MECHANISTIC INSIGHT INTO THE ANTAGONISTIC EFFICACY OF ANTIFUNGAL COMPOUNDS ON *Cryptococcus neoformans* AND *Candida albicans* USING *in vitro* AND *in silico* APPROACHES

Ramya.R

(19MPBCF004)

M.Phil in Biochemistry

A Thesis submitted in

Partial Fulfilment of the

Degree of Master of Philosophy (M.Phil)

Department of Biochemistry, Biotechnology and Bioinformatics

Avinashilingam Institute for Home Science and Higher Education for Women

Coimbatore-641043

December, 2020

CERTIFICATE

MECHANISTIC INSIGHT INTO THE ANTAGONISTIC EFFICACY OF ANTIFUNGAL COMPOUNDS ON *Cryptococcus neoformans* AND *Candida albicans* USING *in vitro* AND *in silico* APPROACHES

Ramya.R

(19MPBCF004)

M.Phil in Biochemistry

A Thesis submitted in

Partial Fulfilment of the

Degree of Master of Philosophy (M.Phil)

Department of Biochemistry, Biotechnology and Bioinformatics

Avinashilingam Institute for Home Science and Higher Education for Women

Coimbatore-641043

December, 2020

21/12/2020

Signature of the Head of the Department

Signature of the Supervisor

DECLARATION

DECLARATION

I declare that the dissertation entitled **Mechanistic Insight into the Antagonistic Efficacy of Antifungal Compounds on *Cryptococcus neoformans* and *Candida albicans* using *in vitro* and *in silico* Approaches** submitted by me for the degree of **Master of Philosophy (M.Phil.)** is the record of work carried out by me during the period from **July, 2019** to **December, 2020** under the guidance of **Dr.D.KAVITHA** and has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship, Titles in this University or any other University or other similar institution of Higher Learning.

Signature of the Candidate

CERTIFICATE FROM THE SUPERVISOR

CERTIFICATE FROM THE SUPERVISOR

I certify that the dissertation entitled **Mechanistic Insight into the Antagonistic Efficacy of Antifungal Compounds on *Cryptococcus neoformans* and *Candida albicans* using *in vitro* and *in silico* Approaches** submitted for the degree of **Master of Philosophy (M.Phil.)** is the record of research work carried out by her during the period from **July, 2019** to **December, 2020** under my guidance and supervision, and that this work has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other Titles in this University or any other University or other institution of Higher Learning.

Signature of the Supervisor with designation

Dr. D. KAVITHA, M.Sc.,M.Phil.,Ph.D.,
Assistant Professor
Department of Biochemistry,
Biotechnology & Bioinformatics
Avinashilingam Institute for
Home Science and Higher Education for Women
Coimbatore - 641 043.

ACKNOWLEDGEMENT

ACKNOWLEDGEMENT

I owe a special tribute to ***God Almighty*** for the opportunity given to take up to complete my work successfully. In addition to the will of supreme divinity, the willingness of many subject experts and erudite scholars to extend their assistance and help for the completion of a work plays, indeed a vitally important role.

I express my deep sense of gratitude to all my higher authorities of Avinashilingam Institute for Home Science and Higher Education for Women for their immense support.

I take the opportunity of expressing my sincere thanks to ***Dr. (Thiru) P.R. Krishna Kumar (Late)*** and ***Dr. (Thiru) S.P. Thiyagarajan,*** Chancellor, Avinashilingam Institute for Home Science and Higher Education for women, Coimbatore, for providing the opportunity and infrastructure to undertake this investigation.

I immensely thank ***Dr. Premavathy Vijayan***, Vice Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the entire facilities essential to carry out and complete the study.

I record my sincere thanks to ***Dr. S. Kowsalya***, Registrar, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for timely help rendered to carry out the work.

I record my sincere thanks to ***Dr. P.R. Padma (Late)***, former Dean and Head, Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the opportunity and timely help rendered to carry out the work successfully.

I express my special gratitude to ***Dr. A. Vijayalakshmi,*** Dean, School of Biosciences, Professor and Head, Department of Botany, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore and her sustained support throughout the study.

I express my reverential thanks to ***Dr. G.P. Jeyanthi***, Director, Research and Consultancy, Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore, for their support and encouragement rendered towards the completion of my thesis work.

I record my sincere gratitude to ***Dr. Anitha Subash,*** Professor and Head, Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore and her immense support and motivation throughout my study.

I owe my indebtedness, profound and deepest thanks to my guide ***Dr. D. Kavitha,*** Assistant Professor, Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her incessant guidance, immense tolerance, meticulous care, good support, creative influence, thoughtful advice, steady encouragement, motherly love throughout the research and motivation right from the selection of topic to the compilation and completion of the work efficiently and effectively.

I submit my sincere thanks to all ***The Staff Members***, of Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for lending a helping hand and invaluable guidance during the course of this thesis work.

I record my sincere thanks to my senior, ***Ramya K*** for their immense support, constant encouragement, patience help and valuable advice throughout my project work.

I place my gratitude to foot of my parents for their immense support and guidance during the course of my study.

I express my sincere heart bound thanks to my friends, Department of Biochemistry, Biotechnology and Bioinformatics, for giving an affectionate advice, unconditional love and incredible supports for the completion of my project work.

I acknowledge the contribution of all other unseen hands during the course of the study for help rendered in the successful completion of the study.

Ramya.R

CONTENTS

CONTENTS

Chapter No.	Title	Page No.
	LIST OF TABLES	
	LIST OF FIGURES	
	LIST OF PLATES	
	LIST OF APPENDICES	
1.0	INTRODUCTION	1
2.0	REVIEW OF LITERATURE	7
3.0	METHODOLOGY	26
4.0	RESULTS AND DISCUSSION	34
5.0	SUMMARY AND CONCLUSION	89
6.0	BIBLIOGRAPHY	93
	APPENDICES	104

LIST OF TABLES

LIST OF TABLES

Table No.	Title	Page No.
1	**Percentage hemolysis of antifungal culture filtrates of *Aspergillus giganteus* using goat red blood cells**	41
2	**Concentration of protein content during purification processes**	52
3	**Ligand structures with their molecular formula and molecular weight**	64 65
4	**ADME predictions for the selected compounds**	66
5	**List of selected pathogenic target proteins**	68
6	**Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Cryptococcus neoformans* target protein 3SFX**	69
7	**Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Cryptococcus neoformans* target protein 4AF0**	70
8	**Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Cryptococcus neoformans* target protein 6CW5**	71
9	**Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Cryptococcus neoformans* target protein 6TZ8**	72

10	**Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Cryptococcus neoformans* target protein 6WHP**	**73**
11	**Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Candida albicans* target protein 1AI9**	**80**
12	**Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Candida albicans* target protein 1IYL**	**81**
13	**Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Candida albicans* target protein 1ZAP**	**82**
14	**Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Candida albicans* target protein 5V5Z**	**83**

LIST OF FIGURES

LIST OF FIGURES

Figure No.	Title	Page No.
1	**Leakage of DNA from pathogenic cell membrane by the action of culture filtrates of *Aspergillus giganteus* at various time intervals**	43
2	**Leakage of RNA from pathogenic cell membrane by the action of culture filtrates of *Aspergillus giganteus* at various time intervals**	44
3	**Leakage of protein from pathogenic cell membrane by the action of culture filtrates of *Aspergillus giganteus* at various time intervals**	47
4	**Leakage of glucose from pathogenic cell membrane by the action of culture filtrates of *Aspergillus giganteus* at various time intervals**	48
5	**Influence of the culture filtrates of *Aspergillus giganteus* on the extracellular pH of the fungal pathogens**	50
6	**Lipid content of pathogenic *C. neoformans* and *C. albicans* cell membrane treated with antagonistic culture filtrates**	51
7	**Leakage of DNA from pathogenic cell membrane by the action of AFCs from *Aspergillus giganteus* at various time intervals**	55
8	**Leakage of RNA from pathogenic cell membrane by the action of AFCs from *Aspergillus giganteus* at various time intervals**	56
9	**Leakage of protein from pathogenic cell membrane by the action of AFCs from *Aspergillus giganteus* at various time intervals**	57
10	**Leakage of glucose from pathogenic cell membrane by the action of AFCs from *Aspergillus giganteus* at various time intervals**	58
11	**Action of AFCs from *Aspergillus giganteus* on the permeabilization of ions and other solute molecules in the pathogenic cell membrane**	61

12	**Lipid contents in the AFCs treated pathogenic *C. neoformans* and *C. albicans* cell membrane**	**62**
13	**Ligand-protein interaction of antifungal compounds docked with the target protein 3SFX showing the 3D and 2D images**	**74**
14	**Ligand-protein interaction of antifungal compounds docked with the target protein 4AF0 showing the 3D and 2D images**	**75**
15	**Ligand-protein interaction of antifungal compounds docked with the target protein 6CW5 showing the 3D and 2D images**	**76**
16	**Ligand-protein interaction of antifungal compounds docked with the target protein 6TZ8 showing the 3D and 2D images**	**77**
17	**Ligand-protein interaction of antifungal compounds docked with the target protein 6WHP showing the 3D and 2D images**	**78**
18	**Ligand-protein interaction of antifungal compounds docked with the target protein 1AI9 showing the 3D and 2D images**	**84**
19	**Ligand-protein interaction of antifungal compounds docked with the target protein 1IYL showing the 3D and 2D images**	**85**
20	**Ligand-protein interaction of antifungal compounds docked with the target protein 1ZAP showing the 3D and 2D images**	**86**
21	**Ligand-protein interaction of antifungal compounds docked with the target protein 5V5Z showing the 3D and 2D images**	**87**

LIST OF PLATES

LIST OF PLATES

Plate No.	Title	Page No.
1	Antagonistic activity of *Aspergillus giganteus* against pathogenic *Cryptococcus neoformans*	35
2	Antagonistic activity of *Aspergillus giganteus* against pathogenic *Candida albicans*	35
3	Hyphal interaction of antagonistic *Aspergillus giganteus* with pathogenic *Cryptococcus neoformans*	37
4	Hyphal interaction of antagonistic *Aspergillus giganteus* with pathogenic *Candida albicans*	37
5	Antagonistic activity of the culture filtrates of *Aspergillus giganteus* on human fungal pathogens	39

LIST OF APPENDICES

LIST OF APPENDICES

Appendix No.	Title	Page No.
1	Composition of czapek yeast extract media	104
2	Hyphal interaction assay by lactophenol cotton blue staining	105
3	Estimation of protein by Lowry's method	106
4	Estimation of DNA by diphenylamine method	107
5	Estimation of RNA by orcinol method	108
6	Estimation of glucose by anthrone method	109
7	Purification of proteins by ammonium sulphate precipitation	110
8	Desalting of proteins by dialysis	111

INTRODUCTION

1.0 INTRODUCTION

Fungal infections are the most dreadful diseases among the human diseases in perspective to their management and treatment. Nearly, 1.7 billion people are suffering with infections caused by fungal pathogens. Invasive fungal infections are described as systemic fungal infections where the fungi has been establishing and infiltrating themselves in the deeper tissues of the host. Even though invasive fungal infections are life threatening, it has occurred in minimum number while comparing with superficial fungal infections which is the reason for high morbidity, mortality and economical burden. The severe fungal infections has been observed in individuals due to major health issues such as acquired immunodeficiency syndrome (AIDS), asthma, cancer, diabetes, organ transplantation and hormone based treatments (Aboody and Mickymaray, 2020).

Yeasts are very large, widespread opportunistic fungus for the cause of harmful infections and associated with various diseases. There are wide spectrum of yeasts where most of the pathogenic fungus has been evolved and cause major illness among human, plants and animals. The opportunistic fungal pathogens belongs to the genus aspergillus, candida, cryptococcus and pneumocystis and have been declared as the most harmful life threatening pathogens leading to the higher incidence of the death worldwide (Butts *et al*., 2018).

Despite the increased incidence of mortality rate recorded with fungal infections, more of antifungal compounds have been discovered. There are a few classes of antifungal drugs available for the prevention and treatment of fungal infections. Unfortunately, most of the fungal pathogens may develop the resistance to antifungal drugs that resulted in the unsuccessful treatment of fungal infections. Numerous investigations have been reported that this antimicrobial resistance is a global threat now causing increased mortality rate and it may go above 10 millions of death by the year 2050. Possibly, this antimicrobial resistance may cause major mortality and morbidity rate compared to other malignancies and metabolic diseases (Nicola *et al*., 2019). Apart from the drug resistance, various factors may also be involved in the poor treatment in patients such as improper distribution of drug or immune dysfunction in the host or influence of other medications on the pathogenic fungi (Tong *et al*., 2020).

The fungal cell wall is considered as the main targets for the antifungal compounds. The cell wall of fungi is the important and key structure for the maintenance of cellular integrity and its function. It plays a number of roles like control of cellular permeability and to protect the

cells from various stresses such as mechanical stress and osmotic stress. Fungal cell wall is mainly composed of polysaccharides, proteins, lipids and pigments. Few components of the pathogenic fungal cell wall are said to be immunogenic that can induce cellular and humoral immune responses during infection processes (Augustinho *et al*., 2018).

Currently, according to the clinicians and veterinarians, the antifungal drug classes for systemic fungal infections belongs to four major classes which includes polyenes, azoles, echinocandins and pyrimidine analogues. These antifungal drugs target various parts in the pathogenic fungal cell. First class of drug, polyene namely, amphotericin B, mainly targets the ergosterol, an important component of the fungal cell membrane. The drug Amphotericin B (AMB) is a cyclic heptaene which binds to the ergosterol thus causing the formation of pore on the cellular membrane results in the release of ions like K^+, Mg^{2+}, Ca^{2+}, Cl^- and glucose molecules. Sequentially, the Amphotericin B induces the accumulation of reactive oxygen species, causing the damage of DNA, RNA, protein and mitochondrial and membrane damages (Revie *et al*., 2018).

In the second class of antifungal drugs, the first and second generation antifungal drugs namely, flucanazole, itraconazole, posaconazole, voriconazole and isavuconazole intensively interrupts the ergosterol especially in lanosterol demethylation steps during biosynthesis. Echinocandins are the third class of antifungal drugs which are reported to be fungicidal and fungistatic against a few pathogenic fungal strains. The echinocandins mainly targets the 1,3,β-D glucans, the main component of fungal cell wall, thus causing the weakening of cell wall leading to the complete lysis of pathogenic fungi. The final class of antifungal drugs namely, pyrimidine analogues flucytosine ultimately targets the nuclear level and affects the protein, RNA and DNA biosynthetic pathways. The over use of antifungal drugs leads to the opportunistic fungal pathogen resistance. The World Health Organization (WHO) declared that these types of antimicrobial resistance might be the biggest threat as per 2019 records (Livengood *et al*., 2020).

The microorganisms may develop the resistance against the antifungal drugs by following three major mechanisms such as reducing the accumulation of the amount of antifungal drugs within the fungal cell, decreasing the binding affinity of the drug molecules to the target site and modifications of the metabolism to counterbalance the effect of drug molecules. Hence, there is a need to discover the antifungal metabolites from natural sources having more potential mode of action compared to the existing antifungal drugs (Nicola *et al*., 2019).

Since it is needed to expand the development of an efficient antifungal compounds for the treatment of fungal infections, scientists and researchers have approached various sources for the development of antifungal compounds with potential activity. The medicinal plant or its bioactive components play an crucial role in providing the great and better fungal treatments with lesser side effects. Most of the studies have reported that the natural phytoconstituents are potent antimicrobial compounds and it can be applied either alone or in combination with other antifungal drugs against a few pathogenic strains (Freitas *et al*., 2020).

Apart from the plant sources, natural peptides are produced from diverse species of bacteria, archaea, fungi and other eukaryotic species that can be obtained from natural sources. In this aspect, most of the antifungal compounds (AFPs) have been discovered and developed by screening its potential antagonistic activity against several bacterial and fungal pathogens using *in vitro* assays. In bacteria, *E.coli* is considered as the host for the heterologous production of various peptides and proteins for therapeutic potential. *Bacillus subtilis* has been explored for AFP production and other bacterial genera including lactobacillus, leuconostoc, pediococcus, streptococcus, and enterococcus. The species Lactobacillus has been producing an antimicrobial peptide namely, bacteriocins that has a greater antimicrobial activity against human pathogens (Ganesan and Xu, 2018).

Filamentous fungi itself can be referred as the best source for the production of various metabolites and enzymes. Filamentous fungi can produce a range of primary metabolites by naturally that including, fatty acids, organic acids and some important secondary metabolites such as antibiotic like penicillin, cephalosporin and griseofulvin and a few cholesterol lowering agent called lovastatin. The filamentous fungi have known to produce a large quantity of proteins into the extracellular medium that can also actively involved in the complex process of posttranslational modifications such as glycosylation, proteolytic cleavage and multiple disulphide bond formation (Tong *et al*., 2020).

AFPs isolated from the natural sources have more advantages than the commercial antifungal drugs. The AFPs have direct antimicrobial activity on the pathogenic cell targets. The compounds have the ability to recognize the multiple targets at the same time, hence it reduce the possibility of the development of antimicrobial resistance. The pathogenic targets for the developed antimicrobial compounds include fungal membrane, cell wall components and molecules involved in the synthesis of macromolecules like DNA, RNA and protein and cell cycle. Most of the developed AFPs have no side effects but it does not ensure the less level

of toxicity. Several AFPs may target the molecules in the fungal cell namely, glucosylceramide, mannosyldiinositol phosphorylceramide, enzymes involved on the synthesis of ergosterol and β-glucan (Ullivarri *et al*., 2020).

Indeed, the AFPs have shown the reduced level of toxicity by two main reasons involves, there is a strong interaction has been formed in the negatively charged fungal membrane with the positively charged peptides in the AFPs and secondly, most of the AFPs have targeted the cell membrane lipids like ergosterol which is very unique in the fungal membrane and absent in human (Pappas *et al*., 2018). The application of AFPs remains a challenging one until the proper characterization was done. Hence, there is an urge for the production of potential AFPs in required and sufficient amounts and need to purify the compounds to study their structure-function relationships, potential activity and its safety in their clinical and medical point of view. There are three main strategies involved to achieve the production of efficient antimicrobial compounds such as direct isolation of AFPs from natural sources, heterologous expression and chemical synthesis (Sewczyk *et al*., 2018).

AFPs have been reported to be more potential against various pathogenic fungi. More than 90% of death rate was observed with the infections caused by *Candida, Cryptococcus, Histoplams, Aspergillus* and *Pneumocystis*. These pathogenic organisms cause harmful effects at specific sites in the host. These opportunistic pathogens contribute to a global death (Adenis *et al*., 2018).

Cryptococcus neoformans is the environmental yeast which can cause cryptococcosis that can be characterized as an asymptomatic pulmonary infection. It can be often observed in childhood but it can be cleared when the person enters into the stage of latency. The disease can be re-emerged in individuals once they become immunocompromised. The infection can cause severe secondary associated problems in human. It is estimated that more than one million infections and nearly 60,000 death rates was reported annually with *C. neoformans* causing cryptococcosis (Crawford *et al*., 2020). The exopolysaccharide capsule is considered as the primary factor for the virulent nature of *Cryptococcus neoformans* and it is anchored to the outer layer of the cell wall. The cell wall component namely, α-1,3-glucan is the important component necessary and was synthesized by *AGS1*. Melanin is also considered as an important factor for the virulence potential present in *Cryptococcus neoformans* cell wall. This important pigment responsible for virulence potential is secreted by the enzyme laccase which

confers resistance to several stress factors and it modulates the immune response of the host for infection process (Zaragoza, 2019).

Candida species are common commensal fungi that colonized in wide range of mucosal surfaces including, oral cavity, gut or vagina in the host. The increasing invasive fungal infection caused by the pathogen, *Candida albicans*, is a global phenomenon which is the causative agents for candidiasis. In healthy patients, *Candida albicans* can causes the mild superficial mucosal infection with significant morbidity considered as oral thrush and vulvovaginal candidiasis. This Candida infection can cause a higher mortality and morbidity rate, especially found in patients who are admitted in ICUs and suffered with HIV (Toth *et al*., 2019). β-glucan, the cell wall component has quickly recognized by the immune system of the host, thus generating an efficient response against the *Candida albicans*. Hence, the candida cleverly masks the β-glucan for pathogenesis. Any disturbances in the organization and biosynthesis of the cell wall components making the β-glucan unmasking that might increase the chance of host cell recognition of β-glucan layer for prevention of infection in the host. Mannoproteins has the fibrillar layer containing O-glycosylated and N-glycosylated polysaccharide moieties of the cell wall of *Candida albicans*. This mannoproteins are needed for masking the layer of β-glucan thus reducing the recognition and identification of pathogenic cell wall by the host immune system. Chitin is another important cell wall component which is responsible for maintain the cell membrane integrity. Disturbances in any of these components synthesis or organization resulted in easy recognition of pathogenic cell by antifungal compounds and human immune system (Wagener *et al*., 2017).

The *in silico* approaches have been used for the prediction of good antimicrobial compounds against various pathogens using molecular docking methods. The selected lead compounds in this method allowed testing their activity in *in vitro* followed by *in vivo* studies. There are various targets on the pathogenic cell which can be docked with the antagonistic fungal compounds and derivatives. With various antifungal compounds the screening of them is a rate limiting step in the current studies and researches. Hence, the virtual screening is the most powerful and important computational technique to accomplish a preliminary identification of compounds having antifungal activity by the application of molecular data bank (Rolta *et al*., 2020; Dar and Mir, 2017).

Molecular docking is the best method to estimate the binding affinity and binding strength by the docking of a ligand molecule with the target proteins from the pathogens. The

docking scores and its comparison is a great indicative of the best activity of compounds which is further considered as the inhibitor for the target enzymes or proteins. Molecular docking has enhanced its applications as a fundamental computational tool for the further insight into the drug development and discovery (Segarra *et al*., 2019).

The present study has dealing with the determination of antifungal potential of antifungal compounds from *Aspergillus giganteus* on emerging human fungal pathogens namely, *Cryptococcus neoformans* and *Candida albicans* using *in vitro* and *in silico* approaches. The antagonistic potential of the compounds from *Aspergillus giganteus* and its mode of action can be investigated with the following objectives.

- ✓ To determine the antagonistic potential of *Aspergillus giganteus* on *Cryptococcus neoformans* and *Candida albicans*
- ✓ To investigate the mode of action of *Aspergillus giganteus* on human fungal pathogens
- ✓ To purify the antifungal compounds responsible for the antagonistic nature of *Aspergillus giganteus*
- ✓ To understand the molecular interactions of antifungal compounds of *Aspergillus giganteus* with the target proteins of pathogenic fungi using *in silico* approaches

REVIEW OF LITERATURE

2.0 REVIEW OF LITERATURE

Invasive fungal infections play a crucial role in mortality and morbidity worldwide, especially in immunocompromised individuals. Many fungal infections in human may lead to various secondary related disorders resulted in severe infections or even a death. The most prominent life threatening fungal infections relies on the *Aspergillus* spp., *Candida* spp., and *Cryptococcus* spp. which resulted in aspergillosis, candidiasis and cryptococcosis. Only a limited number of fungal metabolites have been studied as potential antifungal compounds for specific systemic fungal infections. Hence, discovery of new antifungal compounds for treating fungal infections caused by opportunistic fungal pathogens becomes an emerging area of drug discovery. The pathogenic cell wall and cell membrane act as the primary targets for antifungal compounds (Lozano *et al*., 2018).

This review of literature has been extensively discussed with the following topics;

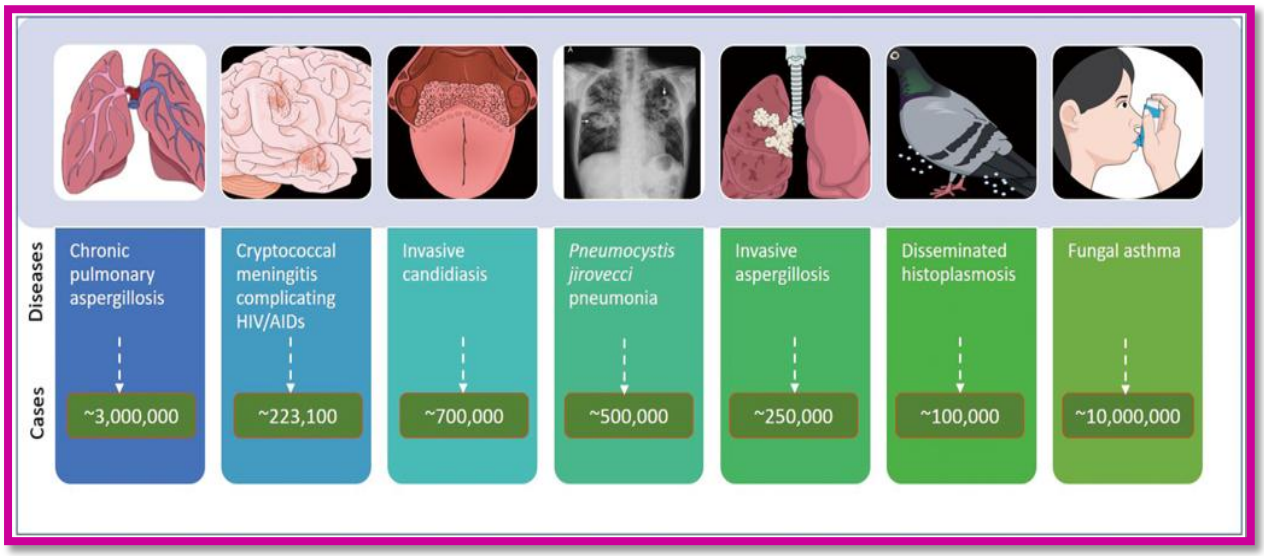
2.1. Fungal infections
2.2. Pathogenic fungi
2.3. Fungal cell wall
    2.3.1. Glucans
    2.3.2. Chitin
    2.3.3. Glycoproteins
2.4. The cell wall of *Cryptococcus neoformans*
2.5. The cell wall of *Candida albicans*
2.6. Antifungal resistance
2.7. Antifungal drugs and its development
2.8. Mechanism of action
    2.8.1. Inhibition of nucleic acid biosynthesis
    2.8.2. Inhibition of ergosterol and protein biosynthesis
    2.8.3. Inhibition of glucan biosynthesis
    2.8.4. Inhibition of chitin biosynthesis
2.9. Antifungal compounds
    2.9.1. Natural peptides
    2.9.2. Semisynthetic or synthetic peptides
2.10. AFPs producing organisms
2.11. *In silico* approaches

2.1. Fungal infections

Fungal kingdom comprises of 6 million eukaryotic species and most of them have a great effect on the global health, biodiversity, ecology, agriculture and biomedical research. Nearly, more than 600 fungal species are found to cause infections in vertebrates, among these 200 are associated with human either as commensals and microbiome or as pathogens. These organisms have been reported as a global threat to the well being of human resulting in increased rate of invasive fungal infections and allergy on the skin. More broadly, emergence of opportunistic pathogenic fungi mainly targets animal and plant diversity. More than 8000 species are associated with plant infections (Fisher *et al*., 2020).

Among infections caused by other microorganisms, infections caused by fungi are responsible for increased incidence of morbidity and mortality worldwide. Fungal diseases have been continuously neglected over three decades despite their harmful impact on human health (Rodrigues and Albuquerque, 2018).

Fungi are important component in microbiota and it promotes homeostasis in ecosystem through interactions with other fungal and bacterial pathogens in lungs, guts, soil and other environments. Generally, fungi belong to the category of auxotrophic, they can harvest the electromagnetic radiation for its survival and growth. It is often found in the complex community and mainly composed of several cell types. The predominant life form of fungi is biofilm formation and it eventually causes infection in host (Rodrigues and Nosanchuk, 2020).



Estimated global cases reported with diverse fungal infections (Bajpai *et al*., 2019).

2.2. Pathogenic fungi

The opportunistic fungal pathogens are major threats to human and animal health and these fungal species are the reason behind deleterious and harmful infections in host. *Aspergillus sp.* may cause Aspergillosis, *Candida albicans* cause Candidiasis in mouth, throat and vagina or the blood stream and *Cryptococcus neoformans* infection leads to crytococcosis in human that affects Central nervous system. Various fungi are possessed as pathogens in nature such as *Aspergillus niger, Candida albicans, Cryptococcus neoformans, Cryptococcus gatti, Pneumocystis jirovecii, Aspergillus flavus, Histoblatoma capsulatum, Blastomyces dermatitidis, Aspergillus fumigatus* and *Penicillium chrysogenum.* These pathogenic organisms cause harmful effects at specific sites in the host. These opportunistic pathogens contribute to a global death (Hembach *et al*., 2020).

Cryptococcus neoformans is a facultative intracellular fungal pathogen and it is addressed as the causative agent for the deleterious disease cryptococcosis. Cryptococcosis disease primarily affects the individual with impaired immunity especially, the person suffered with advanced HIV infection. This pathogen can be seen everywhere around the environment and environmental conditions ranging between the tropical and temperate. *Cryptococcus neoformans* infection can occur through inhalation and subsequently it forms infection in the lungs. The cryptococcosis infection may be clear and formed as granuloma, which may disperse from its initial site, resulting in pneumonia and meningoencephalitis, this can cause death, if it prolonged or untreated. Even though there is a more antifungal therapy and chemotherapy available for treating fungal infections nearly more than 650,000 people die by this cryptococcosis infection caused by *Cryptococcus neoformans* (Crawford *et al*., 2020; Beardsley *et al*., 2019).

Candida species are common commensal fungi that colonized in several mucosal surfaces namely, oral cavity, gut or vagina. The increasing invasive infection in the human caused by *Candida albicans* is a global phenomenon which is the causative agents for candidiasis (Toth *et al*., 2019). In healthy patients, *Candida albicans* can penetrate into the host system and cause mild superficial mucosal infections with significant morbidity considered as oral thrush and vulvovaginal candidiasis. This Candida infection can cause a higher mortality and morbidity rate, especially found in patients who are admitted in ICUs and suffered with HIV. Hence, much attention should be given to understand the basics of their pathobiology,

virulence factors, predisposing conditions along with the immune responses of both healthy and immunocompromised patients (Singh *et al*., 2020).

2.3. Fungal cell wall

The cell wall structure of fungi composed of three layers where the innermost layer is the structure that is highly conserved and the remaining layers can vary from species of fungi to fungi. Fungal cell wall is the important structure which could interfere with human cells and tissues during pathogenesis. The cell wall components of fungi are involved in the protection of fungal cells from various factors and making the host immune response favourable to the growth of fungal pathogens (Sem *et al*., 2016). The fungal cell wall contains a tensile and robust core scaffold where a wide range of proteins and some superficial components with fibrous and gel like carbohydrates from polymers, and everything together formed a strong and flexible structure of fungal cells (Gow *et al*., 2017). The protein kinase C causes high osmolarity glycerol response in the host and involved in the ca^{+2}-calcineurine pathways which have been reported as resulted in the damage of the fungal cells. Calcineurine is considered as a client protein for the genetic understanding of the gene HSP90 and Hsp90 chaperone thus, it reduce the tolerance mechanism (Lima *et al*., 2019).

2.3.1. Glucans

Glucan is considered as the most important fungal cell wall which is composed of polysaccharides and it accounts for 50-60% in the dry weight of the fungal cells. The glucan polymers are composed of 1,3 linkage glucose units it contains nearly, 65-90% of the dryweight. Glucans are mainly composed of β-1,6, β-1,4, α-1,3 and α-1,4 links in the cell wall of *Candida albicans*. The most important form of glucan is β-1,3-D- glucan stated as main structural component with this other components of this structure are covalently attached to it. The β-1,3-D-glucan is synthesized by a series of enzymes found in the plasma membrane known as glucan synthases becoming a target for many antifungal compounds (Wang *et al*., 2018).

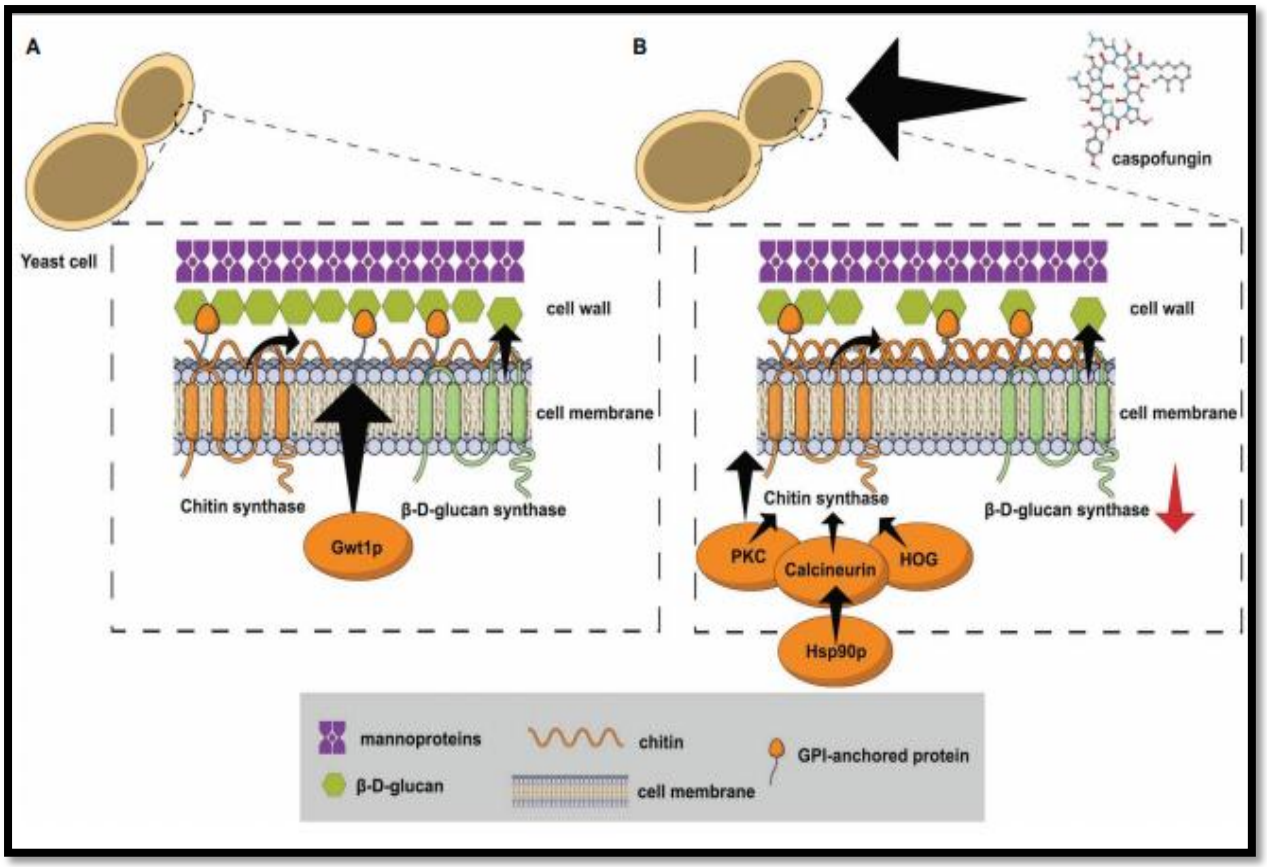
2.3.2. Chitin

The content of chitin in the fungal cell wall differs with the morphological phases of each fungal organism. The chitin content is account for nearly, 1-2% of the dry weight of the cell wall. The enzyme chitin synthase catalyzes the biosynthesis of chitin from N-acetyl glucosamine. The chitin synthase enzyme deposited the chitin polymers in the extracellular

space adjacent to the cytoplasmic membrane which is essential for elongation of the chitin chain thus, it is considered as antifungal target (Camacho *et al*., 2017).

2.3.3. Glycoproteins

Nearly, 30-50% of the dry weight of the cell wall in yeast contains of mainly proteins. Many proteins are found to be associated with carbohydrates either by N or O linkages thus resulting in glycoproteins. These glycoproteins have diversified functions such as, participation in the maintenance of the cell shape, adhesion processes, protection of fungal cell against various substances, and absorption of molecules, signal transmission, synthesis and reorganization of wall components agents (McCarthy *et al*., 2017).

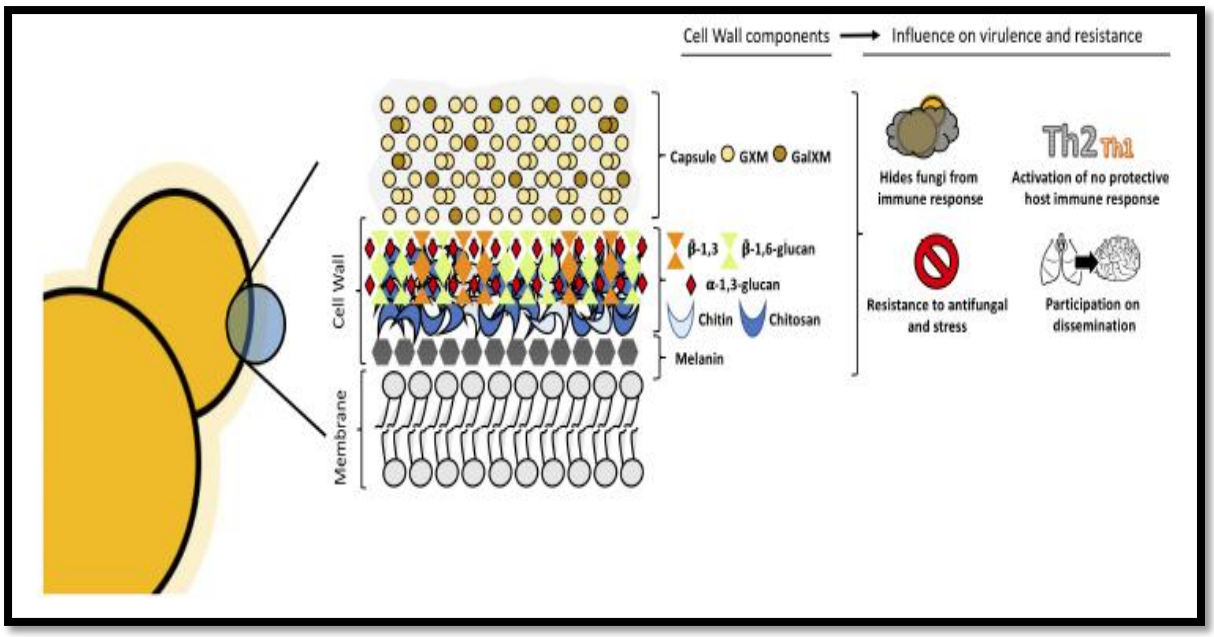


A) The fungal cell wall and possible targets for antifungal compounds;
B) Echinocandin namely, Caspofungin action on pathogenic fungal cell wall (Lima *et al*., 2019).

2.4. The cell wall of *Cryptococcus neoformans*

The cell wall of *Cryptococcus neoformans* is a strong structure that undergoes constant remodeling to change the distribution and cross linking of the cell wall components that are essential for the cell growth and cell division. The cell wall is a double layered structure which is mainly composed of α-1,3- glucan, β-1,3 and β-1,6 glucan, chitin, chitosan, mannoproteins and other GPI- anchored proteins. These are the important components necessary for the maintenance of shape and function of the cell especially for infection processes (Wang *et al*., 2018).

The exopolysaccharide capsule is considered as the primary virulence factor in *Cryptococcus neoformans* and it is anchored to the outer layer of the fungal cell wall. α-1,3-glucan is the important necessary component synthesized by *AGS1*. The main function is to protect the cell against host immune system, acting as a protecting shield and required for the correct organization of the capsule for the infection. β-1,3-glucan is found in less quantity in *C. neoformans* cells but its function is to maintain the cellular shape and its inhibition causes the cell death and changes in the morphology of the cell. Like β-1,3-glucan, chitin is also present in small quantities but it contributes the major strength to the cell wall of fungi. The inhibition of chitin synthase enzyme resulted in the morphological alterations and inability to synthesize the pigment called melanin. Chitosan is referred as a deacetylated form of chitin is referred as a soluble and flexible polymer and it is more abundantly found in cells by two to four times higher than that of chitin. Melanin is the crucial virulence factor present in *Cryptococcus neoformans* cell wall. This important pigment responsible for virulence potential and it is secreted by the enzyme laccase which confers resistance to stress factors and it changes the host immune response for infection process. Apart from all these components, proteins play the crucial role in completing the structure of cell that is embedded in the cell wall carbohydrates. Approximately, the *Cryptococcus neoformans* cell wall has 29 GPI- anchored proteins which including proteases, carbohydrate active enzymes and phospholipase B1. The protein phospholipase B1 is covalently attached to β-1,6 glucans and it is participated in membrane homeostasis, remodeling and maintenance of cell membrane integrity which contributes the survival of fungal pathogens in host environment (Zaragoza, 2019; Fonseca *et al*., 2018; Hommel *et al*., 2018).

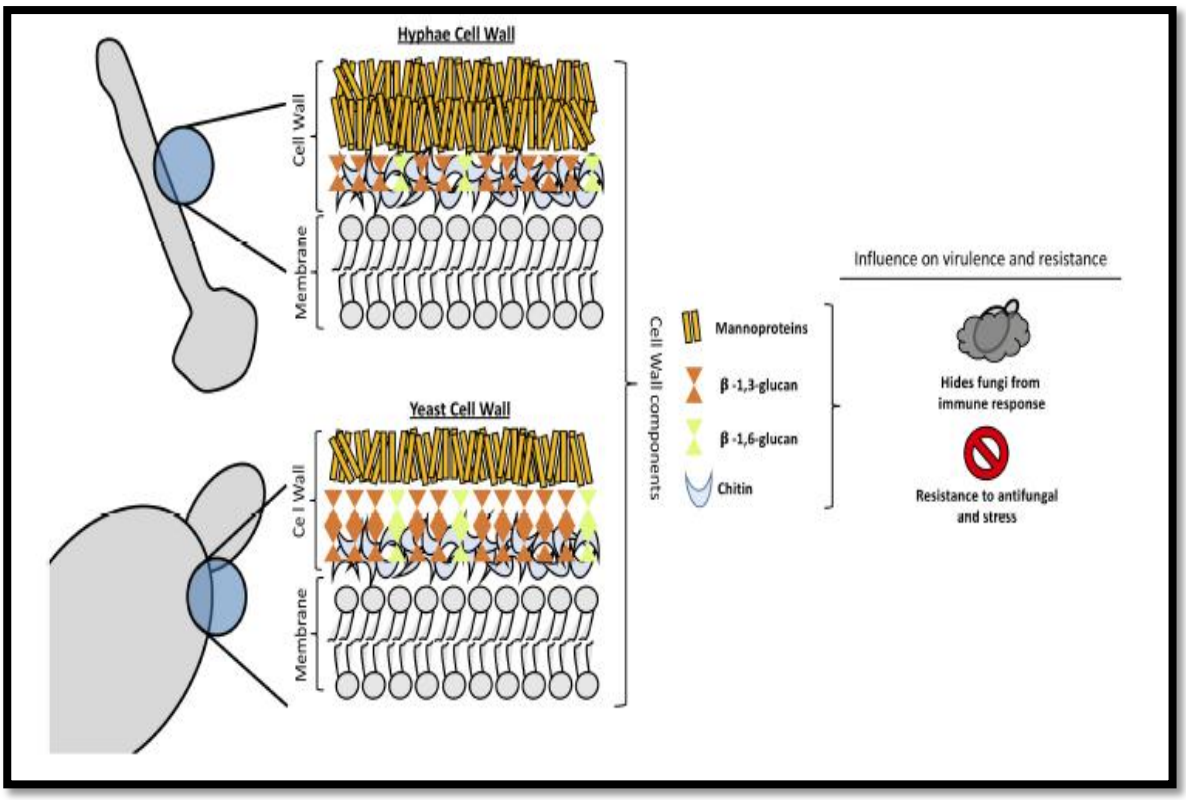


Structural organization of *Cryptococcus neoformans* cell wall (Rubio *et al*., 2020).

2.5. The cell wall of *Candida albicans*

Fungal cell wall is the important one which is actively involved in the formation of interaction with the host cells and tissues during infection process. β-glucan is the important cellular components of *Candida albicans*. This component has been quickly recognized by the host immune system thus generating an efficient signal against the *Candida albicans*. Hence, the Candida cleverly masks the β-glucan for pathogenesis. Any disturbances in the biosynthesis and organization of the fungal cell wall components making the β-glucan unmasking thereby increasing the chance of host cell recognition of β-glucan layer for prevention of infection in the host (Granger, 2018).

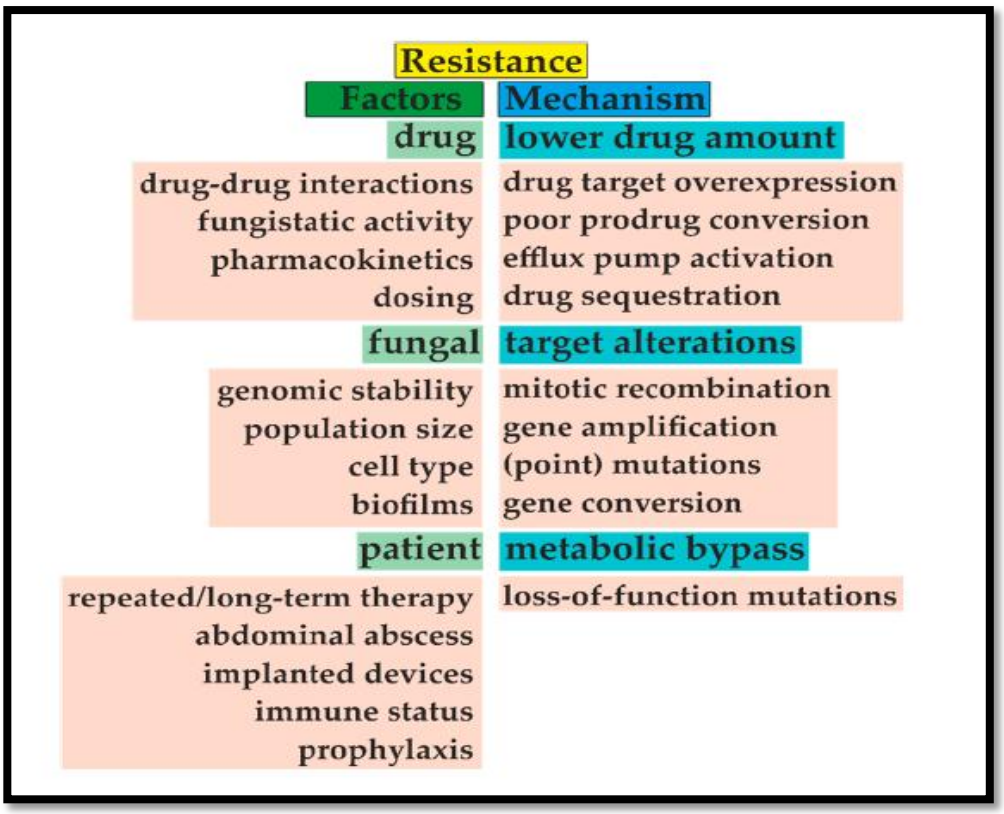
Mannoproteins forms a fibrillar layer which is composed of O-glycosylated and N-glycosylated polysaccharide moieties of the cell wall of *Candida albicans*. This mannoproteins are needed for masking the β-glucan layer thus reducing the easy recognition of pathogenic cell wall by the host immune system. Chitin is another important component of cell wall which is responsible for maintaining the cell membrane integrity. Disturbances in any of these components synthesis or organization resulted in easy recognition of pathogenic cell by antifungal compounds and human immune system (Wagener *et al*., 2017).



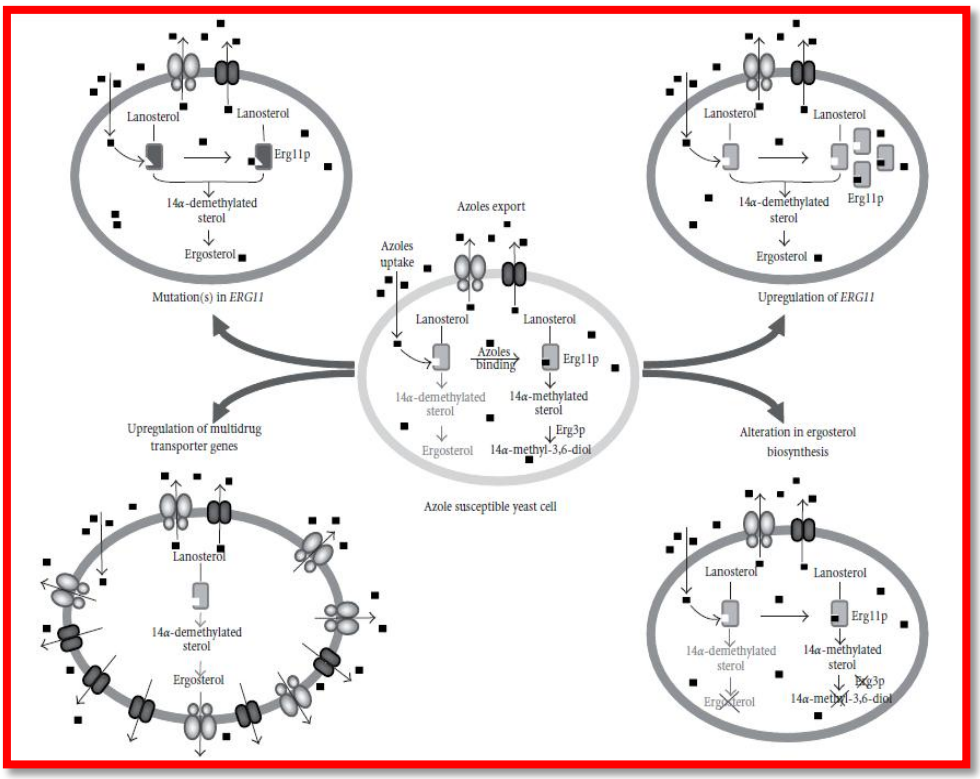
Structural architecture and organization of *Candida albicans* cell wall (Rubio *et al*., 2020).

2.6. Antifungal resistance

Fungal cell wall structures are found to be modified during the stress produced by the host environment and exposure of antifungal drugs. Many *in vitro* studies have reported that the condition exactly mimic the host environment at the site of infection. Hence the fungal cells make modifications in the cell wall structures and antifungal resistance to particular antifungal compounds. Growth of *Candida albicans* cells in serum show major changes in the cell wall structures and observed with the decreased content of mannan chains, chitin and β-glucan. By developing the tolerance nature is the way to mitigate antifungal resistance in the pathogens. Fungal cells survived with the drug exposure can respond to selection and evolve resistance to certain antifungals (Satish *et al*., 2019). The difference between the nature of drug as fungistatic or fungicidal and required dosage is necessary for the successful treatment. Repeated antifungal therapy often resulted in prophylaxis and narrows the selection of drug. Biofilm formation ability of fungi makes this task more complexed. Often, fungi can decrease the concentration of drug by efflux pump activation or target overexpression. In addition, the drug targets can be amplified by various types of mutations including, amino acid substitutions (Houst *et al*., 2020).



Emergence of microbial resistance by multiple factors and various molecular mechanisms (Satish *et al*., 2019).



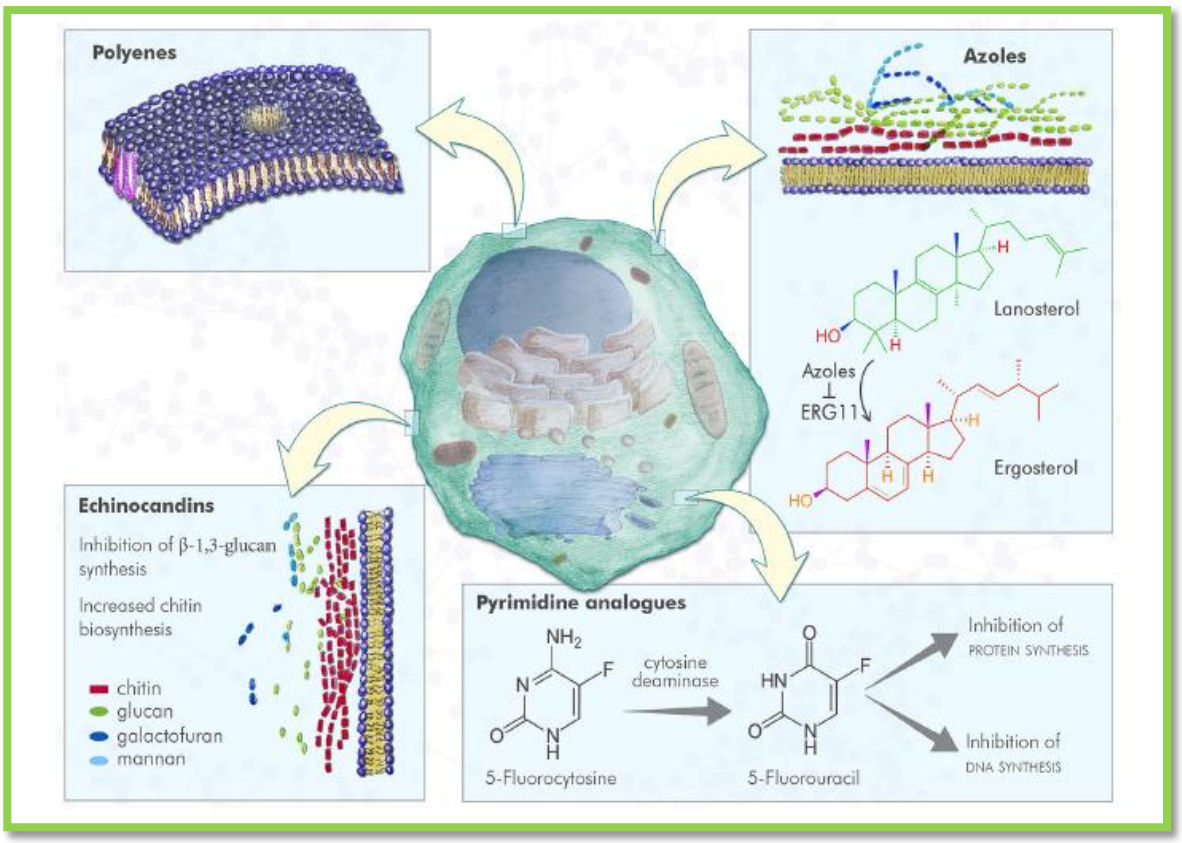
Molecular mechanism of resistance to azole drugs in *Candida albicans* (Houst *et al*., 2020).

Recent years, there has been an increasing evident on response of mammalian immune system to fungal pathogens. Hence, investigation of antifungal mechanism has been discovered in each and every higher organism. The human innate immune system has evolved to recognize conserved microbial structures referred as pathogen-associated molecular patterns (PAMPs) through a wide range of pattern-recognition receptors (PRRs) on their cell surfaces. Recognition of pathogenic fungal cell wall by the human immune system results in the uptake and killing of the pathogens by the process of phagocytosis and induction of innate and adaptive immunity. Fungal cell wall component such as mannans and mannoproteins are highly recognized by mannose receptor and Toll-like receptor 4 (TLR4), phospholipomannan is recognized by TLR2 and β-mannosides by the receptor galectin-3, β-glucan is recognized by the C-type lectin dectin-1 and chitin & chitosan can be detected via the mannose receptor and also by Nod2 & TLR9 (Gow *et al*., 2017).

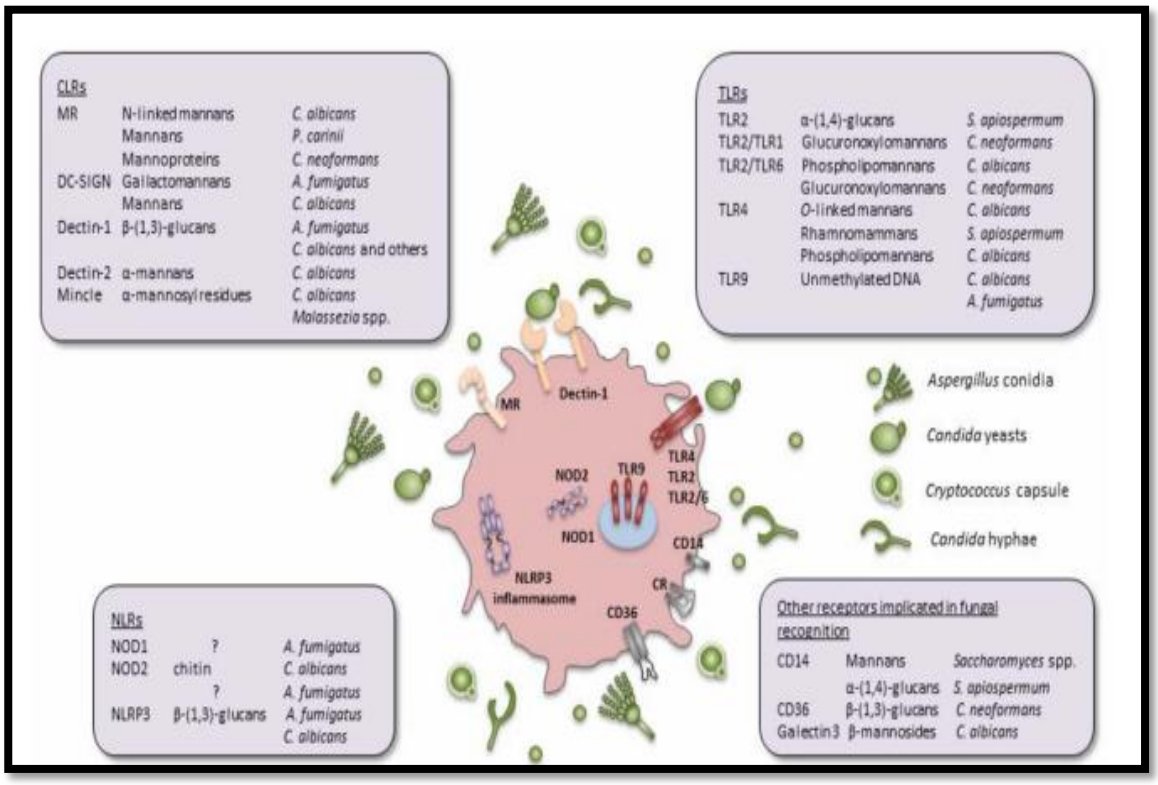
2.7. Antifungal drugs and its development

Since 1950, there are more than 200 polyenes have been discovered with potential antifungal activity. Currently, the treatment for fungal infections depends on the use of antifungal drugs that is already discovered. There are five classes of antifungal drugs that have been used in the treatment of systemic fungal infections such as, polyenes (amphotericin B), azoles (flucanazole, itraconazole, posaconazole, voriconazole and isavuconazole), echinocandins (caspofungin, micafungin and anidulafungin), allylamines (terbinafine) and antimetabolites (flucytosine) (Gintjee *et al*., 2020).

Amphotericin B (AMB) is considered as the single polyene drug for the treatment of fungal infections. 5- flucytosine (5-FC) was the first commercially used antimetabolite used in the treatment of cancer but due to its low antineoplastic activity it is used in synergism with other antifungal drugs for the treatment. In the category of azoles, the first generation of antifungal drug development includes flucanazole (FLC) and itracanazole (ITC) and the second generation drugs are voriconazole (VOR), posaconazole (POS) and isavuconazole (ISV). Echinocandins are the new class of antifungal drugs and it includes caspofungin (CSF), micafungin (MCF), anidulafungin (ANF).

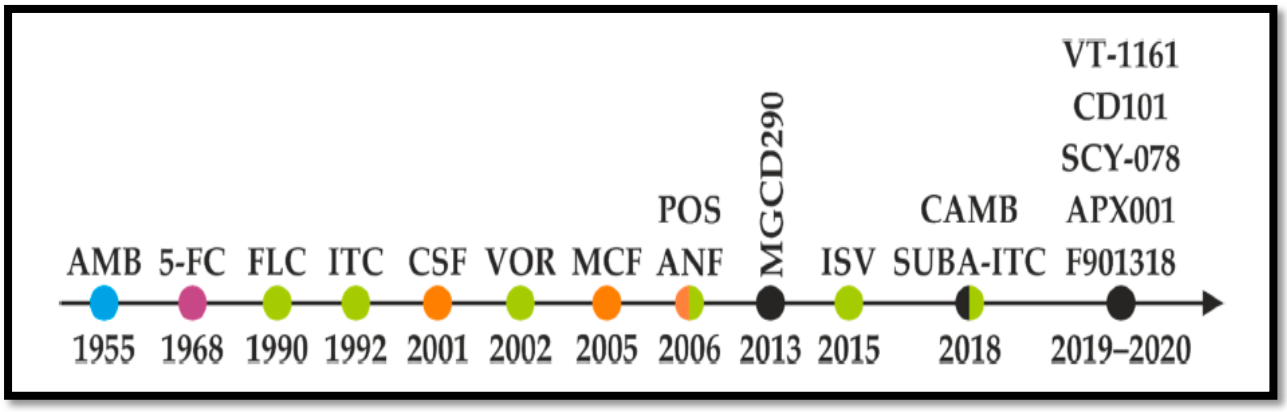


Different class of antifungal drugs and its action (Mambo *et al*., 2019).



Recognition of human fungal pathogens by various receptors of innate immune system (Gow *et al*., 2017).

Currently, the discovered antifungal drugs have been tested and proved the potential activity on various fungal pathogens. Many antifungal compounds and/or drugs showed the spectrum of activity to overcome resistant fungi. However, there is still a gap in the discovery of novel compounds with good activity. MGCD290 have the potential activity when combined with azoles and/or echinocandins. ABA (aureobasidin A), CAMB (encochelated amphotericin B), FOSMANO (fosmanogepix), GSI (glucan synthase inhibitor), IBREXA (ibrexafungerp), NIKZ (nikkomycin Z), REZA (rezafungin) are the novel antifungal drugs (Rauseo *et al*., 2020).



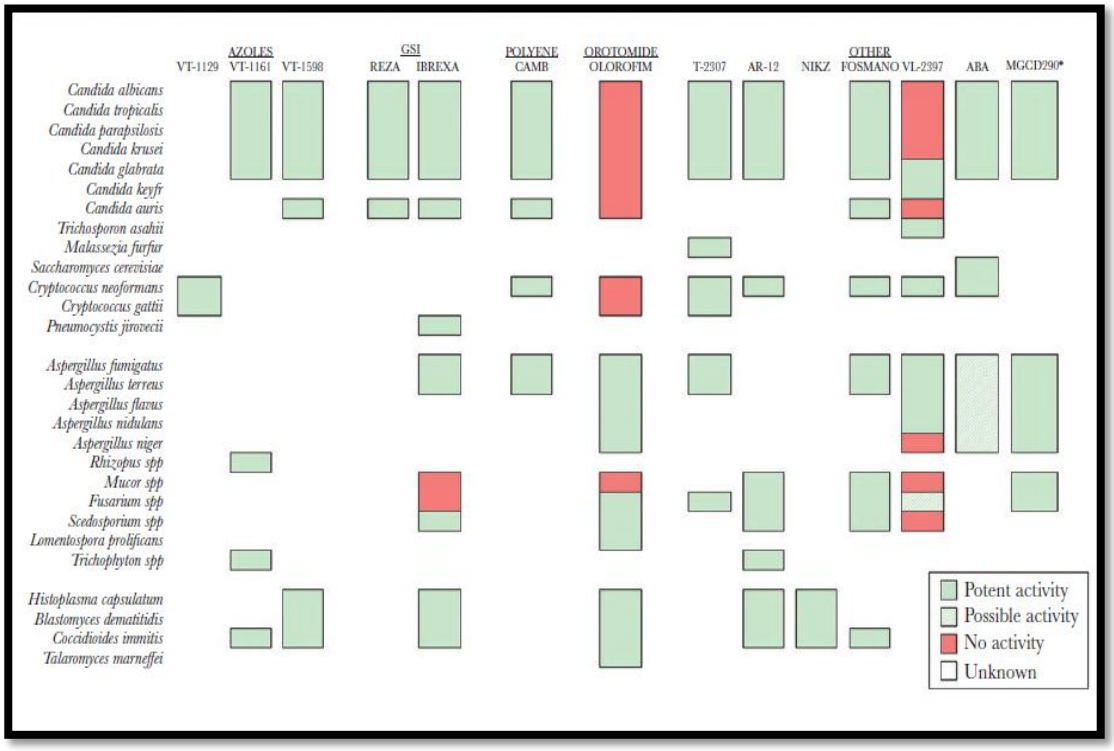
Chronology of antifungal drug development (Houst *et al*., 2020).

In the above mentioned figure, the blue colour indicates polyenes, purple colour was pyrimidine analogues, green colour represents the triazoles, orange colour indicates echinocandins and the black colour represents the antifungal drugs under development and/or testing in clinical trials.

2.8. Mechanism of action of antifungal compounds on the pathogenic cell

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).



Novel antifungal drugs and its potential activity on various human fungal pathogens (Rauseo *et al*., 2020).

2.8.1. 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 is 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 enters into the RNA molecule, causing premature chain termination, and it inhibiting the DNA synthesis via 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 occur at a high frequency. Therefore, flucytosine might deserve more use in the clinical practices (Kathiravan *et al*., 2012).

2.8.2. Inhibition of ergosterol and protein synthesis

Polyenes are the class of antifungal drugs which mainly target the ergosterol biosynthetic pathway. The drug binds to the ergosterol and disrupts the fungal cell membrane leads to the formation of aqueous pores. Like polyenes, sodarins is a drug molecule which suppresses the protein synthesis and retards the cell growth of fungal cells. Ultimately, the fungal cellular permeability is modified and resulting in the leakage of cytosolic components, thus, fungal cell death (Sampinato and Leonardi, 2013).

2.8.3. 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 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).

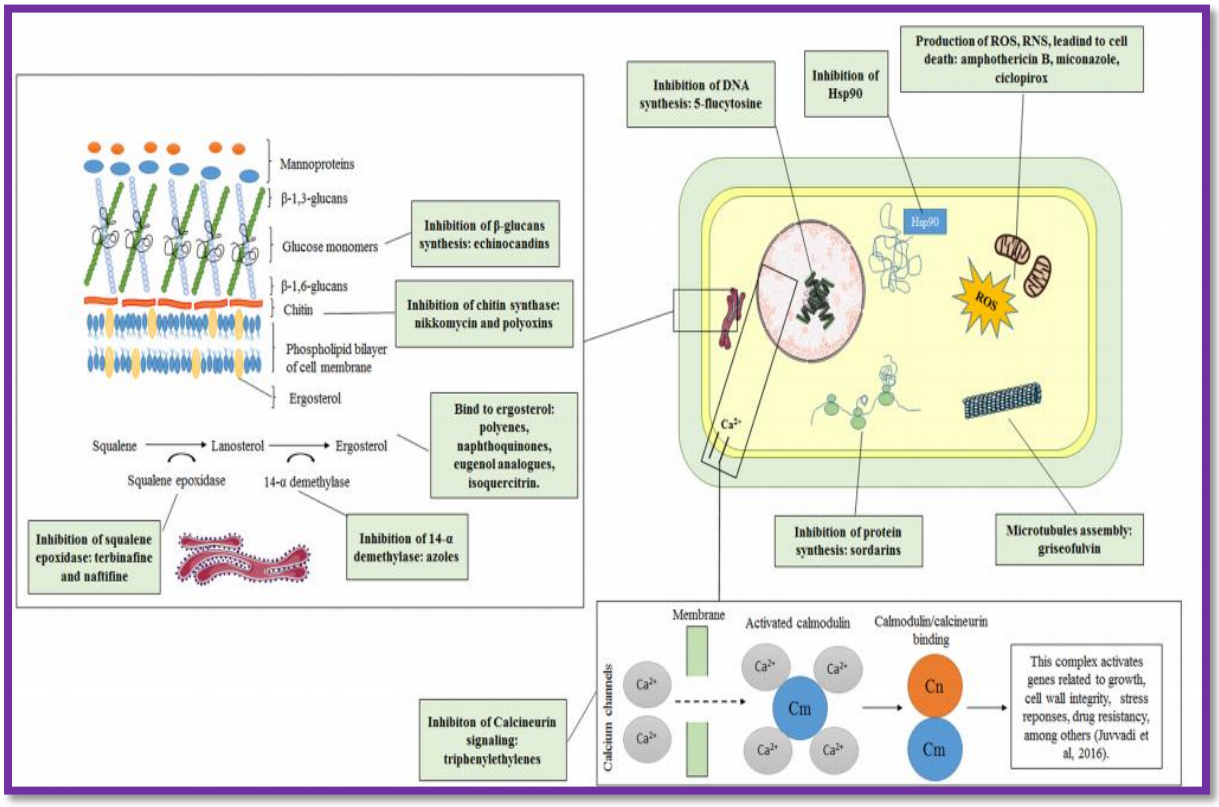
2.8.4. Inhibition of chitin synthesis

Antifungal drugs that are inhibiting chitin biosynthesis in fungal cell wall are nikkomycins, polyoxins and plagiochin. Nikkomycins are referred as peptidyl nucleoside agent that completely inhibits the action of chitin synthase (Cheung and Hui, 2017).

2.9. Antifungal compounds

Antifungal drugs such as azoles are known to inhibit the synthesis of ergosterol, echinocandins primarily inhibits the glucan biosynthesis and fluorinated pyrimidines can

interfere with the pyrimidine metabolism and results in the inhibition of DNA and RNA synthesis. However, high mortality rate was observed with the patients infected with fungal pathogens, long duration and dosage of drugs. Cross- resistance and narrow spectrum activity of antifungal drugs are due to the similar mechanism of action of antifungal drugs that has triggered the search and discovery of novel antimicrobial compounds with reduced toxicity and enhanced activity of already existing antifungal drugs. As of November 2019, in Antimicrobial Peptide Database (APD3) nearly 1,133 peptides with potential antifungal activities were reported and recorded. AFPs have been classified by number of different aspects and criteria including, structure and its mode of action. The most accepted classification is origin of peptide by natural, synthetic or semisynthetic (Ullivarri *et al*., 2020).



Mode of action of antifungal drugs on target sites (Rauseo *et al*., 2020)

2.9.1. Natural peptides

Natural peptides are produced from different species of bacteria, Archaea, fungi and other eukarya that are isolated from natural sources. In this aspect, most of the antifungal compounds (AFPs) have been discovered by screening its potential antagonistic activity by *in vitro* assays against pathogenic fungi. Moreover, the reduced cost for applications due to the rise of sequencing technologies, new strategies for the prediction of antifungal compounds are

emerging now. New strategies and methods includes template-based, docking simulations, hidden Markov model and other sequence based methods, that allows for the novel compound prediction by *in silico* approaches (McNair *et al*., 2018).

Most of the AFPs have α-helix structure, β-hairpin or sheet with two cysteine residues or mixed α-helix/β-sheet structures upon interaction with membranes. Some of the antifungal peptides are rich in specific amino acid residues hence they are referred as glycine-rich, proline-rich, arginine-rich, histidine-rich and tryptophan-rich (Bondaryk *et al*., 2017). The peptide length of AFPs is necessary for its secondary structure and its mode of action. Mostly, number of AFPs has the length of 11-40 residues. Only less than 20 amino acid residues will allow the AFP to form transmembrane structures in fungal cell membrane while longer length of AFP preparation may affect the cytotoxicity, stability and manufacturing cost (Akkam, 2016).

2.9.2. Semisynthetic or synthetic peptides

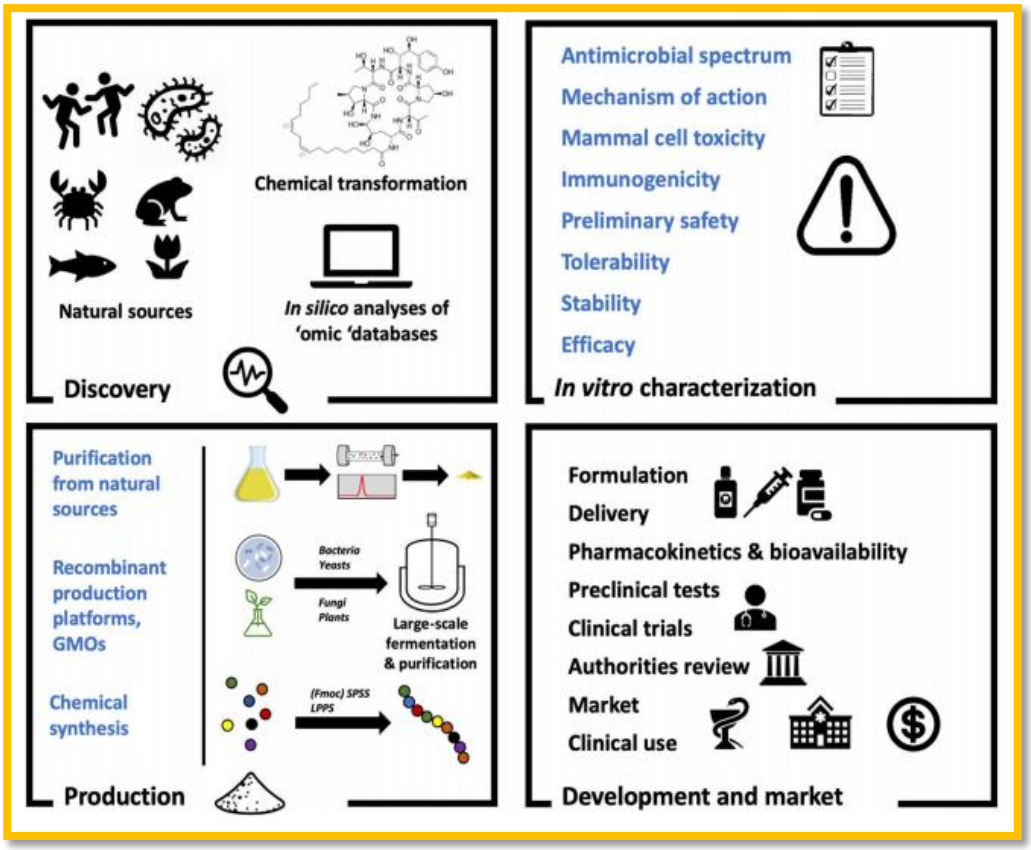
Antimicrobial compounds as synthetic or semisynthetic peptides are generally prepared to improve its pharmacological properties, reducing side effects and lowering the immunogenicity of natural peptides. These formulations also help to increase the stability and bioavailability. For example, synthetic transformation of echinocandin B AFP where the haemolytic activity of this compound was significantly decreased by the replacement of linoleoyl side chain with either octyloxybenzoy (cilofungin) or pentyloxyterphenyl (anidulafungin) side chains (Bondaryk *et al*., 2017).

2.10. AFPs producing organisms

AFP can be produced form number of bacteria, archaea, fungi and plants. In bacteria, *E.coli* is considered asthe host for the heterologous production of peptides and proteins. *Bacillus subtilis* has been explored for AFP production, other bacterial genera includes lactobacillus, leuconostoc, pediococcus, lactococcus, streptococcus, and enterococcus. The species Lactobacillus has been producing an antimicrobial peptide namely, bacteriocins which has a greater antimicrobial activity against human pathogens.

AFP is extensively produced by the fungus, especially by filamentous fungi. Filamentous fungi are a well-known source for the production of various metabolites and enzymes. This can naturally produce a wide range of primary metabolites namely, fatty acids, organic acids and some important secondary metabolites such as antibiotic like penicillin, cephalosporin and griseofulvin and some cholesterol lowering agent called lovastatin. The filamentous fungi have

known to secrete large amounts of proteins into the growth medium. The can also perform complex posttranslational modifications such as glycosylation, proteolytic cleavage and multiple disulphide bond formation. *Aureobasidium pullulans, Penicillium chrysogenum,* and *P. digitatum* have been used for the production of AFPs (Alberti *et al*., 2017). The antifungal compounds lfAFP from *A. giganteus*, NFAP and AFAP2 from *Neosartorya fischeri*, PAF and PAFB from *Penicillium chrysogenum*, AfpA and AfpB from *Penicillium digitatum* and PeAfpA, AfpB and AfpC from *Penicillium expansum* have been found to exert its potential activity on fungal pathogens (Tong *et al*., 2020).



Development of Antifungal compounds with potential antagonistic activity (Ullivarri *et al*., 2020).

One such novel fungus is *Aspergillus giganteus* which produces a small, basic and cysteine-rich antimicrobial protein that allows for antagonism against filamentous and non filamentous fungi. This protein, named antifungal protein (AFP), is secreted as a 91-amino acid inactive precursor containing a signal sequence for secretion and a prosequence that is removed by a protease during the process of secretion The prosequence maintains the protein inactive until it has crossed the plasma membrane. The mature AFP protein contains 51 amino acids and its structure is similar to the plant defensins and Y-thionins. The AFP protein disturbs the

integrity of the plasma membrane and inhibits chitin biosynthesis in sensitive fungi.Additionally, AFP may enter the host cell, binding to fungal nucleic acids and promotes charge neutralization and condensation of DNA (Narvaez *et al.*, 2018).

Aspergillus giganteus is known to secrete the protein namely, α-sarcin which shows ribonucleolytic activity. α-sarcin is the most representative member of a protein family called ribotoxins. The other important protein referred as AFP (the antifungal protein) which is a basic and 51 amino acid long. The AFP from *Aspergillus giganteus* is known to be a potent inhibitor for the pathogenic fungi namely, *Magnaporthe grisea* and *Fusarium verticilloides* (Binder *et al*., 2011).

2.11. *In silico* approaches

Discovery and development of drugs from natural compounds with good antimicrobial activity is very complicated and time consuming process. There are many factors responsible for the failure of discovered drugs including, lack of effectiveness, side effects, poor pharmacokinetics and marketable value. During the past 34 years, the expenditure of this process has been amplified. There has been an emerging development towards the application of *in silico* approaches like molecular modelling for computer-aided drug design and now has gain greater attention. The major application of utilizing *in silico* method is effective in various analysis and development of potential drug. The compound showing dose-dependent target inflection that means certain degree of confidence is further developed as lead compounds. The selected lead compound with good value is being tested on animal models in the laboratories and the compounds showing positive results can be optimized. For suitable drug development, the physicochemical properties and biological safety features should be checked for the positive compounds (Shadrack and Ndesendo, 2017).

2.11.1. Antifungal compounds for drug discovery

For the past 30 years, increase in the demand of drug development from natural sources due to its structural novelty, biocompatibility and functional diversity. Developing a natural based medicine seems to be lengthy and costly due to the difficulties faced in the understanding of its mode of action. In this study, the antifungal compounds from *Aspergillus giganteus* and pathogenic target proteins of *Cryptococcus neoformans* and *Candida albicans* is selected based on the scientific studies. The pharmacological property of the selected compounds exhibits its activity by forming interacting with the pathogenic target proteins. Hence, target protein

identification is the crucial step to elucidate its mechanism for the development of drugs (Chen *et al*., 2020).

2.11.2. Molecular docking

Molecular docking is the important method and it plays a crucial role in the rational design of drugs. In the stream of molecular modelling, docking is the method used to predict the favoured orientation of one molecule to the next molecule in order to form a stable complex. Molecular docking is the best method for the optimization of selected compounds, in other words, it could be considered as the best-fit orientation of a ligand that binds to the protein of our own interest. Docking is the method which is often used to predict the binding affinity of small molecules (ligand) to their target proteins in order to get the affinity and activity of the ligand molecule. The most important computational tools for docking studies are such as DOCK, Schrodinger, AutoDOCK, FTDOCK, Arguslab and CHARMM. There are two important classification based on the known type of inhibitor in molecular docking namely, receptor based methods and ligand based methods (Rolta *et al*., 2020).

The receptor based molecular docking involves docking of compounds into the binding site of the target protein and predicts the electrostatic fit. The positive compounds with good antimicrobial activity were selected by observing the best score and the scores compare with the binding affinity. Ligand based molecular docking applies the information of the known inhibitors to the target receptors. Structures that are very similar to the known inhibitors are recognized and selected from the databases by diversity of methods. Some of the methods extensively used including, comparison and substructure searching, pharmacophore matching and 3D shape matching (Chikhale, 2020).

METHODOLOGY

3.0 METHODOLOGY

Fungal infections became a major health problem worldwide and it has been estimated that more than 300 million people are suffering with a severe fungal infections. Among 13% of people have infections on skin and 25 million populations are landed in severe consequences such as blindness or even death. In the past decades, a fungal infection has tremendously increased among people especially, with the pathogenic fungi namely, *Cryptococcus neoformans* and *Canida albicans* (Beltran *et al*., 2019).

There are many virulence factors involved in the pathogenesis of opportunistic fungi including, polysaccharide capsule, production of pigment, capacity to grow at normal temperature of body and secretion of selective and important extracellular enzymes. Expression of the enzymes is required for the survival and growth of pathogen and damage the host cell. The pathogenic enzymes are the effective target for the newly discovered antifungal compounds (Almeida *et al*., 2015).

Hence, the methodologies were adopted to evaluate the antagonistic activity of antifungal compounds from *Aspergillus giganteus* against pathogenic strains namely *Cryptococcus neoformans* and *Candida albicans*. Also, screening the compounds with best inhibitory activity on the pathogenic cell targets by *in silico* approaches is another milestone for the drug discovery.

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 *Cryptococcus neoformans* and *Candida albicans* 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 has been carried out to explain the antagonistic efficacy of *Aspergillus giganteus* with pathogenic fungus namely, *Cryptococcus neoformans* and *Candida albicans*.

3.2. 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 fungus was inoculated on one end of the plate. On the other end, pathogenic fungi were streaked on the czapek yeast extract agar in opposite direction at equal distance from each other. The pathogenic fungi alone were taken as control. Then the plates were kept incubation at 28^{o}C for four to six

days. The percentage of radial inhibition was calculated. All the experiments were done in triplicates.

The percentage of inhibition was expressed by the following equation:

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

Where,

Control = growth of fungal pathogen (measured in centimetres)

Test = growth of fungal pathogen in the presence of antagonistic fungi (measured in centimetres)

3.3. 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.

3.4. Preparation of culture filtrates of *Aspergillus giganteus*

The fungal strain was cultured in an optimized medium at 30^{o}C with constant shaking at 170 rpm for 8 hours. Then the culture was incubated at 28^{o}C for 4-7 days. The culture was collected and subjected to centrifugation at 10,000g at 4^{o}C for 15 minutes. The supernatant was filtered through 0.22µm membrane filter to obtain cell free culture filtrates. Finally the culture filtrates were stored at 4^{o}C before use. The protein concentration in the prepared culture filtrate was evaluated using Lowry's method and the detailed procedure is given in Appendix III.

The antagonistic activity of prepared culture filtrates were tested on the pathogens, namely, *Cryptococcus neoformans* and *Candida albicans* by overlay assay. The overlay assay was performed with the inoculation of 200µl of culture filtrates onto the czapek yeast extract agar plates and the pathogenic fungi were streaked on the plates. Then the plates were allowed to incubate at 28±2^{o}C for 4-7 days. The pathogenic fungi alone was taken as control. After incubation, the inhibitory percentage was calculated using the formula given in co culture assay. All the experiments were performed in triplicates.

3.5. 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* using goat red blood cells.

To assess the cytotoxicity of antifungal compounds, hemolytic activity was performed to evaluate the percentage hemolysis using 4% suspension of goat red blood cells (gRBCs) treated with the culture filtrates of *Aspergillus giganteus*. The gRBCs were washed thrice with a phosphate buffered saline (PBS). 100µl aliquots of a gRBC suspension were taken in to 96 well microtitre plates, and then 100µl of culture filtrates of *Aspergillus giganteus* in PBS was added into each well. The plates were incubated at 37^0C for 1 hour. Then the mixtures were centrifuged at 1500 rpm for 10 minutes, and aliquots were taken in to a new well in the microtitre plates. The absorbance of aliquots was read at 414 nm by using a microtitre ELISA reader. Hemolytic rates of 0% were determined with PBS and 100% with 0.1% Triton X-100. The following equation is applied for the calculation of percentage hemolysis:

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

3.6. Cell membrane disruption assay

3.6.1. Leakage of cellular materials

Cells from 100ml of czapek yeast extract broth culture of pathogenic strains namely, *Cryptococcus neoformans* and *Candida albicans* were harvested by centrifugation at 4000 g for 20 minutes, washed it for thrice, and resuspended in 100ml phosphate buffered saline with pH 7.0. The collected suspesion was then treated with various concentration of antifungal compounds in the culture filtrates grouped as CF1, CF2, CF3, CF4 and CF5 (Concentration ranging from 100 – 500 µg) and incubated at 28 ± 2^oC under agitation in an environmental incubator shaker for 0, 30, 60 and 120 minutes. Sequentially, 2ml of the sample was taken and centrifuged at 12000g for 2 minutes.

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

3.6.2. Measurement of extracellular pH

100µl of fungal suspensions were added to 20ml czapek yeast extract broth and kept incubation in a moist chamber at 28±2^{o}C for 2 days. Then it was centrifuged at 4000g for 20 minutes and the pellet was harvested and washed twice or thrice with sterilized double distilled water. Then the pellet was resuspended in 20ml sterilized double distilled water. The range of concentration of antifungal compounds in the culture filtrates such as CF1, CF2, CF3, CF4 and CF5 (100-500µg) was added in to the pathogenic cells and the changes in the extracellular pH was evaluated at 0, 30, 60 and 120 minutes.

3.6.3. Cell membrane integrity assay

Total lipid content of pathogenic strains, *Cryptococcus neoformans* and *Candida albicans* cells with different concentration of culture filtrates of *Aspergillus giganteus* namely, 110µg, 220µg, 330µg, 440µg and 550µg were determined using phosphovanillin method. The 2 day old culture from 50ml broth was harvested and subjected to centrifugation at 4000g for 10 minutes. Then the treated culture was dried with a vacuum freeze drier for 4 hours. 0.1g of dry cells was homogenized using liquid nitrogen and then extracted with 4.0ml methanol-chloroform-water mixture (2:1:0.8, v/v/v) with vigorous shaking for 30 minutes. The tubes were then centrifuged for 10 minutes at 4000g. The lipid was found in the lowest phase was thoroughly mixed using 0.2ml saline solution and the sample was centrifuged at 4000g for 10 minutes. About 0.2 ml of chloroform and lipid mixture was taken in a clean and dry tube and 0.5ml of H_2SO_4 was added, heated in a boiling water bath for 10 minutes. After that, 3ml of phosphovanillin solution was added followed by vigorous shaking, and then incubation was carried out at room temperature and kept for 10 minutes. The amount of lipid content in the treated pathogenic cell membrane was read at 520nm and cholesterol can be used as a standard for constructing calibration curve.

3.7. Purification of antifungal compounds from the culture filtrates of *Aspergillus giganteus*

Aspergillus giganteus produces an AFP (Antifungal Protein) that can be characterized by many techniques such as ammonium sulphate precipitation, TCA, acetone, etc. Ammonium sulphate precipitation method is used for the precipitation of proteins from the culture filtrates of *Aspergillus giganteus*.

3.7.1. Precipitation of proteins by ammonium sulphate method

The culture filtrates of *Aspergillus giganteus* was prepared by filtering the supernatant of the *Aspergillus giganteus* culture using 0.22µm membrane filter. The filtrate was then subjected to ammonium sulphate precipitation. The soluble proteins can then be precipitated out of the filtrate. The crude protein fractionized and purified using ammonium sulphate (0-30%, 30-60% and 60-90%). Each fraction obtained in the precipitation process was pooled with the minimum volume of Tris- HCl buffer (pH 7.4±0.2) and dialyzed overnight in the Tris- HCl buffer. The detailed procedure of ammonium sulphate precipitation is explained in Appendix VII.

3.7.2. Dialysis

The precipitated protein was then dialyzed and used for further process. The precipitated protein was dialyzed in 2.5nm dialyzed bag and the fractions were collected and used for the further use. The detailed procedure of dialysis is explained in Appendix VIII.

3.8. Antagonistic potential of partially purified antifungal compounds of *Aspergillus giganteus*

The mechanistic action of purified compounds from *Aspergillus giganteus* was tested against the pathogenic organisms namely, *Cryptococcus neoformans* and *Candida albicans* carried out by the methodologies reported above.

3.9. *In silico* studies

Validation of *in vitro* results, the *in silico* approaches have seemingly important and gives a way for new and novel drug development. The antifungal compounds from *Aspergillus giganteus* have been downloaded and docked against the pathogenic target proteins.

3.9.1. Schrödinger software

Schrodinger is widely recognized as a scientific leader in developing chemical stimulation software for use in pharmaceutical and biotechnology research. Schrodinger has achieved breakthroughs in the fields of quantum chemistry, molecular, drug design and protein structure determination.

3.9.2. MAESTRO

Maestro is the graphical user interface (GUI) for all Schrodinger's computational technology. Maestro also helps researchers organize and analyze data. Maestro's makes setting up calculations easy and straightforward for all Schrodinger's computational programs like

ComboGlide, Epik, Glide, Impact, Jaguar, Liaison, Ligprep, Macromodel, Phase, Prime, Qikprop, Qisite, Sitemap and Strike. Computed results are mechanically returned and incorporated into projects for more study. It contains tools for building, displaying and manipulating chemical structures; for organizing, loading and storing these structures and associated data; and for setting up, submitting, monitoring, and visualizing the results of calculations on these structures. Maestro is a powerful and versatile molecular modeling environment, and portal to the most advanced science in computational chemistry.

3.9.3. Selection of Ligands from *Aspergillus giganteus*

The suitable compounds and it derviatives from *Aspergillus giganteus* and standard drugs currently in practice were obtained from literature search. Three dimensional structures of the selected ligands were retrieved using PubChem databases.

3.9.4. Generation of the ligand library

A set of compounds having inhibitory activity is extracted from the PubChem database for the present docking studies. Maestro version was used to construct models of ligands for docking. After constructing the ligands, the Ligprep 2.0 application was used to prepare the ligand for further study. Ligprep uses force field and produces the low energy 3D conformers of the selected ligands. These ligands were then neutralized and analysed for their ADME predictions using qikprop applications.

3.9.5. ADME prediction using QIKPROP version 5.8

The Absorption, Distribution, Metabolism, Excretion and Toxicity (ADME/Tox) properties are prior important to design the drug molecules. This process plays an important role in clinical phases for the identification of lead compounds. Qikprop 5.8 predicts physically significant descriptors and pharmaceutically relevant properties of organic molecules, either individually or in batches. In addition to predicting molecular properties, Qikprop provides ranges for comparing a particular molecule's properties with those of 95% of known drugs. The Absorption, Distribution, Metabolism, Excretion (ADME) studies of the prepared ligands was done using Qikprop 5.8, Schrodinger.

3.9.6. Selection of the target proteins from *Cryptococcus neoformans* and *Candida albicans*

The target protein from *Cryptococcus neoformans* including, Farnesyl transferase (3SFX), Ionisine 5′-monophosphate (4AF0), Ribokinase (6CW5), Serine/Threonine – protein

phosphate 2B catalytic subunit A1 (6TZ8) and Choline kinase (6WHP) were selected to understand the antagonistic action of *Aspergillus giganteus*. The target protein, 3SFX is an important key enzyme for the virulent nature of *Cryptococcus neoformans* which are actively involved in the catalyzation of post-translational lipidation of signal transduction proteins (Hast *et al*., 2011). Complete disruption of *de novo* synthesis of GTP/ATP in the pathogen leads to the destruction of pathogenic attack in host. Ionisine 5′-monophosphate acts as a rate limiting enzyme in the *de novo* biosynthesis of GTP/ATP (Morrow *et* al., 2012). Ribokinase enzyme, catalyzes the reaction involved in the phosphorylation of ribose at O-5, resulting in the D-ribose-5-phosphate formation. The inhibition of ribokinase in the pathogen might be reduce the pathogenic virulence in the host. The target protein 6TZ8 activation triggers the nuclear translocation of dephosphorylated transcription factors which resulted in fungal virulence (Juvvadi *et al*., 2019).

The target proteins namely, Dihydrofolate reductase (1IYL), N-Myristoyl transferase (1AI9), Lanosterol 14-α demethylase (5V5Z) and Secreted aspartyl transferase (1ZAP) from *Candidaa albicans* were selected for drug-ligand interactions. Dihydrofolate reductase is an important enzyme involved in various cellular functions. It is necessary for the growth of the fungal cell. It is necessary to inhibit this enzyme for the pathogenic progression in the host (Mitchell, 2020). 1AI9 involved in the transfer of the fatty acid myristate from myristoyl-CoA to the N-terminal glycine residue in many eukaryotic and viral proteins. It is considered as an attractive target for antifungal drugs (Sogabe *et al*., 2002). 1ZAP and 5V5Z are effectively involved in the various functions in the pathogenic cell for enhancing its virulence potential in the host (Keniya *et al*., 2018).

3.9.7. Preparation of the protein

The protein preparation wizard accepts a protein from its raw state to a state in which it is properly prepared for docking. A typical PDB structure file consists only of heavy atoms and might include a co crystallized ligand, water molecules, metal ions and Co factors. Some structures are multimeric, and may need to be reduced to a single unit. The intended workflow in protein preparation wizard follows fixing structures first and then deleting unwanted chains and water molecules, then fixing or deleting hetero groups, optimization of the fixed structures followed by minimization of the protein. The refined protein was prepared using protein preparation wizard and outfile was *jobname.impref_ref.out.*

3.9.8. Molecular docking of active compounds with pathogenic target proteins

The Grid was generated at the centroid of selected residues. This was done using Receptor grid generation option in the Glide. The options in each tab of the receptor grid generation panel allow us to define the receptor structure by excluding any co crystallized ligand that may be present, determine the position and size of the active site as it will be represented by receptor grids, and set up glide constraints. The outfile generated was saved as *jobname.zip.* The ligand docking jobs cannot be performed until the receptor grids have been generated.

Glide ligand docking jobs requires a set of previously calculated receptor grids and one or more ligand structures prepared using Ligprep. The ligand docking was carried out with the SP mode of Glide that combines a powerful sampling protocol with the use of a custom scoring function designed to identify ligand poses. The output file was generated with the extension *jobname.Sp_pv.mae.* The docking results were viewed using the pose viewer panel in the Glide.

RESULTS AND DISCUSSION

4.0 RESULTS AND DISCUSSION

Fungi are considered as the major clinical pathogens and the fungal infections are becoming a global threat. The prevention and treatment of both invasive and systemic fungal infections have been a challenge to scientists and medical professionals. There are various reasons behind the increased prevalence of fungal infections in human including overuse of immunosuppressive, antineoplastic drugs, prosthetic graft and its devices, broad spectrum of antibiotic usages and surgery (Bajpai *et al*., 2019). Fungi as such become the natural source for the production of antimicrobial compounds. *Aspergillus giganteus* is the mold, known to secrete the antifungal protein which is antagonistic to many bacterial and fungal pathogens (Alberti *et al*., 2017). Hence, the present study is focused to evaluate the antagonistic potential of the antifungal compounds from *Aspergillus giganteus* on the emerging fungal pathogens such as *Cryptococcus neoformans* and *Candida albicans*. The *in silico* studies further confirmed the interaction of antifungal compounds with the pathogenic target proteins.

4.1. Antagonistic potential of *Aspergillus giganteus* on human fungal pathogens

4.1.1. Co-culture assay

Co-culture method is the best method to identify the interactions between antagonistic fungi and pathogenic fungi. The co-culture assay was carried out to prove the antagonistic potential of *Aspergillus giganteus* against pathogenic fungi, namely, *Cryptococcus neoformans* and *Candida albicans*. The results of the co-culture method for *Aspergillus giganteus* against *Cryptococccus neoformans* and *Candida albicans* are shown in plate 1 and plate 2, respectively.

Inhibition percentage of *Aspergillus giganteus* with *Cryptococcus neoformans* was found to be 99% and the inhibition percentage for *Candida albicans* was observed to be 97%. The results have proved the effective antagonistic activity of *Aspergillus giganteus* against both the selected pathogenic organisms.

Cryptococcus neoformans

***Cryptococcus neoformans* with antagonistic fungi**

Plate 1: Antagonistic activity of *Aspergillus giganteus* against pathogenic *Cryptococcus neoformans*

Candida albicans

***Candida albicans* with antagonistic fungi**

Plate 2: Antagonistic activity of *Aspergillus giganteus* against pathogenic *Candida albicans*

Several literature studies have been reported to prove the inhibitory potential of antagonistic organisms.

Abro *et al*., 2019 have performed the inhibiting potential of 30 different species of endophytic fungi against Fusarium wilt causing species *F. oxysporum* and *F.cucumerinumo* in cucumber, among which 10 of them have shown maximum inhibiting potential against the pathogens.

Choudhary and Ashraf, 2019 have reported that *T. harzianum* have shown the best inhibiting activity against the growth of fungal pathogens while performing the dual culturing of bioagents and organic amendments against fungal pathogens.

Antagonistic efficacy of *Paenibacillus pasadenensis* (strain 16) was performed against three different pathogens namely, *Botrytis cinerea*, *Phomopsis viticola* and *Fusarium verticilloides* by Passera *et al*., 2017 where the strain 16 was found to be ineffective against *Fusarium verticilloides* and effective against *Botrytis cinerea*.

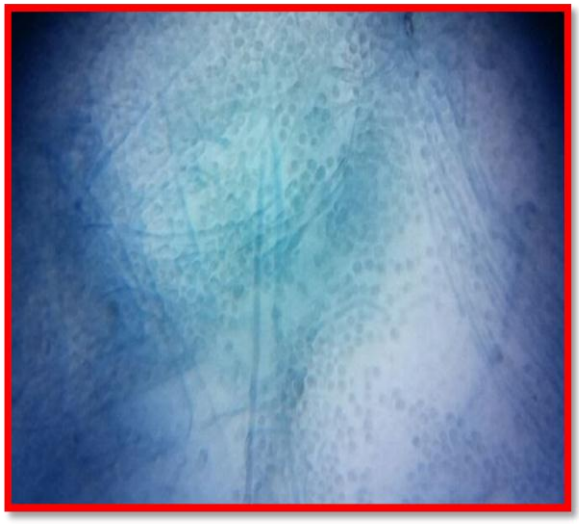
The *in vitro* antagonistic activity of all the 12 isolates of *Trichoderma* against *Rhizoctonia solani*, *Pythium ultimum* and *Alternaria solani* have been proved by Mazrou *et al*., 2020 among which T6 isolate have found to be antagonistic to *Rhizoctonia solani*.

Durairaj*et al*., (2018) have carried out the antagonistic effect of *Pseudomanas* and *Bacillus* strains against ginseng root rot fungal phytopathogens where two strains of bacterial species effectively inhibited the growth of ginseng root fungal pathogens.

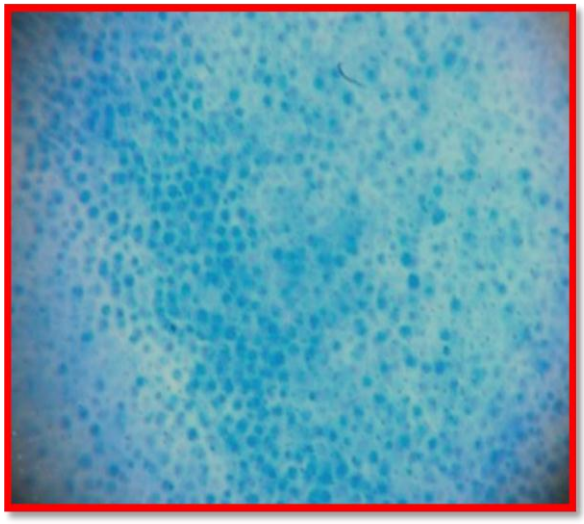
4.1.2. Hyphal interaction assay

Hyphal projection is the important key factor for the pathogenic processes. The antagonistic activity observed in the co culture assay plate was further proved by hyphal interaction assay by lactophenol cotton blue staining method. The results for the interaction of *Aspergillus giganteus* hyphae with the hyphae of *Cryptococcus neoformans* and *Candida albicans* are shown in plates 3 and 4 respectively.

The results have shown that the pathogenic fungi alone was observed with the good morphology and extended hyphae while the fungi treated with *Aspergillus giganteus* have the damaged and reduced hyphal elongation.

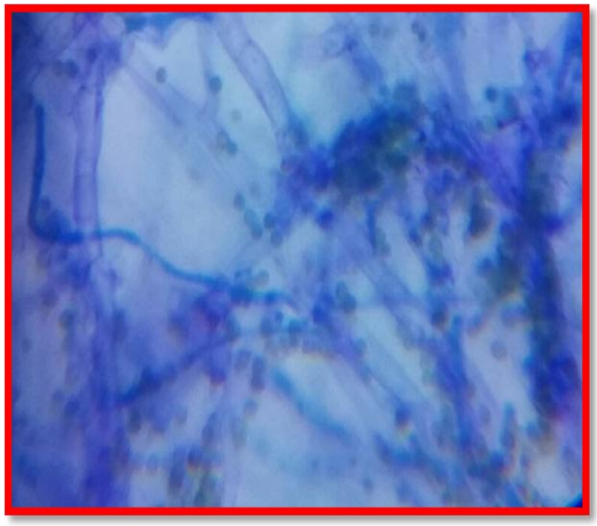


Cryptococcus neoformans

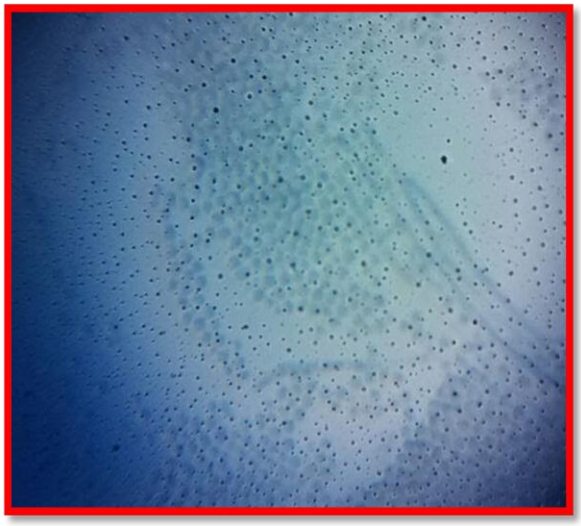


Cryptococcus neoformans with *Aspergillus giganteus*

Plate 3: Hyphal interaction of antagonistic *Aspergillus giganteus* with pathogenic *Cryptococcus neoformans*



Candida albicans



Candida albicans with *Aspergillus giganteus*

Plate 4: Hyphal interaction of antagonistic *Aspergillus giganteus* with pathogenic *Candida albicans*

Scientific evidences are available for the antagonistic potential analyzing the hyphal interactions.

Biocontrol potential of endophyte *Fusarium oxysporum* against pathogen *Rhizoctonia solani* was carried out by Divya *et* al., 2018, where the morphological changes were identified in the treated pathogens.

Morphological changes in the mycelial growth of the pathogen, *Dematophora necatrix* was analysed after interaction with the spectrum of fungal endophytes isolated from Apple and other cross including, *Fusarium equiseti*, *Talaromyces aculeatus*, *Neocosmospora perseae*, *Aspergillus aculeatus*, *Crinipellis tabtim*, *Fusarium circinatum*, (Pal *et al*., 2020).

Anteneh *et al*., 2019 have evaluated the morphological appearances of fungi isolated from marine sponge sources. The yeast like form revealed elliptical conidia occurring in the single – shaped blastospores with the presence of conidogenous cells within the hyphae.

The hyphal interaction of *Bacillus amyloliquefaciens* BAS23 with various pathogenic fungi was performed, where the swell and bulb formation was observed microscopically in the test plate with various pathogenic fungi (Saechow *et al*., 2018). Stefanini *et al*., 2018 have reported that the hyphae of *Aspergillus fumigatus* was damaged by the newly introduced antifungal compound 089.

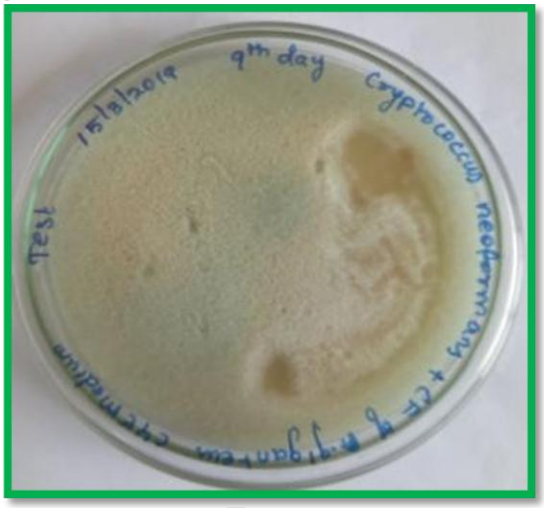
Thus the results of co-culture assay revealed that the *Aspergillus giganteus* was antagonistic to fungal pathogens namely, *Cryptococcus neoformans* and *Candida albicans* and the hyphal interaction assay showed the efficacy of *Aspergillus giganteus* exhibiting damaged and reduced hyphal elongation in the treated pathogens.

4.2. Antagonistic potential of the culture filtrates from *Aspergillus giganteus*

Culture filtrates prepared from the fungal cells contain only the active metabolites which possess the antagonistic activity to various bacterial and fungal pathogens (Khan *et al*., 2018; Parveen *et al*., 2019). The preliminary studies in our laboratory have proved that the culture filtrates prepared from *Aspergillus giganteus* were found to be antagonistic in nature. Hence, the culture filtrates was prepared and tested for its efficiency against the selected human fungal pathogens namely, *Cryptococcus neoformans* and *Candida albicans*.

Cryptococcus neoformans

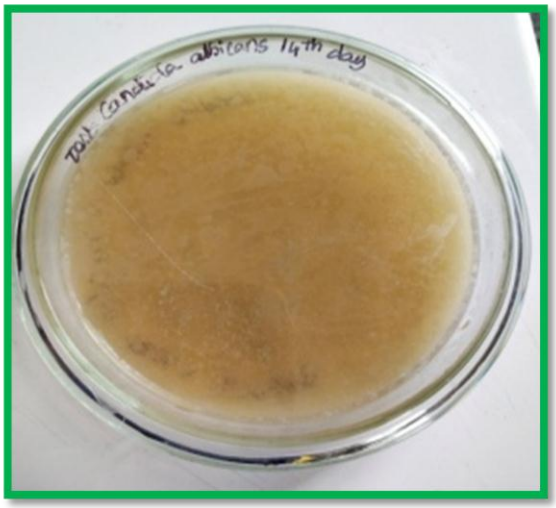
Control



Test

Candida albicans

Control



Test

Plate 5: Antagonistic activity of the culture filtrates of *Aspergillus giganteus* on human fungal pathogens

The percentage of inhibition for the antagonistic activity of the culture filtrates on *Cryptococcus neoformans* and *Candida albicans* was found to be 91% and 93%, respectively. The result for the overlay assay is depicted in plate 5.

The antifungal compounds (AFP) present in *Aspergillus giganteus* are protein in nature and it has been reported in many scientific literatures (Vila *et al*., 2001; Tong *et al*., 2020). Since the antagonistic substance produced by the *Aspergillus giganteus* is protein in nature, the protein estimation was performed to find out the concentration of antifungal proteins present in the culture filtrates. The amount of protein content in the culture filtrate was found to be 1.6 mg/ml.

Scientific evidences have demonstrated the antagonistic potential of culture filtrates against pathogens.

Antagonistic activity of culture filtrates of three *Trichoderma* isolates was examined against several pathogenic fungi namely, *Penicillium expansum, Aspergillus niger, Alternaria alternata, Mucor plumbeus, Penicillium chrysogenum, Trichothecium roseum* and *Rhizoctonia solani* among which *Rhizoctonia solani* was found to be more susceptible to all the three isolates of *Trichoderma* (Koka *et al*., 2019).

The *in vitro* antagonistic activity of cell free culture filtrates of *Pseudomonas aeruginosa* against *Fusarium oxysporum* was carried out by Islam *et al*., 2018, where mycelial growth of *Fusarium oxysporum* was inhibited with the culture filtrates from 48 hours old culture than 12 hours culture.

Endophytic fungi isolated from *Monarda citriodora* was tested against pathogenic fungi namely, *Fusarium oxysporum, Fusarium redolens, Aspergillus flavus* and *Aspergillus fumigatus* where the maximum antagonistic activity was found with *Fusarium oxysporum* (Katoch and Pull, 2017).

Hence, the results revealed that the culture filtrate of *Aspergillus giganteus* has maximum antagonism against pathogenic *Cryptococcus neoformans* and *Candida albicans*.

4.3. Evaluation of *Aspergillus giganteus* for its cytotoxicity

The cytotoxicity of antifungal compounds was determined to evaluate for its safer applications by hemolytic activity using red blood cells. The prepared culture filtrates from the *Aspergillus giganteus* was used to determine the toxicity. The different concentration of antifungal compounds in the culture filtrates such as 100 µg, 200 µg, 300 µg, 400 µg and 500 µg

(CF1, CF2, CF3, CF4 and CF5, respectively) was tested for the determination of cytotoxicity of AFCs (Antifungal Compounds). The results for the evaluation of cytotoxic levels of *Aspergillus giganteus* is represented in Table 1.

Table 1: Percentage hemolysis of antifungal culture filtrates of *Aspergillus giganteus* using goat red blood cells

S.No	Concentration of AFCs in the culture filtrates of *Aspergillus giganteus* (µg)	Percentage of hemolysis			Mean (%)	Standard deviation
		T1	T2	T3		
1	CF1	8.1	9.1	8.4	8.5	8.5±0.41
2	CF2	15.2	15.2	15.5	15.3	15.3±0.14
3	CF3	17.6	16.6	17.0	17.1	17.1±0.41
4	CF4	22.3	21.5	23.0	22.6	22.6±0.61
5	CF5	25.0	25.4	25.0	25.1	25.1±0.18

The results revealed that the percentage hemolysis of *Aspergillus giganteus* and the haemolytic value was increased with the volume of culture filtrates. None of the selected concentrations of AFCs in the culture filtrate showed better hemolysis of red blood cells. This test has proved that the use of antifungal compounds from *Aspergillus giganteus* to treat fungal infections due to their better efficacy and safer use with minimal side effects.

Several scientific studies have supported the cytotoxicity of antifungal compounds for their safer use.

Mapfunde *et al*., 2016 have performed the hemolysis of *C. zeyheri* phytoconstituents and the percentage of hemolysis was directly proportional to the sample concentration. The plant secondary metabolite namely, alkaloids exhibited greater toxicity level than saponins.

The hemolytic activity of *A. arvensis* was carried out by Soberon *et al*., 2017 has proved that the saponins phytoconstituent might be responsible for the toxicity.

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

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

Garrigues *et al*., 2018 carried out the evaluation of hemolytic activity of antagonistic fungal proteins and peptides of *Penicillium expansum*, among which none of the proteins showed hemolytic activity within the concentration range of 1-100µM.

4.4. Mode of action of *Aspergillus giganteus* by membrane disruption

The cellular materials are essential for the maintenance of membrane structure, integrity and are involved in several functions. The pathogenic cellular membrane is a target for many antifungal compounds. The antifungal compounds may disturb and disintegrate the cellular and nuclear membrane using various strategies. The leakage of cellular constituents such as DNA, RNA, protein and glucose from the pathogenic cell was used to monitor the action of antifungal compounds on the pathogenic cell wall and membrane. The quantification of released cellular and nuclear constituents of treated samples was analyzed for the antagonistic nature of culture filtrates of *Aspergillus giganteus* on the pathogenic cells.

4.4.1. Leakage of nuclear constituents from pathogenic cell membranes

The release of genetic material confirmed the disruption of pathogenic cell membrane by the action of culture filtrates of *Aspergillus giganteus*. The pathogenic organisms, *Cryptococcus neoformans* and *Candida albicans* was treated with the action of different concentration of antifungal compounds present in the culture filtrates CF1, CF2, CF3, CF4 and CF5 (ranging from 100 µg – 500 µg) *Aspergillus giganteus,* at various time intervals (0, 30, 60 and 120 minutes). The components released into the medium were read by the absorbance at 600nm for DNA and 660nm for RNA. All the experiments were carried out in triplicates. The results for the leakage of the genetic materials are given in Fig. 1 and 2.

The outcome of cell membrane permeability assay has proved the membrane damage and disruption of pathogenic cells caused by the action of culture filtrates of *Aspergillus giganteus*. The genetic material was estimated in the treated pathogenic strains with various volumes of culture filtrate and interval time.

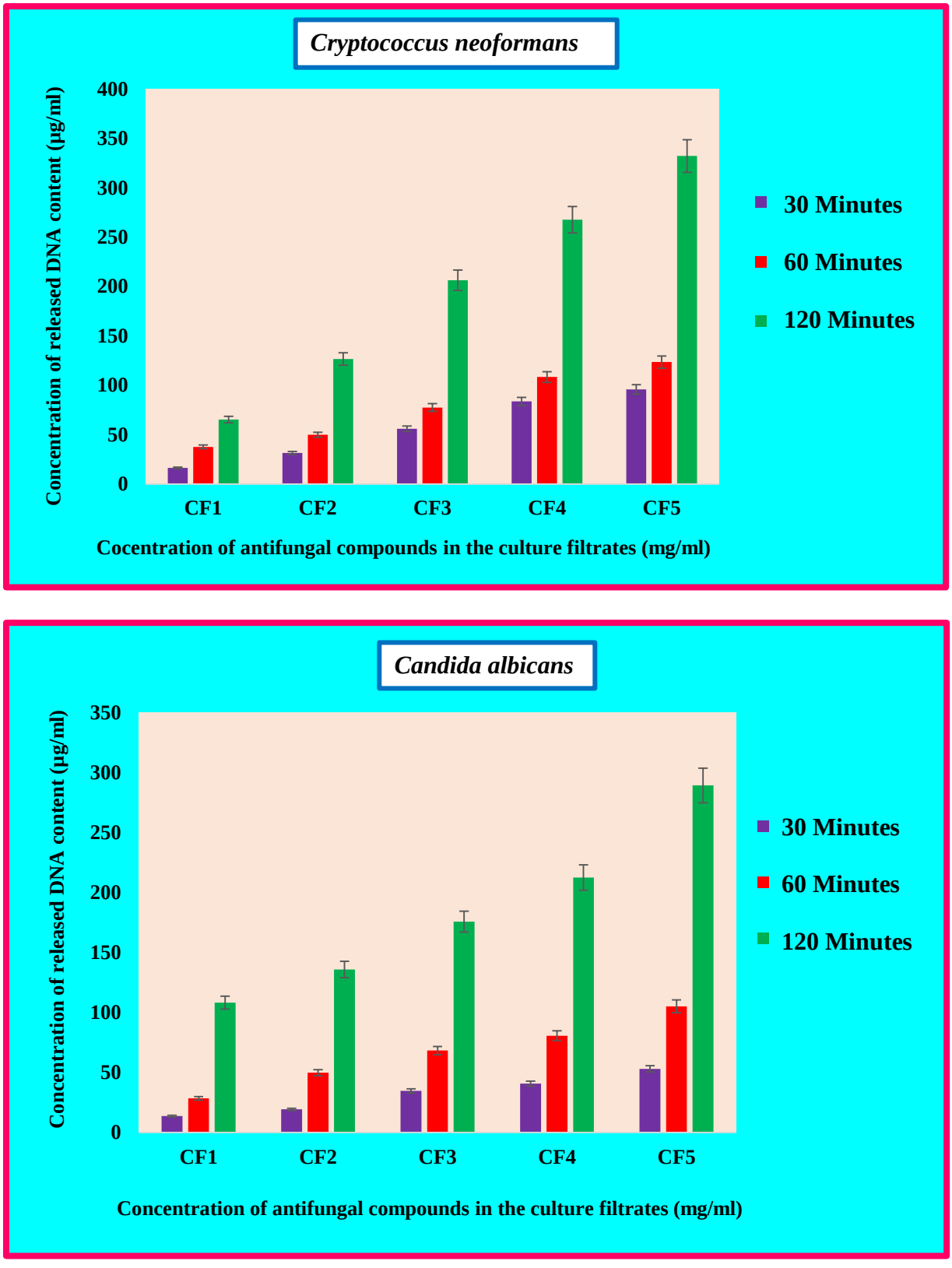


Fig. 1: Leakage of DNA from pathogenic cell membrane by the action of culture filtrates of *Aspergillus giganteus* at various time intervals

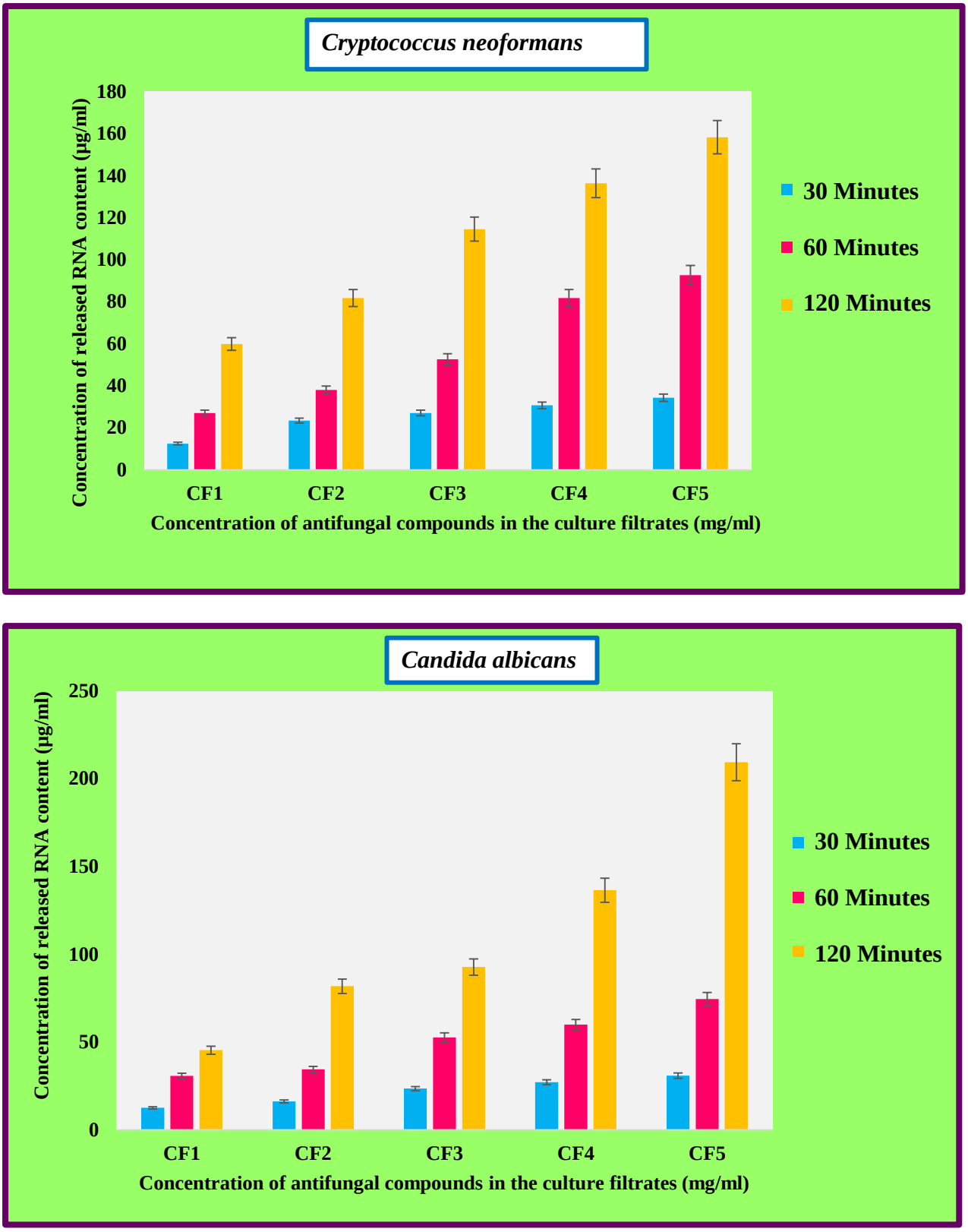


Fig. 2: Leakage of RNA from pathogenic cell membrane by the action of culture filtrates of *Aspergillus giganteus* at various time intervals

In *Cryptococcus neoformans*, the leakage of more amount of DNA was observed at 120 minutes of treatment. The amount of DNA estimated in different groups of culture filtrates at 120 minutes was obtained as 65.3µg/ml, 129.8µg/ml, 212.71µg/ml, 267.98µg/ml and 350.87µg/ml, respectively. No changes were noticed in the control. Like DNA, RNA was also released into the medium from the treated sample and the concentration of released constituents was estimated. The concentration of released RNA was also increased in the medium with the increased concentration of AFCs in the culture filtrates. In *Candida albicans*, the DNA and RNA was also elevated into the medium at 120 minutes of treated sample was found to be increased with the concentration of AFCs and time interval. This has proved that the membrane damage and disintegration had occurred in the pathogenic cell wall and cell membrane due to the action of AFCs.

The results have confirmed that the release of nuclear components from the treated pathogenic cell might be concentration-time dependent.

4.4.2. Leakage of protein from the pathogenic cells

Proteins are the important constituents of the cell membrane and it maintains the structural integrity and involved in various cellular functions. Proteins are considered as the potential cell targets for antifungal activity. The leakage of proteins into the medium via cell membrane by the action of culture filtrates could be quantified to prove the antagonistic activity of *Aspergillus giganteus*.

Hence, the pathogenic cells namely, *Cryptococcus neoformans* and *Candida albicans* were subjected to the action of culture filtrates from *Aspergillus giganteus* to know its potential action. Various concentrations of culture filtrates such as 100 µg, 200 µg, 300 µg, 400 µg and 500 µg were added to the pathogenic cells and the leakage of protein constituents were estimated at various time intervals (0, 30, 60 and 120 minutes). The results of released protein constituents by the action of culture filtrates are given in Fig. 3.

The outcome of the action of culture filtrates on the pathogenic cell membranes has proved that the protein constituents were released into the medium caused by the membrane disintegration and membrane damage of treated pathogenic cells. The more amount of pathogenic protein constituents were released with the increased concentration of culture filtrates and increased time interval. The release of cellular protein constituents in the graph indicates that the leakage of protein from the treated pathogenic cell membrane has proved that the

membrane damage is directly proportional to the concentration of compounds and time needed for its action.

In *Cryptococcus neoformans*, the released protein constituents were found to be increased with the time and concentration of antifungal culture filtrates and the OD at 660 nm were found to be maximum at 60 and 120 minutes of treatment. The release of protein constituents in *Candida albicans* was also increased at 60 and 120 minutes intervals. The results have indicated that the amount of protein released from the damaged pathogenic *Candida albicans* increases with the increase in time exposure of the antifungal compounds. Hence, the culture filtrates of *Aspergillus giganteus* was considered to be effective for causing an ultimate damage to the pathogenic cells namely, *Cryptococcus neoformans* and *Candida albicans*.

4.4.3. Leakage of glucose from the treated pathogenic cell membranes

Glucose is also an important component and it is often found in the form of glycoproteins by the conjugation of glucose with protein molecules. These are also considered as potential antifungal targets. Quantification of released glucose level in the pathogenic cells treated with different concentration of antagonistic culture filtrates has proved the disintegration and damage of pathogenic cells. The estimated glucose content in the treated pathogenic cells is represented in Fig. 4.

The maximum leakage of glucose contents were observed with increased concentration of culture filtrates of *Aspergillus giganteus* and time intervals. The 550 µg of AFCs of culture filtrates have shown maximum activity on the pathogenic cell membranes. The concentration of released glucose content with 550 µg of culture filtrates treated *Cryptococcus neoformans* cell membranes at different time intervals was obtained as 0.0 µg/ml, 39.74 µg/ml, 74.89 µg/ml, 159.83 µg/ml where as for *Candida albicans* 0 µg/ml, 42.67 µg/ml, 74.89 µg/ml, 145.18 µg/ml of glucose respectively.

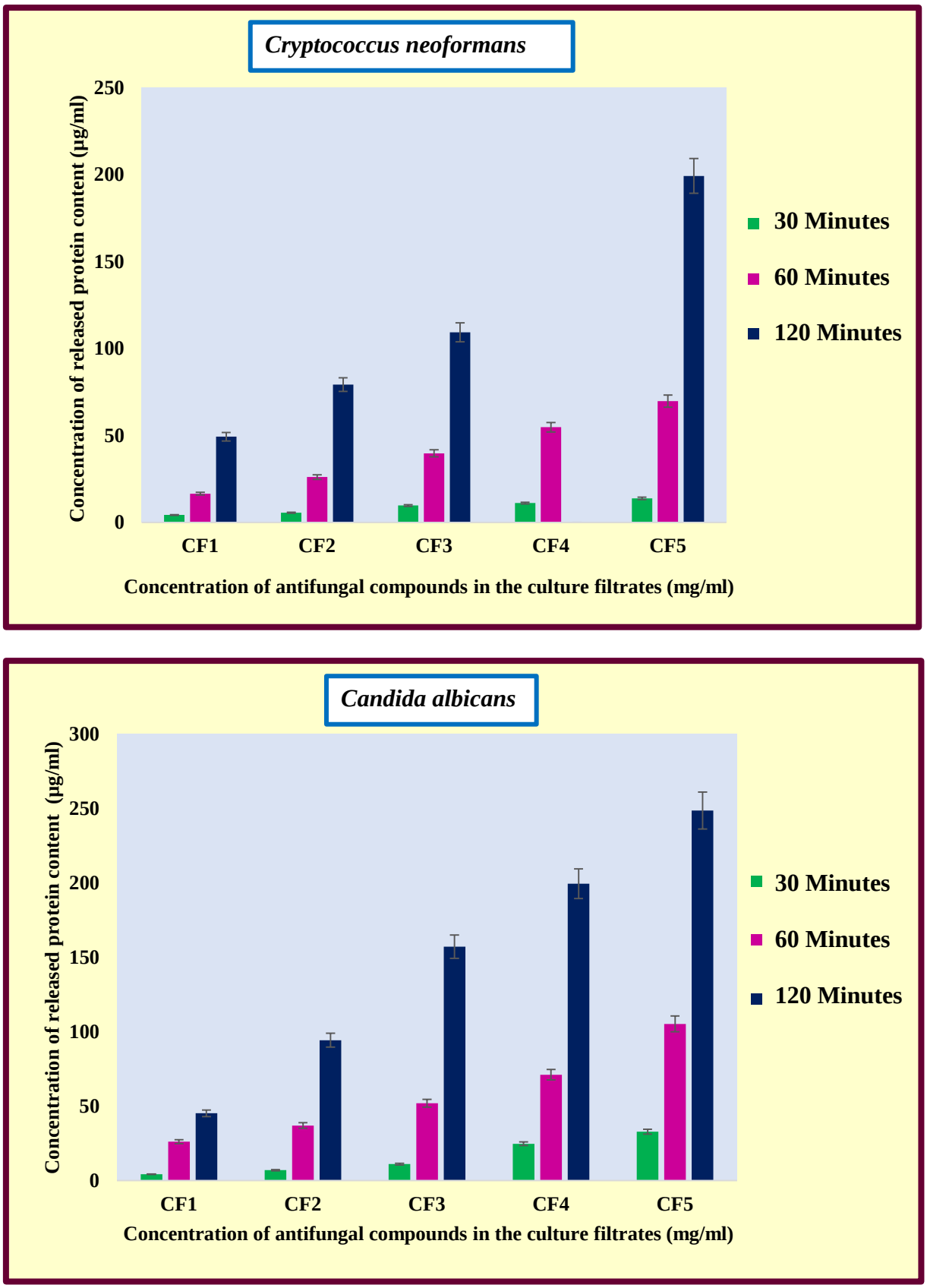
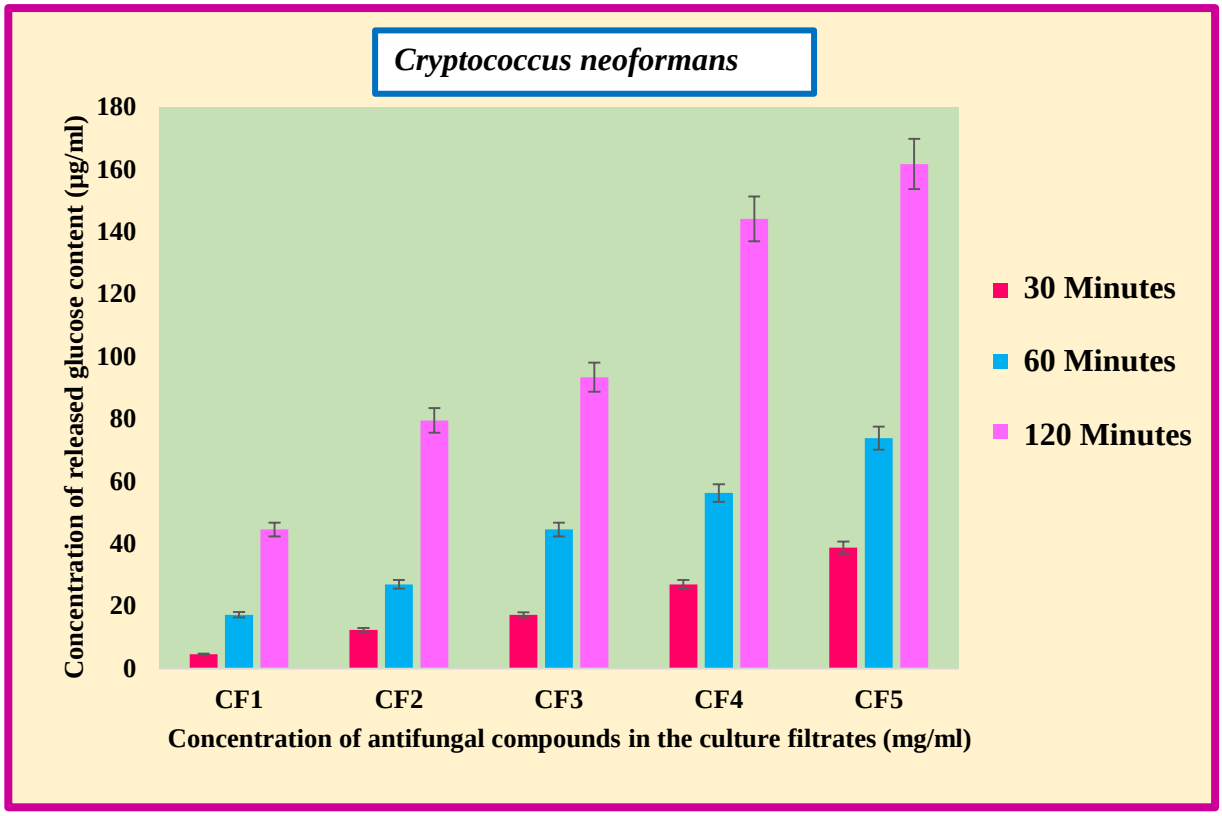


Fig. 3: Leakage of protein from pathogenic cell membrane by the action of culture filtrates of *Aspergillus giganteus* at various time intervals



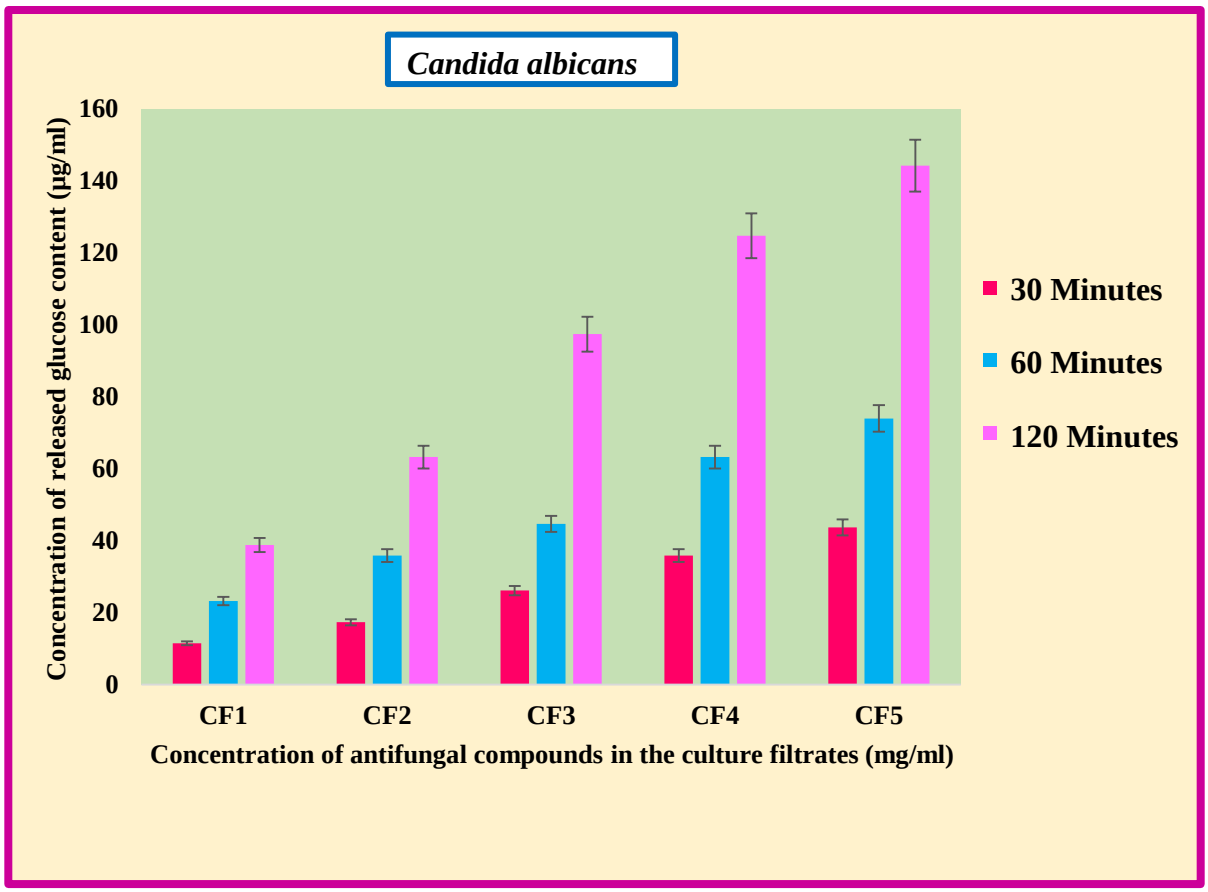


Fig. 4: Leakage of glucose from pathogenic cell membrane by the action of culture filtrates of *Aspergillus giganteus* at various time intervals

4.4.4. Influence of culture filtrates of *Aspergillus giganteus* on the release of substances in the pathogenic cell wall

The cell wall permeability was determined by the pH changes occurred in the treated pathogenic fungi. The results of the pH variance were observed in the treated *Cryptococcus neoformans* and *Candida albicans.* The graphical representation for the changes in the extracellular pH of the control and treated pathogenic *Cryptococcus neoformans* and *Candida albicans* is depicted in Fig.5. Alkali, acids or other substances might be eluted from the treated pathogenic cell wall that can be confirmed by the enhanced pH level in the extracellular medium.

The results revealed that some acids, alkali, ions and/or other metabolic substances might be released from the pathogenic cell wall into the medium upon the addition of culture filtrates of *Aspergillus giganteus.* Hence, the pH changes were observed in the treated pathogens at various time intervals.

The optimal pH for the growth and function of *Cryptococcus neoformans* is ~ 4 to 5. The pathogenic cell wall was subjected to different concentrations of culture filtrates of *Aspergillus giganteus* and the pH changes in the extracellular medium was monitored at various time intervals (0, 30, 60 and 120 minutes). The increased pH value was observed with the increased concentration of culture filtrates and time of treatment. The pH value at 120 minute of treatment with different culture filtrate groups was found with the range between 6.9 and 8.6.

The optimal pH for the better growth and function of *Candida albicans* is around 4 to 7. The *Candida albicans* was also subjected to different culture filtrates groups. The pH level in the extracellular medium varied with the different concentration of AFCs of culture filtrates and time intervals. Changes in the pH level of treated pathogenic strains were found to be inversely proportional to the concentration of culture filtrates and time of intervals.

The results revealed that the change in the extracellular pH for *Candida albicans* was not constant with the different time exposure and different concentration of antifungal compounds.

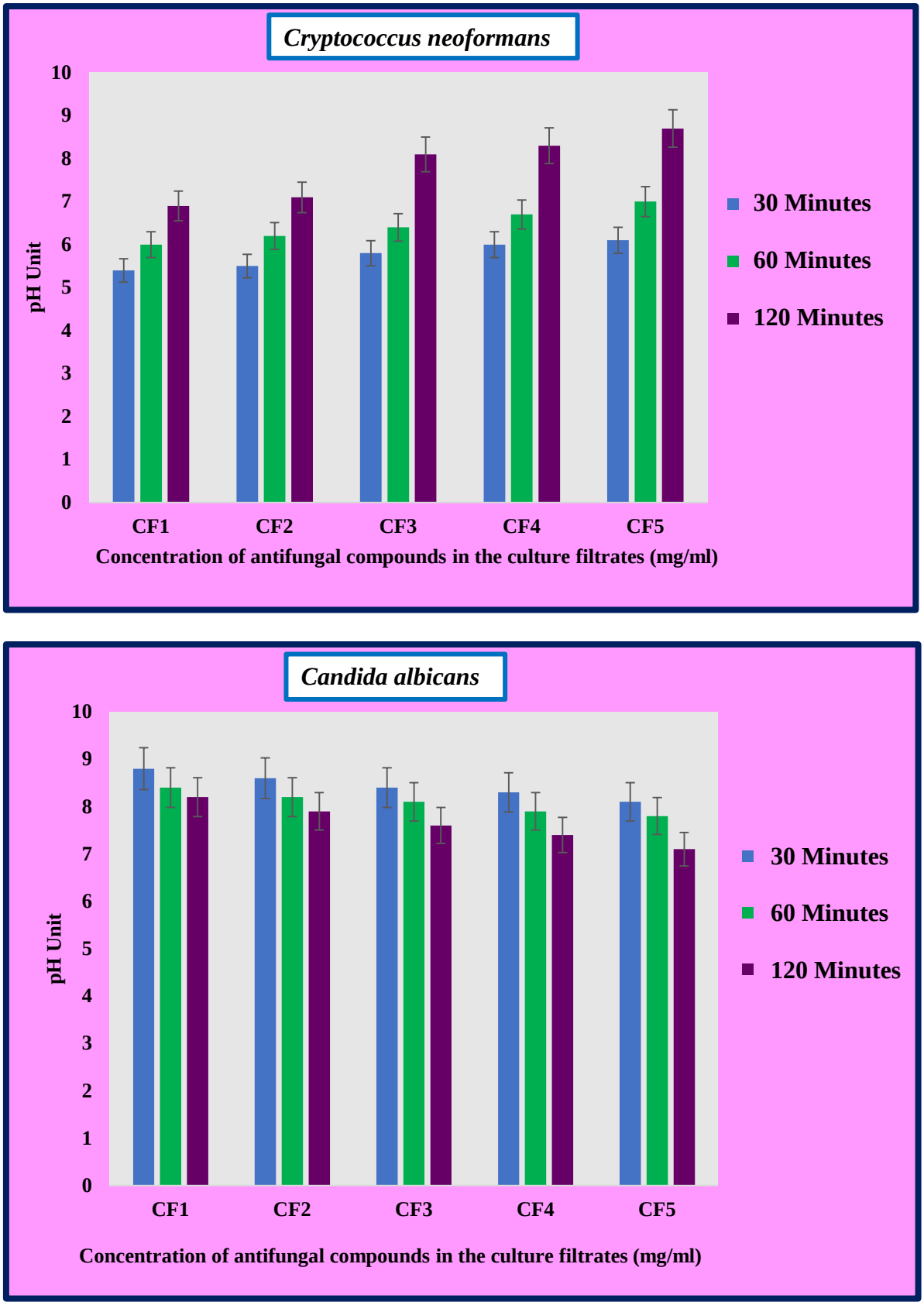


Fig. 5: Influence of the culture filtrates of *Aspergillus giganteus* on the extracellular pH of the fungal pathogens

4.4.5. Membrane integrity assay

Lipids are the major component of the cell membrane to maintain the structure and its integrity. The lipid content in the treated pathogenic cell membrane was determined by phosphovanillin method by the absorbance read at 520 nm. The pathogenic cell wall, *Cryptococcus neoformans* and *Candida albicans* incubated with different concentration of culture filtrates (100 µg, 220 µg, 300 µg, 400 µg and 500 µg) followed by the addition of various solvent, the lipid level of treated pathogenic strains was measured. The lipid level got deduced in the pathogenic cell membranes due to the activity of culture filtrates of *Aspergillus giganteus*. The result is depicted in Fig. 6.

The treated *Cryptococcus neoformans* and *Candida albicans* cell wall has been found to be more sensitive to the antifungal compounds in the culture filtrates of *Aspergillus giganteus*. In the control tube of *Cryptococcus neoformans* and *Candida albicans*, the lipid level was obtained as 2.25 mg/ml and 3.15 mg/ml, respectively. The treated pathogenic cells such as *Cryptococcus neoformans* and *Candida albicans*, the lipid level gets drastically reduced to 0.61 mg/ml and 1.87 mg/ml respectively.

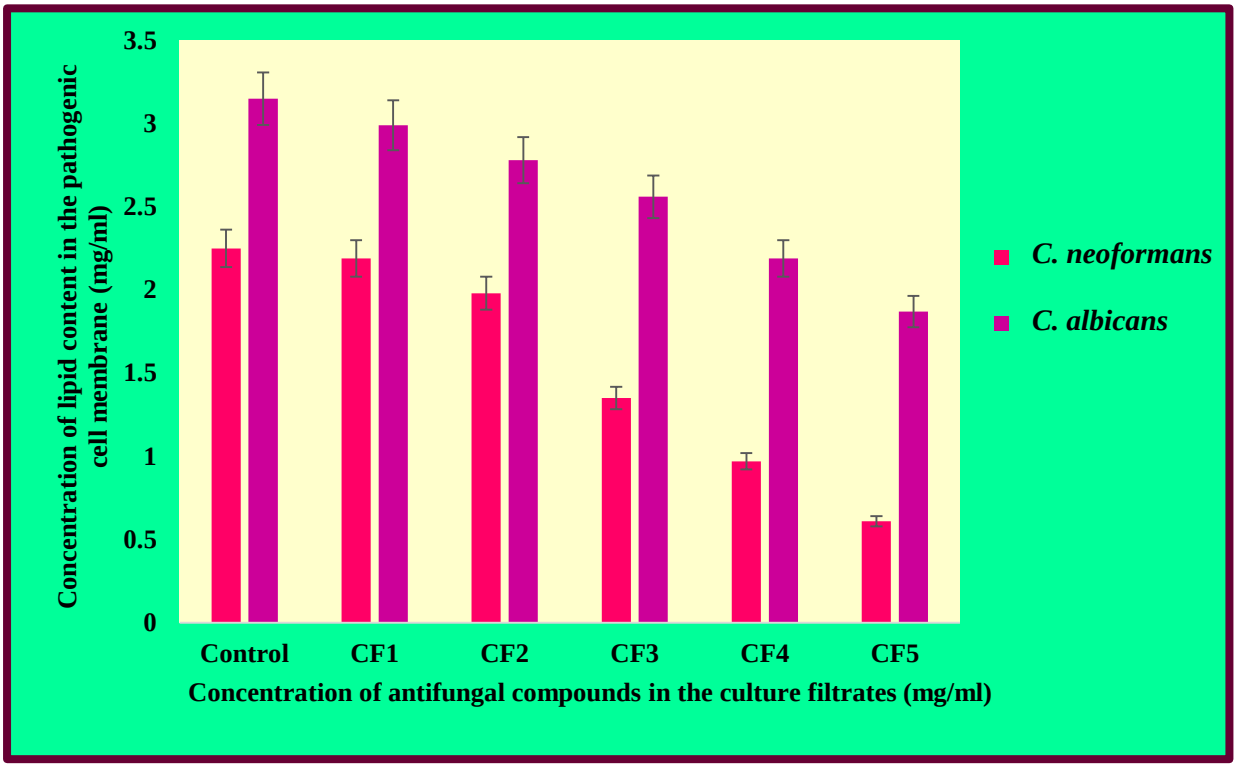


Fig. 6: Lipid content of pathogenic *C. neoformans* and *C. albicans* cell membrane treated with antagonistic culture filtrates

4.5. Characterization of antifungal compounds from the culture filtrates of *Aspergillus giganteus*

The antifungal compounds present in the *Aspergillus giganteus* was further purified by ammonium sulphate precipitation followed by desalting procedure (dialysis). In order to check the nature of culture filtrates containing the antifungal compounds responsible for the antagonistic property of *Aspergillus giganteus*, we performed the ammonium sulphate precipitation to precipitate the protein.

4.5.1. Purification of antifungal proteins

The prepared culture filtrates were taken to perform the purification process. The proteins in the culture filtrates get precipitated by various percentage solution of ammonium sulphate. The results for the protein content in each step of purification are given in Table 2. Different salt cuts such as 0-30%, 30-60% and 60-90% were prepared and the pellets were dissolved in Tris-HCl buffer. The 0-30% of salt cut did not give any visible proteins in the form of pellets. Hence, the other two salt cuts namely, 30-60% and 60-90% were further subjected to dialysis to remove the salts. At each step, the protein concentration was estimated by Lowry's method. The precipitated protein was dissolved using Tris-HCl and stored at 4ºC until further step of purification to be done. Obtained pellets at 30-60% and 60-90% were dialysed and the protein concentration was estimated.

Table 2: Concentration of protein content during purification processes

S.No	Purification process	Protein concentration in mg/ml
1.	Control – culture filtrate	1.6mg
2.	Ammonium sulphate precipitation	
	0-30%	0.3mg
	30-60%	1.4mg
	60-90%	1.8mg
3.	Dialysis	
	30-60%	0.7mg
	60-90%	1.0mg

The results revealed that the protein content in the 30-60% and 60-90% gave the better protein yield compare to 0-30% of precipitation. For further investigation of mode of action of antifungal compounds on the pathogenic cell wall 60-90% of protein fractions were taken.

Purification of antimicrobial compounds from various sources has been reported in scientific literatures.

Lei *et al*., 2019 have precipitated the proteins of *Lactobacillus* in cell free supernatant and ethylacetate extraction where the protein concentration was found to be more in ethylacetate extraction. The antagonistic activity of crude extract was less than that of partially purified compounds.

Pramudito *et al*., 2020 have reported the purification of the compounds in *Burkholderia* strains where 4 fractions namely 20%, 40%, 60% and 80% was obtained. Fraction 2 has the best antagonistic activity against *S. cerevisiae*.

The crude protein has extracted from the mung bean seeds and it has precipitated using 0-30%, 30-60% and 60-90% salt cuts where 30-60% gave the maximum yield of protein and this fraction has found to be antagonistic to two phytopathogens namely, *M. phaseolina* and *M. grisea* (Solanki *et al*., 2018).

The antifungal compounds have precipitated from *Lactobacillus paracollinoides* using saturation ammonium sulphate precipitation of 40%, 60% and 80%, among which 60% was reported to yield higher concentration of proteins after performing dialysis (Sathe *et al*., 2017).

Thus, the partially purified antifungal compounds were assessed for its antagonistic potential to compare the efficacy of culture filtrates and the purified fractions.

4.6. Mode of antagonistic action of purified fraction of *Aspergillus giganteus*

The purified fraction of *Aspergillus giganteus* was used to investigate its mode of antagonistic action on pathogenic *Cryptococcus neoformans* and *Candida albicans* cell wall and cell membrane.

4.6.1. Analysis of membrane damage by purified fractions of *Aspergillus giganteus*

The quantification of released contents in the treated pathogenic fungi such as DNA, RNA, protein and glucose was done to prove the antagonistic nature of partially purified compounds from *Aspergillus giganteus*.

The partially purified fraction of *Aspergillus giganteus* was used to target the pathogenic *Cryptococcus neoformans* and *Candida albicans* cell wall and cell membrane. Thus, it causes the damage to the cellular membrane and also disintegrates the nuclear membrane which releases the DNA, RNA, protein and glucose into the extracellular medium. The pathogenic cell wall was exposed to various concentrations of purified fraction of antifungal compounds (PF1, PF2, PF3, PF4 and PF5) AFCs such as 50 µg, 100 µg, 200 µg, 400 µg and 800 µg and the released contents were estimated at different time intervals (0, 30, 60 and 120 minutes). The result of the quantification of released constituents of DNA, RNA, protein and glucose in the treated fungal pathogens is depicted in Fig. 7, 8, 9 and 10, respectively.

The pathogenic *Cryptococcus neoformans* and *Candida albicans* have exhibited that the cell wall is more sensitive to the partially purified fractions of *Aspergillus giganteus*. The nuclear components have been elevated into the medium with increased concentration and time intervals.

The protein constituents in the pathogenic cell wall get released when treated with the purified antifungal fractions of *Aspergillus giganteus* and the concentration of released protein constituents was increased with the time and concentration of purified antifungal fractions.

The pathogenic cell wall of *Cryptococcus neoformans* and *Candida albicans* treated with a different concentration of partially purified compounds was observed with the damage and disintegration of cell membrane. This was confirmed by the quantification of released glucose contents in the treated pathogenic fungal membrane. No changes were noticed in the control tubes. The concentration of released glucose contents were increased with the concentration of AFCs and time of intervals.

The results have confirmed that the leakage of cellular constituents in both the pathogenic fungi treated with the partially purified compounds is time-concentration dependent. Both the pathogenic fungal cell membranes have been found to be susceptible to the AFCs of *Aspergillus giganteus*.

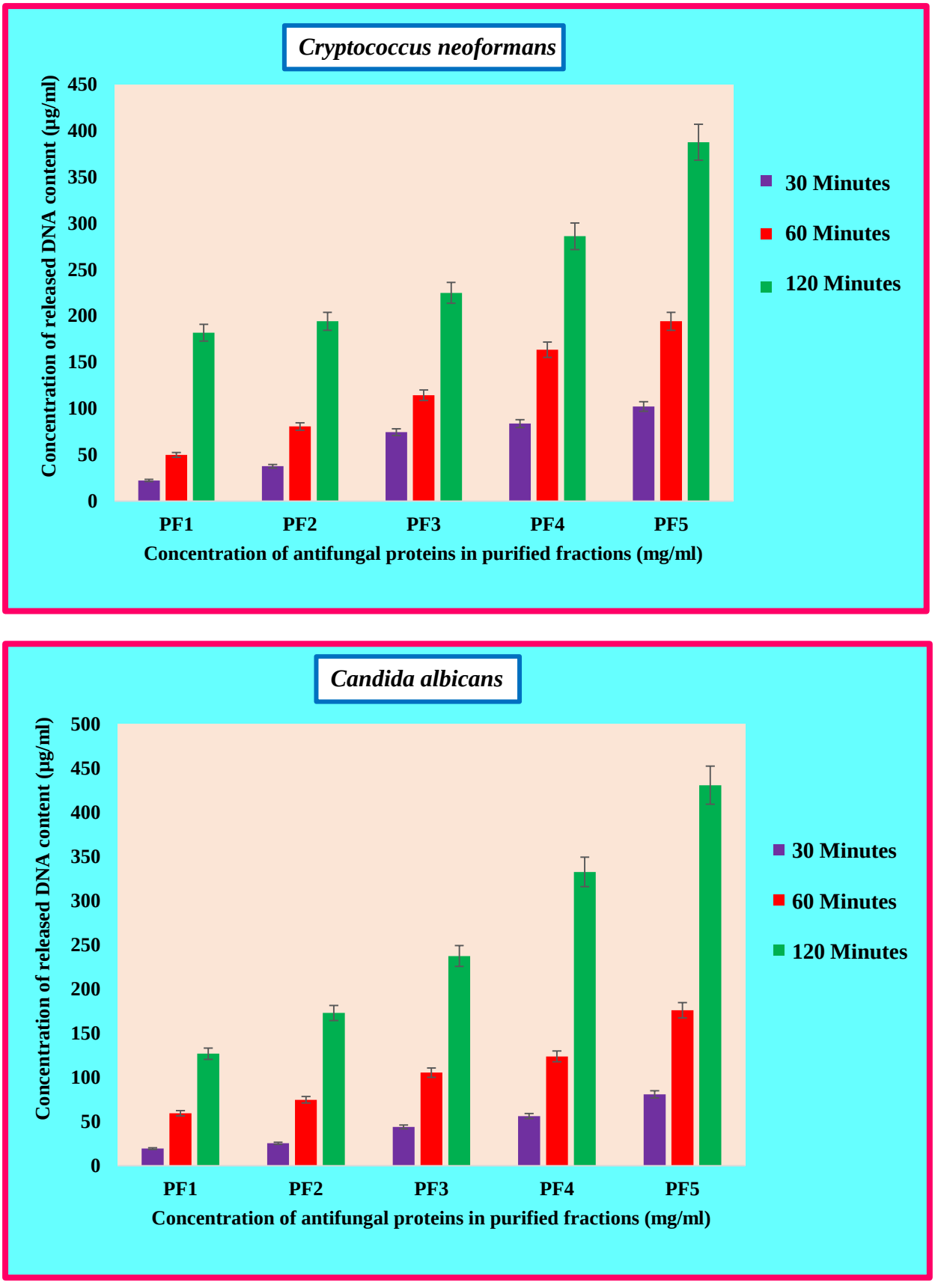


Fig 7: Leakage of DNA from pathogenic cell membrane by the action of AFCs from *Aspergillus giganteus* at various time intervals

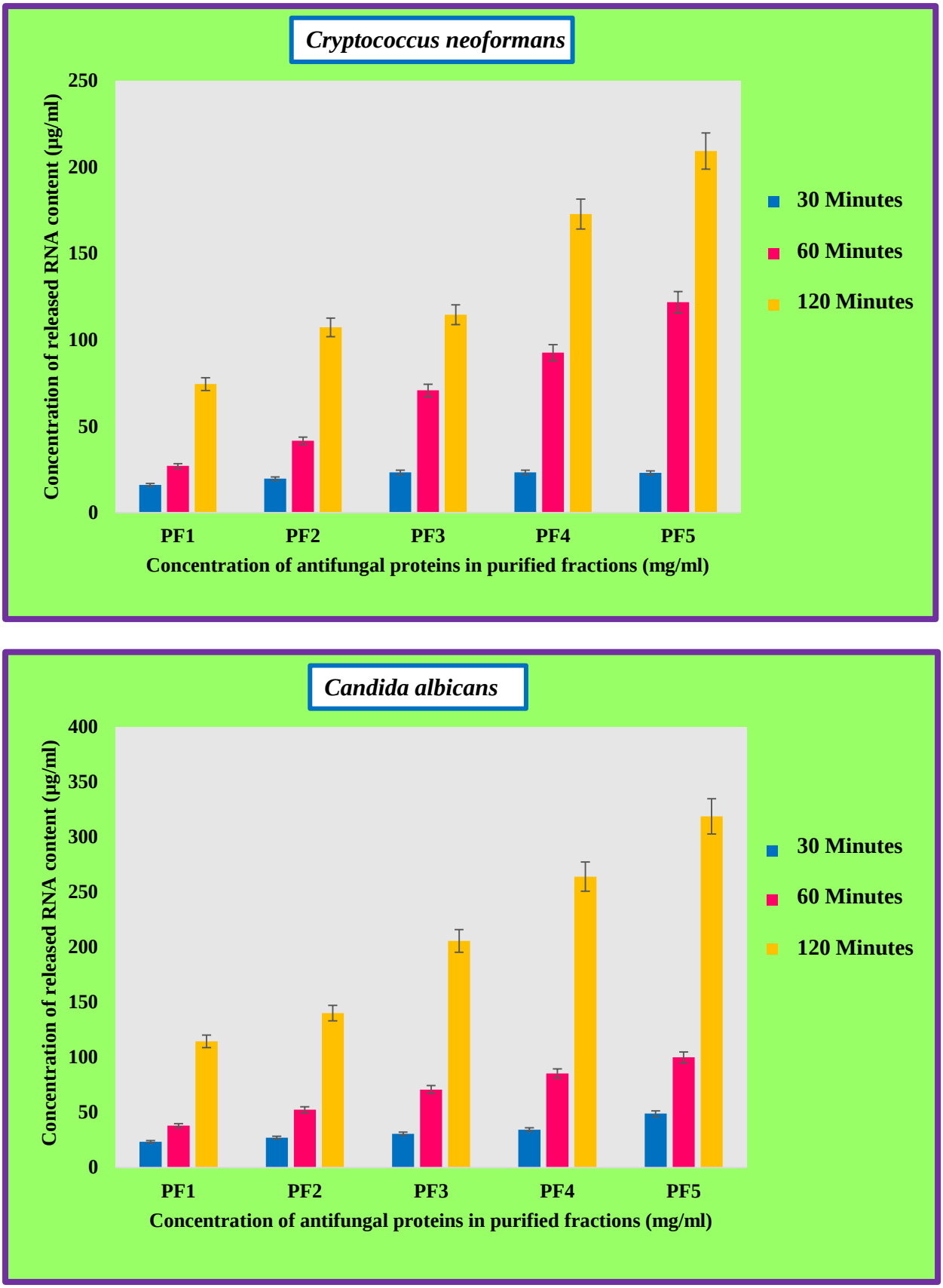


Fig. 8: Leakage of RNA from pathogenic cell membrane by the action of AFCs from *Aspergillus giganteus* at various time intervals

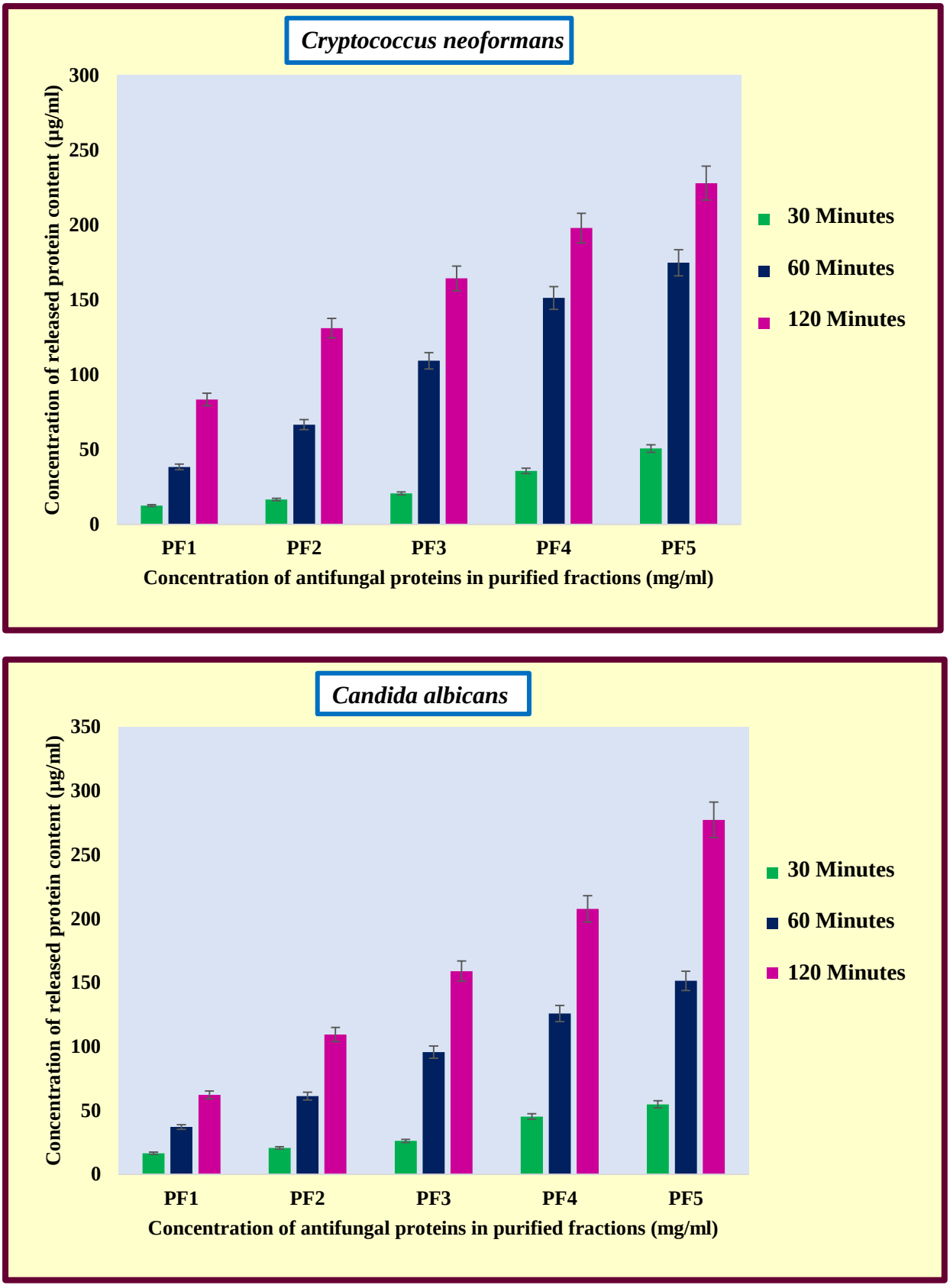


Fig. 9: Leakage of protein from pathogenic cell membrane by the action of AFCs from *Aspergillus giganteus* at various time intervals

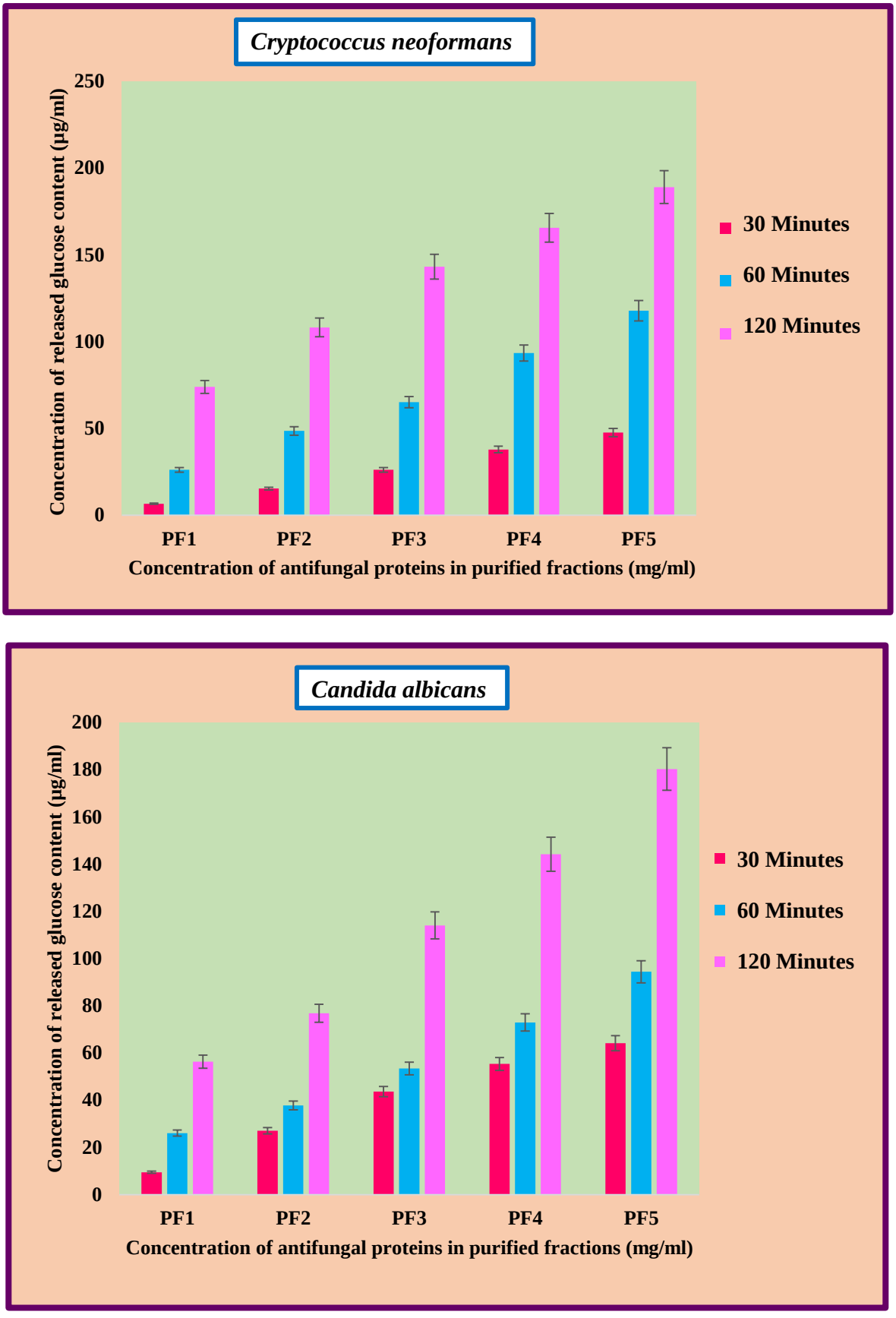


Fig. 10: Leakage of glucose from pathogenic cell membrane by the action of AFCs from *Aspergillus giganteus* at various time intervals

4.6.2. Action of purified fractions on the pathogenic extracellular pH level

The permeability of pathogenic cell membrane treated with the partially purified AFCs was confirmed by monitoring the pH changes in the extracellular medium. The pathogenic *Cryptococcus neoformans* and *Candida albicans* cell wall was exposed to various concentrations of AFCs of *Aspergillus giganteus* and the extracellular pH was checked at different time intervals. The result for the extracellular pH changes in the treated pathogenic fungi is given in Fig. 11.

The optimal pH for the growth and maintenance of pathogenic *Cryptococcus neoformans* and *Candida albicans* is ~ 4 to 5 and ~4 to 7, respectively. The pH level in the extracellular medium was changed at 120 minutes of treatment, ranging from 5.5 to 10.8 for *Cryptococcus neoformans* and 8.7 to 6.1 for *Candida albicans*. The changes in the extracellular pH of treated pathogenic fungus have confirmed that the damage and disturbances was observed in the permeability of cell membrane thus, it causes the leakage of acids, alkalis, ions and/or other substances from the pathogenic cell.

4.6.3. Lipid contents in the AFCs treated pathogenic cell membrane

The cell wall of *Cryptococcus neoformans* and *Candida albicans* was treated with a range of concentrations of AFCs (50 µg, 100 µg, 200 µg, 400 µg and 800 µg) and the lipid content in the treated pathogenic cell membrane was quantified by phosphovanillin method. The graphical representation of lipids in the treated pathogenic fungi is given in Fig. 12.

The cell wall of *Cryptococcus neoformans* was treated with the partially purified antifungal fractions of *Aspergillus giganteus* where the lipid level drastically reduced with the increased concentration of AFCs. The lipid level in the pathogenic cell wall without the addition of AFCs remains same. The lipid content in the treated *Candida albicans* cell wall also got deduced with the increased concentrations of purified fractions. The lipid level in the pathogenic *Cryptococcus neoformans* and *Candida albicans* treated with 800 µg of AFCs was obtained as 0.13 mg/ml and 0.87 mg/ml, respectively.

Several scientific studies reported that the mode of action of antifungal compounds on the pathogenic cell wall and cell membrane.

The leakage of cellular and nuclear components in the *S. aureus* and *P. aeruginosa* treated with the essential oils of *Origanum compactum* was found to be significantly increased by concentration dependent manner. This proved that the irreversible and gross morphological

changes were observed in the pathogenic cell wall and cell membrane due to the activity of antimicrobial substances (Bouyahya *et al*., 2019).

1-alklyl-3-methyl imidazolium ionic liquids and its derivatives have the antagonistic potential against the cell wall and cell membrane of *Candida albicans* where the cellular constituents and ions like potassium and calcium were released into the medium. This study proved that the pathogenic *Candida albiacns* cell wall was susceptible to the antifungal compounds, alkylimidazolium ionic liquids (Reddy and Nanchariah, 2020).

The mechanistic of action of essential oil of *Cinnamomum zeylanicum* bark was investigated on the cell wall of *Candida albicans* where the leakage of constituents from the pathogenic cell membranes was noticed by Shahina *et al*., 2018.

The antagonistic effect of antifungal compound, IVS320 on the membrane of *Candida albicans* was evaluated by Ferriera *et al*., 2016, where the release of cellular materials was quantified which has proved the damage of pathogenic cell membranes.

Knauth and Reichenbach, 2016 have analysed the lipid content in pathogenic fungi treated with ambruticin where the decreased level of lipid content is observed in the pathogen treated with antifungal compounds.

The cell membrane permeability assay has revealed that both the pathogenic organisms were found to be more susceptible to the antifungal compounds present in the purified fractions of *Aspergillus giganteus*. Hence, the antifungal compounds reported in the scientific studies have taken to study its potential targeting activity against pathogenic proteins using *in silico* approaches.

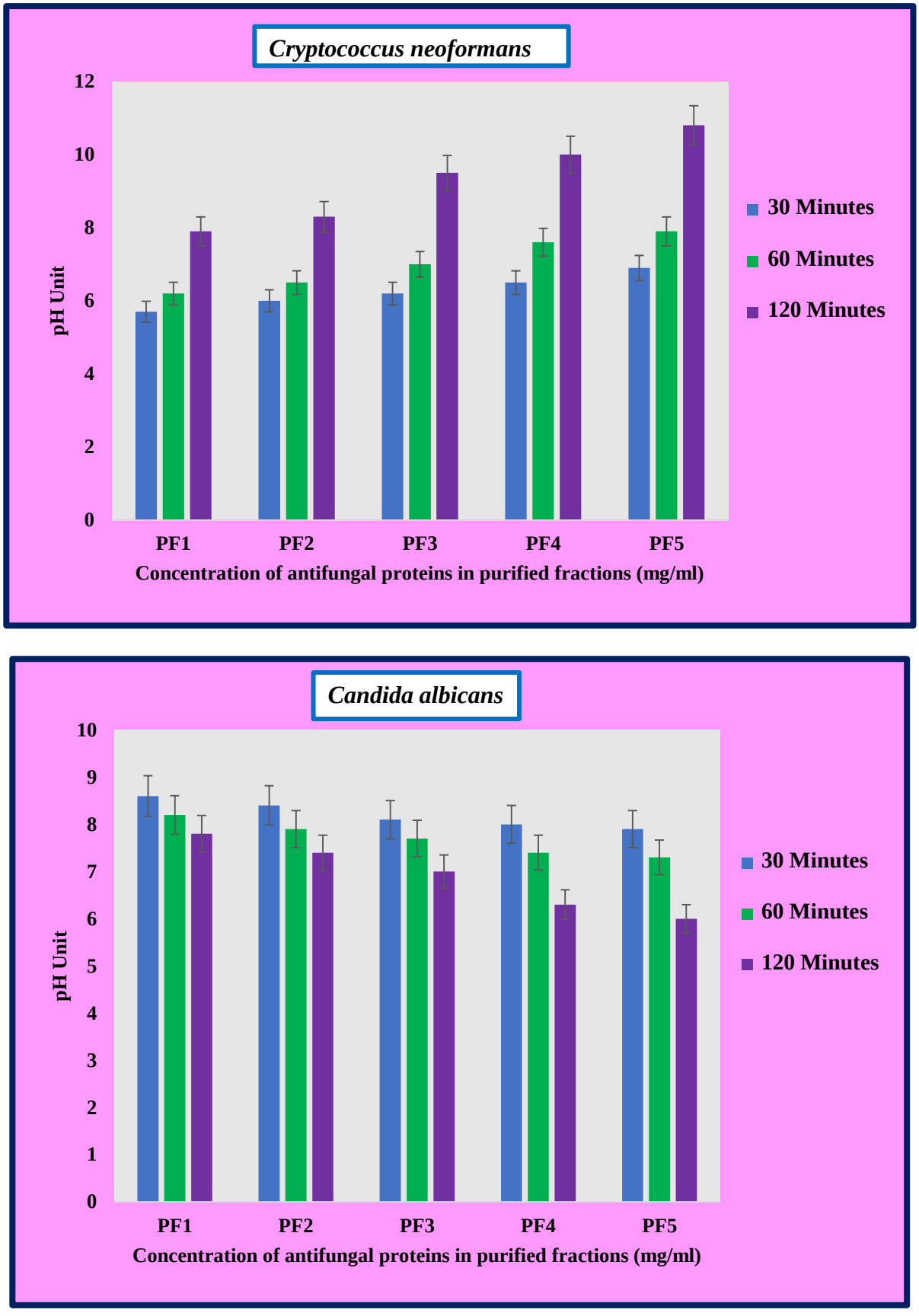


Fig. 11: Action of AFCs from *Aspergillus giganteus* on the permeabilization of ions and other solute molecules in the pathogenic cell membrane

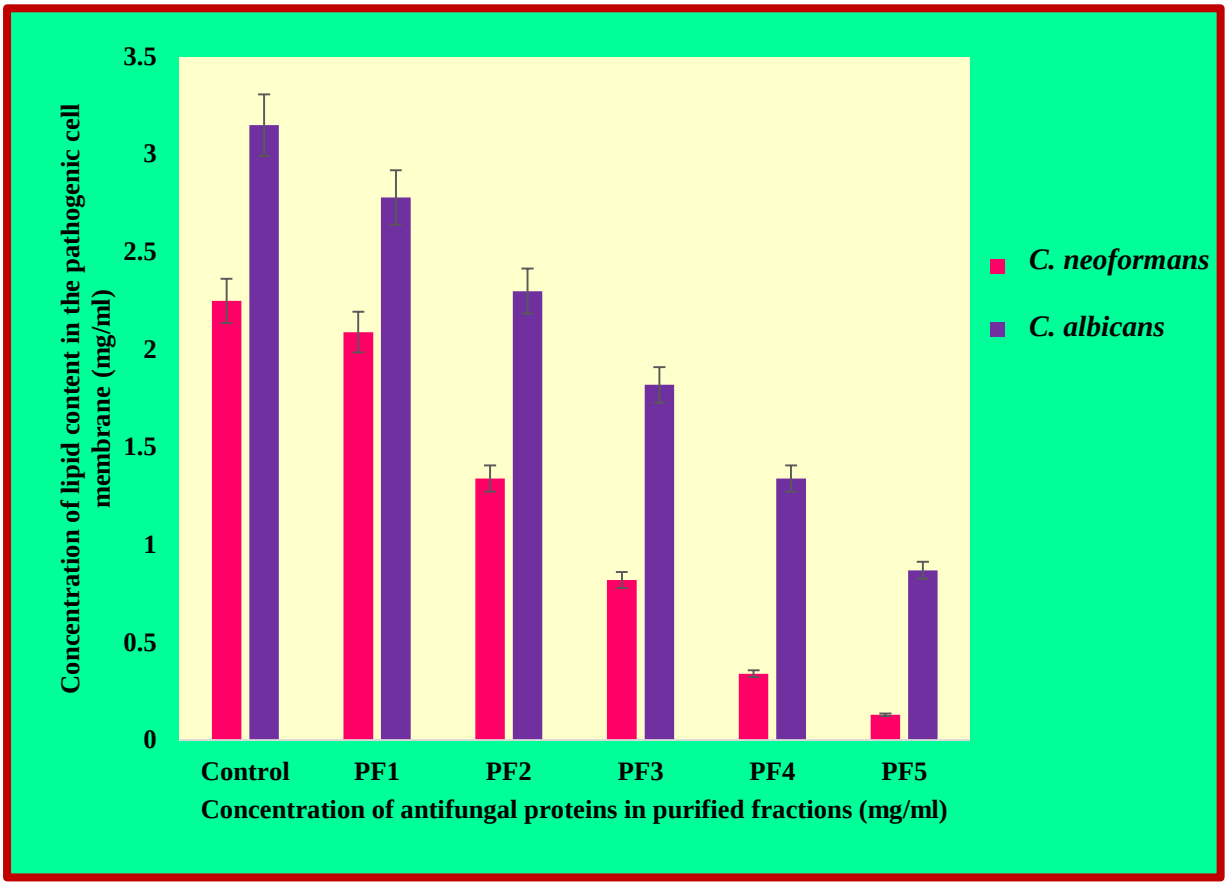


Fig. 12: Lipid contents in the AFCs treated pathogenic *C. neoformans* and *C. albicans* cell membrane

4.7. *In silico* approaches

In traditional method, identification of lead compound is the first and foremost difficult task for the development of drug using *in vitro* and *in vivo* analysis. *In silico* approaches have been constructed as an alternative method to mitigate the various steps and predict the compounds at system level. The pathogenic targets like macromolecules such as DNA, RNA and protein are considered as the primary target for the lead compounds (Srivastava and Neelabh, 2020; Chikhale, 2020). In the present study, the selected antifungal compounds have been docked with the target proteins of pathogenic strains in order to check the drug effectiveness towards the pathogenic attack. The ADME properties has predicted for the drug likeness.

4.7.1. Ligand preparation

The antifungal compounds present in *Aspergillus giganteus* was selected based on several scientific literatures. The structure of the selected compounds and standard drugs were drawn using Maestro window. The structure and details of the selected ligands is given in Table 3 A and B.

4.7.2. ADME prediction

The computational predictions of pharmacokinetic and pharmacodynamic properties of selected drug compounds namely, absorption, distribution, metabolism, excretion (ADME) and also toxicity risk (tox) level is evaluated. This prediction attains a great importance in the discovery and development of drug process. Characterization of compounds for ADME/tox property is predominant for the successful discovery of drug and it is possible to eliminate the compound that does not have an acceptable pharmacokinetic and pharmocodynamic properties. This study provides an opportunity to perform the *in vivo* studies by predicting its ADME and toxicological properties. Physically significant descriptors of pharmaceutically relevant properties of all the ligands were predicted and listed in table 4.

Table 3 A: Ligand structures with their molecular formula and molecular weight

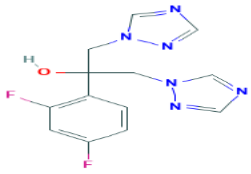
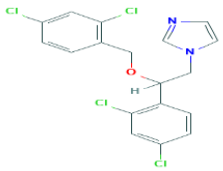
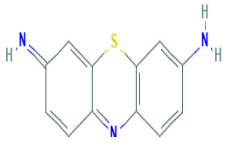
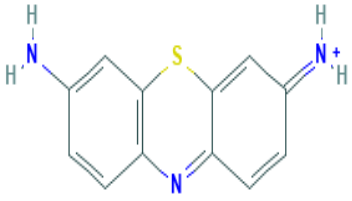
Ligands	Structure	Molecular weight	Molecular formula	PubChem ID
STANDARD DRUG 1		344.842	$C_{22}H_{17}CLN_2$	2812
STANDARD DRUG 2		306.277	$C_{13}H_{12}F_2N_6O$	3365
STANDARD DRUG 3		416.123	$C_{18}H_{14}CL_4N_2O$	4189
COMPOUND 1		181.217	$C_6H_7N_5S$	3032391
COMPOUND 2		227.285	$C_{12}H_9N_3S$	65044
COMPOUND 3		228.293	$C_{12}H_{10}N_3S^+$	462371

Table 3 B: Ligand structures with their molecular formula and molecular weight

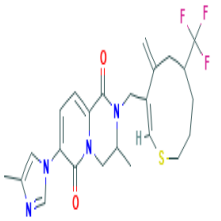
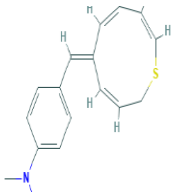
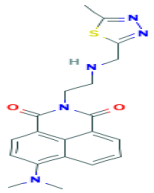
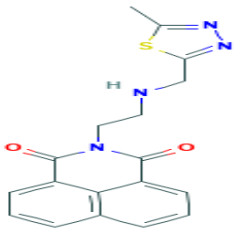
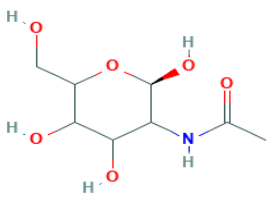
Ligands	Structure	Molecular weight	Molecular formula	PubChem ID
COMPOUND 4		492.561	$C_{24}H_{27}F_3N_4OS$	122678533
COMPOUND 5		269.406	$C_{17}H_{19}NS$	88094842
COMPOUND 6		395.481	$C_{20}H_{21}N_5O_2S$	86223064
COMPOUND 7		352.412	$C_{18}H_{16}N_4O_2S$	86223063
COMPOUND 8		221.209	$C_8H1_5NO_6$	6857375

Table 4: ADME predictions for the selected compounds

Ligand Name	PubChem ID	Molecular Weight (KDa) (130.0/725.0)	H-bond Donor (0.0/6.0)	H-bond Acceptor (2.0/20.0)	Human oral Absorption
Standard Drug 1	2812	344.843	0.000	1.500	High
Standard Drug 2	3365	306.274	1.000	6.750	High
Standard Drug 3	4189	416.133	6.000	3.700	High
Compound 1	3032391	181.215	3.000	5.000	High
Compound 2	65044	227.283	2.500	3.000	High
Compound 3	462371	227.283	2.500	3.000	High
Compound 4	122675833	492.558	0.000	8.000	Low
Compound 5	88094842	269.404	0.000	1.500	High
Compound 6	86223064	395.478	1.000	7.500	High
Compound 7	806223063	352.410	1.000	6.500	High
Compound 8	6857375	221.210	5.833	6.083	Medium

Lipinski's rule of 5 has been used to predict the drug likeness and non drug likeness and it helps to predict the high frequency of failure or success of the drug molecule. The rule describes important molecular properties to predict the pharmacokinetics in the human body along with ADME. This rule is based on following two or more phenomena such as molecular mass less than 500 g/mol, high lipophilicity can be represented as Log Po/w $\leq$ 5, less than 5 hydrogen bond donors, $\leq$10 hydrogen bond acceptors and molar refractivity is ranging from 40 to 130. These are the important properties that need great attention and it helps to avoid expensive late stage preclinical and clinical failures. In this study, the antifungal compounds showed drug like characteristics based on the Lipinski's rule-of-five. The human oral absorption of compound

4 was low. Among all the compounds selected, compound 8 was moderate and the compounds 1, 2, 3, 5, 6, 7, 9, 10, 11 were found to be highly absorbed.

ADME predictions are being an important property for the successful drug discovery and several scientific studies supports the method for pharmacokinetic prediction.

In silico prediction of toxicity performed in imidazole derivatives and the results showed that the compounds 1, 2 and 3 have no risk of mutagenicity, tumerogenicity, irritation or reproduction (Bouchal *et al*., 2019).

Altındag *et al*., 2019 have performed the ADME/tox prediction of imidazole derivatives and it revealed that the compounds have found to be safe for the drug formulations.

James *et al*., 2018 has worked with ALK inhibitors and pharmacophore hit molecules. The properties, such as QPlogKp, QPlogPw,QPlogPoct, QPlogS,QPlog BB were found in the acceptable range.

4.7.3. Preparation of target proteins

Three dimensional structures of the selected target compounds was downloaded from the PDB databases. The proteins were prepared in the maestro following various steps, including, preprocess, analyze workspace, generate states, refinement, optimization and minimization. The target proteins selected for the studies have listed in table 5.

4.7.4. Molecular docking studies

Molecular docking studies were performed to gain more information about the binding efficiency of the compounds to the active site of pathogenic target proteins. In the present study, the selected compounds from *Aspergillus giganteus* and standard drug for the treatment of fungal infections were docked with the target proteins mentioned in table 5. Each compound has specific interaction and activity towards the pathogenic target proteins and the results are discussed below.

Table 5: List of selected pathogenic target proteins

S.No	Pathogenic organisms	Target proteins	PDB ID
1.	*Cryptococcus neoformans*	Farnesyl transferase	3SFX
		Ionisine 5′-monophosphate dehydrogenase	4AF0
		Ribokinase	6CW5
		Serine/Threonine-protein phosphate 2B catalytic subunit A1	6TZ8
		Choline kinase	6WHP
2.	*Candida albicans*	Dihydrofolate reductase	1AI9
		Myristoyl-CoA: protein N-Myristoyl transferases	1IYL
		Secreted aspartyl proteases	1ZAP
		Lanosterol 14-α demethylase	5V5Z

4.7.4.1. Molecular docking of antifungal compounds with target proteins of *Cryptococcus neoformans*

The antifungal compounds and selected drug molecules was docked with the target proteins of *Cryptococcus neoformans* and the results are listed in Table 6, 7, 8, 9 and 10. The table contains all the necessary information about the docking of compounds and target proteins which include, docking score, glide energy, number of hydrogen bonds and interacting residues. The docked image and ligand interaction diagrams of best docked compounds with the each target proteins of *Cryptococcus neoformans* is given in Fig. 13, 14, 15, 16 and 17.

The compound 1 has a good docking score of -5.796 for the target protein 3SFX and -4.081 for 6WHP. For the target proteins 4AF0, compound 7 was found to have a good docking score and docking energy and the values are -8.534 and -56.004, respectively. Compounds 2, 3 and 8 were observed to have a great interacting and binding efficiency towards all the selected target proteins of *Cryptococcus neoformans*. Compound8 was more efficient in making many

hydrogen bonds with the target proteins. The standard drug 2 and 3 formed the great interactions with the target proteins.

Table 6: Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Cryptococcus neoformans* target protein 3SFX

Compounds name	PubChem ID	Docking score	Glide energy	Number of H-bonds	Distance	Interacting residues
Standard Drug 1	2812	-2.273	-22.102	-	-	-
Standard Drug 2	**3365**	**-3.447**	**-28.807**	**2**	**2.23, 1.79**	**GLN64, LEU61**
Standard Drug 3	4189	-2.815	-32.263	1	2.24	ASP491
Compound 1	**3032391**	**-5.796**	**-24.398**	**3**	**1.74, 2.04, 1.99**	**ASP491, ASN492, LEU61**
Compound 2	65044	-4.701	-29.356	2	1.97, 2.03	ASP491, ASN492
Compound 3	462371	-4.701	-29.356	2	1.97, 2.03	ASP491, ASN492
Compound 4	122678533	-2.582	-30.19	0	-	-
Compound 5	88094842	-1.878	-26.591	0	-	-
Compound 6	86223064	-4.185	-40.095	2	2.17, 1.93	ASP491, TYR58
Compound 7	86223063	-2.728	-33.636	0	-	-
Compound 8	**6857375**	**-4.888**	**-26.88**	**3**	**1.99, 1.83, 1.73**	**ASP491, TYR58, LEU61**

Table 7: Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Cryptococcus neoformans* target protein 4AF0

Compounds name	PubChem ID	Docking score	Glide energy	Number of H-bonds	Distance	Interacting residues
Standard Drug 1	2812	-5.321	-20.525	0	-	-
Standard Drug 2	**3365**	**-8.318**	**-54.43**	**5**	**2.34, 2.23, 2.24**	**GLN470, GLY340, SER290**
Standard Drug 3	4189	-5.752	-25.668	0	-	-
Compound 1	3032391	-7.573	-42.325	2	2.10, 2.09	GLY429, MET428
Compound 2	65044	-7.679	-36.815	1	2.15	ASP378
Compound 3	462371	-7.679	-36.815	1	2.15	ASP378
Compound 4	No interactions					
Compound 5	88094842	-6.585	-33.285	0	-	-
Compound 6	86223064	-7.463	-51.823	2	1.90	SER343
Compound 7	**86223063**	**-8.534**	**-56.004**	**1**	**2.28**	**GLY340**
Compound 8	**6857375**	**-7.757**	**-40.177**	**4**	**2.05, 1.57, 2.77, 2.25**	**SER86, ASP378, MET428, GLY429**

Table 8: Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Cryptococcus neoformans* target protein 6CW5

Compounds name	PDB ID	Docking score	Glide energy	Number of H-bonds	Distance	Interacting residues
Standard Drug 1	2812	-4.473	-34.348	1	1.48	GLU190
Standard Drug 2	3365	-5.439	-40	3	2.73, 2.21, 2.37	ASN187, GLY270, ASP273
Standard Drug 3	**4189**	**-5.907**	**-42.47**	**0**	**-**	**-**
Compound 1	3032391	-6.038	-28.158	4	2.73, 1.79, 1.92, 2.40	GLY270, SER165, ASN187, GLU190
Compound 2	65044	-6.669	-32.63	1	1.93	ALA259
Compound 3	462371	-6.669	-32.63	1	1.93	ALA259
Compound 4	**122678533**	**-6.722**	**-44.179**	**0**	**-**	**-**
Compound 5	88094842	-6.049	-30.73	-	-	-
Compound 6	86223064	-6.62	-51.462	2	2.27, 2.57	GLY270, GLY272
Compound 7	86223063	-6.118	-46.283	2	2.74, 2.51	GLY272, LYS43
Compound 8	**6857375**	**-5.493**	**-32.783**	**5**	**2.30, 2.19, 2.01, 1.99, 1.95**	**LYS43, ASP273, ASN163, ASN187, THR238**

Table 9: Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Cryptococcus neoformans* target protein 6TZ8

Compounds name	PDB ID	Docking score	Glide energy	Number of H-bonds	Distance	Interacting residues
Standard Drug 1	2812	-5.272	-26.153	0	-	-
Standard Drug 2	**3365**	**-5.435**	**-25.168**	**1**	**1.92**	**ASP38**
Standard Drug 3	4189	-4.634	-34.076	0	-	-
Compound 1	3032391	-5.725	-29.587	1	2.11	TYR83
Compound 2	65044	-7.46	-28.218	1	2.12	TYR83
Compound 3	**462371**	**-7.46**	**-28.218**	**1**	**2.12**	**TYR83**
Compound 4	122678533	-4.192	-32.102	1	2.33	ILE57
Compound 5	88094842	-5.351	-34.707	0	-	-
Compound 6	86223064	-4.674	-31.538	-	-	-
Compound 7	86223063	-6.014	-35.975	1	1.92	ASP38
Compound 8	**6857375**	**-5.125**	**-48.421**	**3**	**2.23, 2.05, 2.01**	**ILE57, TYR83, ASP38**

Table 10: Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Cryptococcus neoformans* target protein 6WHP

Compounds name	PDB ID	Docking score	Glide energy	Number of H-bonds	Distance	Interacting residues
Standard Drug 1	No interactions					
Standard Drug 2	3365	-2.776	-21.927	3	2.47	ARG442
Standard Drug 3	**4189**	**-2.983**	**-28.963**	**1**	**1.53**	**ASP444**
Compound 1	**3032391**	**-4.081**	**-16.925**	**1**	**1.84**	**GLY440**
Compound 2	65044	-3.944	-20.86	1	1.64	VAL439
Compound 3	462371	-3.944	-20.86	1	1.64	VAL439
Compound 4	No interactions					
Compound 5						
Compound 6	86223064	-1.212	-25.487	2	2.32, 2.53	ARG442, ARG253
Compound 7	No interactions					
Compound 8	**6857375**	**-3.807**	**-21.223**	**4**	**1.87, 1.90, 1.99**	**GLY440, VAL439, ARG442**

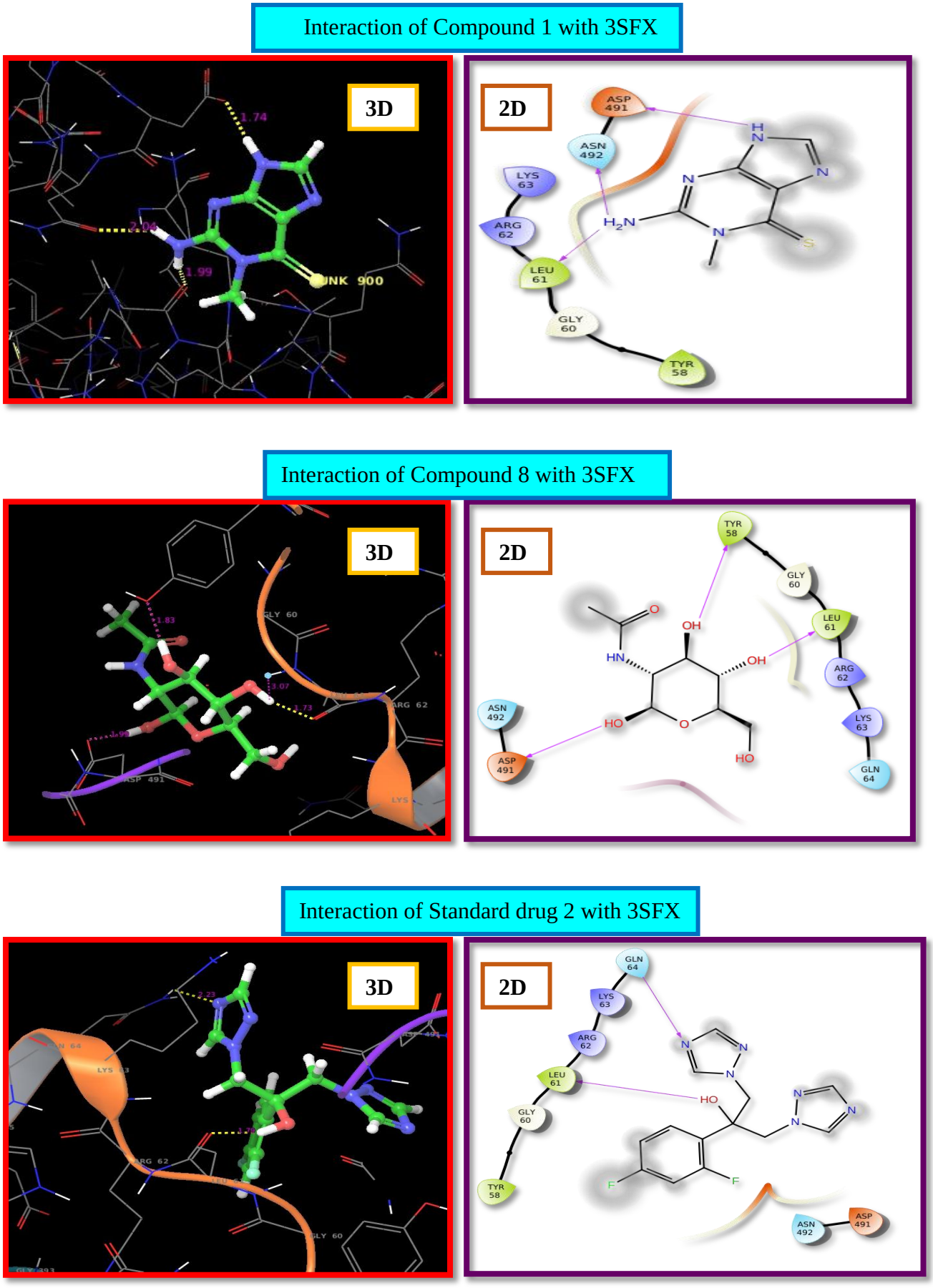
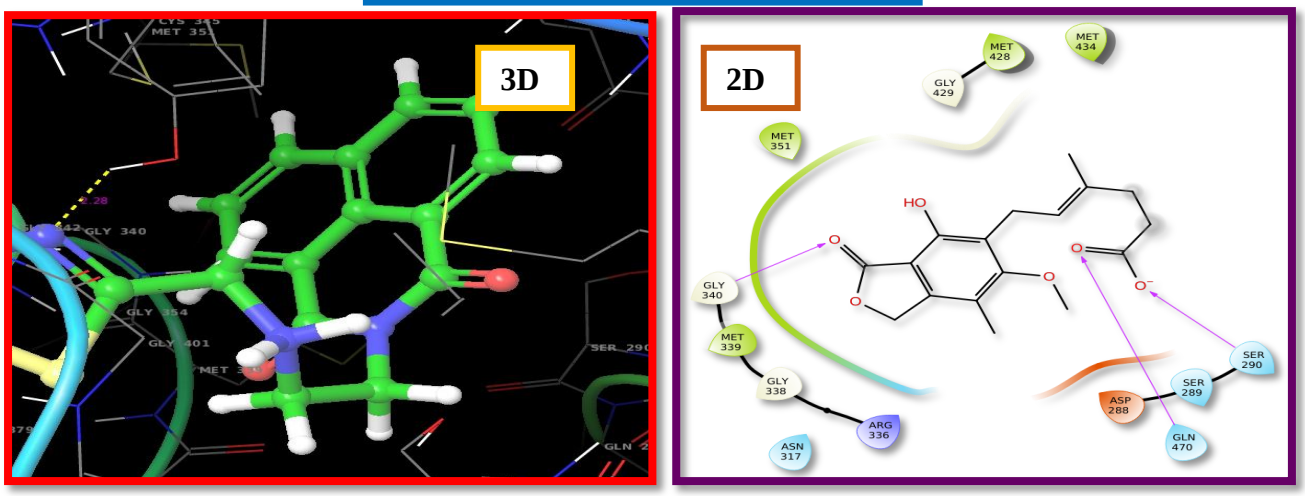
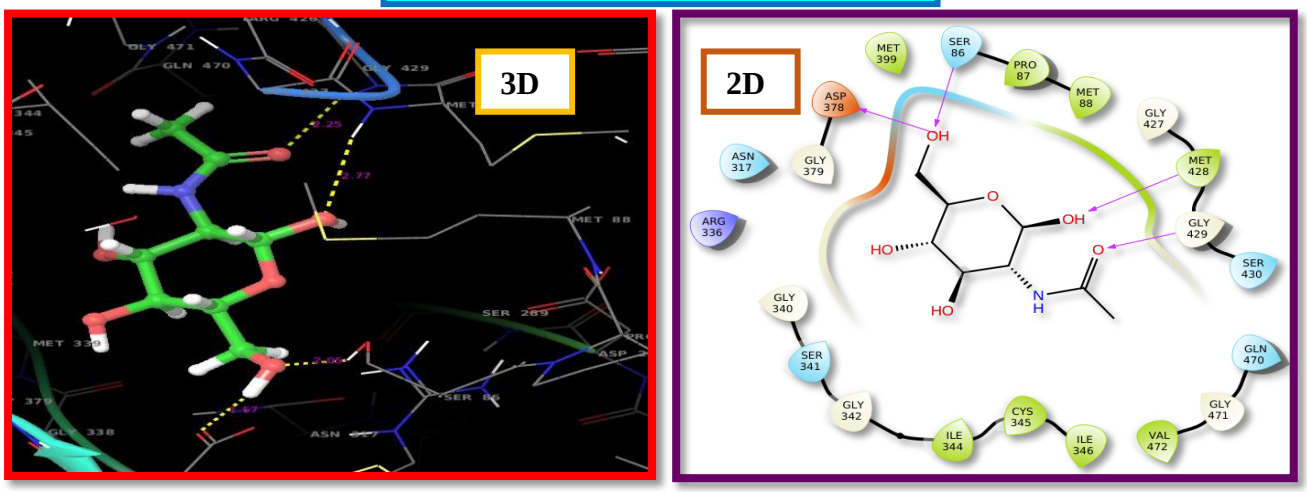


Fig 13: Ligand-protein interaction of antifungal compounds docked with the target protein 3SFX showing the 3D and 2D images

Interaction of Compound 7 with 4AF0



Interaction of Compound 8 with 4AF0



Interaction of Standard drug 2 with 4AF0

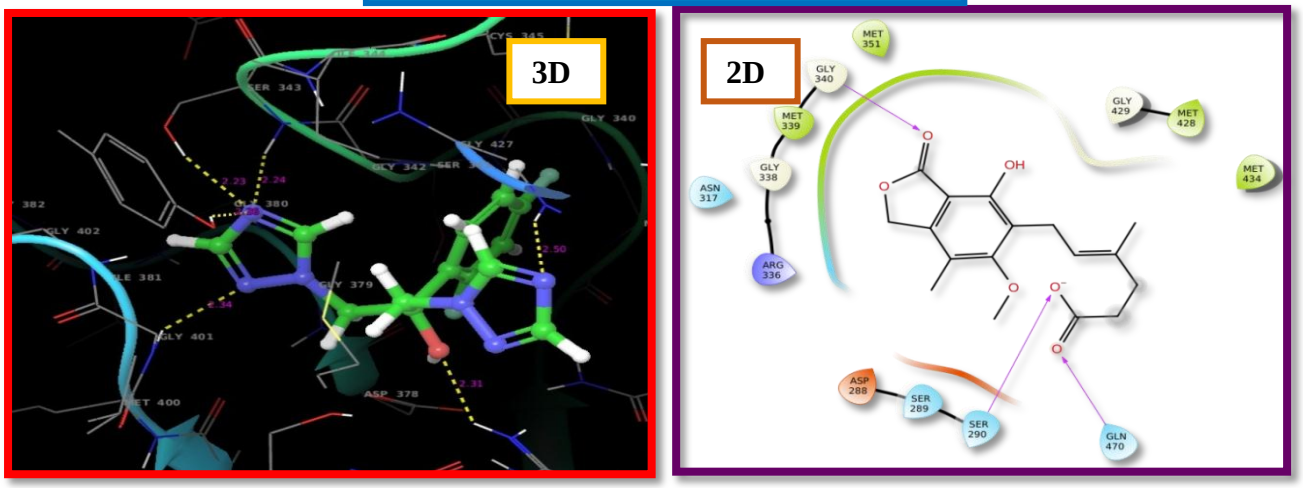
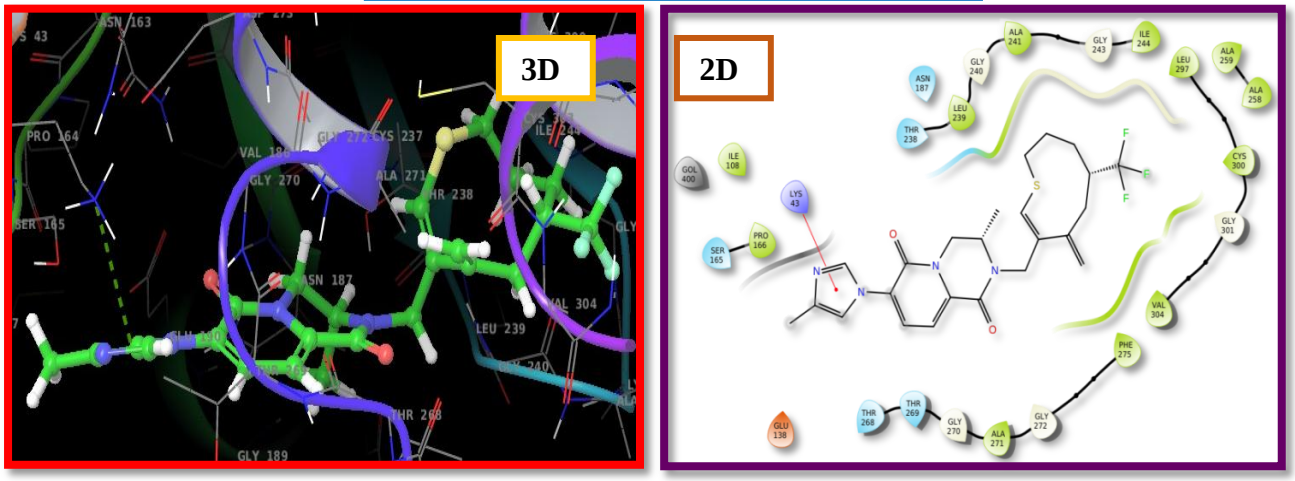
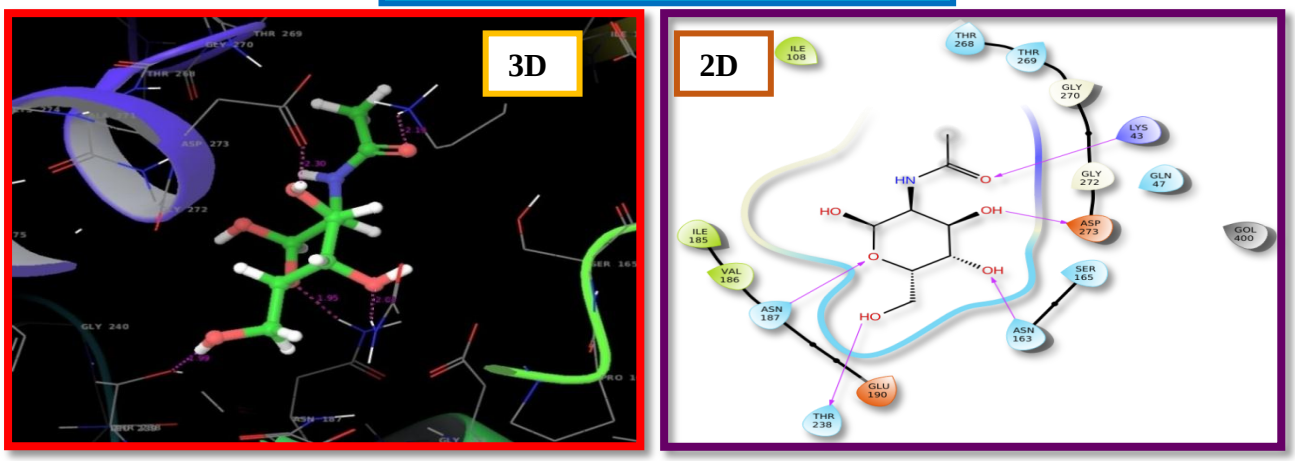


Fig 14: Ligand-protein interaction of antifungal compounds docked with the target protein 4AF0 showing the 3D and 4D images

Interaction of Compound 3 with 6CW5



Interaction of Compound 8 with 6CW5



Interaction of Standard drug 3 with 6CW5

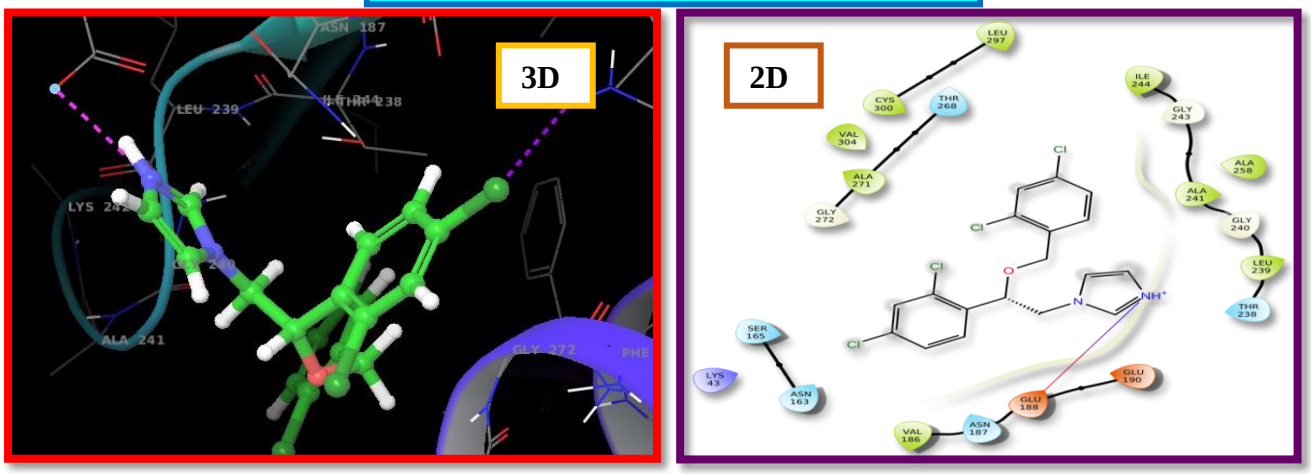


Fig 15: Ligand-protein interaction of antifungal compounds docked with the target protein 6CW5 showing the 3D and 2D images

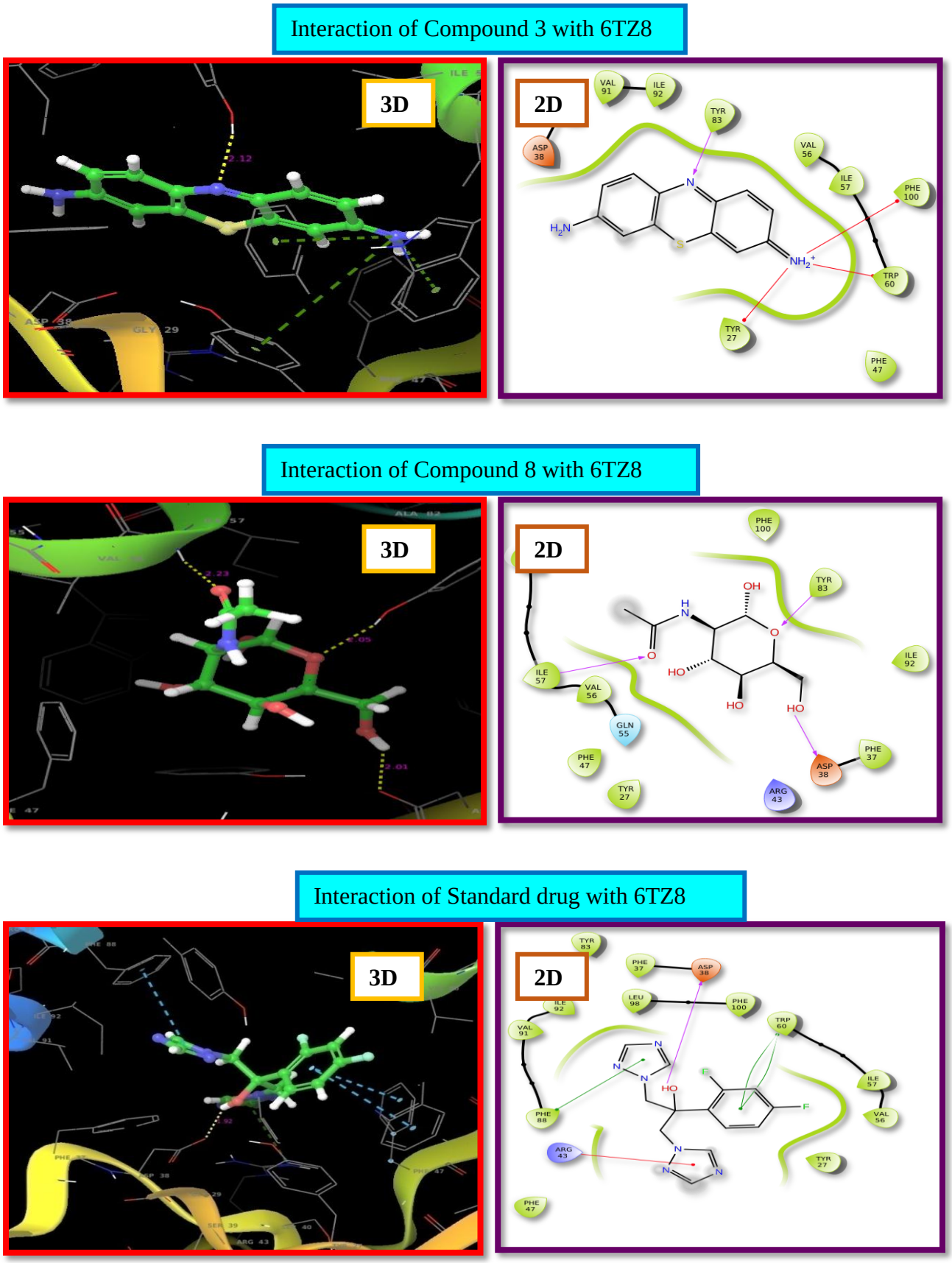


Fig 16: Ligand-protein interaction of antifungal compounds docked with the target protein 6TZ8 showing the 3D and 2D images

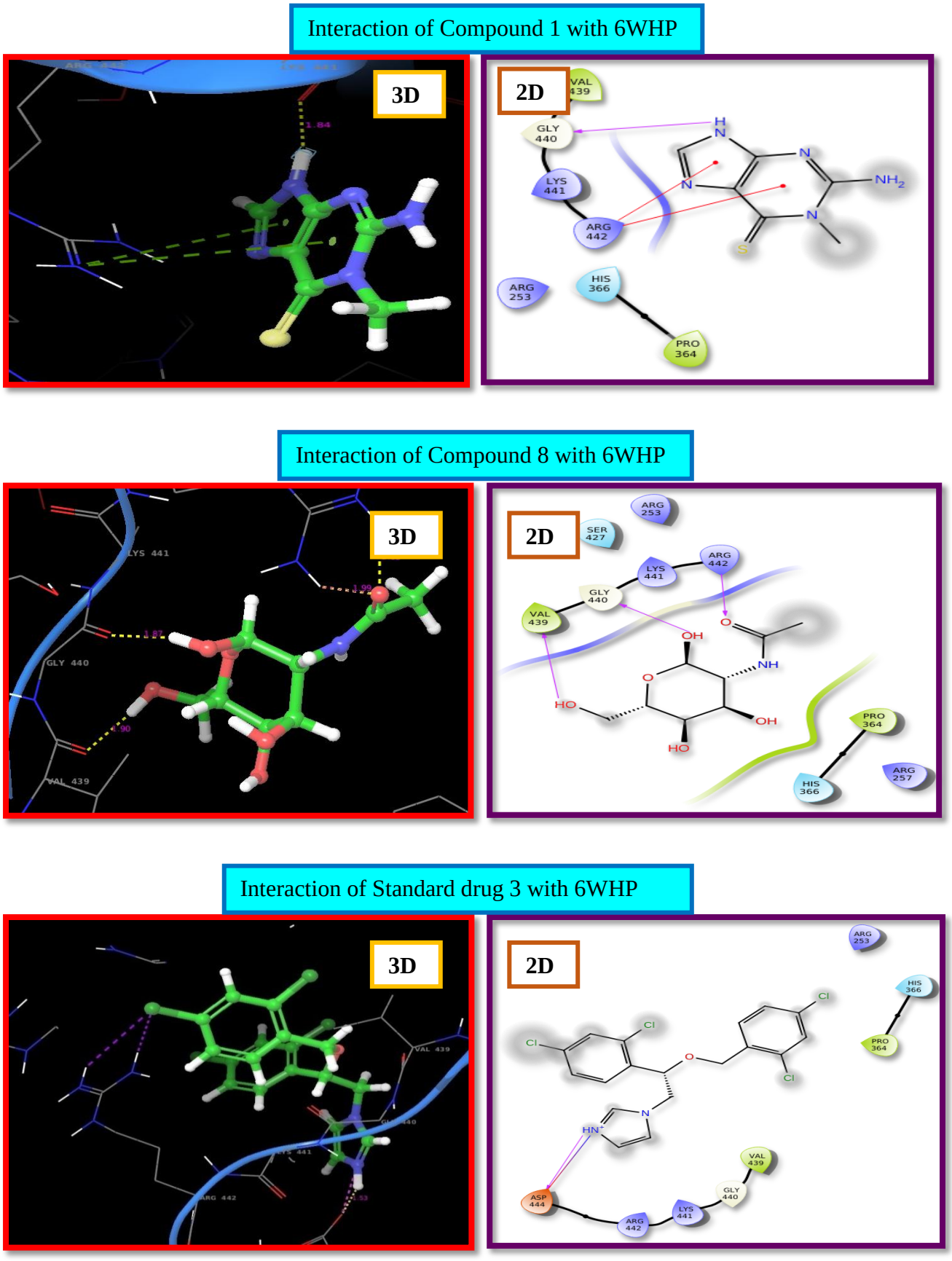


Fig 17: Ligand-protein interaction of antifungal compounds docked with the target protein 6WHP showing the 3D and 2D images

4.7.4.2. Molecular docking of antifungal compounds with target proteins of *Candida albicans*

The antifungal compounds and the selected drug molecules were docked with the target proteins such as 1AI9, 1IYL, 1ZAP and 5V5Z. The docking results including, docking score, glide energy, interacting residues and number of hydrogen bonds are tabulated in Table 11, 12, 13 and 14. The best docking image and ligand interaction diagrams are given in Fig. 18, 19, 20 and 21.

The compounds have a unique interaction and binding with the target proteins of *Candida albicans*. Compound 8 was found to have good and best hydrogen bonds with all the selected target proteins. All the compounds selected for the study has given the good interaction and binding activity to the active site of the target protein namely, Lanosterol 14-α demethylase (5V5Z). Apart from the compounds, standard drug 3 has the best docking score with the selected targets.

Several scientific evidences support the molecular docking studies for the drug development and drug discovery.

Castillo *et al*., 2020 have performed the molecular docking studies for the derivatives of cinnamic and benzoic acids. The results showed that the compound 7 was predicted to bind to ST-PK in its ATP binding grove and making contact mainly with L436, V444, A457, K459, E474, I491, F507, I509, L510, L561, I573, and D574.

Ndaba *et al*., 2020 have done the molecular docking analysis of the *Drimia delagoensis* plant against the target protein of *Aspergillus fumigatus*. Compounds 1 to 9 showed a significant docking scores ranging between -1.313 and -4.431.

Sari and Kart, 2020 have evaluated the molecular interaction studies of two azole derivatives with one indole ring namly, 4a, 4b. In this context, both the compounds have the similar docking scores with the target protein 5TZ1.

Therefore the *in silico* studies have proved the results obtained in the *in vitro* analysis. The antagonistic activity of *Aspergillus giganteus* against the pathogenic *Cryptococcus neoformans* and *Candida albicans* was confirmed by analyzing its mode of action on the cell membrane.

Table 11: Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Candida albicans* target protein 1AI9

Compounds name	PDB ID	Docking score	Docking energy	Number of H-bonds	Distance	Interacting residues
Standard Drug 1	2812	-3.866	-32.987	1	2.15	GLU116
Standard Drug 2	3365	-5.98	-38.104	1	2.23	ALA115
Standard Drug 3	**4189**	**-6.294**	**-46.992**	**1**	**1.81**	**SER61**
Compound 1	**3032391**	**-7.184**	**-30.634**	**3**	**1.71, 2.11, 2.15**	**ILE19, THR58, ILE112**
Compound 2	65044	-6.791	-38.062	2	1.98, 1.82	SER61, GLY23
Compound 3	462371	-6.791	-38.062	2	1.98, 1.82	SER61, GLY23
Compound 4	122678533	-6.62	-49.8	0	-	-
Compound 5	88094842	-6.583	-34.345	0	-	-
Compound 6	86223064	-7.043	-50.097	2	2.47, 2.64	GLY23, ALA115
Compound 7	86223063	-6.622	-48.446	2	2.47, 2.72	ILE19, ALA11
Compound 8	**6857375**	**-6.33**	**-33.655**	**6**	**2.00, 2.30, 1.52**	**LYS57, LEU77, ARG56**

Table 12: Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Candida albicans* target protein 1IYL

Compounds name	PDB ID	Docking score	Docking energy	Number of H-bonds	Distance	Interacting residues
Standard Drug 1	2812	-4.625	-36.462	0	-	-
Standard Drug 2	3365	-6.464	-41.602	1	2.04	TYR107
Standard Drug 3	**4189**	**-7.296**	**-47.724**	**1**	**1.88**	**LEU451**
Compound 1	3032391	-6.381	-35.765	1	2.05	LEU451
Compound 2	65044	-7.834	-38.873	2	2.09, 1.56	LEU450, LEU451
Compound 3	462371	-7.834	-38.873	2	2.09, 1.56	LEU450, LEU451
Compound 4	122678533	-7.042	-44.882	1	2.07	TYR107
Compound 5	88094842	-6.657	-27.556	0	-	-
Compound 6	**86223064**	**-8.427**	**-54.256**	**0**	**-**	**-**
Compound 7	86223063	-8.095	-48.711	2	2.62, 2.01	TYR225, HIE227
Compound 8	**6857375**	**-6.371**	**-35.502**	**5**	**2.11, 1.98, 1.75, 1.74 2.11**	**TYR107, TYR119, LEU451, TYR335**

Table 13: Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Candida albicans* target protein 1ZAP

Compounds name	PDB ID	Docking score	Docking energy	Number of H-bonds	Distance	Interacting residues
Standard Drug 1	2812	-3.399	-32.337	0	-	-
Standard Drug 2	3365	-4.862	-40.434	0	-	-
Standard Drug 3	**4189**	**-5.39**	**-42.488**	**0**	**-**	**-**
Compound 1	3032391	-5.729	-29.739	1	2.13	GLY34
Compound 2	65044	-6.842	-37.931	2	1.59, 2.06	ASP218, ASP32
Compound 3	**462371**	**-6.842**	**-37.931**	**2**	**1.59, 2.06**	**ASP218, ASP32**
Compound 4	122678533	-4.516	-50.396	1	2.56	GLY85
Compound 5	88094842	-4.616	-32.04	0	-	-
Compound 6	86223064	-5.807	-48.266	2	2.02, 1.69	ASP218, ASP32
Compound 7	86223063	-5.908	-45.423	2	1.74, 2.08	ASP32, ASP218
Compound 8	**6857375**	**-5.409**	**-33.306**	**4**	**1.95, 1.51, 1.79, 2.54**	**ASP32, ASP218, GLY220**

Table 14: Docking analysis of antifungal compounds of *Aspergillus giganteus* against *Candida albicans* target protein 5V5Z

Compounds name	PDB ID	Docking score	Docking energy	Number of H-bonds	Distance	Interacting residues
Standard Drug 1	2812	-5.883	-35.433	0	-	-
Standard Drug 2	3365	-6.221	-38.177	1	2.02	HIE377
Standard Drug 3	**4189**	**-7.766**	**-42.857**	**1**	**2.15**	**HIE377**
Compound 1	3032391	-7.063	-32.387	2	1.70, 2.02	SER507, TYR505
Compound 2	65044	-7.355	-40.299	1	1.70	TYR505
Compound 3	462371	-7.355	-40.299	1	1.70	TYR505
Compound 4	**122678533**	**-9.183**	**-53.099**	**0**	**-**	**-**
Compound 5	88094842	-4.738	-31.378	0	-	-
Compound 6	86223064	-8.256	-52.361	2	2.00, 1.93	HIE377, MET508
Compound 7	86223063	-7.888	-49.231	2	1.87, 2.15	HIE377, SER378
Compound 8	**6857375**	**-7.995**	**-36.374**	**5**	**1.80, 1.69, 2.42, 2.16**	**TYR505, MET508, HIE377, SER378**

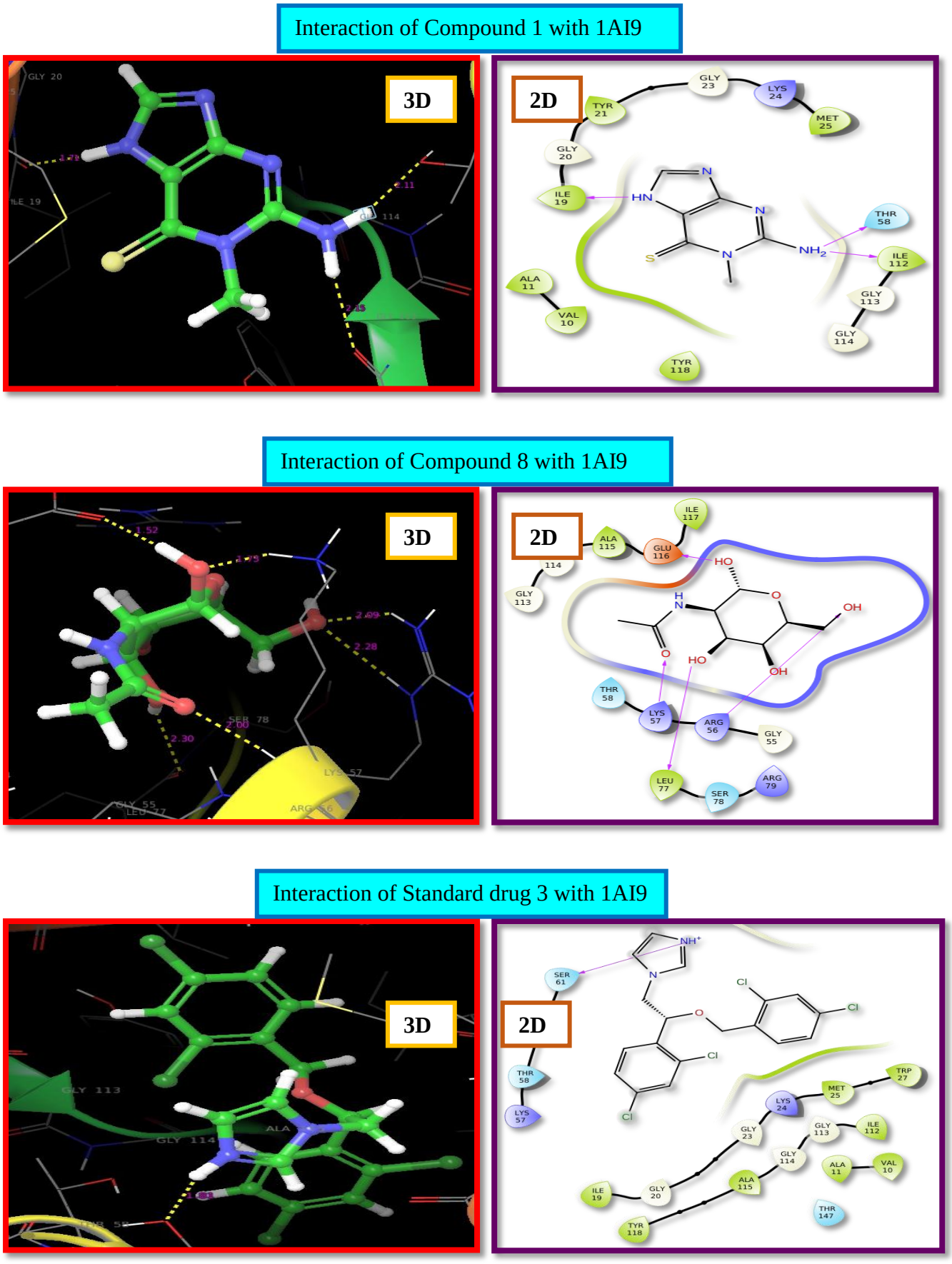
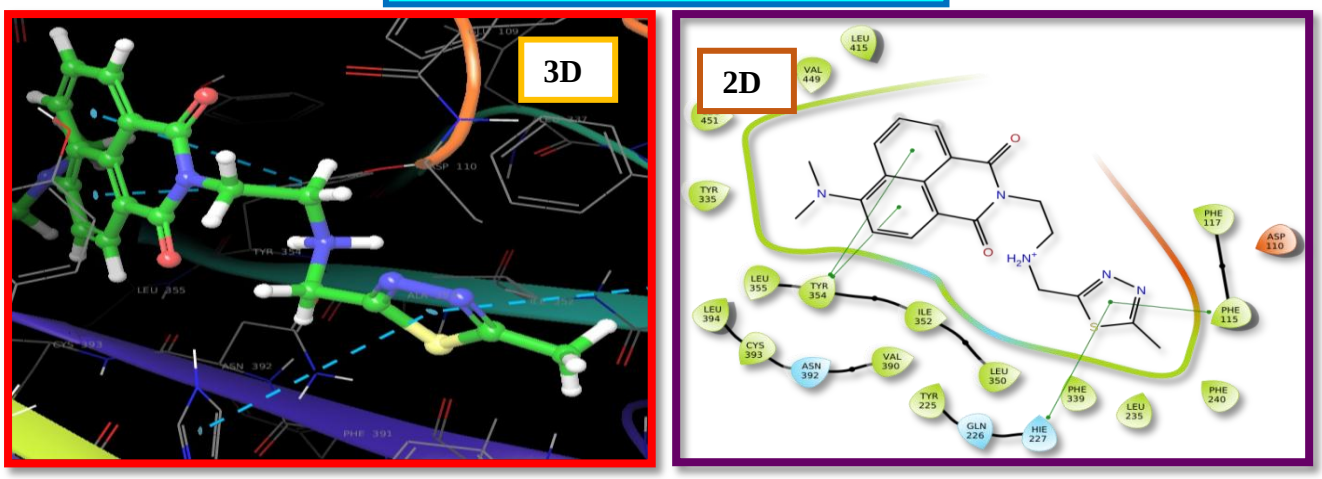
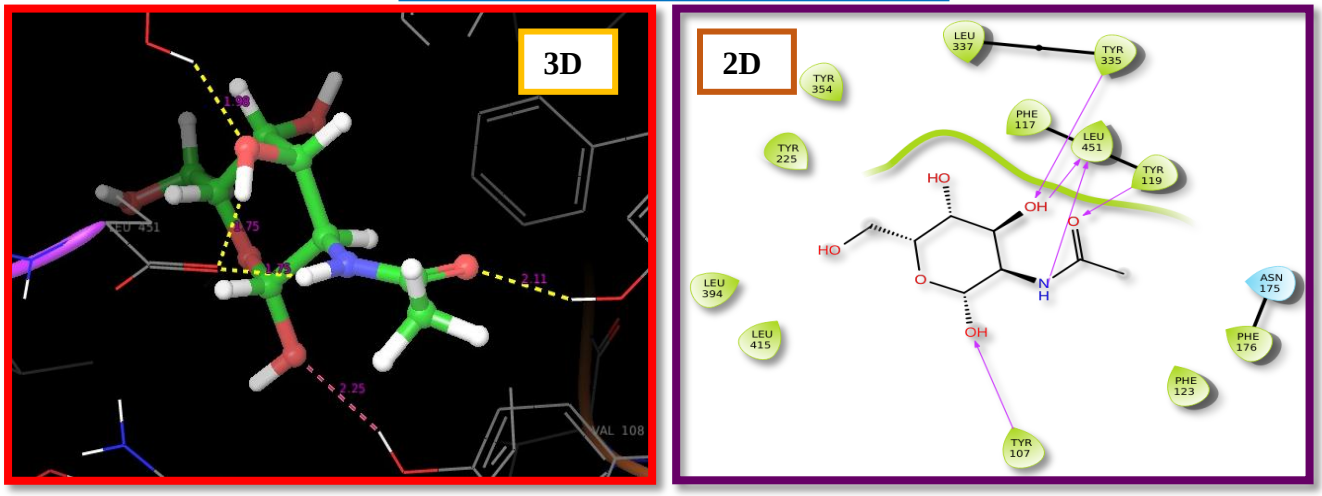


Fig 18: Ligand-protein interaction of antifungal compounds docked with the target protein 1AI9 showing the 3D and 2D images

Interaction of Compound 6 with 1IYL



Interaction of Compound 8 with 1IYL



Interaction of Standard drug 3 with 1IYL

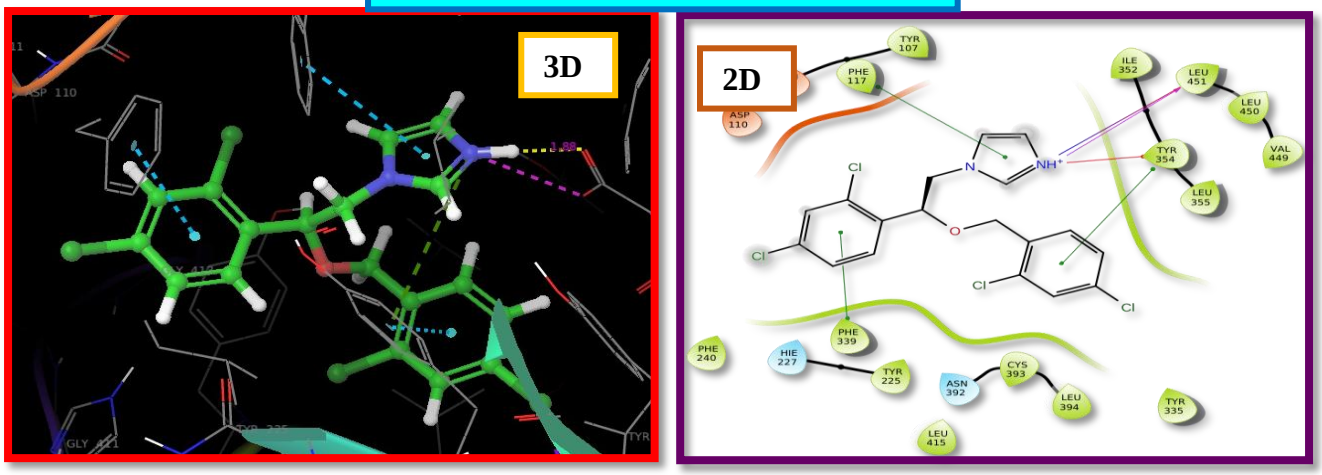


Fig 19: Ligand-protein interaction of antifungal compounds docked with the target protein 1IYL showing the 3D and 2D images

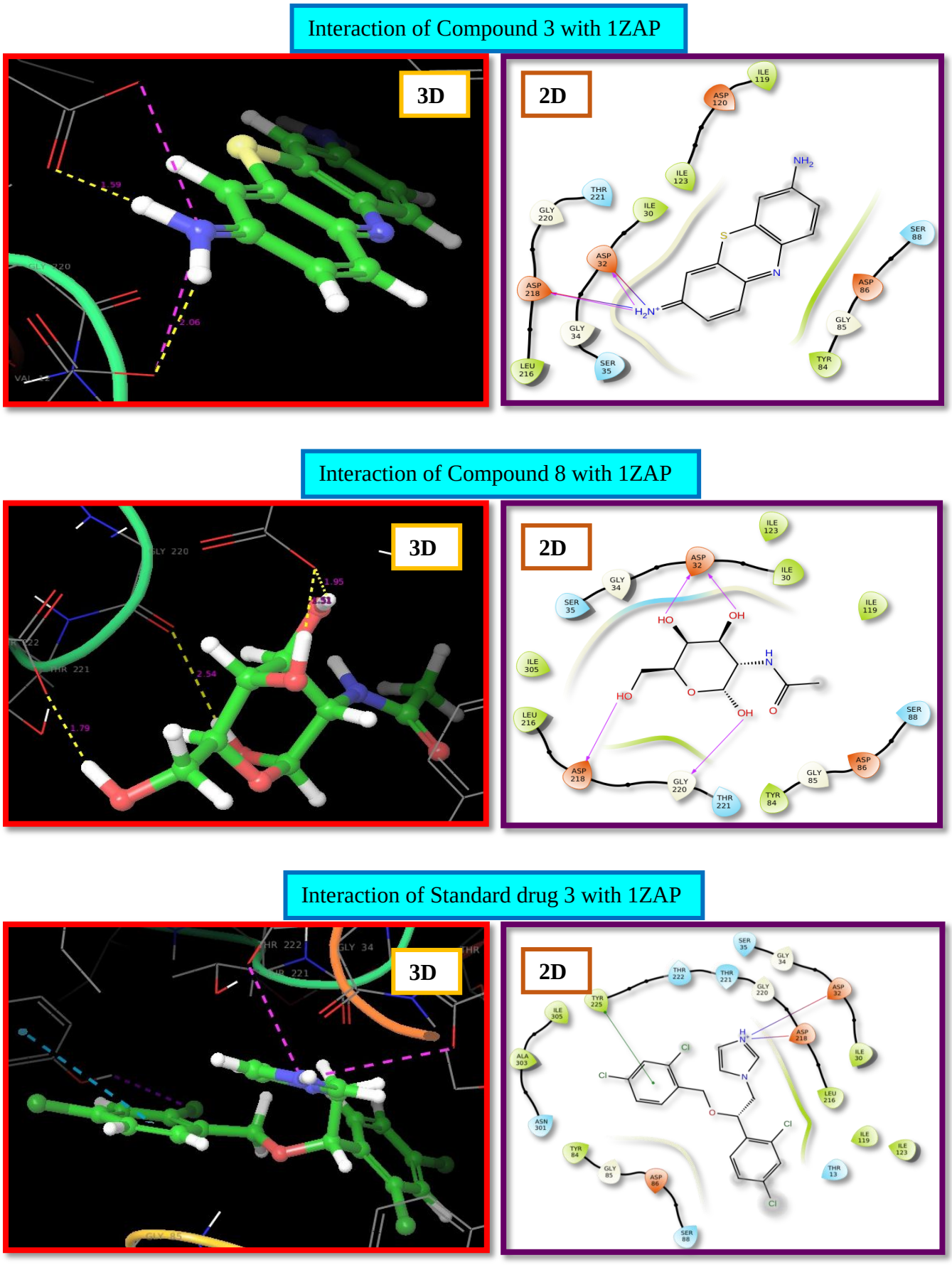


Fig 20: Ligand-protein interaction of antifungal compounds docked with the target protein 1ZAP showing the 3D and 2D images

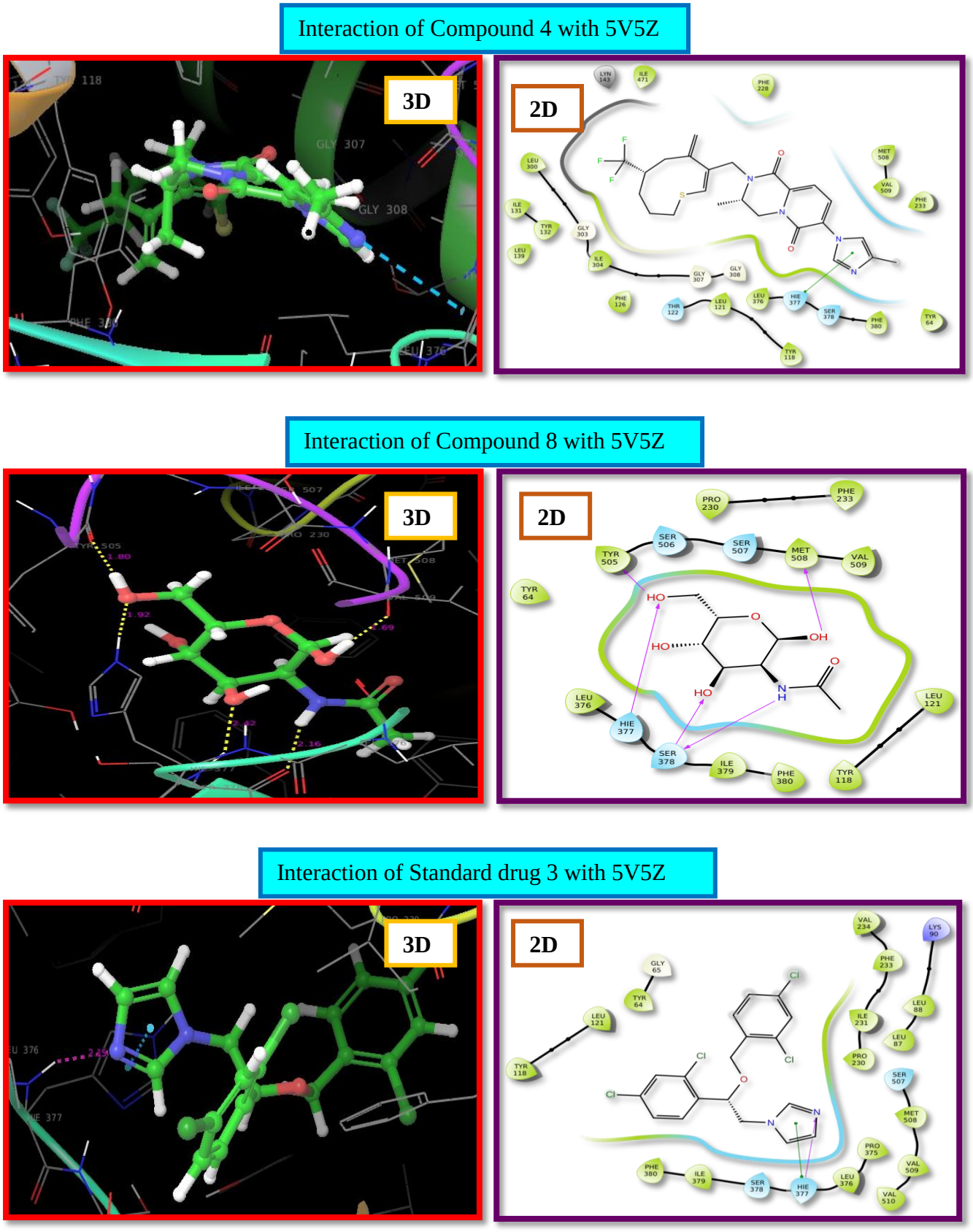


Fig 21: Ligand-protein interaction of antifungal compounds docked with the target protein 5V5Z showing the 3D and 2D images

The present study has revealed that *Aspergillus giganteus* was found to be antagonistic to the selected pathogenic fungi namely, *Cryptococcus neoformans* and *Candida albicans*. The co-culture assay and hyphal interaction assay has proved that the damaged morphology and hyphal elongation was occurred in the treated pathogens. The culture filtrates of *Aspergillus giganteus* were prepared and the antifungal compounds were also estimated. Mode of antagonistic action of antifungal compounds in the culture filtrates and the purified fractions on the pathogenic cell wall and cell membrane was investigated. The results have shown the damage and disruption was occurred in the AFCs treated pathogenic cells by monitoring the release of cellular macromolecules such as, DNA, RNA, protein, glucose and extracellular pH. The concentration of lipids in the treated pathogenic cell membrane was found to be decreased with the increased concentration of antifungal compounds and it showed the potential activity of *Aspergillus giganteus* on the cell membrane integrity of pathogens. Validation of *in vitro* results, the *in silico* approaches have seemingly important and gives a way for new and novel drug development. The antifungal compounds from *Aspergillus giganteus* have been downloaded and docked against the pathogenic target proteins. The molecular interaction study has proved that the compounds were shown to have the best interactions with the target proteins and might be used for the treatment of fungal infections.

SUMMARY AND CONCLUSION

5.0 SUMMARY AND CONCLUSION

Fungal infections are considered as the major threat to human health and leads to the greatest mortality and morbidity rate worldwide. Many fungal pathogens are known to cause deleterious effect to the plants, animals, human and environment when it becomes debilitated or immunocompromised. Indeed, fungal species belongs to aspergillus, candida, cryptococcous, histoblastoma and others have been listed as potential fungal pathogens. Candida includes more than 200 species, but only a few species are classified under pathogens. Among them *Candida albiacns* infection has been causing candidiasis in human which is often referred as a life threatening pathogens. *Cryptococcus neoformans* infection causes cryptococcosis in the host which primarily affects the central nervous system and resulting in various secondary related diseases.

Emergence of fungal infection, the treatment predominantly relies upon the use of antifungal drugs. The increased use of drugs resulted in the development of drug resistance in the host. In this context, there is an urge to develop the antifungal compounds from natural sources rather from synthetic sources. The plant metabolites are well known potential compounds for the treatment of various bacterial and fungal pathogens. Apart from other sources, filamentous fungi itself can act as a good source for the production of antifungal compounds exhibiting antagonism against various fungal and bacterial pathogens.

Aspergillus giganteus is a filamentous fungi, which is known to produce AFP (antifungal protein). Hence, the present study was aimed to determine the antagonistic potential against the pathogenic fungi namely, *Candida albicans* and *Cryptococcus neoformans*. After that the cell free supernatant of the *Aspergillus giganteus* was prepared and its antagonistic efficacy was also evaluated. Purification of antifungal compounds responsible for the antagonistic nature was carried out and the mode of action of antifungal compounds on the pathogenic cell was rather investigated. *In silico* studies have performed to understand the molecular interactions of antifungal compounds with the pathogenic target proteins.

To meet the selected objectives, various methodologies were adopted and performed. The co-culture and overlay assay was performed to exhibit the antagonistic potential of *Aspergillus giganteus* on the pathogenic *Cryptococcus neoformans* and *Candida albicans* and the antagonism was further proved by the hyphal interaction assay. The culture filtrate was prepared and tested its antagonistic activity. The antifungal compounds responsible for the antagonistic nature was further purified using ammonium sulphate precipitation followed by

dialysis. The mode of action of the culture filtrates and the partially purified compounds were investigated by quantifying the released constituents such as DNA, RNA, protein and glucose in the treated pathogenic strains using standard procedures. The pH changes in the extracellular medium were also monitored. The lipid content in the treated pathogenic cell wall was determined by phosphovanillin method. The ADME/Tox predictions were done to predict the pharmacokinetic properties of the antifungal compounds and the possible molecular interactions between the antifungal compounds and the pathogenic cell target proteins were investigated using molecular docking approaches.

The results have shown that the maximum antagonistic activity against the pathogenic strains in terms of percentage inhibition. The inhibition percentage of *Aspergillus giganteus* with *Cryptococcus neoformans* and *Candida albicans* was found to be 99% and 97% respectively. The antagonistic nature was further proved by hyphal interaction assay (lactophenol cotton blue staining) where, in the test plate the damaged and reduced hyphal projection was observed and in the control plate, the good morphology was noticed.

The culture filtrate was prepared and the antagonistic potential was evaluated using overlay assay and the inhibition percentage was found to be 91% for *Cryptococcus neoformans* and 93% for *Candida albicans*. This assay proved that the compounds present in the culture filtrates might be responsible for the growth inhibition of pathogens. Then, the pathogenic fungal cell wall was exposed to the antagonistic culture filtrates.

Membrane damage and disruption assay was performed to test the effect of antifungal compounds on the pathogenic cell wall and cell membrane. The leakage of cellular materials namely, DNA, RNA, protein and glucose in the antifungal culture filtrates treated pathogenic cell was quantified at various time intervals. The results revealed that the increased concentration of antifungal compounds in the culture filtrates and time interval has influenced the release of cellular constituents into the medium, thus, it might be based on the principle of dose-time dependent manner. This mode of action proved that the irreversible and gross morphological changes were occurred in the pathogenic cell wall and cell membrane by the action of antagonistic culture filtrates.

The extracellular pH in the medium was noticed using pH meter. The results have demonstrated that the pH changes in the treated pathogenic cell were observed with the increased concentration of AFCs in the culture filtrates and time interval. Some ions, acids, alkali and/or other metabolic substances may be released into the medium because of the action

of antagonistic culture filtrates, thus, pH changes was noticed. The pH value in the control remains the same under different concentration of AFCs treatment and time interval. The lipid content in the treated pathogenic cell wall was observed to be decreased gradually with the time and increased concentration of AFCs in the culture filtrates. Hence, the membrane integrity of the pathogenic cell wall was disturbed in both the pathogenic strains by the action of culture filtrates.

The antagonistic nature of *Aspergillus giganteus* and the culture filtrates has been proved, and henceforth, the antifungal compounds responsible for the antagonism was purified from the culture filtrates using ammonium sulphate precipitation followed by dialysis. Three salt cults (0-30%, 30-60% and 60-90%) were prepared and the protein content in the pellets was estimated in each step of precipitation. The amount of protein was observed in 30-60% and 60-90% was found to be 1.4 mg/ml and 1.8 mg/ml, respectively. Hence, these two fractions were dialyzed to remove the salts and the protein content, estimated in 30-60% was found to be 0.7mg/ml and 1.0mg/ml in 60-90%. The partially purified protein fraction obtained in the 60-90% of precipitation was further examined for its mode of action on the pathogenic cell wall.

The mode of antagonistic action of partially purified protein fractions was investigated on the pathogenic cell wall and cell membrane by membrane disruption. The leakage of cellular materials (DNA, RNA, protein and glucose) was estimated in the treated pathogenic cells and the release of cellular components was estimated. The results revealed that the leakage of cellular components from the treated pathogenic cells following the concentration-time dependent manner. The pH changes in the treated pathogenic *Cryptococcus neoformans* and *Candida albicans* was monitored and the changes might be the reason of the leakage of some ions and other substances. The lipid content in both the pathogenic cell wall was deduced with the increased concentration of partially purified compounds (AFCs).

The results of the mode of action have proved the good antagonistic nature of *Aspergillus giganteus* and the purified protein fractions against both the pathogenic cells namely, *Cryptococcus neoformans* and *Candida albicans*. Indeed, the antagonistic property of *Aspergillus giganteus* was demonstrated *in vitro,* the *in silico* studies were also performed to understand the molecular interactions of antifungal compounds and pathogenic target proteins.

Nearly, eight compounds present in the *Aspergillus giganteus* was selected based on the scientific studies and some standard drugs used for the treatment of candidiasis and cryptococcosis was downloaded from PubChem databases. The ADME predictions were used to

predict the pharmacokinetic and pharmacodynamic properties of the antifungal compounds. The compounds (ligands) were prepared using ligprep applications in the Schrodinger software and the ADME predictions were done using Qikprop. In this study, the antifungal compounds for the various properties were analyzed and it exhibited drug like characteristics based on Lipinski's rule-of-five. The human oral absorption of compounds 4 was low. In case of compounds 8 was found to be moderate and the compounds 1, 2, 3, 5, 6, 7, 9, 10, 11 were found to be highly absorbed.

The target proteins such as 3SFX, 4AF0, 6CW5, 6TZ8, 6WHP from *Cryptococcus neoformans* and 1IYL, 1AI9, 5V5Z, 1ZAP from *Candidaa albicans* were selected and were downloaded from PDB databases. The target protein was prepared using protein preparation wizard in the schrodinger software. The molecular docking for protein with each selected compounds was performed to study the interactions.

Compound 8 have the best interaction with all the selected target proteins of *Cryptococcus neoformans* and *Candida albicans* by forming more hydrogen bonds with the target proteins. The best docking score was found to be -9.183 for the compound 4 docked with the target protein of *Candida albicans* 5V5Z and -7.46 for the compound 2 and 3 with the target protein 6TZ8 of *Cryptococcus neoformans*. The standard drug 2 has the best molecular interactions with the target proteins of *Cryptococcus neoformans* and standard drug 3 was found to be form a good interaction with the *Candida albicans* target proteins. Some of the compounds did not have the interactions with some target proteins. The molecular interaction study has proved that the compounds were shown to have the best interactions with the target proteins and might be used for the treatment of fungal infections.

Hence, the study concluded that the antagonistic potential of *Aspergillus giganteus* against pathogenic *Cryptococcus neoformans* and *Candida albicans* was proved. The culture filtrates also demonstrated the good inhibitory efficacy on the both the pathogens. The pathogenic cell wall and cell membrane gets damaged and disrupted by the action of antagonistic culture filtrates and partially purified protein fractions. The membrane integrity was also disturbed by the AFCs (antifungal compounds). The antifungal compounds may diffuse into the cell membrane and disturb all the components and the activities of the pathogenic cell. ADME/Tox prediction has proved the antifungal compounds that can be used for the human oral absorption. The molecular interactions study proved the possible interactions of the compounds with the pathogenic target proteins. The outcome of the study exhibited that the antifungal

compounds from the *Aspergillus giganteus* can be an effective lead compound for the pharmaceutical industries to develop the novel drug.

Suggestions for future research:

- The antagonistic potential of *Aspergillus giganteus* against various plant pathogenic fungi can be studied
- The antifungal compounds responsible for the antagonistic nature can be purified and it can be evaluated by gene expression studies
- The *in vivo* studies for the purified antifungal compounds can be exploited in detail

BIBLIOGRAPHY

6.0 BIBLIOGRAPHY

- ✓ Aboody, M.S.A. and Mickymaray, S. (2020) Anti-fungal efficacy and mechanisms of flavonoids, Antibiotics, 9(45), 1-42.
- ✓ Abro, M.A., Sun, X., Li, X., Jatoi, G.H. and Guo, L.D. (2019) Biocontrol potential of fungal endophytes against *Fusarium oxysporum* f. sp. *cucumerinum* causing wilt in cucumber, The Plant Pathology Journal, 35(6), 598-608.
- ✓ Adenis A.A., Valdes A. and Cropet C. (2018) Burden of HIV-associated histoplasmosis compared with tuberculosis in Latin America: a modelling study, Lancet Infectious Diseases, 18, 1150–1159.
- ✓ Agustinho, D.P., Miller, L.C., Li, L.X. and Doering, T. L. (2018) Peeling the onion: the outer layers of *Cryptococcus neoformans*, Memorias do Instituto Oswaldo Cruz, 113(7), 1-8.
- ✓ Akkam, Y. (2016) A review of antifungal peptides: basis to new era of antifungal drugs, Jordan Journal of Pharmaceutical Sciences, 9, 51–75.
- ✓ Alberti, F., Foster, G.D. and Bailey, A.M. (2017) Natural products from filamentous fungi and production by heterologous expression, Applied Microbiology and Biotechnology, 101, 493–500.
- ✓ Almeida, F., Wolf, J.M. and Casadevall, A. (2015) Virulence-associated enzymes of *Cryptococcus neoformans*, American Society of Microbiology, 14(12) 1173–1185.
- ✓ Altındag, F.D., Saglık, B.N., Çevik, U.A., Işıkdag, I., Ozkay, Y. and Gençer, H.K. (2019) Novel imidazole derivatives as antifungal agents: Synthesis, biological evaluation, ADME prediction and molecular docking studies, Phosphorus, Sulfur, and Silicon and the Related Elements, 194(9), 887-894.
- ✓ Anteneh, Y.S., Brown, M.H. and Franco, C.M.M. (2019) Characterization of a halotolerant fungus from a marine sponge, BioMed Research International, 1-9.
- ✓ Bajpai, V.K., Khan, I., Shukla, S., Kumar, P., Rather, I.A., Park, Y.A., Huh, Y.S. and Han, Y.K. (2019) Invasive fungal infections and their epidemiology: measures in the clinical scenario, Biotechnology and Bioprocess Engineering 24, 436-444.
- ✓ Beardsley, J., Sorrell, T. C. and Chen, S. C. (2019) Central nervous system cryptococcal infections in non-HIV infected patients. J Fungi 5:E71
- ✓ Beltran, E.R., Mendez, C.I.B., Mormeneo, M.I.S. and Arias, J.P.L. (2019) The cell wall of *Candida albicans*: a proteomics view, Intech Open, (4) 71-92.

- ✓ Binder, U., Bencina, M., Eigentler, A., Meyer, V. and Marx, F. (2011) The *Aspergillus giganteus* antifungal protein AFPNN5353 activates the cell wall integrity pathway and perturbs calcium homeostasis, BMC Microbiology, 11:209.
- ✓ Bondaryk, M., Staniszewska, M., Zielinska, P. and Lipkowska, Z.U. (2017) Natural antimicrobial peptides as inspiration for design of a new generation antifungal compounds, Journal of Fungi, 3(46):1-36.
- ✓ Bouchal, B., Abrigach, F., Takfaoui, A., Errahhali, M.E., Errahhali, M.E., Dixneuf, P.H., Doucet, H., Touzani, R. and Bellaoui,M. (2019) Identification of novel antifungal agents: antimicrobial evaluation, SAR, ADME–Tox and molecular docking studies of a series of imidazole derivatives, BMC Chemistry, 13(100), 1-12.
- ✓ Bouyahya, A., Abrini, J., Dakka, N. and Bakri, Y. (2019) Essential oils of Origanum compactum increase membrane permeability, disturb cell membrane integrity, and suppress quorumsensing phenotype in bacteria, Journal of Pharmaceutical Analysis, 9, 301-311.
- ✓ Butts, A., Reitler, P., Ge, W., Fortwendel, J.R. and Palmer, G.E. (2018) Commonly used oncology drugs decrease antifungal effectiveness against *Candida* and *Aspergillus* species, Antimicrobial Agents and Chemotherapy, 6(7), 1-9.
- ✓ Camacho, E., Chrissian, C., Cordero, R. J. B., Liporagi-Lopes, L., Stark, R. E. and Casadevall, A. (2017) N-acetylglucosamine affects *Cryptococcus neoformans* cell-wall composition and melanin architecture, Microbiology, 163, 1540–1556.
- ✓ Castillo, Y.P., Lima, T.C., Ferreira, A.R., Silva, C.R., Campos, R.S., Neto, J.B.A., Magalhaes, H.I.F., Cavalcanti, B.C., Junior, H.V.N. and Sousa, H.P. (2020) Bioactivity and molecular docking studies of derivatives from cinnamic and benzoic acids, Biomed Research International, 1-13.
- ✓ Chen, X., Wang, Y., Ma, N., Tian, J., Shao, Y., Zhu, B., Yong, Y.K., Liang, Z., Zou, C. and Wang, J. (2020) Target identification of natural medicine with chemical proteomics approach: probe synthesis, target fishing and protein identification, Signal Transduction and Targeted Therapy, 5(72), 1-13.
- ✓ Cheung, Y.Y. and Hui, M. (2017) Effects of echinocandins in combination with Nikkomycin Z against invasive *Candida albicans* bloodstream isolates and the FKS mutants, Antimicrobial Agents Chemotherapy, 61.
- ✓ Chikhale, H.U. (2020) Review on *in silico* techniques: An approach to drug discovery, Current Trends in Pharmacy and Pharmaceutical Chemistry, 2(1), 24-32.

- ✓ Choudhary, A. and Ashraf, S. (2019) Utilizing the combined antifungal potential of Trichoderma spp. and organic amendments against dry root rot of mungbean, Egyptian Journal of Biological Pest Control, 29(83), 1-8.
- ✓ Crawford, C., Wear, M.P., Smith, D.F.Q., d'Errico, C., Casadevall, A. and Oscarson, S. (2020) Glycan FRET probes for screening catalytic antibodies against *Cryptococcus neoformans* capsule, ChemRxiv, 1-99.
- ✓ Dar, A.M. and Mir, S. (2017) Molecular docking: approaches, types, applications and basic challenges, Journal Bioanalytical Techniques, 8 (2), 1-3.
- ✓ Divya R, Asok, A.K. and Jisha, M.S. (2018) Bio control potential of rice endophyte *Fusarium oxysporum* against rice sheath blight pathogen *Rhizoctonia solani*, Agricultural Research & Technology, 19(1), 1-3.
- ✓ Durairaj, K., Velmurugan, P., Park, J.H., Chang, W.S., Park, Y.J., Senthilkumar, P., Choi, K.M., Lee, J. and Oh, B.T. (2018) An investigation of biocontrol activity *Pseudomonas* and *Bacillus* strains against *Panax* ginseng root rot fungal phytopathogens, Biological Control, 1-10.
- ✓ Ferreira, M.D.P.S.O., Cardoso, M.F.C., Silva, F.C., Ferreira, V.F., Lima, E.S. and Souza, J.V.B. (2016) Antifungal activity of synthetic naphthoquinones against dermatophytes and opportunistic fungi: preliminary mechanism-of-action tests, Annals of Clinical Microbiology and Antimicrobials, 13(26), 1-6.
- ✓ Fisher, M.C., Gurr, S.J., Cuomo, C.A., Blehert, D.S., Jin, H. Stukenbrock, E.H., Stajich, J.E., Kahmann, R., Boone, C., Denning, D.W., Gow, N.A.R., Klein, B.S., Kronstad, J.W., Sheppard, D.C., Taylor, J.W., Wright, G.D., Heitman, J., Casadevall, A. and Cowen, L. (2020) Threats posed by the fungal kingdom to humans, wildlife, and agriculture, American Society of Microbiology, 11(3), 1-17.
- ✓ Fonseca, F.L., Reis, F.C.G., Sena, B.A.G., Jozefowicz, L.J., Kmetzsch, L. and Rodrigues, M.L. (2018) The overlooked glycan components of the Cryptococcus capsule, Current Topics in Microbiology and Immunology, 422, 31–43.
- ✓ Freitas, M.A., Andrade, J.C., Alves, A.I.S., dos Santos, F.d.A.G., Leite-Andrade, M.C., Sales, D.L., Nunes, M., Ribeiro, P.R.V., Coutinho, H.D. M. and Morais-Braga, M.F.B. (2020) Use of the natural products from the leaves of the fruitfull tree *Persea americana* against Candida sp. biofilms using acrylic resin discs, Science Total Environmental, 703, 1-26.

- ✓ Ganesan, K. and Xu, B. (2018) A critical review on phytochemical profile and health promoting effects of mung bean (*Vigna radiata*), Food Science and Human Wellness, 7, 11–33.
- ✓ Garrigues, S., Gandia, M., Castillo, L., Coca, M., Marx, F., Marcos, J.F. and Manzanares, P. (2018) Three antifungal proteins from *Penicillium expansum*: different patterns of production and antifungal activity, Frontiers in Microbiology, 9(2370), 1-15.
- ✓ Gintjee, T.J., Donnelley, M.A. and Thompson, G.R. (2020) Aspiring antifungals: review of current antifungal pipeline developments, Journal of fungi, 6(28), 1-11.
- ✓ Gow, N.A.R., Latge, J.P. and Munro, C.A. (2017) The fungal cell wall: structure, biosynthesis, and function, Microbiology Spectrum, 5, 1-26.
- ✓ Granger, B. L. (2018) Accessibility and contribution to glucan masking of natural and genetically tagged versions of yeast wall protein 1 of *Candida albicans*, PLoS One, 13:e0191194.
- ✓ Hast, M.A., Nicholss, C.B., Armstrong, S.M., Kelly, S.M., Hellinga, H.W., Alspaugh, J.A. and Beese, L.S. (2011) Structures of *Cryptococcus neoformans* protein farnesyltransferase reveal strategies for developing inhibitors that target fungal pathogens, The Journal of Biological Chemistry, 286(40), 35149-35162
- ✓ Hembach, L., Bonin, M., Gorzelanny, C. and Moerschbachel, B.M. (2020) Unique subsite specificity and potential natural function of a chitosan deacetylase from the human pathogen Cryptococcus neoformans, PNAS, 117(7), 3551-3559.
- ✓ Hommel, B., Mukaremera, L., Cordero, R.J.B., Coelho, C., Desjardins, C.A. and Sturny-Leclere, A. (2018) Titan cells formation in *Cryptococcus neoformans* is finely tuned by environmental conditions and modulated by positive and negative genetic regulators, PLoS Pathog. 14:e1006982.
- ✓ Houst, J., Spizek, J. and Havliek, V. (2020) Review Antifungal Drugs, Metabolites, 10(106), 1-16.
- ✓ Islam, M.A., Nain, Z., Alam, M.K., Banu, N.A. and Islam, M.R. (2018) In vitro study of biocontrol potential of rhizospheric *Pseudomonas aeruginosa* against *Fusarium oxysporum* f. sp. *cucumerinum*, Egyptian Journal of Biological Pest Control, 28(90), 1-11.
- ✓ James, N., Surana, R., Thigale, I., Preethi, B., Shanthi, V. and Ramanathan, K. (2018) Exploring novel ALK inhibitors using energy based pharmacophore mapping

and high – throughput virtual screening, Indian Journal of Pharmaceutical Education and Research, 52(4), 707-717.

- ✓ Juvvadi, P.R., Fox, D., Bobay, B.G., Hoy, M.J., Gobeil, S.M.C., Venters, R.A., Chang, Z., Lin, J., Averette, A.F., Cole, D.C., Barrington, B.C., Wheaton, J.D., Ciofani, M., Trzoss, M., Li, M., Lee, S.C., Chen, Y.L., Mutz, M., Spicer, L.D., Schumacher, M.A., Heitman, J. and Steinbach, w.j. (2019) Harnessing calcineurin-FK506-FKBP12 crystal structures from invasive fungal pathogens to develop antifungal agents, Nature Communications, 10(4275), 1-18.
- ✓ Kathiravan, M.K., Salake, A.B., Chothe, A.S., Dudhe, P.B., Watode, R.P. and Mukta, M.S. (2012) The biology and chemistry of antifungal agents: a review. Bioorganic Medicinal Chemistry20, 5678–5698.
- ✓ Katoch, M. and Pull, S. (2017) Endophytic fungi associated with *Monarda citriodora*, an aromatic and medicinal plant and their biocontrol potential, Pharmaceutical Biology, 55(1), 1528-1535.
- ✓ Keniya, M.V., Sabherwal, M., Wilson, R.K., Woods, M.A., Sagatova, A.A., Tyndall, J.D.A. and Monk, B.C. (2018), Crystal structures of full-length lanosterol 14 alpha-demethylases of prominent fungal pathogens *Candida albicans* and *Candida glabrata* provide tools for antifungal discovery, Antimicrobial Agents and Chemotherapy, 62(11), 1-9.
- ✓ Khan, M.R., Ahmad, I. and Ahamad, F. (2018) Effect of pure culture and culture filtrates of *Trichoderma* species on root-knot nematode, *Meloidogyne incognita* infesting tomato, Indian Phytopathology, 71(2), 265-274.
- ✓ Knauth, P. and Reichenbach, H. (2016) On the mechanism of action of the myxobacterial fungicide Ambruticin, The Journal of Antibiotics, 53(10), 1182-1190.
- ✓ Koka, A.J., Wani, A.H. and Bhat, M.Y. (2019) Effect of culture filtrate of isolates of *Trichoderma* species on mycelial growth inhibition of fungi causing rot of tomato and brinjal, World Journal of Pharmaceutical and Life Sciences, 5(4), 161-166.
- ✓ Lei, S., Zhao, R., Sun, J., Ran, J., Ruan, X. and Zhu, Y. (2019) Partial purification and characterization of a broad spectrum bacteriocin produced by a *Lactobacillus plantarum* zrx03 isolated from infant's feces, Food science and nutrition, 8, 2214-2222.
- ✓ Lima, S.L., Colombo, A.L. and Almeida, J.N.J. (2019) Fungal Cell Wall: Emerging Antifungals and Drug Resistance, Frontiers in Microbiology, 10, 1-9.

- ✓ Livengood, S.J., Drew, R.H. and Perfect, J.R. (2020) Combination therapy for invasive fungal infections, Current Fungal Infections Reports, 14, 40–49.
- ✓ Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randell, R. J. (1951) Protein measurement with the folin phenol reagent, Journal of Biological Chemistry,193:265-275.
- ✓ Lozano, V.H., Rangel, T.R.J., Gonzalez, G.M., Rodriguez, P.A., Ortiz, C.A., Reyna, C.L., Alvarado, S.G. and Resendez, M.F. (2018) Clinical evaluation of the antifungal effect of sertraline in the treatment of cryptococcal meningitis in HIV patients: a single Mexican center experience, Infection, 46(1), 25-30.
- ✓ Mahadevan, G, Mohan, G., Vinoth, J. and Ravi, V. (2019) Biotic potential of mucus extracts of giant mudskipper *Periophthalmodon schlosseri* (Pallas, 1770) from Pichavaram, southeast coast of India, The Journal of Basic and Applied Zoology, 80(13):1-7.
- ✓ Mapfunde, S., Sithole, S. and Mukanganyama, S. (2016) In vitro toxicity determination of antifungal constituents from *Combretum zeyheri*, BMC Complementary and Alternative Medicine, 16(162), 2-11.
- ✓ Mazrou, Y.S.A., Makhlouf, A.H., Elseehy, M.M., Awad, M.F. and Hassan, M.M. (2020) Antagonistic activity and molecular characterization of biological control agent *Trichoderma harzianum* from Saudi Arabia, Egyptian Journal of Biological Pest Control, 30(4), 1-8.
- ✓ McCarthy, M.W., Kontoyiannis, D.P., Cornely, O.A., Perfect, J.R. and Walsh, T.J. (2017) Novel agents and drug targets to meet the challenges of resistant fungi, The Journal of Infectious Diseases, 216(S3):S474–S483.
- ✓ McNair, L.K.F., Siedler, S., Vinther, J.M.O., Hansen, A.M., Neves, A.R. and Garrigues, C. (2018) Identification and characterization of a new antifungal peptide in fermented milk product containing bioprotective Lactobacillus cultures, FEMS Yeast Research, 18.
- ✓ Mitchell, A.P. (2020) Dihydrofolate reductase is a valid target for antifungal development in the human pathogen *Candida albicans*, Molecular Biology and Physiology, 5(3), 1-15.
- ✓ Morrow, C.A., Valkov, E., Stamp, A., Chow, E.W., Lee, R., Wronski, A., Williams, S.J., Hill, J.M., Djordjevic, J.T., Kappler, U., Kobe, B. and Fraser, J.A. (2012) De novo GTP biosynthesis is critical for virulence of the fungal pathogen *Cryptococcus neoformans*, PLOS Pathogens, 8(10), 1-18.

- ✓ Narvaez, I., Khayreddine, T., Pliego, C., Cerezo, S., Jiménez-Díaz, R.M., Trapero-Casas, J.L. and Pliego-Alfaro, F. (2018) Usage of the heterologous expression of the antimicrobial gene afp from *Aspergillus giganteus* for increasing fungal resistance in olive, Frontiers in Plant Science, 9.
- ✓ Ndaba, N., Fotsing, M.C., Anku, W.W. and Govender, P.P. (2020) In vitro and in silico studies of the antifungal properties of the bulb and leaves extracts of *Drimia delagoensis* Baker (Jessop), Advances in Traditional Medicine, 20, 373–379.
- ✓ Nicola, A.M., Albuquerque, P. and Paes H.C. (2019) Antifungal drugs: new insights in research & development, Pharmacology Therapies, 195, 21–38.
- ✓ Pal, J., Sharma, S.K., Devi, S., Sharma, R., Raj, H., Karn, M., Verma, S., Vedukola, P.D. and Sharma, A. (2020) Screening, identification, and colonization of fungal root endophytes against *Dematophora necatrix*: a ubiquitous pathogen of fruit trees, Egyptian Journal of Biological Pest Control, 30(112), 1-14.
- ✓ Pappas, P.G., Lionakis, M.S., Arendrup, M.C., Ostrosky-Zeichner, L. and Kullberg, B.J. (2018) Invasive candidiasis, Disease Primers, 4, 1-20.
- ✓ Parveen, S., Wani, A.H. and Bhat, M.Y. (2019) Effect of culture filtrates of pathogenic and antagonistic fungi on seed germination of some economically important vegetables, Brazillion Journal of Biological Sciences, 6(12), 133-139.
- ✓ Passera, A., Venturini, G., Battelli, G., Casati, P., Penaca, F., Quaglino, F. and Bianco, P.A. (2017) Competition assays revealed *Paenibacillus pasadenensis* strain R16 as anovel antifungal agent, Microbiological Research, 198, 16-26.
- ✓ Perfect, J. R. (2016) Is there an emerging need for new antifungals? Expert Opin Emerging Drugs: 1–3.
- ✓ Pramudito, T.E., Agustina, D., Budinarta, W., Nguyen, T.K.N., Prihatna, C. and Suwanto, A. (2020) Identification and characterization of antifungal compounds from a *Burkholderia* strain against plant pathogenic fungi, Malaysian Journal of Microbiology, 16(4), 245-252.
- ✓ Rauseo, A.M., Reilly, A.C. Larson, L. and Spec, A. (2020) Hope on the Horizon: Novel Fungal Treatments in Development, Open Forum Infectious Diseases, 1-19.
- ✓ Reddy, G.K.K. and Nanchariah, Y.V. (2020) Alkylimidazolium ionic liquids as antifungal alternatives: antibiofilm activity against *Candida albicans* and underlying mechanism of action, Frontiers in microbiology, 11(730), 1-15.

- ✓ Revie, N.M., Iyer, K.R., Robbins, N. and Cowen, L. (2018) Antifungal drug resistance: Evolution, mechanisms and impact, Current Opinions in Microbiology, 45, 70–76.
- ✓ Rodrigues, M.L. and Albuquerque, P.C. (2018) Searching for a change: The need for increased support for public health and research on fungal diseases, PLOS Neglected Tropical Diseases, 12(6), 1-5.
- ✓ Rodrigues, M.L. and Nosanchuk, J.D. (2020) Fungal diseases as neglected pathogens: A wake-up call to public health officials, PLOS: Neglected Tropical Diseases, 14(2), 1-9.
- ✓ Rolta, R., Kumar, V., Sourirajan, A. and Dev, K. (2020) Molecular Docking studies of Phytocompounds of Rheum emodi Wall with proteins responsible for antibiotic resistance in bacterial and fungal pathogens: In silico approach to enhance the bio-availability of antibiotics, BioRxiv.
- ✓ Rubio, R.G., Oliveira, H.C., Rivera, J. and Contador, N.T. (2020) The Fungal Cell Wall: Candida, Cryptococcus, and Aspergillus Species, Frontiers in Microbiology, 10, 1-13.
- ✓ Saechow, S., Thammasittirong, A., Kittakoop, P., Prachya, S. andThammasittirong, S.N. (2018) Antagonistic activity against dirty panicle rice fungal pathogens and plant growth-promoting activity of *Bacillus amyloliquefaciens* BAS23, Journal of Microbiology and Biotechnology, 28(9):1527–1535.
- ✓ Sampinato, C. and Leonardi, D. (2013) Candida Infections, Causes, Targets, and Resistance Mechanisms: Traditional and Alternative Antifungal Agents, BioMed Research International, 1-13.
- ✓ Sari, S. and Kart, D. (2020) Design, synthesis, antifungal activity, and QM/MM docking study of two azole derivatives with indole ring, Journal of Research in Pharmacy, 24(5), 681-692.
- ✓ Sathe, S.J., Gajbhiye, M.H. and Doshi, D.V. (2017) Production and extraction of antifungal compound from *Lactobacillus paracollinoides* FG401, International Research Journal of Science & Engineering, A1, 210-214.
- ✓ Satish, S., Jiménez-Ortigosa, C., Zhao, Y., Lee, M. H., Dolgov, E. and Krüger, T. (2019) Stress-induced changes in the lipid microenvironment of β-(1,3)-D-glucan synthase cause clinically important echinocandin resistance in *Aspergillus fumigatus*, mBio 10:e00779-19.

- ✓ Segarra, V.V., Carrion, K.C., Santini-Gonzalez, J.J., Valerio, Y.A.R., Quinones, L.E.V., Lima, A.R., Medina, J.R. and Matos, E.I.P. (2019) Modelling and molecular docking studies of the cytoplasmic domain of Wsc-family, full-length Ras2p, and therapeutic antifungal compounds, Computational Biology Chemistry, 78, 338-352.
- ✓ Sem, X., Le, G. T., Tan, A. S., Tso, G., Yurieva, M. and Liao, W. W. (2016) Beta-glucan exposure on the fungal cell wall tightly correlates with competitive fitness of Candida species in the mouse gastrointestinal tract, Frontiers Cell Infections, Microbiology, 6, 186.
- ✓ Sewczyk, T., Antink, M.H., Maas, M., Kroll, S. and Beutel, S. (2018) Flow rate dependent continuous hydrolysis of protein isolates, AMB Express, 8(18), 1-9.
- ✓ Shadrack, D.M. and Ndesendo, V.M. (2017) Molecular docking and ADMET study of emodin derivatives as anticancer inhibitors of NAT2, COX2 and TOP1 enzymes, Computational Molecular Bioscience, 7 (1), 1–18.
- ✓ Shahina, Z., Ganiny, A.M.E., Minion, J., Whiteway, M., Sultana, T. and Dahms, T.E.S. (2018), *Cinnamomum zeylanicum* bark essential oil induces cell wall remodelling and spindle defects in *Candida albicans*, Fungal Biology and Biotechnology, 5(3), 1-12.
- ✓ Shukla, P.K., Singh, P., Yadav, R.K., Pandey, S. and Bhunia, S.S. (2016) Past, present and future of antifungal drug development, Berlin:Springer, 49, 668–679.
- ✓ Singh, D.K., Toth, R. and Gacser, A. (2020) Mechanisms of Pathogenic *Candida* Species to Evade the Host Complement Attack, Frontiers in Cellular and Infection Microbiology, 10(94), 1-9.
- ✓ Soberon, J.R., Sgariglia, M.A., Pastoriza, A.C., Soruco, E.M., Jager, S.N., Labadie, G.R., Sampietro, D.A. and Vattuone, M.A. (2017) Antifungal activity and cytotoxicity of extracts and triterpenoid saponins obtained from the aerial parts of *Anagallis arvensis* L., Journal of Ethnopharmacology, 203, 233-240.
- ✓ Sogabe, S., Masubuchi, M., Sakata, K. and Fukami, T.K. (2002), Crystal structures of candida albicans n-myristoyltransferase with two distinct inhibitors, Cell Chemical Biology, 9(10), 1119-28.
- ✓ Solanki, D.S., Kumar, S., Parihar, K., Sharma, K., Gehlot, P., Singh, S.K. and Pathak, R. (2018) Purification and characterization of a novel thermostable antifungal protein with chitinase activity from mung bean, Journal of environmental biology, 39, 406-412.

- ✓ Srivastava, A. and Neelabh. (2020) In-silico study of some natural compounds used as antifungal agents against *Candida albicans*, European Journal of Biological Research, 10(3), 188-197.
- ✓ Stefanini, I., Rizzetto, L., Rivero, D., Carbonell, S., Gut, M., Heath, S., Gut, I.G., Trabocchi, A., Guarna, A., Ghazzi, N.B., Bowyer, N., Kapushesky, M. and Cavalieri, D. (2018) Deciphering the mechanism of action of 089, a compound impairing the fungal cell cycle, Scientific Reports, 8(5964), 1-15.
- ✓ Thery, T., Shwaiki, L.N., Callaghan, Y.C.O., Brien, N.M.O. and Arendt, E.K. (2018) Antifungal activity of a *de novo* synthetic peptide and derivatives against fungal food contaminants, Journal of Wiley Peptide Sciences, 25(3137), 1-12.
- ✓ Tong, S., Li, M. Keyhani, N.O., Liu, Y., Yuan, M., Lin, D., Jin, D., Li, X., Pei, Y. and Fan, Y. (2020) Characterization of a fungal competition factor: production of a conidial cell-wall associated antifungal peptide, PLOS pathogens, 2-29.
- ✓ Toth, R., Nosek, J., Mora-Montes, H. M., Gabaldon, T., Bliss, J. M. and Nosanchuk, J. D. (2019) *Candida parapsilosis*: from genes to the bedside, Clinical Microbiology Revision, 32, 111-118.
- ✓ Ullivarri, M.F., Arbulu, S., Garcia-Gutierrez, E. and Cotter, P.D. (2020) Antifungal peptides as therapeutic agents, Frontiers in Cellular and Infection Microbiology, 10, 1-22.
- ✓ Vila, L., Lacadena, V., Fontanet, P., Pozo, A.M. and Segundo, B.S. (2001) A protein from the mold *aspergillus giganteus* is a potent inhibitor of fungal plant pathogens, The American Phytopathological Society, 14(11), 1327-1331.
- ✓ Wagener, J., MacCallum, D.M., Brown, G.D. and Gow, N.A. (2017) *Candida albicans* chitin increases Arginase-1 activity in human macrophages, with an impact on macrophage antimicrobial functions, mBio, 8:01820-16.
- ✓ Wang, Z. A., Li, L. X., and Doering, T. L. (2018) Unraveling synthesis of the cryptococcal cell wall and capsule, Glycobiology, 28, 719–730.
- ✓ Wring, S.A., Randolph, R. and Park, S. (2017) SCY-078 a first in class orally active antifungal glucan synthesis inhibitor: pre-clinical pharmacokinetics and pharmacodynamics target in murine models of disseminated candidiasis, Antimicrobial Agents and Chemotherapy, 59:4308–4311.
- ✓ Zaragoza, O. (2019) Basic principles of the virulence of Cryptococcus, Virulence, 10, 490–501.

APPENDICES

APPENDIX – I

Composition of czapek yeast extract media

- Czapek concentrate – 10.0ml

NaNO3	-	30.0g
KCl	-	5.0g
MgSO4	-	5.0g
FeSO4	-	0.1g
Distilled water	-	100ml

- K_2HPO_4 - 1.0g
- Yeast extract - 5.0g
- Dextrose - 2.0g
- Agar - 15.0g
- Dis.H_2O - 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 that serve as a fungicide, lactic acid serving as a cleaning agent, cotton blue that stains the cytoplasm of the fungus and glycerine, giving a semi-permanent preparation. The periodic acid shift (PAS) and methanamine can also be used for fungal elements in tissue section.

Materials required

AFCs treated pathogenic strains, 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 AFCs treated pathogenic fungi usually with spores and spore bearing structure into the broth using flamed needle. Mixed gently stab with the fungal structures. Finally placed a cover slip over the preparation and examined under microscope.

APPENDIX – III

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-Ciocalteau reagent by the aminoacids tyrosine and tryphtophan 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-Ciocalteau 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 cell free supernatant of *Aspergillus giganteus* was 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^{o}C for 3 minutes. 0.3 ml of solution E was added, mixed well and incubated at 37^{o}C for 3 minutes again. The blue color developed was read at 670 nm against reagent blank. The estimation of released protein content in the treated cultures was also performed.

APPENDIX - IV

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^{o}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 samples 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 – V

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 ($FeCl_3.6H_2O$) 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. 1.0 ml of supernatant of treated pathogenic strains was taken in the clean dry test tubes. 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 – VI

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 100µg 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.

APPENDIX – VII

Purification of proteins by Ammonium sulphate precipitation

Principle:

Ammonium sulphate is a useful salt for the fractional precipitation of proteins. The precipitation is based on the principle of salting out. Ammonium sulphate is available in the highly purified form, has great solubility allowing significant changes in the ionic strength and is also inexpensive. Changes in ammonium sulphate concentration of a solution can be brought either by adding the solid substance or by adding the salt of known saturation, generally a fully saturated solution (100%).

Reagents:

1. Ammonium sulphate
2. Phosphate buffer at pH 7.4

   Solution A: Weighed 21.25 g of disodium hydrogen phosphate and dissolve it in1 litre of distilled water to get 150mM.

   Solution B: Weighed 23.85 g of sodium dihydrogen phosphate and dissolve it in1 litre of distilled water to get 150mM.

3. Crude extract of antifungal culture – Prepared the extract by removed the mat formed by *Aspergillus giganteus* and the supernatant was filtered and used as source of crude extract.

PROCEDURE

1. The ammonium sulphate precipitation such as 0-30%, 30-60% and 60-90% was performed with the culture filtrates of *Aspergillus giganteus*.
2. Dissolved the required ammonium sulphate salt and continued stirring for 30 minutes to allow equilibrium to be reached between the dissolved and the aggregate proteins.
3. Centrifuged the samples at 10,000xg for 10 minutes at 4°C.
4. The supernatant discarded and the pellet was collected.
5. The pellet was dissolved by using phosphate buffer at pH 7.4 and stored at 4°C.

APPENDIX – VIII

Desalting of proteins by dialysis

Principle:

Desalting is commonly used to remove the salt from proteins. The presence of salts in proteins interferes in many ways. Special semi-permeable membranes called dialysis bag have the property to allow compounds with smaller molecular weight to pass through them while those with higher molecular weight, like proteins to be retained.

Reagents:

1. Dialysis bag
2. Magnetic bar and stirrer motors
3. 2% sodium carbonate
4. 0.05% EDTA
5. Phosphate buffer at pH 7.4
6. Ammonium sulphate purified protease

Procedure:

1. Dialysis bag of suitable diameter was selected and cut into required length.
2. The bags were then treated with the boiling distilled water.
3. Submerged the bags into a solution of 2% sodium bicarbonate followed by 0.05% EDTA and boiled for 10 minutes in distilled water.
4. Repeated the process once.
5. Rinsed the dialysis bag with distilled water or buffer, before use.
6. Sealed the bag at one end with a double knot.
7. And then dialysis bag was filled with the solution to be dialysed.
8. Placed the bags in beaker containing phosphate buffer with a magnetic bar and stirrer motor in cold room.

The solution was allowed to reach equilibrium by changing the phosphate buffer for every 3 hours at 4℃. Small molecules pass freely and get diluted by the large volumes of fluids in the external medium.