12, 18)

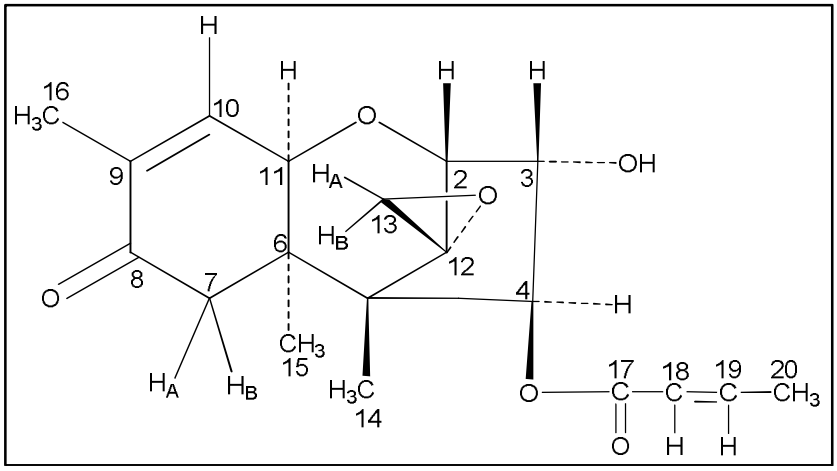


Fig 1. Trichothecinol-A (Mw: 348, $C_{19}H_{24}O_6$)

Trichothecenes are minute, amphiphatic molucules which are extremely powerful inhibitors of protein synthesis as these have their specific site of action located on a very crucial site on the ribosomal RNA (rRNA), thus interfering with the normal polypeptide chain initiation, elongation and termination.(13, 19) As protein synthesis is much more in cells which are rapidly dividing, cells of the integumentary system, gastrointestinal system, liver, etc. are much more susceptible to passive absorption of these molecules and resultant oxidative stress due to generation of hazardous number of free radicals. Unlike other mycotoxins, trichothecenes do not depend upon metabolic activation to be able to exert their biological activity and can thus infest directly through the skin, making them one of the most potential of toxins. So much so that, there are controversial reports stating that trichothecenes like *Fusarium* species were used by the Soviet Union as bioweapons during the Vietnam War where the "Yellow rain attacks" proved to be inhumanely lethal. (7)

Also, to this day, there is no licensed vaccine or antidote available to protect against the extreme toxicity caused by trichothecene mycotoxins. As even toxins are medicinal if administered in right doses, altering the biological activity of trichothecenes has helped in inducing anti-bacterial, anti-viral, insecticidal,

phytotoxic, cytoxic, anti-biotic and anti-tumor properties in the resultant molecules. (1) With a view to obtain anti-cancer compounds from thichothecenes, the fungus *Trichothecium roseum* belonging to the same family was studied, where the bioassay-guided separation of extracts of its culture broth revealed three known (trichothecin, trichodermol and trichothecolone) and three new (trichothecinols A– C) compounds, all showing remarkable potential at inhibiting EBV-EA activation induced by TPA (12-O-tetradecanoylphorbol-13-acetate) in Raji cells which are the human lymphoblastoid cells carrying the EBV genome. (9, 17) Out of these, the most active compound, trichothecinol-A also suppressed TPA-induced tumor promotion on mouse skin initiated with DMBA (7,12-dimethylbenz[a]anthracene) in a two-stage carcinogenesis experiment. (2)

The three most important structural features which rent trichothecenes their mycotoxic properties and other biological activities are the 12,13-epoxy ring a.k.a the EPT core, the presence of either acetyl or hydroxyl groups at appropriate positions on the trichothecene nucleus, and the structure and location of the side-chains. The dynamic properties of trichothecinol-A can thus be attributed to its remarkable structural similarity to trichothecene with insertion of a hydroxyl group at C-3. These findings make trichothecinol-A an excellent candidate to be evaluated as an anti-cancer agent, anti-microbial agent, and in production of various other medicinal drugs and derivatives. (4, 8, 14, 16)

The present work focuses on the isolation, purification and complete characterization of trichothecinol-A from the endophytic fungus *Trichothecium* sp. isolated from the medicinal herb *Phyllanthus* sp. The anti-fungal, anti-cancer (anti-proliferative), anti-metastatic and apoptotic activities of trichothecinol-A revealed its class-apart potential to be developed as a drug for each mentioned field.

Materials

Fermentation media: per liter composition

Glucose 1gm, Sucrose 3gm, Fructose 6gm, Soya peptone 1gm, Zinc sulphate 2.5gm Manganese chloride 5mg, Magnesium sulphate 3.6mg, Cupric nitrate 1mg, Calcium

nitrate 6.8mg, Ferric chloride 2mg, Biotin 1mg, Thiamine 1mg, Pyridoxal 1mg, L-phenylalanine 25mg, Calcium pentothenate 1mg, , Phosphate buffer 1ml (pH 6.8)

MGYP broth: per liter composition

Malt extract 3gm, Glucose 10gm, Yeast extract 3gm, Peptone 5gm.

Solvents used:

Acetonitrile, Methanol, Ethyl acetate, Hexane, Chloroform. All the solvents used were from Qualigens ExceleR grade.

Methods

The fungus *Trichothecium* sp. maintained on PDA slants has optimum growth at pH 7 and temp. 27^{o}c. subculturing was done to maintain the stock cultures at monthly intervals and preserved at 15^{o}c. Starting material for fermentation experiment were taken from an actively growing stock culture, which were subcultured on fresh slants and incubated for 7 days at pH 7 and temp. 27^{o} C.

Isolation and purification of trichothecinol-A:

Fermentation:

Production of trichothecinol-A was studied by a two stage fermentation procedure. In the first stage, the fungal strain was grown in 500ml Erlenmeyer flasks containing 100ml MGYP medium. The flasks were inoculated with agar plugs containing mycelium from 7 days old culture. The inoculated flasks were incubated at 25-27^{0} C on a rotary shaker (240 rpm) for 4-5 days, which was used as seed culture (Ist stage). Later, 10ml seed culture was transferred to 500ml Erlenmeyer flask containing 100ml of fermentation (production) medium called as modified S7 medium and were incubated at 25-27^{0}C as a stationary for 30 days (IInd stage). After which it was harvested and used for further study.

Lyophilization:

Culture filtrate and mycelium were separated with the help of muslin cloth and then lyophilized and stored at 4^{0}C for further processing.

Extraction:

Culture filtrate was extracted with equal volumes of ethyl acetate each time. The organic layer was separated from the aqueous layer using separating funnel. The extraction was repeated thrice and solvent was dried using anhydrous sodium sulphate and concentrated under vacuum using Rotavapor at 40^0C in order to get crude extract.

Purification:

Preparative TLC:

A small amount of (0.5 ml) crude extract was dissolved in ethyl acetate and subjected to Thin Layer Chromatography (Prep TLC) on Silica gel G (0.75mm thickness) using hexane:ethyl acetate 2:1 as solvent system. The developed plates were exposed to iodine vapours. The bands are then cut, eluted with ethyl acetate and tested for their biological activity. The bands showing activity were further processed for their purification.

HPLC analysis:

Small amount of crude (EToAc extract) was dissolved in 1ml of HPLC grade acetonitrile and 10 µl of this solution was further processed to purify active compound by reverse phase HPLC. HPLC analysis performed on a Waters model using a C_{18} symmetry pack column with a flow rate of 0.5 ml/min, and the eluting compound was detected by a dual mode wavelength detector set at 220nm and 240nm. HPLC grade solvents were prefiltered using a Millipore system and analysis was performed. The column was first equilibrated with three column volumes of 100% acetonitrile and eluted with a gradient of 5-95% acetonitrile.

Quantification Of trichothecin By HPLC:

Stock solution of different concentrations (1mg/ml, 3mg/ml, 5mg/ml and 10mg/ml) of a purified trichothecinol-A was prepared in HPLC grade acetonitrile. 10 µl of each stock solution was injected in HPLC to generate a standard graph of peak area v/s concentration. Then, the known volume of (10 µl) crude dissolved in HPLC grade acetonitrile containing unknown concentration of trichothecinol-A was analysed by HPLC and the peak area of trichothecinol-A containing peak was compared to standard graph to calculate its concentration.

Characterization

UV-Visible analysis:

UV-visible spectroscopy measurements were carried out on Jasco dual beam spectrophotometer (model V- 570) operated at resolution of 1nm.

ESI-MS and HRMS analysis:

Molecular mass of the purified compound was determined by M/S Applied Biosystems API QSTAR pulsar (ESI-MS) mass spectrometer. Samples for the analysis were dissolved in HPLC grade methanol. Samples were then analyzed by infusion method / (injected into MS) at a flow rate of 5 µl/min and at a IS voltage of 3800 V in TOF mode. Spectrum from a range of m/z 200 to 500 Daltons was obtained. HRMS analysis was carried out using mass spectrometry instrument (model – Autoconcept) by direct injection probe with resolving power of 6000.

FTIR:

FTIR spectroscopy measurements of purified compound taken in KBr pellet were carried out using a Perkin-Elmer spectrum one instrument. Spectrometer was used in the diffuse reflectance mode of resolution 2 cm^{-1}. To obtain good signal to noise ratio, 128 scans of the film were taken in the range of 450-4000 cm^{-1}.

NMR:

All the NMR measurements were carried out on Bruker AV 500 spectrometer operating at 500 MHz and 125 MHz, respectively for ^{1}H and ^{13}C. Approximately 10 mg of the compound was dissolved in $CDCl_3$ in a standard 5mm NMR tube and the ^{1}H, COSY, NOESY, TOCSY, ^{13}C DEPT, ^{13}C-^{1}H HSQC and ^{13}C-^{1}H HMBC experiments were performed on a standard 5mm BBFO probe at ambient temperature (~ 25^{o}C). Chemical shifts in the ^{1}H spectra were referenced to residual $CHCl_3$ peak (7.26 ppm), while ^{13}C spectra were referenced to $CDCl_3$ (77.0 ppm). 3600 and 2400 transients were collected for ^{13}C CPD and DEPT135 spectra of Trichothecinol-A. Gradient spectroscopic techniques were employed for all the 2D experiments. 256 experiments (t1 increments) of 4, 16 and 24 scans were performed for COSY, NOESY and TOCSY measurements of Trichothecinol-A. COSY and the HMBC spectra were collected in a magnitude mode while a phase sensitive (States-TPPI)

mode was used for HSQC and NOESY measurements. A mixing time of 1 sec and 300 m /sec was employed for NOESY. The number of scans used for each t1 increment for other 2D experiment for Trichothecinol-A were as follows: 16 (^{13}C HSQC), 32 (^{13}C HMBC). The ^{13}C HMBC data were optimized for a long range coupling constant of 6 Hz. A pulse sequence employing a double low pass filter was found to give better results for ^{13}C HMBC due to spread in 1JC-H values (160 -135 Hz). The HMBC spectrum was acquired without proton decoupling during detection. The 90º pulse lengths for ^{1}H and ^{13}C were 13.5 and 10, respectively. Appropriate window functions *viz*. sine squared bell with no phase shift for all magnitude mode and phase shifted (ssb = 2) sine squared bell for phase sensitive mode were used for data processing. In general, a 1Kx1K data matrix size was used for the 2D experiments.

Results:

Production and Purification of Trichothecinol-A by RP-HPLC

The culture filtrate of endophytic fungal strain *Trichothecium* sp extracted with ethyl acetate yielded a brown residue after removal of the solvent. The crude extract of the fungus on preparative TLC on silica gel G using hexane: ethyl acetate (2:1) as solvent system produced 8 bands when the plates were exposed to iodine vapours. All the bands were cut and eluted with ethyl acetate and tested for their biological (antifungal) activity against fungus *Aspergillus niger*. Out of 8 bands produced, band no. 2 and 5 showed strong antifungal activity and were further processed for their purification and complete characterization. The 2nd fraction obtained from preparative TLC was further processed for its purification by reverse phase HPLC.

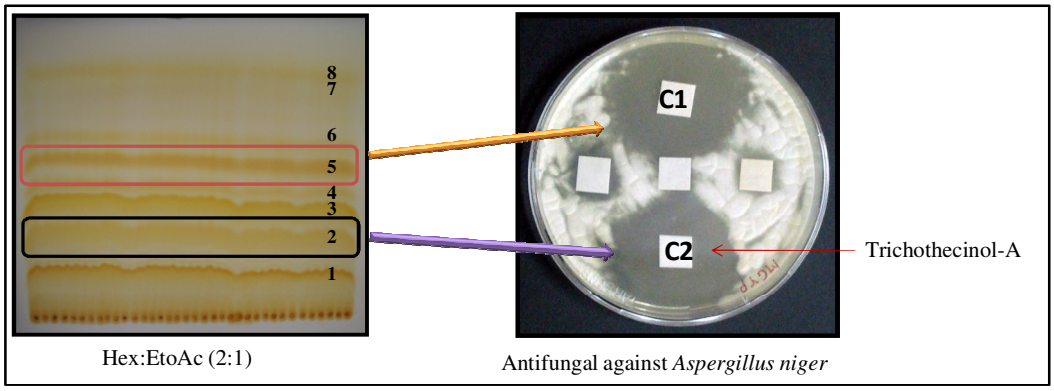


Fig 2. Antifungal activity of crude extract (Band 2) against *Aspergillus niger*.

RP-HPLC of fraction 5 was carried out using Waters separation module. Compound was loaded on analytical C_{18} (4.6 X 250 mm) symmetry pack column with a flow rate of 0.5 ml/min. 40 minutes gradient of milli Q water – acetonitrile (95-5%) were used as a mobile phase. The progress of HPLC was monitored at 220 nm. Each peak was collected separately and tested for antifungal activity. The peak eluted at RT 36 min only showed antifungal activity and processed for its complete characterization. The homogeneity of the purified compound was confirmed by HPLC analysis which showed a single symmetrical peak at RT 34 minute on C18 symmetry coloumn.

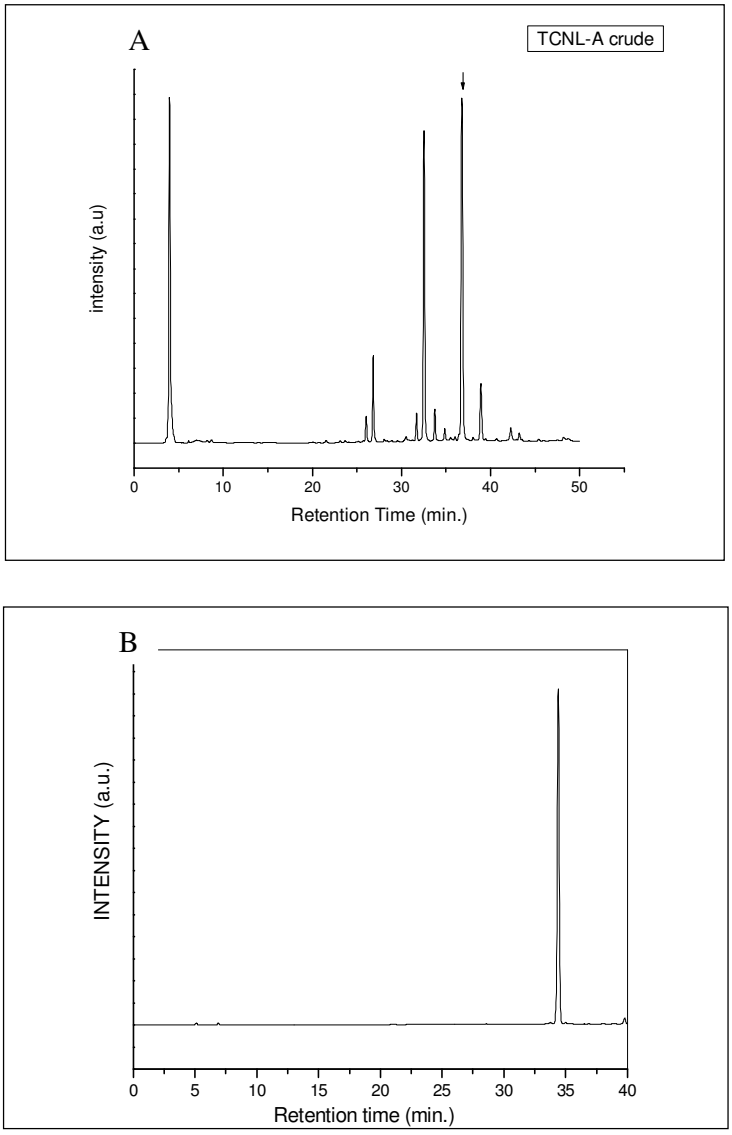


Fig 3. A) RP-HPLC profile of ethyl acetate extract. Peak containing Trichothecinol -A indicated by arrow eluted at retention time of 36 minute. B) HPLC profile of purified Trichothecinol -A shows the peak at retention time of 34 minutes

UV-Vis. Spectroscopy:

UV-visible spectroscopy measurements were carried out on Perkin Elmer spectrophotometer (model lambda - 750) operated at resolution of 1nm. Maximum UV absorption was observed at 251nm in ethyl acetate.

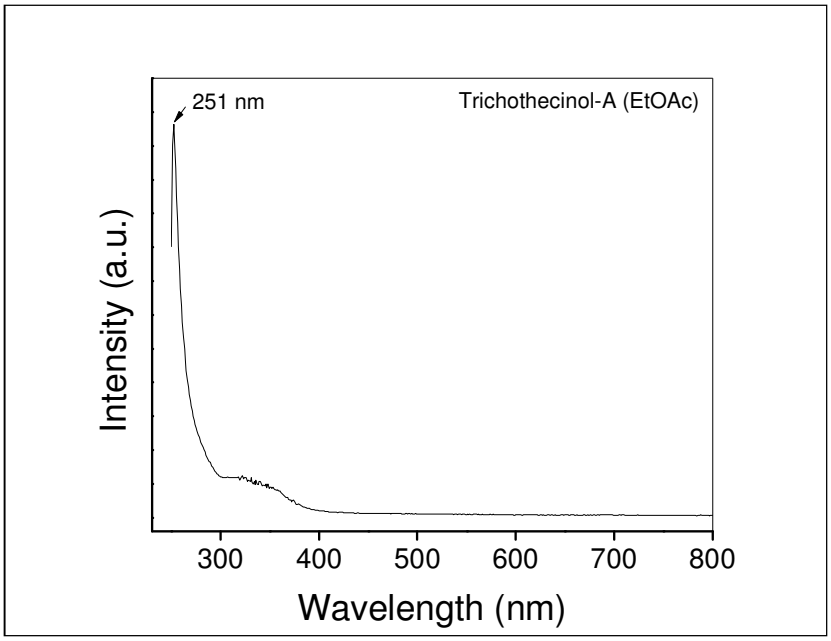


Fig 4. UV-Vis. spectra of Trichothecinol-A.

FTIR spectrum:

FTIR spectroscopy measurements of purified compound taken in KBr pellet were carried out using a Perkin-Elmer spectrum one instrument. Spectrometer was used in the diffuse reflectance mode of resolution 2 cm^{-1}. To obtain good signal to noise ratio, 120 scans of the film were taken in the range of 450-4000 $cm.^{-1}$ Trichothecinol –A shows characteristic 3452 cm^{-1} (OH stretching), 1721 cm^{-1} (C=O stretching) and 1679 cm^{-1} (C=O aromatic stretching). This is similar to the reported value of Trichothecinol-A. (11)

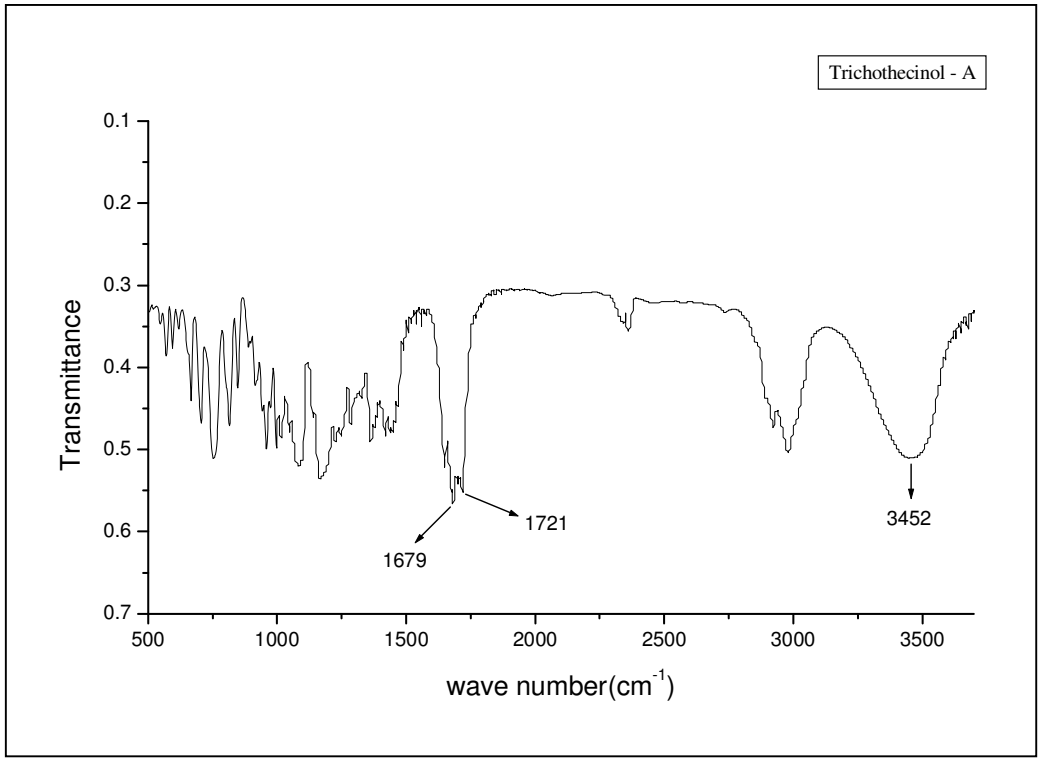


Fig 5. FTIR spectra of purified Trichothecinol-A

ESI-MS and HRMS analysis:

Molecular mass of the compound was determined by M/S Applied Biosystems API QSTAR pulsar (ESI-MS) mass spectrometer. Samples for the analysis were dissolved in HPLC grade methanol and analysed by infusion method / (injected into MS) at a flow rate of 5 µl/min and at an IS voltage of 3800 V in TOF mode. Spectrum from a range of m/z 200 to 500 Daltons was obtained. The spectrum showed molecular ion at *m/z* 349.57 and at *m/z* 371.28 attributing to M+H and M+Na ions of Trichothecinol -A respectively. HRMS of Trichothecinol-A calculated for $C_{19}H_{24}O_6$: 348.1573, found: 348.1683 which confirmed Trichothecinol-A. This is similar to the reported value of Trichothecinol. (11)

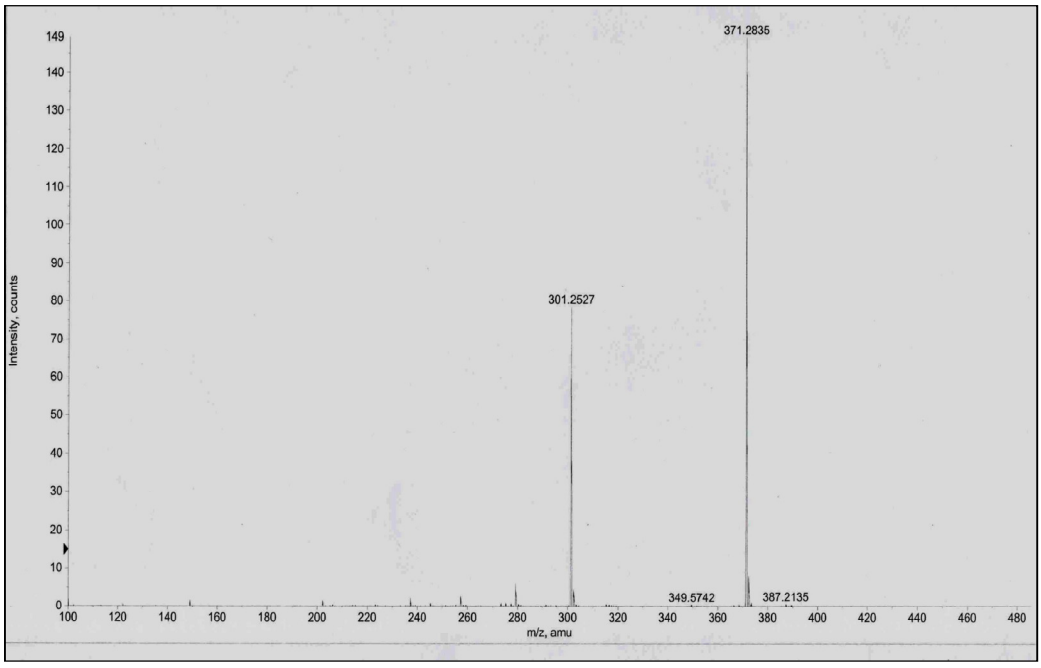


Fig 6. ESI-MS spectra of purified Trichothecinol-A.

Quantification of Trichothecinol-A through HPLC:

The data of area peak vs Trichothecinol-A concentration obtained in case of standard sample was used to estimate the quantity of purified Trichothecinol-A. The isolation of the Trichothecinol-A from 1 litre culture filtrate yielded 4.36 mg of Trichothecinol-A.

NMR analysis:

^{1}H NMR

^{1}H nuclear magnetic resonance spectra (NMR) analyses of Trichothecinol -A were carried out on a Brucker AV 500 Spectrometer operated at 500 MHz. 10 mg of the compound was dissolved in 0.6 ml of 99.8% deuterated $CDCl_3$ in 5-mm NMR tube. Chemical shifts in the ^{1}H spectra were referenced to residual $CHCl_3$ peak (7.25 ppm). The ^{1}H NMR spectrum of the compound isolated is given in Fig.7. This compound also showed presence of same number of protons (19 protons) as trichothecin. A comparison of the ^{1}H NMR spectrum of this compound with that of trichothecin is shown in Fig.8. which shows that the olefinic linkages, the methyl groups, the epoxide part (13a,13b protons) and one of the methylene group (7a, 7b) are intact, though some changes in chemical shifts are noticed. The most noticeable feature of the ^{1}H spectrum is the absence of the multiplet corresponding 3a,3b protons of trichothecin and presence of a broad peak at ~3.5δ.

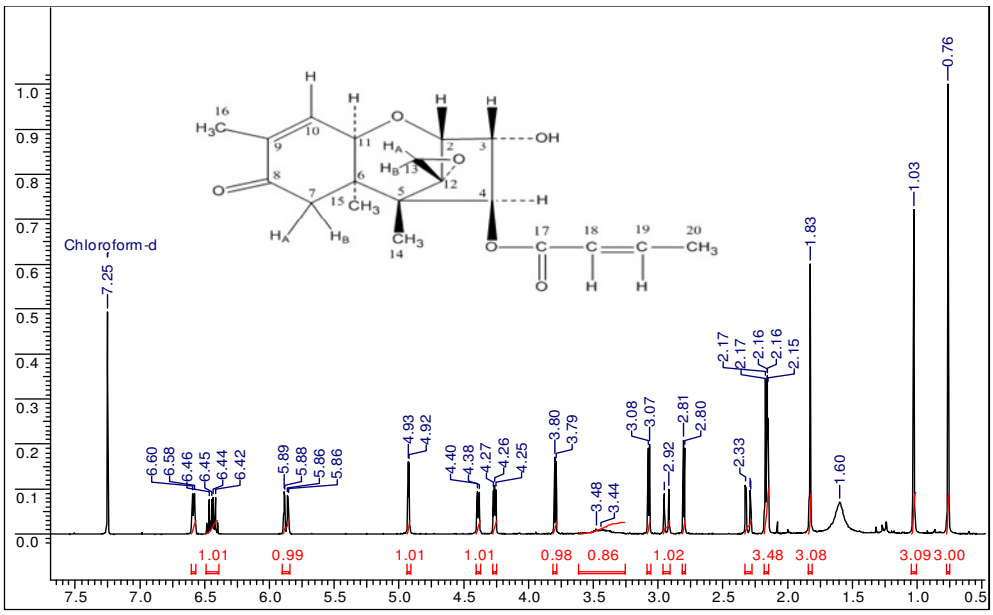


Fig 7. ^{1}H nuclear magnetic resonance spectra (NMR) of Trichothecinol –A.

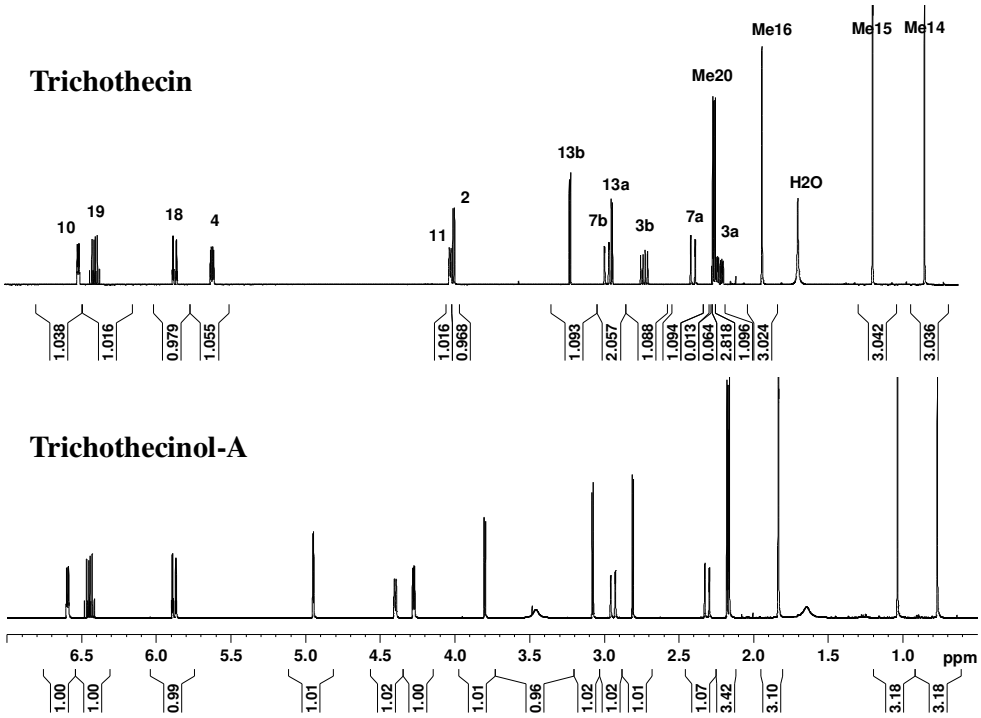


Fig 8. Comparison of ^{1}H NMR spectra of Trichothectin and Trichothecinol –A.

^{13}C NMR

^{13}C nuclear magnetic resonance spectral (NMR) analyses of Trichothecinol -A were carried out on a Brucker AV 500 Spectrometer operated at 125 MHz. ^{13}C NMR spectra were obtained with 5-mm NMR tubes and 99.8% deuterated $CDCl_3$ as the solvent. Chemical shifts in the ^{13}C spectra were referenced to $CDCl_3$ (δc, 77.03 ppm). ^{13}C NMR of this sample (Fig.9, 10) also shows the presence of 19 carbons. The major difference compared to trichothecin being the presence of only two CH_2 groups (one less compared to trichothecin) and 7CH carbons (one more than trichothecin) which clearly indicates substitution of one of the CH_2 carbons, most likely by an OH group since the additional CH appearing in the region 78 to 83 region. Comparisons of the ^{13}C NMR spectra are shown in Fig.11 and 12. The absence of multiplet pattern similar to that of 3a, 3b in trichothecin suggest that the substitution is most likely at this site and the substituent is likely to be an OH group.

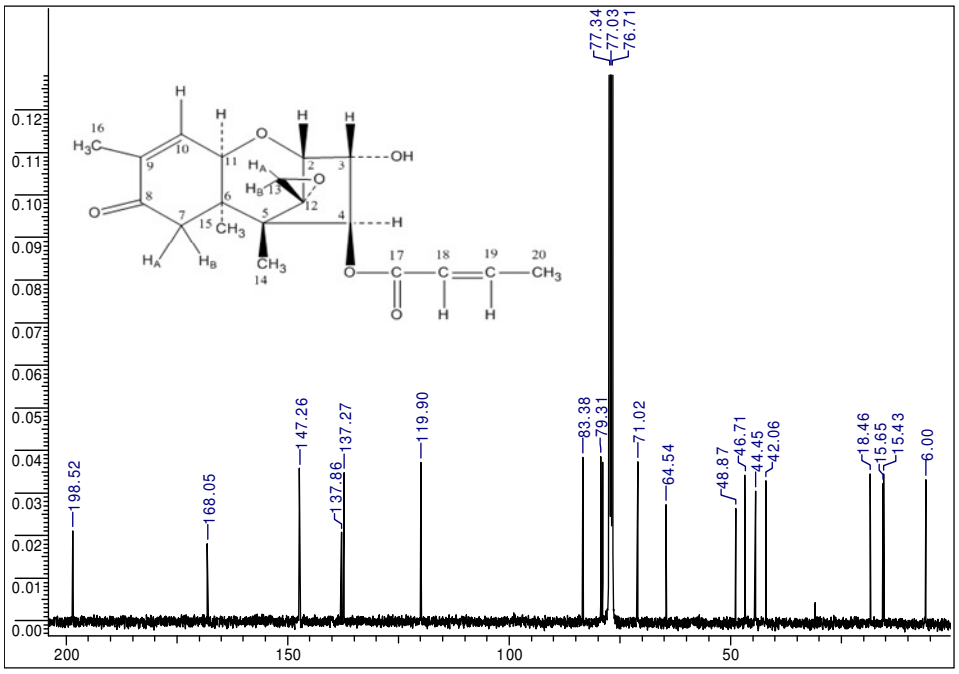


Fig 9. ^{13}C nuclear magnetic resonance spectra (NMR) analyses of Trichothecinol –A.

DEPT

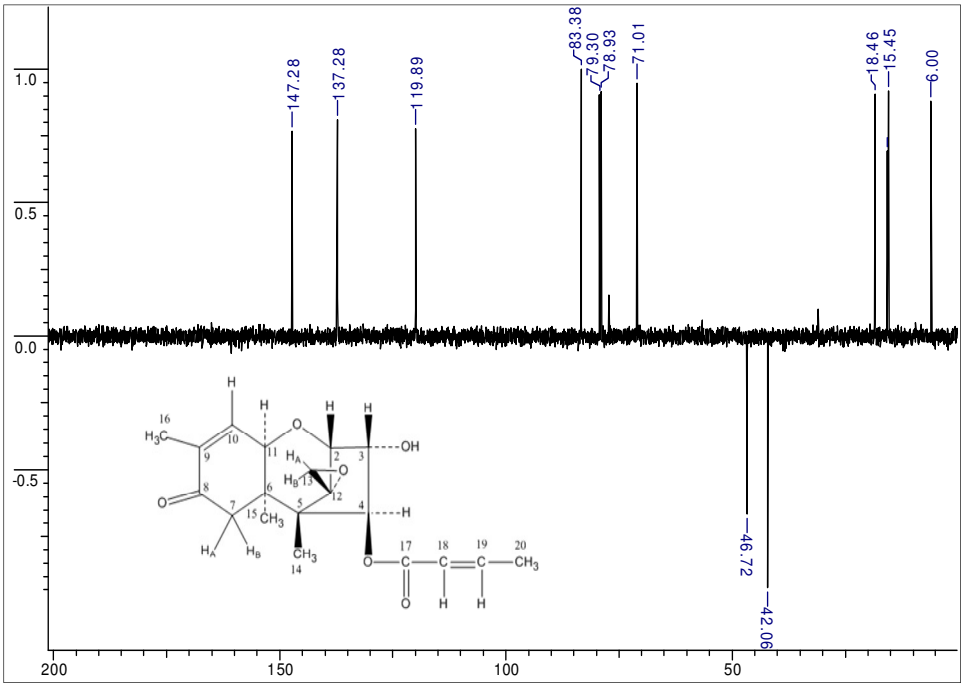


Fig 10 . DEPT 135 spectrum of Trichothecinol –A.

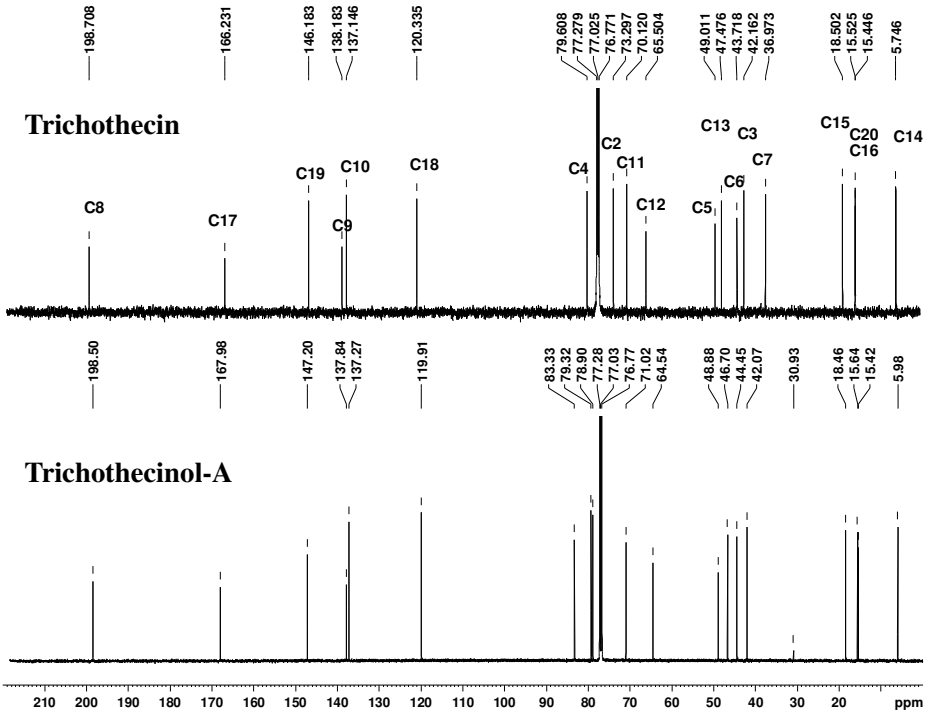


Fig 11. Comparison of ^{13}C NMR spectra of Trichothectin and Trichothecinol –A.

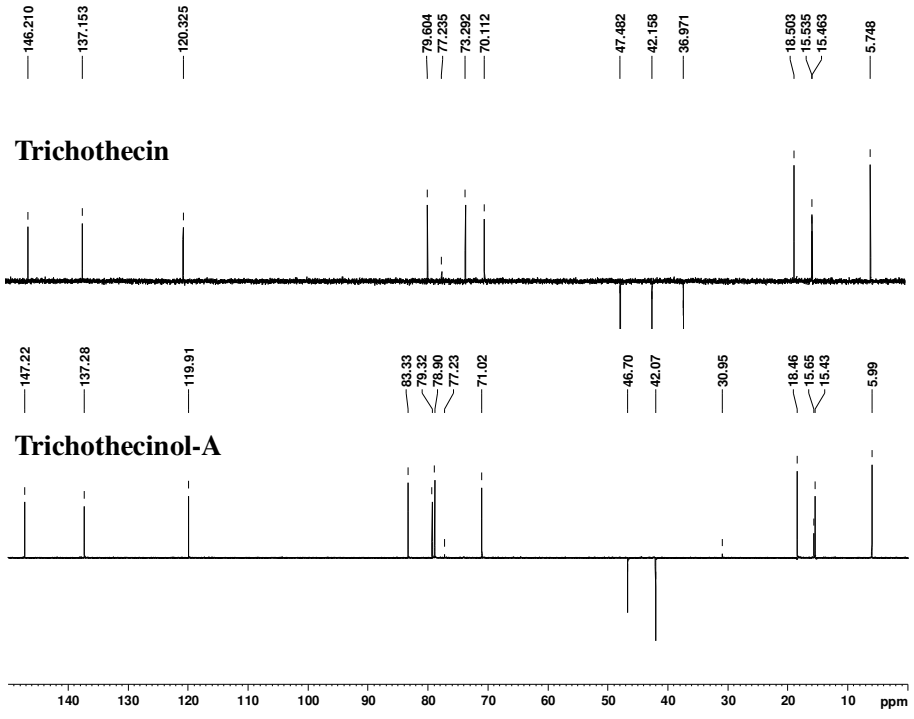
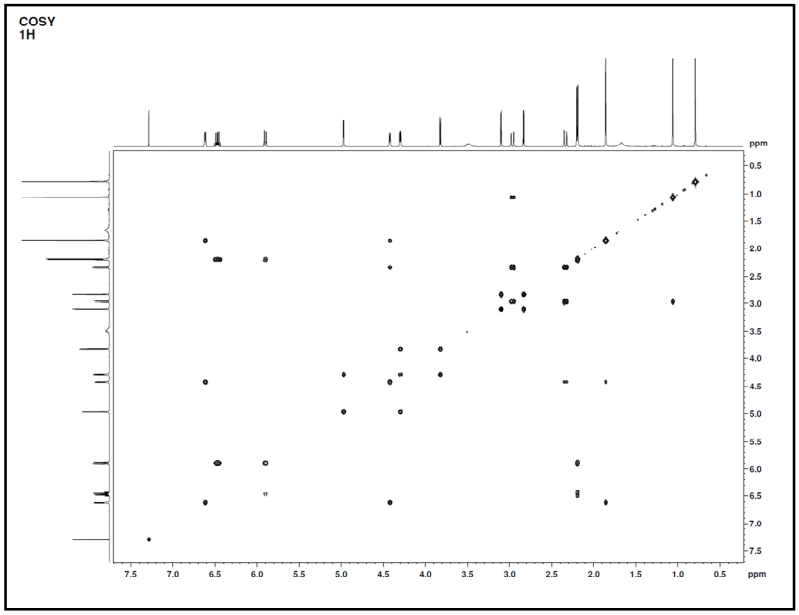


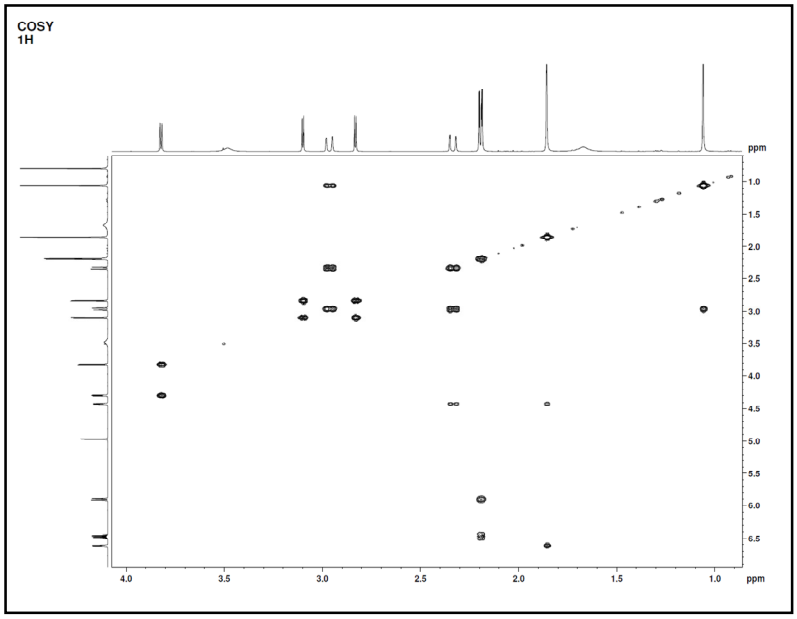
Fig 12. Comparison of ^{13}C NMR spectra of Trichothectin and Trichothecinol –A.

As in the case of trichothecin a detailed structural elucidation was performed with the help ^{1}H, ^{13}C, COSY (Fig.13), TOCSY (Fig.14), ^{1}H-^{13}C HSQC (Fig.15) and ^{1}H-^{13}C HMBC (Fig.16) experiments. The assignments are shown in Tables1 which unambiguously indicates that the H3 appear as a doublet of doublet (J=5Hz, 3Hz), H2 as a doublet (J=5Hz) and H4 experienced an upfield shift (4.95δ from 5.58δ). The structure was further confirmed by the NOESY spectrum (Fig-17) and the important NOESY correlations are depicted in Fig.18.

A



B



C

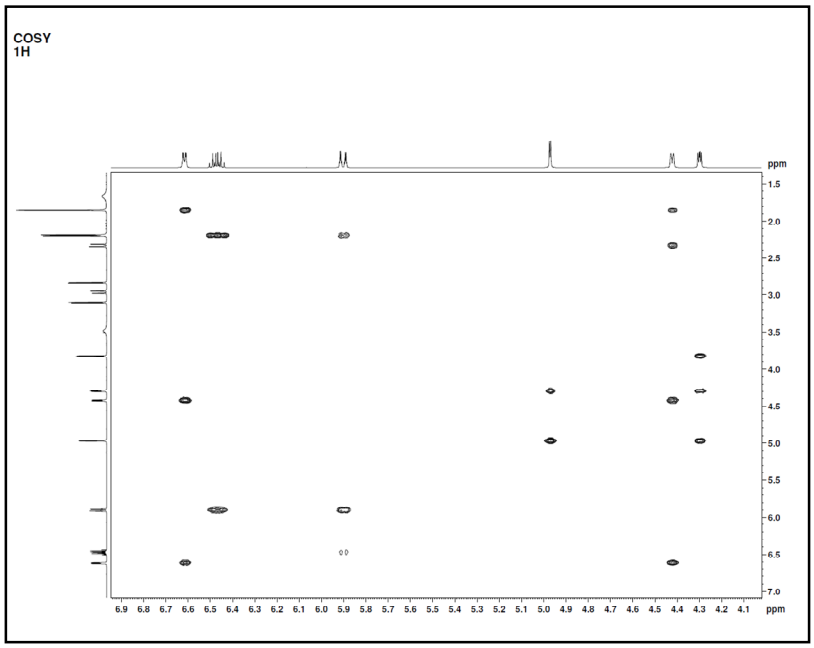
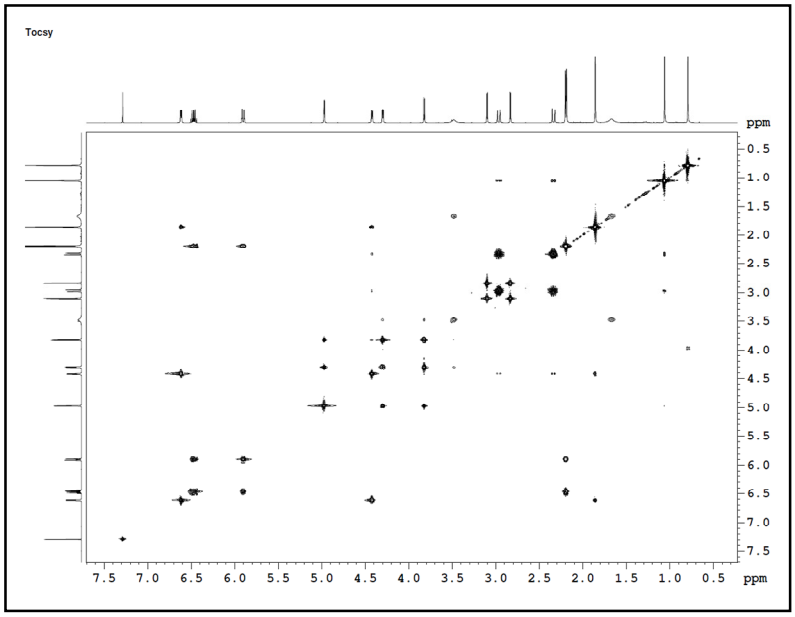


Fig 13. 500 MHz COSY spectrum of the Trichothecinol-A in 99.8% deuterated $CDCl_3$. **A.** complete spectra, **B** and **C** expanded spectra.

A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1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
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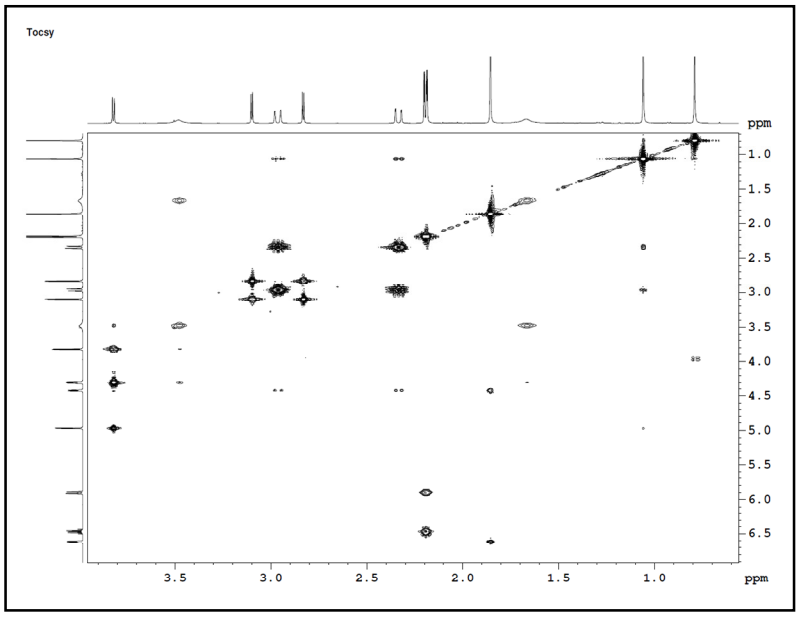
6.0
6.5
7.0
7.5
7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
ppm

B


Tocsy
ppm
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1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
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ppm

C

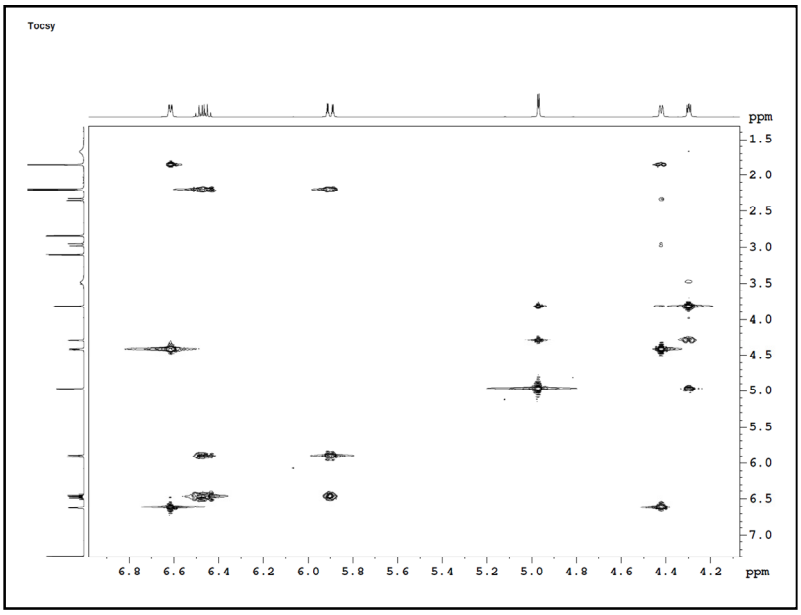


Fig 14. 500 MHz TOCSY correlations of the Trichothecinol-A. **A** shows complete while **B** and **C** indicates expanded spectra.

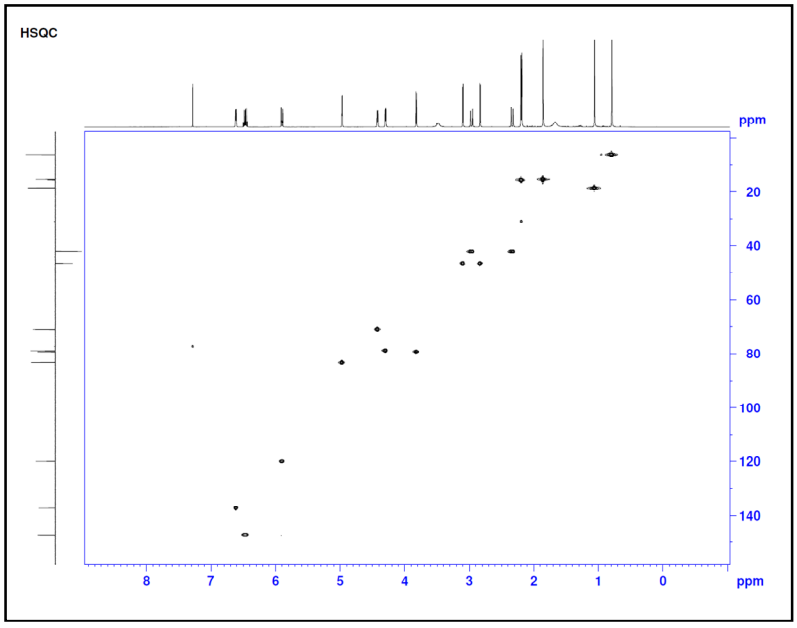
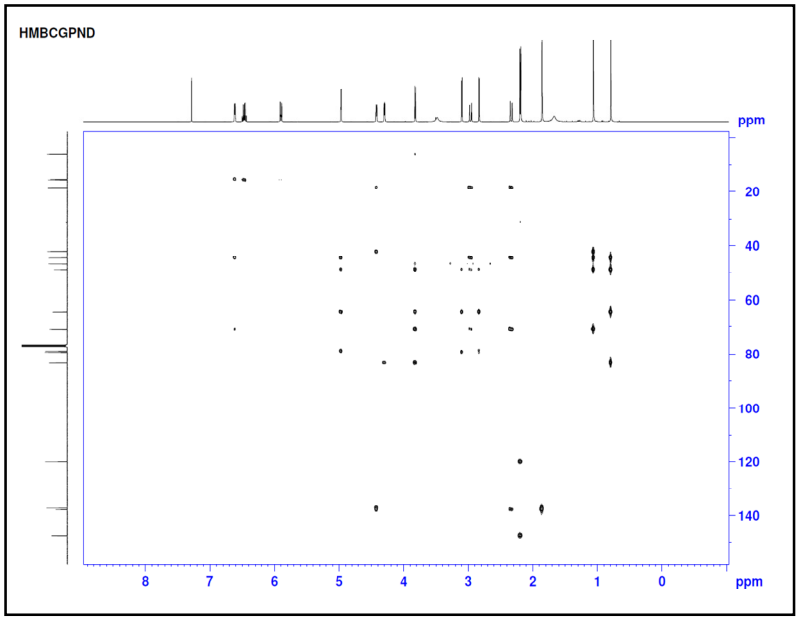


Fig 15. ^{1}H-^{13}C HSQC spectrum of the Trichothecinol-A.

A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80
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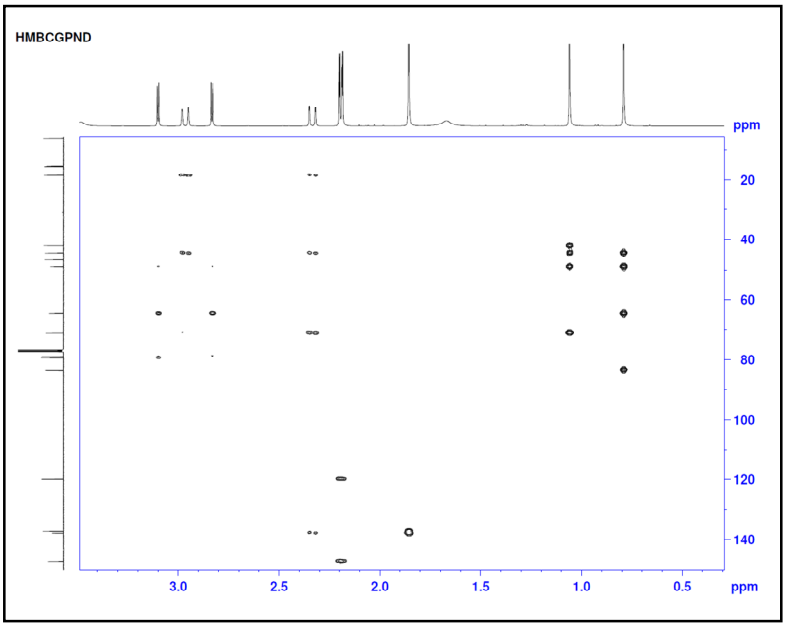
140
8
7
6
5
4
3
2
1
0
ppm

B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2.5
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1.5
1.0
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C

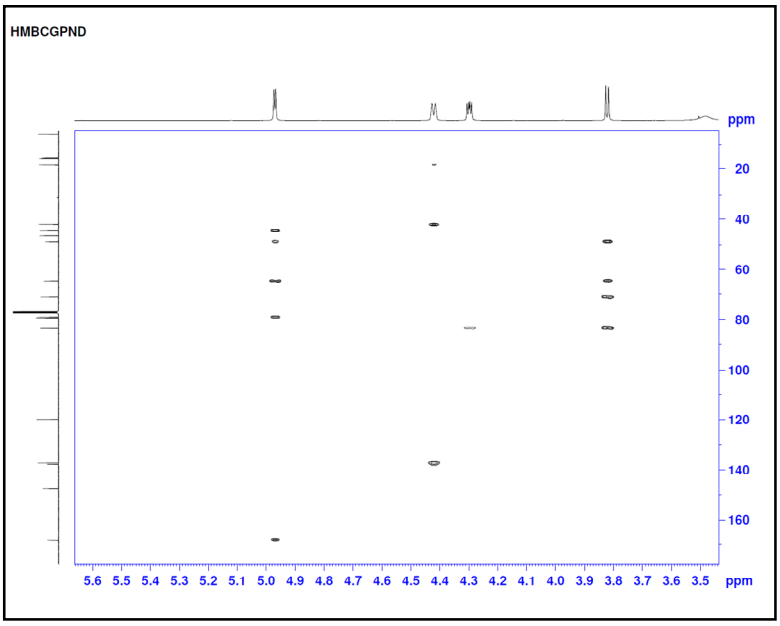
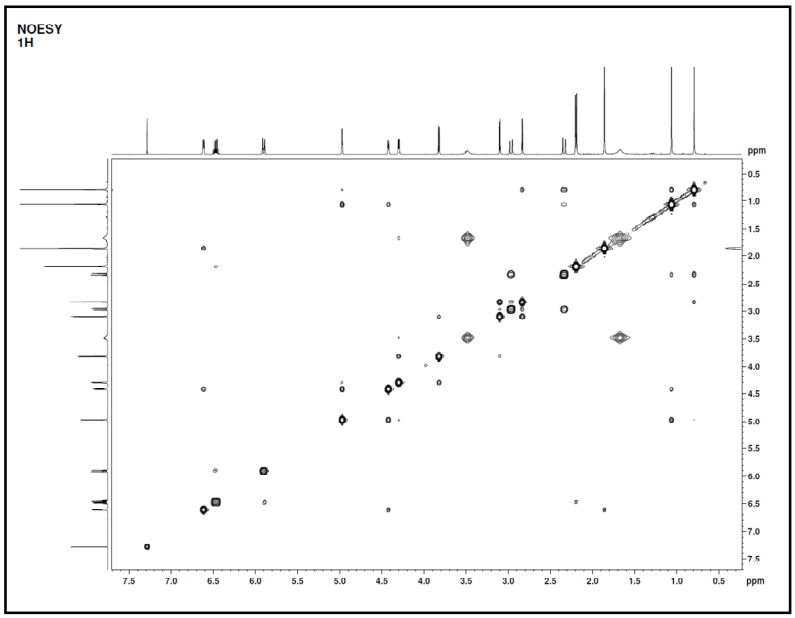
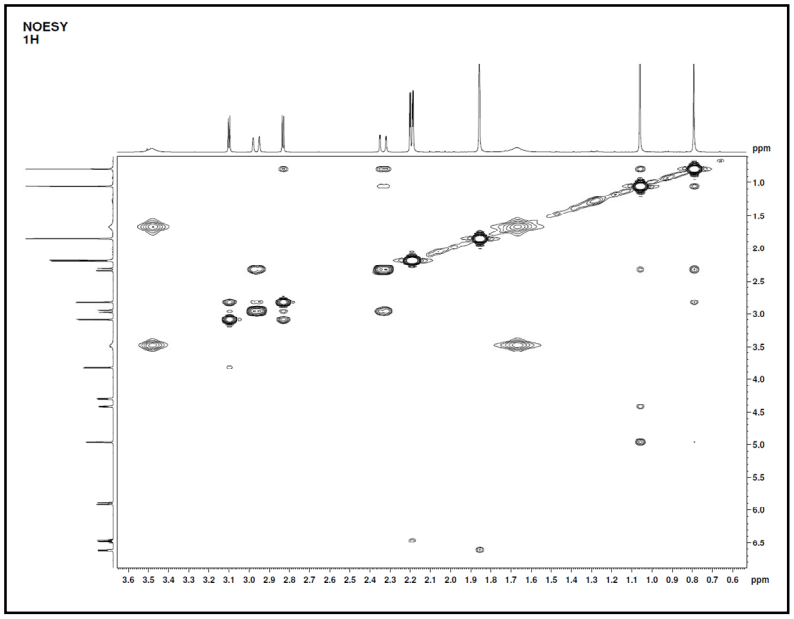


Fig 16. ^{1}H-^{13}C HMBC correlations of the Trichothecinol-A. A shows complete while B and C indicates expanded spectra.

A



B



C

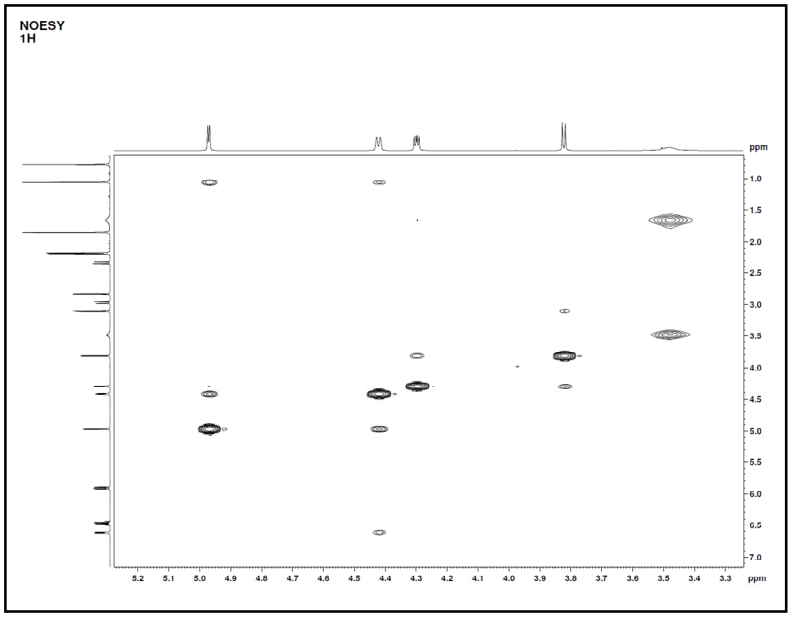


Fig 17. 500 MHz NOESY spectrum of the Trichothecinol-A in 99.8% deuterated $CDCl_3$. **A**. complete spectra, **B** and **C** expanded spectra.

Table-1 ^{13}C and ^{1}H chemical shifts ^{1}H coupling constants

Position	$\delta_{C,\ mult.}$	δ_H [a] (δ)	Numbering of Atom, coupling constant
1	------	------	1
2	79.31 (CH)	3.80, d	C2, H2 ($J_{2,3}$=4.95)
3	78.93 (CH, OH)	4.27, OH- 3.48,brs	C3, H3 ($J_{3,2}$=5.12,$J_{3,4}$=3.01)
4	83.38 (CH)	4.93	C4 ,H4 ($J_{4,3}$=3.07)
5	48.87 (q)	------	C5
6	44.71 (q)	--------	C6
7	42.06 (CH2)	2.33, dd 2.96, dd	C7, $H7_a$ ($J_{7a,7b}$=15.18, $J_{7a,11}$=1.54) $H7_b$, ($J_{7b,7a}$=15.18,$J_{7b,15}$= 1.10)
8	198.52 (q)	--------	C8
9	137.52 (q)	---------	C9
10	137.27 (CH)	6.60,dd	C10,H10 ($J_{10,11}$=5.80,$J_{10,16}$= 1.54)
11	71.02 (CH)	4.40,d	C11, H11 ($J_{10,11}$=5.82)
12	64.54 (q)	------	C12
13	46.63 (CH2)	2.81, d 3.08, d	C13, $H13_a$ ($J_{13b,13a}$=3.88) $H13_b$, ($J_{13a,13b}$=3.96)
14	6.00 (CH3)	0.76, s	C14, Me14
15	18.46 (CH3)	1.03, s	C15, Me15
16	15.43 (CH3)	1.83, s	C16, Me16
17	168.05 (q)	-------	C17
18	119.90 (CH)	5.89, dq	C18, H18 ($J_{18,19}$=11.50, $J_{18,20}$=1.76)
19	147.26 (CH)	6.48, dq	C19,H19 ($J_{19,18}$=14.68,$J_{19,20}$=7.37)
20	15.65 (CH3)	2.17, dd	C20, H20 ($J_{20,18}$=1.80, $J_{20,19}$=7.30)

[a] s – singlet, d – doublet, dd – doublet of doublet, dq – doublet of quadruplet.

[b] w – weaker interactions.

Table-2: Homo nuclear correlations

Sr. No	δH a(δ) , assignment	Observed COSY Cross peaks to protons atδH	Observed TOCSY Cross peaks to protons at δH	Observed NOESY Cross peaks to protons at δH
2	3.80, H2	4.27	3.48(w),4.27, 4.93	3.08, 4.27
3	4.27, H3	3.80, 4.93	3.48,3.80, 4.93	3.80
	3.48,d OH-	--	4.27	--
4	4.93, H4	4.27	3.80, 4.27	1.03, 4.48
7	2.33, $H7_a$	2.96, 4.48	1.03(w), 2.96, 4.48(w)	0.76, 1.03, 2.96.
	2.96, $H7_b$	1.09, 2.33	2.33, 4.48(w)	2.33
10	6.60, H10	1.83, 4.48	1.83, 4.48	1.83, 4.48
11	4.48, H11	1.83, 2.33, 6.60	1.83, 2.33(w), 2.92(w), 3.80(w),6.60	1.03, 4.93, 6.60
13	2.81, $H13_a$	3.08	3.08	0.76, 3.08, 2.96
	3.08, $H13_b$	2.81	2.81	2.81, 2.96, 3.80
14	0.76, Me14	--	3.80 (w)	1.03, 2.33, 2.81
15	1.03, Me15	2.96	2.33(w), 2.96(w	0.76, 2.33, 4.48, 4.93
16	1.83, Me16	4.48, 6.60	4.48(w), 6.60	6.60
18	5.89, H18	2.17, 6.48	2.17, 6.48	6.48
19	6.48, H19	2.17, 5.89	2.17, 5.89	2.17, 5.89
20	2.17, H20	5.89, 6.48	5.89, 6.48	6.48

Table-3: Hetero nuclear correlations

Sr. No	δC, mult.	Observed HSQC Correlations	Observed HMBC Correlations to protons
2	79.31 (C2)	3.80	3.08
3	78.93 (C3)	4.27	4.93
4	83.38 (C4)	4.93	0.76, 3.80, 4.27
5	48.87 (C5)	----------	3.80, 4.93
6	44.71 (C6)	----------	0.76,1.03, 2.33,2.96, 4.93, 6.60
7	42.06 (C7)	2.33, 2.96	1.03, 4.40
8	198.52 (C8)	----------	----------
9	137.52 (C9)	----------	2.33
10	137.27 (C10)	6.60	1.83, 4.40
11	71.02 (C11)	4.40	1.03,2.33, 3.80, 6.60
12	64.54 (C12)	----------	0.76,2.81, 3.80, 4.93
13	46.63 (C13)	2.81, 3.08	0.76, 1.03
14	6.00 (C14)	0.76	----------
15	18.46 (15)	1.03	2.33, 2.96
16	15.43 (16)	1.83	6.60
17	168.05 (17)	----------	4.93
18	119.90 (18)	5.89	2.17
19	147.26 (19)	6.48	2.17
20	15.65 (20)	2.17	6.40

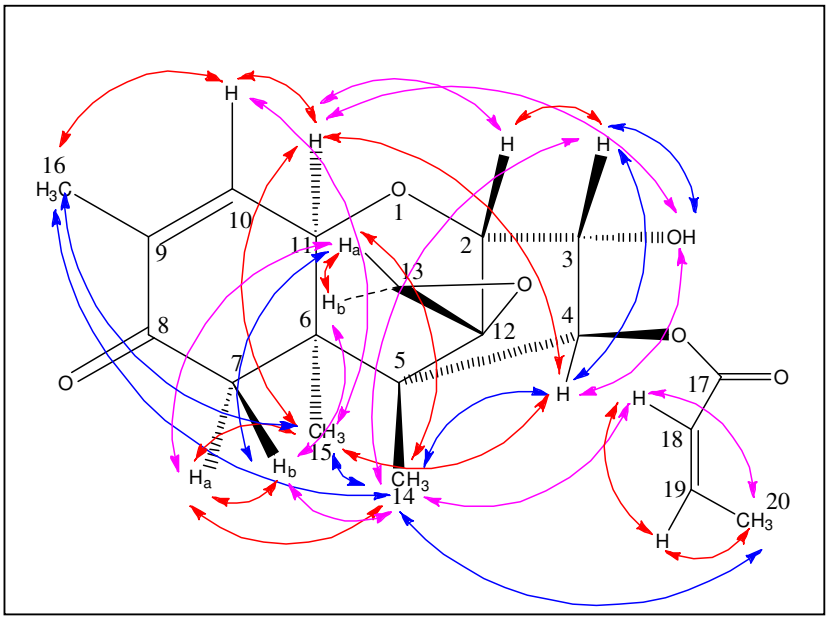


Fig 18. NOE correlations of Trichothecinol–A. Red colour indicates strong interactions, while blue and pink colour indicates medium and weak interactions respectively.

From the structural investigations we observed that the compound isolated is Trichothecinol-A, NMR spectra of which has already been reported. The stereochemistry of the isolated product aslo found to be similar to the reported data.

Discussion:

We have isolated an endophytic fungal culture *Trichothecium* sp. from medicinal herb *Phyllathus* sp. producing Trichothecinol-A extracellularly. Trichothecinol-A was isolated and its antifungal activity against *Aspergillus niger* was estimated. The compound was purified using RP-HPLC equipped with C_{18} symmetry column where it showed a single symmetrical peak at retention time of 34 min that confirmed its homogeneity. Absorption maximum of the purified Trichothecinol-A was found to be at 251 nm in ethyl acetate. FTIR spectrum of purified Trichothecinol-A in KBr pellet shows characteristic stretchings as follows; 3452 cm^{-1} (OH stretching), 1721 cm^{-1} (C=O stretching) and 1679 cm^{-1} (C=O aromatic stretching).ESI-MS spectrum showed molecular ion at *m/z* 349.57 and at *m/z* 371.28 attributing to M+H and M+Na ions of

Trichothecinol -A respectively. HRMS of Trichothecinol-A calculated for $C_{19}H_{24}O_6$: 348.1573, found: 348.1683 which confirmed Trichothecinol-A. ^1H, ^{13}C NMR spectrum was also matching with that of the reported Trichothecinol-A spectrum.(11) This is the first report of isolation of appreciable amount of trichothecinol-A from endophytic fungus *Trichothecium* sp. isolated from medicinal herb *Phyllanthus* sp. However, trichothecinol-A are commonly found in *Trichothecium roseum* and plant infected by this fungus from various parts of the world as well as India. These observations support the present findings. This suggest that the ability to synthesize trichothecinol-A resides not only in *Trichothecium roseum* and other infected plant from various part of the globe but also from *Trichothecium* sp. isolated by us from *Phyllanthus* plant. This compound previously has been characterized by various standard techniques such as ^1H NMR and ^{13}C NMR. Since this compound is reported to have cancer preventive activities, its complete characterization is must. During the present investigation we completely characterized this compound by variety of 1D and 2D NMR spectroscopy.

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Chapter 5

Evaluation of antifungal, antiproliferative and antimetastatic activity exhibited by Trichothecin and Trichothecinol-A.

Summary

Antifungal assay revealed that Trichothecin is more active than Trichothecinol-A. But, contrary to earlier reports, Trichothecinol-A showed prominent activity against yeast *Cryptococcus albidus* var *diffluens* NCIM 3371 and 3372 up to 16 µg/mL after 48h. This is the first report of antifungal activity of Trichothecinol-A. Cytotoxicity studies indicated that B16F10 and HeLa cells shows enhanced sensitivity to Trichothecinol-A and induce 50% cell death at 500nM concentration. Further cell cycle analysis of B16F10 cells revealed that Trichothecinol-A treated cells had higher percentage of apoptotic fraction compared to Trichothecin treated cells at 12h and 24h. Trichothecin treated cells showed a significant increase in G2/M population at 12h suggesting G2/M arrest. Inhibition of wound migration assay performed on MDA MB-231 cells revealed that 500 nm of Trichothecinol-A was able to inhibit wound migration by 50% whereas 2.5 and 5 µM concentration inhibited migration upto 75%, indicating its remarkable antimetastatic property. Being non proteinaceous in nature, Trichothecinol-A could be further developed as drug or used as a scaffold to synthesize novel analogues having more potency.

Introduction

A range of antifungal compounds of different chemical classes such as alkaloids, peptides, terpenoids, steroids, flavonoids, quinones, lignans, phenols, lactones, etc. and several other secondary metabolites have been isolated from plants and their associated microorganisms. (12, 13, 20) We for the first time isolated, purified, and completely characterized two Trichothecene mycotoxins viz. Trichothecin and Trichothecinol-A from endophytic fungus *Trichothecium* sp. isolated from medicinal plant *Phyllanthus* sp. As cases of drug resistance in viruses, pathogenic fungi and bacteria are alarmingly on a rise, new and improved therapeutic agents and derivatives are required for effective treatments of diseases in humans, plants and animals. In order to provide a new class of antifungal compounds to overcome resistance in above mentioned microorganisms, we evaluated the antifungal activity of both these compounds against a range of plant pathogenic and saprophytic fungi and different genera of yeast. Both the compounds are very effective against all the above mentioned fungi and yeast and can be developed as a new class of fungicide.

As per WHO, cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in the year 2000 and are projected to

continue to rise over 11 million in 2030.(1) Every year, billions of dollars are poured into research to find out a way to fight cancer. Much of the efforts have been focused to search the ways to cure the disease by exterminating cancer cells while sparing their normal counterparts. A rational approach to this problem would be inhibition of cancer cell growth. Most of the antiproliferative drugs administered in chemotherapy treatment tend to inhibit growth of cancerous cells or may also results in significant decrease in tumour burden. But unfortunately, the majority of drugs currently available are not specific, which leads to many common side effects associated with chemotherapy.

As, the common approach of all chemotherapy is to decrease the growth rate (cell division) of the cancer cells, the side effects are seen in bodily systems that naturally have a rapid turnover of cells including skin, hair, gastrointestinal and bone marrow. These healthy, normal cells also end up damaged by the chemotherapy program. Inhibition of cancer cell migration could be an effective means of preventing cancer metastasis, thereby enabling confinement of primary tumours in manageable form, making surgical options more viable.(11) During opening phase of the metastatic cascade, cancer cells penetrate into surrounding tissues and blood vessels and it has been shown to be the rate limiting step in an experimental model.(29) Therefore, inhibition of the cell migration represents a potential therapeutic approach for the treatment of tumor metastasis. Hence, it's reasonable to look out for compounds with antimetastatic activity which could be further developed as lead against cancer metastasis.

Historically, natural products have served as novel scaffolds for the majority of drugs and drug leads in various therapeutic areas including cancer. According to Newmann, during 1985 to 2002, 74% of drugs approved for cancer treatment belonged to natural products or mimicked them in one or another way.(23) Compounds like Migrastatin from *Streptomyces platensis* and several of its analogues including Isomigrastatin have shown to have antimetastatic potential.(6, 9, 21, 22, 24, 25, 28) Trichothecenes produced by imperfect fungi such as *Fusarium* sp. and *Trichothecium* sp. and closely related sesquiterpenoids, exhibit a wide array of biological activities such as antifungal, antiviral, insecticidal and phytotoxic behavior.(3, 7, 8, 10, 26) In addition, many of them have cytotoxic behaviour and antitumor activity.(14–19) In this

chapter, we are reporting antifungal, antiproliferative and antimetastastic potential of Trichothecenes namely Trichothecin and Trichothecinol- A produced by endophytic fungus *Trichothecium* sp.

Materials and methods

Cell Culture and Reagents:

Human breast adenocarcinoma (MDA-MB-231), human cervical cancer (HeLa) and murine melanoma (B16F10) cell lines were obtained from American Type Culture Collection and were grown in L-15, DMEM and RPMI media respectively supplemented with 10% fetal bovine serum and 100 U/mL Penicillin and Streptomycin (Invitrogen). All cells were grown in humidified atmosphere containing 5% CO_2 and 95% air at 37^{o}C. Tetrazolium dye and Propidium Iodide were purchased from Sigma.

Cell Viability Assay:

MDA-MB-231, HeLa and B16F10 cells ($2x10^4$) were seeded in 96 well plates. Cells were treated with Trichothecin and Trichothecinol-A for 24h with indicated concentrations. Treatment was terminated by removing media and MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] (0.5 mg/ml) was added. After 4h, crystals were dissolved in isopropanol and A_{570} was recorded.

Flow Cytometry Analysis:

Flow cytometry experiments were performed as described earlier.(27) Murine melanoma B16F10 cells were treated with indicated concentrations of Trichothecin and Trichothecinol-A in RPMI media supplemented with 10% fetal bovine serum for 12 and 24h. DNA analysis was done and apoptotic fractions were determined following propidium iodide staining (FACS Calibar, Becton Dickinson).

Wound Assay:

Wound assay was performed with post confluent MDA-MB-231 cells as described.(5) Briefly, uniform sized wounds were made using micropippet tips. MDA-MB-231 cells were treated with 0-2.5µM of Trichothecin and Trichothecinol-A. DMSO was used as vehicle control. After 18h, photographs were taken and the area of migrated cells was quantified using Image Pro Plus 6.0 software (Nikon).

Statistical Analysis

The data reported in cytotoxicity and cell migration experiments are expressed as mean±S.E. Statistical differences were determined by Student's *t* test. The p value < 0.05 was considered significant.

Antifungal assay:

The purified compounds were analysed for their antifungal activity against different plant pathogenic, saprophytic fungi and yeast. Antifungal assay was performed in microtiter plates as reported earlier.(2, 4) Fungal spores were removed from a 4 days old culture, transferred to YM broth (Glucose 1%, Malt extract 0.3%, Yeast extract 0.3%) and adjusted to $4x10^5$ spores or cells/mL. The compounds were diluted in DMSO to get desired concentrations. 50µL aliquots of spores were mixed with different concentrations of compounds (10 to 100µg/mL in 10µL of DMSO). Final volume was adjusted by adding 140µL YM media. Total assay volume was 200µL. The plates were incubated at 28^{o}C for 48h.

OD 600 measurements:

The OD 600 measurements were carried out on microtiter plate reader (Bio-Rad xMark). The MIC was determined as the lowest concentration of Trichothecin and Trichothecinol-A which completely inhibits visible growth of the microorganism. The dose response curves were obtained by plotting concentration of compound v/s % growth inhibition. The IC_{50} values were determined as the concentration of Trichothecin and Trichothecinol-A which could show 50% of maximum inhibition.

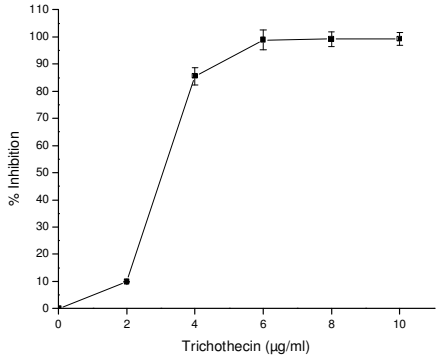
Results

Antifungal activity of Trichothecin and Trichothecinol-A.

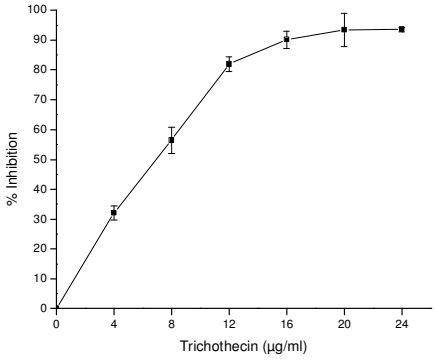
The dose response curves were obtained by plotting concentration of compounds against % growth inhibition. MIC and IC_{50} values were determined as described earlier (Table 1).

Dose response curves of Trichothecin:

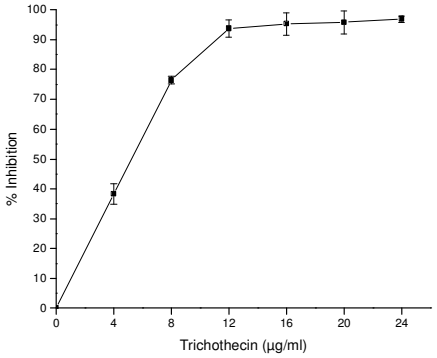
Saccharomyces cerevisiae, NCIM 3045



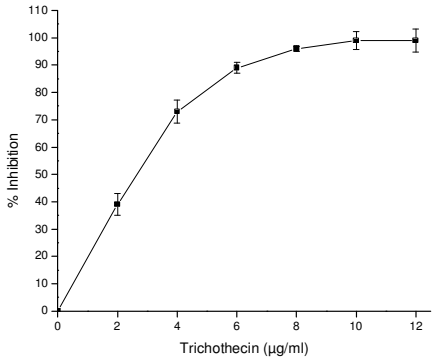
Cryptococcus albidus var *diffluens* NCIM 3371



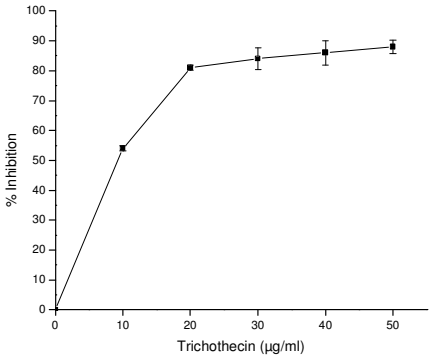
Cryptococcus albidus var *diffluens* NCIM 3372



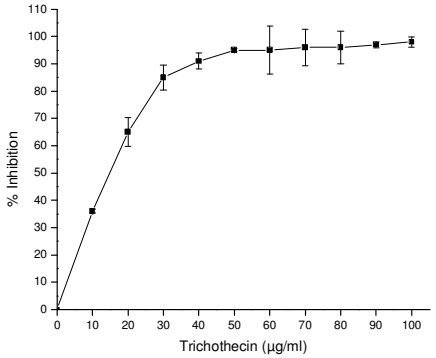
Fusarium oxysporum NCIM 1043



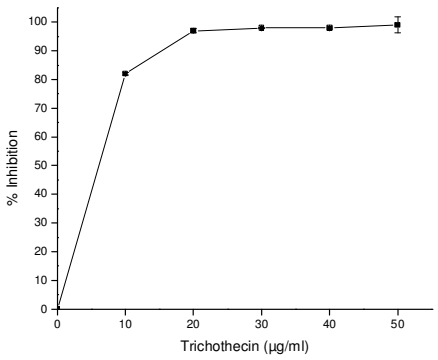
Aspergillus flavus NCIM 535



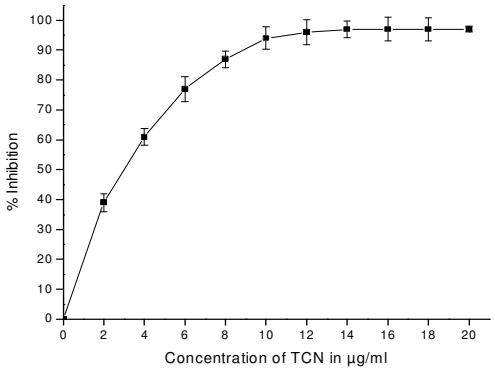
Trichoderma reesei NCIM 992



Penicillium expansum NCIM 939



Aspergillus niger NCIM 773



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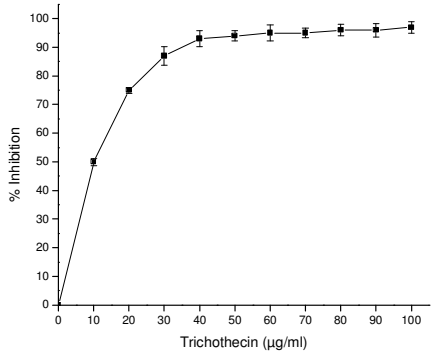
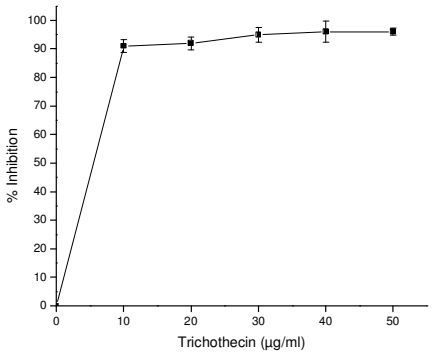
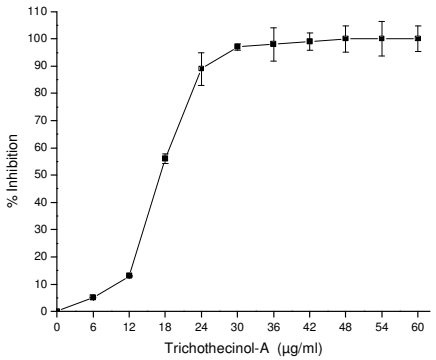


Paecilomyces varioti NCIM 1217

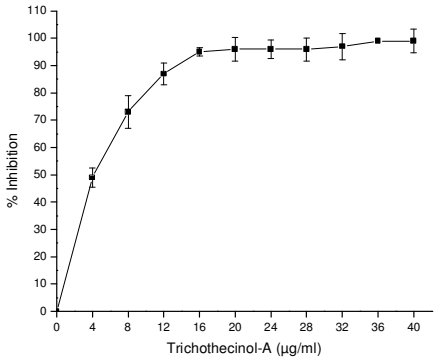


Dose response curves of Trichothecinol-A .

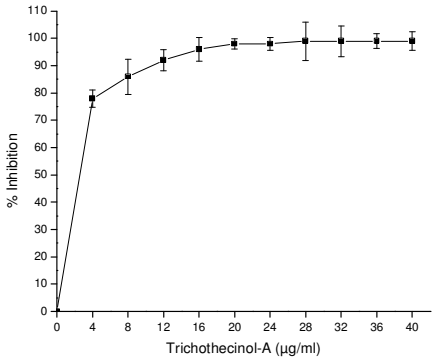
Saccharomyces cerevisiae, NCIM 3045



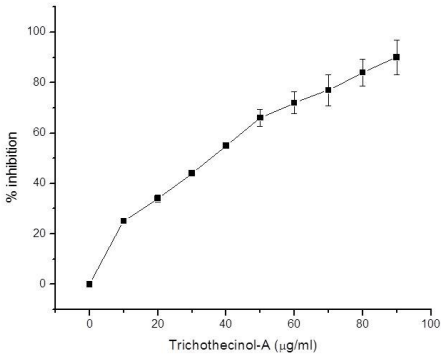
Cryptococcus albidus var *diffluens* NCIM 3371



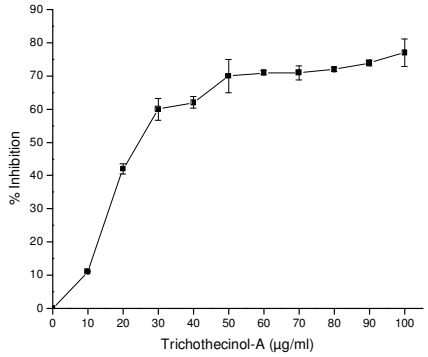
Cryptococcus albidus var *diffluens* NCIM 3372



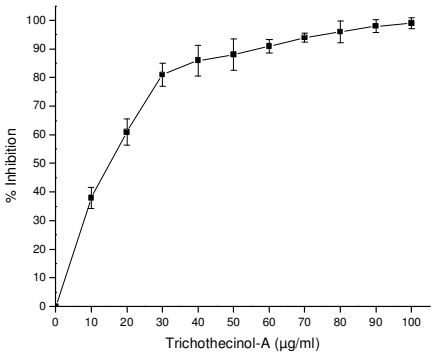
Fusarium oxysporum NCIM 1043



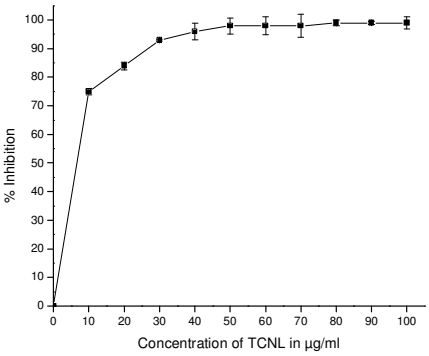
Aspergillus flavus NCIM 535



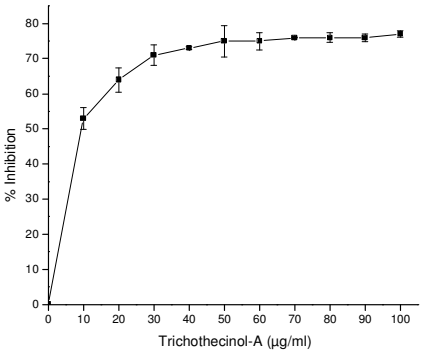
Trichoderma reesei NCIM 992



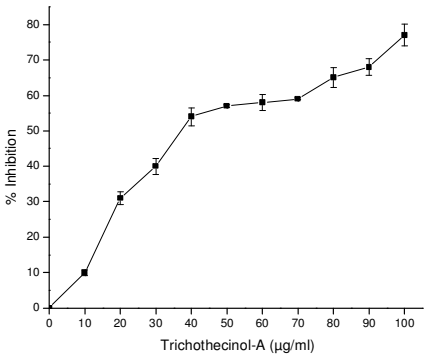
Penicillium expansum NCIM 939



Aspergillus niger NCIM 773



Trichoderma viride NCIM 1051



Paecilomyces varioti NCIM 1217

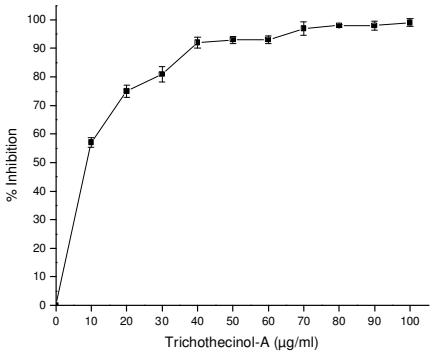


Table 1: MIC and IC_{50} values of Trichothecin and Trichothecinol-A.

Sr.No.	Organism[$]	IC_{50} (µg/mL)		MIC (µg/mL)	
		TCN[*]	TCNL-A[#]	TCN[*]	TCNL-A[#]
1	*Saccharomyces cerevisiae* NCIM 3045	3.05	17.16	6	36
2	*Cryptococcus albidus* var *diffluens* NCIM 3371	6.91	4.08	20	36
3	*Cryptococcus albidus* var *diffluens* NCIM 3372	5.26	2.5	12	20
4	*Fusarium oxysporum* NCIM 1043	2.61	7.16	10	>100
5	*Aspergillus flavus* NCIM 535	8.19	24.3	>100	>100
6	*Trichoderma reesei* NCIM 992	14.4	15	70	90
7	*Penicillium expansum* NCIM 939	6.07	6.61	30	50
8	*Trichoderma viride* NCIM 1051	9.7	36.6	40	>100
9	*Paecilomyces varioti* NCIM 1217	5.18	8.69	20	70
10	*Aspergillus niger* NCIM 773	2.95	9.12	12	>100

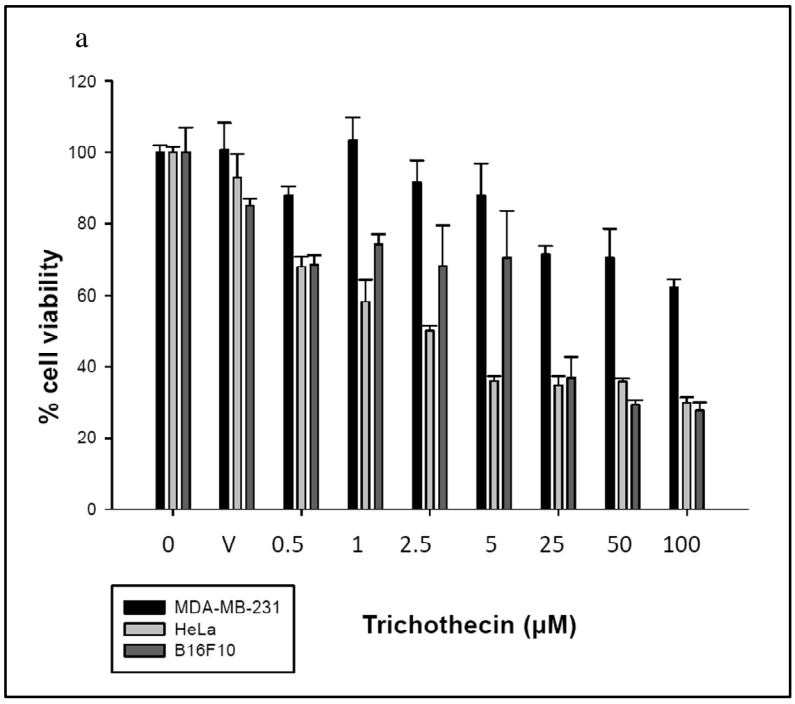
[*]TCN corresponds to Trichothecin.

[#]TCNL-A corresponds to Trichothecinol-A.

[$]NCIM corresponds to National Collection of Industrial Microorganisms, Pune, India.

Trichothecin and Trichothecinol-A exhibit cytotoxicity at nanomolar concentrations against B16F10 and HeLa but not in MDA-MB-231 cells.

Cytotoxicity studies indicated that B16F10 and HeLa cells show enhanced sensitivity to Trichothecinol-A and Trichothecin compared to MDA-MB-231 cells. Trichothecinol-A induced about 50 % cell death in HeLa and B16F10 cells compared to about 25% in MDA-MB-231 cells at 500 nM concentrations (Fig. 1b). Trichothecin had relatively less effect on MDA-MB-231 cells at lower concentration as compared to HeLa or B16F10 cells (Fig. 1a).



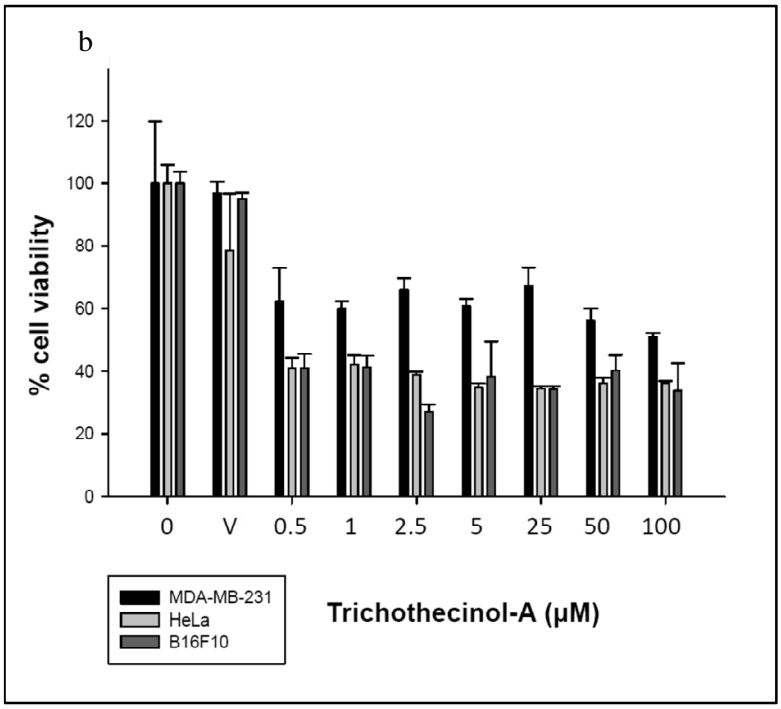
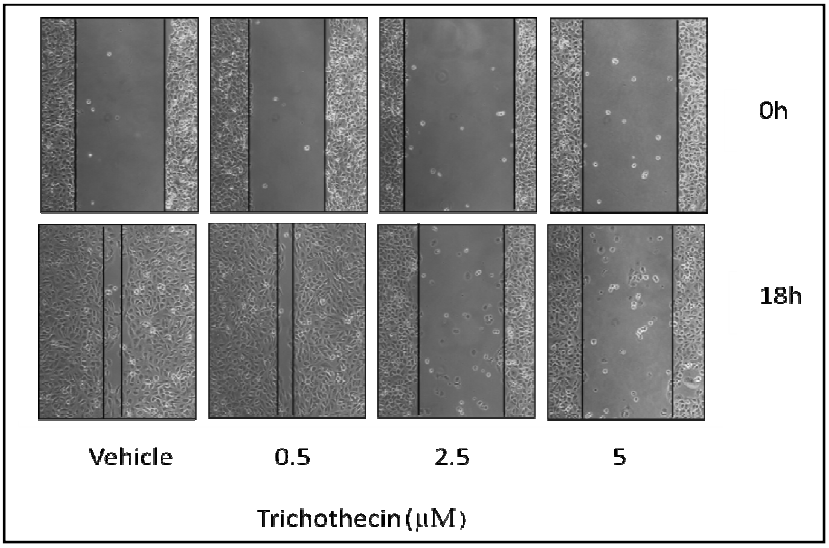


Fig 1: Effect of Trichothecin, Trichothecinol-A on cancer cell proliferation: a & b, MDA MB-231, HeLa and B16F10 cells were treated with DMSO as vehicle control and with Trichothecin or Trichothecinol-A at varying concentrations from 0-100 µM for 24 h and cell viability was determined by MTT assay.

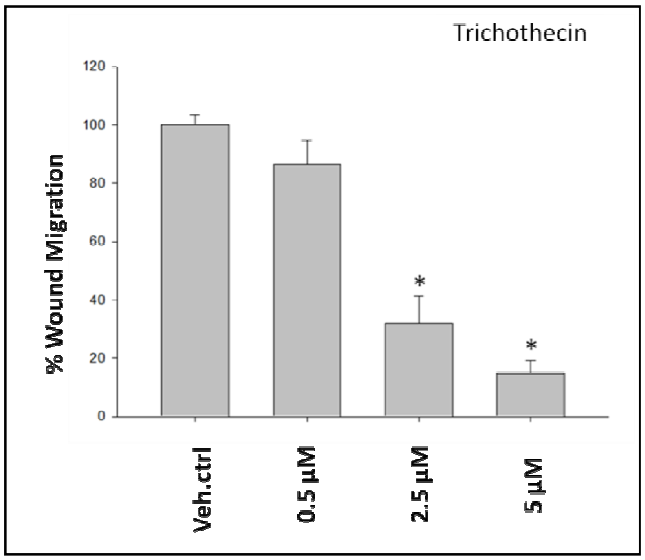
Trichothecin and Trichothecinol-A effectively inhibit MDA-MB-231 cell migration.

Enhanced migratory capacity is one of the hall marks of highly invasive tumor cells. Here, we studied the inhibitory effect of Trichothecin and Trichothecinol-A in MDA-MB-231 cell migration at various concentrations. Percentage of wound closure was determined by the difference in area covered by migrated cells in control versus treated with Trichothecin or Trichothecinol-A at 0 or 18h. The results show that 500 nM of Trichothecinol-A was able to inhibit wound closure by 50% where as 2.5 and 5µM concentrations of Trichothecinol-A inhibited migration upto 75% as compared to vehicle control (Fig. 2c & d). Trichothecin was less effective in inhibiting MDA-MB-231 cell migration at 500 nM, but showed increased inhibition at higher concentrations (Fig. 2a & b).

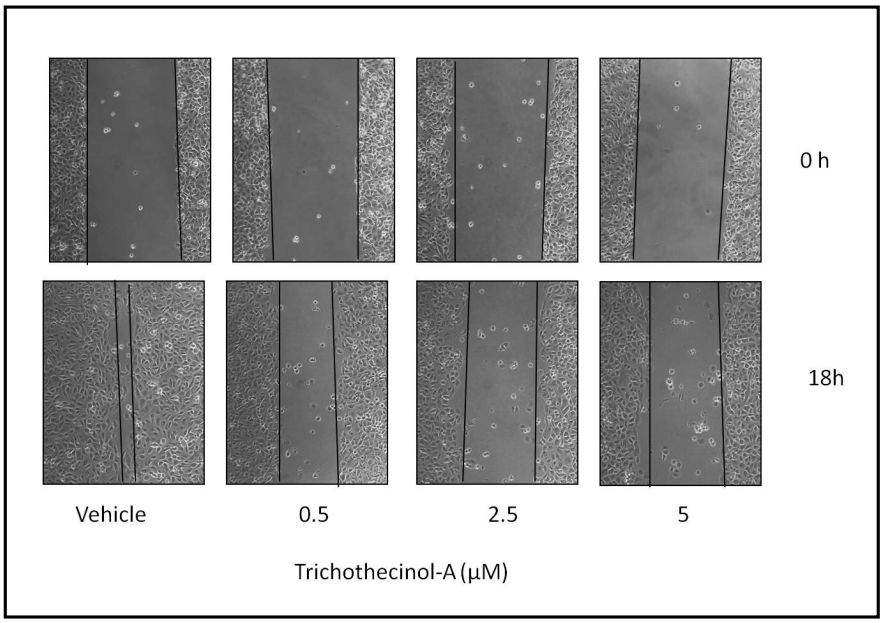
a.


0h
18h
Vehicle
0.5
2.5
5
Trichothecin (µM)

b.


Trichothecin
% Wound Migration
120
100
80
60
40
20
0
*
*
Veh.ctrl
0.5 µM
2.5 µM
5 µM

c.



d.

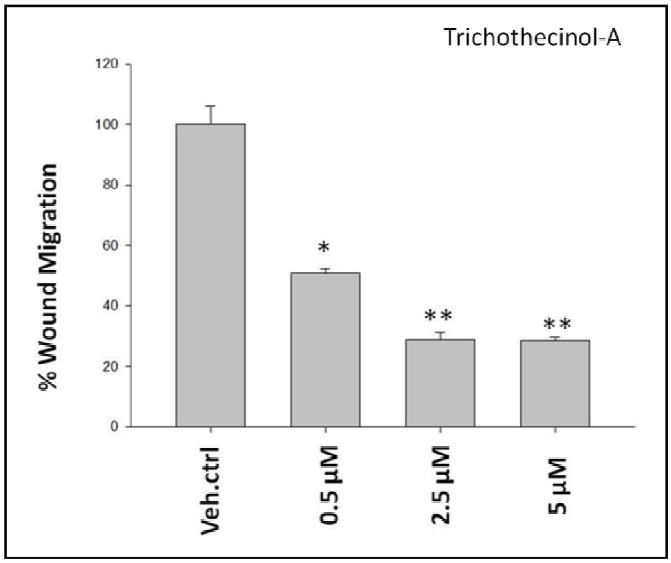


Fig 2: Inhibition of cell migration by Trichothecin and Trichothecinol-A: a. MDA MB-231 cells were treated with DMSO as a vehicle or Trichothecin at concentrations of 0-5 µM for 18 h and its effect on cell migration was determined by wound assay. b. The area of migrated cells were quantified using image pro plus 6.0 software and represented as bar graph. *,p=.002 vs. vehicle control. c. MDA MB-231 cells were treated with DMSO as vehicle or Trichothecinol-A at concentrations of 0-5 µm for 18 h and photographs were taken. d. The area of migrated cells were quantified and represented as bar graph. *,p=.0013, **,p=.0003 vs. vehicle control.

Trichothecin and Trichothecinol-A induce apoptosis in B16F10 cells.

The effect of Trichothecin and Trichothecinol-A on B16F10 cell death was performed by cell cycle analysis. Briefly, cells were treated with Trichothecinol-A in a dose of 0-5 µM for 12 and 24h respectively and then stained with PI. The DNA fluorescence histogram of PI-stained cells revealed a distinct quantifiable region beyond G1 peak (Fig.3).

Significant increase in the sub G_0 population was observed in Trichothecinol-A treated cells in a time dependent manner. 500 nM of Trichothecinol-A had 12.7% sub G_0 population as compared to 3.88% in vehicle control in 12h. However, cells treated with 500 nM of Trichothecinol-A for 24h resulted in an increase of apoptotic fraction to 52.96% versus 16.68% in vehicle control.

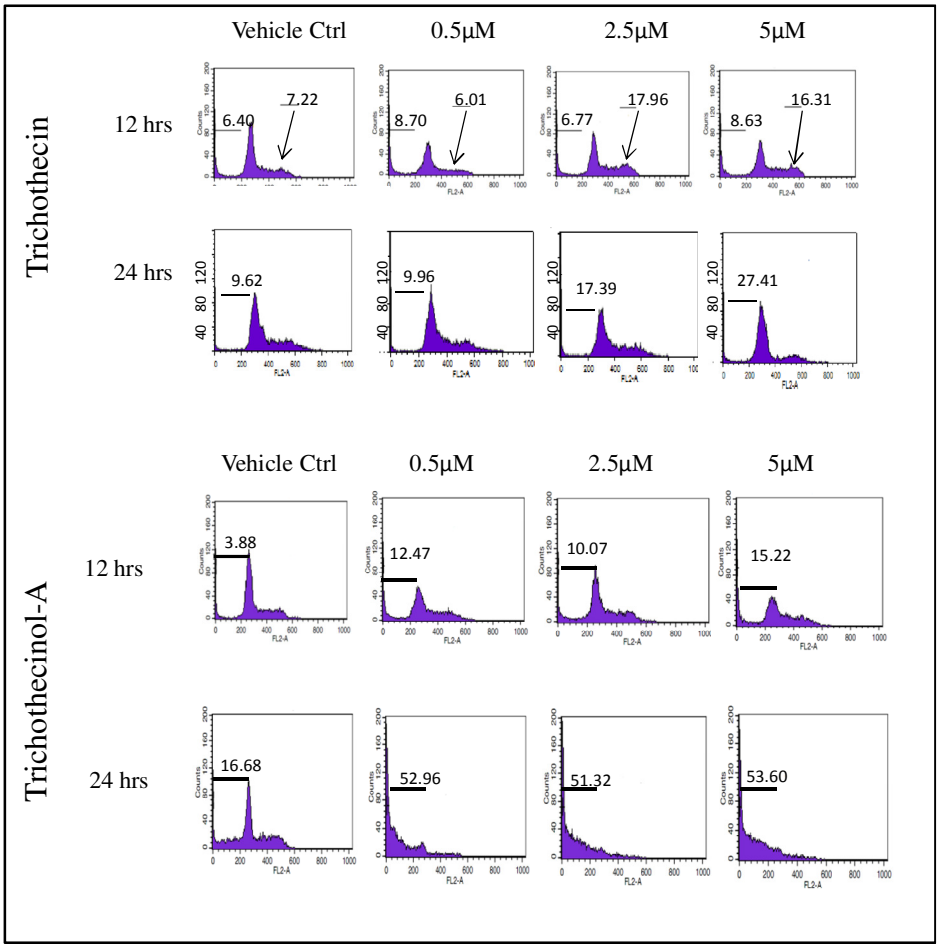


Fig 3: Cell cycle analysis of B16F10 cells by Flow Cytometry: a & b, B16 F10 cells were treated with DMSO as vehicle control or with Trichothecin or Trichothecinol-A at concentrations of 0-5 µM for 12 and 24 h respectively and cell cycle distribution was determined by measuring DNA content using flow cytometry.

Trichothecin treatment did not show any significant sub G_0 fraction in these cells at 12h but with incubation of 24h resulted in an increase in sub G_0 population to 17.39 % and 27.41% at 2.5 and 5µM concentrations respectively. We observed a significant increase in G2/M population at 2.5 and 5 µM doses of Trichothecin at 12h indicating a G2/M arrest.

Discussion:

Antifungal activity of Trichothecin and Trichothecinol-A against different plant pathogenic, saprophytic fungi and yeasts revealed that Trichothecin is a potent antifungal compound than Trichothecinol-A with the exception of *Penicillium expansum* NCIM 939. In contrary to earlier reports, we for the first time demonstrate the antifungal activity of Trichothecinol-A. Trichothecinol-A showed prominent activity against yeast *Cryptococcus albidus* var *diffluens* NCIM 3371 and 3372 upto 16 µg/mL. Cytotoxicity studies suggests that both Trichothecin and Trichothecinol-A induce cellular cytotoxicity. Trichothecinol-A exhibited significant toxicity in HeLa and B16F10 cells from a starting concentration of 500 nM whereas Trichothecin induced a similar effect at 25µM concentration. Further, cell cycle analysis revealed that Trichothecinol-A treated cells had higher percentage of apoptotic fractions compared to Trichothecin treated cells at 12 and 24h time points. Additionally, Trichothecin treated cells showed a significant increase in G2/M populations at 12h clearly suggesting a G2/M arrest. A wound closure inhibition study in MDA-MB-231 cells again revealed that Trichothecinol-A was able to inhibit wound closure upto 50% at 500 nM and is more potent than Trichothecin. Taken together these studies suggest that Trichothecin and Trichothecinol-A showed significant and cell specific antiproliferative and antimetastatic activity. Presence of single -OH group at 3' position makes Trichothecinol-A more hydrophilic and hence potent antiproliferative and antimetastatic compound as compared to Trichothecin but, the lack of same -OH group enhances antifungal potential of Trichothecin than Trichothecinol-A. Trichothecinol-A is showing promising antimetastatic activity against human breast carcinoma (MDA MBA-231) and antiproliferative activity against skin carcinoma (B16F10) at nanomolar concentration. Trichothecin and Trichothecinol-A could not be used as drug owing to their higher toxicity but could be used as template for combinatorial chemistry enabling the generation of libraries of analogs, which might have enhanced drug like properties (e.g. pharmacokinetics, low toxicity, specificity,

solubility, etc.). Further studies are required to understand the mechanism by which these compounds inhibit cancer cell growth and metastasis.

This is the first report of antifungal activity exhibited by both the compounds Trichothecin and Trichothecinol-A isolated by us from the endophytic fungus *Trichothecium* sp. derived from medicinal plant *Phyllanthus* sp. Antifungal activity of Trichothecinol-A extracted by other researchers using *Trichothecium roseum* isolated from plant infected by the fungus was evaluated against a range of fungi and yeast, but unfortunately could not inhibit the growth of the same. Contrary to earlier reports, Trichothecinol-A isolated by us showed prominent antifungal activity against filamentous fungi and yeast and is hence the first report of its kind. This compound, apart from having potential of being used as a drug, may also be used as a food preservative and against food borne diseases which are a serious issue worldwide. Our observations are supported by the fact that Trichothecin isolated by other researchers from *Trichothecium roseum* also exhibited antifungal activity against a range of filamentous fungi and a different genera of yeast.

Anti-cancer studies conducted on both these compounds showed that they significantly inhibit cancer cell migration and can thus be developed as a new class of anti-metastatic drug. This is the first report of anti-metastatic activity exhibit by both these compounds isolated by us. Both the compounds also showed anti-proliferative activity.

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Chapter 6

General discussion and conclusions

Endophytic fungi are symbionts residing within plant species which are now being explored as an alternate source of valuable compounds of plant origin. Endophytic fungi were isolated from the leaves, stem and roots of *Phyllanthus* sp. obtained from Pune, India. Each fungal culture was checked for purity and transferred to agar slants by hyphal tip as well as single spore isolation method. From the fungal population, only the slow growing and unusual fungi were considered for further study. Stock cultures were maintained by subculturing at monthly intervals. After growing at a pH of 7 and 25 ^{o}C for 7 days, the slants were maintained at 15 ^{o}C. From an actively growing stock culture, sub-cultures were made on fresh slants and after 7 days incubation at pH 7 and 25 ^{o}C, they were used as the starting material for fermentation experiments.

Production of bioactive secondary metabolites by the thirty endophytic fungi isolated from different plant parts of *Phyllanthus* sp. was studied by two stage of fermentation procedure. In the first stage these fungi were grown in MGYP medium as submerged culture and in the second stage they were grown in modified s7 medium as stationary culture. After 3 weeks of incubation, the culture was harvested and passed through four layers of muslin cloth to separate the mycelial mat from the culture filtrate. Both the culture filtrate & mycelia were lyophilized to dryness and extracted thrice with equal volumes of ethyl acetate each time. The extracts were pooled and dried with anhydrous sodium sulphate and concentrated at 40 ^{0}C *in vacuo* to yield crude extract. Antifungal activity against *Aspergilus niger* was used as marker assay for detection of bioactive molecules. The crude extracts derived from fermentation of all endophytic cultures were subjected to above mentioned bioassay. One culture was found to show good activity against *Aspergilus niger* and was selected for further studies.

For studying the cultural and morphological characters, the fungus was grown on PDA. 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 Ziess Axiovert 25 Inverted microscope and Nikon Eclipse E200). Mycelia, conidiophores and conidia produced from the fungus in culture were examined under a microscope. The fungus showed slight variation in growth rate when grown on various nutrient media. Results showed that the fungus can be grown on a variety of natural as well as

semi-synthetic media. Among the four different media tested, it was found that V-8 Juice Agar was the best for the growth of the fungus followed by PDA, OMA media and CMA. Excellent sporulation was recorded in PDA and OMA media. Good sporulation was observed in CMA medium and V-8 Juice medium.

Genomic DNA of the fungal culture was extracted by using the Salting out method with some modifications. The quality of the DNA which was isolated was checked on 0.7 % agarose gel stained with ethidium bromide. ITS regions from the fungal strain were amplified using PCR with a Robocycler GRADIENT 96, Stratagene, USA. The primers used were (forward primer) **ITS1-TCCGTAGGTGAACCTGCGG** and (reverse) **ITS4- TCCTCCGCTTATTGATATGC.** The resulting PCR products were analyzed on 1 % agarose gel containing ethidium bromide. Extraction of the fragment DNA was carried out by the protocol as described in Axygen^{TM} GEL elution kit, Biosciences, USA. The gel-eluted fragment was ligated into pGEM-T vector (Promega, USA.). Competent E. coli XL 1 Blue cells were used for transformation. Positive colonies were picked after screening by blue/white selection (disruption of lac Z gene). The alkaline lysis method of Sambrook *et al.* was improvised upon so that samples are processed conveniently for Plasmid DNA extraction. Purified plasmid DNA was given for sequencing. The complete internal transcribed spacer (ITS) sequence of isolate was searched for homology with representative genebank nucleotide sequences using online NCBI blast tool. The internal transcribed spacer (ITS) sequence analysis alongwith morphological and cultural studies confirm that the isolate belongs to genus *Trichothecium.*

Endophytic fungi were isolated from the leaves of *Phyllanthus* sp. plant obtained from different areas of Pune and then screened for trichothecin production. Out of 30 fungal cultures screened, only one AAP-PS-1 was found to produce trichothecin extracellularly in appreciable amounts. Homogeneity of the purified trichothecin was determined by TLC on three different solvent systems. RP-HPLC analysis performed on a Waters model using a C_{18} symmetry pack column with a flow rate of 0.5 ml/min, and the eluting compounds were detected by a dual mode wavelength detector set at 220nm and 240nm. The 1D and 2D NMR (^{1}H, COSY, NOESY, TOCSY, ^{13}C, DEPT, ^{13}C-^{1}H HMBC, ^{13}C-^{1}H HSQC), ESI-MS, HRMS, IR and UV-Vis show conclusively

that the isolated compound was Trichothecin. One liter of *Trichothecium* sp. yielded 4.25 mg of Trichothecin.

Endophytic fungi were isolated from the leaves of *Phyllanthus* sp. plant obtained from different areas of Pune and then screened for trichothecinol-A production. Out of 30 fungal cultures screened, only one AAP-PS-1 was found to produce trichothecinol-A extracellularly in appreciable amounts. Homogeneity of the purified trichothecinol-A was determined by TLC on three different solvent systems. RP-HPLC analyses performed on a Waters model using a C_{18} symmetry pack column with a flow rate of 0.5 ml/min and the eluting compounds were detected by a dual mode wavelength detector set at 220nm and 240nm.The 1D and 2D NMR (^{1}H, COSY, NOESY, TOCSY, ^{13}C, DEPT, ^{13}C-^{1}H HMBC, ^{13}C-^{1}H HSQC), ESI-MS, HRMS, IR and UV-Vis show conclusively that the isolated compound was trichothecinol –A (31). One liter of *Trichothecium* sp. yielded 4.37mg of Trichothecinol-A.

Antifungal activity of Trichothecin and Trichothecinol-A against different plant pathogenic, saprophytic fungi and yeasts revealed that Trichothecin is a more potent antifungal compound as compared to Trichothecinol-A with the exception of *Penicillium expansum* NCIM 939. In contrary to earlier reports, we for the first time demonstrated the antifungal activity of Trichothecinol-A. Trichothecinol-A showed prominent activity against yeast *Cryptococcus albidus* var *diffluens* NCIM 3371 and 3372 up to 36µg/mL and 20µg/mL respectively. MIC and IC_{50} values were determined as described earlier.

Cytotoxicity studies indicate that B16F10 and HeLa cells show enhanced sensitivity to Trichothecinol-A and Trichothecin as compared to MDA-MB-231 cells. Trichothecinol-A induced about 50% cell death in HeLa and B16F10 cells as compared to about 25% in MDA-MB-231 cells at 500 nM concentrations. Trichothecin has relatively less effect on MDA-MB-231 cells at lower concentration as compared to HeLa or B16F10 cells. Cytotoxicity studies suggests that both Trichothecin and Trichothecinol-A induce cellular cytotoxicity. Trichothecinol-A exhibits significant toxicity in HeLa and B16F10 cells from a starting concentration of 500 nM whereas Trichothecin induced a similar effect at 25 µM concentration.

Enhanced migratory capacity is one of the hall marks of highly invasive tumor cells. We studied the inhibitory effect of Trichothecin and Trichothecinol-A in MDA-MB-231 cell migration at various concentrations. Percentage of wound closure was determined by the difference in area covered by migrated cells in control versus treated with Trichothecin or Trichothecinol-A at 0 or 18h. The results show that 500 nM of Trichothecinol-A was able to inhibit wound closure by 50% where as 2.5 and 5µM concentrations of Trichothecinol-A inhibited migration upto 75% as compared to vehicle control. Trichothecin was less effective in inhibiting MDA-MB-231 cell migration at 500 nM, as compared to Trichothecinol-A but showed increased inhibition at higher concentrations.

The effect of Trichothecin and Trichothecinol-A on B16F10 cell death was performed by cell cycle analysis. Significant increase in the sub G_0 population was observed in Trichothecinol-A treated cells in a time dependent manner. 500 nM of Trichothecinol-A had 12.7% sub G_0 population as compared to 3.88% in vehicle control in 12h. However, cells treated with 500 nM of Trichothecinol-A for 24h resulted in an increase of apoptotic fraction to 52.96% versus 16.68% in vehicle control. Trichothecin treatment did not show any significant sub G_0 fraction in these cells at 12h but with incubation of 24h resulted in an increase in sub G_0 population to 17.39 % and 27.41% at 2.5 and 5µM concentrations respectively. We observed a significant increase in G2/M population at 2.5 and 5 µM doses of Trichothecin at 12h indicating a G2/M arrest.

To sum up the entire work done in this thesis, we have isolated two sesquiterpenes viz. Trichothecin and Trichothecinol-A produced by an endophytic fungus *Trichothecium* sp. isolated from medicinal herb *Phyllanthus* sp. Both the compounds were purified and characterized by using different chromatographic and spectroscopic techniques. Presence of single -OH group at C-3 position makes Trichothecinol-A more hydrophilic and hence potent antiproliferative and antimetastatic compound as compared to Trichothecin; but the lack of same enhances antifungal potential of Trichothecin. Trichothecinol-A exhibits promising antimetastatic activity against human breast carcinoma (MDA MBA-231) and antiproliferative activity against skin carcinoma (B16F10) at nanomolar concentrations. Also, Trichothecinol-A **showed** good antifungal activity against *Cryptococcus albidus* var *diffluens* (NCIM 3371 and

3372) and *Penicillium expansum* (NCIM 939). Therefore, these molecules could be used as scaffold in drug discovery programmes to synthesize effective and more specific analogues which can be further developed as drugs. Further studies are required to understand the mechanism by which these compounds inhibit cancer cell growth and metastasis.