Life Science Informatics Publications

Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences

Journal Home page http://www.rjlbpcs.com/

RJLBPCS

ISSN 2454-6348

Original Research Article **DOI: 10.26479/2018.0404.23**

INTERACTION OF CURCUMIN WITH AZOLES AND POLYENES AGAINST *ASPERGILLUS* INFECTIONS

Ritu Pasrija*, Deepika Kundu

Department of Biochemistry, Maharshi Dayanand University, Rohtak, Haryana, India.

ABSTRACT: Fungal infections are already on rise due to limited antifungal available and increased drug resistance against already available drugs. Although, the antifungal activity of curcumin is already reported in many isolated studies, against different fungal pathogens including yeast and filamentous fungi, however, therapeutically it has not gained popularity till now due to its limited activity. Keeping this in mind we are suggesting combination approach as a tool to combat *Aspergillus* infections. In this study antifungal activity of curcumin against various strains of different *Aspergillus* species was studied by employing broth microdilution assay following the CLSI (formerly the NCCLS) guidelines, time–kill assays and germ tube inhibition. Also, the activity of curcumin was tested along with already antifungal available (azoles and polyenes) by chequerboard method. Curcumin showed antifungal activity against various species of *Aspergillus.* Combination studies of curcumin with various azoles and polyenes showed significant additive or synergistic activity. This provides a novel therapeutic strategy to improve activity of already available antifungal and thus can be life saving in cases of drug resistance in patients undergoing chemotherapy and immunosuppressant's.

KEYWORDS: *Aspergillus*, fungi, antifungal, azoles, polyenes

Corresponding Author: Dr. Ritu Pasrija* Ph.D.

Department of Biochemistry, Maharshi Dayanand University, Rohtak, Haryana, India

Email Address: ritupasrija@yahoo.com

1.INTRODUCTION

Aspergillus is the most common type of fungi in the environment and belongs to a group called molds. Its infections are categorized in the group of diseases called aspergillosis. Usually only people with weakened immune systems are susceptible to infection by *Aspergillus* [1], [2], [3],

[4]. Out of more than 200 species reported; about 16 species are known to be dangerous to humans. The different disease causing species includes: *A. fumigatus*, *A. flavus*, *A. niger,* and *A. terreus* etc. High mortality rates from invasive aspergillosis in immunocompromised patients are prompting research toward improved antifungal therapy and better understanding of fungal physiology [5], [6]. *Aspergillus* infection which usually starts from the lungs can normally cause allergic reactions, but people with severe asthma are often sensitive and can suffer asthma attacks because of the spores. The group of diseases includes: allergic bronchopulmonary aspergillosis (ABPA), acute invasive aspergillosis, disseminated invasive aspergillosis (IA) [7]. In addition, mycotoxins are produced by some species. *Aspergillus* molds have a powdery texture. However the color of the mold's surface differs from species to species and can be used to identify the type [8]. *A. fumigatus* causes infection in humans more often than any other *Aspergillus* species. People who handle or who are exposed extensively to *A. fumigatus* often develop a hypersensitivity and severe allergic reactions to the mold [9]. It is the most tolerant species to temperature and can grow in environments between 20-55˚C [10]. It can be identified by the blue-green or gray color of its surface and appears white or tan underneath. *A. flavus* produces the carcinogenic mycotoxin, aflatoxin under stress conditions which often contaminates foods such as nuts [11]. After *A. fumigatus*, it is the second most common *Aspergillus* mold to infect humans. The surface of *A. flavus* is yellow-green in color [12]. *A. niger,* the most abundant species and is characterized by the typical black color. It can grow on a large variety of substances, even in environments with very little nutrients available and often found growing on damp walls. Among different *Aspergillus* species, *A. niger* is the reason behind one third of the infections. The health effect includes hearing problems and even hearing loss [13]. *A. terreus* causes infections to the frequency range from 3–12.5%. The in vitro and in vivo analysis indicates that *A. terreus* is distinguishable from other disease causing *Aspergillus* species in being more resistant to amphotericin B therapy [14]. It gives brownish coloration in culture. Azole antifungals, such as voriconazole and posaconazole are recommended drugs to manage *Aspergillus* diseases. Surveillance studies indicate that azole resistance is increasing in multiple European countries, in the Middle-East, Asia and Africa. Therefore, alternative treatment regimens need to be investigated to improve the outcome of patients with azole-resistant IA. There are basically two alternative options with respect to the management of azole resistant IA: treatment with a new antifungal formulation or combination therapy. Recent data suggest that combination therapy using a triazole and an echinocandin may be a beneficial treatment strategy for triazole resistant isolates. However for voriconazole, both *in vitro* interactions and *in vivo* studies indicated that the level of synergistic effect is lost at high voriconazole minimum inhibitory concentration (MIC) (MIC $\geq$ 8 μg/ml) [15]. As a consequence, this is a major drawback in the treatment of patients with azole resistant IA. Due to limited availability of the antifungals and increasing resistance development against them, it is important

to potentiate the activity of the antifungals by combinatorial therapy. Combination therapy studies are based on the rationale of combining agents that have complementary mechanisms of action. The benefits of combination therapy include broader spectrum of action, greater potency than either of the drugs used in mono therapy and reduction in the number of resistant organisms [16], [17]. In this study, we are exploring the activity of herbal compound curcumin, which by itself also has activity against *Aspergillus*, but can provide synergistic effect with already available drugs as better option to treat infections.

2. MATERIALS AND METHODS

Materials

Media chemicals were obtained from HiMedia (Mumbai, India). The drugs amphotericin B (AMB), itraconazole (ITR), voriconazole (VOR) and curcumin (CUR) were obtained from Sigma Chemical Co. (St Louis, Mo, USA). Caspofungin® (CAS) was procured from Merck (Merck and Co., USA).

Fungal strains and inoculum preparation

All clinical isolates were provided by Diiachi Sankyo, Gurgram, and maintained on Sabouraud Dextrose Agar (SDA) for 5 to 7 days at 35 to 37°C before preparation of the inoculums. The suspensions of conidia were harvested in normal saline containing 0.025% Tween 20. The appropriate dilutions in normal saline were made to obtain final inoculums concentration of 2-5 x 10^5 CFU/ml [18], [19].

In vitro antifungal susceptibility and Interactions of drugs

Drug interactions were assessed by a chequerboard microdilution method that also included the determination of the MIC of each drug alone in the same microtitre plate as per Clinical and Laboratory Standards Institute (CLSI) guidelines (M27-A2) [20], [21]. Drug dilutions in two-fold increments were prepared at four-fold levels above the desired final concentration. Each well contained combination of drugs dispensed at 50 µl each, effectively creating a 2X concentration of each drug. Antifungal agents were placed in the rows or in the columns of the trays to perform all possible combinations, with concentrations from 32 to 0.5 µg/ml for CAS and 16 to 0.015 µg/ml for azoles. The spore suspension was adjusted to 0.5 McFarland (MaF) turbidity. A total of 0.1 ml of each spore suspension was dispensed into serially diluted wells containing the drugs, reaching the final targeted drug concentration. For all the drugs and their combinations, an optically clear (MIC-0) endpoint criterion was used, which was defined as the lowest concentration resulting in 99.9% inhibition of visible fungal growth after 48 h of incubation. Duplicate testing was performed on three different days. The Fractional Inhibitory Concentration Index (FICI) was calculated and used to classify drugs interaction. The FICI is the sum of the FIC of each of the drugs, which in turn was defined as the MIC of each drug when used in combination divided by the MIC of the drug when used alone. The FICI was defined as: synergic if the FICI was ≤0.5;

neither synergistic nor antagonistic if the FICI was >0.5 to ≤4.0; and antagonistic if the FICI was >4.0 [22].

Time- kill kinetics

Time- kill curve studies were performed in standard RPMI 1640 medium, by the method described by Klepser et al. [23]. Briefly, the isolates were sub-cultured at least twice and grow for 24 h at 37^0C on SDA plates. Tubes containing the appropriate concentrations of testing drug either alone or in combination with other drug in 10ml volume with inoculums of 2 - 8 × 10^6 CFU/ml were incubated at 37^0C with agitation. For CFU determination, a 0.1 ml aliquot was removed at predetermined time points (0, 6, 24, 30, 48, 56 and 70h); 10-fold serially diluted in normal saline, and 20 µl was spotted on SDA plates and incubated for 24-48h. When CFU/ml was expected to be less than 1,000; a 50 µl sample was taken directly from the test solution and spread onto a SDA plate.

Germ tube Inhibition assay

Impact on early fungal growth was also examined. *Aspergillus* conidia (10^6 conidia/ml) were incubated for 14h in RPMI 1640, and effect of subsequent addition of drug was monitored on hyphae [24].

3. RESULTS AND DISCUSSION

Drug combinations are a promising strategy to overcome the compensatory mechanisms and unwanted off-target effects that limit the utility of many potential drugs. In this study, In vitro analysis of antifungals including azoles (ITR, VOR and polyene drug, AMB) alone and in combination with CUR was done (Figure 1) against different species of *Aspergillus* was done and results are summarized as follows:

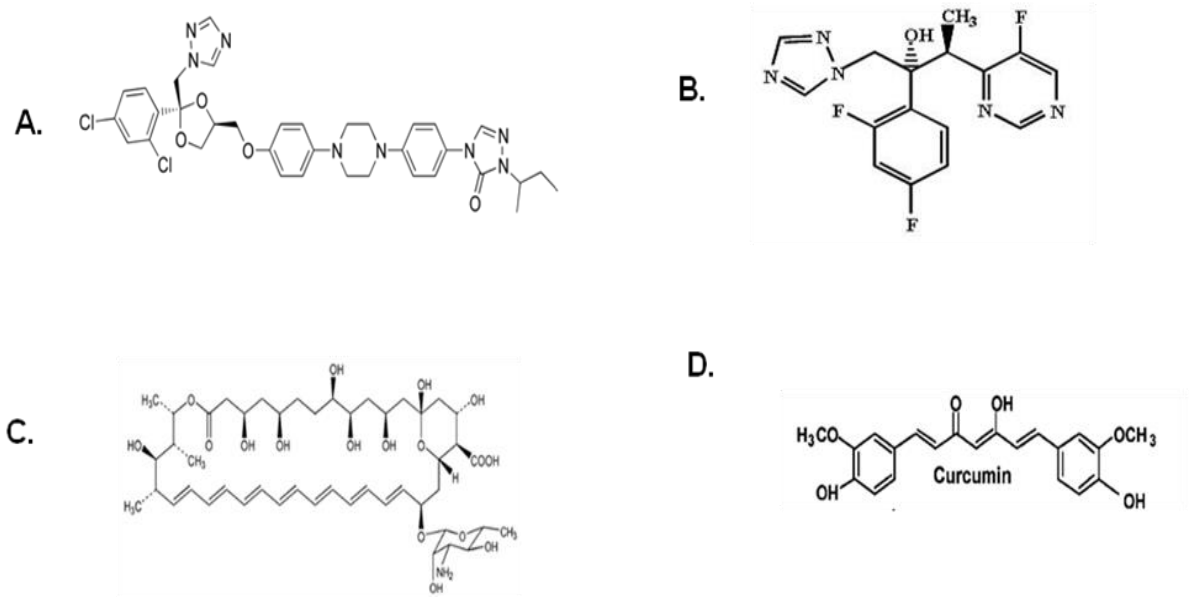

Figure 1: Structures of the drugs used in the study: (A) Itraconazole (ITR); (B) Voriconazole (VORI); (C) Amphotericin B (AMB); (D) Curcumin (CUR).

Chequerboard Microdilution Results

The MIC value of azoles: ITR, VORI and AMB were evaluated alone and in combination with CUR against *Aspergillus sp* including: *A. fumigatus*, *A. flavus*, *A. terreus* and *A. niger* to select the drug concentration range in chequerboard microdilution. The MIC range was in the range of 0.5-2 µg/ml for ITR; 0.03-0.25 µg/ml for VORI; 2 -8 µg/ml for AMB and 16 µg/ml for CUR as given in Figure 2.

	MIC $_{80}$ (µg/ml)				MIC $_{80}$ (µg/ml)			MIC $_{80}$ (µg/ml)		
Strains	**CUR**	**ITR**	**ITR/CUR**	**FICI**	**VORI**	**VORI/ CUR**	**FICI**	**AMB**	**AMB/ CUM**	**FICI**
A. fumigatus	16	0.5	0.25/2	0.63	0.125	0.03/1	0.3	8	8.0/16	2
A. flavus	16	0.5	0.25/0.5	0.53	0.06	0.03/1	0.3	2	2.0/16	2
A. terreus	16	2	0.25/2	0.63	0.03	0.02/0.25	0.52	4	4.0/16	2
A. niger	16	1	0.25/2	0.56	0.25	0.06/0.5	0.27	2	2.0/16	2
MIC Range	**16**	**0.5-2**	**0.25/0.5-2**	**0.53-0.63**	**0.03-0.25**	**0.02-0.03/ 0.25-1**	**0.27-0.52**	**2.0-8.0**	**2.0-8.0/ 16**	**2**

Figure. 2: CUR shows synergy to additive with azoles and polyenes, which was calculated by Fractional Inhibitory Concentration Index (FICI). The FICI is the sum of the FIC of each of the drugs, which in turn was defined as the MIC of each drug when used in combination divided by the MIC of the drug when used alone. The FICI was defined as: synergic if the FICI was ≤0.5; neither synergistic nor antagonistic if the FICI was >0.5 to ≤4; and antagonistic if the FICI was >4. The combination of CUR with antifungal drugs drastically reduced the MIC of VORI in combination suggesting synergy as shown in Figure 2. The FICI was used to classify drug interaction and the results were interpreted as follow: synergy, FICI ≤0.5; no interaction, FICI >0.5 to ≤4.0; or antagonism, FICI >4.0. The FICI was in the range of 0.53-0.63 for ITR and CUR; 0.27-0.52 for VORI (synergy to no-interaction) and CUR and 2 for AMB and CUR as summarized in Figure 2. The results reflect that CUR, which by itself is partially active, enhanced the antifungal activity of VORI against *Aspergillus*, as per their FICI.

Time- Kill Kinetics

To confirm the results of synergy obtained in chequerboard microdilution, time- kill kinetics was performed, which provides better growth kinetic information over time and detailed picture of the effect of drug combinations on rate and extent of fungal killing. As per time- kill kinetics results- CUR, ITR and AMB didn't show any significant decrease in CFU till 24 h, as shown in Figure 3 A & B for *A. fumigatus* and *A. flavus* respectively. However, in the combination drugs kill-kinetics, where CUR along with the combination drug was added together in broth culture at 0-h, clearly

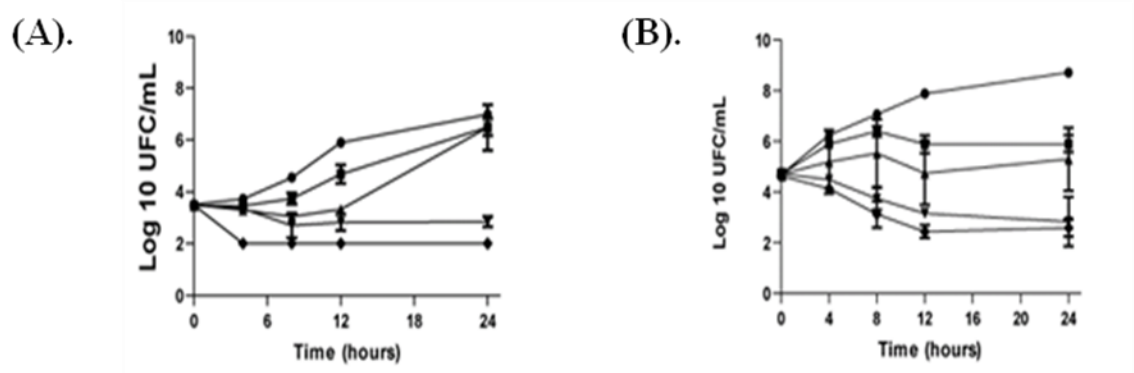

Figure. 3: Time-kill assay to show the effect on (A). *A. fumigatus* (B). *A. flavus* using different drugs alone and in combination. Filled symbols represent growth in presence of CUR alone (filled circles); AMB alone (filled squares); VORI alone (filled inverted triangles); AMB and CUR in combination (filled squares); VORI and CUR in combination (filled diamonds).

show that AMB and ITR display good synergy with CUR (Figure 3). Interestingly, CUR with AMB and ITR showed better fungicidal effect than with CUR as found in chequerboard analysis (Figure 1 A & B). CUR (16µg/ml) by itself show reduction in CFU (Figure 3), but in combination with ITR (1µg/ml) showed 2.79- log reduction (CFU/ml) (16:1 ratio by concentration i.e. 16 µg/ml of CUR: 1 µg/ml of ITR) (Figure 3). CUR (16µg/ml) with VORI (0.125 µg/ml) showed 3-log reduction in 128:1 ratio by concentration (i.e. 16µg/ml of CUR: 0.125µg/ml of VORI) (Figure 3). CUR (16µg/ml) and AMB (4µg/ml) even in the ratio of 4:1 (i.e. 16:4µg/ml), gave 3.18 and 3.77 log reduction in CFU/ml respectively (Figure 1 B). This study makes it clear that the antifungal activity increases in combination with CUR against *Aspergillus.* This suggests that the combinatorial therapy with desirable activities, which encourage lowering the dose of drugs, should be promoted to avoid emergence of resistance.

Germ tube Inhibition assay

For hyphae germination, 10^6 conidia/ml of *A. fumigatus* were incubated for 14h in RPMI 1640. Germination into hyphae was complete by 12h in the untreated controls (data not shown), but germination had only begun in the conidia after 16h in the well where drugs were added in combination (Figure 4). CUR was added to 1X MIC concentration (16 µg/ml) (Figure 4 A); ITR was added to 1X MIC concentration (1µg/ml) (Figure 4 B); AMB was added to 1X MIC concentration (4 µg/ml) and their combinations *i.e.,* ITR & CUR and AMB & CUR (Figure 4 D & E respectively). Thus, combination of the drugs caused a profound initial delay in conidial germination.

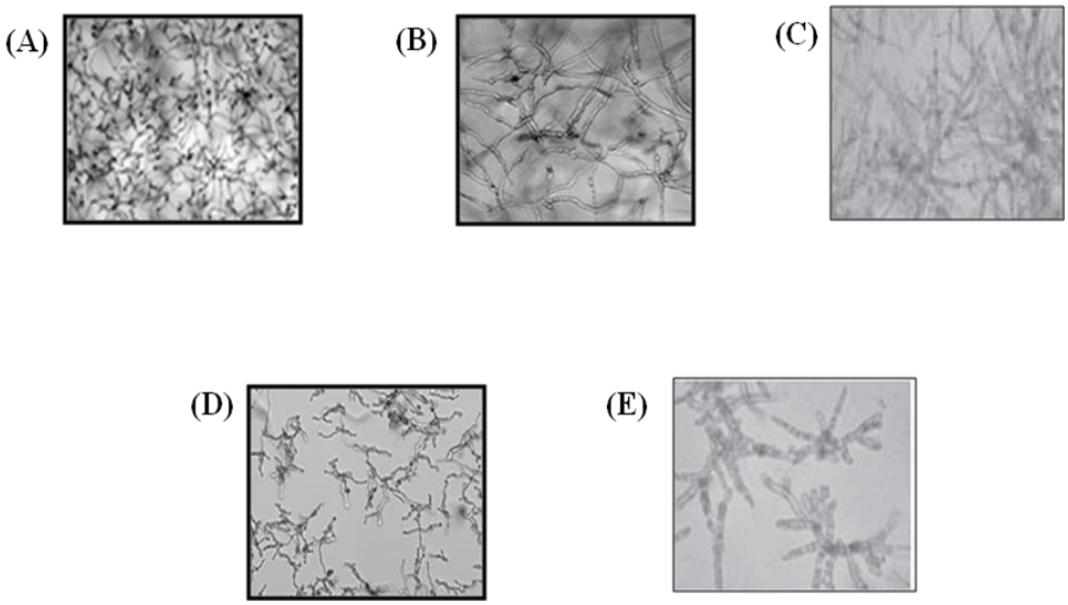

Figure 4: Germ Tube assay show reduction in filamentation in *A. fumigatus* with (A). CUR at 1X MIC concentration (16µg/ml); (B). ITR at 1X MIC concentration (1µg/ml); (C). AMB at 1X MIC concentration (4µg/ml); (D). VORI and CUR in combination (1X MIC each); (E). AMB and CUR in combination (1X MIC each).

4. CONCLUSION

This suggests that combinatorial therapy with desirable activities, which encourage lowering the dose of drugs, increasing the rate of fungal killing, shorten the duration of therapy, avoid the emergence of drug resistance, expand the spectrum of activity and decrease drug related toxicity should be promoted [17], [25].

5. ACKNOWLEDGEMENT

Authors acknowledge Daiichi Sankyo, Gurgaon for providing cultures. This work was supported by the individual grant to R. Pasrija from University Grant Commission (F.No. 42-647(2013)SR) and Department of Science and Technology (SERB/F/4213/2013-14) and Department of Biotechnology IPLS funding (BT/PR4329/INF'22/1444/2011) to Maharshi Dayanand University.
We thank D.J. Upadhyay, Diiachi Sankyo, Gurgram for providing the strains.

6. CONFLICT OF INTEREST

Authors don't have any conflict of interest with anyone.

REFERENCES

1. Bennett JW. An Overview of the Genus *Aspergillus*. In: Machida M, Gomi K, editors. *Aspergillus* Mol Biol Genomics. Norfolk, UK: Caister Academic Press; 2010. p. 1–17.
2. Fairlamb AH, Gow NAR, Matthews KR, Waters AP. Drug resistance in eukaryotic microorganisms. Nat Microbiol. 2016;1:16092.
3. Alastruey-Izquierdo A, Cadranel J, Flick H, Godet C, Hennequin C, Hoenigl M, et al. Treatment of Chronic Pulmonary Aspergillosis: Current Standards and Future Perspectives.

Respir Int Rev Thorac Dis. 2018;1–12.

4. Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. Hidden Killers: Human Fungal Infections. Sci Transl Med. 2012;4:165rv13.
5. Xiong Q, Hassan SA, Wilson WK, Han XY, May GS, Tarrand JJ, et al. Cholesterol Import by *Aspergillus fumigatus* and Its Influence on Antifungal Potency of Sterol Biosynthesis Inhibitors. Antimicrob Agents Chemother. 2005;49:518–524.
6. Gupta AK, Tomas E. New antifungal agents. Dermatol Clin. 2003;21:565–576.
7. Brandt M, Brown C, Burkhart J, Burton N, Cox-Ganser J, Damon S, et al. Mold prevention strategies and possible health effects in the aftermath of hurricanes and major floods. MMWR Recomm Rep Morb Mortal Wkly Rep Recomm Rep. 2006;55:1–27.
8. Dilendra C, Sao S, Deashmukh YK, Verma L, Sahu PK. Isolation of *Aspergillus niger* from *Allium cepa* bulb and production of Citric Acid from it. 2013. 5:144–147.
9. Dagenais TRT, Keller NP. Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. Clin Microbiol Rev. 2009;22:447–465.
10. Bhabhra R, Miley MD, Mylonakis E, Boettner D, Fortwendel J, Panepinto JC, et al. Disruption of the *Aspergillus fumigatus* gene encoding nucleolar protein CgrA impairs thermotolerant growth and reduces virulence. Infect Immun. 2004;72:4731–4740.
11. Krishnan S, Manavathu EK, Chandrasekar PH. *Aspergillus flavus:* an emerging non-*fumigatus Aspergillus* species of significance. Mycoses. 2009;52:206–222.
12. Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW. *Aspergillus flavus*: human pathogen, allergen and mycotoxin producer. Microbiol Read Engl. 2007;153:1677–1692.
13. Palencia ER, Hinton DM, Bacon CW. The Black *Aspergillus* Species of Maize and Peanuts and their Potential for Mycotoxin Production. Toxins. 2010;2:399–416.
14. Steinbach WJ, Benjamin DK, Kontoyiannis DP, Perfect JR, Lutsar I, Marr KA, et al. Infections due to *Aspergillus terreus*: A Multicenter Retrospective Analysis of 83 Cases. Clin Infect Dis. 2004;39:192–198.
15. Seyedmousavi S, Meletiadis J, Melchers WJG, Rijs AJMM, Mouton JW, Verweij PE. In Vitro Interaction of Voriconazole and Anidulafungin against Triazole-Resistant *Aspergillus fumigatus*. Antimicrob Agents Chemother. 2013;57:796–803.
16. Mukherjee PK, Sheehan DJ, Hitchcock CA, Ghannoum MA. Combination treatment of Invasive Fungal Infections. Clin Microbiol Rev. 2005;18:163–194.
17. Spitzer M, Robbins N, Wright GD. Combinatorial strategies for combating invasive fungal infections. Virulence. 2017;8:169–185.
18. Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing. EUCAST Technical Note on the method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for

conidia-forming moulds. Clin Microbiol Infect Dis. 2008;14:982–984.

19. Gomez-Lopez A, Aberkane A, Petrikkou E, Mellado E, Rodriguez-Tudela JL, Cuenca-Estrella M. Analysis of the Influence of Tween Concentration, Inoculum Size, Assay Medium, and Reading Time on Susceptibility Testing of *Aspergillus* spp. J Clin Microbiol. 2005;43:1251–1255.
20. Wayne P. Reference method for broth dilution antifungal susceptibility testing of yeasts, Approved standard-second edition. CLSI Doc M27-A2 2002.
21. Li Y, Sun S, Guo Q, Ma L, Shi C, Su L, et al. In vitro interaction between azoles and cyclosporin A against clinical isolates of *Candida albicans* determined by the chequerboard method and time–kill curves. J Antimicrob Chemother. 2008;61:577–585.
22. Kumar A, Dhamgaye S, Maurya IK, Singh A, Sharma M, Prasad R. Curcumin Targets Cell Wall Integrity via Calcineurin-Mediated Signaling in *Candida albicans*. Antimicrob Agents Chemother. 2014;58:167–175.
23. Klepser ME, Wolfe EJ, Jones RN, Nightingale CH, Pfaller MA. Antifungal pharmacodynamic characteristics of fluconazole and amphotericin B tested against *Candida albicans*. Antimicrob Agents Chemother. 1997;41:1392–1395.
24. Mowat E, Butcher J, Lang S, Williams C, Ramage G. Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. J Med Microbiol. 2007;56:1205–1212.
25. Shin S, Lim S. Antifungal effects of herbal essential oils alone and in combination with ketoconazole against *Trichophyton* spp. J Appl Microbiol. 2004;97:1289–1296.