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Research Article

Combined effect of furanone fluconazole and amphotericin B against biofilms formed from *Cryptococcus neoformans*

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Abstract:
It is of interest to document the combined effect of furanone fluconazole and amphotericin B against the biofilm formed by *Cryptococcus neoformans*. The MIC values of amphotericine B and Fluconazole were observed as 20µg/ml and 60µg/ml, respectively. The MIC for the Combination (Amphotericin B/ Fluconazole) was found to be at (15/20) µg/ml drug concentration. Thus, data shows the combined effect of furanone fluconazole and amphotericine B derivative against *C. neoformans*.

Keywords: *Cryptococcus neoformans,* fluconazole, amphotericin B, biofilm

Background:
Cryptococcus neoformans are the encapsulated fungus groups that cause meningoencephalitis in immuno-compromised individuals **[1, 3]**. Cryptococcal spores and/or dried yeast cells are very small in size and can deposit deep in the respiratory tract following inhalation **[2]**. Amphotericin B is an antifungal drug given intravenously for serious fungal infections. Amphotericin B is effective with side effects to lungs and kidneys **[4-10]**. Polysaccharide capsule of *C. neoformans* enlarge the size of capsule during infection and the mechanism of growth was unknown **[11-13]**. Therefore, it is of interest to document the combined effect of furanone fluconazole and amphotericin B against the biofilm formed by *Cryptococcus neoformans.*

Materials and Method:
C. neoformans strain ATCC 14116 (isolated from pigeon dropping contaminated soil) was maintained in Potato Dextrose Agar (PDA) (Himedia-M096) slants and Potato Dextrose Broth (PDB) (Himedia-M403). This was inoculated at 37°C for 48 hours.

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Preparation of drug stock solutions:
20mg of amphotericin B and 20mg of fluconazole was weighted and dissolved in 1ml of Dimethyl sulfoxide (DMSO) and stored at 2ºC in vials. Drug stock solution was diluted according to the culture and the DMSO concentration was equally maintained in all experiments (<1%) as shown in **Table 1**.

Table 1: Data on working drug

Treatment	Experimental type	Drug	Working stock (µg/ml of DMSO)
Individual drug	Biofilm	AMPB	20µg/ml
		FLC	100µg/ml
	Biofilm	AMPB	40µg/ml
Combinational drug		FLC	200µg/ml

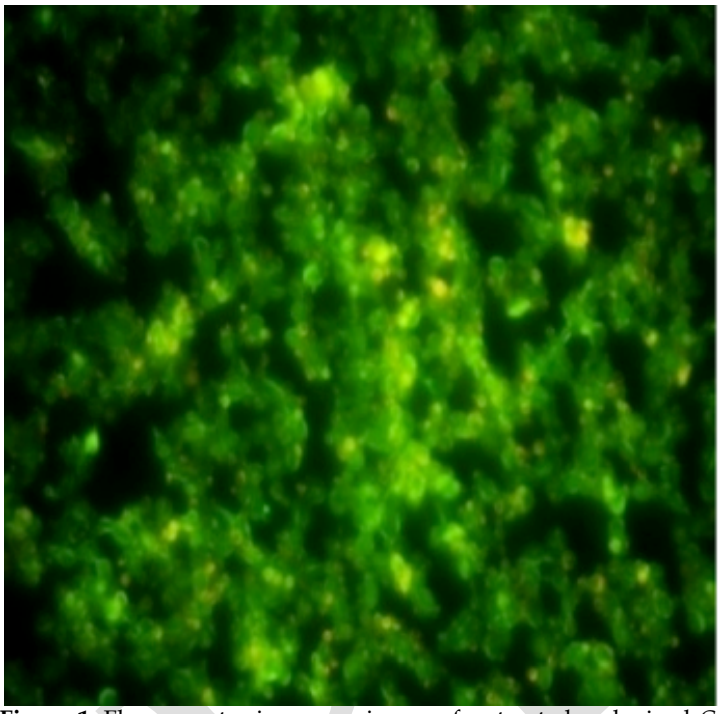

Figure 1: Fluorescent microscopy image of untreated melanized *C. neoformans* (ATCC strain) biofilm

Individual drug Treatment:
The MIC of antifungal drugs against *C. neoformans* biofilm is inoculated in a 96-well plate along with positive and negative control. This was incubated at 37ºC for 7days. The 96 well plates are washed twice with Phosphate Buffer Saline (PBS) to remove free-living cells from the plate after the biofilm formation. Drugs were then added into the wells and incubated at 37ºC for 48 hours. This was washed twice with PBS and the absorbance was measured using ELISA plate reader at 620nm.

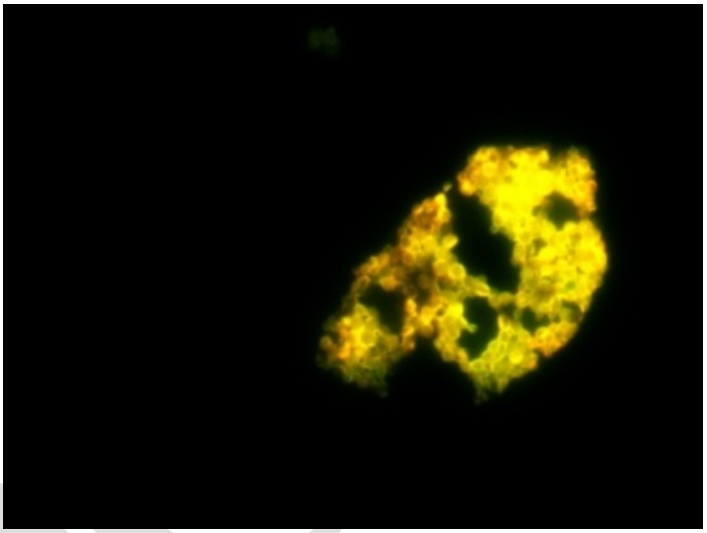

Figure 2: Fluorescent microscopy image of the combined treatment of melanized *C. neoformans* biofilm with the concentration of Amphotericin B and Fluconazole (15/20µg/ml)

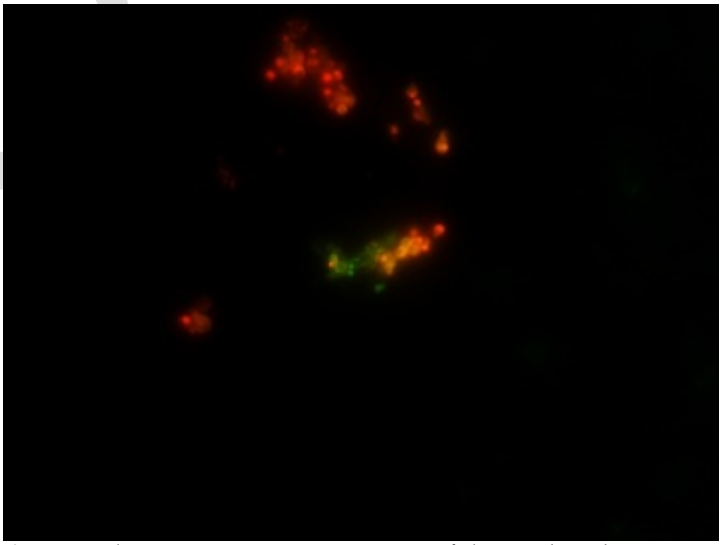

Figure 3: Fluorescent microscopy image of the combined treatment of melanized *C. neoformans* biofilm with the concentration of Amphotericin B and Fluconazole ((17.5/20µg/ml)

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Combined drug treatment:
Two fold dilution method of the drug both above and below the MIC value were prepared. The individual MIC value of amphotericin B and Fluconazole is shown in **Table 2**. The antifungal drugs were serially diluted. Equal volume of inoculums was added using the Checkerboard method. The culture were added equally to the all wells and incubated at 37ºC for 48 hours.

Table 2: MIC values of individual drugs

Strain	Drugs	Biofilm
ATCC 14116	Amphotericin B	20µg/ml
	Fluconazole	60µg/ml

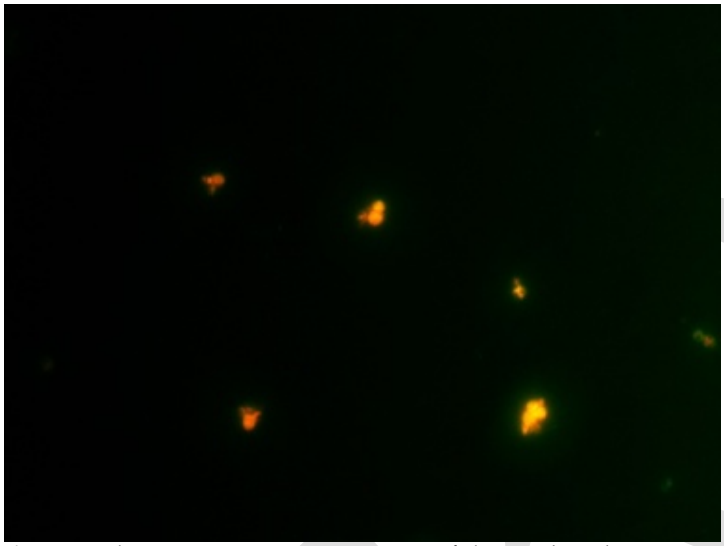

Figure 4: Fluorescent microscopy image of the combined treatment of melanized *C.neoformans* biofilm with the concentration of Amphotericin B and Fluconazole (20/20µg/ml)

Concentration of amphotericin B (µg/ml):
Amphotericin B was added in the concentration range of 0-5µg/ml. Fluconazole was added in the range of 0-25µg/ml. The plates are then incubated at 37ºC for 48 hours. The incubation plates are washed twice with phosphate buffer saline (PBS) and the absorbance was measured using a ELISA plate reader at 620nm (Automatic ELISA Reader - Sunrise)

Fluorescent Microscopy:
The cell were washed twice with PBS, the cells were stained with FITC and propidium Iodine that were prepared at the concentration of 30µg/ml respectively. The staining solutions were added and let to standing for 15min in dark. The staining solution was removed and washed with PBS. The samples were then analyzed using a Nikon Trinocular microscope (Nikon Eclipse Ni-U Japan) **[14]**.

Results & Discussion:
The concentration range of Amphotericin B and Fluconazole used for ATCC strain was 16µg/ml to 240µg/ml and 60µg/ml to 100µg/ml. The MIC value was found to be 25/15 µg/ml (Amphotericin B/Fluconazole) (**Figure 1**). The MIC value in combination is slightly higher or equal to the individual drug MIC value. The concentration of Amphotericin B increased. This may be because of the resistance shown by the organism towards the single drug. MIC of amphotericin B and fluconazole were found to be 20µg/ml and 60µg/ml respectively. The MIC for the combination (amphotericin B/fluconazole) was found to be at (15/20) µg/ml drug concentration. The control of both the strains showing green fluorescent indicating live cells as shown in **Figure 2 to 4**. It was observed that the combination drug treatment is capable of disrupting the biofilm with killing the cells as well as disintegrate the biofilm. Biofilm formation has an important role for the *C.neoformans* to survive within the macrophages and to colonize in the central nervous system (CNS) **[15].** Thus, disrupting the biofilm help to reducing the virulence nature to control the pathogen at the site of infection **[16-17]**.

Conclusion:
The data shows the combined effect of furanone fluconazole and amphotericine B derivative against *C. neoformans* by disrupting the formation of biofilms.

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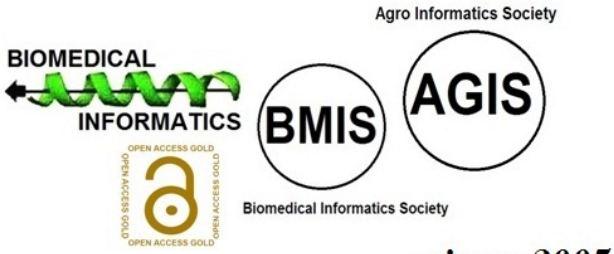

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