

**Efficacy of *Aspergillus giganteus* on Human Fungal Pathogen
for its Antagonistic Potential under Various Culture
Conditions and its Mode of Action**

**Ramya.R
(17PBC013)**

**A Thesis submitted to Avinashilingam Institute for
Home Science and Higher Education for Women,
Coimbatore-641 043**

**In partial fulfilment of the requirement for the degree of
Master of Science in Biochemistry**

April, 2019

Certífcate



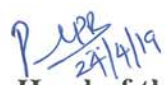
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Signature of the Head of the Department


Signature of the Supervisor

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Introduction

1.0 INTRODUCTION

Microorganisms are ubiquitous in nature and have variety of applications in various fields. With different kinds of applications of microorganisms it may have some deleterious effects to the environment which include plants, animals and humans. In developing countries it may potentially cause infectious disease in a higher proportion. Microorganisms include bacteria, fungi, yeast, protozoan etc., which may be responsible for both beneficial as well as harmful effects to environment. Among those organisms fungi have potential role to cause infectious diseases in humans which are predominant cause for the higher rate of morbidity in worldwide (Aminah *et al.*, 2018).

There are more than billion species of fungi present in worldwide. If those fungal species which may cause harmful effects to the environment then they are collectively called as pathogens. Most of the pathogenic fungi are phytopathogens but some may be pathogenic to humans as well. Mycology is the division for the complete study of fungi which may give the knowledge and more information about fungal species. The study of fungal as pathogens to humans and animals is generally said to be medical mycology. The ability of microorganism to evolve and to adapt to changing populations environment, cause infectious diseases and it creates threats to health throughout the world causing illness and death (Svetaz *et al.*, 2017).

Over the past three decades fungal infection plays a critical role for the cause of increased mortality and morbidity rate in immunocompromised patients mainly by AIDS, organ transplantation or patients who have a long term invasive medical practices and posing a big threat to human health. Currently, invasive fungal infections in humans may have 20% to 30% mortality rates and nearly 1.5 million peoples are losing their life annually, it might have associated with tuberculosis and malaria. Most of the pathogenic fungi are polymorphic in nature and they have the capacity to undergo reversible morphological transitions between yeasts in both pseudohyphal and hyphal growth forms. The hyphal form mainly plays a key role in the infection processes by fungal species as well as it couples with many virulence factors such as adherence and secretion of some endolytic enzymes. The diagnosis of fungal infections is a challenging one in the clinical

field due to the emergence of a new kind of diseases caused by the different strains of fungi (Garrigues *et al.*, 2018).

Therefore, antifungal treatment is initiated when infection is identified in patients clinically, and the diagnostic tests should be performed to confirm the fungal infections in humans so that some unnecessary treatment should be avoided. Despite the emergence of invasive fungal infections, therapies mainly relies upon the use of antifungal drugs mainly from synthetic sources namely, Amphotericin B, Azole antifungal compounds, Echinocandins. These are the three important classes of antifungal drugs used for the treatment of systemic fungal infections (Mircus *et al.*, 2015).

Fungal cell walls are dynamic and very complex structures and they are responsible for maintaining the shape and the integrity of the fungal cell. Some of the external factors that contribute the changes associated with the fungal cell wall such as polarized growth, development, environmental stimuli and stress. An antifungal drug such as Amphotericin B that binds to the fungal membrane ergosterol causes the lysis of cell wall of fungal species. Azoles are other kind of antifungal drugs which includes fluconazole, itraconazole, voriconazole and posaconazole which are involved in the inhibition of biosynthesis of ergosterol. Finally the newly introduced antifungal drug namely, Echinocandins such as caspofungin inhibits the glucan biosynthesis in fungi. Most of these drugs are currently used in clinical practices. But unfortunately, the antifungal drugs may interact with other medications and possibly cause side effects, resistance problem, and most of them are fungistatic rather than fungicidal and some of them are often toxic to the patients. These antifungal drugs inhibit a target unique to fungi and they shows good therapeutic ratios but their oral bioavailability is considerably low. Much more efforts have been taken to improve the oral absorption, but they can use only parenterally (Scorzoni *et al.*, 2017).

More over the synergistic effects of antifungal drugs will be helpful in the treatment and management of fungal infections (i.e) combination treatments. This combinatorial treatment may be attractive due to their cost affordability and decreased toxicity but it rarely may diminish the effectiveness of drug response in the body. For example, the drug namely 5-flucytosine and amphotericin B can be used for the treatment

of cryptococcal meningitis on the basis of synergism. The earliest discovery of those synergistic combinations of drugs may provide alternatives in pharmacotherapy and it may help to study their stability, toxicity and resistance as well as to understand the molecular basis for synergistic mistranslation. However, this combinational treatment may offers a best solution to the medicinal field but still some problems faced by every individual who are exposed to antifungal drugs and more importantly the reduced effectiveness of drugs may be observed in patients having combinatorial drug (Vallieres *et al.*, 2018).

With the limited applications of existing antifungal drugs there is a need for the development of better antifungal compounds to treat systemic infections caused by fungal pathogens. So it is widely accepted to create many antifungal compounds from natural sources rather than from synthetic sources. The urgency for the demand of antifungal compounds from natural sources are expanded due to the low number of existing drugs, and their increased toxicity and higher rate of infections caused by fungal strains with natural and acquired resistance. The scientist mainly focussed on the discovery of novel compounds from natural sources like plants and microbes. The plant is the excellent source antibacterials and antifungals. The plant contains variety of secondary metabolites that are involved in various mechanism of action to kill pathogens. At the same time the microorganisms like bacteria, fungi, etc., can also produce some metabolites which may have better killing actions against pathogenic organisms. Some of the fungal species also secretes some kind of metabolites like proteins and show best antifungal activity against both phytopathogens and human pathogens (Butts *et al.*, 2018).

Currently, the world mainly focussed to utilize new strategies to produce an efficient antifungal compounds which differ in their properties and mode of action from already existing synthetic antifungal drugs. On earth, more beneficial fungal species are present showing better antagonistic effects on pathogenic organisms. For example, *Aspergillus niger* may produce ANAFP (A Novel Anti Fungal Peptide), *Aspergillus giganteus* produce AFP (Anti Fungal Protein), *Aspergillus clavatus* contains ACLA and *Neosartorya fischeri* contains NFAP respectively as an antifungal compounds. With various potential antifungal compound producing fungi, *Aspergillus giganteus* is highly effective in restricting the growth of major plant as well as human pathogenic

filamentous fungi. Antifungal compound is abundantly secreted by *Aspergillus giganteus* which exhibits higher level of antifungal activity against species of the genera *Aspergillus* and *Fusarium* by inducing cell wall stress by disturbing plasma membrane integrity and inhibiting cell wall components synthesis in sensitive fungi (Varadi *et al.*, 2019).

Antifungal protein is produced from filamentous fungi such as *Aspergillus giganteus* and it should have some characteristics to give a better result to fungal infections. Antifungal proteins or peptides are small, cationic and cysteine rich proteins. This compound should be stable in every condition like pH, temperature and proteolysis and it should exhibit higher range of antagonism with different kinds of mechanisms against opportunistic human pathogens (Li *et al.*, 2018).

Pathogenic fungal species to humans may produce some harmful effects in any parts of the body. For example, *Aspergillus sp.* affects the people with lung disease and may cause Aspergillosis, *Candida albicans* which may cause Candidiasis in mouth, throat and vagina or the blood stream. There are numerous fungi as human pathogens present in nature such as *Aspergillus niger*, *Candida albicans*, *Cryptococcal neoformans*, *Aspergillus flavus*, *Cryptococcus gatti*, *Pneumocystis jirovecii*, *Aspergillus fumigatus*, *Histoblatoma capsulatum*, *Blastomyces dermatitidis* and *Penicillium chrysogenum*. They are the important human pathogenic organism causing harmful effects at the specific site in the host (Richardson and Naglik, 2018).

Aspergillosis is an emerging fungal infection caused by *Aspergillus spp.* and the progression of this infection further result in respiratory failure in immunocompromised patients. *Aspergillus* genomes are present in everywhere around the environment and it contains variety of species that may potentially cause severe illness in humans. *Aspergillus fumigatus* causes 1- Allergic bronchopulmonary aspergillosis (ABPA) which is an allergic response in individuals with asthma due to the presence of *Aspergillus* antigen in the lungs. Other parts of the body such as the skin, peritoneum, kidneys, bones, eyes and gastrointestinal tract, fungal infections has been identified in normal or immunocompromised host. This *Aspergillus* may group as a colony and grows to produce a fungal ball that might turn into a mass of entangled hyphae. Further it leads to an infection in humans when the spores of fungus spread from the lungs to other parts (Charrakh *et al.*, 2018).

With the emergence of invasive fungal infections, the optimization of culture conditions is necessary for the better growth of antagonistic fungi. Media containing high carbohydrate source and nitrogen source are required for the growth of fungi at pH range of 5 to 6, and a temperature range from 15°C to 37°C. Mainly there are two types of fungal culture media used for the growth of the fungi namely natural and synthetic media. The natural media has been prepared easily but problems with the unknown concentration of the compounds whereas the synthetic media composed of known concentration of the compounds are preferred. These types of media can be duplicated with precision each time they are made and contain defined amounts of carbohydrates, nitrogen and vitamin sources. For the growth of fungi, various synthetic media can be used. Growth conditions such as carbon source, nitrogen source, pH and temperature should optimized for the better growth of antagonistic fungi. These conditions are eventually required for the best antifungal activity of antagonistic fungi (Basut *et al.*, 2015).

The objectives of the present study was

- ❖ To optimize the culture conditions for the antagonism of *Aspergillus giganteus* against human pathogens
- ❖ To evaluate the antagonistic property of fungal culture filtrates of antagonistic fungi
- ❖ To determine the mechanism of antifungal culture filtrates on human fungal pathogens.

Review of Literature

2.0 REVIEW OF LITERATURE

Microorganisms are almost omnipresent, very diverse and indispensable to human survival. Fungi are major decomposers in certain ecosystems and essential associates of many organisms. In 1991, a landmark paper estimated that there are 1.5 million fungi on earth. Most fungi lack flagella and have filamentous bodies with distinctive cell wall carbohydrates. They can easily interact with all major groups of organism. Fungi constitute a major eukaryotic lineage equal in numbers to animals and exceeding plants. The group of fungi includes molds, yeasts, mushrooms, polypores, plant parasitic rusts and smuts, and *Penicillium chrysogenum*, *Neurospora crassa*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Blackwell, 2011).

The topic entitled “Efficacy of *Aspergillus giganteus* on human fungal pathogen for its antagonistic potential under various culture conditions and its mode of action” has been discussed under the following headings:

2.1 Infectious diseases

2.2 Antifungal resistance

2.3 Antifungal targets

2.3.1 Cell wall

2.3.2 Cell membrane

2.4 Mechanism of action

2.4.1 Inhibition of nucleic acid synthesis

2.4.2 Inhibition of protein synthesis

2.4.3 Inhibition of microtubule synthesis

2.4.4 Reactive Oxygen Species

2.4.5 Inhibition of Heat Shock Protein 90 (Hsp 90)

2.4.6 Inhibition of Calcineurin Signaling

2.4.7 Inhibition of Sterol biosynthesis

2.4.8 Inhibition of glucan biosynthesis

2.5 Antifungal compounds

2.6 Antagonistic fungi

2.7 Pathogenic fungi

2.9 Aspergillosis

2.10 Optimization

2.1 Infectious diseases

Infectious diseases are mainly dependent on the nature of the organisms and the complexity of human system. Depending on this, the pathogenic organisms can be classified into five main types namely viruses, bacteria, fungi, protozoa, and worms. These organisms are evolutionarily dynamic and different infectious agents cause markedly different diseases thus reflecting the diverse processes of the action. Among those fungal infections compose a significant proportion of the global infectious disease hence giving a negative impact on human welfare and the economy (Jones *et al.*, 2008).

Emerging infectious diseases are caused by fungi and it has been recognized as an important outcome of host-pathogen evolution leading to serious public health consequences. Infectious diseases are the leading cause of morbidity and mortality in worldwide, especially in developing countries. Fungi possess the genetic ability to acquire and to transmit resistance to therapeutic agents. Due to the excessive use of antifungal drugs for the treatment of fungal infections in humans, fungi have developed several resistance mechanisms such as target site modification, expression of the efflux pumps and metabolic inactivation, which contribute to the multidrug resistance fungi (Racaniello, 2015).

Among infections caused by other microorganisms, fungal infections are responsible for higher morbidity and mortality rate which are accompanied by high costs to the health system in worldwide. Fungal diseases have been continuously neglected over three decades despite their harmful impact on human health (Rodrigues and Albuquerque, 2018). Some reliable and cost-effective diagnostic tools are available for a very limited number of infections caused by fungi, and therapeutic options are restricted

to a few classes of poorly effective, toxic, and expensive pharmaceutical agents. There are no licensed antifungal vaccines can be used to treat fungal infections. Hence, antifungal chemotherapy is commonly employed to treat various systemic fungal infections (Roacha *et al.*, 2017).

Currently the fungal infections poses a unique problem because both the mammalian host and the invading fungi are eukaryotic in nature, this property making it difficult to develop a specific antifungal aimed only at the pathogen. So, there is an increasing demand for the development of new antifungal agents that are more effective and less toxic than those already in use. This is the major factor that initiates an intensive search among various sources for the discovery of new antifungal compound from natural sources (Leite *et al.*, 2015).

2.2 Antifungal Resistance

Like other organisms, there are some helpful fungi as well as harmful fungi are present in the environment. When these harmful fungi invade the body, they can be difficult to destroy and kill it posing a lifetime threat to human health (Johnson *et al.*, 2018).

Antifungal resistance can be defined as microbiologic or clinical resistance, or as a composite of the two. Microbiologic resistance is occurs when growth of the infecting organism or pathogen is inhibited by an antimicrobial agent. Clinical resistance is defined by the situation in which the infecting organism is inhibited by a concentration of an antimicrobial agent that is associated with a high likelihood of therapeutic failure. In other words, the pathogen is inhibited by an antimicrobial concentration that is higher than could be safely achieved with normal dosage. By the composite definition, antifungal resistance is present when fungal isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or when they demonstrate minimum inhibitory concentrations that fall in the range where specific resistance mechanisms are likely, and where clinical efficacy against the isolate has not been reliably shown in treatment studies (Pfaller *et al.*, 2016).

In clinical perspective the drug resistance occurs when the appropriate drug therapy is not effective or causing persistence/ progression of an infection. However, the molecular mechanisms that lead to antifungal resistance are always complex. Fungal cells

must commonly adapt to the presence of some toxic drugs and the primary molecular survival strategies includes

- (1) Mutation of drug targets that reduces its affinity for the drug
- (2) Overexpression of the targeted protein by modification of the promoter region of the gene
- (3) Expression of an efflux system
- (4) Degradation of the drugs, and pleiotropic drug responses

The different kinds of mechanisms of antifungal drug resistance are shown in Fig 1. The similarity of the biology of the eukaryotic host and fungal pathogens has led to a drug development focused on the specific characteristics of fungal cells. For example, the fungal cell wall is a specific antifungal drug target. In addition, ergosterol is present in fungal membranes and can be used as a drug target, because it is distinct from the cholesterol present in mammalian cells. Commercial antifungal drugs that act on the cell wall (i.e) ergosterol are azoles, echinocandins, and polyenes (Sanglard, 2016).

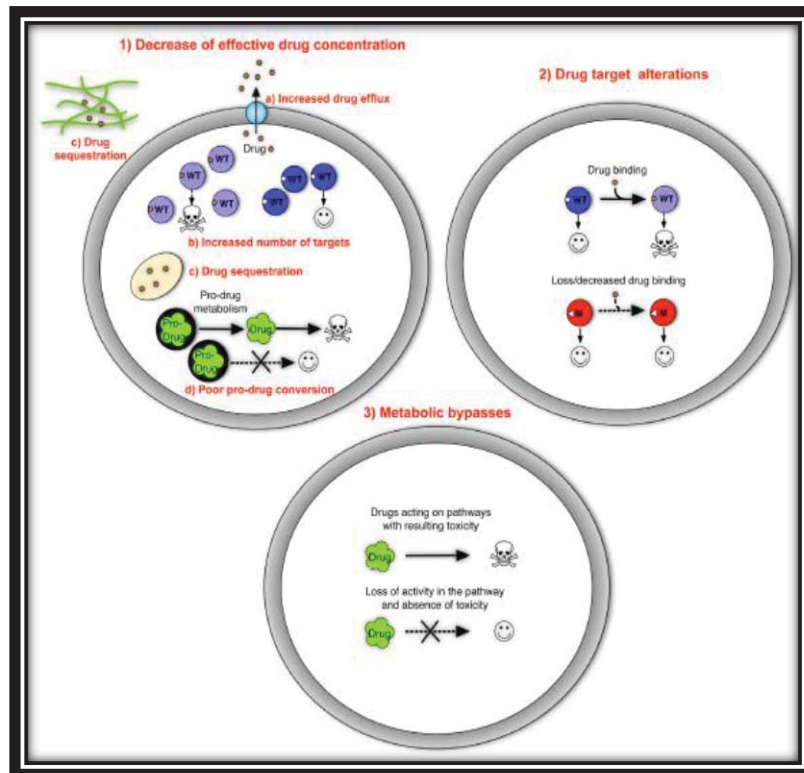


Fig 1: Mechanism of antifungal drug resistance

2.3 Antifungal targets

The scientists proposed that some macromolecules present in the fungi which is essential for fungal survival, growth, virulence or cellular morphogenesis that have been recognised as potential targets for novel antifungal agents. However, the antifungal discovery process based on screening the antifungal compounds against molecular targets, it takes time to generate practically useful results from fundamental biomedical research, and it is probably still too early to expect new antifungal agents to be discovered this way. However, the shift from random screens for antifungal effects towards screens against specific molecular targets has led to a new development in the field of medicine (Odds *et al.*, 2003).

The targets of all antifungal agents used in the clinic and of some agents that entered or approached clinical development but have not been marketed. The considerable diversity of antifungal targets already exists and the targets for different antifungal drugs are depicted in fig 2. Nevertheless, in terms of numbers of classes of agents that can be used to treat lifethreatening fungal infections, the targets are heavily focused either directly or indirectly which should be act on the cell envelope namely, cell wall and plasma membrane, and particularly on the fungal membrane sterol, ergosterol, and its biosynthesis. The antifungal targets elsewhere in the cell would therefore be a welcome innovation for systemically bioavailable antifungal agents (Vega *et al.*, 2013).

2.3.1 Cell wall

The fungal cell wall is essential for fungi and it is absent from the mammalian host which making it the most easily accessible fungal specific drug target. The anchoring of proteins to the plasma membrane through covalent attachment to glycosylphosphatidylinositol (GPI) provides a captivating potential target for the development of antifungal agents (McCarthy *et al.*, 2017).

The fungal cell wall, which is primarily composed of chitin, glucans, mannans, and glycoproteins, is essential for adhesion and fungal pathogenesis and also serves as a protective barrier, limiting the access of molecules to the plasmamembrane. The two main mechanisms of action of antifungals targeting the cell wall are related to the inhibition of chitin and beta-glucan synthesis (Weerden *et al.*, 2013).

2.3.2 Cell Membrane

Glycosphingolipids (GSLs) are a type of glycolipid found in cell membranes of fungal species that have emerged as key regulators of pathogenicity in a variety of fungi. When yeast and mold lack the glycosphingolipids called glucosylceramide (GlcCer), they are unable to replicate. Two newly identified compounds namely N'- (3-bromo-4-hydroxybenzylidene)- 2 methylbenzohydrazide (BHBM) and its derivative, 3-bromo-N'- (3-bromo-4 hydroxybenzylidene) benzohydrazide (D0), have been found to decrease the levels of GlcCer in fungi but not mammalian cell (McCarthy *et al.*, 2017). The molecular architecture of fungal cell has shown in Fig 3.

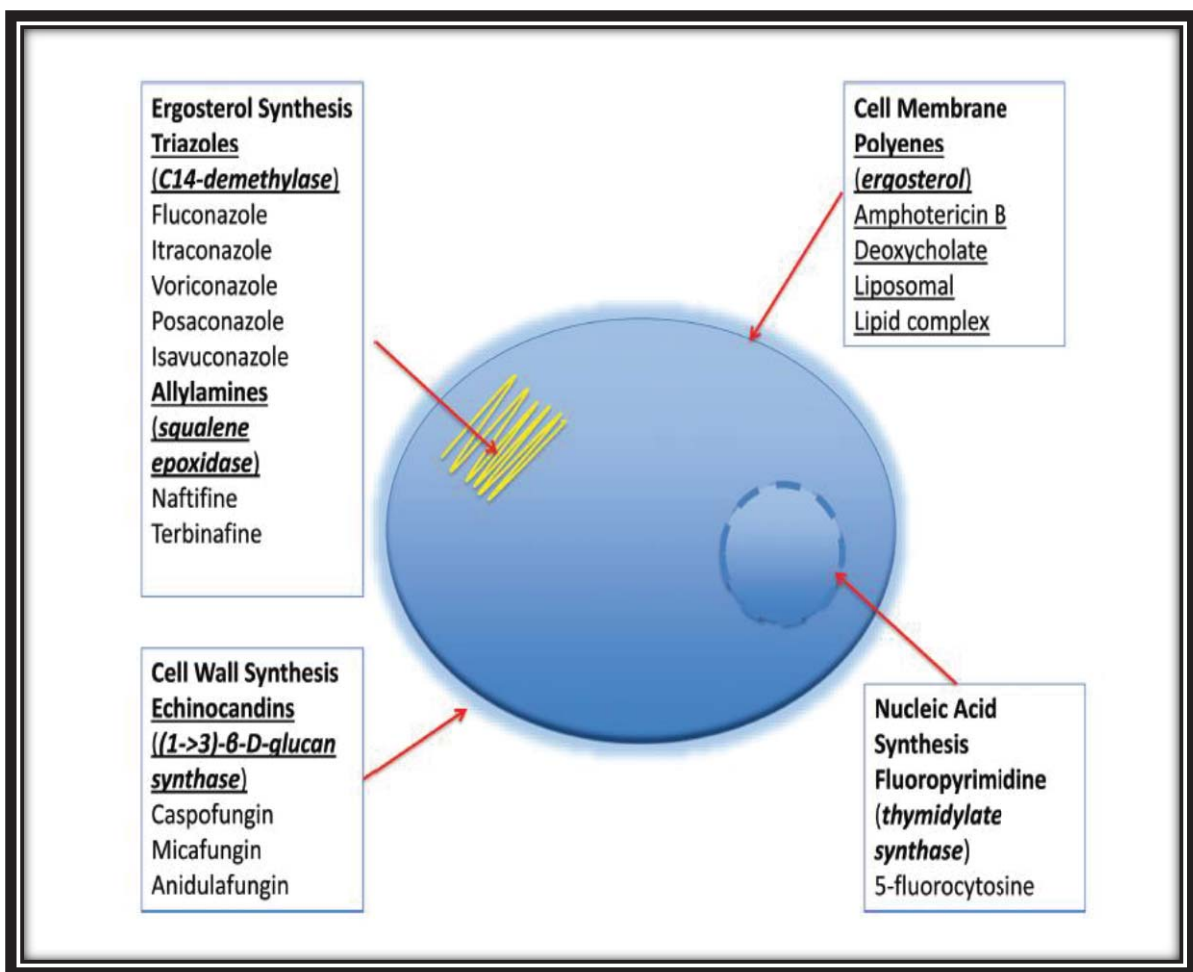


Fig 2: Cellular and biochemical targets for antifungal drugs

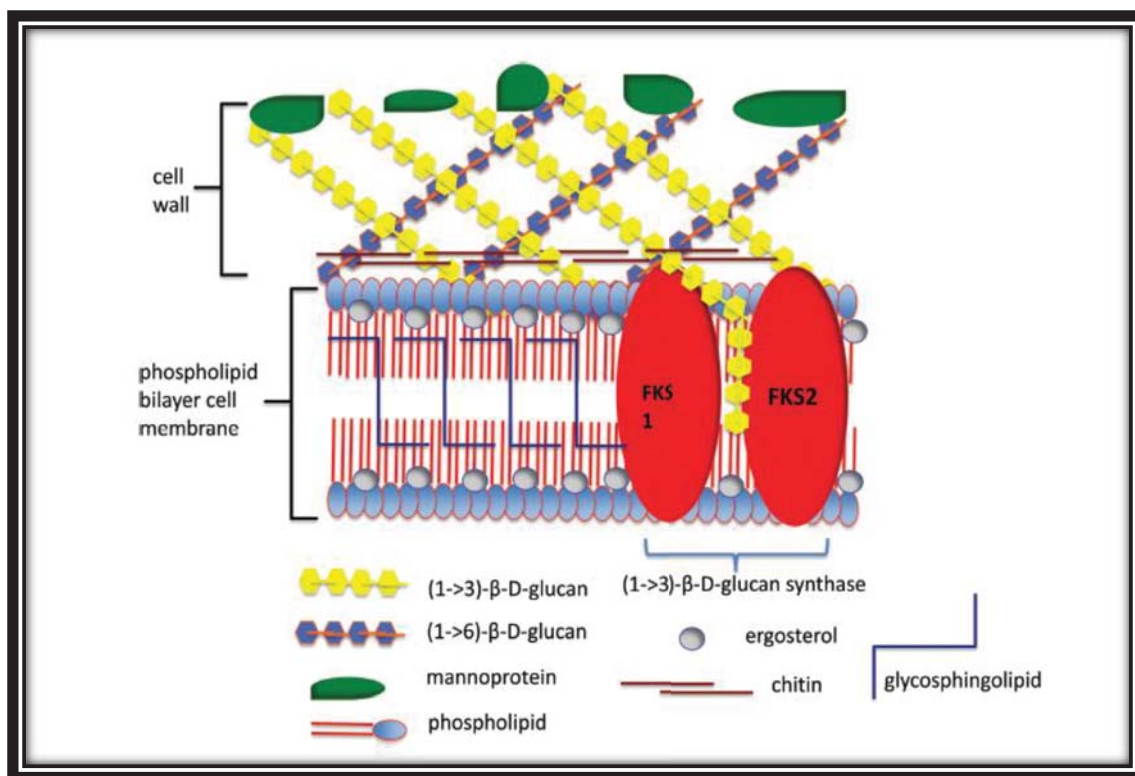


Fig 3: Molecular architecture of fungal cell

Ergosterol

Ergosterol is an important component of fungal cell membrane. It is a lipid component responsible for membrane fluidity and permeability and for the function of fungal integral membrane proteins. Accordingly, this sterol is essential for cell viability. Several antifungals primarily target ergosterol, either by inhibiting its biosynthesis or by binding to it, causing formation of pores in the membrane of fungal cells (Song *et al.*, 2016).

Chitin

Chitin, α β -1-4-linked *N*-acetylglucosamine polymer, is an essential component of the fungal cell wall, though it is only present in very small amounts in yeasts (1–2%) but in considerable quantities in filamentous fungi (10–20%). Nikkomycin and polyoxins are antifungal agents that target chitin synthase, which is responsible for elongation of the chitin chain and, therefore, is considered an attractive target (Morozov and Likhoshway, 2016).

β- glucan

β-glucan is another important component of the fungal cell wall, and recently inhibitors of its synthesis have been reported. The inhibitors of both β-1,6-glucan synthesis and β-1-3-glucan synthesis were identified (Reta, 2017).

2.4 Mechanism of action

The slow development of antifungal agents is the fact that fungi are eukaryotic, with a close evolutionary relationship with human hosts, which complicates the search for antifungal targets. Nonetheless, detailed knowledge regarding the structure, composition and biochemistry of fungal cells, in addition to various facets of fungal infections, has contributed to our understanding about the mechanism of action of many antifungal agents. Typically a long period of 8 to 10 years is required for an antifungal to be approved for clinical use. Reducing toxicity, enhancing bioavailability, improving the antifungal spectrum and combating resistance are efforts that are expected to increase the efficacy of the available antifungals. Indeed, elucidation of the mode of action of a potential antifungal compound can shorten the time from lead to candidate drug. Small antifungal molecules from natural products could represent structural templates for structure-activity relationship studies, thus providing more information to optimize potential new antifungal agents (Perfect, 2016).

2.4.1 Inhibition of nucleic acid synthesis

Inhibition of nucleic acid synthesis is related to the action of 5-flucytosine, which is converted to 5-fluorouracil by the enzyme cytosine deaminase and then it converted to 5-fluorouridylic acid by UMP pyrophosphorylase. Although 5- flucytosine was synthesized in 1957, its antifungal property was not discovered until 1964. The antifungal activity of this drug is established after seven years of its discovery (Shukla *et al.*, 2016).

Fluorouracil becomes incorporated into RNA, causing premature chain termination, and it inhibits DNA synthesis through the action of thymidylate synthase. For this mechanism of action, the target cells must possess cytosine permease to internalise the flucytosine molecule so that the cytosine deaminase can convert it to 5-fluorouracil, and uracil phosphoribosyl transferase which converts 5-fluorouracil into a

substrate for nucleic acid synthesis. Most filamentous fungi lack these enzymes and so that the useful spectrum of flucytosine is restricted to pathogenic yeasts. Flucytosine is used as adjunctive, rather than primary therapy, in the clinic, because primary and secondary resistance that resulting from defects in the permease, deaminase and/or phosphoribosyl transferase enzymes were occur at a high frequency. Therefore, flucytosine might deserve more use in the clinical practices (Kathiravan *et al.*, 2012).

2.4.2 Inhibition of protein synthesis

Sordarins suppress protein synthesis, which retards cell growth. Essentially, two fungal proteins have been described as target of sordarins such as translation elongation factor 2 (eEF2) and the large ribosomal subunit protein rpP0 (Botet *et al.*, 2008). Attempting to elucidate the mechanism of action, performed genetic assays using *Saccharomyces cerevisiae* mutants to demonstrate the fungal specificity of sordarins and proved that eEF2 is a target (Shukla *et al.*, 2016).

Different sordarin derivatives have different spectra of susceptible species, for these reasons that are not yet clear. This might reflect some problems of penetration of these agents into target fungi. Nevertheless, their high specificity for the fungal target and the relative ease with which new sordarin variants can be synthesised and it holds promise for positive future developments with effective actions (Scorzoni *et al.* 2017).

2.4.3 Inhibition of microtubule synthesis

The earliest inhibitory agent specific to fungal species was griseofulvin. The exact mechanism of action of this compound is still unknown, but the favoured explanation is that it may interfere with microtubule assembly. The selective toxicity of griseofulvin for fungi is only moderate and its spectrum of action is restricted mainly to the dermatophyte fungi that cause ringworm and athlete's foot. However, other types of compound are known to interfere with microtubule assembly and function in pathogenic fungi. Effects on microtubules explained the antifungal activity of early compounds, such as benzimidazole. However, unlike the situation in fungicide research against plant pathogens, relatively little research attention seems to have been paid in recent years to microtubules as possible antifungal targets for clinical use. With respect to the synthesis

of microtubules, it is known that griseofulvin interferes with intracellular production, thus inhibiting fungal mitosis (Botet *et al.*, 2008).

2.4.4 Reactive Oxygen Species

It is known that treatment with some antifungals such as Amphotericin B and itraconazole can cause more than one effect on fungal cells. Mitochondria naturally produce free radicals. However, under adverse conditions, such as in the presence of oxidants and UV light, these free radicals are produced in abundance, causing damage to proteins, lipids and DNA and leading to cell death. Accordingly, ROS production is also associated with apoptosis. Treatment with Amphotericin B is able to induce oxidative and nitrosative bursts in pathogenic fungi thus enhancing its fungicidal effect (Ferreira *et al.*, 2013).

Amphotericin B belongs to polyenes family. Polyenes are fungicidal and it mainly acts on the membrane. These molecules are natural fermentation products of *Streptomyces*, and the most used polyene drug is amphotericin B (used in systemic fungal infections). For many years, it was believed that polyenes (amphipathic drugs) bind strongly to ergosterol, destroying the proton gradient across the cell membrane and allowing leakage of ions. Now, it is known that drug-lipid complexes extract ergosterol from phospholipid in the membrane, depleting ergosterol in the cell. Amphotericin B is refractory to the development of resistance, despite its use for 50 years in clinical treatment. In *Candida tropicalis*, low ergosterol content in the membrane is associated with reduced susceptibility to amphotericin B. Because amphotericin B increases the level of reactive oxygen species (ROS) in fungal cells, amphotericin B-resistant *Candida tropicalis* produces less ROS and alters mitochondrial activity. In *Aspergillus terreus*, the oxidative damage caused by amphotericin B is more important in causing cell injury than membrane permeation (Roacha *et al.*, 2017).

Imidazoles and triazoles ('azoles') are the largest class of antifungal agents in clinical practices. Their main effect is to inhibit 14 α -demethylation of lanosterol in the ergosterol biosynthetic pathway, but in some fungal species, they can also inhibit the subsequent D22-desaturase step. With ergosterol depleted and replaced with unusual sterols, the normal permeability and fluidity of the fungal membrane is altered, with secondary consequences for membrane bound enzymes, such as those involved in cell

wall synthesis. Resistance to azoles can occur by mutations that modify the target molecule or by overexpression of membrane efflux pumps that export the antifungals from the cell (Mesa-Arango *et al.*, 2014).

2.4.5 Inhibition of Heat Shock Protein 90 (Hsp90)

Heat shock protein 90 (Hsp90) is a molecular chaperone of the heat shock protein (Hsp) family. It can be synthesized as an adaptive response to noxious condition these proteins contribute to the survival of pathogenic microorganisms in the host. Hsp90 has been related to fungal pathogenicity, phase transition in dimorphic fungi and antifungal drug resistance which makes it a potential target for antifungal therapy. The transcription profiles of *Trichophyton rubrum* under different stress conditions, such as interaction with nail and skin cells and molecules, nutrients and treatment with antifungal drugs. In addition to suggesting the role of Hsp 90 in the pathogenesis and susceptibility to dermatophytosis antifungal agents, the scientists also related this protein to the regulation of other heat shock proteins (Juvvadi *et al.*, 2016).

2.4.6 Inhibition of Calcineurin Signaling

Calcineurin is defined as a conserved Ca^{2+} -calmodulin (CaM)-activated protein phosphatase 2B that belongs to the phospho-proteinphosphatase family. This protein is involved in calcium-dependent signalling and regulation of several important cellular processes in yeasts (*Candida* spp., *Cryptococcus* spp.) and filamentous fungi (*Aspergillus fumigatus*), including growth, cell wall integrity, transition between morphological states, cation homeostasis, stress responses, and drug resistance. Most important is the role of calcineurin in maintaining the integrity of fungal cell wall. Findings suggested that triphenylethylenes, are a novel class of antifungal drugs that act on calcium homeostasis in *Cryptococcus neoformans* via direct inhibition of the calcineurin activator calmodulin (Li *et al.*, 2018).

2.4.7 Inhibition of sterol biosynthesis

The ergosterol biosynthetic pathway is the target for two other classes of antifungal agent. The allylamines, notably terbinafine that is involved in the inhibition of squalene epoxidase is an early step in the ergosterol biosynthetic pathway, with

fungicidal consequences in susceptible species. These include many filamentous fungi. Although terbinafine is not approved for treatment of visceral fungal infections, there is interest in the possibility of combining terbinafine with other ergosterol synthesis inhibitors to achieve synergistic inhibitory effects. The phenylmorpholine class, of which amorolfine is the sole representative in human medicine, affects two targets late in the ergosterol pathway such as Erg24p, the D14 reductase enzyme, and Erg2p, the D8-D7 isomerase enzyme. Amorolfine can be used only for topical treatment of superficial fungal infections (Seyedmousavi *et al.*, 2013).

2.4.8 Inhibition of glucan biosynthesis

The target for the echinocandins is the complex of proteins responsible for synthesis of cell wall β -1,3 glucan polysaccharides. In *Saccharomyces cerevisiae*, where the enzyme complex has been best studied, two proteins namely, Fks1p and Fks2p are regulated by a GTP-binding peptide, Rho1p, and by elements of the calcineurin pathway. Homologues for all three gene products have been found in *Candida albicans*, but it seems that the Fks2p homologue is not expressed in growing cells. Mechanistic details of glucan synthesis and its inhibition by echinocandins still remain not clear, largely because a membrane-associated protein complex is involved. There is no doubt that the component to which echinocandins bind is Fks1p, but their non-competitive inhibitory effects on glucan synthesis do not necessarily imply that Fks1p itself is the catalytic subunit, nor is it clear whether the echinocandin-binding site on Fks1p is external or internal to the cell membrane. β -1,3-glucan synthase echinocandin inhibition leads to cell wall defects, which in turn cause cellular stress, and several genes are expressed to adapt to this stress condition. Dimorphic fungi have a natural resistance to echinocandins during the pathogenic phase (Wring *et al.*, 2017).

2.5 Antifungal compounds

Currently, the antifungal drugs are commonly used to treat fungal infections but they have limited role in the treatment and have considerable disadvantages. Hence there is an urgency to develop a novel and more efficient antifungal drugs. In the development of new antifungal strategies, antifungal compounds that target the cell wall which is unique to fungi are highly preferable. In addition, the ultimate aim is to obtain a drug that

combines major desirable properties such as sustainability, high efficacy, limited toxicity, and low cost of production. So the strategies are shifted to produce more efficient antifungal compounds from natural sources like plants and microbes. Fungi as such (i.e) beneficial fungi can produce some metabolites and have a potential antifungal activity. Fig 4 explains the development of antifungal drugs from the first compound to till now. The development of antifungal drugs still proceeds for the production of best drugs that can be efficient in the treatment of fungal infections (Ngo *et al.*, 2016).

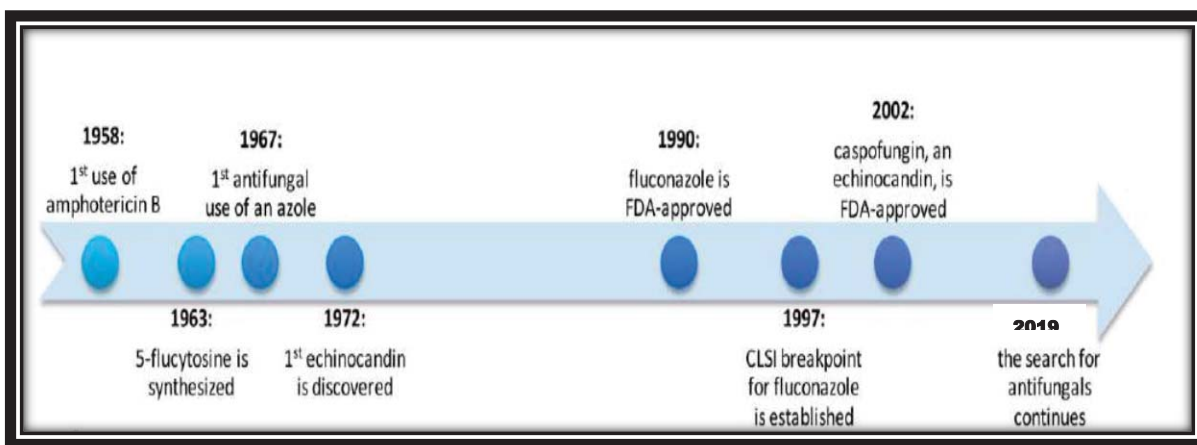


Fig 4: Development of antifungal drugs

AFP is abundantly secreted by *Aspergillus giganteus* and which exhibiting high levels of antifungal activity against species of the genera *Aspergillus* and *Fusarium*. In addition, AFP has been demonstrated to have neither cytotoxic nor immunogenic effects on different types of mammalian cells, which is indicative of its marvellous potential for medical applications. Based on their degrees of susceptibility to AFP, filamentous fungi are classified into sensitive species *Aspergillus niger*, *Fusarium oxysporum*, and *Fusarium moniliforme*, moderately sensitive species (e.g., *Aspergillus nidulans*), and resistant species (e.g., *Penicillium chrysogenum*) (Butts and Krysan, 2012).

AFP is a small protein that is composed of 51 amino acids. It contains five antiparallel β strands define a β barrel topology which is stabilized by four disulfide bridges, providing remarkable resistance to heat and protease degradation. The positive net charge of AFP can be attributed to the presence of 12 lysine residues, which are

partially involved in the formation of a protein domain (K9, K10, and K32) referred to as the cationic site. This site, in conjunction with a hydrophobic domain (Y29, V30, Y45, and V50) may account for the amphipathic character of AFP. Electron microscopic analyses have demonstrated that AFP accumulates at distinct areas within the cell wall of the AFP-sensitive fungus *A. niger*. In addition, small amounts of AFP localized at the plasma membrane and severe membrane alterations have been observed. In accordance with this, it has been shown that AFP readily permeates the plasma membranes of sensitive fungi (Hagen *et al.*, 2007).

2.7 Antagonistic fungi

The genome of antagonistic fungus has evolved specifically to attack other fungi which are pathogenic in nature and they represent a potential source of antifungal genes of non-plant origin. For example, *Trichoderma* spp., which has been repeatedly, used as biocontrol agents that producing fungal cell wall degrading enzymes (Bondaryk *et al.*, 2017).

A relatively new group of antifungal proteins secreted by filamentous fungi are small, cationic and cysteine-rich proteins. So far, only few antifungal proteins have been characterized, namely AFP from *Aspergillus giganteus*, ANAFP from *Aspergillus niger* PAF from *Penicillium chrysogenum* and NAF from *Penicillium nalgiovense*. Nevertheless, there is evidence, that their toxicity is mediated by interaction with distinct molecules or receptors at the outer layers of the cell, e.g. cell wall or plasma membrane. The *Aspergillus giganteus* antifungal protein AFP was found to modulate the cell wall composition by enhancing the expression of the α -1,3-glucan synthase A gene (*agsA*), possibly by the activation of the cell wall integrity pathway (CWIP), and inhibiting chitin synthesis in sensitive fungi (Binder *et al.*, 2011).

2.8 Pathogenic fungi

Fungi are eukaryotic organisms that can cause both endemic and severe infections, predominantly in the immunocompromised and debilitated host. Structurally, fungi are usually formed by a cell membrane surrounded by a fungal cell wall (Oliver *et al.*, 2016)

There are more than billion fungal species present in the world. They have both beneficial role as well as the deleterious effect to the environment. The fungi that are responsible for causing disease in humans, plant and animals are collectively called as pathogens. Mostly the pathogenic fungi are plant pathogens but there are comparatively few fungal species that are pathogenic to humans and animals. More than 400 fungal species in the environment that may cause harmful effects to the animals, and far fewer of these species will specifically cause disease in humans. Thus, there are not many species of fungi that are pathogenic to human that will be fatal. The study of fungi as human and animal pathogens is said to be Medical Mycology (Datta and Hamad, 2015).

There are number of diseases that specifically cause human diseases. However, fungi can vary in their host specificity. The majority of most human pathogenic fungi appear to be soil inhabiting species where they live as saprobes, but given the appropriate conditions, i.e., if the person is not healthy, an open wound is present, direct injection of fungus into your system will aggressively attack people. These fungal infections are caused by the fungal species that are collectively known as opportunistic fungi (facultative parasites). For example, *Coprinus cinereus*, a common mushroom has been recorded as causing endocarditis and *Schizophyllum sp.* cause several different types of infections, including meningitis and lung disorders (Shao *et al.*, 2007).

There are various fungal species that cause some pathogenic effects to the humans. For example, *Aspergillus sp.* affects the people with lung disease and may cause Aspergillosis, *Candida albicans* which cause Candidiasis in mouth, throat and vagina or the blood stream. There are numerous fungi as human pathogens present in nature such as *Aspergillus niger*, *Candida albicans*, *Cryptococcal neoformans*, *Aspergillus flavus*, *Cryptococcus gatti*, *Pneumocystis jirovecii*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Penicillium chrysogenum*. These pathogenic organisms cause harmful effects at specific sites in the host (Boutheina *et al.*, 2017).

Among various pathogenic fungi, filamentous fungi represent an increase threat to human life. The medically important fungal species of the genus *Aspergillus*, strain *Aspergillus fumigatus* is considered as one of the most prominent aspergillosis causing organism. Other filamentous fungi namely, *Fusarium oxysporum* are considered

major cause of plant diseases and crop loss. The naturally derived compounds should have some desirable properties such as sustainability, high efficacy, restricted toxicity and low cost of production (Mayr and Florl, 2011).

2.9 Aspergillosis

Aspergillus fumigatus is the most common fungal pathogen of humans causing severe and frequently fatal infection in patients with immunosuppression or underlying structural lung disease. An estimated 200,000 death cases occur each year worldwide. Left untreated, mortality rates from invasive pulmonary aspergillosis (IPA) exceed 90% and even following aggressive antifungal treatment, fatality rates of 50% are common in heavily immunosuppressed hosts such as those with leukemia and transplant recipients. This high rate of mortality following *A. fumigatus* infection is a result of the suboptimal diagnostic tools available, leading to late diagnosis and the relative ineffectiveness of existing antifungal drugs against established *Aspergillus* infections (Osherov and Kontoyiannis, 2017).

Invasive Aspergillosis is a common fungal infection that primarily affects severely immunocompromised patients, particularly those with prolonged neutropenia and those receiving cancer chemotherapy, high-dose corticosteroids, or broad-spectrum antibiotics. Patients undergoing solid organ or bone marrow transplantation with associated immune suppression and patients with AIDS are also at increased risk of aspergillosis (Krel *et al.*, 2014).

Aspergillus fumigatus, are able to cause a wide range of diseases, including 1- Allergic bronchopulmonary aspergillosis (ABPA), as its name suggests, an allergic response to the presence of *Aspergillus* antigen in the lungs and occurs in patients with asthma. ABPA occurs in some 10% of cystic fibrosis patients, 2-Aspergilloma in patients with pre-existing lung cavities or chronic pulmonary disorders. Aspergilloma occurs when the conidia that been inhaled enter a pre-existing cavity, germinate, and produce abundant hyphae in the abnormal pulmonary space. Those patients with previous cavitory disease (e.g. tuberculosis, sarcoidosis, emphysema) are at risk (Brooks *et al.*, 2014).

2.10 Optimization

Optimization is the important key factor for the antagonistic activity of organisms against pathogens. Variation in the type of carbon and nitrogen sources as well as changes in pH, temperature, incubation period, shaking and inoculum size of the antagonistic fungal strain can greatly influence biosynthesis of antagonistic substances (Thakur *et al.*, 2014).

Antifungal compound production can also decrease when media is deficient in appropriate carbon and nitrogen sources and optimal physiological conditions like pH, temperature and incubation time. Therefore, optimization and maintenance of proper culture conditions are necessary criteria to achieve maximum production of bioactive metabolites by an antagonistic microbial strain (Gogoi *et al.*, 2008).

There is also an increasing need of alternative novel drugs to control the dominant infectious diseases and multidrug resistant microorganisms. Besides, there are almost no investigations as regards to optimization of cultural conditions for better growth and production of bioactive metabolite by antagonistic fungal strains (Jain and Pundir, 2011).

Methodology

3.0 METHODOLOGY

Fungi are non-motile eukaryotic protists and they are characterized by the absence of chlorophyll and the presence of rigid cell wall. The pathogenicity of the fungi results in many infectious diseases among different organisms such as plants, animals and humans. Fungi can cause significant number of human infectious diseases represented as pathogens such as *Aspergillus fumigatus*, *Cryptococcal neoformans*, *Candida albicans* and *Histoblastoma sp.* There is a tremendous increase in the development of novel antifungal compounds to treat several infectious diseases caused by the pathogenic fungi. Hence, our study has focussed on the screening of antifungal compounds from the beneficial fungi to act against the pathogenic fungi.

3.1 Collection and maintenance of fungal strains

The fungal strain of *Aspergillus giganteus* was obtained from MTCC – IMTECH Chandigarh and the strain number is MTCC 8408. The pathogenic fungi *Aspergillus fumigatus* was obtained from PSGIMS, Coimbatore.

The czapek yeast extract medium was used for the growth of fungal species. The composition for the preparation of czapek yeast extract medium is given in Appendix I. The co-culture assay is a preliminary experiment which has been carried out to explain the antagonistic efficacy of *Aspergillus giganteus* with pathogenic fungus namely, *Aspergillus fumigatus*.

3.1.1. Co-culture assay

Co-culture systems have an effective test to identify the interactions of antagonistic fungi with pathogenic fungi. 5mm diameter of actively grown mycelia from antagonistic fungi and pathogenic fungi were inoculated on petriplates containing czapek yeast extract agar in opposite direction at equal distance from each other. The mycelia from pathogenic fungi alone can be inoculated in the plate was used as a control. Then the plates were incubated at 28°C for four to six days. The percentage of radial inhibition was calculated.

The percentage of inhibition was expressed by following equation:

$$\text{Percentage of inhibition} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

Where,

Control = growth of fungal pathogen

Test = growth of fungal pathogen in the presence of antagonistic fungi.

3.1.2 Hyphal interaction assay

The hyphae of fungal species play a key role in the infectious processes of any pathogen. The interaction of hyphae of antagonistic fungi and pathogenic fungi were visualized by this assay. The detailed procedure of hyphal interaction assay is given in Appendix II.

3.2 Optimization of culture conditions

Optimization of various factors namely, pH, temperature carbon sources and nitrogen sources in the czapek yeast extract medium were checked for the better growth of antagonistic fungi. Optimization follows the co-culturing technique and the radial inhibition percentage was measured using above mentioned formula. All the experiments were carried out in triplicates.

3.2.1 Optimization of carbon sources

The antagonistic efficacy of *Aspergillus giganteus* against *Aspergillus fumigatus* was evaluated using different carbon sources namely, sucrose, fructose, maltose and dextrose. The czapek yeast extract agar was prepared with 3% of sucrose while the concentration of fructose, maltose and dextrose was used in the concentration as 2%. Co-culturing of *Aspergillus giganteus* with *Aspergillus fumigatus* was performed in czapek basal media by varying the carbon sources. These plates were inoculated with 5mm plugs from 7 day old culture of all fungal strains and incubated at 28°C for 4 days after which the percentage of radial growth inhibition was calculated.

3.2.2 Optimization of nitrogen sources

The organic nitrogen sources selected for the optimization of culture conditions were urea and peptone whereas the inorganic nitrogen sources are sodium nitrite, ammonium sulphate and ammonium dihydrogen orthophosphate. Czapek basal media was prepared with 3% of both organic and inorganic nitrogen sources. This was inoculated with 5mm mycelial growth discs of 7 day old *Aspergillus giganteus* and *Aspergillus fumigatus*. This was incubated at 28°C for 4 days. The radial growth of *Aspergillus fumigatus* in control plates and in dual culture was measured and percentage of growth inhibition was calculated.

3.2.3 Optimization of pH

Three different pH conditions namely, pH 5.0, pH 6.0 and pH 7.0 were chosen for the identification of better antagonistic activity of *Aspergillus giganteus*. The czapek yeast extract medium was prepared with optimized carbon and nitrogen sources and it was inoculated with 5mm mycelial growth discs of 7 day old culture of *Aspergillus giganteus* and *Aspergillus fumigatus*. The radial growth of *Aspergillus fumigatus* in control plates and in dual culture was measured after the incubation period. Finally, the percentage of growth inhibition was calculated.

3.2.4 Optimization of temperature

The basal media was prepared with optimized carbon, nitrogen and pH conditions for the inhibitory effect of *Aspergillus giganteus* and incubated at different temperatures such as 25°C, 30°C and 35°C. The radial growth of *Aspergillus fumigatus* in control plates and in test plates was measured for calculating the percentage of radial inhibition.

3.3 Preparation of culture filtrates

The fungal strain was cultured in an optimized medium at 30°C with constant shaking at 170 rpm for 8 hours. Then the culture was incubated at 28°C for 4-7 days. The culture was collected and subjected to centrifugation at 10,000g at 4°C for 15 minutes. The supernatant was filtered through 0.22µm membrane filter to obtain cell free culture filtrates. Finally the culture filtrates was stored at 4°C before use.

3.3.1 Detection of antifungal metabolite

The antifungal metabolite produced from *Aspergillus giganteus* might be intracellular or extracellular. *Aspergillus giganteus* culture was centrifuged at 10000 rpm for 20 minutes. The supernatant was collected and then the pellet was washed two times with phosphate buffered saline. The pellet was resuspended in phosphate buffered saline. Then the supernatant and pellet were tested for their antifungal activity against *Aspergillus fumigatus* by overlay assay.

The detailed procedure for the overlay assay is explained in Appendix III.

3.3.2. Estimation of protein in culture filtrates

The protein content present in the prepared culture filtrates was estimated by Lowry's method. The experimental procedure has been given in Appendix IV.

3.3.3 Optimization of culture conditions for culture filtrates

The optimization of carbon sources, nitrogen sources, pH and temperature was done for the *Aspergillus giganteus*. It was further proved that the culture filtrates of *Aspergillus giganteus* has better antagonistic potential by overlay assay.

3.3.4 Hyphal interaction assay

The hyphae of fungal species play a key role in the infectious processes of any pathogen. The morphological changes of *Aspergillus fumigatus* with antifungal compounds present in the culture filtrate of *Aspergillus fumigatus* was identified by hyphal interaction assay.

3.3.5 SEM analysis

The 4 day old fungal culture of *Aspergillus fumigatus* grown on czapek yeast extract medium treated with culture filtrates of antagonistic fungi along with the control was prepared. Then these samples were subjected to SEM structural observations. About 5x10mm segments were cut from cultures growing on czapek yeast extract medium and promptly placed in vials containing 4.0% glutaraldehyde in 0.05 mol/l phosphate

buffered saline at 4°C. Samples were then placed in this solution for 4 to 6 hours for fixation and then washed with distilled water three times for 20 minutes each. They were dehydrated in various ethanol dilutions namely, 30%, 50%, 70% and 90% for 20 minutes and finally with absolute ethanol for 45 minutes. Samples were then allowed to dry. Following drying, the samples prepared were mounted on standard ½ in SEM stubs using double stick adhesive tabs and coated with gold-palladium, and analysed under different magnifications.

3.4 Microbroth dilution assay

3.4.1 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration

Cultures of *Aspergillus fumigatus* were placed on czapek yeast extract agar and incubated for 24 to 72 hours at 37°C. Colonies of this culture were suspended in sterile 0.85% NaCl. Czapek yeast extract broth containing antifungal compounds at different concentrations 500µg, 250µg, 125µg, 62.5µg and 31.25µg were added to a 96 well plate. The plates were incubated at 37°C for 24 to 48 hours. 10µl of each of the wells with no fungal growth were seeded on plates containing czapek yeast extract agar, which were then incubated at 37°C for 24 to 48 hours for the determination of minimum fungicidal concentration.

Minimum inhibitory concentration was determined by adding 20µl of 0.5% triphenyl tetrazolium Chloride to each well and then the plate was further incubated for 24 hours. MIC was defined as the lowest antifungal compound concentration that inhibited visible growth of the microorganism, as also indicated by reading 96 well plates.

3.5 Mechanism of action of antifungal compounds

3.5.1 Release of cellular materials

Mycelia from 100ml czapek yeast extract broth culture of *Aspergillus fumigatus* were collected by centrifugation at 4000g for 20 minutes, washed three times, and resuspended in 100ml phosphate buffered saline (pH 7.0). The suspensions were then treated by AFC at various concentrations (50-250µg) and incubated at 28±2°C under

agitation in an environmental incubator shaker for 0, 30, 60 and 120 minutes. Subsequently, 2ml of the sample was collected and centrifuged at 12000g for 2 minutes.

The concentration of released constituents such as DNA, RNA, protein and glucose were determined by measuring the absorbance of treated cultures. The procedures for the estimation of DNA, RNA, and glucose are given in Appendix V, VI and VII respectively and also the released proteins of treated cultures were estimated by Lowry's method.

3.5.2 Sorbitol Assay

Sorbitol act as fungal cell wall osmotic protective agent, when this sorbitol added to the culture medium it protects the cell wall of pathogenic fungi. This sorbitol assay implicated the cell wall as one of the possible cell targets for the product tested.

The different concentrations of antifungal compounds in culture medium along with pathogenic fungi were taken in 96 well microtitre plates. Then sorbitol was added to the culture medium to give a final concentration of 0.8M. Then the plate was incubated at 25°C. The plates were read after 48 hours and 7 days at 595nm.

3.5.3 Measurement of extracellular pH

100µl of fungal suspensions were added to 20ml czapek yeast extract broth and incubated in a moist chamber at 28±2°C for 2 days. The mixtures were centrifuged at 4000g for 20 minutes and the resulting pellet was collected and washed for 2-3 times with sterilized double distilled water, and resuspended in 20ml sterilized double distilled water. After the addition of antifungal compounds at different concentrations, the extracellular pH of fungal pathogen was determined at 0, 30, 60 and 120 minutes.

3.5.4 Determination of total lipid content

Total lipid content of *Aspergillus fumigatus* cells with antifungal compound at various concentrations 50µg, 100µg, 150µg, 200µg and 250µg were determined using phosphovanillin method. The 2 day old mycelia from 50ml broth was collected and centrifuged at 4000g for 10 minutes. Then the samples were dried with a vacuum freeze

drier for 4 hours. About 0.1g of dry mycelia was homogenized with liquid nitrogen and extracted with 4.0ml methanol-chloroform-water mixture (2:1:0.8, v/v/v) in a clean dry test tube with vigorous shaking for 30 minutes. The tubes were centrifuged at 4000g for 10 minutes. The lowest phase containing lipids was thoroughly mixed with 0.2ml saline solution and centrifuged at 4000g for 10 minutes. Then, an aliquot of 0.2 ml chloroform and lipid mixture was transferred to a clean and dry tube and 0.5ml H₂SO₄ was added, heated for 10 minutes in a boiling waterbath. After that, 3ml phosphovanillin was added followed by vigorous shaking, then incubation was carried out at room temperature for 10 minutes. The absorbance at 520nm was utilized to calculate total lipid contents from the standard calibration curve using cholesterol as a standard.

3.6 Cytotoxicity assay

Cytotoxicity assay was performed to determine the toxicity level of antifungal compounds. It was evaluated by analyzing the hemolytic properties of the antifungal compounds present in the culture filtrate of *Aspergillus giganteus* against goat red blood cells.

3.6.1. Hemolytic activity against erythrocytes

To assess the cytotoxicity of antifungal compound against erythrocytes, hemolytic activity was evaluated by the percentage of hemolysis of a 4% suspension of goat red blood cells (gRBCs) treated with various concentrations (50 to 250µg) of AFC. The gRBCs were washed three times with a phosphate buffered saline. 100µl aliquots of a gRBC suspension were added to 96 well microtitre plates, and then 100µl of culture filtrates of *Aspergillus giganteus* in PBS was mixed into each well. After incubating the mixtures for 1 hour at 37°C, the mixtures were centrifuged at 1500 rpm for 10 minutes, and aliquots were transferred to new well in the microtitre plates. The absorbance of aliquots was measured at 414 nm by using a microtitre ELISA reader. Hemolytic rates of 0% and 100% were determined in PBS and 0.1% Triton X-100, respectively. The percentage of hemolysis was calculated by employing the following equation:

$$\text{Percentage Hemolysis} = \frac{[\text{Abs}_{414\text{nm}} \text{ in the compound solution} - \text{Abs}_{414\text{nm}} \text{ in PBS}]}{[\text{Abs}_{414\text{nm}} \text{ in 0.1\% Triton X 100} - \text{Abs}_{414\text{nm}} \text{ in PBS}]} \times 100$$

Results and Discussion

4.0 RESULTS AND DISCUSSION

The main aim of our study is to test the potential antifungal activity of *Aspergillus giganteus* against *Aspergillus fumigatus* and investigate its mode of actions. The pathogenic fungi may cause Aspergillosis in immunocompromised individuals. The pathogenicity of fungal strains may pose an ever threat to human health and environment. There is an emergence of the discovery of novel antifungal drug for their better effects on human health.

4.1 Co-culture Assay

Co-culture systems have an effective test to identify the interactions of antagonistic fungi with pathogenic fungi. It is a possible tool to stimulate and produce an antifungal compound for the discovery of new molecules for industrial applications. The traditional methods like growing single microbial strain in a plate have produces some bioactive compounds whereas the co-culturing method have potential applications for the discovery of enormous production of antifungal compounds. In dual culture one organism may senses the presence of other organisms which triggers antagonistic effect that promotes the induction of antifungal compounds and some enzymes (Serrano *et al.*, 2017).

The growth inhibiting ability of *Aspergillus giganteus* against pathogenic fungi has been assayed with dual culture which is the preliminary step to find antagonism between two fungal cultures. The plates containing the pathogenic fungal mycelium may serve as control. The coculturing of antagonistic fungi with the pathogenic fungi namely, *Aspergillus giganteus* is depicted in plate 1.

The results have proved the antagonistic activity of *Aspergillus giganteus* against *Aspergillus fumigatus* and the inhibition percentage was found to be 81.2%.

There are several literatures to prove the inhibitory action of antagonistic organisms, by co-culture assay.

Kunova *et al.*, (2016) have done a dual culture assay to determine the antagonistic activity of 130 *Streptomyces* strains against six soil borne fungal pathogens which are categorized into three groups depending on their growth rate of mycelium namely fast

growing, medium growing and slow growing. The high inhibition was found in group 2 when compared to other groups of fungal pathogens.

A study was conducted by Khan *et al.*, (2017) to identify the fungal species which have potential antagonistic effect against wilt causing phytopathogenic bacterial and fungal species where *Aspergillus flavus* has better antagonistic efficacy against selected phytopathogenic bacteria and fungi among the selected six fungal species.

Durairaj *et al.*, (2018) had conducted the co-culturing assay to prove the antagonistic effect of *Pseudomonas* and *Bacillus* strains against *Panax ginseng* root rot fungal phytopathogens where two strains of this species effectively inhibit the growth of ginseng root fungal pathogens.

In another study, the dual culture was done to explore the antifungal potential of endophytic fungi with the medicinal plant *Mirabilis jalapa* for their ability to produce antagonistic substances. The antagonistic capacity of *Aspergillus clavatonanicus* strain MJ31 was performed against five phytopathogenic fungi namely, *Fusarium oxysporum*, *Fusarium culmorum*, *Fusarium graminearum*, *Aspergillus flavus* and *Trichophyton mentagrophytes*. The prominent antagonistic activity was found with *Fusarium graminearum* and *Fusarium culmorum* followed by *Fusarium oxysporum* while the minimum inhibition was obtained against *Aspergillus flavus* (Mishra *et al.*, 2017).

Suresh and Nelson, 2016 have done co-culture assay to determine its antagonistic potential against pathogenic fungi where the higher level of inhibition was obtained with *Trichoderma harzianum* against *Colletorichum falcatum*.

4.1.1. Hyphal interaction assay

Hyphae of pathogenic fungi play crucial role in the process of infections. The antagonistic efficacy of *Aspergillus giganteus* was further proved by hyphal interaction assay. The inhibition of pathogenic fungal growth by *Aspergillus giganteus* was observed microscopically and the results are shown in plate 2.

The results reveals that the pathogenic fungi in the control plates showed good morphology with extended mycelium whereas the pathogenic fungi with *Aspergillus giganteus* showed reduced hyphal elongation in the test plate.

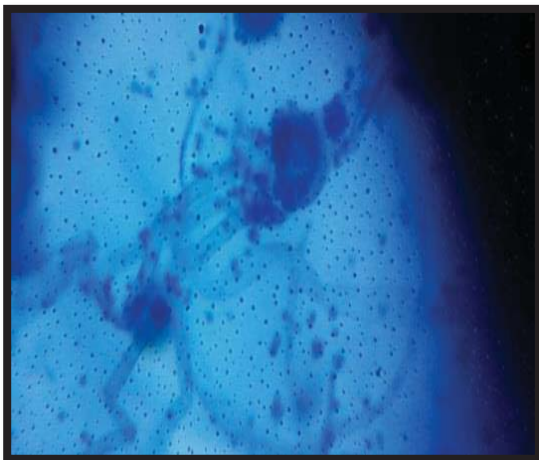


Aspergillus fumigatus

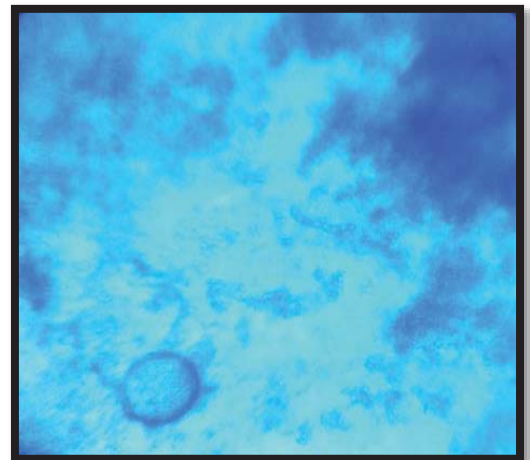


Aspergillus fumigatus with
Aspergillus giganteus

Plate 1: Co culture of *Aspergillus giganteus* and *Aspergillus fumigatus*



Aspergillus fumigatus



Aspergillus fumigatus with *Aspergillus*
giganteus

Plate 2: Hyphal interaction of *Aspergillus giganteus* with *Aspergillus fumigatus*

The hyphal interaction assay was carried out by many researchers to find out the inhibition activity of antagonistic fungi with pathogenic fungi.

Saechow *et al.*, 2018 have done hyphal interaction assay to identify the effect of *Bacillus amyloliquefaciens* BAS23 against various pathogenic fungi. The swell and bulb formation was observed microscopically in the test plate with various pathogenic fungi.

In another study by Debaiky, 2017 the hyphal interaction was performed with antagonistic fungi namely *Trichoderma harzianum* and *Aspergillus piperis* against various phytopathogenic fungi where the damaged hyphae of pathogenic fungi were observed with antagonistic fungi.

Waing *et al.*, 2015 have performed the hyphal interaction assay which proved that *Aspergillus*, *Penicillium*, *Fusarium* and *Neosartorya* shows antagonism against selected pathogenic fungi where the hyphal folding, bending and coiling were found in pathogenic fungi due to antagonistic interactions.

Stefanini *et al.*, 2018 have reported that the hyphae of *Aspergillus fumigatus* was damaged by the newly introduced antifungal compound 089.

4.2 Optimization of culture conditions

The culture conditions for the growth of fungi play a crucial role in the production of important bioactive components. Hence it is necessary to optimize the culture conditions such as carbon sources, nitrogen sources and appropriate environment with pH and temperature for the better growth of antagonistic fungi (Verma *et al.*, 2018).

4.2.1 Optimization of carbon sources

Carbon Sources:

Four different carbon sources such as sucrose, fructose, maltose and dextrose were used to optimize the medium to check the independent antagonistic effect of *Aspergillus giganteus* against *Aspergillus fumigatus*. The results are shown in fig 5 and plate 3.

Among the various carbon sources used, the maximum inhibition was seen in the dextrose medium and percentage of radial inhibition was found to be 81.2%. The inhibition was found to be very less in medium with sucrose as carbon source. Hence the czapek yeast extract medium was prepared with dextrose as carbon source for further studies.

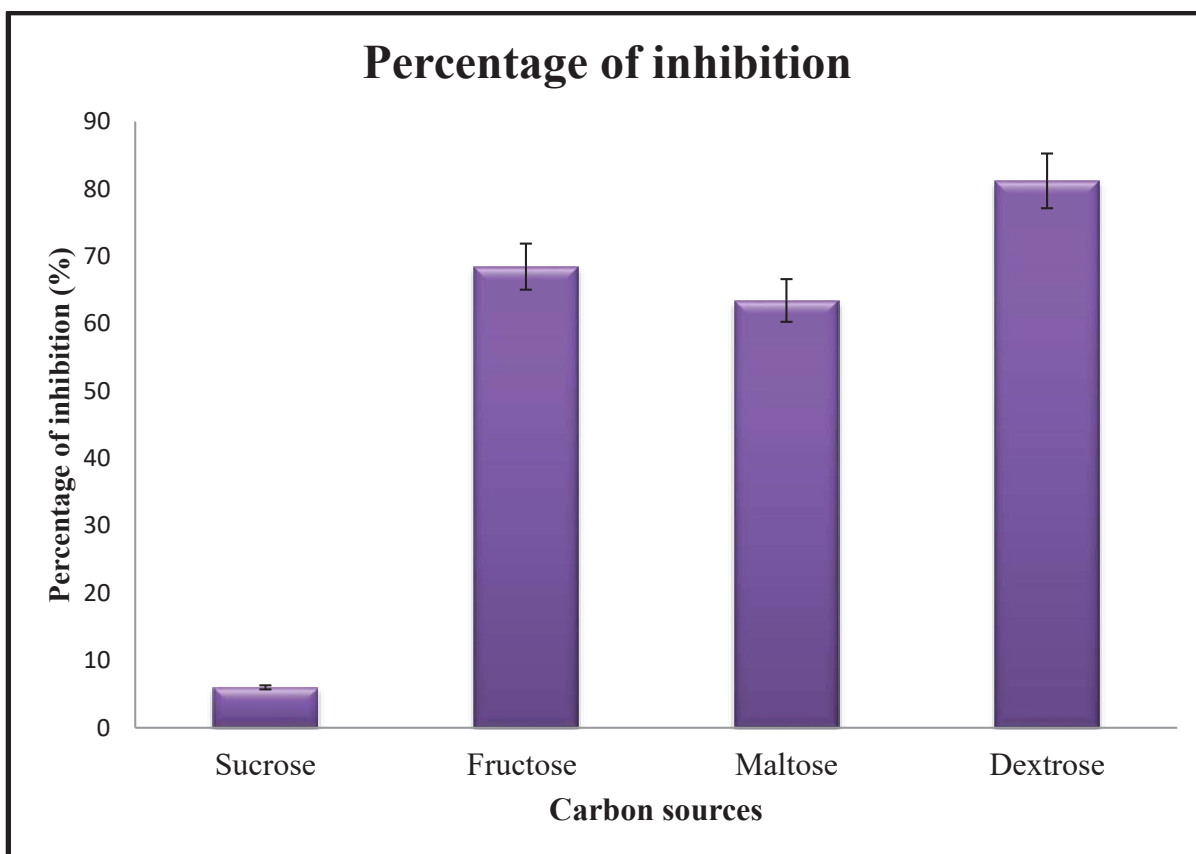


Fig 5: Percentage inhibition of *Aspergillus giganteus* during co-culture with *Aspergillus fumigatus* on various carbon sources

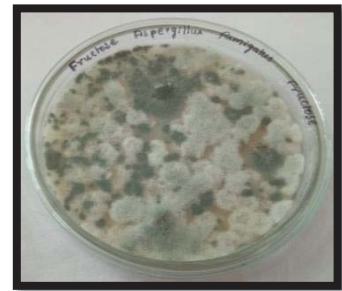
Aspergillus fumigatus



Aspergillus fumigatus with
Aspergillus giganteus



A) Co-culture assay with sucrose as carbon source



B) Co-culture assay with fructose as carbon source



C) Co-culture assay with maltose as carbon source



D) Co-culture assay with dextrose as carbon source

Plate 3: Percentage inhibition of *Aspergillus giganteus* during co-culture with
Aspergillus fumigatus on various carbon sources

4.2.2 Optimization of nitrogen sources

The antagonistic efficacy of *Aspergillus giganteus* against selected pathogenic fungi was determined using various organic and inorganic nitrogen sources. The organic nitrogen sources including urea, peptone and the inorganic nitrogen sources such as sodium nitrite, ammonium sulphate and ammonium dihydrogen orthophosphate were selected for the optimization of better nitrogen source for antagonistic fungal growth. The results are shown in fig 6, plates 4 and 5.

The results revealed that the antagonistic efficacy of *Aspergillus giganteus* was found to be higher in inorganic nitrogen sources than organic nitrogen sources. The best inhibition was found in medium with ammonium sulphate as inorganic nitrogen source and peptone as organic nitrogen and the inhibition percentage was found to be 99.3% and 79.3% respectively. Hence, ammonium dihydrogen ortho phosphate was used as a nitrogen source in the czapek yeast extract medium for further assays.

4.2.3 Optimization of pH

The inhibitory effect of *Aspergillus giganteus* was determined by varying the pH conditions. The pH range 5.0, 6.0 and 7.0 was selected for the optimization to have a better antagonism against human pathogenic fungus namely, *Aspergillus fumigatus*. The results are shown in fig 7 and plate 6.

The results showed that the favourable condition for the growth of antagonistic fungi was observed at pH 7.0 and the minimum antagonistic activity was observed in pH 5.0 and the moderate level of inhibition was recorded at pH 6.0.

4.2.4 Optimization of temperature

Optimization of temperature is an important factor for the growth of organisms and their metabolite production. The fungal cultures were treated in three different temperature range namely 25°C, 30°C and 35°C for the determination of better growth of antagonistic fungi. The results are depicted in fig 8 and plate 7.

The results revealed that the optimum temperature for the growth and antagonistic effects of *Aspergillus giganteus* against *Aspergillus fumigatus* was found at 25°C and the percentage of inhibition was 69.7%. The inhibition was less at temperature 37oC with 28.8% of inhibition.

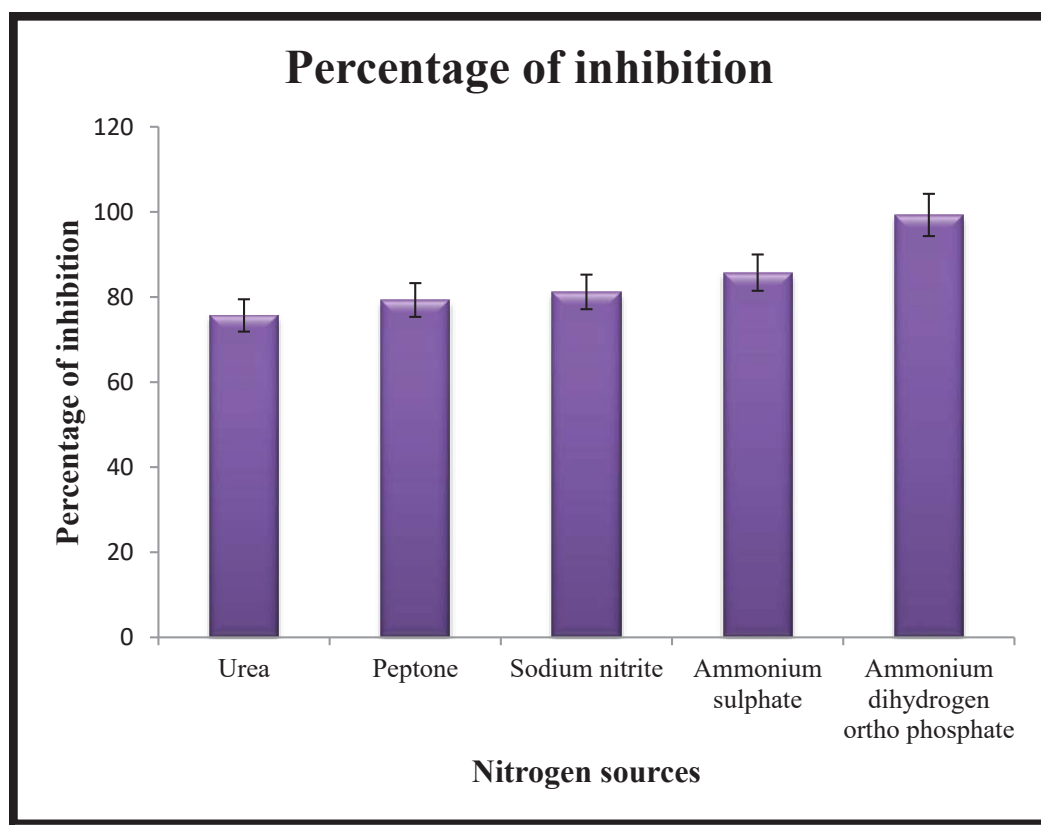


Fig 6: Percentage inhibition of *Aspergillus giganteus* during co-culture with *Aspergillus fumigatus* on various organic and inorganic nitrogen sources

Aspergillus fumigatus



Aspergillus fumigatus with
Aspergillus giganteus



A) Co-culture assay with urea as nitrogen source



B) Co-culture assay with peptone as nitrogen source

Plate 4: Percentage inhibition of *Aspergillus giganteus* during co-culture with *Aspergillus fumigatus* on various organic nitrogen sources

Aspergillus fumigatus



Aspergillus fumigatus with
Aspergillus giganteus



A) Co-culture assay with sodium nitrite as nitrogen source



B) Co-culture assay with ammonium sulphate as nitrogen source



C) Co-culture assay with ammonium dihydrogen ortho phosphate as nitrogen source

Plate 5: Percentage inhibition of *Aspergillus giganteus* during co-culture with
Aspergillus fumigatus on various inorganic nitrogen sources

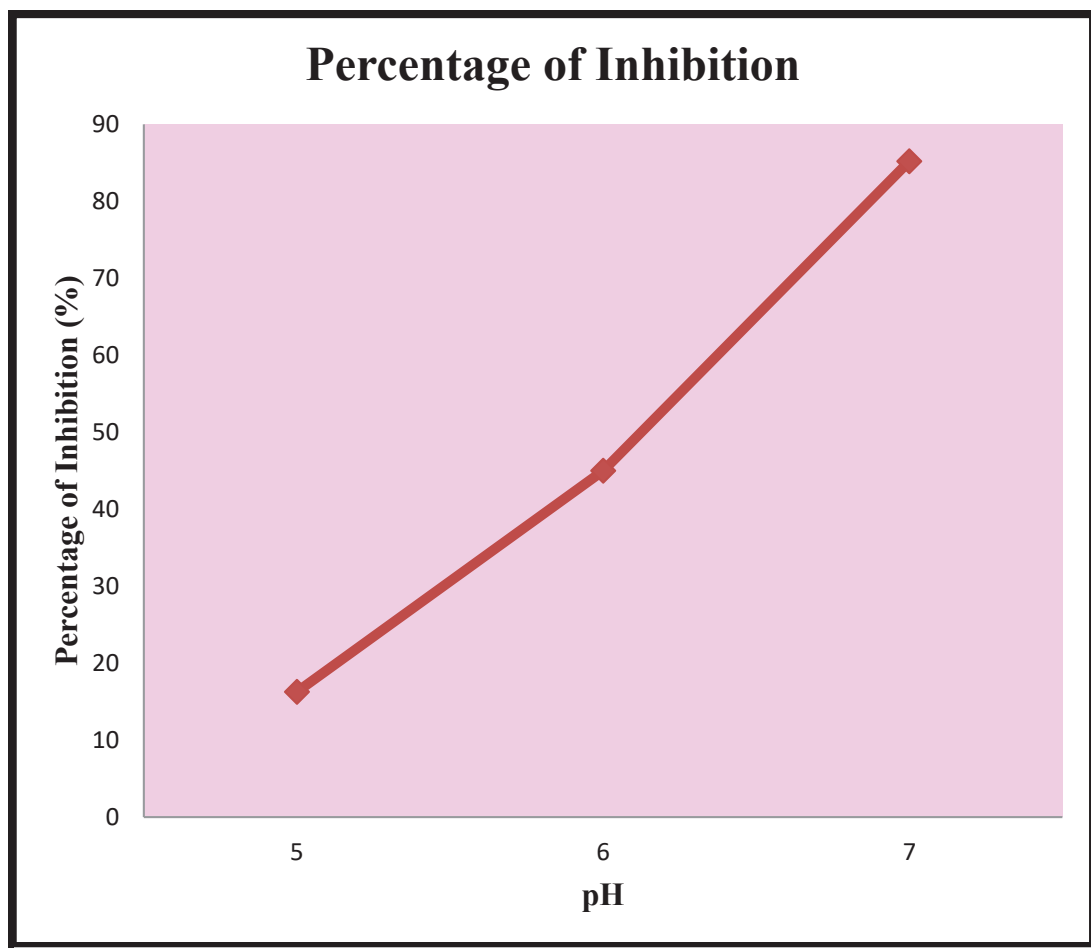


Fig 7: Percentage inhibition of *Aspergillus giganteus* during co-culture with *Aspergillus fumigatus* on various pH

Aspergillus fumigatus



Aspergillus fumigatus with
Aspergillus giganteus



A) Co-culture assay with pH 5.0



B) Co-culture assay with pH 6.0



C) Co-culture assay with pH 7.0

Plate 6: Percentage inhibition of *Aspergillus giganteus* during co-culture with
Aspergillus fumigatus on various pH

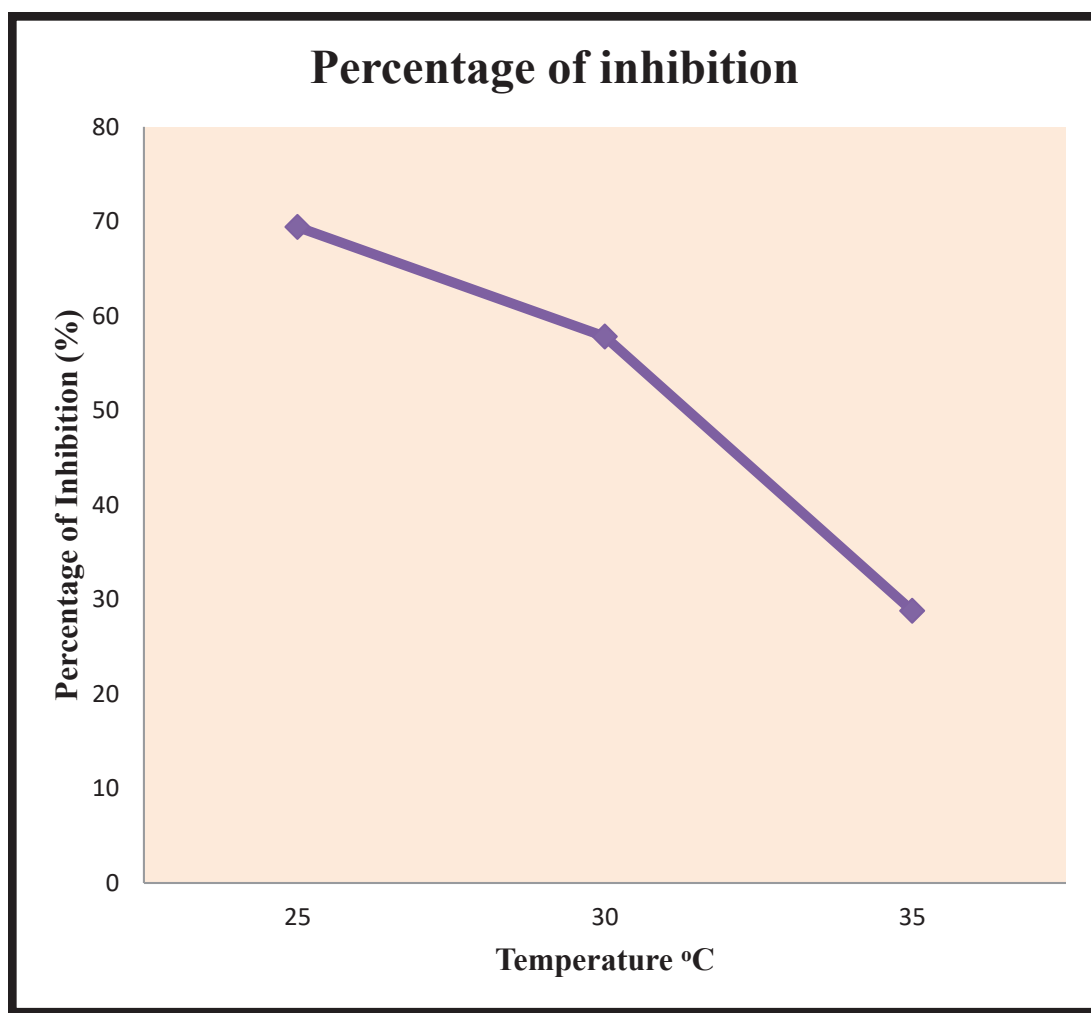


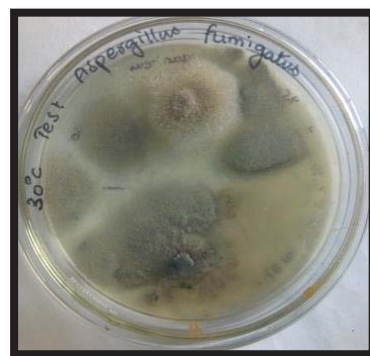
Fig 8: Percentage inhibition of *Aspergillus giganteus* during co-culture with *Aspergillus fumigatus* at different temperatures

Aspergillus fumigatus

Aspergillus fumigatus with
Aspergillus giganteus



A) Co-culture assay with 25°C



B) Co-culture assay with 30°C



C) Co-culture assay with 35°C

Plate 7: Percentage inhibition of *Aspergillus giganteus* during co-culture with
Aspergillus fumigatus at different temperatures

Several studies have been carried out to optimize the culture conditions for the better growth, antagonism and production of secondary metabolites from antagonistic organisms.

Kim *et al.*, 2016 showed that the optimization of culture conditions like pH and temperature was done to determine the growth and antagonistic effect of *Paenibacillus polymyxa*. The better growth for antagonism was found in pH 8.0. The optimum temperature was recorded at 30°C for better antagonism.

In a study by Bhattacharya and Jha, 2017, optimization was performed with different ranges of temperatures (10°C to 15°C) for the antagonistic activity of TSF146 strain of *Aspergillus Spp.* against various pathogenic fungi and bacteria. The maximum growth and zone of inhibition was observed at 30°C whereas the lowest level of inhibition and growth was recorded at 15°C and 55°C. No growth and antagonistic activity of TSF146 strain of *Aspergillus Spp* were noticed at $\leq 10^{\circ}\text{C}$.

Also optimized the culture conditions such as carbon and nitrogen sources for the better antagonistic activity of TSF146 strain of *Aspergillus Spp.* and also induce the production of antifungal compounds where sucrose acts as a carbon source for the growth of antagonistic fungi among selected carbon sources. The better antagonistic activity and production of antifungal compounds was observed in the media supplemented with asparagine as a nitrogen source.

Verma *et al.*, 2018 have done their work to determine the effects of different culture conditions on growth and production of antifungal compounds by two different fluorescent *Pseudomonas sp.* viz., An 2-nali and Pn-2-kho against *Dematophora nectrix* and *Phytophthora cactorum*. They have evaluated the culture conditions such as pH and temperature for the antagonism where decreased antifungal activity was seen at above and below 28°C. The optimum temperature for the best antagonism was recorded at 28°C. The pH 7.0 was observed as a best culture condition for the production of antifungal compounds against fungal pathogens.

In another study by Goutam *et al.*, 2014 have done the optimization of culture conditions like temperature, carbon sources and nitrogen sources were determined for the metabolite production by *Emericella quadilineata*. The maximum amount of metabolite

production was recorded at temperature 25°C followed by 20°C. Among selected carbon and nitrogen sources used, starch and ammonium chloride has been acts as a better supplement for the growth and production of antifungal compounds

Optimization of temperature for the growth and antagonistic activity of *Rhizoctonia solani* were done by Ritchie *et al.*, 2016. The temperatures selected for its growth and antagonism was 4°C to 30°C. The best growth and antagonistic activity was found at 28°C.

Another study was carried out by Anwar and Iqbal (2017) to optimize the culture conditions for the growth and antagonistic effect of *Trichoderma harzianum* against phytopathogens where the optimal conditions were found with pH 5.0 and temperature at 25°C for the better antagonistic activity of *Trichoderma harzianum* against pathogens.

Optimization of culture conditions reveals that the optimum conditions for the growth of *Aspergillus giganteus* was found to dextrose as carbon source, ammonium dihydrogen ortho phosphate as nitrogen source with pH 7.0 and at temperature 25°C. The czapek yeast extract medium was prepared with these optimum conditions and used for further assays.

4.3 Determination of antifungal metabolite

The antifungal metabolites are efficient in treating fungal diseases caused by pathogens. The antagonistic potential of antifungal metabolites from *Aspergillus giganteus* which might be either extracellular or intracellular was confirmed by overlay assay. The control plate was inoculated with pathogenic fungi alone. The inhibition percentage of supernatant and pellet of *Aspergillus giganteus* is given in table 1.

Table 1: Antagonistic potential of fungal culture filtrates on *Aspergillus fumigatus*

S. No	Culture filtrate	Inhibition in cm				Percentage of inhibition (%)			Mean (%)	Standard deviation
		C	T-1	T-2	T-3	T-1	T-2	T-3		
1	Supernatant	4.5	0.4	0.5	0.4	91.1	88.8	91.1	90.3	90.3±1.3
2	Pellet	4.5	3.5	3.4	3.4	22.2	24.4	24.4	23.6	23.6±1.2

From the results, the maximum inhibition was found in supernatant of antagonistic fungal culture filtrates and the inhibition percentage was found to be 90.3%. This study further proved that the antagonistic fungal metabolite must be extracellular.

Similar results were obtained with Song *et al.*, 2015 for the determination of antagonistic effect of *Bacillus* species against plant fungal pathogen where the supernatant of culture filtrates showed better antagonistic effect against phytopathogenic fungi.

Another study by Serrano *et al.*, 2017 showed the supernatant of *Botrytis cinerea* have better antagonistic activity against selected pathogenic fungi.

Bosmans *et al.*, 2017 have reported that the antagonistic compound of *Paenibacillus clade* is intracellular or extracellular where the supernatant of the culture filtrates shows better inhibitory activity against pathogens.

Several literature studies confirmed that antifungal compound of antagonistic fungi are protein in nature. The protein content present in the culture filtrates of *Aspergillus giganteus* was estimated by Lowry's method. The amount of protein present in 1ml of culture filtrate was found to be 0.5mg.

4.3.2 Optimization of culture conditions for culture filtrates

The overlay assay was performed to check the inhibitory efficacy of culture filtrates of *Aspergillus giganteus* under optimized culture conditions. The results are given in plate 8.

The results confirmed the antagonistic potential of culture filtrates with pathogenic fungi. The inhibition percentage of antagonistic fungal culture filtrates was found to be 95.5%.

Similar results were obtained in other studies by performing overlay assay with antagonistic fungi.

Kivanc *et al.*, 2014, showed that the inhibitory potential of lactic acid bacteria against selected fungal pathogens where the best inhibition was found with ten of 22 isolates of bacterial strain against pathogenic fungi by overlay method.

Matar *et al.*, 2017 have done overlay assay to determine the antagonistic efficacy of *Bacillus subtilis* against selected plant pathogenic fungi where maximum inhibition was found with *Rhizoctonia solani* among other plant pathogenic fungi.

Talla *et al.*, 2015 have reported that the antagonistic effect *Trichoderma* spp. against pathogenic fungal spp. where the maximum inhibition was found in *Macrophomina phaseolina* among selected fungal pathogens.

4.3.3. Hyphal interaction assay with antagonistic fungal culture filtrates

The hyphae of fungal pathogens shows good morphological appearance in control plate and this form play a key role in spreading infectious diseases. The results of hyphal interaction of culture filtrates of *Aspergillus giganteus* with pathogenic fungi were observed microscopically and the images are depicted in plate 9.

The results showed the damaged hyphal elongation of *Aspergillus fumigatus* with culture filtrates of *Aspergillus giganteus*. It indicates that the antifungal culture filtrate interferes in the elongation of hyphal forms involved in infectious processes whereas the good hyphal forms were observed in plate containing only pathogenic fungi.

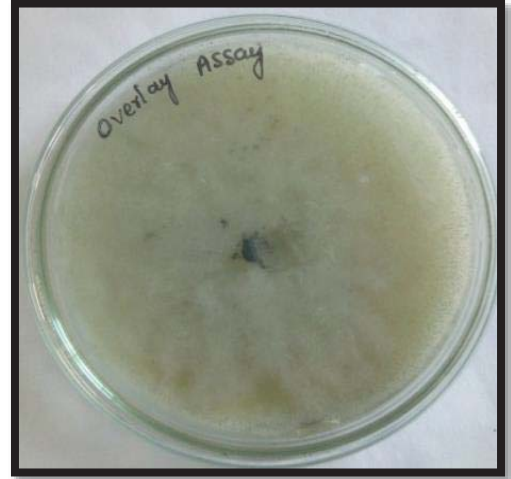
4.3.4. Scanning Electron Microscopical analysis (SEM)

Morphological changes of pathogenic fungi treated with antagonistic fungi were well established by Scanning Electron Microscopy. The inhibitory potential of *Aspergillus giganteus* with *Aspergillus fumigatus* was observed by SEM under 10µm and 2µm magnification. The medium inoculated with pathogenic fungi alone can act as control. The results are given in plate 10.

The results revealed that the antagonistic activity of *Aspergillus giganteus* culture filtrates against *Aspergillus fumigatus*. In control plates regular shaped homogenous hyphae was observed whereas the plate containing pathogenic fungi with antagonistic fungal culture filtrates shown collapsed structure and hyphal morphological changes of mycelium.

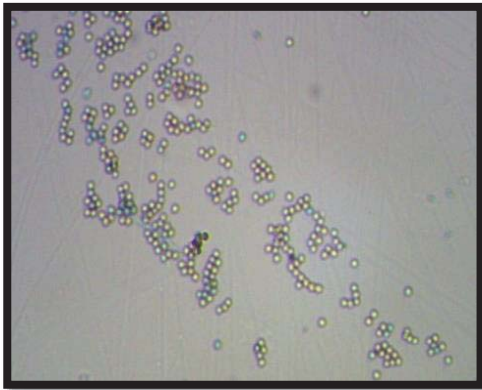


Aspergillus fumigatus

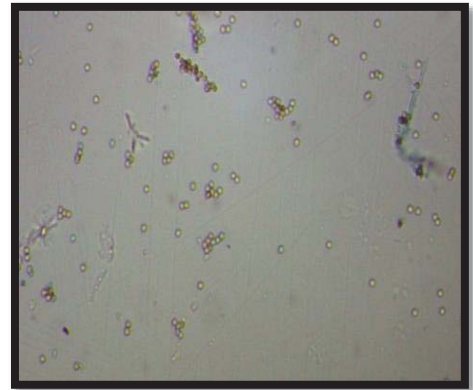


Aspergillus fumigatus with
Aspergillus giganteus

**Plate 8: Overlay assay for antagonistic potential of culture filtrates on
*Aspergillus fumigatus***



Aspergillus fumigatus



Aspergillus fumigatus with
Aspergillus giganteus

**Plate 9: Hyphal interaction assay of antifungal culture filtrates with
*Aspergillus fumigatus***

Several literature studies follow the SEM analysis for the identification of morphological changes caused by antagonistic organisms.

Da *et al.*, 2019 showed that the non-treated fungal cells remain normal, expected hyphal form and no extracellular material was observed in scanning electron microscopy whereas shrinkage, cracking of fungal filaments and degenerated cytosol were observed in pathogenic fungi treated with Bicaein bioactive compound from *Scutellaria* root extract.

Kadi *et al.*, 2018 have done scanning electron microscopic analysis to evaluate the morphological characteristics of *Aspergillus flavus*, isolated from cake samples.

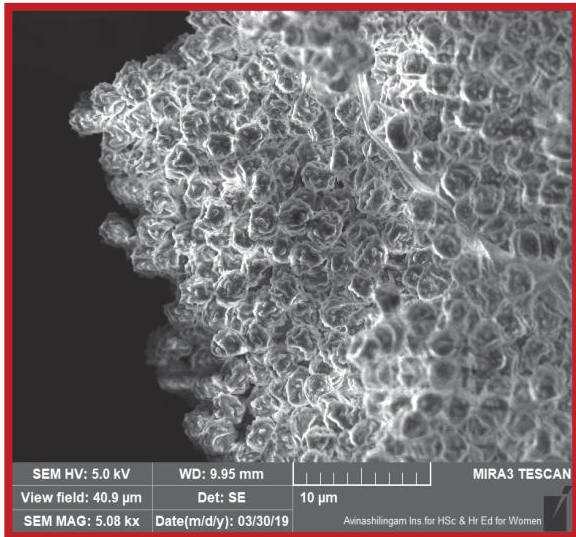
Jung *et al.*, 2018 have done the antagonistic activity of *Streptomyces* spp. against wood decay fungi *Gleophyllum trabeum*. This was subjected to SEM analysis for the determination of its inhibitory potential. *Gleophyllum trabeum* became thinner and degradation of cell wall was observed due to the action of *Streptomyces* spp.

Lipinska *et al.*, 2018, have reported that the morphological changes were observed in *Alternaria brassicicola* and *Candida vini* with antagonistic substances of *Lactobacillus pentosus* where the disorganized mycelium and hyphal form was recorded in the pathogens.

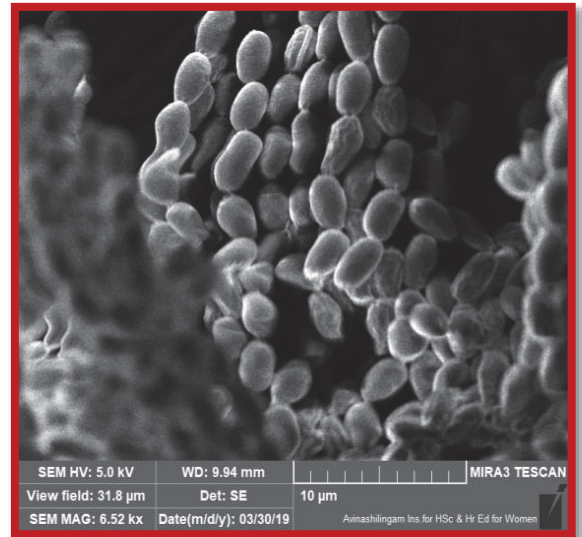
Another study done by Tao *et al.*, 2014 have reported that morphological change of *Penicillium italicum* was observed with antifungal drug, citral where considerable changes in the hyphal morphology, severely collapsed cytoplasm, shrunken and distorted mycelia were observed in treated pathogens.

4.4 Microbroth dilution assay

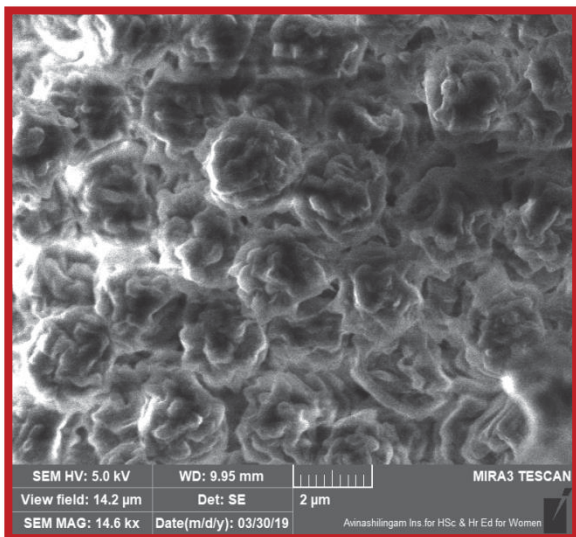
The microbroth dilution methods are the important microbial susceptibility test for the determination of minimum inhibitory concentration and minimum fungicidal concentration of antimicrobial compounds (Balouiri *et al.*, 2016).



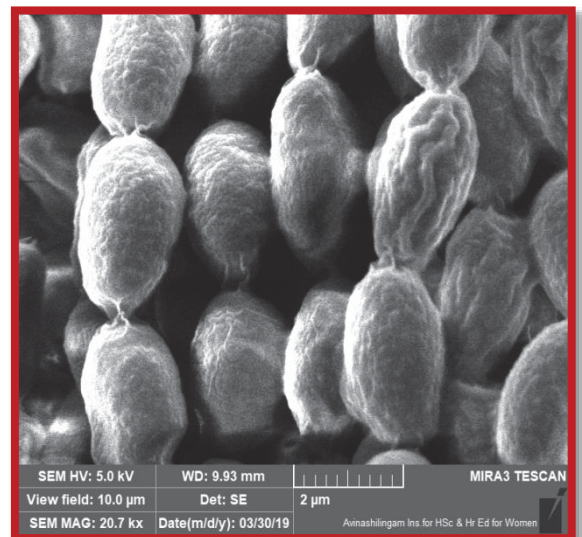
Aspergillus fumigatus



Aspergillus fumigatus with
Aspergillus giganteus



Aspergillus fumigatus



Aspergillus fumigatus with
Aspergillus giganteus

**Plate 10: Morphological changes observed with antagonistic fungal culture filtrates
using SEM analysis**

4.4.1 Minimum Inhibitory Concentration (MIC)

MIC was considered as the lowest inhibitory concentration of antagonistic substances that completely inhibits the growth of pathogenic organisms. The MIC values for the culture filtrate of *Aspergillus giganteus* with *Aspergillus fumigatus* was carried out by microbroth dilution methods. The effect of antifungal culture filtrate with pathogenic fungi for MIC determination has shown in table 2.

Table 2: MIC of culture filtrates of *Aspergillus giganteus* with *Aspergillus fumigatus*

S.No	Concentration of antifungal compounds in the culture filtrates (µg)	Growth of the pathogenic organism
1	0 (Control)	+
2	500	-
3	250	-
4	125	+
5	62.5	+
6	31.25	+

“+” indicates the growth of fungal pathogen

“-” indicates the inhibition of pathogenic fungal growth by antagonistic fungal culture filtrates.

Control = Inoculation of pathogenic fungi alone in the czapek yeast extract medium.

From the results, the MIC values were found with 125µg of antifungal compounds present in the culture filtrates of *Aspergillus giganteus*. The inhibition was not observed in concentration less than 125µg.

4.4.2 Minimum Fungicidal Concentration (MFC)

MFC is considered as the minimum concentration of antifungal compound that exhibits nearly 98 to 99.9% of killing effect on pathogens. The MFC is also determined by microbroth dilution methods. The fungicidal effect of antagonistic fungal culture filtrates was observed and the results are shown in plate 11.

The results revealed that the maximum killing action on *Aspergillus fumigatus* was found with 250µg of antifungal compounds present in the culture filtrate of *Aspergillus giganteus*. The MFC value was expressed as 2xMIC concentration. The control plates were prepared with only pathogenic organisms. There is no fungal growth observed in medium containing 250µg of antifungal compound with *Aspergillus fumigatus*.

There are various studies done to determine the MIC and MFC for antagonistic potential of fungi.

Leite *et al.*, 2015 have determined the MIC and MFC for the antagonistic effect of geraniol against *Candida albicans*. The minimum inhibitory concentration was found to be 16µg/ml of geraniol and the fungicidal activity was observed in 2xMIC which prove that the fungicidal activity of geraniol is completely dose dependent.

Another study by Sanchez *et al.*, 2016 performed MIC for the determination of inhibitory potential of plant extracts against nosocomial microorganisms where 0.7 to >15mg/ml was considered as the MIC of methanolic plant extract against various pathogens.

Medeiros *et al.*, 2016 showed the MIC and MFC of (S)-(-)-Citronellal was found to be 64µg/ml and 128µg/ml respectively against *Candida tropicalis* strains.

Gucwa *et al.*, 2018 have done the experiments to determine the MFC of ethanolic extract of antifungal compounds derived from honey bee namely, propolis against 69 clinical isolates of *Candida albicans* where 0.08–1.25% (v/v) could be considered as MFC.



Aspergillus fumigatus



Aspergillus fumigatus with
Aspergillus giganteus

Plate 11: MFC of antagonistic fungal culture filtrates with *Aspergillus fumigatus*

4.5 Mechanism of action of antifungal compounds

A detailed knowledge regarding the structure, composition and biochemistry of fungal cells has contributed to our understanding about the mechanism of action of many antifungal compounds.

4.5.1 Release of cellular materials

The cellular materials comprised of mainly nucleic acids like DNA and RNA, protein, glucose and ergosterol. The pathogenic fungal cell wall and cellular materials get damaged when disruption had occurred by antagonistic fungal substances. The released substances were estimated to confirm the damage caused by antifungal compounds acted upon the pathogenic fungal cell wall.

Quantitative analysis of released DNA in the treated *Aspergillus fumigatus*

DNA is the important cellular material present in the nucleus of fungal cell. The released DNA contents in the treated *Aspergillus fumigatus* was estimated in four different time intervals namely, 0min, 30mins, 60mins and 120mins and using five different concentration of antifungal compounds in the culture filtrates of *Aspergillus giganteus*. The results are depicted in fig 9.

The results concluded that the amount of released constituents are directly proportional to time and concentration of the antifungal compounds from culture filtrates of *Aspergillus giganteus*. It was confirmed that the antifungal metabolites damages fungal cell wall and release the cellular materials into the culture medium.

Quantitative analysis of released RNA in the treated *Aspergillus fumigatus*

The cellular material gets damaged and released the cellular constituents into the medium when antagonistic fungal compounds acted on the cell wall of pathogenic fungi. The RNA was released into the medium and estimation of RNA was done to find out the leakage of cellular materials from the pathogenic fungal cell. Control was performed without the addition of antagonistic fungal culture filtrates. The results of released RNA content from the pathogenic cell when treated with culture filtrates of *Aspergillus giganteus* is depicted in fig 10.

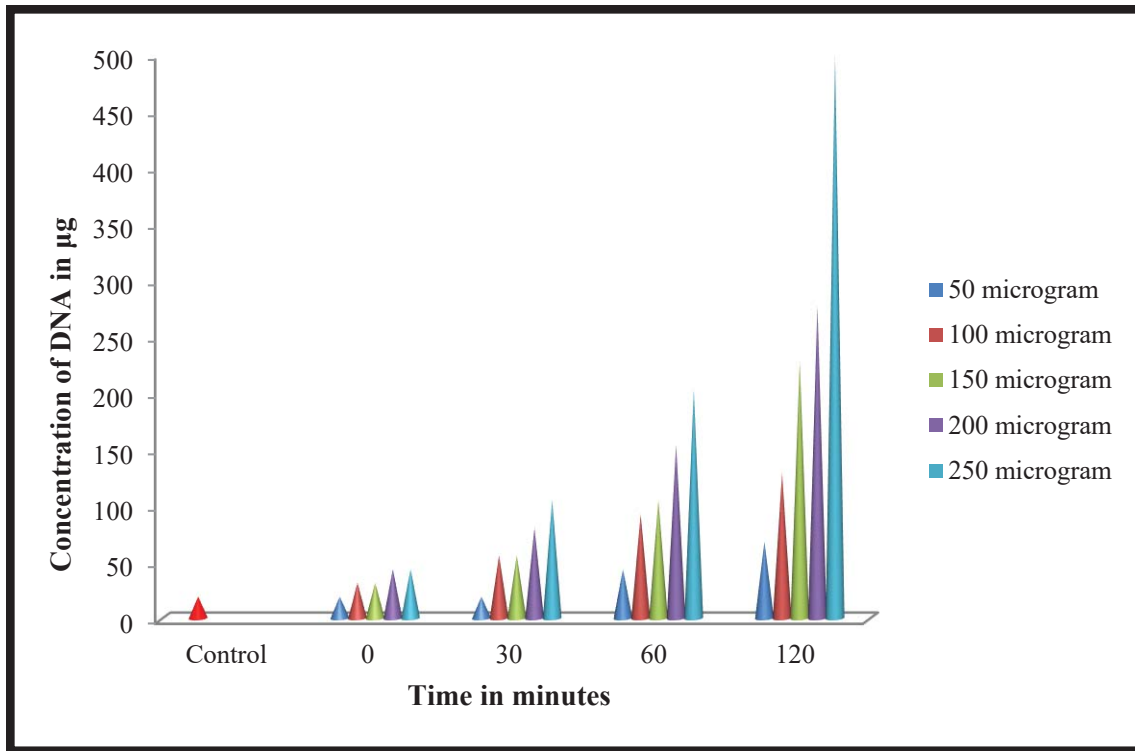


Fig 9: Quantitative analysis of released DNA in the treated *Aspergillus fumigatus*

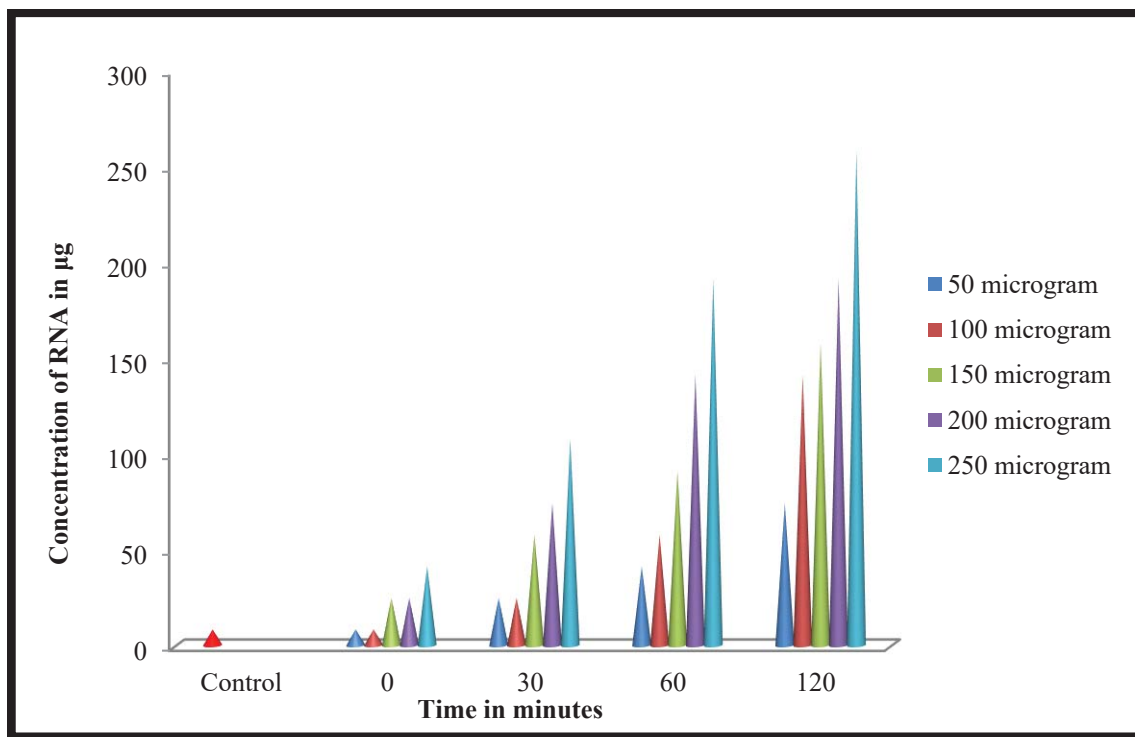


Fig 10: Quantitative analysis of released RNA in the treated *Aspergillus fumigatus*

From the results, the damage caused by the culture filtrates of *Aspergillus giganteus* treated on the *Aspergillus fumigatus* cell wall was proved by the estimation of RNA in the medium. The release of cellular constituents into the medium was increased with time and concentration of antagonistic fungal substances in the culture filtrates of *Aspergillus giganteus*.

Quantitative analysis of released protein in the treated *Aspergillus fumigatus*

Protein is the necessary compounds present in the fungal cell wall. When antagonistic fungal compounds enter into the fungal cell it damages the cell wall and then releases an abundant protein into the medium. The released proteins were estimated by Lowry's method and the damage caused by antagonistic fungal substances was proved by the estimation of cellular compounds into the medium. The results are given in fig 11.

The results indicated that the cellular constituents released into the medium was increased with time and concentration of the antagonistic fungal compounds in the culture filtrates of *Aspergillus giganteus* when acted upon the pathogenic fungal cell wall.

Quantitative analysis of released glucose in the treated *Aspergillus fumigatus*

The fungal cell wall was composed of repetitive sugar units of glucose. The antagonistic fungal compounds enters into the pathogenic fungal cell, it damages the fungal cell wall and release the sugar units in terms of glucose into the medium. The mode of action of antagonistic fungal compounds was confirmed by the release of cellular materials from the pathogenic fungal cell wall. The results of released glucose content from the pathogenic cell wall treated with antifungal compounds of *Aspergillus giganteus* culture filtrates is given in fig 12.

The results revealed that the cell wall of fungal cell gets damaged and released it cellular constituents into the medium when treated with the antagonistic fungal substances present in the culture filtrates of *Aspergillus giganteus*. The cellular leakage assay was proved that the gross and irreversible damage was caused to the cytoplasm and plasma membrane of the pathogenic fungal cell wall.

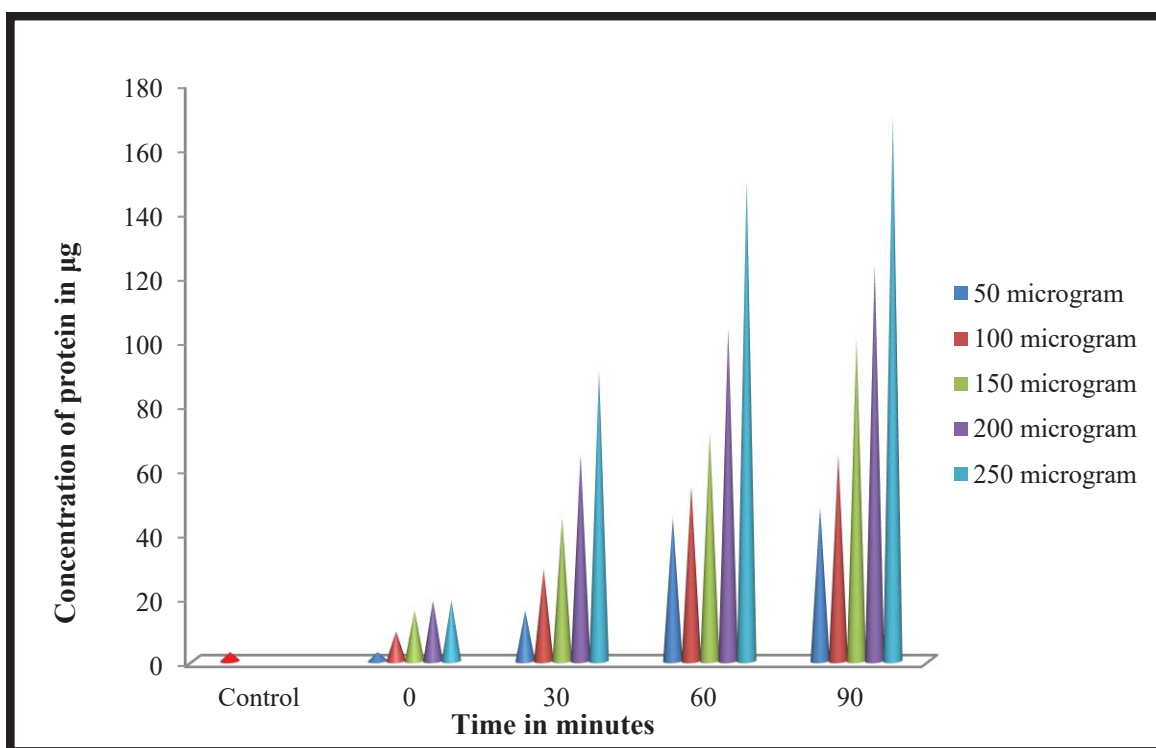


Fig 11: Quantitative analysis of released protein in the treated *Aspergillus fumigatus*

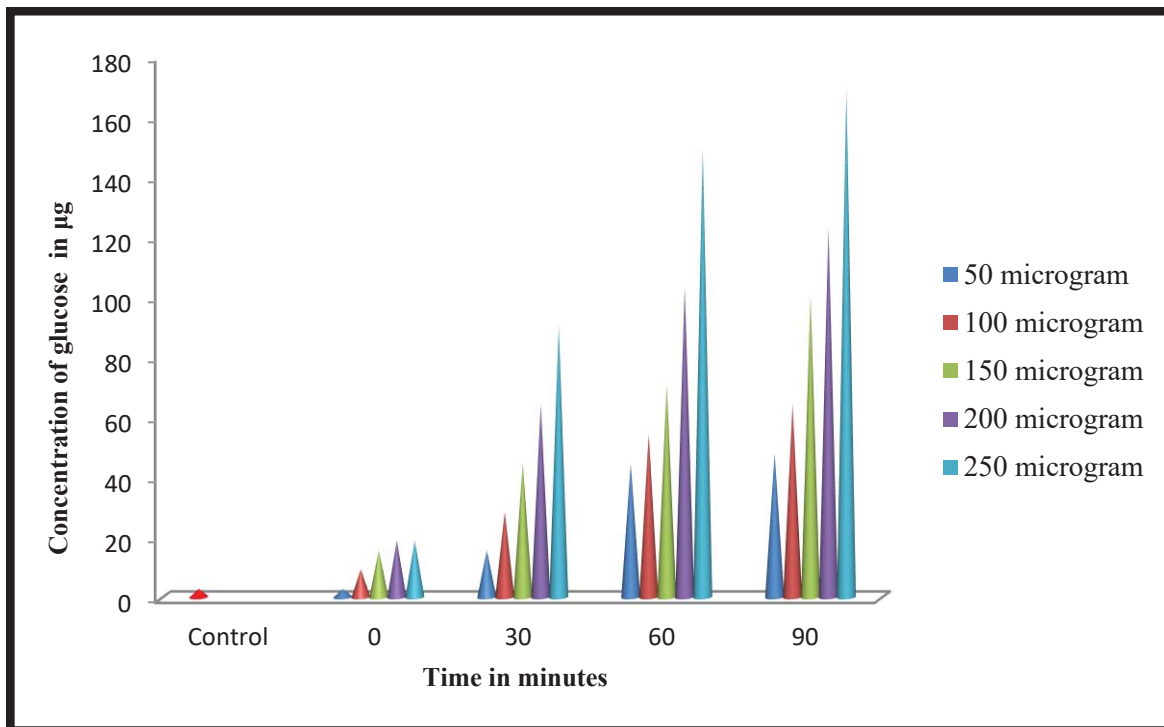


Fig 12: Estimation of released glucose in the treated *Aspergillus fumigatus*

Several studies on release of cellular constituents while treating with antagonistic compounds have reported.

Ferriera, *et al.*, 2016 have performed the cellular leakage assay for the determination of the effect of antifungal compound, IVS320 on the membrane of *Candida albicans* where the release of cellular materials were evaluated by the absorbance at 260nm which conformed the membrane rupture causes the leakage of intracellular components from the pathogenic fungi.

Shahina *et al.*, 2018 have demonstrated that the essential oil of *Cinnamomum zeylanicum* bark acted on the cell wall of *Candida albicans* resulting in the compromising the cell membrane and allowed to release the cellular constituents into the medium.

In another study by Tao *et al.*, 2014 experiments were performed to determine the release of cellular constituents of *Penicillium italicum* when treated with citral where the release of cellular constituents has gradually increased with the increased concentration of citral.

4.5.2 Sorbitol Assay

Sorbitol is an osmotic protectant of fungal cell wall which is used to stabilize the fungal protoplasts. When the fungal cell was protected by sorbitol the effect of antifungal compounds are reversed. Cells protected with sorbitol can grow in the medium with the presence of antifungal compounds while the growth of the fungi will be inhibited when there is no sorbitol in the medium. The assay was carried out to investigate whether the antagonistic fungal substances have effect on the pathogenic fungal cell wall in the presence and absence of sorbitol in the medium.

The results revealed that the sorbitol can act as a protective barrier for fungal cell wall of *Aspergillus fumigatus*. The medium incorporated with sorbitol can promote the growth of fungal pathogen and it proved that the antagonistic fungal culture filtrates of *Aspergillus giganteus* doesnot inhibit the growth of *Aspergillus fumigatus*.

4.5.3 Measurement of extracellular pH

The extracellular pH indicates the normal functioning of any cell. If any changes occurs in the extracellular pH of the cell that might be due to the changes in the cell permeability, morphological changes of cell wall, leakage of cellular materials into the environment and disturbances in the cellular metabolism. The results of changes in the extracellular pH of fungal pathogen when treated with antagonistic fungal culture filtrates of *Aspergillus giganteus* are given in fig 13.

From the results, it is concluded that there is a change in the extracellular pH when there is a gradual increase in the concentration of the antagonistic fungal culture filtrates of *Aspergillus giganteus* and incubation time. Decreased pH levels are observed after 30 minutes of incubation and then gradual fall of extracellular pH of the pathogenic organisms was recorded. This declined pH range proved that the cellular damage and release of some ions, acids and metabolites into the medium.

4.5.4 Determination of total lipid content

Lipids are one of the important components of fungal cell membrane. Ergosterol is the lipid compound present in the fungal cell wall and membrane. Lipids mainly act as a protective barrier and it maintains the integrity of cellular membrane. The assay was carried out to estimate the amount of lipid content present in the treated *Aspergillus fumigatus*. Controls were done without the addition of antifungal culture filtrates of *Aspergillus giganteus*. The result is given in fig 14.

The results revealed that the decreased lipid content of *Aspergillus fumigatus* was observed in the pathogenic fungi and no changes in the level of lipid content in control plate as well as in 50µg and 100µg of antagonistic substances in the culture filtrates of *Aspergillus giganteus*. The very low level of lipid content was recorded in the pathogenic fungi with 200 and 250µg of antifungal compounds of the *Aspergillus giganteus*. This further proved that the membrane permeability and integrity of cellular membrane of pathogenic fungi has disturbed by the antifungal compounds present in the culture filtrates of *Aspergillus giganteus*.

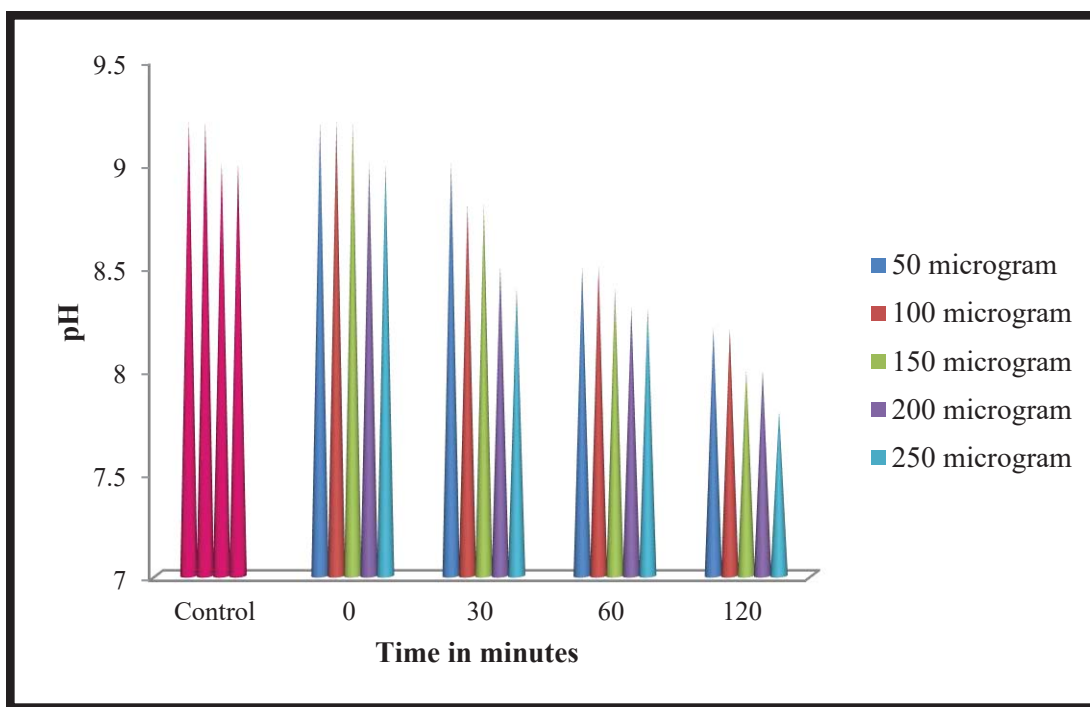


Fig 13: Variation in extracellular pH in the treated *Aspergillus fumigatus*

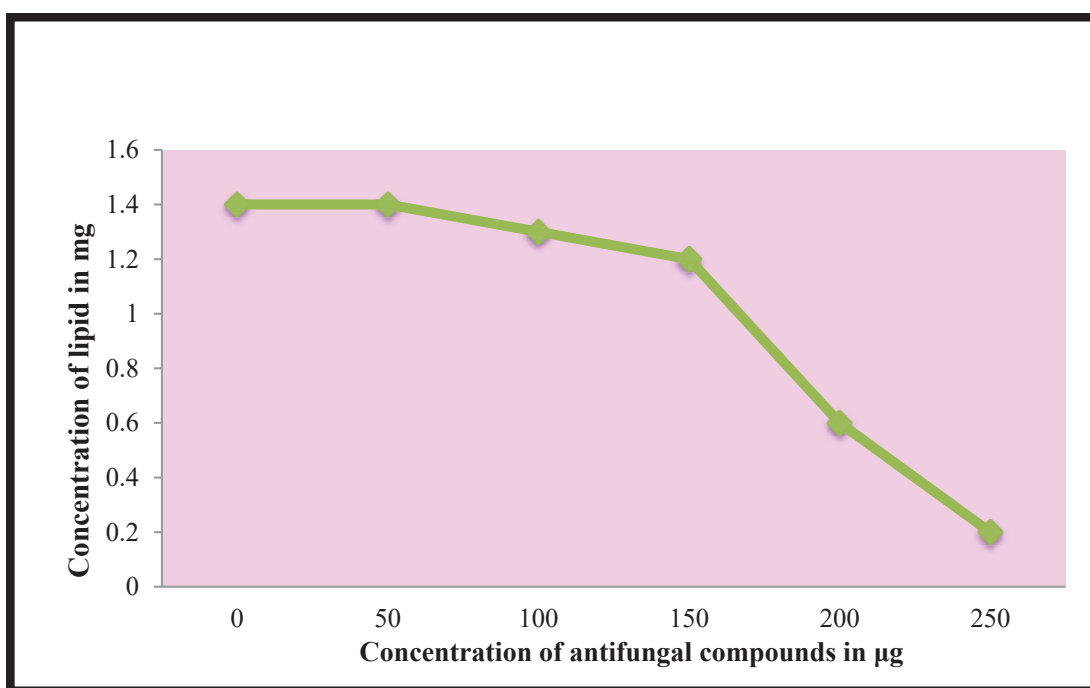


Fig 14: Lipid content of *Aspergillus fumigatus* treated with antifungal culture filtrates of *Aspergillus giganteus*

Several literatures have done the determination of lipid content in the pathogens after treated with antagonistic substances.

Knauth and Reichenbach, 2016 have performed the lipid determination assay to check the lipid content in pathogenic fungi treated with ambruticin where the decreased level of lipid content is observed in pathogen with antifungal compound.

Tao *et al.*, 2014 have reported that the lipid content of *Penicillium italicum* was significantly decreased with increasing citral concentration in the initial 30 minutes of treatment.

Li *et al.*, 2018, have done estimation of lipid content of pathogenic fungi where decreased level of lipid content was found in treated pathogens.

4.6 Cytotoxicity Assay

Cytotoxicity assay was performed to find out the toxicity level of antimicrobial compounds for their safer use.

4.6.1 Hemolytic activity against erythrocytes

The hemolytic assay was done with the antifungal culture filtrates of *Aspergillus fumigatus* goat red blood cells. The percentage of hemolysis was measured using five different concentration of antifungal compounds present in the culture filtrates namely 50µg, 100µg, 150µg, 200µg and 250µg on goat red blood cell suspensions. The percentage of hemolysis has depicted in table 3.

Table 3: Percentage of hemolysis of antifungal culture filtrates of *Aspergillus giganteus* on goat red blood cells

S. No	Concentration of antifungal compounds in the culture filtrates (µg)	Percentage of hemolysis			Mean (%)	Standard deviation
		T1	T2	T3		
1	50	8.1	9.1	8.4	8.5	8.5±0.41
2	100	15.2	15.2	15.5	15.3	15.3±0.14
3	150	17.6	16.6	17.0	17.1	17.1±0.41
4	200	22.3	21.5	23.0	22.6	22.6±0.61
5	250	25.0	25.4	25.0	25.1	25.1±0.18

The results showed that the percentage of hemolysis was increased with the concentration of antifungal compounds present in the culture filtrates of *Aspergillus giganteus* where no better hemolysis of goat red blood cells was observed in any of the selected concentration ranges 50µg to 250µg of antifungal compounds. This proved that the use of antifungal compounds from *Aspergillus giganteus* to treat fungal infections due to their efficacy and safer use with minimal side effects.

Several studies have performed hemolytic assay to find out the cytotoxicity of antifungal compounds for their safer use.

Garrigues *et al.*, 2018 have reported that the hemolytic activity of antagonistic fungal proteins and peptides of *Penicillium expansum* where none of the proteins showed hemolytic activity in the concentration of 1-100µM.

Lum *et al.*, 2015 have done hemolytic assay on human red blood cells using synthetic peptides where peptides demonstrated low hemolytic activity but it showed significant toxicity on two normal human epithelial cell lines.

In another study the hemolytic activity of *Tityus serrulatus* venom and its major Ts1 showed non-cytolytic on human erythrocytes done by Santussi *et al.*, 2017.

Thery *et al.*, 2018 have determined the hemolytic activity of four synthetic peptides where no peptides showed a hemolytic activity up to 200µg/ml but had different behaviours of cytotoxicity.

Mahadevan *et al.*, 2019 have showed the hemolytic activity of different extracts of *Periophthalmodon schlosseri* against human and chicken red blood cells where maximum hemolysis was observed in dichloromethane extract of *Periophthalmodon schlosseri*.

Summary and Conclusion

5.0 SUMMARY AND CONCLUSION

Microorganisms may produce both beneficial as well as harmful effects to the environment. Among those organisms fungi have potential role to cause infectious diseases in humans which are predominant cause for the higher rate of morbidity in worldwide. The treatment available for curing the emerging fungal infections is not appropriate due to the toxicity nature, side effects and poor absorption of existing drugs. Many strategies are currently in progress to treat fungal infections because of the limited applications of those existing drugs. So it is widely accepted to create many antifungal compounds from natural sources rather than from synthetic sources. *Aspergillus giganteus* is an antifungal compound producing fungi, which could be selected for the study to treat fungal infections caused by *Aspergillus fumigatus*. The study mainly focussed to study the mode of action of antagonistic fungal culture filtrates and to optimize the culture conditions for the better growth of antagonistic fungi.

The antagonistic activity of *Aspergillus giganteus* against *Aspergillus fumigatus* was determined by adopting various materials and methods. Primarily, the optimization of culture conditions namely, carbon sources, nitrogen sources, pH and temperature was done to find out the better growth of antagonistic fungi. The hyphal interaction study was conducted to determine the damage of *Aspergillus fumigatus* hyphae when treated with *Aspergillus giganteus*. The antagonism was further proved for the antifungal compounds present in the culture filtrates of *Aspergillus giganteus* by overlay assay. Scanning electron microscopy was performed to identify the changes in the fungal morphology. Microbroth dilution assay such as minimum inhibitory concentration and minimum fungicidal concentration was also determined for the antifungal compounds in the culture filtrates. Then the mechanism of antifungal compounds were studied by estimating the release of cellular components, variations in the extracellular pH, sorbitol binding assay, determination of lipid contents of *Aspergillus fumigatus* when treated with the antifungal compounds present in the culture filtrates of *Aspergillus giganteus*.

Culture conditions of carbon sources, nitrogen sources and appropriate environment conditions such as pH and temperature are the important factors for the growth of any organisms. So we need to optimize it for the antagonistic fungal growth.

The results showed that the czapek yeast extract medium was prepared with dextrose as carbon source, ammonium dihydrogen ortho phosphate as nitrogen source, pH at 7.0 and temperature at 25°C for further analysis.

The hyphae of fungal pathogen play an important role in the process of infection. The hyphal morphological changes were observed with *Aspergillus giganteus* proved the inhibition of hyphal growth of *Aspergillus fumigatus* was occurred.

The antagonistic fungal culture filtrates of *Aspergillus giganteus* was prepared and it was checked for their antagonistic potential against *Aspergillus fumigatus* by overlay assay and hyphal interaction assay which proved the antagonistic efficacy of antifungal compounds present in the culture filtrates of *Aspergillus giganteus*.

The antagonistic substances are mostly protein in nature and the amount of protein present in the culture filtrate of *Aspergillus giganteus* was estimated by Lowry's method.

Scanning electron microscopy analytical results revealed that the *Aspergillus fumigatus* showed collapsed structure and hyphal morphological changes of mycelium when treated with the antagonistic fungal culture filtrates of *Aspergillus giganteus*.

The minimum inhibitory concentration and minimum fungicidal concentration were determined for the antifungal compounds present in the culture filtrates of *Aspergillus giganteus* to identify the minimum concentration that showed complete inhibition and killing effect on *Aspergillus fumigatus*.

A detailed knowledge regarding the structure, composition and biochemistry of fungal cells has contributed to our understanding about the mechanism of action of many antifungal compounds.

The results of mechanistic action of culture filtrates of *Aspergillus giganteus* on *Aspergillus fumigatus* revealed that the cell wall of fungal cell gets damaged and released its cellular constituents into the medium when treated with the antagonistic fungal substances. The cellular leakage assay was proved that the gross and irreversible damage was caused to the cytoplasm and plasma membrane of the pathogenic fungal cell wall due to the antifungal compounds present in the culture filtrates of *Aspergillus giganteus*.

Sorbitol is an osmotic protectant of fungal cell wall which is used to stabilize the fungal protoplasts. The results of this assay showed that the sorbitol can act as a protective barrier for fungal cell wall of *Aspergillus fumigatus*. The medium incorporated with sorbitol can promote the growth of fungal pathogen and it proved that the antagonistic fungal culture filtrate of *Aspergillus giganteus* does not inhibit the growth of *Aspergillus fumigatus*.

The extracellular pH indicates the normal functioning of any cell. The antagonistic fungal culture of *Aspergillus giganteus* acted on *Aspergillus fumigatus* which results in the gradual decrease in the extracellular pH of fungal pathogen with increased concentration of antifungal compounds. This can further proved that the cellular damage and release of some ions, acids and metabolites into the medium.

Lipids mainly act as a protective barrier and it maintains the integrity of cellular membrane. The lipid content of pathogenic fungi was determined after treating with antagonistic fungal culture filtrates of *Aspergillus giganteus*. The lipid content of pathogenic fungi was decreased with the gradual increase in the concentration of antifungal compounds in the culture filtrate. This further proved that the membrane permeability and integrity of cellular membrane of pathogenic fungi has been distorted by the antifungal compounds from *Aspergillus giganteus*.

Cytotoxicity assay was performed to find out the toxicity level of antimicrobial compounds for their safer use. The haemolytic activity was performed to check the toxicity level of antifungal compounds present in the culture filtrates of fungi on goat red blood cells. The results showed that the percentage of hemolysis was increased with the concentration of antifungal compounds present in the culture filtrates of *Aspergillus giganteus* where no better hemolysis of goat red blood cells was observed in any of the selected concentration of antifungal compounds. This proved that the use of antifungal compounds from *Aspergillus giganteus* to treat fungal infections due to their efficacy and safer use with minimal side effects.

This study proved that the antagonistic potential of *Aspergillus giganteus* against human pathogenic fungus, *Aspergillus fumigatus*. The complete metabolite profile of

antifungal compound present in the *Aspergillus giganteus* will provide valuable information in the field of medicine to treat fungal infections.

Suggestion for future studies:

- ❖ The complete metabolite study of *Aspergillus giganteus* and purification of the metabolite can be followed.
- ❖ The molecular level of action of antifungal compounds present in the *Aspergillus giganteus* against various fungal pathogens can be studied.
- ❖ The efficacy of antifungal drug from *Aspergillus giganteus* and can be confirmed by *in vivo* studies.

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Appendices

APPENDIX – I

PREPARATION OF CZAPEK BASAL MEDIA

➤ Czapek concentrate	–	10.0ml
NaNO ₃	-	30.0g
KCl	-	5.0g
MgSO ₄	-	5.0g
FeSO ₄	-	0.1g
Distilled water-		100ml
➤ K ₂ HPO ₄	-	1.0g
➤ Yeast extract	-	5.0g
➤ Dextrose	-	2.0g
➤ Agar	-	15.0g
➤ Dis.H ₂ O	-	1000ml

APPENDIX – II

HYPHAL INTERACTION ASSAY BY LACTOPHENOL COTTON BLUE STAINING

Principle

Lactophenol Cotton Blue is a stain commonly used for making semi-permanent microscopic preparation of fungi. It stains the fungal cytoplasm and provides a light blue background against which the walls of the hyphae can readily be seen. It contains 4 constituents namely phenol - which serve as a fungicide, lactic acid – which serve as a cleaning agent, cotton blue – which stains the cytoplasm of the fungus and glycerine – which gives a semi-permanent preparation. The periodic acid shift (PAS) and methanamine can also be used for fungal elements in tissue section.

Materials required

Young culture of mold, mounting needles, glass slides, cover slip, 75% alcohol.

Procedure

A drop of lactophenol cotton blue was placed on a clean glass slide. Transfer a small part of fungus usually with spores and spore bearing structure into the broth using flamed needle. Mixed gently stab with the mold structures. Finally placed a cover slip over the preparation and examined under microscope.

APPENDIX – III OVERLAY ASSAY

200µl of culture filtrates of *Aspergillus giganteus* was poured into the czapek yeast extract culture medium. About 5mm disc of *Aspergillus fumigatus* was inoculated in the culture medium and incubated at 28°C for 4 days.

APPENDIX – IV ESTIMATION OF PROTEIN BY LOWRY'S METHOD (Lowry *et al* .,1951)

Principle

The intensity of the blue colour developed by the reduction of phosphomolybdic and phosphotungstic components in the Folin-Ciocalteu reagent by the aminoacids tyrosine and tryptophan present in the protein and the colour developed by the Biuret reaction of the protein with alkaline cupric tartarate are measured spectrophotometrically at 670nm.

Reagents

1. Solution A: 1% Copper Sulphate
2. Solution B: 2% Sodium Potassium Tartarate
3. Solution C: 2% Sodium Carbonate in 0.1N Sodium Hydroxide
4. Solution D: Mixed just before use, 1ml of solution A, 1ml of solution B and 100ml of solution C.

5. Solution E: Folin-Ciocalteu reagent (Mixed equal volumes of commercially available reagent and distilled water just prior to use). Stored and protected from light.

6. Standard BSA:

a) Stock solution: A stock solution containing 50mg BSA in 50ml was prepared. 1ml of this solution contains 1mg of protein.

b) Working standard: Diluted the stock solution in the ratio 1:10 for use as working standard. 1 ml of this solution contains 100 µg of protein.

Procedure:

Pipetted out the working standard solution (0.2 to 1.0ml) and the supernatant of treated *Aspergillus fumigatus* were taken into clean dry test tubes. The tubes were then made up to 1ml with 0.1N sodium hydroxide and shaken well to treat the protein with alkali. Then 3ml of solution D was added, mixed well and incubated at 37°C for 3 minutes. 0.3 ml of solution E was added, mixed well and incubated at 37°C for 3 minutes again. The blue color developed was read at 670 nm against reagent blank.

APPENDIX - V

ESTIMATION OF DNA BY DIPHENYLAMINE METHOD

Principle

Under extreme acid condition, DNA is initially depurinated quantitatively followed by the dehydration of sugar to hydroxy levulinyl aldehyde. This aldehyde condenses in the acidic medium with diphenylamine to produce a deep blue coloured condensation product with absorption maximum at 595nm.

Reagents

1. DNA standard: 50mg of DNA was dissolved in 100ml of distilled water (warmed slightly if necessary).
2. DNA working standard: 20ml of the DNA standard was taken and made up to 100ml in order to contain 100µg/ml.

3. Diphenylamine reagent: Mixed 5g of fresh or re-crystallized diphenylamine, 500ml of glacial acetic acid and 13.75ml concentrated sulphuric acid (stable for 6 months at 2°C. Warmed to room temperature, swirled to remix before use).

Procedure

Pipetted out 0.2-1.0 ml of working standard DNA solution and 1.0 ml of supernatant of treated sample were taken into clean dry test tubes. The tubes were made upto 1.5ml with distilled water. Added 3ml of diphenylamine reagent to each tube and mixed. Heated the tubes in a boiling water bath for 10 minutes and cooled. Read the blue colour developed at 600nm against the blank.

APPENDIX – VI

ESTIMATION OF RNA - ORCINOL METHOD

Principle

The method depends on the conversion of pentose (ribose) in the presence of hot acid to furfural which then reacts with orcinol to yield a green color which can be measured spectrophotometrically at 660nm.

Reagents

1. Standard RNA – 50 µg/ml in ice cold TE buffer (10mM Tris, 1mM EDTA, pH 7.2) or any other buffer for dissolving RNA completely.
2. Acid orcinol reagent: Added 2ml of 10% solution (w/v) of ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) to 400ml of Conc.HCl (mixed freshly).
3. Alcoholic – orcinol: Dissolved 6g orcinol in 100ml of 95% ethanol in a brown bottle (mixed freshly).

Procedure

0.5-3.0 ml of working standard RNA was pipetted out into clean test tubes. Took 1.0ml of the supernatant of treated *Aspergillus fumigatus* were taken into a clean dry test tube. The tubes were made upto 3ml with distilled water. Added 6ml of acid-orcinol reagent followed by 0.4 ml alcoholic orcinol reagent to each tube and mixed well and heated all the tubes in a boiling water bath for 20 minutes. Cooled and read the absorbance at 660nm against a reagent blank.

APPENDIX – VII

ESTIMATION OF GLUCOSE BY ANTHRONE METHOD

Reagents:

1. Anthrone reagent: Dissolve 200mg anthrone in 100ml of ice- cold 95% Sulphuric acid. Prepare fresh before use.
2. Standard Glucose: Stock- Dissolve 100mg in 100ml distilled water. 1.0ml of this solution contains 1.0 mg of glucose.
3. Working Standard: 10ml of stock diluted to 100ml with distilled water. Store refrigerated after adding a few drops of toluene. 1.0ml of this solution contains 100 μ g of glucose.

Procedure:

Pipetted out 0.2 to 1.0 ml of working standard glucose solution and supernatant of treated samples were taken into a clean dry test tubes. All the tubes were made up to 1ml with distilled water. 4ml of anthrone reagent was added to all the tubes and heated for 8 minutes in a boiling water bath and allowed to cool rapidly. The dark green colour developed was read at 630 nm against reagent blank.