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In vitro antifungal activity of Clotrimazole, Miconazole and Ketoconazol by binary mixture pattern against hospital isolates of *Candida*

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ABSTRACT

The lack of variation in antifungal drugs, and the misuse or inappropriate use usually causes resistant strains of the yeast in human's normal flora. Unfortunately, a large number of *Candida* infection cases in immunosuppressed patients with insufficient treatment eventually can cause patient's death. The aim of this study was to evaluate the in vitro conventional antifungal azole compounds with binary mixture with appropriate ratios by susceptibility test using the laboratory as a mixture of two in vitro conditions. In present study, 10 isolates of *Candida* were admitted from patients that were referred to mycology laboratory of Faghihi Hospital at Shiraz with signs of cutaneous and mucosal infections. In the present study we used the methods of the binary mixure of common antifungal drugs Clotrimazole, Ketoconazole and Miconazole on equally proportion have been used. The drugs were solved in various concentrations on the SDA medium and then Cndida isolates were cultured in the SDA plates. The minimum inhibitory concentration (MIC 90) and minimum fungicidal concentration (MFC) was determined. The results of this study suggest that a binary mixture of these drugs inhibited the growth of most strains of pathogenic Cndida isolates. The combination with Clotrimazole and ketoconazole in equal proportion had more effective than other drug mixtures against all isolates with the exception of isolate 3. In contrary, the combination of Miconazole and Clotrimazole had the least effect, and the MIC was calculated in the range of 3.12 to 50 µg/ml. Evaluation of MFC showed almost the same results. Lowest values of the MFC belonged to the combination of clotrimazole and ketoconazole which was obtained 6.25 µg/ml. It is concluded that the use of the combination of Clotrimazole and Ketoconazole in equal ratios has better antifungal effects against cutaneous and mucosal isolates of the *Candida* infection.

1. Introduction

The azole-derivative antifungal agents include a large number of ergosterol synthesis inhibitors currently used to treat human fungal infections, including the imidazoles (clotrimazole [CZ], miconazole [MZ], ketoconazole [KZ], etc.) and the newer systemic triazoles (fluconazole [FZ] and itraconazole

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[IZ]) (Fromtling,1988). Candidiasis is one of the most common opportunistic fungal infections caused by *Candida* in humans which has a worldwide distribution and is caused by several species of the genus. *Candida* is a yeast-like fungus, mainly in the normal flora of the skin and mucosal surface. The most important pathogenic species is *Candida albicans*, but the reports show that the increasing prevalence of non-albicans in the patients with immunosuppression disease. Host immune responses with some of the body's natural micro organism keeps yeast population a reasonable level. Long-term use of broad spectrum antibiotics, corticosteroids, chemotherapy, oral contraceptives, excessive wrinkling of skin diseases associated with impaired immune systems (including AIDS), pregnancy and diabetes can cause yeast overgrowth. Candidiasis is known as the most common systemic mycosis in humans and the fourth cause of fungal bloodstream infections in hospitalized patients, which causes 40 percent of deaths in hospital (Anaissie et al., 2003; Saporiti et al., 2001; Ostrosky-Zeichner et al., (2003; Rezaeian et al., 2005). Most of the *Candida* species are sensitive to antifungal drugs including azole and Polyene. Polyene antifungal such as amphotericin B and nystatin bind to ergosterol within fungal cell walls and makes holes and lead to destroy the fungus. Azole prevents the synthesis of ergosterol. However, the unsuitable use of the drug, and the lack of variation in common antifungal drugs, lead to resistant strains of fungi and makes the treatment of these infections difficult (Rex et al., 1993; Sheehan et al., 1999). The proposed method is very convenient to prevent or delay in the development of drug resistance in the infections. In this case, the use of lower doses of the individual drug decreases the side effects and toxicity to humans (Sheehan et al., 1999; Johnson et al., 2004; Shams Ghahfarokhi et al., 2007). Antifungal susceptibility testing has become an important tool in the management of patients with invasive candidiasis, since both in vitro resistance and toxicity issues must be considered when selecting an antifungal agent (Ghannoum et al., 1996; Hoffman et al., 2001; Martins et al., 1996; Pfaller et al., 2001; Rex et al., 2002). National Committee for Clinical Laboratory Standards (NCCLS) has developed the standardized and reproducible M27-A2 method for the yeasts (Pfaller et al., 2000). Research also showed significant differences in the distribution and drug susceptibility test of *Candida* species in the country and different regions (Bakhtiari et al., 2007; Espinel-Ingroff, 2003, Rezaeian et al., 2005; Hussein far et al., 2007; Soleimani Pour et al., 2008). In the present study, we investigated the in vitro antifungal activity of clotrimazole, miconazole and ketoconazol by binary mixture pattern against groups of recent clinical isolates of *Candida*.

2. Material and Methods

In this research agar-based techniques have been used extensively to investigate azol antifungal activity since they are simple, economical, and easy to perform simultaneously on large numbers of organisms. The antifungal drugs which can be solved in various concentrations on the SDA medium, was used in this study, then the fungus were cultured in the SDA plates. Each plate was examined for the growth after 24 to 48 hours of incubation at 35 to 37°C.

10 pathogenic isolates of *Candida* were admitted from patients was used in this study which were referred to mycology laboratory of Faghihi Hospital at Shiraz, with signs of coetaneous and mucosal infections. After culture, the samples were analyzed by microbiological techniques (microscopic and biochemical test), the isolated species were determined and stored in the tubes containing a culture medium at 2 to 8°C, until used. In this case the maximum storage time is four weeks.

2.1. Preparation of drug solution

Pure powders of Ketoconazol (Sobhan pharmaceutical companies), Miconazol (Sobhan pharmaceutical companies), and clotrimazol (Aboureihan pharmaceutical companies) were dissolved in suitable solvents to obtain stocks concentration of 6400 mg/ml. Serial dilutions were made to 0.39 mg/ml. Drug dilutions in twofold increments were prepared at fourfold

levels above the desired final concentration for drug tested. Drug concentrations were mixed to Sabouraud dextrose agar medium (SDA) with the ratio of 1;1 at 40-45°C. Concentrations of drug were used in this study were 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 µg/ml. (Hashemi et al.,1999)

The isolated strain was cultured individually in SDA medium at 35 to 37°C for 24 hours before use. The colony were solved in tubes containing sterile saline and shaked. Yeast suspension was adjusted in spectrophotometric method. Adjustment by matching the turbidity at a 530 nm of a 0.5 Mc Farland ($10^6 \times 1$ cell per ml). 20 µl of the suspension was poured over the surface of the medium by sterile loop and incubated (Falahati et al., 2007; Rex et al., 1993).

2.2. Determination of Minimum Inhibitory Concentration (MIC) and minimum fungicidal concentration (MFC)

MIC was defined as the lowest concentrations of drug at which there was no visible growth of organisms for each test was considered as the control group. MFC the lowest concentration of drug that resulted in a 99.9% reduction in CFU of the initial inoculums) was determined by counting the contents of plates that showed no visible growth of fungi onto Sabouraud dextrose medium (Merck, Darmstadt, Germany) and were incubated at 35 to 37° C for 18 h. The MFC was considered the lowest concentration of drug that prevented any colony formation in solid media (Falahati et al., 2007; Rex et al.,1993).

2.3. Statistical Analysis

All tests were repeated three times. Available data on the results of independent tests were performed. All the statistical analyses were performed by the SPSS 11.0 (SPSSFW, SPSS Inc., Chicago, USA) statistical package.

3. Results

Morphological and biochemical tests of fungal isolates showed that 9 of the isolates belong to the albicans species. Germ tube is a well characterized to the albicans species. Other Candida species not capable of producing germ tubes or making a short germ pipes. Agar-based techniques were used to evaluate the activity of synergy of binary mixture azole drug against Candida isolates. The mean MIC and MFC values of binary mixture drugs against clinical isolates of candida species have been shown in Table 1. The MIC90 of a binary combination of Miconazole plus Clotrimazole against all *Candida* isolates, were 3.12 to 50 µg/ml, with the exception of isolate 3 that was more than 100 µg/ml. The lowest concentration of the combination of drugs in the cases of non-albicans, was 0.39 µg/ml. The MFC of a binary combination of Miconazole and Cotrimazole for all *candida* isolates were more than 50 µg/ml. Only non-albicans strain did not show any growth in the lowest concentration of drug mixture. The MIC of a binary mixture of Miconazole plus Ketoconazole against all albicans strains were 0.78 to 25µg/ml, with the exception of isolate 3 that was more than 100 µg/ml. The MFC of a binary combination of Miconazole plus Ketoconazole for all *candida* isolates were more than 12.5 µg/ml with the exception of two isolate which was 6.25 µg/ml. The only non-albicans strain did not show any growth in the lowest concentration of drug mixture. The MIC of a medicinal binary mixture Clotrimazole plus Ketoconazole against of all albicans strains were 0.39 to 6.25µg/ml, with the exception of isolate 3 that was more than 100 µg/ml. The MFC of combination of Clotrimazole plus Ketoconazole for all *Candida* isolates, were more than 6.25 µg/ml, with the exception of three isolates which was more than 100 µg/ml. Also the only non-albicans did not show any growth at lowest concentration of three anti-fungal drugs in some binary mixture. Results of statistical analysis showed that the values of the MIC equal to zero and the MFC equals 0.47 with standard error 0.05 and the relative frequency of the MIC and MFC in some of binary combinations of three drugs were significantly different. Thus a manner that the effect of a binary combination of Ketoconazole plus Clotrimazole in concentration of 0.39 µg/ml, with 16.7% of the total sample and 50% of the every sample are significantly higher. The values of MIC frequencies of different binary

mixure azole drugs against of *Candida* isolates have been shown in Table 2. The fungicide effect of a binary combination of Miconazole plus Clotrimazole, and Ketoconazole plus Clotrimazole, respectively at the concentrations of 100, 50 and 6.25 μg/ml with 16.7% ,13.3%, and 13.3% of the total sample and 50%,40 %, and 40 % of the every sample were significantly higher. The values of MFC frequencies of different binary mixure azole drugs against of Candida isolates was shown in Table 3.

4. Discussion

Unfortunately drug susceptibility testing of fungi in some cases does not provide useful clinical information. The results are very different and even performing a test does not show the same results in different laboratories. It seems that the work is difficult because the diversity of host and strains and also the lack of access to an acceptable result of drug concentration in vitro. Nevertheless, sue to increasing prevalence of drug resistance and severe recurrent of fungal infections, especially in the people who have had extensive chemotherapy immune deficiency or physiological diseases such as diabetes. Many studies were made on this issue and a number of standard tests have been done to determine the susceptibility of the antifungal drug in the world. These studies were performed as an international project. The results of the susceptibility test of antifungal binary combination drug against pathogenic yeasts are in agreement with the findings of the Espinel-Ingrof in 2003. They studied the drug resistance of pathogenic fungi on more than twelve thousand isolates from different countries and have shown that 10637 isolates of these were yeasts and yeast-like fungi (Espinel-Ingroff et al., 2003). Pfaller and colleagues separated and analyzed 4169 isolates of *Candida* and Cryptococcus from patients in an international project of centers in different countries, in Asia, Europe and the U.S. (Pfaller et al., 2002). The results of the susceptibility test of antifungal drug against 2000 *Candida* isolates were investigated in 2003 by Ostrosky-Zeichner and colleagues (Ostrosky-Zeichner et al., 2003). The results of the susceptibility test of antifungal drug against 312 *Candida* isolates were investigated (Pfaller et al., 2000). The susceptibility test was performed in 279 isolates (Maxwell et al., 2003) and the experiment of 272 cases was done. (Linares et al., 2004). Identification and drug sensitivity of 200 strains of pathogenic fungi was undertaken (Figueiredo et al., 2007). Susceptibility of 100 pathogenic isolates was made (Espinel-Ingroff et al., 2004). Different results obtained in these studied may be due to using different methods and collection of isolates in different geographical locations. Similar studied have been performed in Iran. The antifungal activity of Itraconazole, Miconazole, Fluconazole and Flucytosine, by microdilution method, was done against 191 *Candida* species isolated from patients with vulvovaginal candidiasis between the years 2006 to 2008 by Mahmoudi Rad et al. at the hospital in Tehran (Mahmoudi Rad et al., 2009). Antifungal effect of Ketoconazole against *Candida* strains isolated from 28 vulvovaginal candidiasis cases with broth dilution method was made (Moghadasi et al., 2008) compared to the effects of Ketoconazole, Clotrimazole Miconazole, fluconazole against 10 albicans isolates from patients with suspected vaginal candidiasis (Hussein far et al., 2007). Comparing the antifungual activity of Fluconazole, Clotrimazole, Miconazole and amphotericin B, with micro broth dilution method and flow cytometry was made against 6 different *Candida* species (Falahati et al., 2007). The effect of temperature and hydrogen ion concentration on the antifungual activity of Ketoconazole against of *Candida albicans* isolates from 10 patients with suspected vulvovaginal candidiasis was undertaken by Sadoughifar et al. (Zarinfar et al., 2008). The drug sensitivity of 106 local isolated

Table 1. mean (range) MIC and MFC values at 24-48 hours of *Candida* isolates

	mean					
	MZ + CZ		MZ + KZ		KZ + CZ	
	MIC	MFC	MIC	MFC	MIC	MFC
Candida albicans (1)	6.25	50	12.50	12.50	6.25	6.25
Candida albicans(2)	3.12	50	25	12.5	6.25	12.5
Candida albicans (3)	>100	>100	>100	>100	>100	>100
Candida albicans (4)	50	>100	0.78	>100	0.39	>100
Candida albicans (5)	3.12	>100	0.78	6.25	0.39	6.25
Candida albicans (6)	6.25	50	0.78	6.25	0.39	6.25
Candida albicans (7)	50	>100	25	>100	6.25	>100
Candida albicans (8)	50	>100	12.5	25	6.25	12.5
Candida albicans (9)	3.12	50	0.78	12.5	0.39	6.25

(Clotrimazole [CZ], Miconazole [MZ], Ketoconazole [KZ])

Table 2. The values of *MIC* frequencies of different binary mixure of azole against the *Candida* isolates.

	%MFCµg/ml								
	0.39	0.78	3.12	6.25	12.5	25	50	>100	Total
MZ +CZ count	1	0	3	2	0	0	3	1	10
%within Drug	10	0	30	20	0	0	30	10	100
% of total	3.3	0	10	6.7	0	0	10	3.3	33.3
MZ + KZ count	1	4	0	0	2	2	0	1	10
%within Drug	10	40	0	0	20	20	0	10	100
% of total	3.3	13.3	0	0	6.7	6.7	0	3.3	33.3
KZ + CZ count	5	0	0	4	0	0	0	1	10
%within Drug	50	0	0	40	0	0	0	1	10
% of total	16.7	0	0	13.3	0	0	0	3.3	33.3
total count	7	4	3	6	2	2	3	3	10
%within Drug	23.3	13.3	10	20	6.7	6.7	10	10	100
% of total	23.3	13.3	10	20	6.7	6.7	10	10	33.3

Chi - Square tests for MIC

	Exact Sig. (2-sided)	Df	Value
Pearson Chi - Square	17.527	10	.041
Fisher's Exact Test	15.157		.041

Table 3. The values of *MFC* frequencies of different binary mixture of azole drugs against of *Candida* isolates.

	Binary Mixture Drugs						
	%MFCµg/ml						
	0.39	6.25	12.5	25	50	>100	Total
MZ +CZ count	1	0	0	0	4	5	10
%within Drug	10	0	0	0	40	50	100
% of total	3.3	0	0	0	13.3	16.7	33.3
MZ + KZ count	1	2	3	1	0	3	10
%within Drug	10	20	30	10	0	30	100
% of total	3.3	6.7	10	3.3	0	10	33.3
KZ + CZ count	1	4	2	0	0	3	10
%within Drug	10	40	20	0	0	30	100
% of total	3.3	13.3	6.7	0	0	10	33.3
total count	3	6	5	1	4	11	10
%within Drug	10	20	16.7	3.3	13.3	36.7	100
% of total	10	20	16.7	3.3	13.3	36.7	33.3

Chi - Square tests for MFC

	(2-sided) Exact Sig.	Df	Value
Pearson Chi - Square	36.571	14	.000
Fisher's Exact Test	6.746		.000

Candida albicans and non-albicans species from patients in Tehran during 2004-2005 with disk diffusion and dilution methods (Abrahim et al., 2007). The simultaneous use of antifungal drugs against fungal infections is a relatively new technique, especially in the treatment of severe systemic infection, or dealing with resistance cases (Johnson et al., 2004). Ghahfarokhi investigated antifungal activity of Fluconazole, Itraconazole and ketoconazole separately and mixed into each other, against two pathogenic strains of *Candida albicans* PTCC5057, *Candida dubliniensis* CD36, Cryptococcus neoformance CNE1, and Malassezia furfur MF1 by broth dilution method. In this study, they found that all the mixtures of drugs have pharmacological synergy as compared with of individual modes. These results indicate that combination of Itraconazole and Fluconazole or Fluconazole mixed with Ketoconazole had the best influence in the MIC. The synergistic effect of the combination with antifungal drugs and some other plant extracts was studied (Nodoushan et al., 2007). In the present study, a synergistic effect of the binary mixure of common azole compounds are examined against skin and mucosal *Candida* infections, and their effectiveness was determined. Azole is the most common drugs that are used in the country. The combinations of Clotrimazole 1% and 2% Ketoconazole and Miconazole are used locally for treatment of fungal infections. In severe cases of *Candida* infections systemic and oral Fluconazole tablets are used in the amount of 100 mg. Ketoconazole tablets are also available, but it causes liver problems, anemia, and increased sensitivity (Bakhtiari et al., 2007). The results of our study suggest that these binary mixture of azole drugs in most strains inhibits the growth of pathogenic fungus *Candida* isolates in a dose-dependently manner.

5. Conclusions

The results of this study showed the synergetic effects of combination of azole antifungal drugs against Cndida spices. It is concluded that the use of the combination of Clotrimazole and Ketoconazole in equal ratios has better antifungal effects against cutaneous and mucosal isolates of the *Candida* infection

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