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Studies of *In-Vitro* Susceptibility of Clinical Fungal Isolates from Immunocompromised Patients.

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Research Article

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ABSTRACT

Clinical fungi were isolated from the blood samples of immunocompromised patients from Subhash Chandra Medical College Jabalpur in SDA with chloramphenicol @ 30µg/ml. The common isolates were *Candida albicans*, *Aspergillus fumigatus*, *A. glaucus*, *A. terreus*, *A. niger*, *A. flavus*, *Penicillium* sps. and *Curvularia* sps. The isolates were then subjected to *in vitro* susceptibility testing against different antifungals viz Amphotericin B, Ketocaonazole, itraconazole, clotrimazole and fluconazole and also against natural herbs like neem, tulsi, garlic ginger, aloe vera, turmeric etc by DDM method. Ketocaonazole was found to be highly effective amongst all antifungals and garlic was most effective amongst all natural antifungals.

INTRODUCTION

During the past twenty five years there has been alarming increase in the fungal diseases with increasing use of chemotherapeutic agents, irradiation, immunosuppressive agents, broad spectrum antibiotics etc. there are many antimycotics which are used in treating fungal infections. The isolates collected in this program can be used to assess the activities of new antimicrobial agents and to aid in the development and validation of new susceptibility methods [1, 2, 3]. The treatment of choice for infected patients remains amphotericin B, itraconazole, voriconazole etc. the appropriate susceptibility is decided by various *in vitro* susceptibility methods [4,5,6,7,8]. The studies to date that have documented the efficacies of agar based methods for the testing of susceptibilities to fluconazole or voriconazole have generally included adequate number of *Candida albicans* species but few *Candida glabrata* isolates. From susceptibility testing one can determine the resistivity of the clinical isolate to various antifungals by determining the MIC range against a sufficiently large number of isolates.

The resistance towards the drugs is increasing day by day. The overuse of antifungal agents makes the fungi resistant towards it and simultaneously leads to chronic conditions in the patients. The drugs may even cause fatal problems. In order to avoid these effects alternative therapy has to be adopted which includes treatment through natural products. The antifungal activities of some spices and herbs have been reported by Pai et. al. 1995, Christina et. al, 2003, Anupama et. al. Apart from the synthetic antifungal antibiotic the naturally occurring herbal products and spices are known to have powerful antifungal properties. Research has proved their antifungal activities. Herbs like goldenseal, myrrh, walnut, licorice, lemongrass and spices like turmeric, cinnamon, clove, ajwain and medicinal plants like neem, tulsi, eucalyptus etc. have a great antifungal profile. Even the combination of these works in synergy and gives better results than a single herb [9].

MATERIALS AND METHODS

Organisms

A total of 36 clinical isolates of filamentous fungi were isolated from blood samples collected from Subhash Chandra Medical College Jabalpur from 2004 to 2008. The clinical isolates included 10 isolates of

Aspergillus fumigatus, 5 of *A.flavus*, 10 of *A. niger*, 3 of *A. terreus*, 4 of *A. glaucus*, 2 of *Penicillium* and 2 of *Curvularia sps.*

Antifungal agents

Voriconazole (Pfizer), fluconazole (Pfizer), itraconazole (Jansen), flucytosine (Sigma), amphotericin B (Hi media). Serial two fold dilutions were prepared exactly as outlined in NCCLS document M 27 A [11]. Final dilutions were prepared in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165M morpholine propane sulphonic acid (MOPS) buffer (Sigma). The dilutions were kept in Eppendorf's tubes until used and stored at -4 °C in Quick freezer.

Natural products

Turmeric, cinnamon, clove, ginger, garlic, neem, tulsi were used for the present study. The natural products were dried, powdered and then alcoholic extracts were prepared. The discs of natural products were prepared by soaking the discs in alcoholic extracts for a period of 24 hours and then dried in air.

Susceptibility testing

Reference antifungal Susceptibility testing of clinical isolates were performed by disc diffusion method as described by Barry et al [10]. MIC's were determined with RPMI 1640 agar. An inoculum suspension adjusted to the turbidity of a 0.5 McFarland standard (~10⁶cells/ml), and incubation at 35 °C for 48 hours. MIC interpretive criteria were those published by Rex et al and the NCCLS document M 27 A and were as follows: susceptible, MIC ≤8µg/ml; susceptible-dose dependent, MIC = 16 to 32µg/ml; resistant, MIC ≥ 64µg/ml. The interpretive criteria for disc test were those published by Barry et al [10]. and the NCCLS document M 27 A (11): susceptible, zone diameter of ≥ 19mm; susceptible-dose dependent, zone diameter of 15 – 18 mm; resistant, zone diameter of ≤ 14mm.

Quality control

QC was performed for disc diffusion method in accordance with NCCLS document M 27 A (11) by using *C. albicans* 3809 as reference culture.

Analysis of results

The diameters of the zone of inhibition for the test antifungals were measured in mm by disc diffusion method and their respective BMD MIC's were also studied. The errors were identified as susceptibility by one method and resistivity by other method.

RESULT AND DISCUSSION

Amongst the 75 clinical samples studied 36 were positive for fungal growth. The common fungi isolated were *Candida albicans*, *Aspergillus fumigatus*, *A. glaucus*, *A.terreus*, *A. niger*, *A. flavus*, *Penicillium sps.* and *Curvularia sps* . The in vitro sensitivity results of the clinical isolates obtained by disc diffusion method against antifungals are expressed in table no. 1. The size of zone indicates the sensitivity of the culture to the antifungal used. From the table it is evident that ketoconazole is the most effective antifungal against all the clinical isolates. Amphotericin B is the most effective antifungal against *Penicillium spp.* followed by *A. niger* and *Candida albicans* and least effective to *A. flavus* and *A. fumigatus*. Ketoconazole is effective against *Candida albicans* followed by *A. fumigatus*, *A. flavus* and *A. niger*. Fluconazole is effective against *Penicillium spp.* and *Curvularia sps* followed by *Candida albicans*, *A. niger* and *A. fumigatus*. *A. flavus* was found to be resistant against fluconazole. 5FC was effective against, *A. glaucus* followed by *Candida albicans*, *A.terreus* and *A. fumigatus*. *A. flavus* and *A. niger* was found to be slightly sensitive to 5FC.

The clinical isolates were also subjected to in vitro sensitivity tests against spices and the results obtained are expressed in table 2. The extent of zone of inhibition of the fungal growth indicates the sensitivity of the fungi to the plant extract studied. It is evident from the results that garlic is most effective antifungal agent as the zone of inhibition is largest with the garlic disc followed by onion, ginger, cinnamon, neem, tulsi, aloe vera. Lemon grass was the least effective plant with antifungal property against all the clinical isolates tested. The present results are similar to those obtained by Ali et. al. 1999. From the table it is clear that garlic is the most effective spice against the clinical isolates tested and turmeric is the least effective spice. The order of susceptibility of *Candida albicans* to various spices are garlic > turmeric > ginger > onion > cinnamon. Though from zone of inhibition obtained it is evident that *Candida albicans* is susceptible to all the spices tested. The orders of susceptibility of *A. fumigatus* to various spices are garlic > onion > cinnamon > ginger > turmeric. *A. fumigatus* is highly susceptible to garlic, intermediately susceptible to onion, cinnamon and ginger while weakly susceptible to turmeric. The orders of susceptibility of *A. flavus* to various spices are garlic > onion > cinnamon > ginger. *A. flavus* is resistant to turmeric. The orders of susceptibility of *A. niger* to various spices are garlic > onion > cinnamon = ginger > turmeric. Though

from zone of inhibition obtained it is evident that *A. niger* is susceptible to all the spices tested. The orders of susceptibility of *Penicillium spp.* to various spices are onion >garlic>cinnamon>turmeric>ginger.

It is clearly illustrated from the table that the common spices used in our daily life and the extracts of natural herbs effects the fungal growth adversely and therefore might be considered to control fungal diseases. The bioactive compounds in these plant extracts must be analyzed, purified and then used as drug for controlling fungal pathogens without the development of drug resistance in pathogens and also without any side effects.

The results obtained are in accordance with those obtained by Abraham et al 1999, Bassiouny et al 1986, and Stern et al, 1987. The antifungal activities of some spices have also been reported by Anupan et. al 2005 [12], Dorman et. al. 2000 [13], Erdogan et. al.2004 [14], Nuzrat et al 2013[15] and Hammer et. al. 1999 [16] which are coherent with the results obtained in the present investigation.

Table 1: Results of in vitro sensitivity tests of clinical isolates against antifungals expressed as zone of inhibition in cms.

Sr. No	Fungal isolate	Zone of inhibition in centimeters				
		Amp B	Ket	Itra	Flu	5FC
1	<i>Candida albicans</i>	0.5	2.3	1.6	1.5	1.2
2	<i>Aspergillus niger</i>	1.0	1.8	1.6	1.3	0.8
3	<i>Aspergillus flavus</i>	-	2.0	1.3	-	0.3
4	<i>Aspergillus fumigatus</i>	-	2.1	1.5	1.1	1.2
5	<i>Aspergillus glaucus</i>	0.5	1.8	1.1	2.0	1.8
6	<i>Aspergillus terreus</i>	0.5	1.3	1.0	1.5	1.2
7	<i>Penicillium spp</i>	1.8	1.2	1.2	1.6	-
8	<i>Curvularia pallescans</i>	-	1.8	1.8	1.6	0.6

Table no. 2: In vitro susceptibility of fungal isolates to plant extracts by disc diffusion method (Kirby Bauer) expressed as zone of inhibition in cms.

S.N	Fungal isolate	Neem	Tulsi	Lemon grass	Aloe vera	Cinnamon	Garlic	Ginger	Onion
1	<i>Aspergillus fumigatus</i>	0.5	0.4	-	-	1.3	2.0	0.8	1.5
2	<i>Aspergillus flavus</i>	0.4	0.4	-	0.7	1.2	1.8	1.0	1.5
3	<i>Aspergillus niger</i>	-	-	-	-	1.2	1.9	1.1	1.3
4	<i>Aspergillus terreus</i>	-	-	-	0.5	1.2	2.0	1.0	1.2
5	<i>Aspergillus glaucus</i>	0.7	0.5	-	0.5	1.3	1.8	1.0	1.2
6	<i>Curvularia pallescans</i>	0.7	0.5	0.2	0.3	-	0.6	0.8	-
7	<i>Penicillium spp.</i>	-	-	-	-	1.4	0.4	1.1	0.9
8	<i>Candida albicans</i>	1.0	0.8	0.5	0.3	-	0.8	-	0.9

CONCLUSION

It is clearly illustrated from the study that the most effective antifungal against the mycotic agents studied is ketoconazole and therefore is of clinical relevance in controlling mycosis. In vitro susceptibility profile of mycotic agents obtained against spices suggests that the common spices used in our daily life affects fungal growth adversely and therefore might be considered to control fungal diseases. The bioactive compounds in these spices must be analyzed, purified and then used as drug for controlling fungal pathogens without the development of drug resistance in pathogens and also without any side effects.

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