ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

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Role of *Ganoderma lucidum* against triazole drugs resistant Aspergillus species

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Article History:

Received on: 02.06.2018
Revised on: 20.08.2018
Accepted on: 22.08.2018

Keywords:

Ganoderma lucidum, Triazole Group antibiotics, Aspergillus Species, Minimal Inhibitor Concentration

ABSTRACT

Edible Medicinal mushroom *Ganoderma lucidum* have an established in use of traditional new antimicrobial drugs and oriental therapies. The current study helps to describe the development of acquired resistance and efficacy of certain antifungal agents against different *Aspergillus species.* A total of 150 clinical isolates of different Aspergillus spp. were obtained from tertiary care center, Puducherry. Various drug concentrations (Posaconazole, Voriconazole, Itraconazole and Ganoderma extract) are based on the measurement of the fungal growth in the presence, so as to determine the minimum inhibitory concentration (MIC) of antifungals was performed according to the CLSI guidelines. *Ganoderma lucidum* full part fruiting body were collected and prepared extraction with help of ethanol at various concentrations 0.008 to 8 µg/ml. Triazoles and Ganoderma extract of MIC distribution among different Aspergillus species were as follows: *A. fumigatus* - Ganoderma extract, 0.12; itraconazole, 1; posaconazole, 0.25; voriconazole, 0.5; *A. flavus* - Ganoderma extract, 0.25; itraconazole, 1; posaconazole, 0.25; voriconazole, 0.5; *A. niger* - Ganoderma extract, 0.12; itraconazole, 1; posaconazole, 0.5; voriconazole, 1; and *A. terreus* - Ganoderma extract, 0.25; itraconazole, 1; posaconazole, 0.25; and voriconazole, 0.5. Ganoderma extract, posaconazole, voriconazole, and itraconazole. Majority of Aspergillus species exhibited in vitro resistance to voriconazole followed by itraconazole.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v9i4.1653

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INTRODUCTION

Advanced clinical practice in China, Japan, Korea, and other Asian countries initiated to impart on medicinal mushroom *Ganoderma lucidum* and its derivatives (Rakhee *et al.,* 2017, Nahata, A *et al.,* 2013). Mainly in Immunocompromised and Immunosuppressive patients are high level to get secondary infections to like fungal infections. The serious infections due to *Aspergillus spp.* are emerging and causes infectious high morbidity and mortality worldwide (Pfaller M.A, *et al.,* 2002, Naveenkumar C, *et al.,* 2018, Ng How Tseung KSY, *et al.,*

2016) High mortality rate associated with systemic mycoses (Aspergillosis) which primarily affect the lungs (Garbino D.J., 2004). Effective treatment requires both Immunocompromised and Immunosuppressive patients to treat invasive fungal infections were limited due to the development of resistance, spectrum activity, etc. (Maertens J.A., 2004). A recent study shows that Ganoderma show vital role in antitumour, immunomodulatory antibacterial activity and very few studies in Antifungal Activity. Along with several studies displays triazole group antifungals (Itraconazole, Posaconazole and Voriconazole) have a wide spectrum of in vitro resistance against moulds and also important in therapeutic agents (Meletiadis J *et al.,* 2002, Vermeulen E, eta., 2015, Paul E, *et al.,* 2016). The activity of the various antifungal agents has been described as fungistatic or fungicidal, mainly by in vitro testing methods and also depending on the drug activity, various isolates, and test environment. Conversely, the azoles group drugs such as itraconazole and voriconazole as exhibit concentration-dependent fungistatic activity and fungicidal activity against many isolates (Klepser, M.E, *et al.,* 1997, Manavathu, E.K, *et al.,* 1997, Johnson, E.M, *et al.,* 1998, Pfaller, M.A, *et al.,* 2004). The minimum inhibitory concentrations (MICs) with the exception for Aspergillus spp. with the triazoles (Moore, C.B *et al.,* 2000), few correlates between in vivo outcome and MIC have been described in the previous studies (Rex, J.H, *et al.,* 2001). The in vitro relationships between MIC and MFC for a limited number of fungal species have attempted to examine in previous studies, by employing non-standardized methodologies (Espinel-Ingroff, 2001, Espinel-Ingroff, A, *et al.,* 2002, Sutton, D.A, *et al.,* 1997). However, the fungistatic effects on clinical outcome remain unproven so far. However, the fungicidal activity has proven significance by the in vitro methods (Lewis, J.S *et al.,* 2008, Andes, D et al. 2013). The Current study discusses the various Aspergillus species and its antifungal activity by Minimum inhibitory concentrations (MIC) to measure the fungal growth among different drug concentrations (Wanger A, 2012). In addition, the Cutoff values of the MIC value were compared with epidemiologic cut-off values (ECVs) of the MIC as per CLSI guidelines (Espinel I.A *et al.,* 2011).

MATERIAL AND METHOD

Clinical Isolates for MIC Distribution analysis

Totally 150 clinical isolates of various *Aspergillus* spp. were obtained from Sri Lakshmi Narayana Institute of Medical Sciences, Puducherry from January to December 2017. The isolates were obtained from different sources, including bronchoscopy sputum, and tissue biopsy specimens, etc. The isolates as follows: 102 of *Aspergillus fumigatus*, 20 of *Aspergillus flavus*, 16 of *Aspergillus niger* and 12 of *Aspergillus terreus.* The various isolates were collected and screened for identification and susceptibility testing. All isolates were identified by standard microscopic and macroscopic in Sabouraud dextrose agar and potato dextrose agar to ensure purity and viability of the isolates.

Antifungal Agents and Drug Concentration Ranges

Posaconazole, voriconazole and itraconazole were obtained from the manufacturers (Himedia). Fully fruit body developed *Ganoderma lucidum* were obtained from MKV Organic, Puducherry for the current study. 100 ml of ethanol were used to mix 05 g of mushroom powder with the help of Soxhlet apparatus and Whatman No. 4 filter paper used for filtration process, to obtained ethanol extract of Ganoderma. Polyethene glycol (Posaconazole and Itraconazole), Dimethyl sulfoxide (Voriconazole) and ethanol (*Ganoderma lucidum*) are used to prepare stock solutions. The various concentrations of drugs in the wells ranged from 0.008 to 8 µg/ml. At overnight incubated at 35°C, the inoculated microdilution wells were measured for OD values. The MIC endpoint for the Ganoderma extract and triazoles was defined by screening complete inhibition of growth at the lowest concentration.

CLSI Broth Microdilution Determination of Minimum Inhibitory Concentration

The various Aspergillus species have been tested antifungal susceptibility testing by triazole activity against by performing standards for broth microdilution method, based on the guidelines of Clinical and Laboratory Standards Institute (CLSI) (Pfaller M *et al.,* 2011, NCCLS Standards 2000). The inoculum density (0.4 to 5 X 104 CFU/ml), the glucose content of the medium (0.2%), in the round-bottom microdilution 96 wells-XTT assay (Lass F.C, *et al.,* 2010, Arno Schmalreck, *et al.,* 2012, Anita karma, *et al.,* 2016) 100 μL of Aspergillus species of cell suspension was transferred into 96 well of a microtiter plate, with both positive and negative control, the well contains Roswell Park Memorial Institute (RPMI) 1640 media and the plate was placed in a shaker at 75 rpm to allow for adherence to the surface of the wells for the incubation period for 2 hours at 37°C (Kumar NC, *et al.,* 2017). Control wells are placed subsequently at three microtiter plate, with the absence of *Aspergillus spp* suspensions. The cell suspensions were aspirated with the help of pipette and with the help of 150 μL of Phosphate Buffer Saline (PBS) washed two times to remove loosely adherent cells. 100 μL of RPMI 1640 media was then transferred into each washed wells subsequently and the plates were incubated at 37°C in a shaker at 75 rpm. After the incubation, media has been aspirated with the help of pipette

and various concentrations like 0.008 to 8 µg/ml, of both extracts, has been added separately as mentioned earlier. The 96 wells were incubated for overnight at 37°C with 5% CO_2 atmosphere (Pasetto S *et al.,* 2014). XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)- 2H-tetrazolium-5-carboxanilide) added and incubated in the dark area 2 – 3hrs at 37°C, after the supernatant aspiration of each well has been performed (Hawser SP *et al.,* 1998, Honraet K, *et al.,* 2005). A gradient of orange colour has been typically demonstrated by visual inspection of the microtitre plates.

Data Analysis

Various drug concentrations with different ranges of MIC required to inhibit or kill the various Aspergillus species, values were determined by microtiter plate reader at OD 450-650 nm to find the significant activity of Ganoderma extract and triazole group.

RESULT

The minimal inhibitory concentrations (MICs) activity of Ganoderma ethanolic extract showed the minimal MIC compared with the other 03 drugs and hence the greatest activity against most of Aspergillus species. These results are the highest compared to all the other medications used in this study, followed by Ganoderma extract, Posaconazole, voriconazole, itraconazole and in order of decreasing activity. Highest activities shown by Ganoderma extract against all the Aspergillus species, in the series of *Aspergillus fumigates* 33% (34) at the point of 0.12 µg/ml, *Aspergillus niger* 32% (05) at the intensity of 0.12 µg/ml*, Aspergillus terreus* 33% (04) at the level of 0.25 µg/ml and *Aspergillus flavus* 30% (06) at the plane of 0.25 µg/ml*. Aspergillus fumigates* (102) shows MIC for Ganoderma extract 33% (34) at the plane of 0.12 µg/ml, Posaconazole 31% (32) at the rank of 0.25 µg/ml, Itraconazole 27% (28) at the point of 1 µg/ml and Voriconazole 27% (26) at the intensity of 0.5 µg/ml. *Aspergillus flavus* (20) shows MIC for Posaconazole 35% (07) at the plane of 0.25 µg/ml, Itraconazole 35% (07) at the level of 1 µg/ml, Ganoderma extract 30% (06) at the amount of 0.25 µg/ml and Voriconazole 25% (05) at the level of 0.5 µg/ml the highest MIC value shown in Posaconazole and Itraconazole and lowest in the Ganoderma extract and Voriconazole. *Aspergillus niger* (16) shows MIC for Ganoderma extract 31% (05) at the point of 0.12 µg/ml, Posaconazole 31% (05) at the point of 0.5 µg/ml, Itraconazole 25% (04) at the range of 1 µg/ml and Voriconazole 25% (04) at the series of 1 µg/ml the highest MIC value shown in both of Ganoderma extract and Posaconazole and the lowest in Itraconazole and Voriconazole. *Aspergillus terreus* (12) shows MIC for Ganoderma extract 33% (04) at the level of 0.25

Table 1: MIC distributions of Triazoles and Ganoderma Ethanolic extract for different Aspergillus spp.

Species	Antifungal agents	No. of isolates MIC (µg/ml)									
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8
A. fumigatus (n = 102)	Ganoderma extract	~	~	29	34	18	12	06	03	~	~
	Itraconazole	~	~	~	15	18	21	28	16	04	~
	Posaconazole	~	~	10	28	32	19	14	09	~	~
	Voriconazole	~	~	~	16	23	27	16	11	09	~
A. flavus (n = 20)	Ganoderma extract	~	~	~	02	06	05	04	03	~	~
	Itraconazole	~	~	~	03	03	04	07	02	1	~
	Posaconazole	~	~	03	04	07	05	04	~	~	~
	Voriconazole	~	~	03	03	04	05	03	02	~	~
A.niger (n = 16)	Ganoderma extract	~	~	~	05	04	03	03	01	~	~
	Itraconazole	~	~	01	02	03	03	04	03	~	~
	Posaconazole	~	~	02	03	04	05	02	~	~	~
	Voriconazole	~	~	02	03	03	03	04	1	~	~
A.terreus (n = 12)	Ganoderma extract	~	~	~	03	04	02	02	01	~	~
	Itraconazole	~	~	01	01	02	03	04	01	~	~
	Posaconazole	~	~	02	03	04	02	01	~	~	~
	Voriconazole	~	~	~	02	03	04	02	01	~	~

Table 2: Epidemiological cut-off values (µg/ml) for Ganoderma extract, Itraconazole, Voriconazole and Posaconazole according to the Aspergillus species 6, 10

Species	Posaconazole	Itraconazole	Voriconazole	Ganoderma extract
Aspergillus fumigates	0.5	1	1	2
Aspergillus flavus	0.5	1	1	2
Aspergillus niger	0.5	2	2	2
Aspergillus terreus	0.5	1	1	2

µg/ml, Posaconazole 33% (04) at the sequence of 0.25 µg/ml, Voriconazole 33% (04) at the level of 0.5 µg/ml and Itraconazole 33% (04) at the level of 1 µg/ml shows similar MIC value at various concentrations. Among the obtained result fourteen isolates were harbouring resistance to Voriconazole (09) and Itraconazole (05) range in between 2 – 8 µg/ml as per the CLSI guidelines. The Ganoderma extract has shown the good significant activities even in the resistant strains of the Aspergillus were explained in Table1.

The epidemiologic cut-off values (ECVs) of the MICs were obtained from previous studies and considered in our study as a starting point of decreasing susceptibility and the emergence of resistance to antifungal agents Table 2.

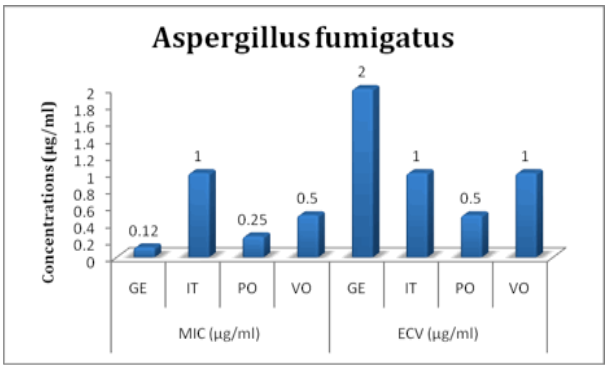

Figure 1: Comparison of ECVs and MIC distributions for *Aspergillus fumigatus*

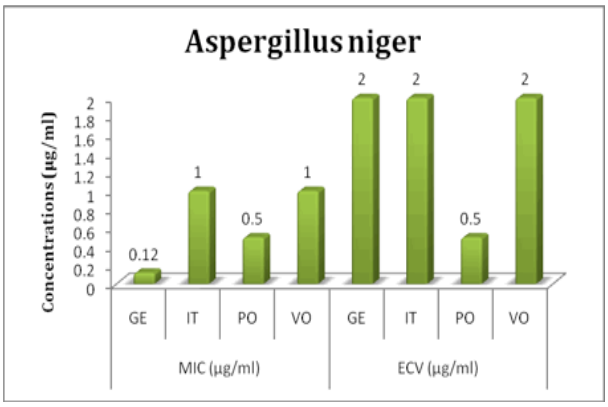

Figure 2: Comparison of ECVs and MIC distributions for *Aspergillus niger*

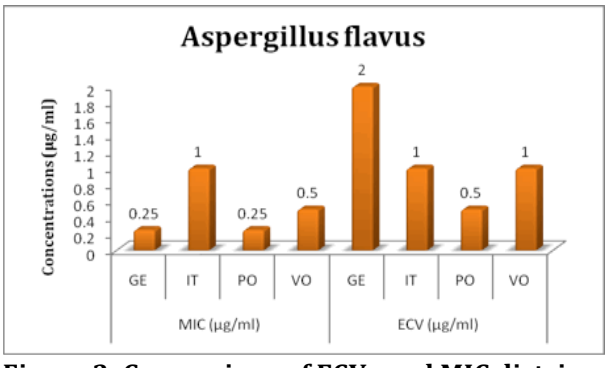

Figure 3: Comparison of ECVs and MIC distributions for *Aspergillus flavus*

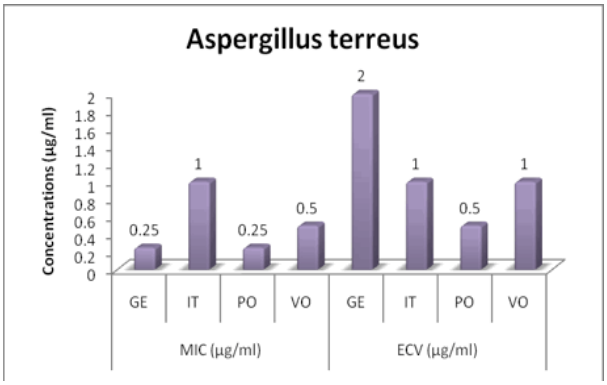

Figure 4: Comparison of ECVs and MIC distributions for *Aspergillus terreus*

The Epidemiologic cut-off values (ECVs) were compared with the obtained MIC value. The comparative result for *Aspergillus fumigatus* for Ganoderma extract of the ECV shows 2 µg/ml and obtained MIC value was 0.12 µg/ml, subsequently for Itraconazole ECV shows 1 µg/ml and obtained MIC value was 1 µg/ml, Posaconazole ECV shows 1 µg/ml and obtained MIC value was 0.25 µg/ml and Voriconazole ECV showed 1 µg/ml and obtained MIC value was 0.5 µg/ml were explained in Fig. 1. *Aspergillus flavus* for Ganoderma extract of the ECV shows 2 µg/ml and obtained MIC value was 0.25 µg/ml, subsequently for Itraconazole ECV shows 1 µg/ml and obtained MIC value was 1 µg/ml, Posaconazole ECV shows 1 µg/ml and obtained MIC value was 0.25 µg/ml and Voriconazole ECV shows 1 µg/ml and obtained MIC value was 0.5 µg/ml were explained in Fig. 2. *Aspergillus niger* for Ganoderma extract of the ECV shows 2 µg/ml and obtained MIC value was 0.12 µg/ml, subsequently for Itraconazole ECV shows 2 µg/ml and obtained MIC value was 1 µg/ml, Posaconazole ECV shows 1 µg/ml and obtained MIC value was 0.5 µg/ml and Voriconazole ECV shows 2 µg/ml and obtained MIC value was 1 µg/ml were explained in Fig. 3. *Aspergillus terreus* for Ganoderma extract of the ECV shows 2 µg/ml and obtained MIC value was 0.25 µg/ml, subsequently for Itraconazole ECV shows 1 µg/ml and obtained MIC value was 1 µg/ml, Posaconazole ECV shows 1 µg/ml and obtained MIC value was 0.25 µg/ml and Voriconazole ECV shows 1 µg/ml and obtained MIC value was 0.5 µg/ml were explained in Fig. 4.

DISCUSSION

The medicinal and pharmacological activities of *G.lucidum* depend on the abiotic factors like altitude, temperature, humidity as well as abiotic factors like substrate which may in turn account for the presence of differential levels of primary and secondary metabolites (Morath, S. U, *et al.,* 2012). Therefore, for the first time, we ventured to evaluate a detailed comparative characterization of the Indian variety of *G. lucidum* in terms of Antifungal activity and compared with the commercially

available drugs. The presence of these various phytochemicals had the medicinal importance of *G. lucidum,* which is very much in alignment with the earlier reported literature (Ren, A *et al.,* 2017). So far the antifungal susceptibility testing results were not much sensitivity and specificity to determine the proper treatment for an infection caused by a particular organism. Preferably, to predict the therapeutic outcome by comparing the clinical breakpoints (CBPs) were for the betterment of the therapeutic. As most of the immunocompromised patients were lack of adjunctive host response is likely to have an additional impact on the probable outcome of therapy from the infections invasive mould. Even though more research work carried out in the several decades, unfortunately, for many filamentous fungal (mould) infections, the development of CBPs is currently not standardized/impossible, due to lacking sufficient clinical trial data for some of the rarer, emerging pathogens. The current data may help clinicians dealing with infections with rarer moulds to decide which antifungal drug may be most likely to be effective which has been compared with the CBPs. The value of susceptibility testing depends on when a specific agent result can define the susceptibility or resistance of the organism being tested. In our study Ganoderma extract had the lowest MICs compared to other medications and accordingly the greatest activity against different Aspergillus species in order of *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus* and *Aspergillus niger* in order of increasing MIC and decreasing activity. The antifungal activity of the Ganoderma extracts shown significant activity against the various Aspergillus species at the minimal concentration level while comparing with the other commercial available antifungal triazole group of antifungal drugs such as Posaconazole, Itraconazole and Voriconazole. According to the previous studies show that posaconazole had the greatest activity against the majority of Aspergillus species followed by voriconazole and itraconazole in order of decreasing activity (Aldorkee S.Y. 2017). The determination of acquired resistance to particular antifungal agents by identifying the "non-wild-type" in the absence of CBPs, ECVs for the particular isolate. With a large number of clinical isolates by analysis various concentrations of antifungal agents by performing antifungal susceptibility testing by using analyzing methods of CBPs and ECVs were projected in the present study for the future development. MIC range higher than the ECV of the MICs may help to identify acquired resistance mechanisms organisms. *cyp51A* gene plays a major role in the drug resistance of the Aspergillus species. The present study reveals the importance of the ECVs to assist the evaluation of clinical isolates by identifying those strains with reduced triazole susceptibility due to mutations and also determine the early warning of emerging subtle changes in the susceptibility patterns of these organisms. This study reveals that isolates of Voriconazole (09) and Itraconazole (05) probability of harbouring a triazole resistance mechanism, Ganoderma extract shows the significant value against the Voriconazole and Itraconazole resistant strains too. Overall, the obtained data in this study confirm that fungicidal activities of Ganoderma lucidum had a more significant activity while compared with the commercially available triazole drugs for isolates of Aspergillus species.

CONCLUSION

The Triazoles and Ganoderma extract had shown significant activity in vitro against most of Aspergillus species in the following order of various drugs: Ganoderma extract, Posaconazole, Voriconazole and Itraconazole. The comparative result ECVs from the previous study and MIC shows the more significant ratio of Ganoderma extract while compared with triazole activity from the current study. Few of Aspergillus species exhibited in vitro resistance to voriconazole followed by itraconazole have been well treated by the Ganoderma extract for the future health betterment.

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