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# Evaluation of Amphotericin B in Combination with Other Compounds against Aspergillus Species

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Invasive aspergillosis is concomitated with high mortality and morbidity. The situation worsened after the COVID-19 pandemic as Aspergillus co-infections emerged in critically ill patients, further complicating outcomes and highlighting the need for enhanced diagnostic and therapeutic strategies. Current guidelines recommend voriconazole and isavuconazole for treating Aspergillus infections. However, the efficacy of these treatments is compromised by the emergence of azole-resistant Aspergillus species, posing significant challenges to effective management and requiring alternative therapeutic strategies. In this scenario, combination therapy is a potential approach for the management of Aspergillus infection. In our study, we investigate in-vitro Amphotericin B interaction with other compounds against various Aspergillus strains. The minimum Inhibitory Concentrations of the tested compounds were determined by micro-broth dilution assay of the tested and for Amphotericin B it ranged between 0.488-1.95mg/L for Aspergillus fumigatus, 0.488-.1.95 mg/L for Aspergillus niger and Aspergillus flavus and .976 mg/L for Aspergillus terreus. The interaction of Amphotericin B with other compounds was evaluated by Chequerboard assay, Fractional Inhibitory Concentration Index. The efficacy of the synergy and cytotoxicity were evaluated by Time-kill kinetics and haemolytic assay, respectively. Amphotericin B combinations with other compounds exhibit variable activity, offering the potential for enhanced antifungal efficacy, and optimized treatment strategies.
**Keywords:** Aspergillosis, Combination Therapy, Time-Kill Kinetics, Chequerboard Assay, Haemolysis Assay

## 1. Introduction

Invasive fungal infections (IFIs) are rapidly increasing throughout the world, especially in immunocompromised patients with underlying haematological disease, solid organ transplant (SOT) recipients, cancer, autoimmune diseases, and critically ill patients, including with respiratory viral infections like COVID-19, and AIDS (Acquired Immune Deficiency Syndrome) which extremely compromise the host immune system and causes life-threatening mycoses and mortality [[1]]. Invasive fungal infections affect approximately 12 million lives of people every year, and the death rate is approximately 1.5 million [[1]]. Fungal species such as Aspergillus, Candida, Mucor and Cryptococcus are the common fungal pathogens concomitant with human health [[2]]. Aspergillus species are ubiquitous and one can easily get infected by simply inhaling fungal spore and can survive a broad range of temperatures and pH, and their hydrophobic cell wall allows spore dispersal by air [[3]]. Aspergillus species such as Aspergillus fumigatus, Aspergillus niger, Aspergillus flavus, and Aspergillus terreus are the mainly involved in the invasive aspergillosis. Recently, WHO listed Aspergillus fumigatus as a critical pathogen in the public health care system [[4]]. Aspergillosis mainly affects the lungs, and general symptoms are cough, fever, shortness of breath, chest pain, and haemoptysis and sometimes it can spread to other body organs [[5]]. Management of infection includes early detection, immunosuppressive therapy reduction, antifungal treatment and sometimes surgery also used to manage the infection.

According to the recent treatment guideline of aspergillosis azoles such as voriconazole and isavuconazole are used as first-line antifungal agents for treatment, however, the excess use of azole in agriculture (azole fungicides) and clinics induces resistance to azoles. The selective pressure causes

azole resistance in Aspergillus fumigatus and subsequently, azole-resistant invasive aspergillosis and that further limits the treatment [[3]]. Therefore, combination therapy provides a new insight into infection management. New treatment guidelines favour the use of Amphotericin B (AmB) in clinical settings with high incidences of azole resistance. Amphotericin B is a polyene, which inhibits the biosynthesis of fungal cell membranes by binding with ergosterol resulting in leakage of cellular components and cell lysis [[6]]. In clinics, antibiotic synergetic interactions were used for the treatment of Malaria, Cancer and HIV. The successful treatment of cryptococcal meningitis by using Amphotericin B in combination with 5-flucytosine provides a new area to explore for researchers [[1]]. A study of isavuconazole in combination with cyclosporin A (antibacterial) shows synergy against Aspergillus niger isolates, whereas against other Aspergillus isolates combination shows indifference [[7]]. In a recent study, fluconazole in combination with doxycycline acetate (antibacterial) was tested against dual species culture of Candida albicans and Staphylococcus and showed synergy against the tested pathogens [[8]]. Therefore, we intend to investigate the AmB interaction with other compounds against Aspergillus strains. The drug interaction with other compounds was determined by using a chequerboard assay, for the cytotoxicity study, a haemolytic assay was performed and the time-kill kinetics assay was used to evaluate the efficacy of the tested combination.

## 2. Material Methods

**Antifungal Tested:** The Amphotericin B (AmB) (polyene), the Sulfamethoxazole (Sul) (Sulfonamides), Folic Acid (FoA), Sodium Salicylate (SoS), (HiMedia Laboratories Pvt. ltd., India) and Ebselen (Eb), Acetylsalicylic acid (AA), Farnesol (Fa) were obtained (Sigma-Aldrich) and used for antifungal testing. The 2mg/ml stock solution of the drugs was prepared by dissolving in 4% DMSO and diluting further to get the desired concentration in distilled water. It was established DMSO did not affect the studied species growth.

**Pathogens and Inoculum Preparation:** In our study, we use the Sabouraud Dextrose Agar and Broth media (SDA and SDB), and RPMI-1640 medium for fungal culture (HiMedia laboratories Pvt. ltd., India). The pathogens were obtained from ITCC (IARI, Delhi) and clinical isolates from PGIMS (Rohtak) India. The strains employed in study were Aspergillus fumigatus ITCC 4517, ITCC 6050, ITCC 4448, ITCC 1628, and Clinical isolate PGIMS, Aspergillus niger ITCC 3002, ITCC 6219, ITCC 5405, Clinical isolate PGIMS, Aspergillus flavus ITCC 5076, ITCC 5192, Clinical isolate PGIMS, and Aspergillus terreus Clinical isolate PGIMS. All the strains were cultured at 37$^{o}$C for 48 hours in SDA. The spores were isolated from SDA plates, suspended in tween-20 0.25%, and 0.85% NaCl solution. The spore concentration was calculated as per EUCAST and CLSI protocols, the working concentration of spores being set to $10^3$-$10^4$ spore/ml.

**In-vitro antifungal and combination testing:**

**Disk diffusion Assay:** The drug solution was diluted and used to test its activity against various strains of A. fumigatus, A. niger, A. flavus, and A. terreus by employing CLSI protocol with some modifications. The Petri plates of SD Agar media (90mm diameter) were inoculated in a spore solution of $10^3$-$10^4$ spores/ml concentration and the plates were allowed to dry. The Whatman grade 4 filter paper 6mm diameter disk was positioned on the SD agar plate, and the discs were saturated with 20µl tested compounds. For the assessment of combinatory effects, the discs were saturated with 10µl of each drug, so the final volume of the drug combination was 20µl on the disc. The plates were incubated at 37$^{0}$C for 24 hours. The area surrounding the disk with no fungal growth was considered the zone of inhibition (ZOI). The efficacy test was performed in triplicates for reproducibility and the ZOI was calculated as means ± standard deviations [[9],[10],[11]]. The results of the disk assay were stated as the ZOI percentage.

$$\text{ZOI} = \frac{\text{Zone Of Inhibition in mm}}{\text{90mm}} \times 100\%$$

**Broth microdilution assay:**

This assay determines the Minimal inhibitory concentration (MIC) values. MICs are defined as the lowest concentration of an antifungal that inhibits the growth visibly of fungal culture after incubating overnight. The 90µl volume of diluted concentration was added to a 96-well microtiter plate containing

90µl RPMI-1640 media with MOPS ([3-(N-morpholino)]-propane sulfonic acid) and then carried out serial dilution. The first and second lane of the 96-well plate was taken as negative (media only) and positive (spore + media) control, respectively. The wells were supplemented with 20µl of fungal culture $10^3$-$10^4$ spores/ml concentration and the plates were incubated at 37°C for 24 hours. The optically clear well concentration was considered as the MIC. For the reproducibility of the experiment, the test was carried out in triplicates [[9],[10],[11]].

**Chequerboard Assay:**
In-vitro interaction of Drug A with Drug B determined by microdilution chequerboard assay. For a 2-D chequerboard, 45 µl of each Drug A concentration was mixed with 45 µl of each concentration of the second tested compound. The dilution of the drug was prepared 2X of the desired concentration. In the chequerboard assay, 45 µl each concentration of Drug A was added into columns 3 to 11 in 96-well microtiter plates and 45 µl of each concentration of the second tested compound was added to the A to G row. Column 12 contained only Drug A and the second tested compound only in row H. Row 1 and 2 were taken as negative (spore and drug-free) and positive control (drug-free), respectively. Afterwards, 20 µl of fungal culture $10^3$-$10^4$ spores/ml concentration inoculum was added to each well and the plates were incubated at 37°C for 24 hours. Each combination experiment was tested in triplicates. For visualization, each well was dispensed with 20 µl resazurin dye (concentration 1mg/ml).

**Fractional inhibitory concentration index (FICI):**
The interaction among the tested compounds was evaluated using a non-parametric approach: the FICI model [[7]]. The FICI was calculated by the formula given below.

$$\text{FICA} = \frac{\text{MIC of A in presence of B}}{\text{MIC of A alone}}$$

$$\text{FICB} = \frac{\text{MIC of B in presence of A}}{\text{MIC of B alone}}$$

FICI=FICA+FICB
The Fractional Inhibitory Concentration Index (FICI) assesses drug interactions: Synergy (FICI ≤ 0.5): Combined effects exceed the sum of individual effects, showing enhanced activity. Additivity (0.5 < FICI ≤ 1): Effects are equal to the sum of individual effects, indicating independent but cumulative action. Indifference (1 < FICI ≤ 4): Combined effects fall between additive and antagonistic, showing no significant interaction. Antagonism (FICI > 4): Combined effects are less than individual sums, indicating interference.

**Spore Germination Inhibition Assay (SGIA):**
The SGI assay was performed in 50ml conical flasks. The 1/2 MIC, MIC, ½ FICI and FICI of the tested drugs and combination were added in flasks containing $10^3$-$10^4$ spores/ml in SD broth and the flasks were incubated at 37°C 130rpm for 7-8 hours. The spore suspension of 10 µl (100 ± 8 spores) was then poured on the haemocytometer and inspected under an inverted microscope for spore germination. The germinated and non-germinated spores number counted in each grid of the haemocytometer. The percentage of spore germination inhibition (PSGI) was calculated by:

$$\text{PSGI} = 100 - \frac{\text{No. of spores germinated in drug treated well}}{\text{No. of spores germinated in control well}} \times 100\%$$

The lowest concentration of compounds, which inhibits ≥90% spore germination, was considered $MIC_{90}$ [[12]].

**Time-Kill Kinetics Assay:**
For time-kill kinetic analysis, 50 µl spore suspension ($5x10^3$-$10^4$ spore/ml) in RPMI-1640 media was added to the 96-well microtiter plate. After that 50µl of ½ MIC, MIC of Drug A and Drug B were dispensed in the wells and for combination ½ FICI, FICI and 2FICI were added in the wells and finally, $10^3$-$10^4$ spore/ml concentration of the fungal spores were exposed to Drug A and Drug B alone and in combination. The readings were taken on 490nm wavelength at 2, 4, 6, 12, 16, 24, 30 and 48 hours. The activated XTT reagent (HiMedia EZcountTM XTT cell assay kit) volume of 50 µl was added to each well before 2 hours of the readings. The wells without drugs were taken as a positive control. The experiment was repeated in triplicates and the results were expressed in spore/ml. The kill curve plotted

spore/ml against the time. The decrease in $\log_{10}$ spore/ml >3 log units indicates the fungicidal effect of the tested combination [[14],[15],[16],[17],[17]].
Statistical analysis of the time-kill kinetic assay was performed by using GraphPad Prism (version 10.0.2). The results were expressed as means of three replicates ± standard deviation (SD) and subjected to two-way ANOVA. For statistical significance, the P-value < .05 was considered.

**Hemolytic Assay:**
The compatibility of the tested combination was assessed by using a hemolytic assay [[18][18]]. The erythrocytes from the healthy human volunteer were taken and stabilized with heparin. The 3ml of heparin stabilized blood sample was added with 14ml of Dulbecco's Phosphate-Buffered Saline (D-PBS) and the mixture was centrifuged at 5000rpm for 10min for isolation of the erythrocytes. After this, the erythrocytes were 4-times washed with 15ml of D-PBS. The 1ml of 2% diluted erythrocytes suspension was exposed to 2ml of the tested drugs alone and in combination dissolved in D-PBS, at different concentrations, the positive control was 10% triton-X and the negative control was D-PBS. After incubating at room temperature for 3 hours, further centrifuged at 5000rpm for 10min and at 540nm absorbance was taken on UV-Vis Spect Lambda Bio 20 Perkin-Elmer.

$$\text{Haemolysis Ratio} = \frac{(\text{OD of test combination} - \text{OD of negative control})}{(\text{OD of positive control} - \text{OD of negative control})} \times 100\%$$

The results were expressed as a percentage of haemolysis and 10% haemolysis was taken as the endpoint (more than 10% haemolysis was taken as toxic).

## 3. Results

**In-vitro antifungal Testing and Combination Testing:**

**Disk Diffusion Assay:**
The antifungal activity of drugs AmB, Eb, SoS, AA, Fa, and Sul was evaluated on thirteen Aspergillus strains as stated in Table 1. The ZOI shown by AmB ranges from 21.2±0.3 to 29.7±0.1, Eb shows ZOI ranges from 16.7±0.2 to 29.3±0.2, whereas Sul shows ZOI ranges 9.5±0.1 to 13.9±0.1. The compound AA, Fa, FoA, and SoS against Aspergillus strains show no visible zone of inhibition. The ZOI of AmB in combination with Sul ranges from 33.4±0.1 to 39.3±0.1. The ZOI results in mm with Mean Standard Deviation (SD) are expressed in Table 1 and the percentage of ZOI of compounds alone and AmB + Sul is summarized in Figure 1.

Table 1: Zone of Inhibition of tested compounds against Aspergillus strains.

| **Aspergillus Strain** | **Zone of Inhibition Mean Diameter (mm) ±SD** | | | |
|---|---|---|---|---|
| | **AmB** | **Eb** | **Sul** | **AmB + Sul** |
| **ITCC 4448** | 26.7±0.1 | 24.7±0.2 | 13.5±0.2 | 36.4±0.1 |
| **ITCC 1628** | 23.2±0.3 | 16.7±0.2 | 12.1±0.1 | 38.3±0.2 |
| **ITCC 4517** | 21.5±0.2 | 25.2±0.3 | 10.1±0.2 | 36.3±0.1 |
| **ITCC 6050** | 22.9±0.2 | 28.2±0.2 | 13.6±0.1 | 35.5±0.2 |
| **ITCC 3002** | 21.7±0.1 | 23.2±0.2 | 10.5±0.2 | 33.4±0.1 |
| **ITCC 6219** | 21.5±0.2 | 21.7±0.1 | 10.1±0.1 | 34.3±0.2 |
| **ITCC 5405** | 21.2±0.3 | 17.7±0.1 | 9.5±0.1 | 34.7±0.1 |
| **ITCC 5076** | 22.7±0.1 | 19.1±0.1 | 13.8±0.2 | 35.2±0.2 |
| **ITCC 5192** | 21.3±0.3 | 29.1±0.2 | 13.9±0.1 | 36.4±0.1 |
| **A. fumigatus PGIMS** | 22.7±0.2 | 19.7±0.1 | 11.9±0.2 | 39.1±0.1 |
| **A. niger PGIMS** | 23.2±0.3 | 22.1±0.1 | 10.2±0.1 | 38.1±0.2 |
| **A. flavus PGIMS** | 21.7±0.2 | 29.2±0.1 | 11.1±0.1 | 34.8±0.1 |
| **A. terreus PGIMS** | 29.7±0.1 | 29.3±0.2 | 12.9±0.1 | 39.3±0.1 |

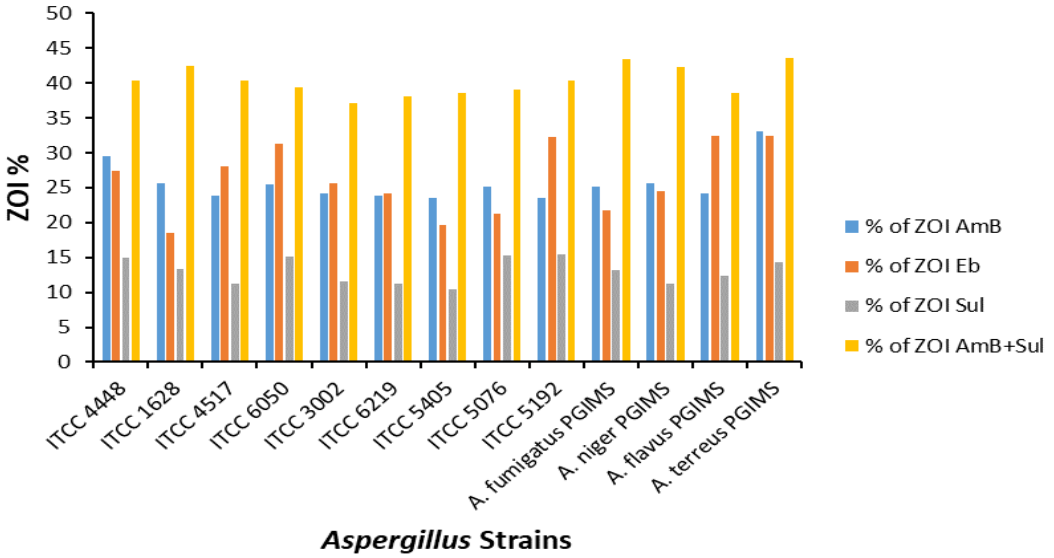



Figure 1: The percentage of ZOI of Amphotericin B (AmB), Ebselen (Eb), Sulfamethoxazole (Sul), and Amphotericin B + Sulfamethoxazole (AmB + Sul).

**Broth Microdilution Assay:** The MIC of AmB, Eb, SoS, AA, Fa, FoA, and Sul was determined by using a broth microdilution assay. The MIC of various tested compounds is summarized in Table 2. SoS, FoA and Fa show no visible growth inhibition against the tested Aspergillus strain. The MIC of AmB ranges between 0.488-1.95mg/L for A. fumigatus, 0.488-.1.95 mg/L for A. niger and A. flavus and .976 mg/L for A. terreus, whereas Eb MIC ranges between 0.244-7.81 mg/L for A. fumigatus, 0.244-3.906 mg/L for A. flavus, 0.976-7.81 mg/L for A. niger, and 0.244 mg/L for A. terreus. AA shows activity at 1000mg/L against A. fumigatus ITCC 4448 and A. niger ITCC 3002 and 5405, whereas AA shows no activity against other Aspergillus strains. The MIC of Sul against A. fumigatus 62.5 mg/L, .125-.250 mg/L for A. niger, 62.5 mg/L for A. flavus and .125 for A. terreus. In our study, we found that SoS, Fa, and FoA did not show any activity against any tested Aspergillus strain.

Table 2: Minimum Inhibitory Concentration in mg/L of various tested compounds against Aspergillus strains.

| **Aspergillus Strains** | **Minimum Inhibitory Concentration in mg/L** | | | |
|---|---|---|---|---|
| | **AmB** | **Eb** | **AA** | **Sul** |
| **ITCC 4448** | 0.488 | 0.976 | 1000 | 62.5 |
| **ITCC 1628** | 0.488 | 7.81 | - | 62.5 |
| **ITCC 4517** | 0.976 | 0.488 | - | 62.5 |
| **ITCC 6050** | 0.488 | 0.244 | - | 62.5 |
| **ITCC 3002** | 0.488 | 0.976 | 1000 | .125 |
| **ITCC 6219** | 0.976 | 0.976 | - | .125 |
| **ITCC 5405** | 0.976 | 7.81 | 1000 | .250 |
| **ITCC 5076** | 0.488 | 3.906 | - | 62.5 |
| **ITCC 5192** | 1.95 | 0.244 | - | 62.5 |
| **A. fumigatus PGIMS** | 1.95 | 3.906 | - | 62.5 |
| **A. niger PGIMS** | 1.95 | 0.976 | - | .250 |
| **A. flavus PGIMS** | 1.95 | 0.244 | - | 62.5 |
| **A. terreus PGIMS** | 0.976 | 0.244 | - | .125 |

**Chequerboard Assay:**
The interaction among AmB and Eb, AA, SoS, Fa, FoA, and Sul were interpreted by the FICI model. AmB in combination with Eb shows indifference ($1<FICI\leq4$) against A. fumigatus ITCC 4448, 1628, 6050 and clinical isolate, A. niger ITCC 3002, 5405 and 6219, A. flavus ITCC 5076 and 5192 and

clinical isolate A. terreus, whereas against A. fumigatus ITCC 4517, clinical isolate A. niger and A. flavus shows antagonism (FICI>4) (Table 3). AmB in combination with SoS, FoA, AA and Fa shows antagonism (FICI>4) against all tested Aspergillus strains (Table 3).

Table 3: FICI of Tested Combination against Aspergillus Strains.

| **Aspergillus Strain** | **FICI= FIC A+ FIC B (FICI ≤0.5 = synergy; 0.5<FICI≤1= additive; 1<FICI≤4 = indifferent, FICI>4 = antagonistic)** | | | | |
|---|---|---|---|---|---|
| | **AmB + Eb** | **AmB + SoS** | **AmB + FoA** | **AmB + AA** | **AmB + Fa** |
| **ITCC 4448** | 3.12 | 8.12 | 4.12 | 8.62 | 6.37 |
| **ITCC 1628** | 2.56 | 5.45 | 4.94 | 4.20 | 6.12 |
| **ITCC 4517** | 4.12 | 6.20 | 5.72 | 4.45 | 6.25 |
| **ITCC 6050** | 2.25 | 8.12 | 5.25 | 8.50 | 6.12 |
| **ITCC 3002** | 3.02 | 8.62 | 6.12 | 8.37 | 6.54 |
| **ITCC 6219** | 2.50 | 4.40 | 6.62 | 4.55 | 6.12 |
| **ITCC 5405** | 2.15 | 5.25 | 8.12 | 6.70 | 6.94 |
| **ITCC 5076** | 2.12 | 8.12 | 4.50 | 8.50 | 8.02 |
| **ITCC 5192** | 2.50 | 8.62 | 4.82 | 4.25 | 8.42 |
| **A. fumigatus PGIMS** | 3.95 | 8.72 | 5.12 | 7.12 | 9.12 |
| **A. niger PGIMS** | 4.12 | 8.14 | 8.66 | 9.25 | 9.44 |
| **A. flavus PGIMS** | 4.82 | 8.25 | 8.82 | 5.20 | 8.94 |
| **A. terreus PGIMS** | 3.62 | 8.82 | 7.12 | 8.37 | 8.76 |

FICI of AmB in combination with Sul ranges from .18 to .25 for A. fumigatus, .18 to .40 for A. niger, .18 for A. flavus and .25 for A. terreus. The value of FICI ≤ .5 shows the interaction between two compounds is synergistic, so, AmB in combination with Sul against all tested Aspergillus strains shows synergy (Table 4).

Table 4: Interaction of AmB with Sul against Aspergillus Strains.

| **Aspergillus Strain** | **FICI= FIC A+ FIC B (FICI ≤0.5 = synergy; 0.5<FICI≤1= additive; 1<FICI≤4 = indifferent, FICI>4 = antagonistic)** | **Interpretation** |
|---|---|---|
| **AmB + Sul** | | |
| **ITCC 4448** | **.06+.12=.18** | **Synergy** |
| **ITCC 1628** | **.125+.125=.25** | **Synergy** |
| **ITCC 4517** | **.06+.12=.18** | **Synergy** |
| **ITCC 6050** | **.06+.12=.18** | **Synergy** |
| **ITCC 3002** | **.15+.25=.40** | **Synergy** |
| **ITCC 6219** | **.06+.12=.18** | **Synergy** |
| **ITCC 5405** | **.03+.25=.28** | **Synergy** |
| **ITCC 5076** | **.06+.12=.18** | **Synergy** |
| **ITCC 5192** | **.06+.12=.18** | **Synergy** |
| **A. fumigatus PGIMS** | **.06+.12=.18** | **Synergy** |
| **A. niger PGIMS** | **.06+.25=.31** | **Synergy** |
| **A. flavus PGIMS** | **.06+.12=.18** | **Synergy** |
| **A. terreus PGIMS** | **.125+.125=.25** | **Synergy** |

**Spore Germination Inhibition Assay:**

The AmB, and Sul alone and in combination inhibited the germination of clinical isolate of A. fumigatus spores (figure 2). The spore germination was studied under an inverted microscope and the spore number was counted by haematocytometer. The germinated spore count in control was (100±5) 94, whereas germinated spore count in ½ MIC AmB, ½ MIC Sul and ½ FICI AmB + Sul were 31, 43, and 22, respectively, and in MIC AmB, MIC Sul and FICI AmB + Sul were 8, 9 and 4, respectively. So, it was evaluated that ½ MIC AmB, ½ MIC Sul and ½ FICI AmB + Sul inhibit 67.02%, 54.26% and 76.60% spore germination, respectively and MIC AmB, MIC Sul and FICI AmB + Sul inhibit 91.49%, 90.43% and 95.75% spore germination, respectively. The inhibited concentration was ≥90% of spore germination, which is considered $MIC_{90}$.

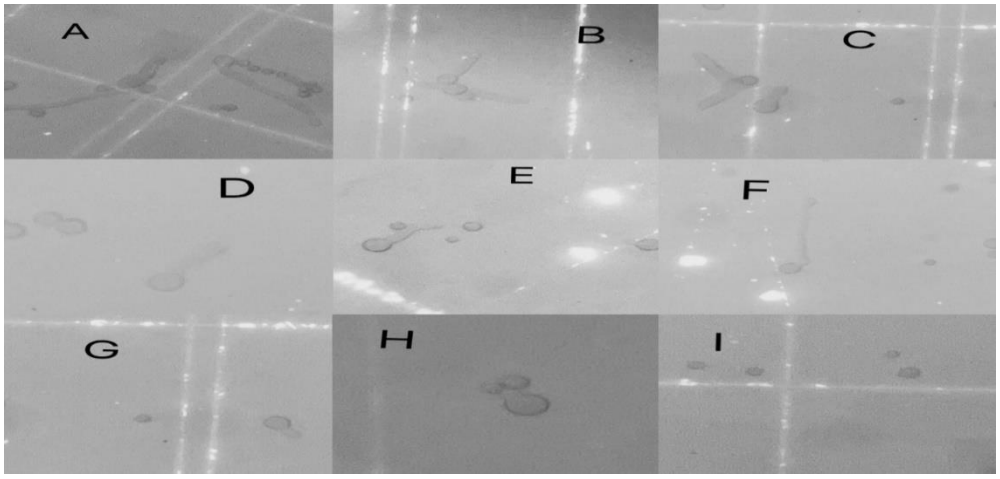
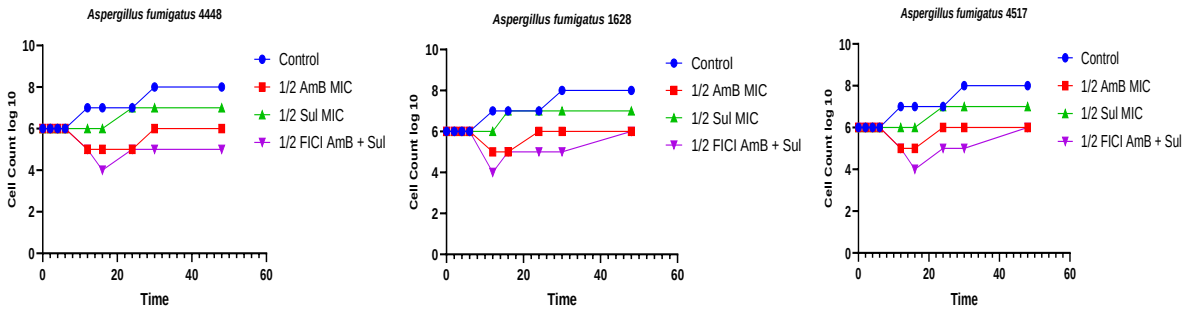


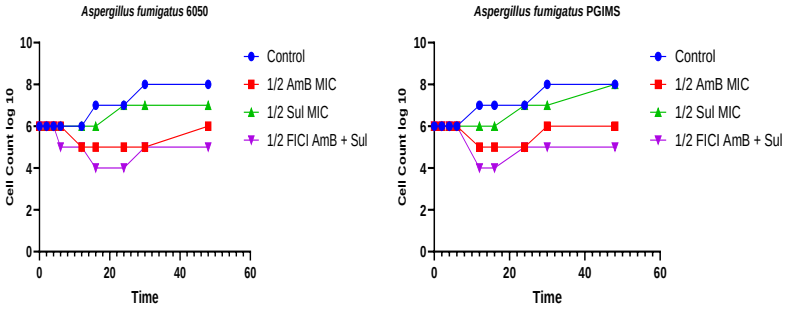

Figure 2: Germinating and non-germinating spore of Aspergillus fumigatus clinical isolate. Figure 2.A Control, 2.B ½ MIC Sul, 2.C ½ MIC AmB, 2.D ½ FICI AmB+Sul, 2.E MIC Sul, 2.F MIC AmB and 2.G, 2.H and 2.I are the FICI AmB+Sul.

**Time-Kill Kinetics Assay:**

The synergistic interaction between AmB and Sul was established by using the time-kill kinetics assay. The representative plot of the log10 spore/ml versus time for AmB and Sul alone and in combination shows a synergistic interaction between the drugs. The time-kill curve of ½ FICI AmB+Sul shows fungistatic results (figure 3-6), but at FICI of the combination, there was a decrease in log10 conidia/mL > 3 log units after 24h which indicates the combination is fungicidal (7-10). Figure 3-10 shows the efficacy of AmB in combination with Sul over AmB and Sul alone.

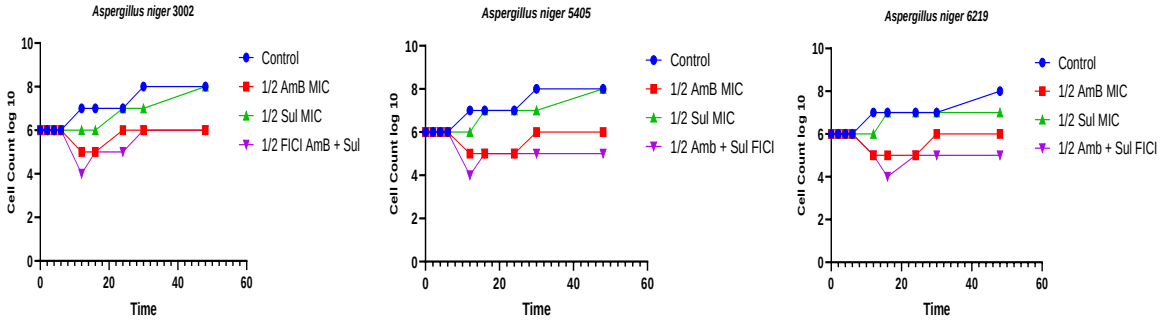



**A** **B** **C**

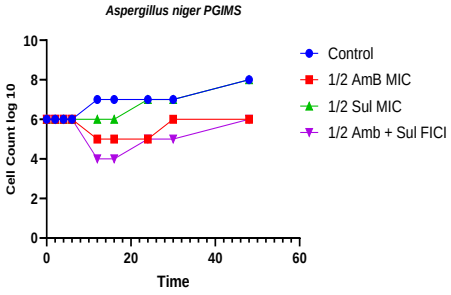



**D** **E**

Figure 3: Time Kill Curve of ½ MIC of AmB and Sul alone and ½ FICI AmB + Sul against Aspergillus fumigatus strains.

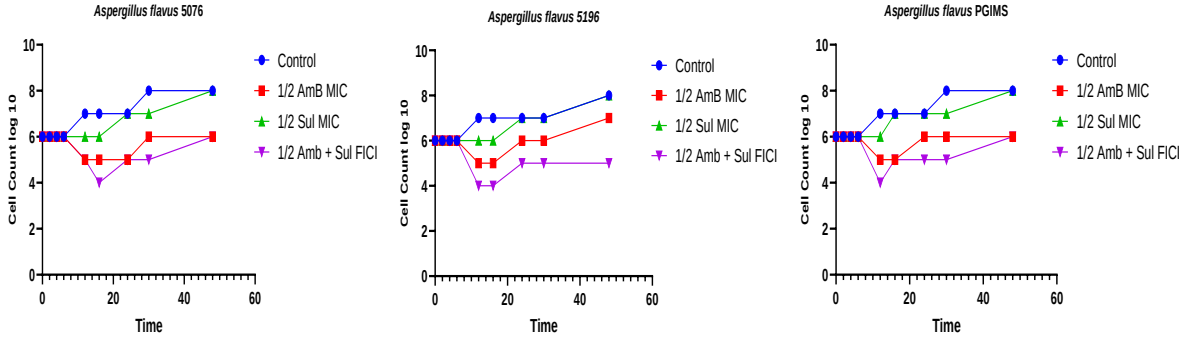



**A** **B** **C**



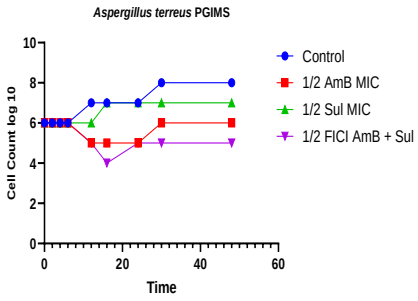

**D**

Figure 4: Time Kill Curve of ½ MIC of AmB and Sul alone and ½ FICI AmB + Sul against Aspergillus niger strains.



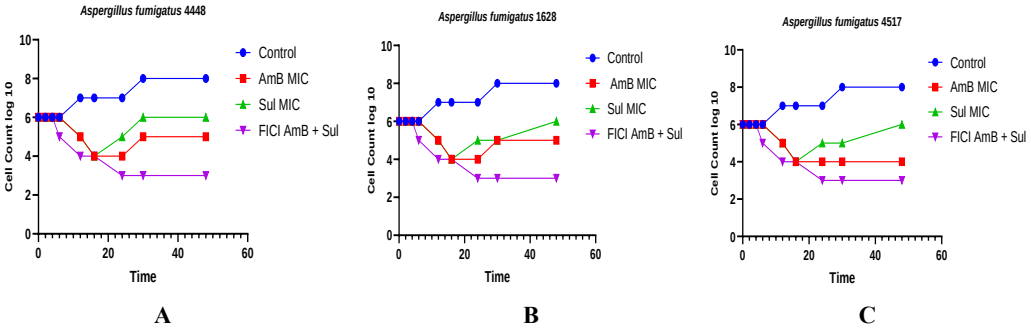

**A** **B** **C**

Figure 5: Time Kill Curve of ½ MIC of AmB and Sul alone and ½ FICI AmB + Sul against Aspergillus flavus strains.

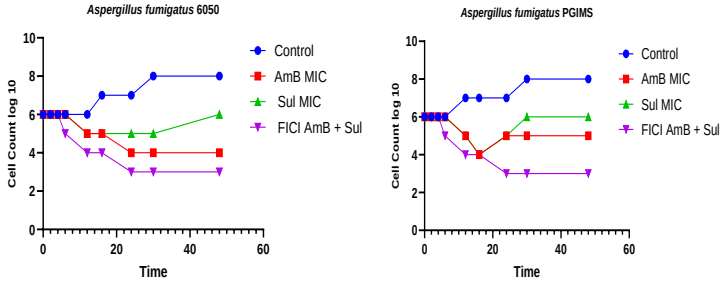



**A**

Figure 6: Time Kill Curve of ½MIC of AmB and Sul alone and ½ FICI AmB + Sul against Aspergillus terreus.



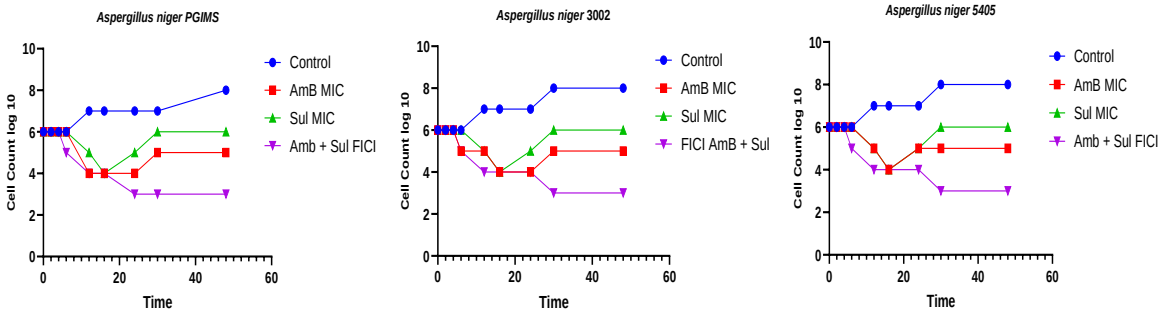

**A** **B** **C**

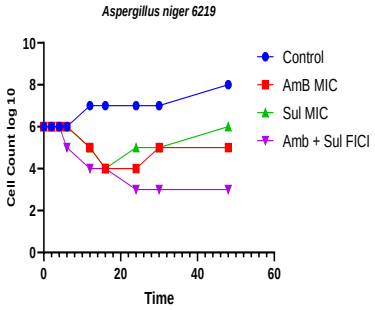



**D** **E**

Figure 7: Time Kill Curve of MIC of AmB and Sul alone and FICI AmB + Sul against Aspergillus fumigatus strains.

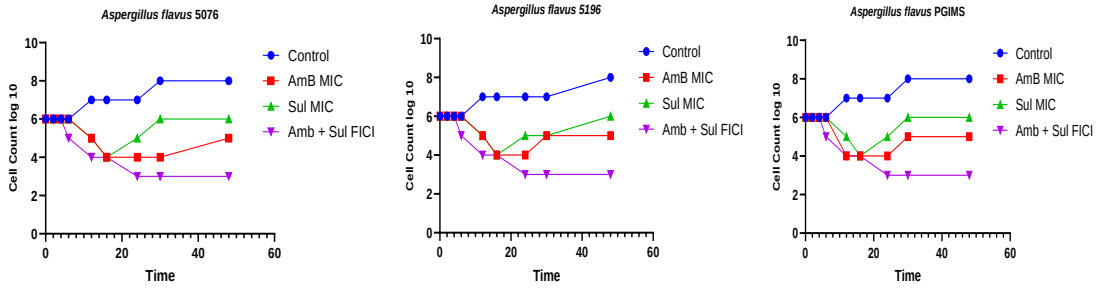
**A** **B** **C**





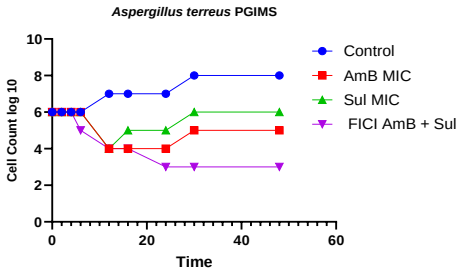

**D**

Figure 8: Time Kill Curve of MIC of AmB and Sul alone and FICI AmB + Sul against Aspergillus niger strains.




**A** **B** **C**

Figure 9: Time Kill Curve of MIC of AmB and Sul alone and FICI AmB + Sul against Aspergillus flavus strains.




**A**

Figure 10: Time Kill Curve of MIC of AmB and Sul alone and FICI AmB + Sul against Aspergillus terreus.

**Hemolytic Assay:**

In-vitro hemolytic assay was performed to determine the cytotoxicity of AmB and Sul alone and in combination for different concentrations ranging from 2mg/ml, 1mg/ml, .5 mg/ml, .25 mg/ml, .125 mg/ml, .0625 mg/ml, .03125 mg/ml, .0156mg/ml and .00781mg/ml of Amphotericin B and Sulfamethoxazole alone and in combination. Triton-X 10% was taken as a positive control which show 100% haemolysis and D-PBS was taken as a negative control. The haemolytic assay shows AmB lyse 100% of erythrocytes up to .05mg/L, Sul is nontoxic up to 2mg/L, whereas the combination of AmB and Sul lyse less than 10% of erythrocytes up to 500mg/L. So, the combination of AmB and Sul is non-toxic up to 500mg/L (less than 10% of the haemolysis was considered non-toxic). The plot of haemolysis versus concentration is in Figure 11.

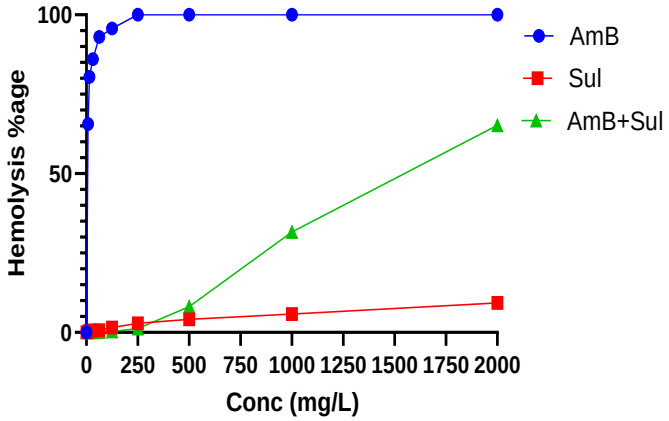



Figure 11: Hemolytic Assay of AmB and Sul alone and in combination.

## 4. Conclusion and Future Perspective:

Aspergillus species are opportunistic fungal pathogens and cause aspergillosis in immunocompromised patients. The excessive use of azole antifungal in clinical settings and agronomy results in azole resistance in A. fumigatus. Since, the past few decades, there has been an upsurge in aspergillosis cases and after the sudden outbreak of COVID-19, the situation become grave due to its association with Aspergillus co-infection (COVID-19-associated pulmonary aspergillosis) which is concomitant with high mortality. So, there is an urgent need for an effective infection management approach. The combination therapy provides an increased potency and broad-spectrum antifungal approach for infection management while reducing the chances of resistance emergence. The successful treatment of cryptococcal meningitis by using a combination of Amphotericin B and 5-flucytosine advocates combination therapy for other fungal infections [[19]]. The clinical data supported the potential use of voriconazole and echinocandins combination against A. fumigatus [[20]]. In-vitro and in-vivo, polyenes and echinocandins combination also show synergy [[20]]. The use of antifungal drugs with antibacterial drugs shows synergy which implies that the use of these two in combination can increase the treatment efficacy. There are various studies related to combination therapy against fungal and bacterial infections that provide insight into the mentioned study [[7],[8],[21]].

In our study, we tested AmB combination with other compounds against various Aspergillus strains by using a chequerboard assay. For antifungal susceptibility testing we performed Disk diffusion (DDA) and Broth microdilution assay (MDA), results of DDA and MDA were summarized in Table 1, figure 1 and Table 2, respectively. According to our study, AmB in combination with Eb (FICI = 2.25-4.12), FoA (FICI = 4.12-8.82), SoS (FICI = 4.40-8.72), AA (FICI = 4.20-9.25) and Fa (FICI = 6.12-9.44) shows indifference and antagonism as summarized in table 3. AmB in combination with Sul shows synergistic interaction with the FICI value = .18-.25 against A. fumigatus,.18-.40 against A. niger, .18 against A. flavus and FICI = .25 against A. terreus as shown in table 4. We evaluated our combination efficacy by time-kill kinetic assay and found that there is a decrease in log10 spore/mL > 3 log units at FICI value, which shows our combination is fungicidal (figure 7-10), but when we tested ½ FICI concentration its shows fungistatic results (figure 3-6). We also perform a spore germination inhibition assay on a clinical isolate of A. fumigatus, and evaluate inhibition of spore germination at ½ FICI (.09) and FICI (.18) is 76.60% and FICI 95.75%, respectively. Cytotoxicity study of combination evaluated by haemolytic assay, which advocates the tested combination (AmB+Sul) is non-toxic up to 500mg/L (figure 11), whereas AmB shows 100% cell lysis at .05mg/L concentration. We also tested our combination on Escherichia coli MTCC 433 (obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India), and found our combination additive with a FICI value of .62 (FIC AmB 0.50 and FIC Sul 0.12). So, with these results, we can say that the combination of AmB with Sul is a potent way to control Aspergillus infection, and the combination also reduces the toxicity of AmB. Thus, the combination of AmB with Sul provides insight into antifungal combination therapy and it should be explored in vivo in future.

It should be noted that a combination of these two may be involved with myopathy. However, the study proves that the activity of bacterial drugs can be synergistic in combination with antifungal agents and can provide a direction for developing new drugs or therapy for IA management with less toxic effects.

**Conflict of Interest**
The authors have no conflict of interest.

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**Authors' Contributions:**
PC led the design and writing of the manuscript and data analysis. AKC did final approval for this research article publication.

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