In-vitro interaction of terbinafine alone and in combination against isolates of Aspergillus Species

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KEYWORDS

Aspergillus species, aspergillosis, combination therapy, terbinafine, antibiotic activity, chequerboard assay.

ABSTRACT

INTRODUCTION: The infection associated with *Aspergillus* species causes high morbidity and mortality in immunocompromised patients and the sudden outbreak of the COVID-19 pandemic worsened the situation in the healthcare system. Recent guidelines recommend the use of voriconazole and isavuconazole for the treatment of aspergillosis, but the emergence of Azole-resistant *Aspergillus* species limits the azole-based treatment approach. The use of antifungal combination therapy might become an emerging alternative strategy for aspergillosis. In our study, we evaluate the terbinafine interaction against *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus* alone and in combination with other compounds.

MATERIAL AND METHODS: *In-vitro* terbinafine and other compounds activity against the *Aspergillus* species was assessed using the Disk Diffusion Assay and micro-broth dilution assay, and the Zone of Inhibition results were measured in millimetres with Mean ± Standard Deviation. The assessment of interaction was done by a chequerboard assay, and the interaction was quantified using the Fractional Inhibitory Concentration Index. These methods provide insights into the nature of the interaction, such as synergy, additivity, indifference, or antagonism.

RESULTS: The Zone of inhibition of terbinafine against *Aspergillus* strains ranges between 22.7±0.2 to 41.3±0.2, Ebselen 16.7±0.2 to 29.3±0.2, Amphotericin B 21.2±0.3 to 27.7±0.2, Aerosporine 6.1±0.2 to 12.5±0.2, Ciprofloxacin 6.3±0.3 and Gentamycin sulphate 6.2±0.2. Terbinafine and other compounds show variable activity and the interaction between these compounds is an interesting approach in the treatment of fungal infections.

CONCLUSION: The interaction between terbinafine and other compounds is interesting and might be a potential therapeutic approach against invasive *Aspergillus* infections.

'ARTICLE HIGHLIGHTS'

- **Terbinafine combination studies were analysed for antifungal efficacy with other compounds against Aspergillus species.**
- **Insights into interactions (synergy, additivity, indifference, or antagonism) were evaluated to improve antifungal strategies.**
- **Disk diffusion, micro-broth dilution, and FICI methods were used to assess combination effects.**
- **Results revealed intriguing interactions, encouraging further testing of these novel combinations and other compounds.**
- **This study may provide valuable insights into the potential of antifungal combination therapies in the future.**

1. INTRODUCTION

After the sudden Coronavirus disease 2019 (COVID-19) outbreaks, various fungal infections upsurged as a complication in COVID-19 patients and *Aspergillus* species causes COVID-19-associated pulmonary aspergillosis (CAPA) in critically ill patients [1]. *Aspergillus* species are concomitant with a wide range of infections ranging from non-invasive to invasive aspergillosis (IA), which significantly contributes to mortality and morbidity in immunocompromised patients. Globally, nearly 2,00,000 cases of IA are reported every year, which may be half of the actual cases due to misdiagnosis, which leads to a variable rate of mortality from 50-100% [2]. Recently, WHO listed *Aspergillus fumigatus* as a critical pathogen in public health care [3]. Aspergillosis commonly affects the human lungs, signs and symptoms of the infection include chest pain, cough (sometimes cough with Blood), fever, shortness of breath and haemoptysis, however, sometimes it can spread to other organs [4]. The optimal infection management of aspergillosis includes early diagnosis, reduction in immunosuppressive therapy, early antifungal treatment and in some cases, surgery opted-in infection management. As per standard treatment guidelines of aspergillosis, voriconazole and isavuconazole are used as first-line drugs for treatment, but the excessive use of azole in agriculture and clinics resulted in resistance emergence, which may limit the treatment approach. In this scenario, combination therapy may significantly affect infection management [4].

Terbinafine belongs to the allylamine drug class and is available for oral and topical use. It binds non-competitively and inhibits the fungal cell membrane enzyme squalene epoxidase (also known as squalene monooxygenase), a key enzyme in ergosterol biosynthesis [7]. It is widely used to treat dermophyte infections and its activity for *Aspergillus* species, *Cryptococcus* species, *Candida* species, *Penicillium marneffei* (now known as *Talaromyces marneffei*), and some other filamentous fungi have also been reported [5,6,7]. The U.S. Food and Drug Administration approved it in the form of terbinafine hydrochloride (LAMISIL) for oral doses [7]. According to the studies, terbinafine in combination with other antibiotic compounds shows synergy against various fungal infections [8,9,10,11]. A study of isavuconazole in combination with cyclosporin A shows synergy against *Aspergillus niger* isolates, whereas with other *Aspergillus* isolates drugs combination shows indifference [12]. In a recent study, fluconazole (antifungal) in combination with doxycycline acetate (antibacterial) interaction was determined against dual species culture of *Candida albicans* and *Staphylococcus* and shows synergy against pathogens [13]. Therefore, we intend to investigate the terbinafine hydrochloride interaction with other compounds against 13 different *Aspergillus* species. The drug interaction with other compounds is determined by chequerboard assay.

2. MATERIAL METHODS

2.1 Tested compounds: Terbinafine hydrochloride (TRB)**,** Ebselen (Eb), Acetylsalicylic acid (AA), Farnesol (Fa) obtained from Sigma-Aldrich and Amphotericin B (AmB), Aerosporine (Aer), Penicillin (Pen), Streptomycin sulphate (SS), Doxycycline hydrochloride (DH), Cefotaxime sodium salt (Cef), Ampicillin sodium salt (AS), Folic Acid (FoA), Amoxycillin (Am), Ciprofloxacin (Cip), Erythromycin (Ery), Gentamycin sulphate (GS), Sodium Salicylate (SoS), Cycloheximide (Chx) obtained from HiMedia Laboratories Pvt. ltd., India used for testing.

2.2 Culture medium and fungal species: In our study, we use the Potato Dextrose Agar and Broth media (PDA and PDB), 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 PGIMS (Rohtak) India. The strains employed in the study, this 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 37ºC for 48 hours in PDA.

2.3 Inoculum preparation and antifungal testing:

The Sabouraud Dextrose Agar/Broth media was used for the assay. 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 M38-A2 protocol. The stock solution of the tested compounds 2mg/ml was prepared by dissolving in 4% DMSO and for the study diluted further to get the desired concentration.

2.4 *In-vitro* antifungal testing by Disk Diffusion Assay:

The drug solution was diluted and used to test its activity against various strains of *Aspergillus fumigatus, Aspergillus niger, Aspergillus flavus,* and *Aspergillus terreus* by employing CLSI M38-A2 protocol with some modifications. The Petri plates of SDA media (90mm diameter) were inoculated in a spore solution of 10^{3-4} 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. The plates were incubated at 37ºC 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 duplicates for reproducibility and the ZOI was calculated as means ± standard deviations [14,15]. The results of the disk assay were stated as the ZOI percentage.

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

2.5 *In-vitro* antifungal testing by Broth microdilution assay:

This assay is used to determine 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 90 µl volume of diluted concentration was added in 96 well microtiter plate containing 90 µl RPMI-1640 media with MOPS ([3-(N-morpholino)]-propane sulfonic acid) and then carried out serial dilution [14,16]. 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 20 µl of fungal culture 10^{3-4} spores/ml concentration and the plates were incubated at 37ºC 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 duplicate.

2.6 *In-vitro* combination study between TRB and other tested compounds by Chequerboard Assay:

In-vitro interaction of TRB with other compounds determined by microdilution chequerboard assay. For a 2-D chequerboard, 45 µl of each TRB concentration was mixed with 45 µl of each concentration of the second tested compound. The drug was prepared at twice the desired concentration (2x: 2MIC)). In the chequerboard assay, 45 µl of each of the concentrations of terbinafine was added into columns 1 to 11 in 96-well microtiter plates and 45 µl of each of the concentrations of the second tested compound was added to the A to G row. Column 12 contained only TRB and row H only has the second tested compound. The drug-free H12 well was taken as a growth control. Afterwards, 20 µl of fungal inoculum with 10^{3-4} spores/ml concentration was added to each well and the plates were incubated at 37ºC for 24 hours, then fungal growth was evaluated in each well having single and combination of drugs and compared with control well (H12 well). Each combination experiment was tested in duplicates for reproducibility.

2.6.1 Fractional Inhibitory Concentration Index (FICI):

The FICI model (a non-parametric approach) is used to evaluate interaction among the tested compounds [12]. The FICI was calculated by the formula given below. (Here, A was drug TRB and B was other tested compounds used in combination testing).

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

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

FICI=FICA+FICB

According to this: Synergy (FICI ≤0.5): The combined effect of the drugs is greater than the sum of their individual effects, indicating that the agents enhance each other's activity significantly. Additivity (0.5<FICI≤1): The effects of the combination are equal to the sum of the effects of each drug taken separately. The agents work independently, with cumulative but not enhanced effects. Indifference (1<FICI≤4): The combined effect lies between additive and antagonistic, showing neither enhancement nor substantial interference between the agents. Antagonism (FICI>4): The combined effect is less than the sum of the individual effects, indicating that the agents interfere with each other's activity.

3. RESULTS

3.1 In-vitro antifungal testing by Disk Diffusion Assay:

The antifungal activity of drugs TRB**,** Eb, AA, Fa, AmB, Aer, Pen, SS, DH, Cef, AS, FoA, Am, Cip, Ery, GS, SoS, and Chx was evaluated against thirteen isolates of *Aspergillus* species including five strains of *A. fumigatus* ITCC 4517, ITCC 6050, ITCC 4448, ITCC 1628, and Clinical isolate PGIMS, four strains of *A. niger* ITCC 3002, ITCC 6219, ITCC 5405, Clinical isolate PGIMS, three strains of *A. flavus* ITCC 5076, ITCC 5192, Clinical isolate PGIMS, and a strain of *A. terreus* Clinical isolate PGIMS because of their pathogenicity in humans. As shown in Table 1 the drugs show variable activity for *Aspergillus* species. The ZOI shown by TRB ranges from 20.7±0.1 to 41.3±0.2, AmB shows 21.2±0.3 to 27.7±0.2, Eb shows 16.7±0.2 to 29.3±0.2, Aer shows 6.1±0.2 to 12.5±0.2, Cip shows activity against *A. fumigatus* ITCC 4448 (6.3±0.3) and 6050 (6.1±0.2), *A. niger* ITCC 3002 (6.0±0.1), 6219 (6.3±0.2) and 5405 (6.4±0.2), *A. flavus* ITCC 5076 (6.3±0.2) and 5192 (6.1±0.3) and *A. terreus* Clinical isolates (6.0±0.1), whereas against ITCC 1628, 4517, and clinical isolates of *A. fumigatus*, *A. niger,* and *A. flavus* shows no visible growth inhibition. GS shows activity against all ITCC *Aspergillus* strains ranging from 6.0±0.1 to 6.2±0.2, no visible growth inhibition was exhibited against clinical isolates of *Aspergillus.* The compounds AA, Fa, Pen, SS, DH, AS, FoA, Am, Ery, SoS, and Chx show no zone of inhibition against any of the *Aspergillus* strains. ZOI results were expressed in mm in Table 1.

The ZOI percentage of TRB ranges from 25.2-45.9, Eb 18.5-32.5, AmB 23.6-30.7, Aer 6.7-13.8, Cip 6.6-7.1 and GS 6.6-6.8. The ZOI results in mm with Mean Standard Deviation (SD) are presented in Table 1.

Table 1: Zone of Inhibition (ZOI) in mm of various tested compounds against *Aspergillus* strains. (NVGI=No Visible Growth Inhibition)

Strain		Zone of Inhibition Mean Diameter (mm) ±SD					
		TRB	**Eb**	**AmB**	**Aer**	**Cip**	**GS**
A. fumigatus	ITCC **4448**	29.2±0.2	24.7±0.2	26.7±0.1	12.3±0.2	6.3±0.3	6.1±0.1
	ITCC **1628**	28.7±0.2	16.7±0.2	23.2±0.3	11.7±0.1	NVGI	6.0±0.1
	ITCC **4517**	37.2±0.3	25.2±0.3	21.5±0.2	12.4±0.1	NVGI	6.1±0.2
	ITCC **6050**	24.2±0.1	28.2±0.2	22.9±0.2	12.1±0.2	6.1±0.2	6.2±0.2
A. niger	ITCC **3002**	41.3±0.2	23.2±0.2	21.7±0.1	11.3±0.3	6.0±0.1	6.1±0.2
	ITCC **6219**	40.7±0.1	21.7±0.1	21.5±0.2	11.6±0.1	6.3±0.2	6.1±0.1
	ITCC **5405**	41.1±0.2	17.7±0.1	21.2±0.3	12.1±0.2	6.4±0.2	6.1±0.2
A. flavus	ITCC **5076**	39.1±0.2	19.1±0.1	22.7±0.1	12.5±0.2	6.3±0.2	6.2±0.1
	ITCC **5192**	38.3±0.3	29.1±0.2	21.3±0.3	12.1±0.3	6.1±0.3	6.1±0.1
***A. fumigatus* PGIMS**		23.2±0.3	19.7±0.1	27.7±0.2	6.3±0.2	NVGI	NVGI
***A. niger* PGIMS**		22.7±0.2	22.1±0.1	23.2±0.3	6.2±0.1	NVGI	NVGI
***A. flavus* PGIMS**		35.1±0.3	29.2±0.1	21.7±0.2	6.1±0.2	NVGI	NVGI
***A. terreus* PGIMS**		40.2±0.1	29.3±0.2	23.2±0.1	11.3±0.1	6.0±0.1	NVGI

(Note: TRB: Terbinafine, Eb: Ebselen, AmB: Amphotericin B, Aer: Aerosporine, Cip: Ciprofloxacin, and GS: Gentamycin sulphate.)

Here, according to the formula stated in section 2.4, the ZOI percentage was calculated (i.e. ZOI shown by the individual drugs in the Petri plate was divided by the diameter (90 mm) of the Petri plate and multiplied by a hundred) and presented in Table 2 and Fig.1.

Table 2: The Zone of Inhibition (ZOI) percentage of various tested compounds against *Aspergillus* strains.

Strain		Zone of Inhibition Percentage					
		TRB	**Eb**	**AmB**	**Aer**	**Cip**	**GS**
A. fumigatus	ITCC **4448**	32.4	27.4	29.6	13.6	7	6.7
	ITCC **1628**	31.9	18.5	25.7	13	-	6.6
	ITCC **4517**	41.3	28	23.8	13.7	-	6.7
	ITCC **6050**	26.9	31.3	25.4	13.4	6.7	6.8
A. niger	ITCC **3002**	45.9	25.7	24.1	12.5	6.6	6.7
	ITCC **6219**	45.2	24.1	23.8	12.8	7	6.7
	ITCC **5405**	45.6	19.6	23.5	13.4	7.1	6.7
A. flavus	ITCC **5076**	43.4	21.2	25.2	13.8	7	6.8
	ITCC **5192**	42.5	32.3	23.6	13.4	6.7	6.7
***A. fumigatus* PGIMS**		25.7	21.8	30.7	7	-	-
***A. niger* PGIMS**		25.2	24.5	25.7	6.8	-	-
***A. flavus* PGIMS**		39	32.4	24.1	6.7	-	-
***A. terreus* PGIMS**		44.6	32.5	25.7	12.5	6.6	-

Note: TRB: Terbinafine, Eb: Ebselen, AmB: Amphotericin B, Aer: Aerosporine, Cip: Ciprofloxacin, and GS: Gentamycin sulphate.

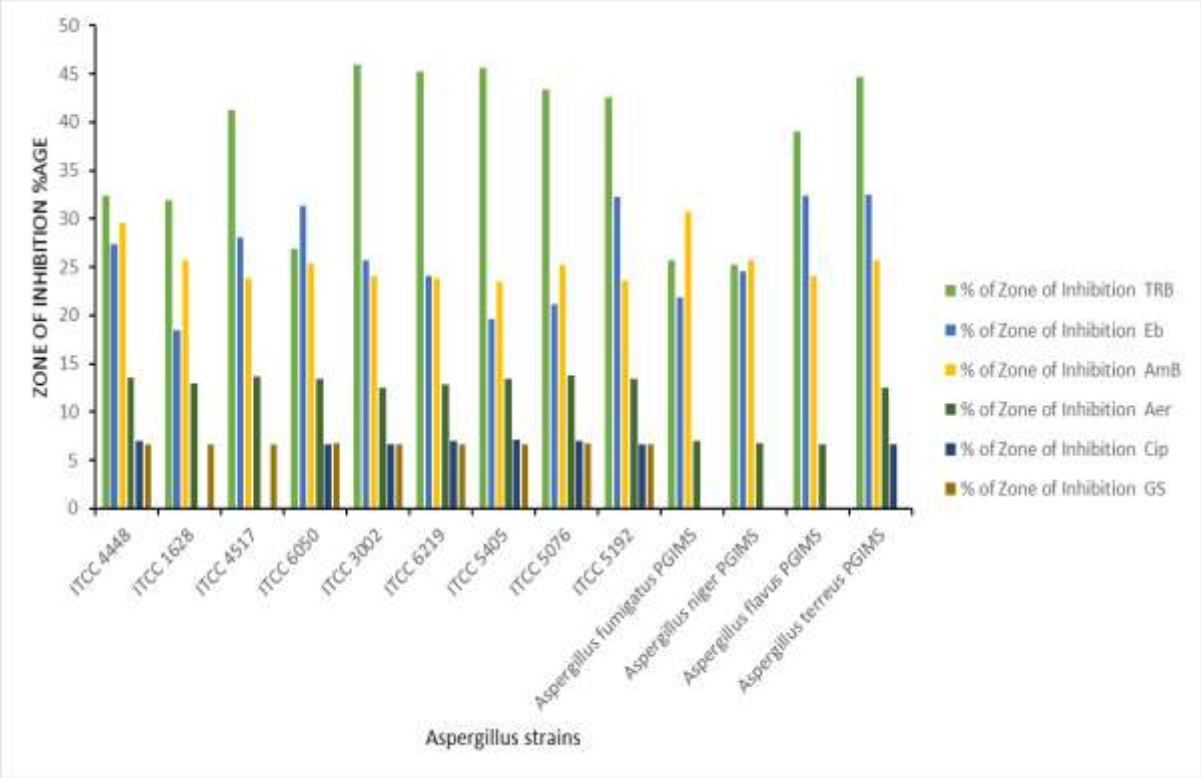

Figure 1: Zone of Inhibition Percentage of tested compounds against *Aspergillus* strains.

3.2 *In-vitro* antifungal testing by Broth microdilution assay:

The MIC of TRB, Eb, AmB, SoS, AA, Fa, Aer, Pen, SS, DH, Cip, AS, FoA, Am, Cef, Ery, Chx and GS were determined by using a broth microdilution assay. The results are summarized in Table 3. SoS, Pen, FoA and Fa showed no visible growth inhibition against the tested *Aspergillus* strain. The MIC value of TRB for *A. fumigatus* varied in the range of 0.0305-0.488mg/L, for *A. flavus* 0.0305-0.061mg/L, for *A. niger* 0.0305-0.976mg/L, and *A. terreus* 0.0305mg/L. Eb MIC ranges between 0.244 and 7.81mg/L for *A. fumigatus*, for *A. flavus* between 0.244 and 3.906mg/L, for *A. niger* between 0.976 and 7.81mg/L, and for *A. terreus* 0.244mg/L and AmB MIC ranges between 0.976-1.95mg/L. AA shows activity at 1000mg/L only for *A. fumigatus* ITCC 4448 and *A. niger* ITCC 3002 and 5405. Aer MIC ranges between 62.5 and 125mg/L; SS MIC was 500mg/L for *Aspergillus* strains except for *A. fumigatus* and *A. flavus* clinical isolates. DH MIC for *Aspergillus* strains ranges between 250 and 500 mg/L except for the *A. flavus* clinical isolate. Cip MIC ranges between 125 and 250mg/L and GS MIC ranges from 125 to 500mg/L for all *Aspergillus* strains. AS MIC is 500mg/L against most *Aspergillus* strains except for the *A. fumigatus* clinical isolate. Am shows MIC of 500mg/L only for ITCC 4448, ITCC 3002, and ITCC 5405. Cef has a MIC of 500mg/L for *Aspergillus* strains except for the clinical isolate of *A. fumigatus.* Ery MIC ranges between 250 and 1000mg/L for most *Aspergillus* strains except for *A. fumigatus* and *A. terreus* clinical isolate. Chx shows MIC 500mg/L only against *A. fumigatus* ITCC 4448, 1628 and 6050 and *A. niger* ITCC 3002, 6219 and 5405.

Table 3: Minimum Inhibitory Concentration of various tested compounds against *Aspergillus* strain.

Strain	Minimum Inhibitory Concentration in mg/L													
	TRB	Eb	AmB	AA	Aer	SS	DH	Cip	AS	Am	Cef	Ery	GS	Chx
4448	0.244	0.976	0.976	1000	62.5	500	500	125	500	500	500	500	125	500
1628	0.244	7.81	1.95	-	62.5	500	500	250	500	-	500	500	125	500
4517	0.0305	0.488	1.95	-	62.5	500	500	250	500	-	500	500	125	-
6050	0.488	0.244	0.976	-	62.5	500	500	125	500	-	500	500	125	500
3002	0.0305	0.976	1.95	1000	62.5	500	250	125	500	500	500	500	125	500
6219	0.0305	0.976	1.95	-	62.5	500	500	125	500	-	500	500	125	500
5405	0.0305	7.81	1.95	1000	62.5	500	500	125	500	500	500	500	125	500
5076	0.0305	3.906	0.976	-	62.5	500	500	125	500	-	500	500	125	-
5192	0.0305	0.244	1.95	-	62.5	500	500	125	500	-	500	250	125	-
***A. fumigatus* PGIMS**	0.488	3.906	0.976	-	125	-	500	250	-	-	-	-	500	-
***A. niger* PGIMS**	0.976	0.976	1.95	-	125	500	500	125	500	-	500	1000	500	-
***A. flavus* PGIMS**	0.061	0.244	1.95	-	125	-	-	250	500	-	500	500	250	-
***A. terreus* PGIMS**	0.0305	0.244	1.95	-	62.5	500	500	125	500	-	500	-	250	-

3.3 *In-vitro* combination study between TRB and other tested compounds by Chequerboard Assay:

The interaction of TRB with Eb, AA, AmB, Aer, SS, DH, Cip, AS, Am, Cef, Ery, GS, Chx, Fa, FoA, Pen and SoS was interpreted by the FICI model. The summary of interaction among tested compounds in combination with TRB is summarised in Table 4 and Table 5 against the various *Aspergillus* strains. No tested compounds show synergistic interaction with TRB (i.e., no FICI ≤ 0.5).

Table 4: FICI of Terbinafine with Tested Compounds against *Aspergillus* strains.

Strain	**Fractional Inhibitory Concentration Index,** FICI ≤0.5 = synergy; 0.5<FICI≤1= additive; 1<FICI≤4 = indifferent, FICI>4 = antagonistic.															
	TRB+Eb	**TRB+AmB**	**TRB+AA**	**TRB+Aer**	**TRB+SS**	**TRB+DH**	**TRB+Cip**	**TRB+AS**	**TRB+Am**	**TRB+Cef**	**TRB+Ery**	**TRB+GS**	**TRB+Chx**	**TRB+Fa**	**TRB+FoA**	**TRB+Pen**	**TRB+SoS**
4448	5.17	4.40	7.2	.72	.59	.62	.75	.64	1.3	4.1	4.45	1.27	4.92	4.2	5.2	1.7	8.1
1628	5.17	4.02	7.80	1.62	.97	.67	.62	.66	1.01	4.46	4.14	1.06	6.84	4.12	5.95	1.96	7.96
4517	4.45	4.02	8.62	1.06	.68	2.67	.62	.62	1.6	4.9	4.02	1.03	6.28	4.95	6.12	1.25	8.12
6050	4.20	4.02	6.32	.82	.76	.67	.57	.62	2.1	5.21	4.6	1.12	6.61	5.12	4.45	1.18	7.62
3002	8.62	1.4	5.17	1.03	.82	.82	.74	.82	2.3	6.3	.62	1.62	8.32	4.92	5.18	1.81	6.98
6219	8.62	1.4	4.18	1.25	.89	1.52	.53	.86	3.4	5.51	5.6	1.72	7.65	5.01	9.8	1.62	5.42
5405	6.53	1.4	7.78	2.12	.78	.64	.62	.67	3.01	7.21	.68	1.08	7.36	5.23	9.93	2.58	6.14
5076	4.40	5.3	6.22	1.02	.98	2.12	.62	.59	2.96	8.26	5.62	1.36	6.6	4.41	8.24	2.1	9.8
5192	4.45	4.93	6.22	2.31	.81	2.61	.75	.54	3.12	7.31	5.26	1.63	6.53	4.18	8.94	2.6	8.16
***A. fumigatus* PGIMS**	8.62	8.1	9.36	1.18	.68	3.11	.53	.62	3.4	7.53	5.62	2.92	9.8	6.38	11.3	3.4	16.6
A. niger	8.62	7.36	9.69	1.08	.58	4.15	.92	.78	3.02	7.36	5.46	2.63	11.4	6.36	17.2	2.62	11.1

PGIMS																	
***A. flavus* PGIMS**	8.94	7.58	8.23	1.81	.79	4.09	.84	.86	3.06	6.69	5.62	2.36	7.78	7.42	16.69	2.18	15.2
***A. terreus* PGIMS**	8.94	6.9	8.18	.94	.84	.71	.79	.65	3.4	8.62	5.62	2.95	9.7	7.95	18.1	2.3	11.7

Table 5: Mode of *in-vitro* interaction of terbinafine with tested compounds.

Combination Tested against *Aspergillus* strain	**Result (≤0.5 = synergy; >0.5≤1 = additive; >1≤4 = indifferent, >4 = antagonistic)**
Terbinafine hydrochloride + Ebselen	Antagonism
Terbinafine hydrochloride + Sodium Salicylate	Antagonism
Terbinafine hydrochloride + Acetylsalicylic acid	Antagonism
Terbinafine hydrochloride + Farnesol	Antagonism
Terbinafine hydrochloride + Aerosporine	Additive and Indifference
Terbinafine hydrochloride + Penicillin	Indifference
Terbinafine hydrochloride + Streptomycin sulphate	Additive
Terbinafine hydrochloride + Doxycycline hydrochloride	Additive, Indifference and Antagonism
Terbinafine hydrochloride + Ciprofloxacin	Additive
Terbinafine hydrochloride + Ampicillin sulphate	Additive
Terbinafine hydrochloride + Folic Acid	Antagonism
Terbinafine hydrochloride + Amoxicillin	Indifference
Terbinafine hydrochloride + Cefotaxime sodium	Antagonism
Terbinafine hydrochloride + Erythromycin	Additive and Antagonism
Terbinafine hydrochloride + Gentamycin sulphate	Indifference and Antagonism
Terbinafine hydrochloride + Cycloheximide	Antagonism
Terbinafine hydrochloride + Amphotericin B	Indifference and Antagonism

4. DISCUSSION:

Aspergillus is a genus of filamentous fungi and opportunistic pathogens in humans, which generally infect people with compromised immune systems. The first-line antifungals are triazole for the treatment of aspergillosis [3,4]. Recently, there has been an escalation in azole

resistance in *Aspergillus fumigatus* and other associated cryptic species due to the excessive use of azole in agriculture and clinics. The resistance undermines the efficacy of azole-based monotherapies, potentially limiting their role in managing *Aspergillus*-associated infections. In this scenario, combination therapy emerges as a promising approach [10,11]. By leveraging the synergistic effects of multiple antifungal agents, combination therapy can enhance therapeutic efficacy, delay or prevent the further development of resistance and broaden the spectrum of antifungal activity by improving outcomes in severe and refractory cases. In summary, antifungal combinations against *Aspergillus* species have garnered significant scientific interest over the years. While promising results have been observed, the viability of this approach as a reliable treatment option requires further validation. To achieve this, additional *in-vitro* studies and robust clinical data are essential to comprehensively evaluate the efficacy, safety, and practicality of combinatorial therapies in managing *Aspergillus*-associated infections. There are various studies related to combination therapy against fungal and bacterial infections that provide insight into the mentioned study [4,8,9,10,11,12,13], building on these findings, we aim to investigate the interaction of terbinafine with other compounds against *Aspergillus* species. We explore the outcomes of combining terbinafine with antifungal agents and other chemical compounds against *Aspergillus* species, the combinations employed in our study are novel and not studied against the *Aspergillus* species.

In our study, we investigate *in-vitro* Terbinafine interaction with other compounds. According to our investigations, we find TRB in combination with Eb (FICI = 4.20-8.94), FoA (FICI = 4.45-18.1), Cef (4.1-8.62), Chx (FICI = 4.92-11.4), SoS (FICI = 5.42-16.6), AA (FICI = 4.18-9.69), and Fa (FICI = 4.12-7.95) shows antagonistic interactions, whereas with GS (FICI = 1.03-2.95), Pen (FICI = 1.7-2.3) and Am (FICI = 1.7-2.3) shows indifference against tested isolates. TRB with AmB shows indifference for ITCC 3002, 6219 and 5405 (FICI = 1.4) and antagonism against other tested isolates (FICI = 4.02-8.1). TRB with AS (FICI = .54-.86), SS (FICI = .58-.98), and Cip (FICI = .53-.92) shows additive interaction against tested isolates. TRB with Aer shows additive interaction against ITCC 4448, 6050 and *A. terreus* PGIMS (FICI = .72-.94) and indifference against other isolates (1.02-2.31). TRB with DH shows additive results against ITCC 4448, 1628, 6050, 3002, 5405 and *A. terreus* PGIMS (FICI = .62-.82), indifference against ITCC 4517, 6219, 5076, 5192 and *A. fumigatus* PGIMS (FICI = 1.52-3.11) and antagonism against *A. niger* PGIMS and *A. flavus* PGIMS (FICI = 4.15 and 4.09 respectively). TRB with Ery shows additive interaction against ITCC 3002 and 5405 (FICI = .62-.68) and antagonism against other isolates FICI =4.02-5.62). No tested compounds show synergistic interaction with TRB (i.e., no FICI $\leq$ 0.5). These results show the variable and surprising interaction among various drugs and compounds, which support the further testing of more compounds to understand the combined action mechanism to combat pathogenic fungal species for better healthcare management. In future, maybe this study will provide valuable insights into the potential of antifungal combination therapies to improve treatment strategies for *Aspergillus*-associated infections.

5. CONCLUSION:

Fungal infection in immunocompromised individuals (due to various reasons and conditions) is an unnerving proposition. The development of resistance against conventional drugs used in monotherapy presents a significant challenge for infection management in the healthcare system. A simple yet explicitly efficient combination approach is urgently needed to combat *Aspergillus*-associated infections, given the severity of the disease caused by this pathogen. The challenges posed by *Aspergillus* infections, such as invasive aspergillosis, are often insurmountable with conventional monotherapy due to intrinsic resistance, rapid adaptation and complex pathogenesis. These challenges highlight the urgency for robust and innovative

treatment strategies, such as combination therapies, which leverage multiple mechanisms of action to enhance efficacy and reduce resistance, providing a more effective solution for managing *Aspergillus*-associated infections**.** Under the above-mentioned investigation, using chequerboard assay we conclude that TRB in combination with various compounds shows variable results ranging from additive to antagonism. While promising results have been observed, the viability of this approach as a reliable treatment option requires further validation. To achieve this, continued research in this direction may hold promising results to develop new therapeutic insights in the treatment of fungal infections with enhanced safety and efficacy.

6. ABBREVIATIONS:

TRB: Terbinafine hydrochloride, Eb: Ebselen, AA: Acetylsalicylic acid, Fa: Farnesol, AmB: Amphotericin B, Aer: Aerosporine, Pen: Penicillin, SS: Streptomycin sulphate, DH: Doxycycline hydrochloride, Cef: Cefotaxime sodium salt, AS: Ampicillin sodium salt, FoA: Folic Acid, Am: Amoxycillin, Cip: Ciprofloxacin, Ery: Erythromycin, GS: Gentamycin sulphate, SoS: Sodium Salicylate, Chx: Cycloheximide, PDA: Potato Dextrose Agar, PDB: Potato Broth media, SDA: Sabouraud Dextrose Agar, SDB: Sabouraud Broth media, RPMI-1640: Roswell Park Memorial Institute-1640, A.: *Aspergillus*, ITCC: Indian Type Culture Collection, EUCAST: The European Committee on Antimicrobial Susceptibility Testing, CLSI: Clinical and Laboratory Standards Institute, DMSO: dimethyl sulfoxide, ZOI: zone of inhibition, MIC: Minimal inhibitory concentration, MOPS: [3-(N-morpholino)]-propane sulfonic acid, FICI: Fractional Inhibitory Concentration Index, NVGI: no visible growth inhibition.

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8. AUTHORS’ CONTRIBUTIONS:

PC led the design and writing of the manuscript and SK aids in data analysis. AKC did final approval for this research article publication.

9. CONFLICT OF INTEREST

The authors have no conflict of interest.

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