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ANTAGONISTIC MECHANISM OF ANTI-FUNGAL COMPOUNDS FROM *ASPERGILLUS GIGANTEUS* ON HUMAN FUNGAL PATHOGENS

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ABSTRACT: *Cryptococcus neoformans* and *Candida albicans* are the major causative agents for infections, cryptococcosis, and candidiasis in human. Anti-fungal drugs for the treatment of fungal infections may show less effective activity on the pathogens due to the poor absorption or inadequate drug distribution, and some pathogens may evolve anti-fungal drug resistance. The development of anti-fungal drugs from natural sources with potential activity is of main global concern. Some filamentous fungi themselves can secrete anti-fungal compounds, which show effective antagonism against numerous pathogens. One such fungus, *Aspergillus giganteus* is known to produce anti-fungal proteins (AFP). In this study, the AFP is purified and its antagonistic activity was determined by analyzing its pathogenic membrane disruption ability. Extracellular pH and the lipid level in the AFP treated pathogenic cell membrane were also evaluated. The results have proved the membrane damage and disintegration of the pathogenic cell membrane by AFP and were observed in a dose-time-dependent manner. The fluctuation in the extracellular pH was monitored in the treated pathogens. This study has identified a novel drug from *Aspergillus giganteus* against fungal pathogens.

INTRODUCTION: Fungal infections are the most dreadful diseases among human diseases in perspective to their management and treatment. Nearly 1.7 billion people are suffering from infections caused by fungal pathogens. Invasive fungal infections are described as systemic fungal infections where the fungi have been establishing and infiltrating themselves in the deeper tissues of the host [1-2]. Yeasts are very large, widespread opportunistic fungus for the cause of harmful infections and associated with various diseases.

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There is a wide spectrum of yeasts where most of the pathogenic fungus has been evolved and cause major illnesses among humans, plants, and animals. The opportunistic fungal pathogens belong to the genus aspergillus, candida, cryptococcus, and pneumocystis and have been declared as the most harmful life-threatening pathogens leading to a higher incidence of death worldwide [3-4].

Cryptococcus neoformans is the environmental yeast that can cause cryptococcosis and it 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 immune-compromised. The infection can cause severe secondary associated problems in humans. It is estimated that more than one million infections and

nearly 60,000 death rates were reported annually, with *C. neoformans* causing cryptococcosis [5-6]. Candida species are common commensal fungi that colonized a wide range of mucosal surfaces including, the oral cavity, gut, or vagina in the host. The increasing invasive fungal infection caused by the pathogen, *Candida albicans*, is a global phenomenon that is the causative agent for candidiasis. In healthy patients, *Candida albicans* causes the mild superficial mucosal infection with significant morbidity considered as oral thrush and vulvovaginal candidiasis [7-8]. Despite the increased incidence of mortality rate recorded with fungal infections, more anti-fungal compounds have been discovered. There are a few classes of anti-fungal drugs available for the prevention and treatment of fungal infections. Unfortunately, most of the fungal pathogens may develop resistance to anti-fungal 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 an increased mortality rate and it may go above 10 million death by the year 2050 [9].

The plant metabolites are well-known potential compounds for the treatment of various bacterial and fungal pathogens. Apart from other sources, 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 [10]. 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 anti-fungal protein (AFP), is secreted as a 91-amino acid inactive precursor containing a signal sequence for secretion and a pro sequence that is removed by a protease during the process of secretion. The pro sequence 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 [11-13]. Hence, the present study focused on purifying the AFPs from *Aspergillus giganteus* and investigate its mechanism to disrupt and damage the cell membranes of *Cryptococcus neoformans* and *Candida albicans*.

MATERIALS AND METHODS:

Collection and Maintenance of Fungal Strains: The antagonistic strain, *Aspergillus giganteus* (8408) was procured from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, and the pathogenic fungi namely, *Cryptococcus neoformans* and *Candida albicans* was collected from PSGIMS, Coimbatore. The fungal strains were maintained on czapek yeast extract medium (CYE) at 4 ºC for storage. Before usage, the fungal strains were revived and subcultured on the same medium at 28 ± 2ºC in dark conditions for 4-7 days.

Purification of Antifungal Compounds from the Culture Filtrates of *Aspergillus giganteus*: The culture filtrates of *Aspergillus giganteus* was prepared by filtering the supernatant of the fungal culture using 0.22 µm membrane filter. The proteins in the culture filtrate were precipitated by adding ammonium sulphate using three salt cuts namely, 0-30%, 30-60%, and 60-90%. At each step of precipitation, the mixture was kept on ice for one hour and then centrifuged at 10,000 g for 20 minutes. The pellet was collected, and the amount of protein was also estimated by Lowry's method [14]. 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 precipitated protein was dialyzed in 2.5 nm dialyzed bag to remove the salts, and the fractions were collected and used for further use.

Cell Membrane Disruption Assay:

Leakage of Cellular Materials through the Pathogenic Cell Membrane: The damage and disintegration of pathogenic cell membrane have been monitored by the leakage of nuclear and cellular constituents. Cells from 100 ml 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 thrice, and resuspended in 100 ml phosphate-buffered saline with pH 7.0. The collected suspension was then treated with various concentration of purified fractions of anti-fungal

compounds grouped as PF1, PF2, PF3, PF4, and PF5 (Concentration ranging from 50-800 µg) and incubated at 28 ± 2 ºC under agitation in an environmental incubator shaker for 0, 30, 60 and 120 min. Sequentially, 2 ml of the sample was taken and centrifuged at 12000 g for 2 minutes. The concentration of leaked constituents such as DNA, RNA, protein, and glucose was determined by measuring the absorbance of treated cultures.

Measurement of Extracellular pH: The pH of the medium could be monitored to understand the damage and disruption that has occurred in the AFP-treated pathogenic strains. 100 µl of fungal suspensions were added to 20 ml czapek yeast extract broth and kept incubated in a moist chamber at 28 ± 2 ºC for 2 days. Then it was centrifuged at 4000g for 20 min, and the pellet was harvested and washed twice or thrice with sterilized double-distilled water. Subsequently, the pellet was resuspended in 20 ml sterilized double-distilled water. The range of concentration of anti-fungal proteins in the purified fractions such as PF1, PF2, PF3, PF4 and PF5 (50, 100, 200, 400 and 800 µg) was added in to the pathogenic cells and the changes in the extracellular pH was evaluated at 0, 30, 60 and 120 minutes.

Cell Membrane Integrity Assay: Lipids are the important cell membrane component, and it is necessary for the maintenance of cell membrane integrity. Total lipid content of pathogenic strains, *Cryptococcus neoformans* and *Candida albicans* cells with different concentration of purified fractions of *Aspergillus giganteus*, namely, 50 µg, 100 µg, 200 µg, 400 µg and 500 µg were determined using phosphovanillin method. The 2 day old culture from 50 ml broth was harvested and subjected to centrifugation at 4000 g for 10 min. Then the treated culture was dried with a vacuum freeze drier for 4 h. 0.1g of dry cells were homogenized using liquid nitrogen and then extracted with 4.0 ml methanol-chloroform-water mixture (2:1:0.8, v/v/v) with vigorous shaking for 30 min. The tubes were then centrifuged for 10 minutes at 4000 g. The lipid was found in the lowest phase was thoroughly mixed using 0.2 ml saline solution, and the sample was centrifuged at 4000 g for 10 min. About 0.2 ml of chloroform and lipid mixture was taken in a clean and dry tube, and 0.5 ml of H_2SO_4 was added, heated in a boiling water bath for 10 minutes. After that, 3 ml of phosphovanillin solution was added, followed by vigorous shaking and then incubation was carried out at room temperature for 10 min. The amount of lipid content in the treated pathogenic cell membrane was read at 520 nm, and cholesterol can be used as a standard for constructing a calibration curve.

Statistical Analysis: All the data were processed from triplicates and represented as mean ± SD.

RESULTS:

Purification of Antifungal Protein (AFP) from *Aspergillus giganteus*: The anti-fungal proteins in the *Aspergillus giganteus* have numerous applications in the field of clinical medicine. The proteins in the culture filtrates of *Aspergillus giganteus* get precipitated by various percentage solution of ammonium sulphate such as 0-30%, 30-60%, and 60-90%. **Table 1** explains the amount of protein concentration obtained by various steps of purification. 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, whereas the 30-60% has yielded 1.4 mg of protein concentration, and 1.8mg of proteins were obtained in the 60-90% of precipitation.

TABLE 1: CONCENTRATION OF PROTEINS DURING VARIOUS STEPS OF PURIFICATION

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

Hence, 30-60% and 60-90% precipitated pellets were further subjected to dialysis to remove the salts. Obtained pellets at 30-60% and 60-90% were dialyzed, and the protein concentration was estimated. 30-60% of dialyzed fractions were found to have a protein concentration of 0.7 mg, and 1.0 mg was obtained in the 60-90% of a dialyzed fraction. Of all, 60-90% of dialyzed fractions were taken to study the antagonistic activity by

determining the membrane damage and disruption of pathogenic strains.

Cell Membrane Disruption by AFPs of *Aspergillus giganteus*: 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 anti-fungal compounds. The anti-fungal 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 anti-fungal compounds on the pathogenic cell wall and membrane.

Leakage of Nucleic Acids through the AFP treated Pathogenic Cell Membranes: The leakage of genetic material from the pathogenic strains could be monitored to understand the mode of action of AFPs.

The partially purified fraction of *Aspergillus giganteus* was targeted the pathogenic *Cryptococcus neoformans* and *Candida albicans* cell wall and cell membranes.

The components released into the medium were read by the absorbance at 600 nm for DNA and 660 nm for RNA. The genetic material was estimated in the treated pathogenic strains with various concentrations of AFP and interval time **Fig. 1** and **2**.

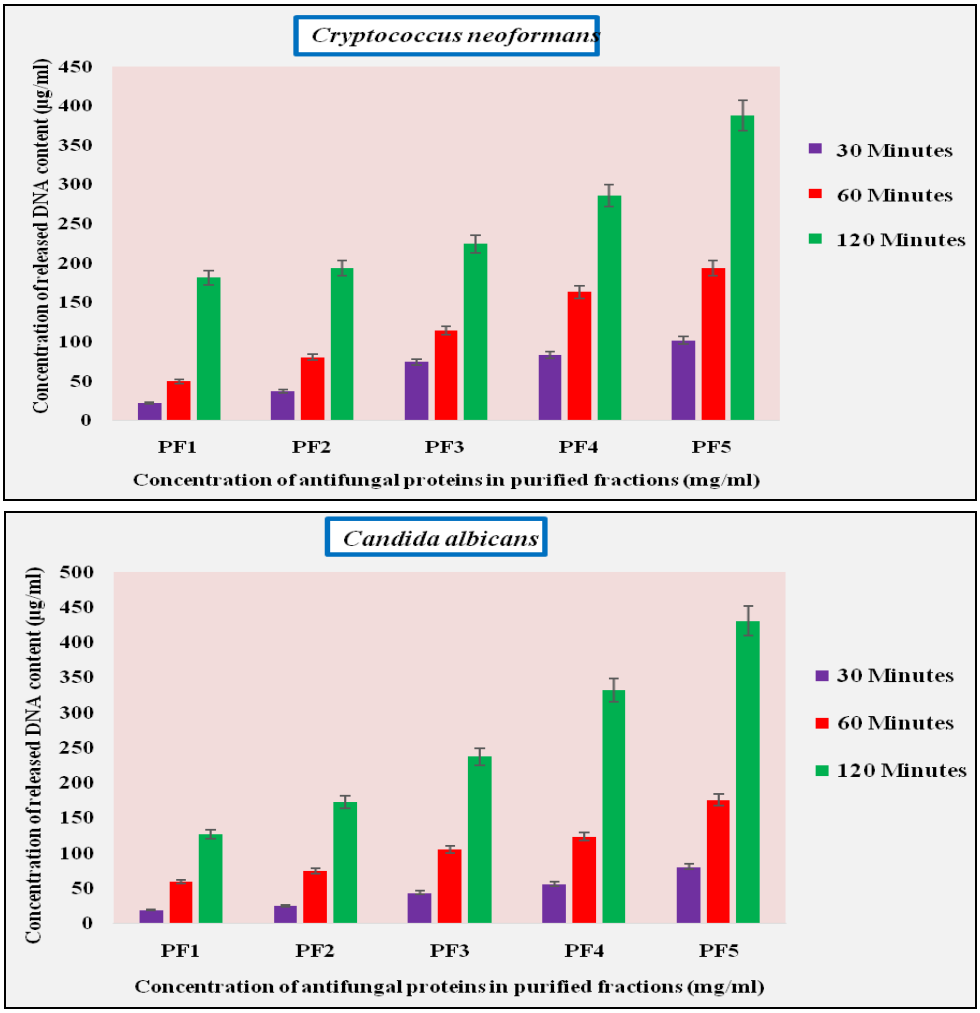

FIG. 1: LEAKAGE OF DNA FROM PATHOGENIC CELL MEMBRANE BY THE ACTION OF AFCS FROM *ASPERGILLUS GIGANTEUS* AT VARIOUS TIME INTERVALS

Leakage of more amount of DNA was observed at 120 min of treatment in the treated pathogenic *Cryptococcus neoformans*, and the concentration of elevated DNA obtained as 182.01 ± 10.63µg/ml, 194.29 ± 0µg/ml, 224.99 ± 10.6 µg/ml, 286.2 ± 16.2 µg/ml and 387.71 ± 31.91µg/ml, respectively. For treated *Candida albicans* the released DNA content at 120 min of treatment was found to be

126.73 ± 5.3 µg/ml, 172.8 ± 5.3 µg/ml, 237.28 ± 5.3 µg/ml, 332.45 ± 15.9 µg/ml and 430.64 ± 21.2 µg/ml. Like DNA, RNA was also released into the medium from the treated sample, and the concentration of released constituents was also increased in the medium with the increased concentration of AFP in the *Aspergillus giganteus*. In *Candida albicans*, the DNA and RNA were also elevated into the medium at 120 min of the treated sample and were 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 shown the membrane damage and disruption of pathogenic cells caused by the action of AFPs of *Aspergillus giganteus*.

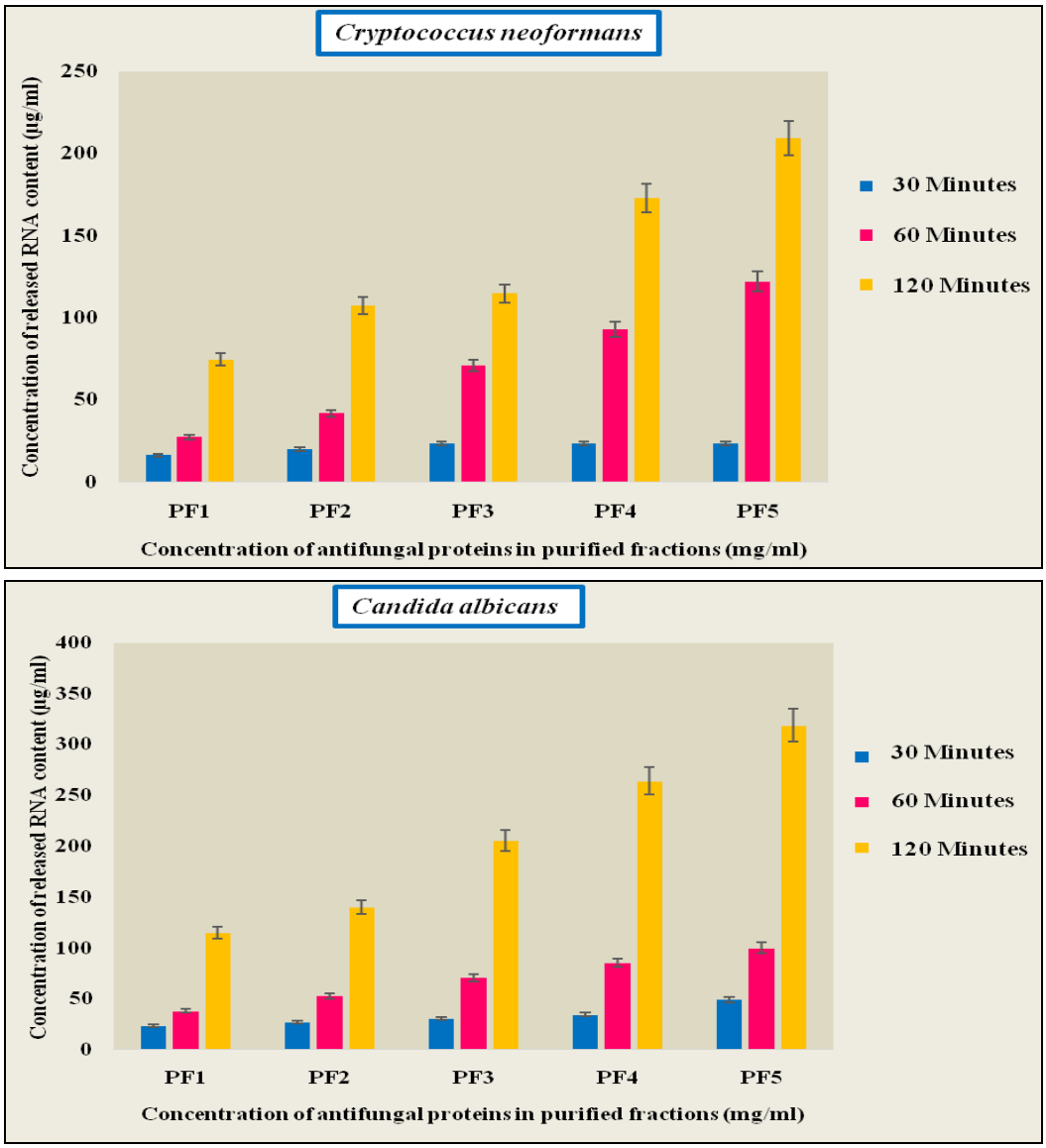

FIG. 2: LEAKAGE OF RNA FROM PATHOGENIC CELL MEMBRANE BY THE ACTION OF AFCS FROM *ASPERGILLUS GIGANTEUS* AT VARIOUS TIME INTERVALS

Leakage of Proteins from treated Pathogenic Cell Membranes: 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 anti-fungal activity. The leakage of proteins from the pathogenic cell membranes is represented in **Fig. 3**. The release of cellular protein constituents in the graph indicates the leakage of protein from the pathogenic cell membrane by the action of AFP. In this context, the result has proved that the membrane damage is directly proportional to the concentration of compounds and time needed for its action.

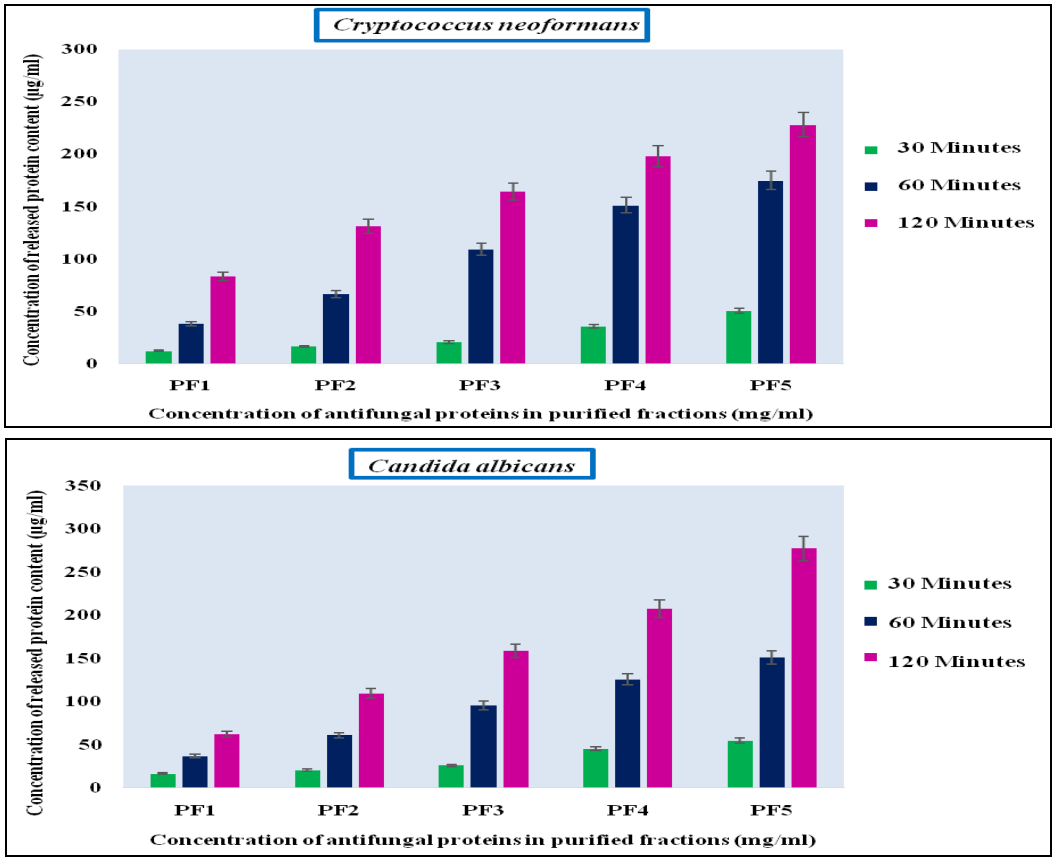

FIG. 3: LEAKAGE OF PROTEIN FROM PATHOGENIC CELL MEMBRANE BY THE ACTION OF AFCS FROM *ASPERGILLUS GIGANTEUS* AT VARIOUS TIME INTERVALS

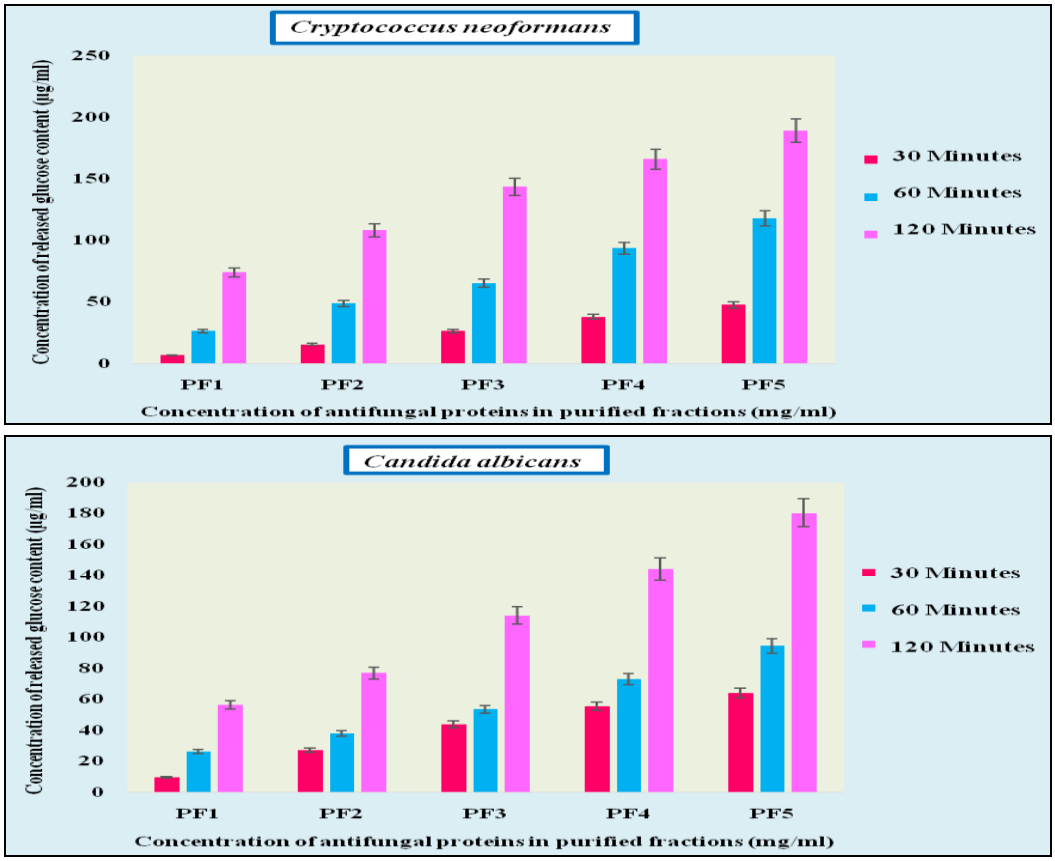

FIG. 4: LEAKAGE OF GLUCOSE FROM PATHOGENIC CELL MEMBRANE BY THE ACTION OF AFCS FROM *ASPERGILLUS GIGANTEUS* AT VARIOUS TIME INTERVALS

Leakage of Glucose through the Treated Pathogenic Cell Membranes: Glucose is considered as 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 potential anti-fungal targets. Quantification of released glucose levels in the pathogenic cells treated with different concentrations of antifungal proteins has proved the disintegration and damage of pathogenic cells **Fig. 4**. Leakage of glucose contents were increased with increased concentration of AFP of *Aspergillus giganteus* and time intervals. The 800 µg of AFPs of *Aspergillus giganteus* have shown maximum activity on the pathogenic cell membranes. Glucose level was gradually increased at 800 µg of AFP treatment in *Cryptococcus neoformans* cell membranes at different time intervals and the released glucose concentration was found to be 47.5±1.6 µg/ml, 117.84±1.6 µg/ml, 189.12±0 µg/ml whereas for *Candida albicans* 64.14±1.6 µg/ml, 94.41±1.6 µg/ml, 180.33±0 µg/ml of glucose respectively was released into the medium.

Influence of AFPs on the Extracellular pH of the Pathogenic Cells: The optimal pH for the growth and function of *Cryptococcus neoformans* is ~ 4 to 5 and for *Candida albicans* is around 4 to 7. The extracellular pH indicates the normal functioning of the cell. If any disturbances occurs in the cell that might be due to the changes of cell membrane permeability, morphological changes of the cell wall, leakage of cellular materials and disturbances in the cellular metabolism. **Fig. 5** explained the pH fluctuations in the pathogenic cells were observed with different concentration of AFP and time. Decreased pH level was noticed in the treated *Cryptococcus neoformans* with increased concentration of AFP and time interval. The pH variations were observed at 120 min of treatment was found to be 6.9 ± 0.05, 7.0 ± 0.05, 8.1 ± 0.1, 8.3 ± 0.05, and 8.7 ± 0.1 for the different concentrations of AFP. The pH was not consistent in the treated *Candida albicans* and the pH variations at 120 min for the different concentrations obtained as 8.2 ± 0.05, 7.9 ± 0.05, 7.6 ± 0.05, 7.4 ± 0.05, and 7.1 ± 0.05 respectively.

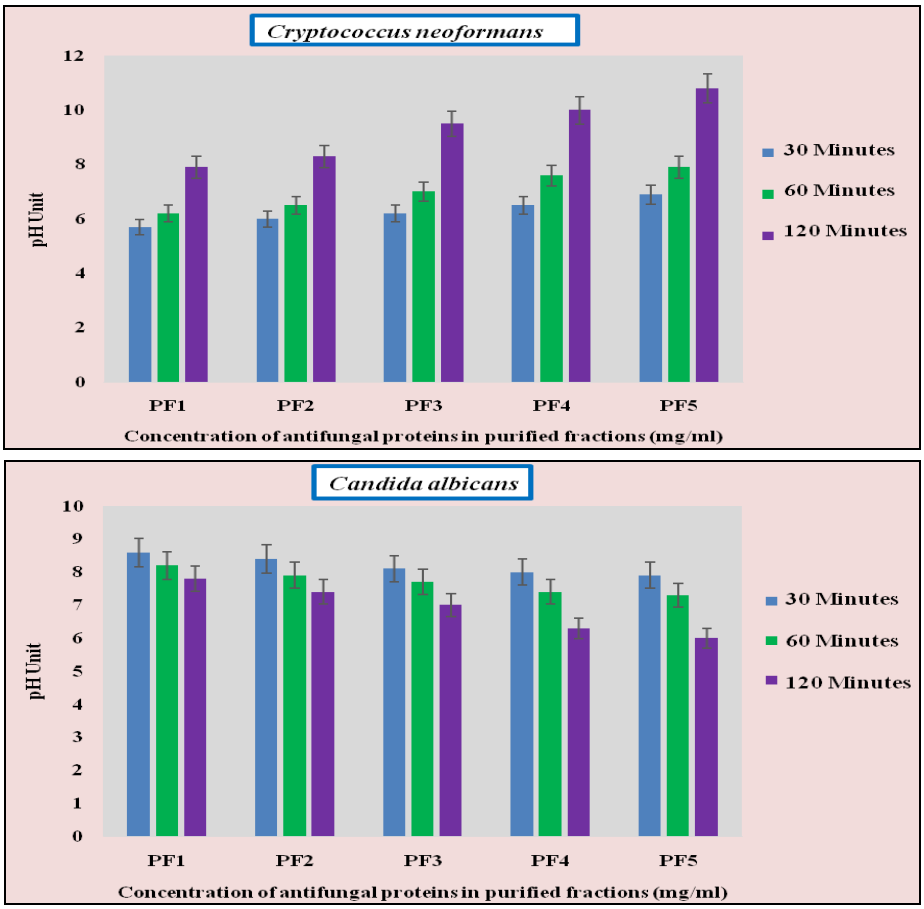

FIG. 5: ACTION OF AFCS FROM *ASPERGILLUS GIGANTEUS* ON THE PERMEABILIZATION OF IONS AND OTHER SOLUTE MOLECULES IN THE PATHOGENIC CELL MEMBRANE

Cell 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 **Fig. 6**. The cell wall of *Cryptococcus neoformans* was treated with the partially purified anti-fungal fractions of *Aspergillus giganteus* where the lipid level drastically reduced with the increased concentration of AFPs. The lipid level in the pathogenic cell wall without the addition of AFPs remains same. The lipid content in the treated *Candida albicans* cell wall also got deduced with the increased concentrations of purified fractions.

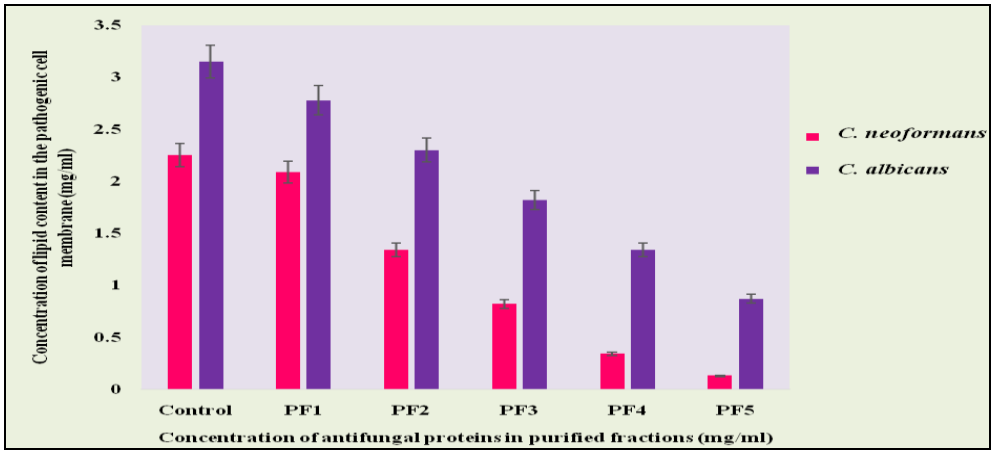

FIG. 6: LIPID CONTENTS IN THE AFCS TREATED PATHOGENIC *C. NEOFORMANS* AND *C. ALBICANS* CELL MEMBRANE

DISCUSSION: Fungal infections are considered as the major threat to human health and leads to the greatest mortality and morbidity rate worldwide. Many fungal pathogens cause deleterious effects on plants, animals, humans, and the environment when it becomes debilitated or immunocompromised [15]. The plant metabolites are well-known potential compounds for the treatment of various bacterial and fungal pathogens. Apart from other sources, filamentous fungi themselves can act as a good source for the production of anti-fungal compounds exhibiting antagonism against various fungal and bacterial pathogens [16, 2]. *Aspergillus giganteus* is a filamentous fungus, which is known to produce AFP (anti-fungal protein). Several scientific evidences also claimed that the AFP is secreted by the *Aspergillus giganteus* [17-19].

In this study, the AFP from the *Aspergillus giganteus* was purified and evaluated its antagonistic activity by determined the disruption and damage of pathogenic cell membranes. Ammonium sulphate precipitation method was used to precipitate the proteins from the *Aspergillus giganteus* and maximum yield was obtained in the 60-90% of precipitation. Numerous studies support the purification of antimicrobial from the different sources [20-23]. Membrane damage and disruption assay was performed to test the effect of anti-fungal compounds on the pathogenic cell wall and cell membrane. The leakage of nuclear and cellular components was increased with the concentration of purified proteins and time interval. The results revealed that the increased concentration of anti-fungal proteins 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 release of cellular and nuclear constituents from the treated pathogenic strains has proved the irreversible and gross morphological changes occurred in the pathogenic cell wall and cell membrane. Many studies have supported the techniques used to determine the mode of action of antagonistic compounds [19, 24].

The extracellular pH should be constant for the normal functioning of any cell. The fluctuations in the pH level of both the pathogenic fungi were noticed while treating with the AFP of *Aspergillus giganteus*. The pH changes were observed in the extracellular medium indirectly revealed that some ions, acids, alkali and/or other metabolic substances may be released into the medium due to the action of AFP. The pH value in control remains the same under different concentrations of AFCs treatment and time intervals. 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 AFPs.

CONCLUSION: The purified fractions from the *Aspergillus giganteus* showed good antagonistic activity on the pathogens by disrupting and damaging the cell wall and cell membranes. Nuclear and cellular disintegration has proved that the compounds were found to have an effect on the pathogenic progression of *Cryptococcus neoformans* and *Candida albicans*. The outcome of the study exhibited that the anti-fungal compounds from the *Aspergillus giganteus* can be an effective lead compound for the pharmaceutical industries to develop the novel drug.

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