ANTIFUNGAL DRUGS SUSCEPTIBILITY AGAINST *ASPERGILLUS TERREUS* AND *ASPERGILLUS FLAVUS*

Dissertation submitted in partial fulfilment of the requirement for the degree of

MASTERS OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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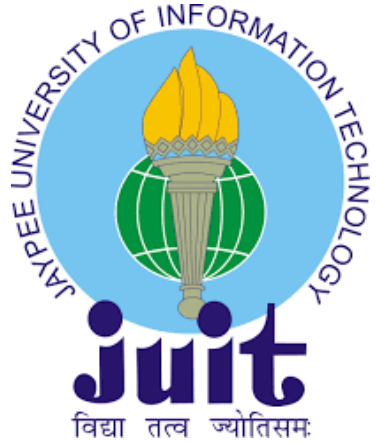
UNDER THE GUIDANCE OF

DR. JATA SHANKAR



JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

MAY 2018

DECLARATION

I hereby declare that the work reported in this M.Tech thesis entitled **"ANTIFUNGAL DRUGS SUSCEPTIBILITY AGAINST *ASPERGILLUS TERREUS* AND *ASPERGILLUS FLAVUS"*** submitted at Jaypee University of Information Technology, Waknaghat India**,** is an authentic record of my work that was carried out under the supervision of **Dr. Jata Shankar**. I have not submitted this work elsewhere for any other degree or diploma.

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CERTIFICATE

This is to certify that the work which is being presented in the project title, **"ANTIFUNGAL DRUGS SUSCEPTIBILITY AGAINST *ASPERGILLUS TERREUS* AND *ASPERGILLUS FLAVUS* "** for the end semester of Masters of Technology and submitted in Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat is an authentic record of work carried out by Deepak Kashyap (162556) during a period from July 2017 to May 2018 under the supervision of **Dr. Jata Shankar**, Associate Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat.

The above statement made is correct to the best of my knowledge.

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M.Tech Biotechnology

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List of abbreviations

S.no.	Abbreviations	Full form
1	IA	Invasive Aspergillosis
2	PBS	Phosphate Buffer Saline
3	PBST	Phosphate Buffer Saline Tween
4	DNA	Deoxyribose Nucleic Acid
5	RNA	Ribose Nucleic Acid
6	PDA	Potato Dextrose Agar
7	PDB	Potato Dextrose Broth
8	ng/ml	Nanogram per microlitre
9	µl	Microlitre
10	Amp b	Amphotericin B
11	PCR	Polymerase Chain Reaction
12	BMT	Bone Marrow Transplantation
13	MIC	Minimum Inhibitory Concentration

14	*A. Terreus*	*Aspergillus terreus*
15	*A. Flavus*	*Aspergillus flavus*

ABSTRACT

Aspergillus terreus resistant to Amphotericin B antifungal drug and is associated with significant morbidity and mortality in immune compromised patients. Local incidence is influenced by the density of airborne *Aspergillus spp*. spores. *Aspergillus terreus* which is a fungus a pathogenic fungus which is filamentous capable of producing different types of conidia which are very small in size and flask in shape mainly present at the tip of filaments. Aspergillus isolates able to cause many diseases in humans, plants and animals. It causes infections in individuals who lacks proper function of immune system and immunosuppressive patients like asthmatic patients having respiratory tract problems in breathing it can also cause the death of the individual and in case of cancer patients and in case of organ transplantation and in case of HIV patients. Whereas in case of plants it destroys crops like nuts, pulses, wheat, rice soya, maize etc. To check the effect of drugs on the following strains of *Aspergillus species "Aspergillus terreus* (NCCPF860035) and *Aspergillus flavus* (MTCC 11866)*"* were used at check at different duration of time with different concentration of drugs "Fluconazole and Itraconazole" and also MIC calculated of these antifungal drugs.

CHAPTER – 1

INTRODUCTION

INTRODUCTION

Fungus word is taken from Latin word *fungus* which means “mushroom” and the Greek word *sphongos* which means “sponge”. Eukaryotic creature the filamentous fungi include microorganisms such as “moulds”, “yeasts”. Following organisms are comes below fungi “kingdom” and their specific characteristics separate them from other kingdoms eukaryotic organisms such as presence of chitin in their cell wall which is not present in the cell wall of plants and cell membrane of animals. Fungi are heterotrophy like animals and photosynthesis is also absent, so they acquire their food by absorbing dissolved molecules with the help of producing secreting digestive enzyme. *Aspergillus* is one of the well explored genus comprising economically, medically and industrial organism.

Aspergillus terreus which is a fungus a pathogenic fungus which is filamentous capable of producing different types of conidia i.e. asexual conidia which are of two types in this case of *Aspergillus terreus* one is “Phialide type conidia” which are very small in size and flask in shape mainly present at the tip of filaments and the another one is the “accessory conidia” which emerge laterally from the hyphae [3].

Aspergillus terreus is beneficial in many productions of products in many industries i.e. production of lovastatin, gliotoxin and bioethanol etc. *Aspergillus terreus* cause damage in agriculture and also creates problem in human health. It is also the reason behind the loss of crops, million tons of many crops face loss because of this pathogenic strain it causes loss in crops of rice, crops of maize, crops of soya bean, crops of potato, crops of wheat [3]. *Aspergillus terreus* acts a significant character in industry in the production of many organic acids and also help in the production of secondary metabolites such as lovastatin which is an agent of anti-hypercholesterolemia. It also causes spoilage of the food mainly of the cereals, nuts in different climatic conditions mainly in the tropical and subtropical regions. *Aspergillus terreus* takes place in the production of variety of mycotoxins [4].

Aspergillus terreus is the third most filamentous pathogenic strain which causes invasive aspergillosis in humans [4]. In filamentous fungi *Candida* species are the most common species which causes mycotic infections which is caused by invasive

opportunistic. Also the *Aspergillus* species are responsible for invasive infections cause which leads to the increase in the mortality all over the world. At present the treatment which is available failed to find out the problems creating by these filamentous species. Amphotericin B remains the standard drug used against fungal infections and for therapy purpose but the results are not up to the mark because of resistance of these species because of prolonged used of this drug, so there must be alternative drugs to cure the infections [5].

Last few years the diseases caused by fungus have increased at a very high rate due to lack of proper medication and the failure of available medicines because of resistance by their prolonged used. It will become serious because these fungal infections infect the person with weak immune system like HIV patients, asthmatic patients and also in case of organ transplantation which leads to the death of the patients. As the drug Amphotericin B is well known and standard drug for all kind of fungal infection, but at present the drug is not workable on all kind of infection because there are more than thousands species of fungus is available in our environment and these species now becoming resistant to this drug. New drugs azoles and echinocandin and caspofungin and micafungin are discovered and the research is still in process on these drugs, but the main problem with these drugs are that as fungi and humans both are eukaryotic organisms and the antifungal treatment is long term and these drugs show cytotoxic effect which harms also human cells and to overcome this problem we can use plant derived secondary metabolites (phytochemicals) with these drug to decreases the concentration of drug given to the patients so that the cytotoxic effect will be very less and useful to treat the patients properly.

CHAPTER - 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 *Aspergillus*

Aspergillus is a type of fungus which is filamentous and this species is present in our environment in soil and in air all over the world. It is useful to some industries and has clinical applications also [6]. Variety of filamentous fungi present in our environment cause diseases to both animals wild as well as domestic and main reason behind the loss of crops by causing diseases in different crops in tropical and subtropical regions, and in case of humans infects those who have weak immune system or immunosuppressive patients [7]. *Aspergillus* is second most diseases causing species. There are hundreds of identified and known species of *Aspergillus* and after year by year new strains are characterized from environment which are resistant against the available drugs [8]. All the species of *Aspergillus* are not dangerous some of the species are useful but in all some species like *A. Terreus, A. flavus, A. Niger, A. fumigatus* are mostly responsible for the infections [9]. The main habitats of these species are soil and air. Once they come in the contact of air the reproduction of the spores and hyphae takes place and hundreds of spores inhaled by an individual and they remain alive for longer period of time and if the individual is immunocompetent than it will not good for the individuals health especially in case of HIV patient and the individual who is taking immunosuppressive drugs or asthmatic patient [10].Because of showing resistant against Amphotericin B *Aspergillus. terreus* becomes the most infection causing strain causing mostly invasive aspergillosis (IA) [11]. Three types of diseases is caused by these species in case of humans which are one is infection caused by invasion of the spores second one is without invasion can be caused by colonization and the third one is due to the weak immune system [9]. Mostly the asthmatic patients faces the problem of fungal infection because after invasion very firstly spores and hyphae attacks on our respiratory tract and creates problem for the asthmatic patients and also the patients who has tuberculosis diseases and also the cancer patients because of therapies and the chemotherapy and the immune suppressive drugs given to them [11].

2.2 *Aspergillus terreus* taxonomy

Kingdom : Fungi
Phylum : Ascomycota
Order: Eurotiales
Family: Trichocomaceae
Genus: *Aspergillus*
Species : *terreus*
Strain: NCCPF 860035

2.3 *Aspergillus terreus* taxonomy

Kingdom: Fungi
Division: Ascomycota
Class: Eurotiomycetes
Order: Eurotiales
Family: Trichocomaceae
Genus: *Aspergillus*
Species: *A. flavus*
Strain: BT-05(MTCC 11866)

2.4 Distribution and morphology of *Aspergillus. terreus*

Aspergillus. terreus is present in all over the world. It is present in the soil and the air in tropical and subtropical regions. *Aspergillus. terreus* is present in the environment in the form of conidia and spores which can grow from room temperature to the high bearable temperature ranges from 11 to 48° C and also present in the sand and salt marsh due to its tolerance to the NaCl higher concentration, that is why it is also used in industries in the production of some organic products. *Aspergillus terreus* which is a fungus a pathogenic fungus which is filamentous capable of producing different types of conidia i.e. asexual conidia which are of two types in this case of *Aspergillus terreus* one is

"Phialide type conidia" which are very small in size and flask in shape mainly present at the tip of filaments and the another one is the "accessory conidia" which emerge laterally from the hyphae. These are very tiny in size so that humans are inhaling them daily because they always remain in the environment at any temperature pressure and concentration and causing infections [4].

2.5 Diseases and infections caused by *Aspergillus. terreus*

Eukaryotic creatures Fungi are right through in our surroundings, with the entire of the satisfactory human breath approximate to manage up to 10 fungal spores. A broad improvement of these mediator bound infects epidemic of yield and other flora, and confined to a small area or total infectious sleaze in bond, bug and badlandserness and cultivated unattractive species. Eukaryotic creature's fungal organisms are more and more recognizable as a matched ingredient of the tuskers micro biome, trophobiosis of the fungal area is connected by all of a sierra of causes. Though, in relationship to viral, bacterial, or parasitic pathogens, it realises distinctly tiny of the fungal sickness, the pathological processes and tissue paleopathology linked to fungal cause, and the character of the reaction of the immune to those organisms. Eukaryotic creature's fungal organisms are typically seemed as speculator infectious agents – infects in immune deficient or immune suppressed persons, instance, humans through number one immune deficiency or hematologic chaos otherwise the ones undergo treatment for cancer and creature medically immune suppressed. Effective cures for infection caused by fungus are small number in quantity and around are fresh distress approximately growth of conflict to a few cure conflicts is linked to a change in the fungal Cyp51 gene and the elevated occurrence can also relate to greater significant and additional extended expression use of azoles pills in person medication and in crop manufacturing [7].

2.5.1 Invasive aspergillosis (IA)

"IA" is a complete contamination because of aspergilli within immune compromised sufferers. Invasive aspergillosis has grow to be a most important reason of dying, mostly in the middle of hematologic victims the common occurrence of "Invasive aspergillosis (IA)" is approximate 10 to 26% in victims with acute leukaemia, 6 to 11%

following allergenic “Bone marrow transplantation”, and 0.6 to 6% following cytotoxic healing of blood illnesses or autonomous “Bone marrow transplantation” and solid-organ transplantation. Amongst “*Aspergillus”* species, “*A. fumigatus”,* “*A. flavus*” and “*A.terreus”* are the major reason of IA. “Invasive aspergillosis (IA)” which track solid-organ transplantation is maximum not unusual in heart-lung transplant victims (18 - 25%) and is situated, in minor order, in liver, coronary heart, lung, and kidney recipients (1 to 10%) not unusual in heart-lung transplant victims (18 - 25%) and is situated, in lesser order, in liver, coronary heart, lung, and kidney recipients (1 - 11%).

Acute Bronchopulmonary Aspergillosis a form of allergic reaction ensuing from species like “*Aspergillus”*. ABPA is one of the most brutal difficulties of respiratory tract and often get position in people having allergies and unnatural with cysts and fibrosis.

2.5.2 Acute Bronchopulmonary Aspergillosis

“Aspergillus. fumigatus” is the foremost aspergilli infect Acute Bronchopulmonary Aspergillosis but currently “*Aspergillus.terreus”* has too received significance as a causal agent of Acute Bronchopulmonary Aspergillosis. Acute Bronchopulmonary Aspergillosis in patients with asthma and patients with monogenic diseases is characterised as an eosinophilia, pulmonary opacity and bronchial asthma. Readily available are varied re-evaluate that guide “*Aspergillus. terreus”* as an approaching purpose of Acute Bronchopulmonary Aspergillosis. Publications of Acute Bronchopulmonary Aspergillosis headache are parallel to the allergies having precise pathophysiological situation resulting from “t-cell” immune reaction. But the Acute Bronchopulmonary Aspergillosis is left over natural; it ends in failure of the respiratory in infected persons. As a consequence, analysis of Acute Bronchopulmonary Aspergillosis is vital, and also its miles imperative to recognize the function of lung epithelial cells for the duration of their interplay with aspergilli for better treatment [14, 15].

2.5.3 Mycetoma

Mycetoma is largely a globe of initial mycelia of fungi that develop in pre-existing hollow of lungs. These hollow are occurring following *Mycobacterium tuberculosis* and its cure, lungs issues and disruptive sinuses. Mycetoma is not a invasive form. Mycetoma can as well widen within immune competent mass. It includes fungal hyphae which are able to be fixed in protein template contain sporulating fungal shape on the outer edge within hollow space of internal lungs. Mycetoma stated once haemoptysis, which is as a result of rupturing of blood vessel, occasionally it is able to be lethal [16, 17].

2.6 Diagnosis

Inspection of the lung tissue samples from lungs are the usual techniques for prognosis of "Invasive aspergillosis". "Invasive aspergillosis" (IA) prognosis stays a hard painting, and its miles difficult to early Diagnose IA in immune compromised sufferers [18]. Histopathological test of the tissues of the lungs allowed finding out the hyphae in combination through very well technique of life of the sample of biopsy for species of "*Aspergillus"* [19]. Though the explanation of histological exam can differ by affected individual type includes the ones holding transplantation and cancers. In calculation the immune compromised persons including sputum section measured for invasive aspergillosis, particularly persons having leukaemia or experienced stem movable transplantation [20]. Cultures of blood of organisms or victims of invasive aspergillosis barely still unbelievable and do not have huge use [21]. The terrific section of the sputum since immune competent host is not important because species of "*Aspergillus"* are not continuously exposed with a clinical indication in immune competent hosts [22]. Moreover trunk nine radiographs are not significant for confirmation of "Invasive aspergillosis" before time, since unlike filamentous fungal contamination of lungs also gives same radiographs of trunk in combination with *Mycobacterium tuberculosis* [23]. This time the technique of elisa i.e. sandwich elisa are using for the recognition of additives from aspergilli all along with "beta-glucan" and "glactomannan". Most recent development approved that the revealing of antigens of aspergilli in body fluids [24]. And FDA has practised the "glactomannan" recognising assay of elisa technique

purposefully to identifying the IA with threshold rate of 0.49 ng/ml concentration in the patients' serum. PCR is a technique used to diagnose this invasive aspergillosis, which detected the *Aspergillus* DNA in patients or victims serum [27, 28]. The sensitivity and the specificity of this attempt ranged from 56-96%. Principal barriers behind this assay not very much sensitivity wrong effects of outcomes because of the presence of glactomannan in food commodities which are impossible to tell apart among other fungi inclusive of "fusarium" and "zygomycetes" [25, 26]. Essential disadvantage about this method is that it can't be decide in migration and infectivity of aspergilli. Similarly, the approach of this is restricted to the reserved laboratory. There also require to widen different or unique species method of diagnosis. The FDA in addition accepted that the recognition of beta-glucan within patients serum of inflamed patients, and also take a look at is exceptionally sensitive and matchless to approach across profound fungal's invasive infections. Nevertheless, its miles for all time really useful to have diagnosis of serology at the side of the trunk radiographs since a few situations copying the Mycetoma situations together with granulomatosis, neoplasm through polyangiitis and cyst which is hydatid. But, at rest it's far tough to identify the "aspergilli" at the variety level, with a purpose to further assist for higher therapeutic remedies. The prognosis of Mycetoma is totally based on the trunk radiographs jointly with methods of serology. The trunk radiograph lacking an uncertainty which shows the prevalence of fungal hyphae in existing cracks. For the analysis of ABPA, radiography and the serological checks is used. In results of medical, the ABPA victims repeatedly have asthma, pain in the chest, fever, and out of breath. The criteria used to diagnose ABPA is character with allergies, fantastic casing take a look at for *Aspergillus* antigens, high level of Ige in serum [29- 32].

2.7 *A. terreus* infections Treatment and diagnosis

2.7.1 Therapy of invasive aspergillosis

Due to the unique property of aspergilli it is hard to deal with the invasive aspergillosis with the fact that makes *Aspergillus* species differ and susceptible to the pills of antifungal [33]. Final decade, the voriconazole supporter drug was used to take care of the invasive aspergillosis together with other drug like Amphotericin b (amp b) [34, 35].

The opportunity treatment is lipid formulation of amp b and micafungin [36]. No matter antifungal remedy of invasive aspergillosis, the consequences are disappointing; with excessive mortality charge starting from distinctive efforts is for setting up for the medical breakpoints (cbps) for antifungal capsules likewise azoles, echinocandin and amp b [37]. Last couple of years, eu committee on susceptibility of antimicrobial trying out to set up the ruin point for drugs of antifungal for their susceptibility and their resistance to *Aspergillus* species [39]. Though cbps is not present in those capsules for combinational use, and their significances are yet incompletely unstated. The choice of antifungal drug for the treatment of IA from many years was amp b. Though, "*A. terreus"* has resistance against this drug and also the reason and the mechanism behind resistance isn't always known. Freshly, it has been stated that the heat-shock proteins and the strain which are oxidative can also contain in *A. terreus* resistance in opposition to amp b. The minimal inhibitory awareness of amp b for a. terreus is >2 mg/l. accordingly, amp b has bad efficacy towards invasive aspergillosis caused by a. terreus. In addition, amp b has critical aspect consequences consisting of reduction in filtration, low level of potassium in the circulating blood, toxicity to the liver and toxicity to the cell. The experimental studies have counselled the extreme charge of failure of amp b treatment against "Invasive aspergillosis (IA)" resulting from *A. terreus*. Similarly, the fats components of amp b widely not increased the success of this drug therapy. As a consequence, amp b isn't always a preference of solution for the infection of *A. terreus*. The azoles drug "Voriconazole" is the most important cure against "Invasive aspergillosis" infected by *Aspergillus. terreus*. The azoles drug "voriconazole" failure of the treatment is in contrast to other tablet shaving a malfunction price is 64%. The "*Aspergillus. terreus*" obtained the resistance against the azoles drug "voriconazole". "*Aspergillus. terreus"* gets a mutation in cyp51 ends up in high MIC of this drug in opposition to "*Aspergillus. terreus"*. The other alternatives of medicine used for the remedy of "Invasive aspergillosis" due to "*Aspergillus. terreus"* are "posaconazole" and "echinocandin" and those drugs are accounted as better capable against "Invasive aspergillosis" as a result of "*Aspergillus. terreus"*. The rate of unsuccessful of cures of antifungal drugs for the therapy of "Invasive aspergillosis" because of "*Aspergillus. terreus"* is complicated. Victims who infects from "Invasive aspergillosis" due to "*Aspergillus. terreus"* are immunocompromised and on co-mediation. Therefore co-

meditation results interactions between drugs and drugs that provide you by the failure of treatment of antifungal [37-43]

2.7.2 Acute Bronchopulmonary aspergillosis therapy

"Acute Bronchopulmonary aspergillosis (ABPA)" or different disorders like hypersensitive aspergillosis all along among allergy and allergic rhinitis and the influence of allergic sinusitis individuals carrying allergies or cystic fibrosis. And they are able to affect immunocompetent host. The treatment by using steroids permits in discount of Ige and eosinophils. So to deal with and manipulate allergic aspergillosis drugs like oral steroidal is used to suppressing the inflammatory or hyper immune response. The problems like hypersensitive are the problems particularly takes place because of reactions of hyper-immune or response of inflammatory immune. Primary hazard of corticosteroids use for immunosuppressant, it makes a person's susceptible to fungal infections. Alongside steroids, azoles drug "Itraconazole" additionally gives to the patients infected with ABPA to reduce the load of aspergilli. Now a day, voriconazole tried with steroids and its use notably improve the ABPA circumstance in cystic fibrosis and asthmatic patients [44, 45].

2.7.3 Latest drugs against fungal infection caused by *Aspergillus species* Infections

The top mortality and dreariness of contaminations on account of contagious pathogens, particularly *Aspergillus* species are identified with constrained antifungal medications and the poisonous quality to sufferers. The most focuses for antifungal cases are cell divider or layer added substances alongside interpretation hindrance. Correspondingly, to choose new medication objectives for antifungal tablets contrary to parasitic pathogens are troublesome because of contagious cell likenesses with human cells. Likewise, most extreme of the pathogenic organisms got the protection systems against existing antifungal medications by method for advancement of contagious bio films furthermore, finished articulation of efflux pump protein. Consequently, there's a need of most recent procedures to enhance antifungal cure, for example, by means of developing new arrangement of existing medications, disclosure of most recent tablets and higher supplier atoms like nano particles can be used to supply an antifungal

medication at the locales of disease. Thusly, it's miles the need of time to inquire about novel medication targets contrary to contagious pathogens. Calcineurin flagging pathway developed as another hostile to aspergillus objective. Calcineurin protein is identified with initiation of calcineurin flagging pathway that related with different organic advancements in aspergilli or previous contagious infectants which incorporate candida. It likewise manages the biosynthesis of ergosterol, β-glucan and chitin. of late, triphenylethylene prompted a new intensify that represses the calcineurin pathway with the guide of following up on calmodulin, which actuates calcineurin moreover, in definite decade new antifungal plans and shape changes had been utilized to grow significantly less noxious antifungal tablets. It controls the contagious versatile morphogenesis, strain response and antifungal protection. A spinoff of amp b, n-methyl-n-d-fructosyl amp b methylester has been progressed with significantly less danger to people. Echinocandin are the most recent antifungal tablets that have been acknowledged for antifungal medications. Additionally new azoles mixes were combined to manage aspergillosis what's more, to battle protection a. fumigatus strains which incorporate pc945 and pc1244. This brilliance of pharmaceutical restrains the biosynthesis of portable divider glucan of parasitic pathogens. These mixes are intended to take through inward breath to battle obtrusive aspergillosis. In expansion, a novel echinocandin, cd101 has been developed to battle contrary to extreme contagious diseases which incorporates intrusive aspergillosis. Despite the across the board activities to control antifungal protection or on the other hand grow new antifungal mixes and revision in current cases are deficient to determine poisonous quality of pharmaceutical and protection get by method for contagious pathogens. In any case, this medication is beneath restorative improvement and indicated promising outcomes against candidemia. Besides, another novel antifungal compound is underneath clinical improvement for the cure of lethal contagious contaminations. Consequently, there's a need of chance treatment alternatives in blend with antifungal cases alongside immunotherapy.

2.8 *Aspergillus flavus*

Aspergillus flavus is a filamentous saprophytic fungus. It is present in all over the world in our environment. This strain is most common and mostly this strain contaminated our food products like cereals, nuts, walnuts, maize, rice and many more. It produces

harmful toxic aflatoxins. Before the collection of the crops and after the collection of crops it causes diseases in the crops like yellow mould in the peanuts and ear rot in the corn crops. The contamination by this strain sees in the crops in the storage time also because of the moisture and the temperature which is high. It causes cancer in the humans mainly the liver cancer and also creates problems in the respiratory tract of the humans and also the animals. This strain is greenish in colour. Also this strain contaminated our other food products. The germinating time of this type of fungus is 24-48 hours.

2.9 Azoles antifungal drug

2.9.1 Azoles drugs history

In 1939 the first azoles drug was agent of antifungal was reported and in the year 1944 and 1949 the first azoles drug was discovered which was used clinically. Then after first azoles drug the drug and the well known antifungal drug Amphotericin B was discovered in 1960. After that in the year 1969 two more drugs was discovered miconazole and clotriconazole and in the year 1974 the drug econazole was discovered. In the year 1980 more drugs were discovered after the large scale use of penicilin. Then for 10 years these drugs were used then the Fluconazole and Itraconazole were introduced in 1990s. That time there were increase in the immunocompressive patients and individual in the HIV patients and asthmatic and the individuals with organ transplantation.

2.9.2 Mode of action and mechanism of drug

These antifungal drugs azoles inhibits the production of the ergosterol which is an important component of plasma membrane and inhibits the $cycp_{450}$ dependent enzyme. This enzyme plays a very important role in the production of cholesterol in individual. Introducing of azoles against these fungal infection deletes the ergosterol of slow down its process so that it will not multiplies in the individuals' body. Also the drug

breakdowns the structure of the ergosterol so that it will not work properly and functions of the ergosterol disables.

2.9.3 Fluconazole

Fluconazole is an antifungal drug. The molecular weight of the fluconazole is 306.277 g/mol, and the molecular formula of the fluconazole is $C_{13}H_{12}F_2N_6O$. The biological half life of this drug is 20-40 hours. This drug is used for antifungal infections. It can be secreted from human milk. It can be given up to 400mg/day for an adult.

2.9.4 Itraconazole

Fluconazole is an antifungal drug. The molecular weight of the fluconazole is 705.641 g/mol, and the molecular formula of the fluconazole is $C_{35}H_{38}Cl_2N_8O_4$. Its mainly comes in the capsules form and vaccines also available. It can be given up to 300mg/day for an adult.

2.9 Phytochemicals

Phytochemicals are the secondary metabolites of the plants. These are good for human's consumptions because these are not toxic to humans. So we can use them with these drugs to decrease the concentration of these drugs. These can be used with drugs or can be used as antifungal separately.

CHAPTER - 3

OBJECTIVES

OBJECTIVES

- To check the inhibition of *Aspergillus terreus* (NCCPF 860035) in the presence of antifungal drugs.
- To calculate the MIC50 of each antifungal drugs against *Aspergillus terreus* (NCCPF 860035).
- To check the influence of these antifungal drugs on terrelysin gene expression.

The main rationale of the project is that as the prolong use of these drugs the different strains of filamentous fungi which are toxic become resistant to these drugs. So new drug or some innovation with drugs needed. As fungi and humans both are eukaryotic organisms so that when these drugs are used against any fungal diseases and cause done by fungi these antifungal drugs are use at very high concentration and the time interval and the duration of the treatment is 1-2 years so these drug of action shows cytotoxic effect which harms individuals cells also so to lower down the concentration of the drug we can use plant derived secondary metabolites phytochemicals with these drugs to decrease the concentration of the drug.

CHAPTER - 4

MATERIALS AND METHODS

MATERIALS AND METHODS

4.1 Culture growth

- Preparation of PDA HiMedia, Pvt, Ltd, Mumbai India (Potato Dextrose Agar) plates and PDA (Potato Dextrose Agar) slants.
- Cultures of *Aspergillus terreus* (NCCPF 860035) and *Aspergillus flavus* (MTCC 11866) (revive from older cultures) were grown on potato dextrose agar slants and potato dextrose agar plates.
- Incubation for 72 hours at 37°C.
- Stored at 4°C for longer period of time.

Fig 1: culture growth plate of *Aspergillus Terreus*

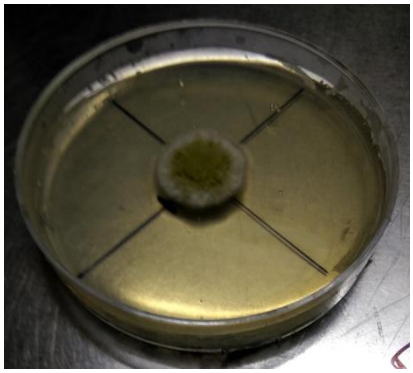


Fig 2: culture growth plate of *Aspergillus flavus*

4.2 Conidia harvesting

- Revive culture from older cultures and spread on the PDA plates and slants.
- Incubation at 37°C for 72 hours without disturbing the plates and slants.
- Harvest conidia after 72 hours in PBST (phosphate buffer saline tween buffer) PBST(Sodium Chloride 8g/l, Potassium Chloride 0.2g/l, Sodium Hydrogen Phosphate 1.42g/l, Potassium dihydrogen Phosphate 0.24g/l)
- 3 times PBS washing.
- Stored in PBS for longer period of time at 4°C.

4.3 Antifungal Agents and phytochemicals

The azoles derivatives used in the present learning were Fluconazole and Itraconazole. And the polyene was Amphotericin B and the phytochemicals used were Ascorbic acid and Quercetin. At concentrations of 100 times the stock solution were prepared in the respective highest concentration to be tested, and were stored at 4°C till use. The azoles used were dissolved in distilled autoclaved water and phytochemicals Ascorbic acid and querecetin in 90 % methanol and DMSO respectively.

4.4 Cells count haemocytometer

It is a cell counting instrument which can be used manually. It is made up of a thick glass slides and rectangular in shape. The cells are calculated by observing under microscope by observing the columns and rows both side and count the cells present in the column and then calculated by the formula and get cells 1×10^6

$$concentration\ of\ cells\ in\ original\ mixture = \{(\frac{number\ of\ cells\ counted}{proportion\ of\ chamber\ counted\ \times\ volume\ of\ square\ counted}) \times (\frac{volume\ of\ diluted\ sample}{volume\ of\ original\ mix\ of\ sample})$$

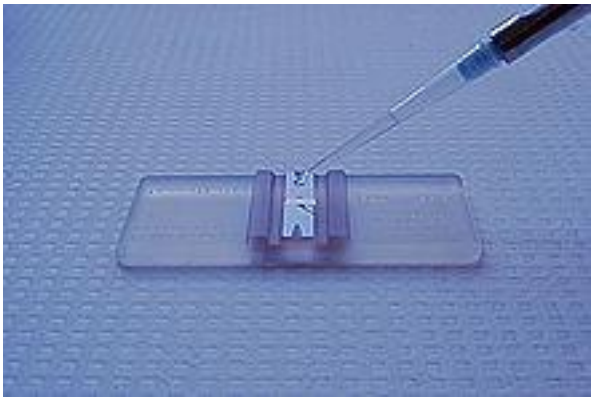


Fig 3: haemocytometer (adapted from internet source)

4.5 Antifungal susceptibility assay (Mycelial inhibition testing)

- After calculating the cells by using haemocytometer preparation of PDA plates was done with different concentration of drugs adding to it.
- Inoculate conidia at the centre of the plate equal volume to each by taking one as control which is without drug.
- All the work was done under aseptic condition in laminar air flow hood.
- Incubation of plates for different interval of times i.e. 24, 48 and 72 hours was done respectively.
- Diameter was taken after different interval of times 24, 48 and 72 hours to calculate the Mycelial inhibition by using the formula

$$Mycelial\ inhibition = \{(\frac{control - test}{control}) \times 100$$

4.6 Minimum inhibitory concentration assay (MIC)

MIC in case of fungus determined by the method which beat spectators' partiality and count the hyphae of the fungi. Because of only turbidity we cannot measure the hyphae possibly present in, there must be a quantitative method to count the hyphae, so the best and workable method is by (MTT) 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide assay. MTT is yellowish in colour and it slash by dehydrogenases enzyme in the mitochondria and other cellular site, and it gives purple than which can

be observed under spectrophotometer at 550 nm. MTT is cut by all living, metabolically on the go fungi free of production and irrespective of unicellular or multicellular growth and is a compute of metabolic action. A technique based on MTT implemented for susceptibility testing of fungi. The MTT method confirmed brilliant harmony with the standard macro dilution way for the antifungal susceptibility testing of yeasts. In direct to conquer the failing of optically MIC reason for filamentous fungi and to extend a unfailing, slanted, and less uneven technique for the antifungal susceptibility testing of filamentous fungi, assess the performance of a technique using MTT and evaluate the results.

4.7 qRT PCR

4.7.1 Isolation of RNA

- Conidia were harvesting after 24 hours incubation in PDB for both drug treated and without drug.
- Culture was taken and homogenize with pestle and mortar after freeze drying in liquid nitrogen.
- Trizol reagent was added and 5 min incubation at room temperature.
- Chloroform was added (0.5ml / 1ml of trizol) under the fume hood.
- Tube was shaken for 10-15 min minutes by hand after adding chloroform and followed by incubation at room temperature for 5 minutes.
- The samples were centrifuged for 15 minutes at 4°C at 12,000 rpm.
- Upper aqueous phase were taken and transferred to fresh tube.
- Precipitation of RNA was done from aqueous phase by mixing with isopropanol alcohol (0.5mp / 1ml of trizol).
- Incubation of sample at room temperature for 10 minutes.
- Centrifuged at 12,000rpm for 10 minutes at 4°C.
- Supernatant was discarded.
- RNA pellet was washed 2 times with 70% of ethanol.
- Centrifuged for 5 minutes at 7500 rpm at 4°C.
- Supernatant was discarded and the RNA pellet at the bottom of the tube was observed.

- Pellet was dried at room temperature for 10-20 minutes and dissolved in DEPC treated water.
- Incubation at 37°C for 5 minutes.
- Stored at -80°C for longer period of time.

4.7.2 Agarose Gel electrophoresis

Agarose gel electrophoresis was performed to check the RNA and cDNA on 1.2% gel.

- Weighing 1.2 g of agarose and add it to the 100ml of TAE buffer.
- Hot the flask till agarose dissolved properly in the buffer.
- Leave it to cool down temperature which can be bearable and then EtBr was added and the comb was put up in the casting tray and then slowly pour the gel in the casting tray and then leave it to solidified.
- After solidification the casting tray was put in the assembly and then loads the sample with ladder and then run the gel and visualised in gel doc.

4.7.3 cDNA synthesis

4.7.3.1 Components of cDNA synthesis (Thermo Fischer kit)

S.no.	Components	Volume (µl)
1	5x primer script buffer	2
2	Rtase	0.5
3	Oligo dT	1
4	dNTP	1
5	Rnase inhibitor	0.5
6	Rnase free water	4
7	Template (RNA)	1

Table 1: cDNA synthesis kit components

4.7.3.2 Steps of cDNA synthesis

At 65°C the PCR vials were incubated in the water bath for about 5 min.

- The reaction mixture was prepared for both the samples by adding the following components.

- Template RNA primer mixture
- 5X Prime Script Buffer
- RNase inhibitor
- Prime Srcipt RTase
- Depc treated water/ nucleus free water

- Then these vials were mixed and positioned in the thermal cycler to bring out cycle for the synthesis of cDNA by following the steps and time-temperature profile.

- 30°C used for 10 min.
- 42°C used for 1 hour.
- 95°C used for 5 minutes.
- Cool down.

- At the end, we acquired cDNA and the product we check on gel doc by following PCR reaction and run on gel electrophoresis and check on gel doc whose amplification had to be carried out.

4.7.3.3 Quantitative Real time PCR

Components of real time PCR

s.no.	Components	Volume(µl)
1	Syber green	6.5
2	Reverse primer	0.5
3	Forward primer	0.5
4	cDNA	1
5	Autoclaved distilled water	12.5

Table 2: qRT PCR components

Protocol of qRT PCR

- Step 1: 95°C for 00:10 seconds
- Step 2: 55°C for 00:45 seconds
- 35 cycles.

CHAPTER – 5

RESULTS

RESULTS

5.1 Antifungal Susceptibility test

Antifungal Susceptibility test was performed on the PDA plates. After giving the incubation at 37°C at different interval of times the diameter of the growth of the conidia was calculated to calculate the mycelial inhibition. Azole drug Fluconazole was inhibiting the *A.terreus* but *A. flavus* was not affected under the presence of tested drug as observed in the results.

5.1.1 *Aspergillus. flavus* (MTCC11866) Antifungal Susceptible results with Azole drug Fluconazole after 24 hours

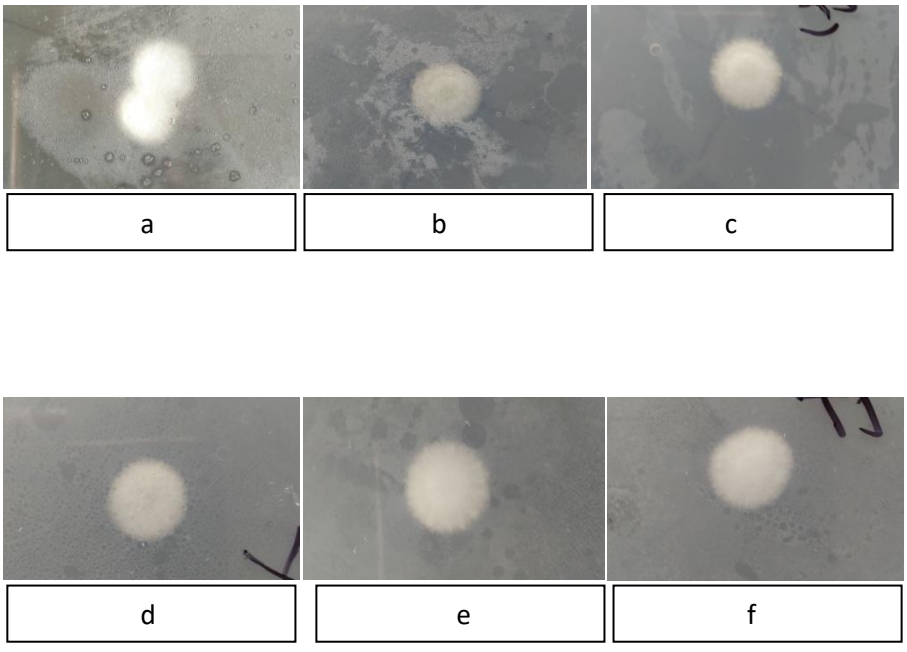


Fig 4: Mycelial inhibition by disk diffusion assay for *A.flavus* (MTCC11866) treated with fluconazole after 24 hours a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.1.2 *Aspergillus. terreus* (NCCPF 860035) Antifungal Susceptible results with Azole drug Fluconazole after 24 hours.

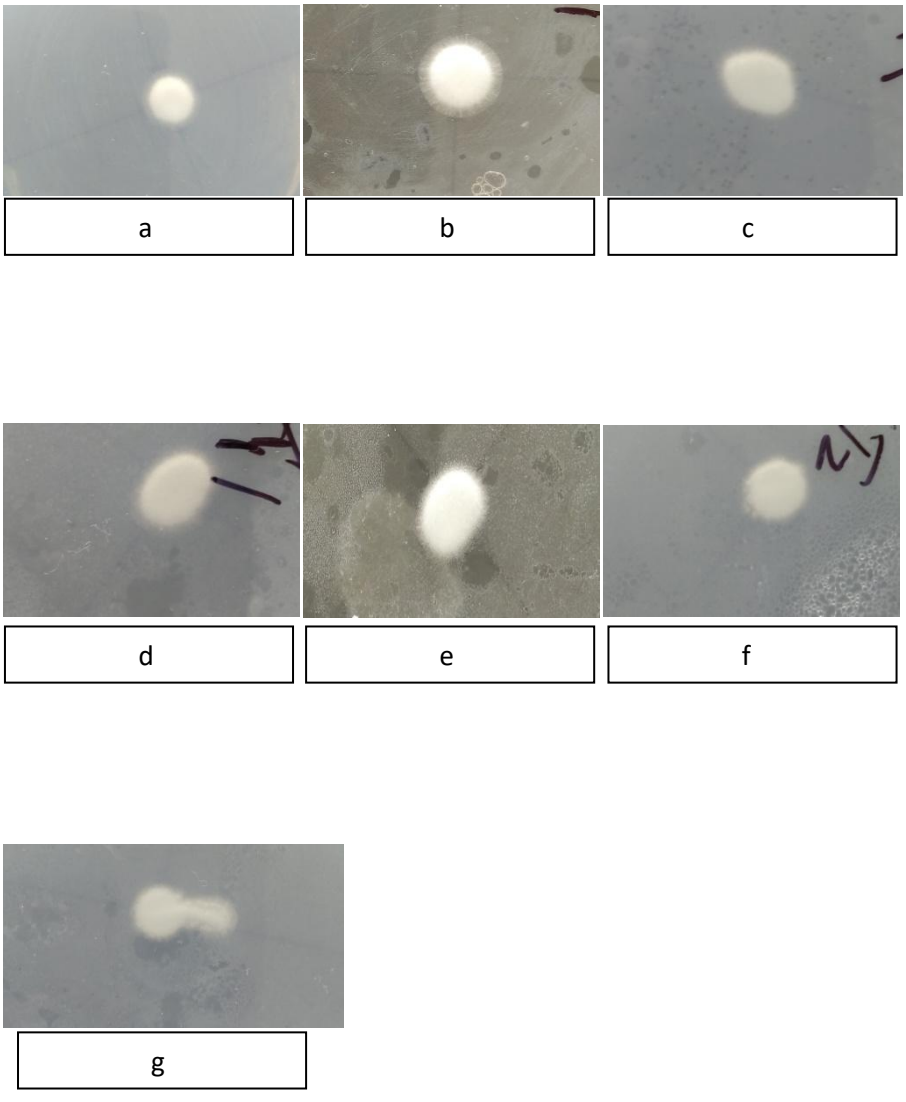


Fig 5: Mycelial inhibition by disk diffusion assay for *A.terreus* (NCCPF 860035) treated with fluconazole after 24 hour a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug g) 64 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.1.4 *Aspergillus. terreus* (NCCPF 860035) Antifungal Susceptible results with Azole drug Itraconazole after 24 hours.

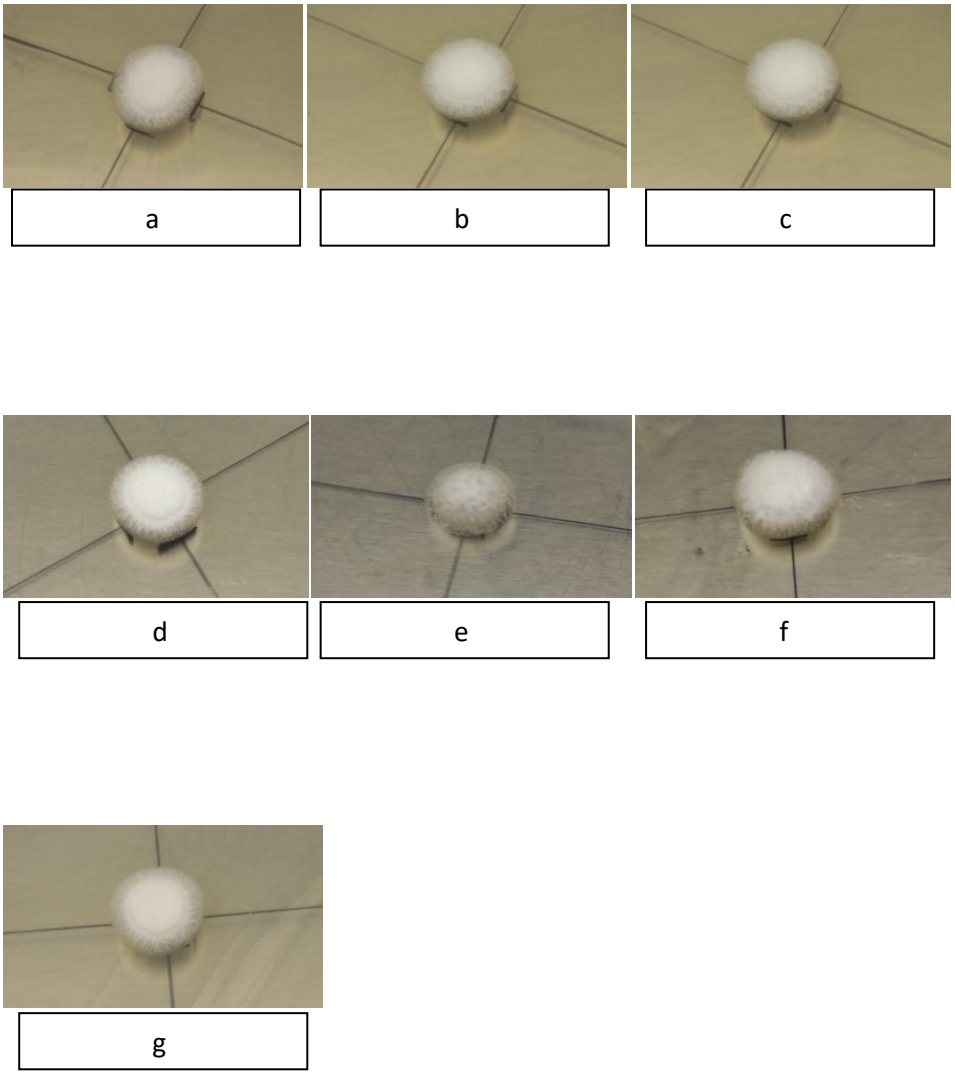


Fig 6: Mycelial inhibition by disk diffusion assay for *A.terreus* (NCCPF 860035) treated with fluconazole after 24 hour a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug g) 64 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.1.4 *Aspergillus flavus* (NCCPF 860035) Antifungal Susceptible results with Azole drug Itraconazole after 24 hours.

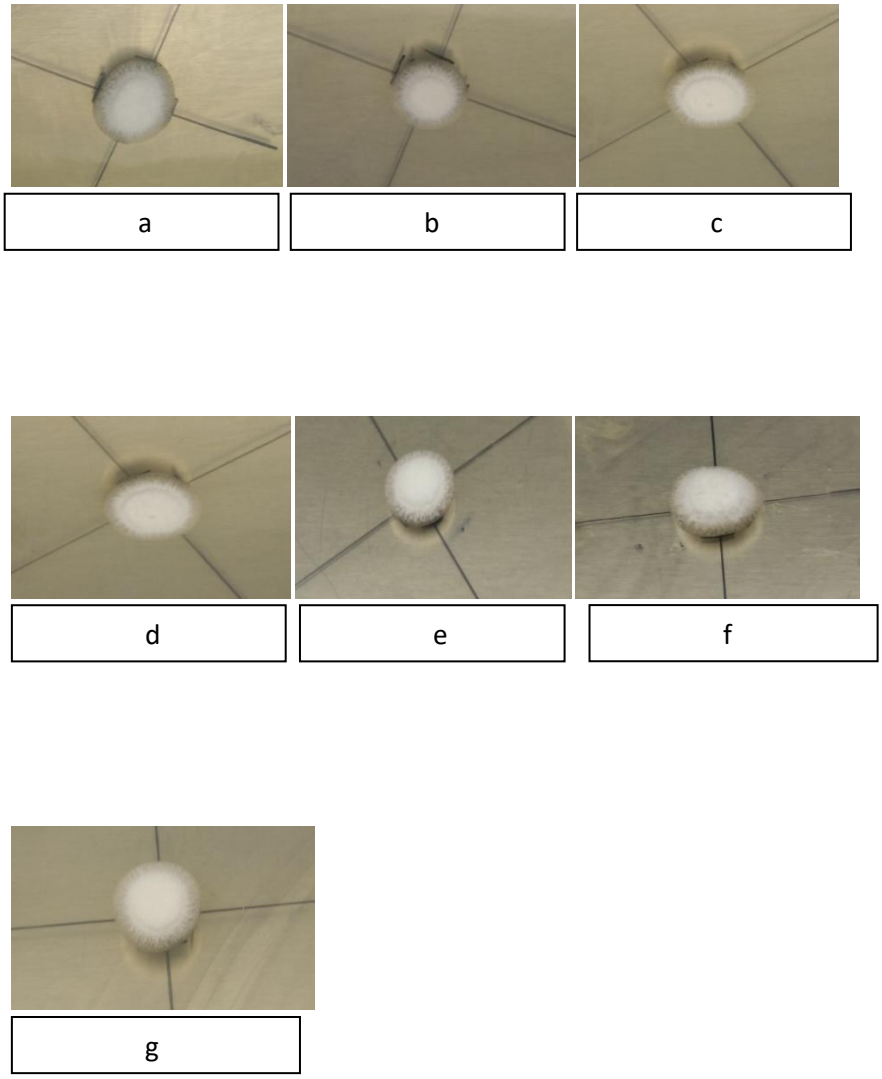


Fig 7: Mycelial inhibition by disk diffusion assay for *A.flavus* (MTCC 11866) treated with fluconazole after 24 hour a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug g) 64 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.1.5 *Aspergillus. flavus* (MTCC 11866) Antifungal Susceptible results with Azole drug Fluconazole after 48 hours.

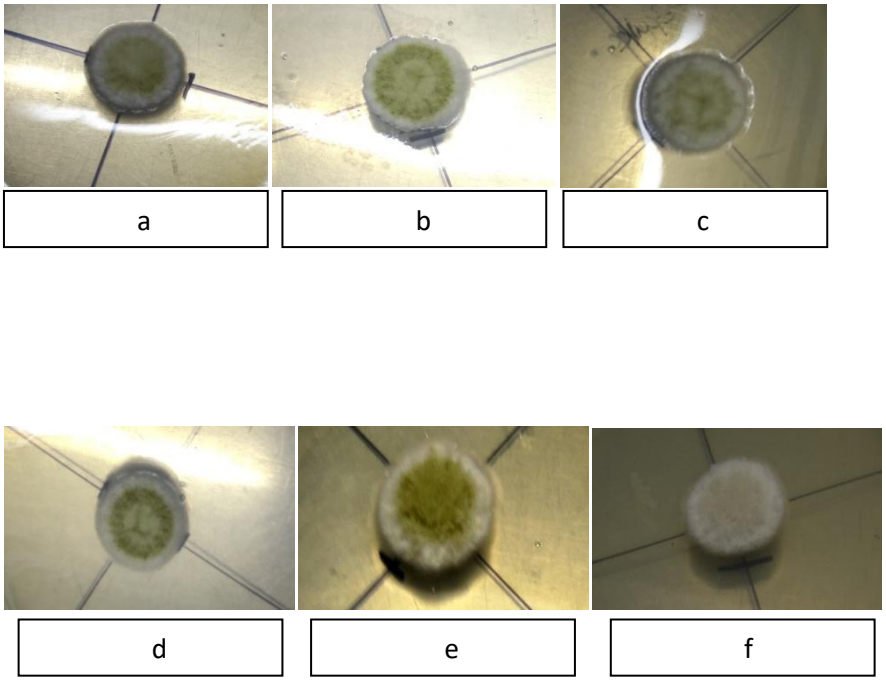


Fig 8: Mycelial inhibition by disk diffusion assay for *A.flavus* (MTCC 11866) treated with fluconazole after 48 hour a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.1.6 *Aspergillus. terreus* (NCCPF 860035) Antifungal Susceptible results with Azole drug Fluconazole after 48 hours.

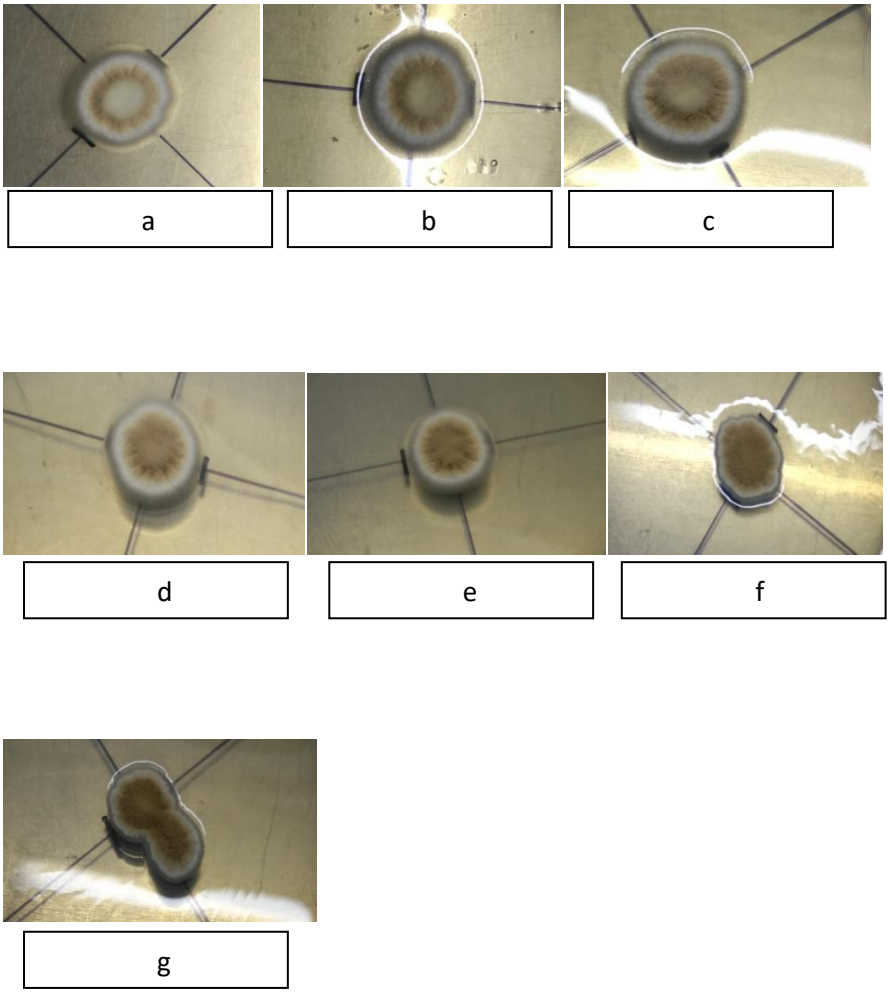


Fig 9: Mycelial inhibition by disk diffusion assay for *A.terreus* (NCCPF 860035) treated with fluconazole after 48 hour a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug g) 64 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.1.7 *Aspergillus. flavus* (MTCC 11866) Antifungal Susceptible results with Azole drug Itraconazole after 48 hours.

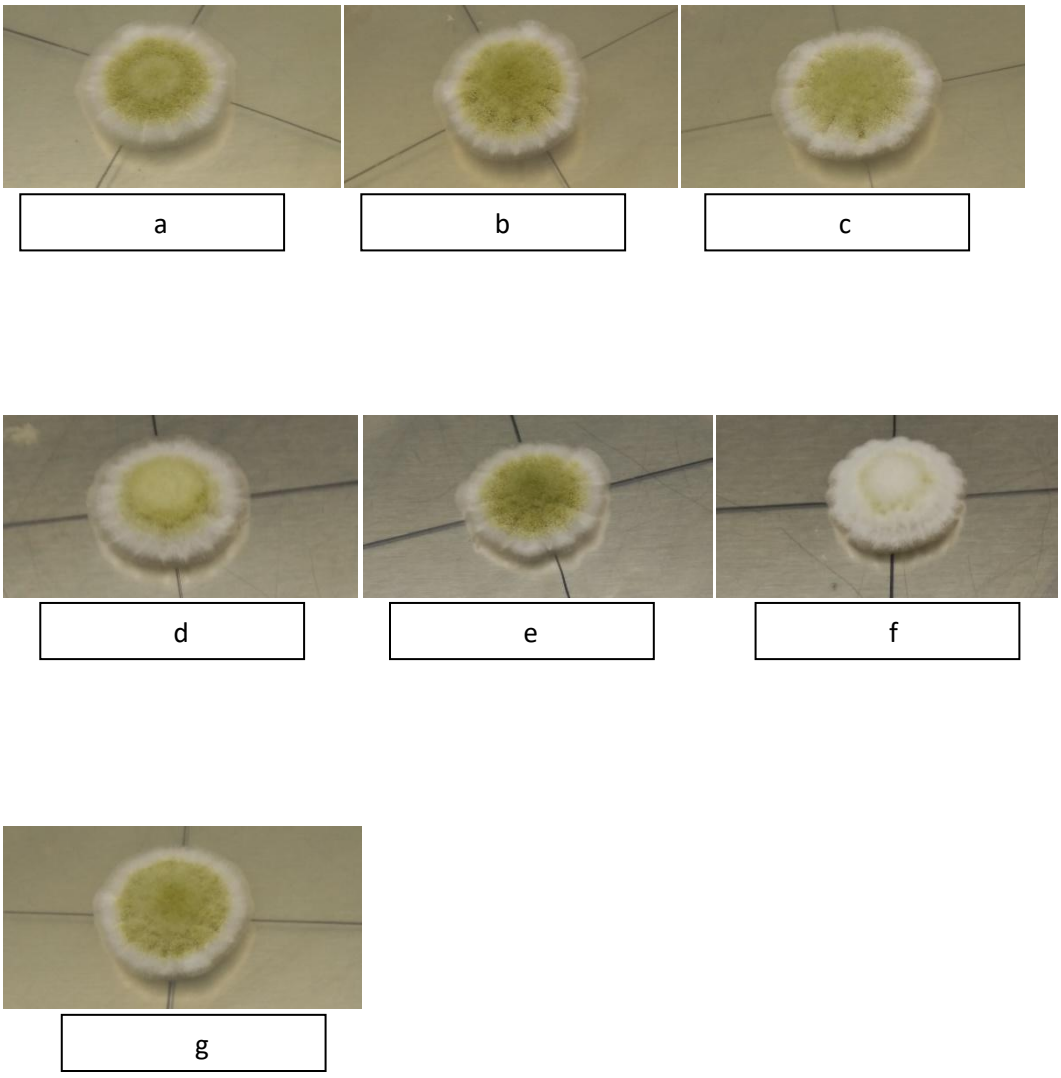


Fig 10: Mycelial inhibition by disk diffusion assay for *A.flavus* (MTCC 11866) treated with Itraconazole after 48 hour a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug g) 64 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.1.8 *Aspergillus. terreus* (NCCPF 860035) Antifungal Susceptible results with Azole drug Itraconazole after 48 hours.

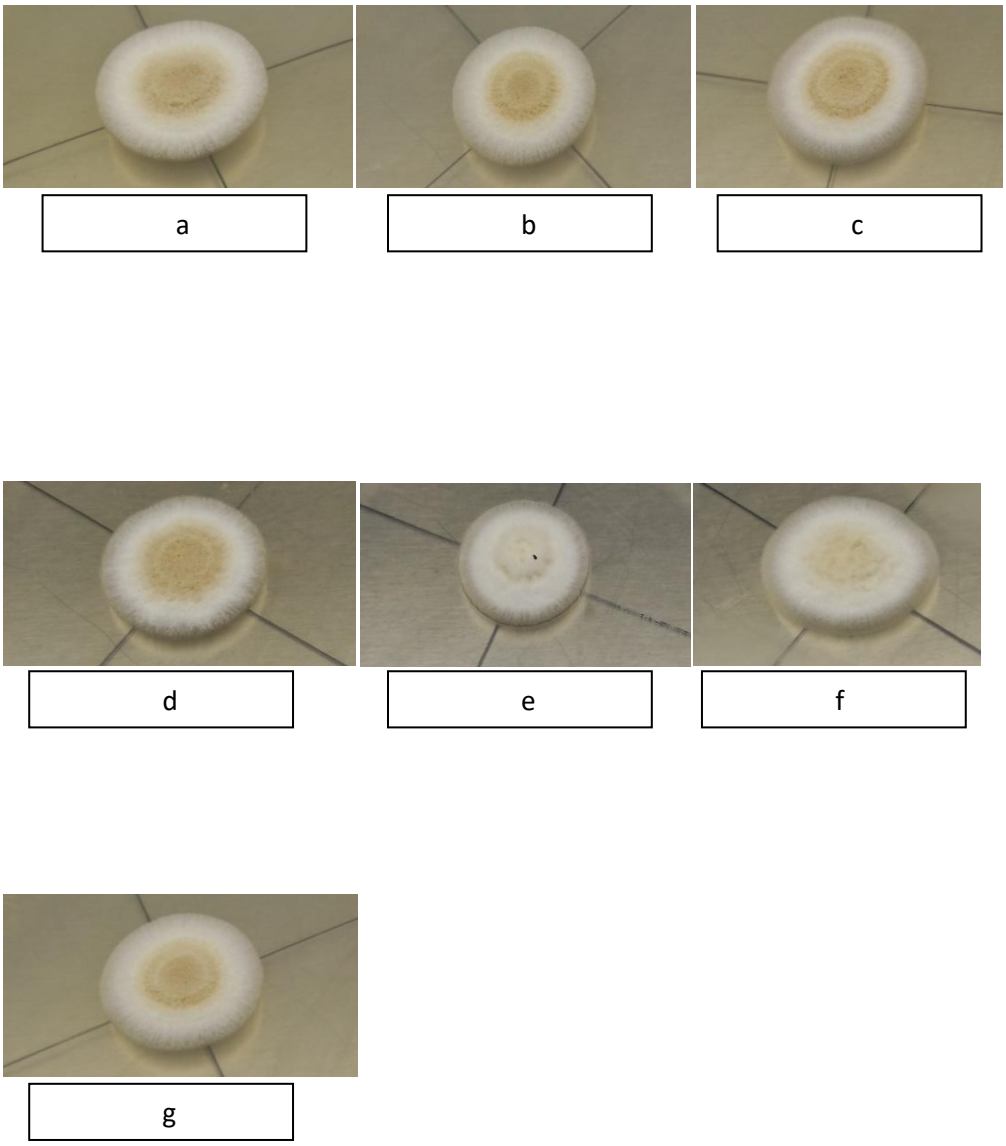


Fig 11: Mycelial inhibition by disk diffusion assay for *A.terreus* (NCCPF 860035) treated with Itraconazole after 48 hour a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug g) 64 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.1.9 *Aspergillus. flavus* (MTCC 11866) Antifungal Susceptible results with Azole drug Fluconazole after 72 hours.

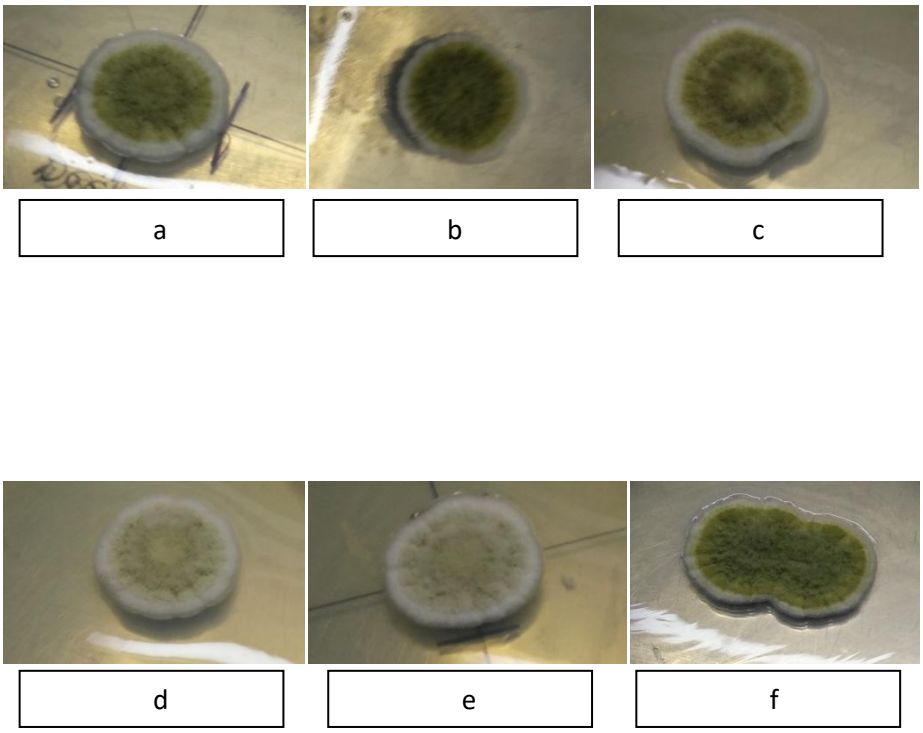


Fig 12: Mycelial inhibition by disk diffusion assay for *A. flavus* (MTCC 11866) treated with fluconazole after 48 hour a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.1.10 *Aspergillus. terreus* (NCCPF 860035) Antifungal Susceptible results with Azole drug Fluconazole after 72 hours.

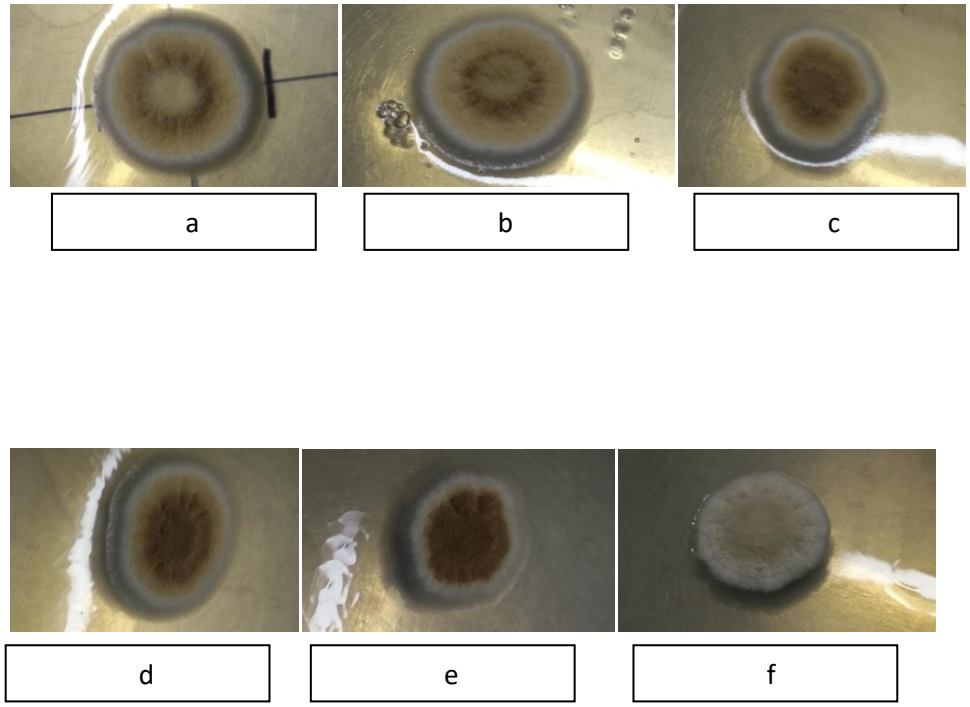


Fig 13: Mycelial inhibition by disk diffusion assay for *A. terreus* (NCCPF 860035) treated with fluconazole after 48 hour a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.1.11 *Aspergillus. flavus* (MTCC 11866) Antifungal Susceptible results with Azole drug Itraconazole after 72 hours.

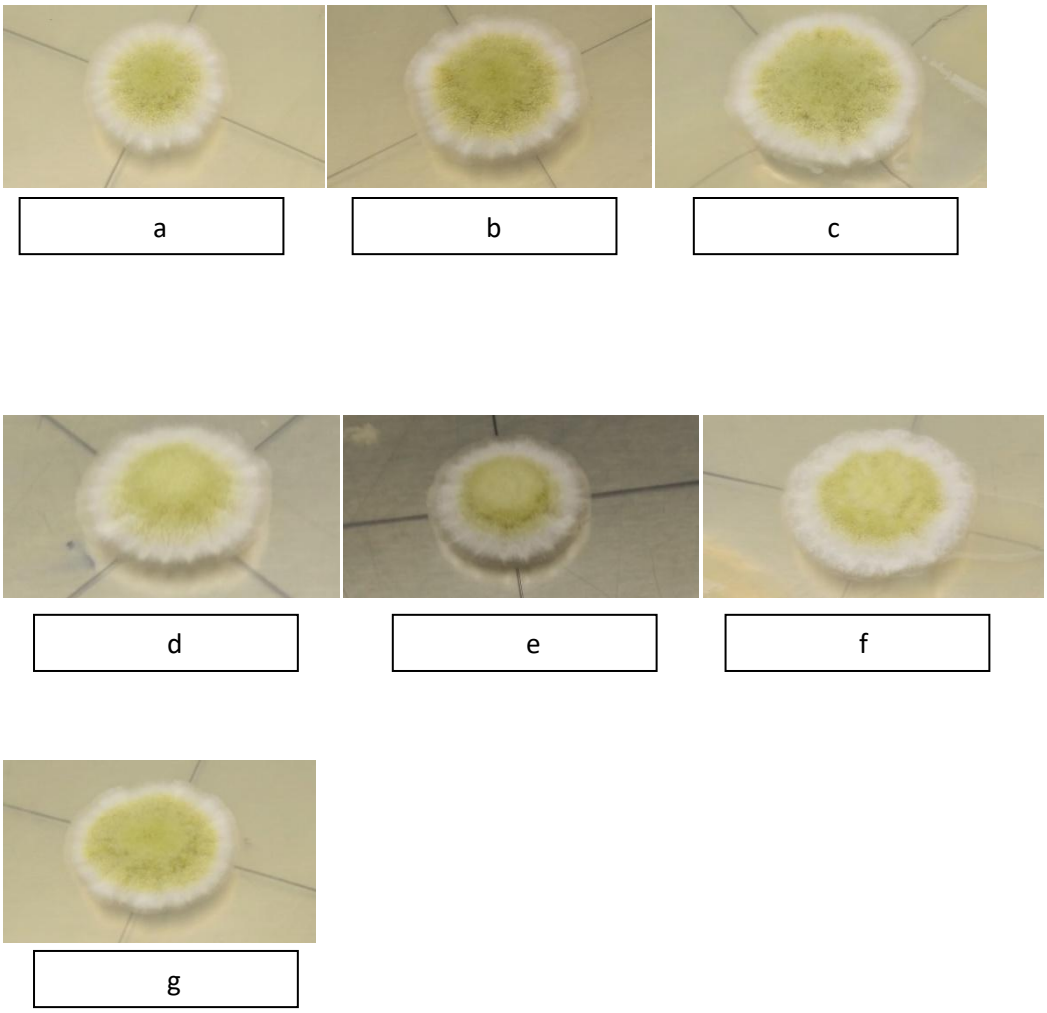


Fig 14: Mycelial inhibition by disk diffusion assay for *A.flavus* (MTCC 11866) treated with Itraconazole after 72 hour a) no drug b) 1 µg drug c) 4 µg drug d) 8 µg drug e) 16 µg drug f) 32 µg drug g) 64 µg drug. The assay was performed on Agar media. Mycelial inhibition was calculated by taking the diameter at different concentration with (a) as control and others at different concentrations of drug.

5.2 Mycelial Inhibition Results

Table 3: Fluconazole drug with *Aspergillus terreus* antifungal susceptibility test result after 24 hours observed by measuring the diameter and calculated by mycelial inhibition formula.

Fluconazole drug *Aspergillus terreus* After 24 hours				
Concentration	experiment1(mm)	experiment2(mm)	mean(mm)	mycelial inhibition %
0 µg	15	15	15	0
1 µg	15	15	15	0.00%
4 µg	13	13	13	13.33%
8 µg	11	12	11.5	23.33%
16 µg	11	11	11	26.66%
32 µg	11	11	11	26.66%
64 µg	10.5	11.5	11	26.66%

Table 4: Fluconazole drug with *Aspergillus terreus* antifungal susceptibility test result after 48 hours observed by measuring the diameter and calculated by mycelial inhibition formula.

Fluconazole drug *Aspergillus terreus* After 48 hours				
Concentration	experiment1(mm)	experiment2(mm)	mean(mm)	mycelial inhibition %
0 µg	24	24	24	0%
1 µg	23	24	23.5	2.08%
4 µg	23	21	22	8.33%
8 µg	20	22	21	12.50%
16 µg	17	17	17	29.16%
32 µg	12	13	12.5	47.91%
64 µg	10.5	11.5	11	54.16%

Table 5: Fluconazole drug with ***Aspergillus terreus*** antifungal susceptibility test result after 72 hours observed by measuring the diameter and calculated by mycelial inhibition formula.

Fluconazole drug *Aspergillus terreus* After 72 hours				
Concentration	experiment1(mm)	experiment2(mm)	mean(mm)	mycelial inhibition %
0 µg	32	34	33	0%
1 µg	33	30	31.5	4.54%
4 µg	30	29	29.5	10.60%
8 µg	28	27	27.5	16.66%
16 µg	27	27	27	18.18%
32 µg	22	23	22.5	31.81%
64 µg	20	20	20	39.39%

Table 3, 4 & 5: In the observation and results the highest mycelial inhibition is 26.66% after 24 hours and 54% after 48 hours and 40% after 72 hours. Which means that the drug is not that much effective or it may be not showing inhibition because of excipients present in the drug which slow down the metabolism of drug in the human's body. The MIC_{50} of this drug comes at 64 µg after 48 hours but after 72 hours the drug becomes less effective.

Table 6: Fluconazole drug with *Aspergillus flavus* antifungal susceptibility test result after 24 hours observed by measuring the diameter and calculated by mycelial inhibition formula.

Fluconazole drug *Aspergillus flavus* after 24 hours				
Concentration	experiment1(mm)	experiment2(mm)	mean(mm)	mycelial inhibition %
0 µg	17	17	17	0
1 µg	15	17	16	5.88%
4 µg	17	17	17	0.00%
8 µg	16	17	16.5	2.94%
16 µg	15	15	15	11.76%
32 µg	14	14	14	17.64%
64 µg	13	13	13	23.52%

Table 7: Fluconazole drug with *Aspergillus flavus* antifungal susceptibility test result after 48 hours observed by measuring the diameter and calculated by mycelial inhibition formula.

Fluconazole drug Aspergillus flavus after 48 hours				
Concentration	experiment1(mm)	experiment2(mm)	mean(mm)	mycelial inhibition %
0 µg	25	24	24.5	0%
1 µg	21	25	23	6.12%
4 µg	23	24	23.5	4.08%
8 µg	23	24	23.5	4.08%
16 µg	24	24	24	2.04%
32 µg	24	24	24	2.04%
64 µg	21	21	21	14.28%

Table 8: Fluconazole drug with *Aspergillus flavus* antifungal susceptibility test result after 72 hours observed by measuring the diameter and calculated by mycelial inhibition formula.

Fluconazole drug Aspergillus flavus after 72 hours				
Concentration	experiment1(mm)	experiment2(mm)	mean(mm)	mycelial inhibition %
0 µg	33.5	32.5	33	0%
1 µg	33	26	29.5	10.60%
4 µg	32	30	31	6.06%
8 µg	30	32	31	6.06%
16 µg	31	29	30	9.09%
32 µg	30	30	30	9.09%
64 µg	22	25	23.5	28.78%

Table 6, 7 & 8: In the observation and results the highest mycelial inhibition is 23% after 24 hours and 14% after 48 hours and 28% after 72 hours. Which means that the drug is not that much effective or it may be not showing inhibition because of excipients present in the drug which slow down the metabolism of drug in the human's body. It was observed that the drug is not effective against this strain (*A.flavus)* and this strain (*A.flavus)* is found to be resistant against this drug.

Table 9: Itraconazole with *Aspergillus flavus* antifungal susceptibility test result after 24 hours observed by measuring the diameter and calculated by mycelial inhibition formula.

Itraconazole *Aspergillus flavus* After 24 hours				
Concentration	experiment1(mm)	experiment2(mm)	mean(mm)	mycelial inhibition %
0 µg	14	14	14	0
1 µg	13	14	13.5	3.57%
2 µg	13	13	13	7.14%
4 µg	10	13	11.5	17.85%
8 µg	10	15	12.5	10.71%
16 µg	11	13	12	14.28%
32 µg	14	10	12	14.28%

Table 10: Itraconazole with *Aspergillus flavus* antifungal susceptibility test result after 48 hours observed by measuring the diameter and calculated by mycelial inhibition formula.

Itraconazole *Aspergillus flavus* After 48 hours				
Concentration	experiment1mm)	experiment2(mm)	Mean(mm)	mycelial inhibition %
0 µg	22	21	21.5	0
1 µg	20	21	20.5	4.65%
2 µg	18	19	18.5	13.95%
4 µg	15	17	16	25.58%
8 µg	15	19	17	20.93%
16 µg	15	20	17.5	18.60%
32 µg	18	17	17.5	18.60%

Table 9 & 10: In observation and results the highest mycelial inhibition is 18% after 24 hours and 25% after 48 hours. The strain *A. flavus* was found to be resistant against this Itraconazole drug.

Table 11: Itraconazole with *Aspergillus terreus* antifungal susceptibility test result after 24 hours observed by measuring the diameter and calculated by mycelial inhibition formula.

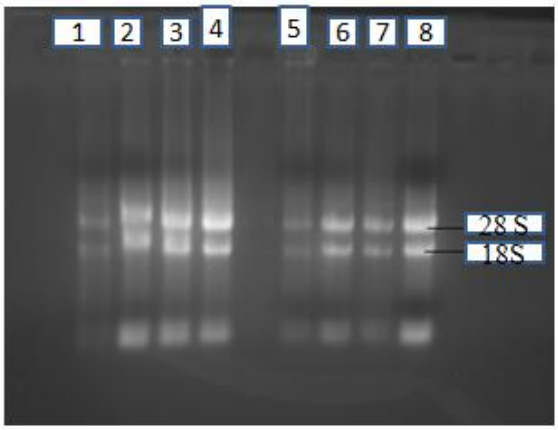
Itraconazole *Aspergillus terreus after 24 hours*				
Concentration	experiment1(mm)	experiment2(mm)	mean(mm)	mycelial inhibition %
0 µg	15	17	16	0
1 µg	13	15	14	12.50%
2 µg	15	15	15	6.25%
4 µg	14	13.5	13.75	14.06%
8 µg	12	15	13.5	15.62%
16 µg	12	13	12.5	21.87%
32 µg	13	13	13	18.78%

Table 12: Itraconazole with *Aspergillus terreus* antifungal susceptibility test result after 48 hours observed by measuring the diameter and calculated by mycelial inhibition formula.

Itraconazole *Aspergillus terreus after 48 hours*				
Concentration	experiment1(mm)	experiment2(mm)	mean(mm)	mycelial inhibition %
0 µg	25	20	22.5	0
1 µg	20	23	21.5	4.44%
2 µg	18	20	19	15.50%
4 µg	19	19	19	15.50%
8 µg	19	20	19.5	13.33%
16 µg	17	18	17.5	22.22%

Table 11 & 12: In the observation and results the highest mycelial inhibition is 22% after 24 hours and 22% after 48 hours. The strain *A. terreus* was found to be resistant against this drug.

RNA of fluconazole treated after 24 hours



Gel picture of RNA of *A terreus* after 24 hours treat with fluconazole in 1 and 2 well it is a control sample and others are fluconazole treated samples for 24 hours.

qRT PCR result

CT values For control (no drug) with 40s gene is 26.18 and in case of drug treated the value is 26.72. For control (no drug) with terrelysin gene is 27.72 and in case of drug treated the value is 28.61. Fold change is 1.27.

Chapter 6

DISCUSSION

DISCUSSION

6.1 Strains used

Aspergillus terreus (NCCPF 860035) and *Aspergillus flavus* (MTCC 11866) were used in this project and these strains were taken from Chandigarh *Aspergillus terreus* is taken from induced sputum and the *Aspergillus flavus* were taken from rice blast (characterized in our laboratory). As the drug given to the patients is without screening of the strain causing the infection. So there must be drugs which are applicable on multiple types of strains almost all the strains or against the strains of same species. For that purpose we were using different types of strains of same species with *Aspergillus terreus* as a main strain and *Aspergillus flavus* as a control.

6.2 Mycelial inhibition

Mycelial inhibition was calculated by antifungal susceptibility testing. Diameter of growth of the strains was measured after different interval of time i.e. 24, 48 and 72 hours. And the mycelial inhibition was measured by the formula. It was observed that the highest mycelial inhibition in case of *Aspergillus terreus* with Fluconazole drug is 26.66% after 24 hours and 54% after 48 hours and 40% after 72 hours, the drug is not that much effective or it may be not showing inhibition because of excipients present in the drug which slow down the metabolism of drug in the human's body. The MIC_{50} of this drug comes at 64 µg after 48 hours but after 72 hours the drug becomes less effective. On the other side *Aspergillus flavus* had the highest mycelial inhibition was 23% after 24 hours and 14% after 48 hours and 28% after 72 hours. The conclusion is that the drug is not effective against this strain (*A.flavus)* and this strain (*A.flavus)* is found to be resistant against this drug. In observation and results the highest mycelial inhibition is 18% after 24 hours and 25% after 48 hours. The strain *A. flavus* was found to be resistant against this Itraconazole drug.

6.3 qRT PCR

Terrelysin gene was used for qRT PCR because Terrelysin gene is specific for *Aspergillus terreus* (NCCPF 860035) and used to characterize the clinical strain. And on the other hand *Aspergillus flavus* (MTCC 11866) was already characterized in our laboratory. As the terrelysin gene which is a virulent factor didn't affect the expression level of antifungal drug in the experiment performed, or may be it only shows its activity during the germinating stage of the strain used.

Chapter 7

SUMMARY AND FUTURE ASPECTS

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7.1 Summary

Aspergillus terreus which is a fungus a pathogenic fungus which is filamentous capable of producing different types of conidia which are very small in size and flask in shape mainly present at the tip of filaments. *Aspergillus terreus* is beneficial in many productions of products in many industries for the production of lovastatin, gliotoxin and bioethanol etc. It cause damage in agriculture and also creates problem in human health. It is also the reason behind the loss of crops, million tons of many crops face loss because of this pathogenic strain it causes loss in crops of rice, crops of maize, crops of soya bean, crops of potato, crops of wheat. It also causes spoilage of the food mainly of the cereals, nuts in different climatic conditions mainly in the tropical and subtropical regions. *Aspergillus terreus* is the third most filamentous pathogenic strain which causes invasive aspergillosis in humans. This strain is causing and able to cause many diseases in humans, plants and animals. It causes infections in individuals who lacks proper function of immune system and immunosuppressive patients like asthmatic patients having respiratory tract problems in breathing it can also cause the death of the individual and in case of cancer patients and in case of organ transplantation and in case of HIV patients. And in case of plants it destroys crops like nuts, pulses, wheat, rice soya, maize etc. At present the treatment which is available failed to find out the problems creating by these filamentous species. Amphotericin B remains the standard drug used against fungal infections and for therapy purpose but the results are not up to the mark because of resistance of these species because of prolonged used of this drug, so there must be alternative drugs to cure the infections. It will become serious because these fungal infections infect the person with weak immune system like HIV patients, asthmatic patients and also in case of organ transplantation which leads to the death of the patients. As the drug Amphotericin B is well known and standard drug for all kind of fungal infection, but at present the drug is not workable on all kind of infection because there are more than thousands species of fungus is available in our environment

and these species now becoming resistant to this drug. The risk of infection is more in immunosuppressive patient so the treatment must be good because immunosuppressive patients or individual are with very weak immune system their immune system is not able to fight against these fungal infections, and day by day the new-new species of these filamentous fungi are available and known now but they are resistant to older drugs because the prolong use of these drugs and the strains are making mutation in their generations and evolving the resistant characteristics in their new generation which is not known yet that what characteristics are helping these strains against these antifungal drugs and why they are resistant this is not known yet. In 1939 the first azole drug was agent of antifungal was reported and in the year 1944 and 1949 the first azole drug was discovered which was used clinically. Then after first azole drug the drug and the well known antifungal drug Amphotericin B was discovered in 1960. In the year 1980 more drugs were discovered after the large scale use of penicilin. Then for 10 years these drugs were used then the Fluconazole and Itraconazole were introduced in 1990s. Introducing of azoles against these fungal infection deletes the ergosterol of slow down its process so that it will not multiplies in the individuals' body. But the drugs we are using at present also shows cytotoxic effect and also harming the normal cells also.

7.2 Future aspects

To overcome the problem of cytotoxic effect of these drugs as these drugs are used for therapy for a longer period of time because of duration time of antifungal drugs and these drugs are used at very high concentration to reduce the concentration of the drugs we can use plant derived secondary metabolites phytochemicals along with azoles in a combination so that decrease the concentration and reduce the cytotoxic effect. This approach could be useful in characterizing the susceptible or resistance Aspergillus isolates in clinical isolates. Patients should be screen for drug susceptibility before prescribing antifungal drug.

Chapter 8

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REFERENCES

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