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Research Article

IN VITRO EFFECTIVENESS OF FUNGAL DRUGS ON FUNGAL ISOLATES CAUSING MUCORMYCOSIS IN POSTCOVID PATIENTS

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ABSTRACT

Covid-19 pandemic triggered Mucormycosis as fast progressing dreadful multidrug resistant fungal coinfection in post covid patients. The present study aimed to compare the efficacy of eight commercially available antifungal tablets on fungal pathogen of Mucormycosis isolated from two postcovid patients clinical samples. In this study eight antifungal tablets were used i.e. Zocon 150 mg, Nuforce 150 mg, Flucanzole 150 mg, Fussy 150 mg, Itrostred 200 mg, Candiforce 200 mg, Terbinaforce 250 mg and Grisovin FP 250 mg. Fungal pathogens were isolated on SDS agar by spread plate technique and identified. We observed Multipal Fungal Rare Mix Infection. Along with *Mucor spp*. and *Candida spp*. we observed rare *Fusarium spp*. and *Alterneria Spp*. and *Aspergillus spp*. were stored in pure culture on SDS agar slant. The speed of growth of *Mucor spp*. was reported as one and half times more than usual. The antifungal activity was studied by paper disc diffusion method on the lawn culture of *Mucor spp*. and *Candida spp*. The zone of inhibition (ZOI) of above eight antifungal tablets against *Mucor spp*. was 21mm, 27mm, 32mm, 21mm, 25mm, 11mm, 56mm and 37mm respectively. But for the *Candida spp*. out of eight tablets only one i.e. Grisovin FP 250mg showed 15mm zone of inhibition, remaining all were resistant. Thus Terbinaforce 250 mg and Grisovin FP 250 mg can be used as alternatives in antifungal therapy of mucormycosis in postcovid patients. Cocktail of Terbinaforce 250 mg and Grisovin FP 250 mg gives very good results against *Mucor spp*.

Keywords: Antifungals, Multidrug resistant microorganisms, Paper disc diffusion method, Postcovid patient's Mucormycosis.

1. INTRODUCTION

The organisms of order Mucarales are omnivorous in nature as these are found in dead and decaying organic matter and soil. They grow rapidlly with the production of large no. of spores. The fungal infection due to these spores is known as Mucormycosis. Types of mucormycosis are 1) Rhinocerebral (sinus and brain) 2) Pulmonary (lung) 3) Gastrointestinal 4) Cutaneous 5) Disseminated. Though humans usually exposed to these spores, progression of infection not allowed due to strong immune system. It causes disease only when immune system is critically lowered. Mycormycosis known to occur in low immunity such as cancer treatments, post organ transplant surgeries and uncontrolled diabetes. Mucormycosis is rare disease but its consequences are devastating since it is associated with unacceptably high mortality rates, ranging from 20 to 50% if localized up to 70 to 90% in cases of disseminated disease. Suddenly mucormycosis in postcovid patients is reported [1-3].

The emergence of fungal infection due to the fungi of order Mucorales of the class Zygomycetes is increasing in postcovid patients. Mucor infection occurs during Covid-19 infection or few days after the treatment of Covid-19. Mucormycosis is life threatening infection leads to invasion of the bone and soft tissue [3]. Rhinocerebral mucormycosis infection generally starts by breathing spores through nose. It starts in nose and progress to the eye and brain with facial abnormalities. Nose and sinus mucor infection is early disease and eye mucor infection is advanced disease. Facial puffiness with fever, severe headache, nasal congestion and nasal discharge, loss of sensation of face muscles, loosening of teeth, drooping eyelid, eye swelling or redness, blurr vision or loss of vision etc. are the different symptoms of mucormycosis in postcovid patients.

Covid 19 leads patients towards diabetes also in previously normal peoples. The severity of Mucor infection depends on patient's health and immunity. In Covid 19 patient immunocompressive treatments to avoid cytokine storm and increased blood suger level are the riskfactors for mucormycosis. Diabetes has been reported as risk factor for mucormycosis in 73.5% of cases in India [4]. Poorly controlled diabetes leads to Ketoacidosis high level of blood acids. The overload of iron supplements in patients also leads to progress the infection fastly. Mucor are able to use excess iron to grow and spread.

Proper diagnosis is important with patients history so treatment can be started as early as possible. Early and proper diagnosis of mucormycosis is important for increasing survival rate and reduce the need of surgery [5, 6]. Antifungal medications which inhibit and destroy the growth of fungal infection is involved in treatment. First line therapy of mucormycosis is amphotericin-B and its lipid formulations [7]. *In vitro* and *in vivo* data, researchers proposed to treat mucormycosis with high dose of LAMB (>5mg/kg/day) [8]. In the start of treatment high doses of intravenous medication are given. Following improvements for some weeks, doctors may step dawn to oral medications such as Pasaconazole. But the availability and cost of these two medications in Covid-19 pandamic era is so difficult for suffering patients. Removal of the infected part, dead tissue, damaged skin, and Subcutoneous tissue is called surgical debridement. Surgical resection of necrotic tissue is the core of mucormycosis therapy [9, 10]. Surgery is also costly and also changes facial shape. Research needed for the long term safty of patients in low cost medications.

So this study was aimed to search proper diagnosis of Mucormycosis using postcovid patient's clinical sample and to check the efficacy of commercially available antifungal medications for finding the alternatives for treatments.

2. MATERIAL AND METHODS

2.1. Clinical sample collection

Clinical samples of two postcovid patients suffering from Rhinocerebral mucormycosis were collected at the time of surgery from Dr. Tarang Shaha; ENT Surgeon, Lila Nursing Home, Barshi, Solapur, Maharashtra. Rhinosinus scrappings of both patients were collected in the sterial plastic air tight container containing 10 ml saline. The clinical samples were safly handled with all necessary precautions for the isolation of fungal pathogen.

2.2. Isolation of fungal pathogens

For the present study 2 clinical samples i.e. Rhinosinus scrappings collected from 2 postcovid patients suffering from mucormycosis were enriched in sabouraud's broth for 24-48 hrs at room temperature. The enriched samples were then used for isolation of the fungal pathogen. Total 7 fungal pathogens were isolated by the spread plate technique on Sabroud's Dextrose Sugar Agar medium (Glucose - 4g, Peptone - 1g, Agar - 2g, Water - 100ml, pH - 5.4) after incubation at ambient temp. for 2 to 3 days.

2.3. Identification of fungal pathogens

Patient's history with identification of characteristic symptoms through clinical evaluation leads to proper diagnosis of mucormycosis. Identification of fungal pathogens were carried out by Light microscopic examination. Both cases suggestive of Zygomycets with Multiple Fungal Rare Mix Infection. Cultural characteristics were studied on SDS agar. Morphological characters were studied. The growth pattern of fungi on the water agar and aerial and substrate mycelium studied. Germ tube test for *Candida spp.* was also carried out.

2.4. Study of efficacy of antifungal tablets against *Mucor spp.* and *Candida spp.*

Paper disc diffusion method was used to check the efficacy of commercially available antifungal tablets against *Mucor spp.* and *Candida spp*. Total eight tablets i.e. Zocon 150 mg, Nuforce 150 mg, Flucanzole 150 mg, Fussy 150 mg, Itrostred 200 mg, Candiforce 200 mg, Terbinaforce 250 mg, Grisovin FP 250mg were used against fungal pathogens. The test was carried out by spreading the surface of an SDS agar plate with fungi isolated from postcovid patients clinical sample. Antifungal containing paper disks were then applied to the lawn culture spreaded on agar plate and the plate was incubated at room temp. Zone of inhibition for each antifungal tablets was measured in mm. By comparing the size of zone of inhibition the most effective antifungal drug was easily pointed out.

2.5. Study of use of cocktail of two antifungal tablets against *Mucor spp.*

Terbinaforce 250 mg and Grisovin FP 250mg showed good zone of inhibition against *Mucor spp.* hence we studied the effect of cocktail of these two antifungal tablets aginst *Mucor spp.* For the first time we made the cocktail of Terbinaforce 250 mg and Grisovin FP 250mg and used by paper disc diffusion method against fungal pathogen of mucormycosis. Also compared it with agar well diffusion method.

3. RESULTS AND DISCUSSIONS

Total two rhinosinus scrappings clinical samples were analyzed from two postcovid patients. First patient was male (age 42) from Madha, Solapur. He got increased blood sugar after Covid-19 treatment. Second patient was female (age 74) from Shrigonda, Ahamadnagar. She already had blood sugar before the Covid-19 treatment. The details of symptomatic condition were as per Table 1.

Multiple Rare Mix Fungal Infection was reported in these postcovid patient's clinical samples. Total seven isolates of fungal pathogen were isolated from two clinical samples of these two postcovid patients suffering from mucormycosis. From the Sample - 1, three fungal pathogens were isolated i.e. *Mucor spp., Candida spp*. and Fusarium spp. on SDS agar. The Sample - 2 gives total four fungal pathogens i.e. *Mucor spp., Candida spp., Aspergillus spp*. and *Alterneria spp.* respectively.

The growth rate of *Mucor spp*. were found increased and found one and half times more than usual growth rate. There were no reports in the literature regarding rare mix fungal infection in postcovid patient suffering from Mucormycosis. Thus our finding of *Fusarium spp.* and *Alterneria spp.* in clinical samples of postcovid patients suffering from Mucormycosis represent maiden report.

No one also had reported increased rate of growth of *Mucor spp.* In present investigation, we reported increase in growth rate by one and half time more than usual which also a maiden report.

Results of efficacy of antifungal tablets in *Mucor spp.* are presented in table 3.The results clearly indicate that all eight used antifungal tablets were effective to *Mucor spp*. The zone of inhibition due to action of zocon, nuforce, Flucanzole, Fussy, Itrostred, Candiforce, Terbinaforce and Grisovin were measured as 21, 27, 32, 21, 25, 11, 56, and 37mm respectively. Thus terbinaforce was reported as most effective on *Mucor spp.* causing Mucormycosis.

On the other hand, same all tablets except Grisovin was found ineffective on *Candida spp.* Grisovin was only tablet found effective on *Candida spp***.** and the zone of inhibition was reported as15mm only.

Table 1: Information of postcovid patient

Native of postcovid patient	Gender of postcovidpatient	Age	Symptoms	Clinicalsample collected	Day of operation after Covid Positive
Madha, Solapur. (M.S)	Male	42	Severe head ache, Swollen face, black discharge from nose	Rhinosinus scrappings	17
Shrigonda, Ahmadnagar.(M.S)	Female	74	Lt. eye blurr vision, Swollen face, loosening of teeth	Rhinosinus scrappings	53

Table 2: Fungal pathogens isolated from two postcovid patients clinical samples

Native and Gender ofpostcovid patient	Clinical sample	Fungal pathogens isolated
Male Madha, Solapur,(M.S).	Sample - 1	***Mucor spp.***
		Candida spp.
		Fusarium spp.
Female Shrigonda, Ahmadnagar, (M.S).	Sample - 2	***Mucor spp.***
		Candida spp.
		Aspergillus spp.
		Alterneria spp.

Table 3: Efficacy of Antifungal Tablets on *Mucor spp. and Candida spp.*

Brand of Tablets	Ingredients	Diameter of zone of inhibition against ***Mucor spp***.in mm.	Diameter of zoneof inhibition against ***Candida spp.*** in mm.
Zocon 150 mg	Fluconazole	21 (Sensitive)	0 (Resistant)
Nuforce 150 mg	Fluconazole	27 (Sensitive)	0 (Resistant)
Flucanzole 150mg	Fluconazole	32 (Sensitive)	0 (Resistant)
Fussy 150 mg	Fluconazole	21 (Sensitive)	0 (Resistant)

Itrostred 200 mg	Itraconazole	25 (Sensitive)	0 (Resistant)
Candiforce 200mg	Itraconazole	11 (Sensitive)	0 (Resistant)
Terbinaforce250 mg	Terbinafine hydrochloride	56 (Sensitive)	0 (Resistant)
Grisovin-FP 250mg	Grisofulvin	37 (Sensitive)	15 (Sensitive)

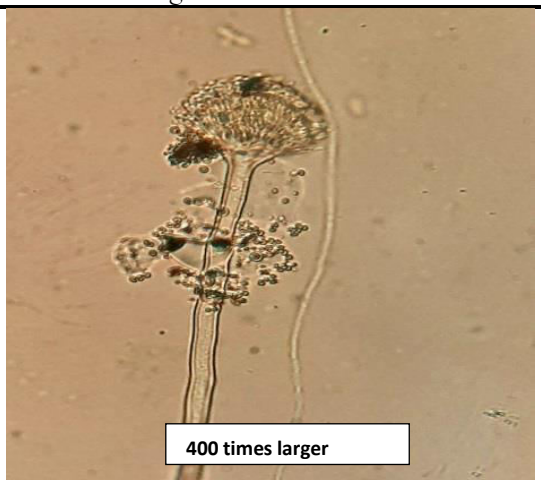


Fig. 1: ***Mucor spp.***

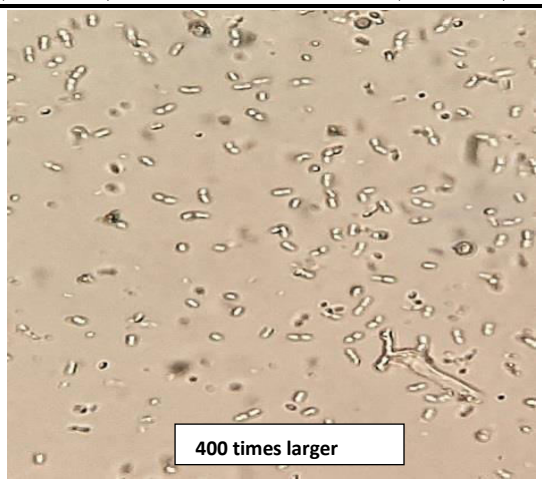


Fig. 2: ***Candida spp.***

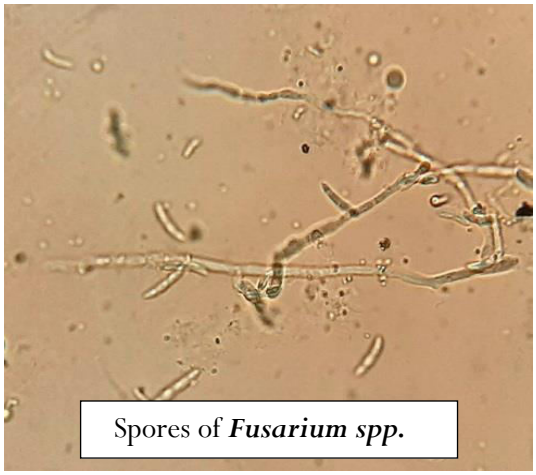
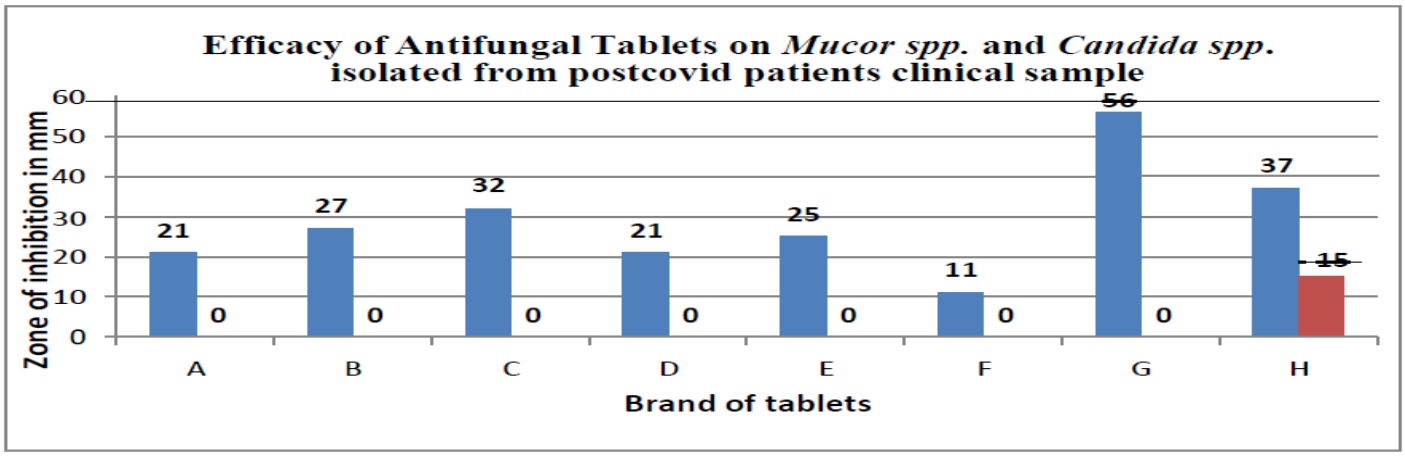


Fig. 3: ***Fusarium spp.***

Fig. 4: ***Alterneria spp***



Where,

A	B	C	D	E	F	G	H
Zocon	*Nuforce*	*Flucanzole*	*Fussy*	*Itrostred*	*Candiforce*	*Terbinaforce*	*Grisovin-FP*
150 mg	*150 mg*	*150 mg*	*150 mg*	*200 mg*	*200 mg*	*250 mg*	*250mg*

As Terbinaforce 250 mg and Grisovin FP 250 mg showed clear and best results, we used cocktail of these 2 antifungal tablets. This cocktail was applied against lawn culture of fungal pathogen of mucormycosis by using both the methods i.e. Paper disc diffusion method and Agar well diffusion method. Both methods gave quite similar outputs. The result of Cocktail clearly showed best zone of inhibition against Mucormycosis causative agent. This was the first report of use of cocktail of antifungal tablets against *Mucor spp. in vitro* test. Hence this study provides alternatives to treatment of Mucormycosis in postcovid patients. Also it would help doctors for the exact treatment according to fungal culture test reports.

Table 4: Effect of cocktail of antifungal tablets against Mucormycosis fungus isolated from postcovid patients clinical sample

Brand of tablets	Ingredients	Diameter of zone of inhibition in mm.	
		Paper discdiffusion method	Agar welldiffusion method
Terbinaforce 250 mg(T)	Terbinafine hydrochloride	56 (Sensitive)	54 (Sensitive)
Grisovin 250 mg(G)	Grisofulvin	37 (Sensitive)	36 (Sensitive)
Terbinaforce 250 mg + Grisovin FP 250 mg(T+G)	Terbinafine Hydrochloride+ Grisofulvin	61 (Sensitive)	60 (Sensitive)

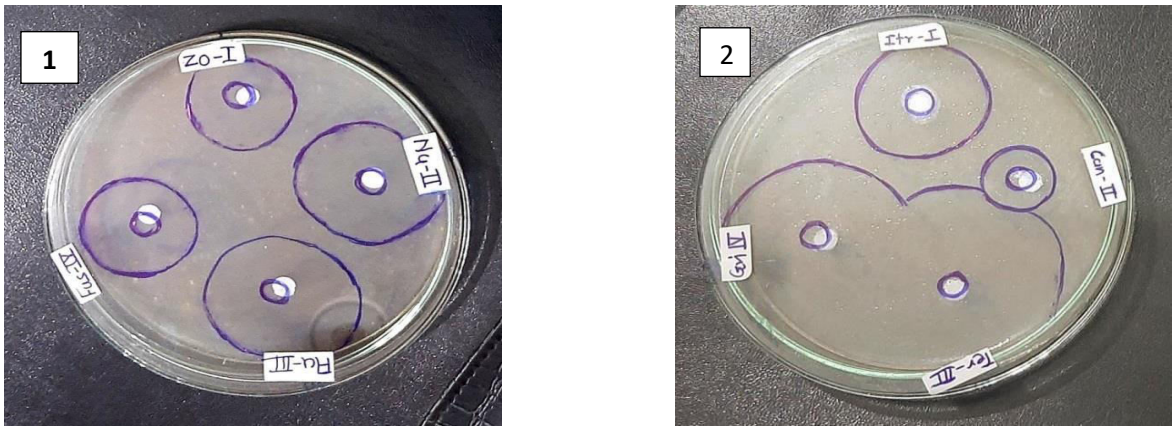


Fig. 5: Efficacy test of antifungal tablets against *Mucor spp.* Isolated from postcovid patients clinical sample

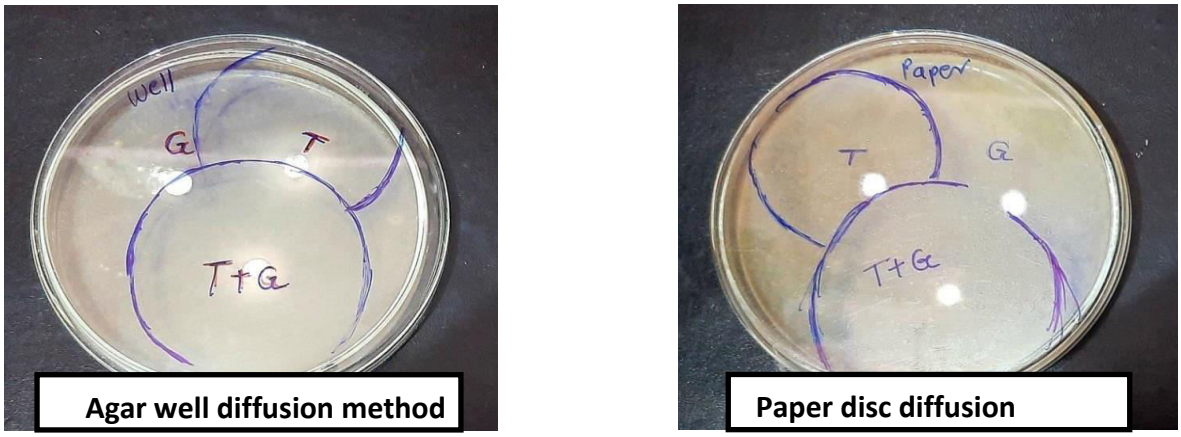


Fig. 6: Cocktail sensitivity test of 2 antifungal tablets against *Mucor spp.* isolated from postcovid patients clinical sample

Earlier researchers reported about the dreadfulness of Mucormycosis in immunocompressed patients and pointed towards the proper and challenging thing of diagnosis for right treatment. It includes following reports.

Roden *et al.* reported the different types of Mucormycosis 1) Rhinocerebral (sinus and brain) 2) Pulmonary (lung) 3) Gastrointestinal 4) Cutaneous 5) Disseminated, with a review of 929 cases of patients [1].

Kontoyiannis and Lewis reported about the use of high doses of LAMB according to weight of patient in the treatment of mucormycosis [8].

Walsh et al. reported that early detection may reduce the need of surgical resection and hence patients from sufferings [6].

Cornely A *et al.* reported that mucormycosis is life threatning fungal infection and leads to invasion of bone and soft tissue [3].

Marty *et al.* reported about the side effects of isavuconazole treatment against mucormycosis and discontinuation of treatment as there is no relation between serum levels of isavuconazole, fungal MICs and outcomes [7].

Tissot *et al.* reported that the limited clinical data and *in vitro/in vivo* data is available showing the activity of amphotericin B (AMB), pasaconazole etc. against *Mucorales.* No validated minimum imhibitory concentration (MIC) breakpoints exist for any of these agents [10].

Patel *et al.* reported Diabetes as a risk factor for mucormycosis in 73.5% of cases in India and Haematopoietic Stem Cell Transplantation (HSCT) counters 1% risk in India [4].

Munot S. *et al.* reported that COVID-19 situations increase the need of early, rapid and reliable diagnosis of fungal infection which can be obtained on cytopathological examination aiding the microbiological stains, using noninvasive methods of sample collection [11].

By comparing above all studies we found the alternatives with their efficacy against Mucormycosis in postcovid patients. Terbinaforce 250 mg and Grisovin FP 250 mg showed good results against *Mucorales* isolated from postcovid patients clinical sample.

4. CONCLUSION

In the present study we concluded that cocktail of Terbinaforce 250 mg and Grisovin-FP 250 mg can be used as alternative treatment for Mucormycosis in postcovid patients. It will reduce the expenditure and patients suffering rather than waiting for amphotericin B (AMB).

5. AKNOWLEDGEMENT

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Conflicts of interest

The authors declare no conflict of interest. The findings and conclusions in this report are those of the authors.

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