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## Effect of Antimicrobial Agents Against Fungal Isolates from Monuments of Bhopal

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### ABSTRACT

Many historical limestone and sandstone monuments in Bhopal are seriously threatened by bio-deterioration and are in need of investigation and conservation. Bio-deterioration processes result from complex interactions of surface-invading microbes (such as fungi) with the surface material. The present investigation focuses on the conservation of monuments by determining the antifungal effect of azoles against the fungal isolates isolated from the monuments of Bhopal: *Aspergillus niger*, *A. flavus*, *A. fumigatus*, *Rhizopus arrhizus* and *Penicillium* sp. We determined the minimum inhibitory concentrations (MICs) of antimicrobial agents using the guidelines of National Committee for Clinical Laboratory Standards (M38-A). To determine MICs, the inoculums of the above isolates were exposed to itraconazole, ketoconazole, fluconazole, griseofulvin and clotrimazole. We found that the order of *in vitro* activity of these antifungal agents against the fungal isolates is Itraconazole > Ketoconazole > Clotrimazole = Fluconazole = Griseofulvin. This result suggests that the use of Itraconazole and Ketoconazole should be a primary consideration in the conservation of monuments. Spraying or painting with these antifungal drugs could protect the monuments from fungal biofilm development.

**Keywords:** Monuments, Biodeterioration, Fungi, Antifungal drugs, MIC, Conservation of Monuments.

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## INTRODUCTION

Many historical limestone and sandstone monuments in Bhopal are seriously threatened by bio-deterioration and are in need of investigation and conservation. Bio-deterioration is a problem associated with the bio-corrosion of the paint coating on the monuments. The paint coatings of these historically important monuments are damaged by complex communities of microorganisms such as fungi<sup>1, 2</sup>. Fungi and all the major groups of microbes such as bacterial spores, nematodes, cyanobacteria and protozoa which continuously impact on substrates, are contained by the atmosphere. Therefore, air acts as a vehicle for the dispersion of fungi leading to bio-deterioration<sup>3, 4</sup>.

The bio-deterioration of monuments facilitates after degradation of paints<sup>2</sup>. Fungi multiply by utilizing their growth nutrients and form fungal bio-film on the substrates of monuments. The fungal cells produce cellulase enzymes which hydrolyse cellulose present in paints<sup>2</sup>. Fungal cells degrade surfaces of the monuments by producing organic pigments such as carotenoids, melanins, and some organic acids such as gluconic, succinic, malic, and oxalic acid<sup>3, 5-9</sup>.

In this study we tried to develop treatments to prevent bio-film colonization on the stone substrates of monuments. For this purpose, we investigated the *in vitro* activities of the antifungal agents against filamentous fungi using broth microdilution susceptibility testing.

Previously, Kavita Sharma et. al. (2010, 2011)<sup>3, 5, 6, 15</sup> and A.K. Pandey et.al (2011)<sup>1</sup> have isolated various fungi from the monuments of Raipur(C.G.), and Gualior (M.P.) respectively. However they did not test the isolates against antifungal drugs. Other authors only tested various fungi against antifungal azoles and reported their results<sup>10-12</sup>. In this study we did both the things together.

## MATERIALS AND METHODS

### Microorganisms and source of samples

We analyzed a group of 60 samples collected from the surface of different monuments of Bhopal, which are Gohar mahal (10 samples), Kamla mahal (20 samples), Takery (15 samples), Bhimbetka (5 samples) and Bhojpur (10 samples). The samples were collected in sterile, air-tight, polythene bags by the wall scraping technique. Since moisture and temperature influence the growth of fungi, we collected the samples in the months of February and March (during spring), when the temperature was around 23.5°C to 36.7°C. After collection, all the strains were cultured on sabouraud dextrose agar (SDA) and potato dextrose agar (PDA) and were identified by microscopic morphological characterization<sup>5, 6</sup>. The identified strains of *Aspergillus niger*,

*Aspergillus fumigatus*, *Aspergillus flavus*, *Rhizopus* and *Penicillium sp.* were then tested for minimum inhibitory concentrations (MICs).

### **Antifungal agents**

We used five antifungal agents for MIC testing: ketoconazole, itraconazole, griseofulvin, fluconazole, and clotrimazole. Ketoconazole and itraconazole were dissolved in dimethyl sulfoxide (DMSO), whereas griseofulvin, fluconazole, and clotrimazole were dissolved in distilled water. These agents were then prepared as stock solutions. For the preparation of stock solutions, the antifungal agents were diluted in RPMI-1640 broth medium (with l-glutamine and without sodium bicarbonate). We buffered the medium of the solutions to pH 7.0 at 25°C with 0.165 M morpholinepropanesulfonic acid<sup>13</sup>. The final drug concentrations ranged from 0.03 to 16µg/ml for itraconazole, clotrimazole, and ketoconazole and from 0.03 to 64µg/ml for fluconazole, and griseofulvin in the stock solutions<sup>14</sup>.

### **Antifungal broth microdilution susceptibility testing**

The broth microdilution susceptibility testing method was used to determine the minimal concentration of the antimicrobial agents for inhibiting or killing the microorganisms. We performed MIC testing as described in the *National Committee for Clinical Laboratory Standards Guidelines (NCCLS)*. The inoculum suspensions of isolates were prepared from the cultures grown for seven days on potato dextrose agar or sabouraud dextrose agar at 24°C. In the cultures, the fungal colonies were covered with 1 ml of sterile 0.85% saline. We made suspensions by gently probing the surface with the tip of a pasteur pipette. Further, we added 1 drop (approximately 0.01ml) of tween-20 or tween-80, thus facilitating the preparation of fungal inocula. After preparation of the fungal inocula, the resulting mixture of conidia and hyphal fragments was withdrawn and transferred to a sterile tube. The heavy particles were allowed to settle in 3 to 5 min and when the heavy particles settled, we collected the upper homogeneous suspensions in a sterile tube and mixed with a vortex mixer for 15 sec at 2000 rpm. The densities of these suspensions were adjusted with the spectrophotometer (ELISA Reader) at a wavelength of 630 nm to obtain two types of standardized inocula: (i) 80 to 85% transmission (T) for all *Aspergillus sp.*, (ii) 65 to 70% transmission for *Penicillium* and *Rhizopus sp.* The suspensions were then diluted to the ratio of 1:50 in RPMI medium to obtain the final inoculum sizes, which ranged from  $0.4 \times 10^4$  to  $5 \times 10^4$  CFU/ml<sup>14, 15</sup>.

We inoculated aliquots of 100 µL of diluted fungal suspensions in each test well of a microtiter plate containing 100 µL of specific antifungal drug concentrations. Each test plate had one drug-

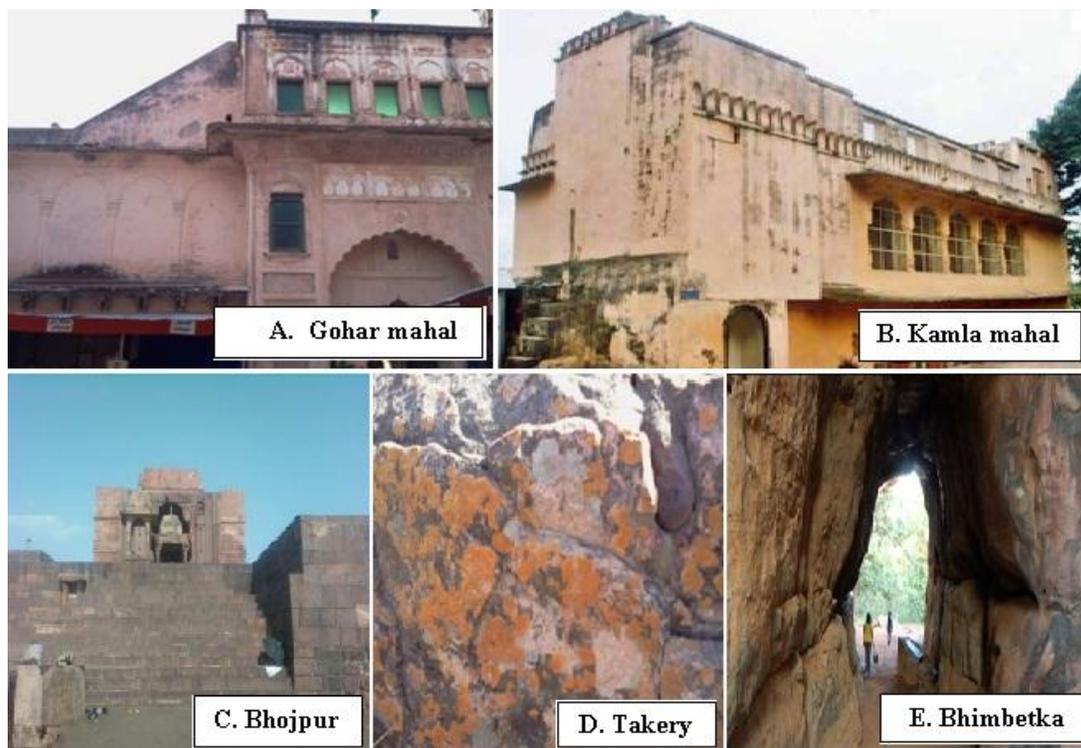
free growth control containing 100  $\mu$ L of diluted inoculum suspension and 100  $\mu$ L of drug diluent without the antifungal agent.

### Incubation and determination of MIC

The MICs were determined by the comparison of measured optical densities of test wells with that of the corresponding growth control (drug-free) wells. As per NCCLS guidelines, growths were checked for *Rhizopus* after 24 h, for *Aspergillus sp.* after 48 h, and for *Penicillium* after 72 h of incubation. MIC ranges were obtained for each species and drug combination tested. To facilitate comparisons of the activities of the drugs, the MICs were reported as the concentration at which 50% (MIC<sub>50</sub>) and 90% (MIC<sub>90</sub>) of the isolates were inhibited<sup>11</sup>.

## RESULTS AND DISCUSSION

In the present study, we collected 60 samples from monuments in Bhopal, and isolated three species of *Aspergillus* and one species each of *Rhizopus* and *Penicillium*. The isolated fungi were *A. niger*, *A. flavus*, *A. fumigatus*, *Rhizopus arrhizus* and *Penicillium*.



**Figure 1: Different sampling sites of monuments.**

We tested the *in vitro* susceptibility or resistant pattern of the fungal isolates to itraconazole, ketoconazole, fluconazole, clotrimazole and griseofulvin and calculated the MIC ranges as MIC<sub>50</sub> and MIC<sub>90</sub>, the drug concentrations required to inhibit 50% and 90% of the isolates of each species respectively.

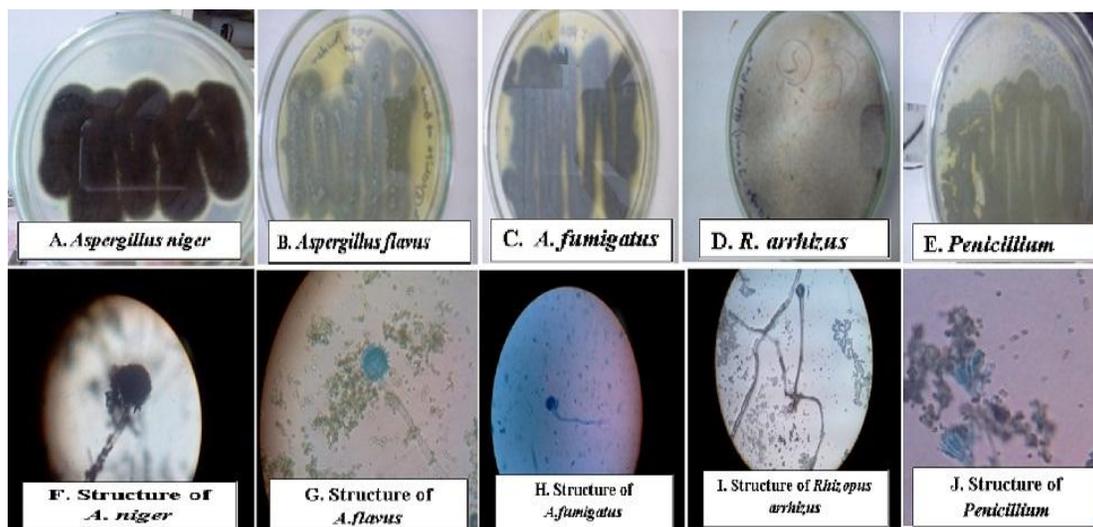


Figure 2: (A-E); Culture plates of different isolates and (F-J); Microscopic slide showing the structure of isolates.

Table 1: Showing the prevalence of an organism in different sites

Monument	samples collected	Types of stone	<i>A. niger</i>	<i>A. flavus</i>	<i>A. fumigatus</i>	<i>Rhizopus</i>	<i>Penicillium</i>
Gohar mahal	10	Sand stone	+	-	+	-	-
Kamla mahal	20	Sand stone	+	+	-	+	+
Takery	15	Sand and Lime stone	+	-	+	+	-
Bhojpur	10	Sand and Lime stone	+	+	-	+	-
Bhimbetka	5	Lime stone	+	-	+	+	-

where + and – symbols are indicating presence and absence of isolated fungal species respectively.

Table 2: MICs of drugs against all the isolates

Fungal isolates	Antifungal drug	Range (µg/ml)	MIC50 (µg/ml)	MIC90 (µg/ml)
<i>A. niger</i>	ITCZ	0.03-16	0.25	0.5
	FLU	0.03-64	>64.0	>64.0
	KCZ	0.03-16	8.0	16.0
	GRIS	0.03-64	>64.0	>64.0
	CL	0.03-16	>16.0	>16.0
<i>A. flavus</i>	ITCZ	0.03-16	0.125	0.5
	FLU	0.03-64	>64.0	>64.0
	KCZ	0.03-16	4.0	8.0
	GRIS	0.03-64	>64.0	>64.0
	CL	0.03-16	>16.0	>16.0
<i>A. fumigatus</i>	ITCZ	0.03-16	0.5	1.0
	FLU	0.03-64	>64.0	>64.0
	KCZ	0.03-16	4.0	8.0
	GRIS	0.03-64	>64.0	>64.0
	CL	0.03-16	>16.0	>16.0

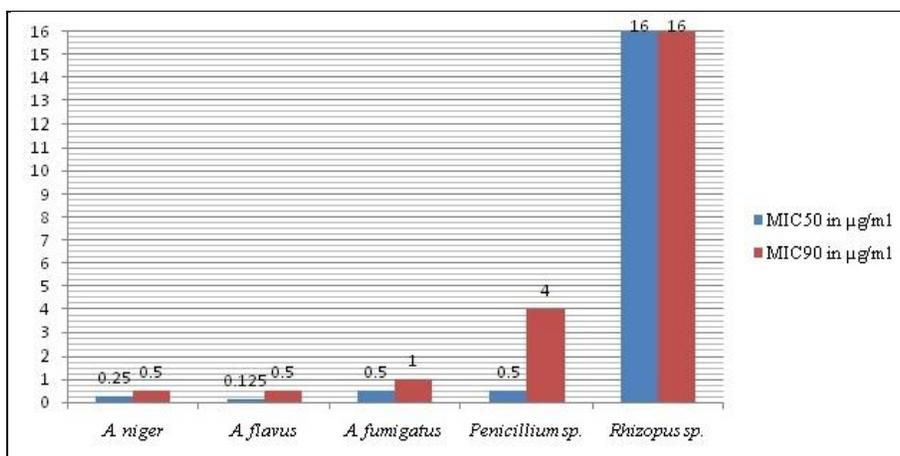
<i>Penicillium sp.</i>	ITCZ	0.03-16	0.5	4.0
	FLU	0.03-64	>64.0	>64.0
	KCZ	0.03-16	0.5	1.0
	GRIS	0.03-64	>64.0	>64.0
	CL	0.03-16	>16.0	>16.0
<i>Rhizopus sp.</i>	ITCZ	0.03-16	>16.0	>16.0
	FLU	0.03-64	>64.0	>64.0
	KCZ	0.03-16	4.0	16.0
	GRIS	0.03-64	>64.0	>64.0
	CL	0.03-16	>16.0	>16.0

Where ITCZ: Itraconazole, FLU: Fluconazole, KCZ: Ketoconazole, GRIS: Griseofulvin, CL: Clotrimazole

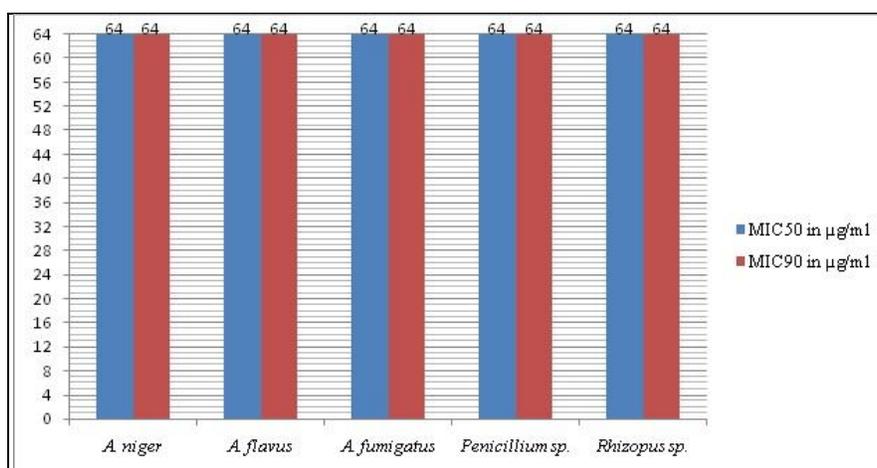
If MIC is  $\leq 1.0$ , it is susceptible

If MIC is = 2.0, it is intermediate

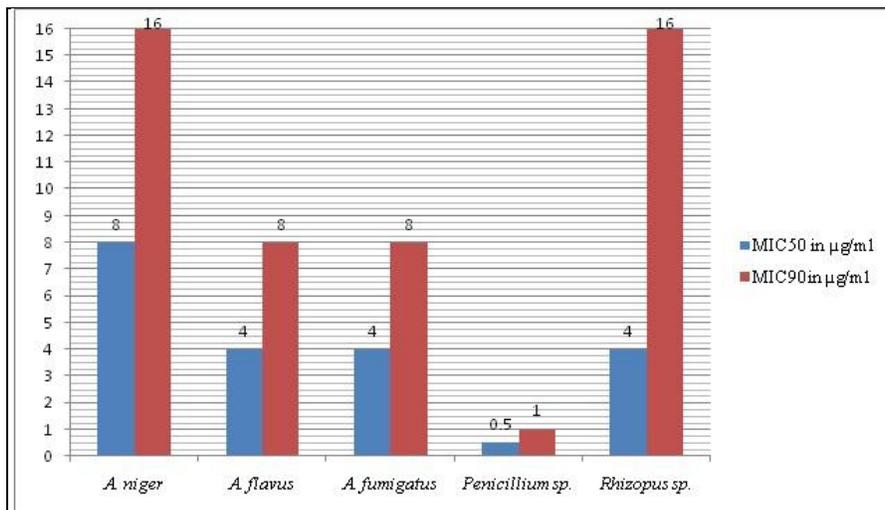
If MIC is  $\geq 4.0$ , it is resistant



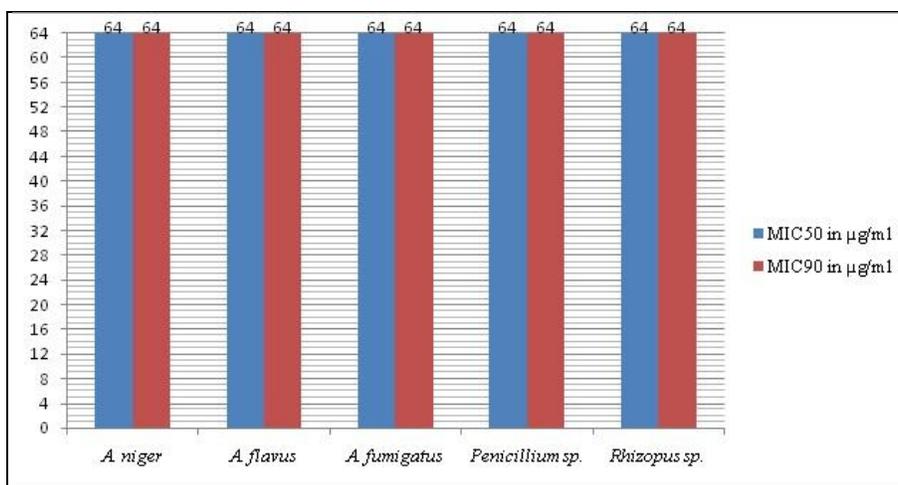
**Figure 3; The MICs of all the isolates against Itraconazole.**



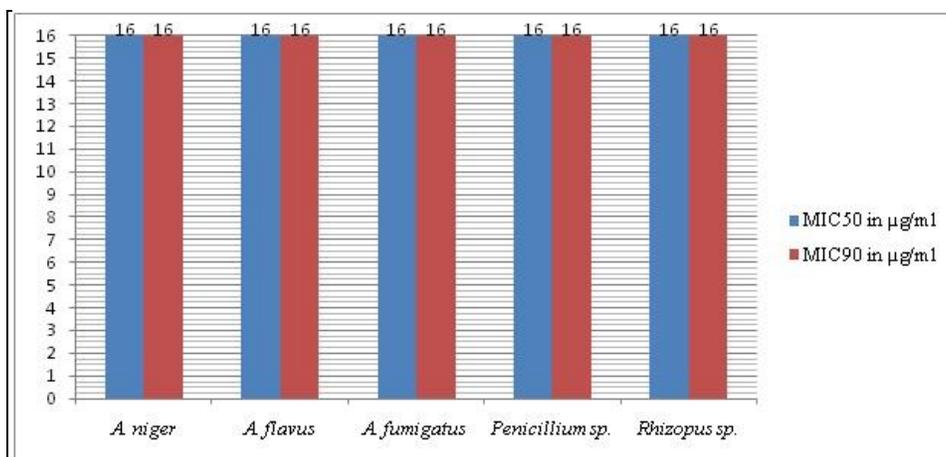
**Figure 4: The MICs of all the isolates against Fluconazole.**



**Figure 5: The MICs of all the isolates against Ketoconazole.**



**Figure 6: The MICs of all the isolates against Griseofulvin.**



**Figure 7: The MICs of all the isolates against Clotrimazole.**

In this study, we identified five fungal species that caused deterioration of the monuments in Bhopal. The fungal species were *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*,

*Rhizopus* and *Penicillium*. Our results show that *Aspergillus niger* was the most common species isolated from the mineral substrates.

On broth microdilution susceptibility testing, we found that all the isolates were resistant to griseofulvin, fluconazole and clotrimazole. *Aspergillus niger*, *A. flavus*, *A. fumigatus* were found to be susceptible to itraconazole. However, *Penicillium sp.* was significantly less susceptible, and *Rhizopus sp.* was resistant to itraconazole. The MIC<sub>90</sub> of ketoconazole for all the isolates except *Penicillium sp.* ranged from 4.0 to 16.0 µg/ml. This indicates that *Penicillium sp.* is more susceptible to ketoconazole than all the other species are. Therefore, we can conclude that the order of *in vitro* activity of the antifungal agents against the fungal isolates is itraconazole > ketoconazole > clotrimazole = fluconazole = griseofulvin.

The results of present investigation reveal with the work done by various researchers. Kavita Sharma et. al. (2010, 2011)<sup>3-6</sup> reported similar results at Raipur (C.G.). They noted a considerable number of the same genus and species along with *Cladosporium*, *Fusarium*, *Mucor*, *Curvularia*, *Trichoderma*, *Aspergillus scalrotium*. Another work done by A.K. Pandey et.al (2011)<sup>1</sup> at Gualior (M.P.), noted *Curvularia*, *Cladosporium*, *Chrysosporium*, *Ulocladium* along with the species which we identified in our investigation.

*In vitro* Susceptibility testing showed that itaconazole was the most active drug against *Aspergillus niger*, *A. flavus*, *A. fumigatus*. Ketoconazole was also found effective against *penicillium sp.* However, the other testing drugs were found ineffective against the fungal isolates. Other authors also have reported similar results for itaconazole and fluconazole<sup>10-12</sup>. However, to our knowledge, we did not find any report on the *in vitro* activity of ketoconazole, griseofulvin and clotrimazole.

*In vitro* test results reveal that most of the isolates could not increase their number when assayed with itraconazole. Azoles that disrupt the cell membrane of fungi do so by targeting ergosterol either by forming pores and causing the membrane to become leaky or by inhibiting ergosterol biosynthesis. Itraconazole prevents fungi from producing the ergosterol that is why it is the most effective drug against the large number of species.

Our results suggest that antifungal agents could be used to protect the surfaces of monuments from fungal attack. All the isolated fungal species have great biochemical decay potential. The painted surfaces of monuments undergo damage or become discolored due to the growth and activity of these fungi<sup>2</sup>. Therefore, biocides or antifungal agents must be added to paint formulations to maintain the product's integrity from microbial attack and to provide protection in the dry film against any fungal growth. *In vitro* susceptibility testing results suggest that the

use of itraconazole and ketoconazole should be a primary consideration in the conservation of monuments. Spraying or painting with these antifungal drugs could protect the monuments from fungal biofilm development.

To our knowledge, this is the first report of the fungal isolates and broth microdilution susceptibility testing of the monuments in Bhopal. In this study, we assumed that all the isolated fungi damage the surface of monuments uniformly. Therefore, further studies are necessary to elucidate the role of particular fungal species in the biodeterioration of monuments. Some molecular methods for example DNA sequencing could provide data for detection of responsible gene of fungi and by gene silencing and DNA methylation, we can regulate the expression of that harmful gene.

## CONCLUSION

To conclude, the present study indicates that most of the isolates could not increase their number when assayed with itraconazole. Therefore itaconazole was the most active drug against *Aspergillus niger*, *A. flavus*, *A. fumigatus*. Ketoconazole was also found effective against *penicillium sp.* Our results suggest that antifungal agents could be used to protect the surfaces of monuments from fungal attack and the use of itraconazole and ketoconazole should be a primary consideration in the conservation of monuments. Spraying or painting with these antifungal drugs could protect the monuments from fungal biofilm development.

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