EFFECT OF ASCORBIC ACID AND PIMECROLIMUS (ELIDEL®) ON THE ACTIVITY OF SOME ANTIFUNGAL AGENTS AGAINST MULTIPLE RESISTANT CLINICAL CANDIDA ALBICANS

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ABSTRACT

Abstract : One hundred patients [60 women with vulvovaginal candidiasis (VVC) and 40 with skin candidiasis] were included in the present study. The VVC and skin candidiasis were detected by both clinical and microbiological examination. The susceptibility of a total 100 *Candida albicans* isolates to 7 common antifungal agents was determined. The incidence of resistance to the tested drugs ranged from 12% to 61% . Lowest percentage of resistance was to tioconazole while the highest one was to fluconazole. The effects of combining ascorbic acid, Pimecrolimus (SDZ ASM 981, Elidel®) with different antifungal agents against *C. albicans* were investigated. The results of combination with ascorbic acid revealed that the percent reduction of resistacne was in the range of 12.7% to 78.1%. The highest value was observed with miconazole (78.1%) while nystatin showed the lowest value (12.7%). Time killing curve of *C. albicans* American Type Culture Collection ($ATCC_{32354}$) was conducted in presence and absence of ascorbic acid with miconazole. The results revealed about 5 log reduction in the number of viable cells. the vaginal and skin miconazole ascorbic acid cream was found to be highly effective in eradicating the candidiasis and curing the clinical symptoms of the disease when used once daily for seven days. On the other hand, the in vitro activity of combination of pimecrolimus (Elidel) cream with miconazole and ascorbic acid showed higher activity against *C. albicans* than miconazole ascorbic acid cream. These results revealed the synergistic activity of ascorbic acid or pimecrolimus to antifungal agents against *C. albicans* resistant tisolates.

INTRODUCTION

Candidiasis of vaginal mucous membrane, vulva and skin are common[1]. Vaginitis affects all age groups including infants, children, adolescents, fertile age, menopausal and postmenopausal women. This disease is the most common gynaecologic problem facing the female today. Penetration of the gastrointestinal mucosa by *Candida* species is thought to be the most frequent, leading to systemic dissemination[2].

Candida albicans possesses a variety of factors that could be involved in the invasive process. Putative virulence is attributed to adhesions, dimorphism, phenotypic switching, and secretion of hydrolytic enzymes as proteinases and phospholipases[3,4,5].

there are relatively few specific antifungal agents available for the treatment of systemic mycoses, yet the incidence of such infection, particularly of those caused by *Candida* species among immuno compromised patients, is generally

considered to have become extremely high. Amphotericin B, flucytosine and three azole derivatives fluconazole, itraconazole and ketoconazole are the currently available drugs of value in systemic yeast infections[6].

In the last decade, the widespread use of azoles and other antifungal agents had led to rapid development of drug resistance in patients with recurrent candidiasis[7]. Several molecular mechanisms that contribute to drug resistance have been described as efflux pump, alteration in target and secretion of the lanosterol demethylase enzyme[8,9].

The rising prevalence of serious fungal infections and the emergence of resistance to commercially available antifungal compounds is increasing and there is much interest in the development of new antifungal agents or combination with other agents to improve efficacy and /or decrease toxicity[10].

Ascorbic acid (ASC) is known to carry out a number of biochemical functions that are a consequence of its ability to donate one or two electrons. Some known or proposed functions of ASC include its utilizaion as a free radical scavenger and as cofactor for a number of enzymes[11]. Pimecrolimus (ASM 981), (Elidel^{R}) a newer macrolactam ascomycin derivative and calcineurin inhibitor closely related to tacrolimus, is also being developed for atopic dermatitis therapy[12,13]. Tacrolimus is an immunosuppressive cytotoxic drug which can potentially reverse the multidrug resistnce (MDR) phonotype[14,15]. It is used clinically in the prevention of allograft rejection[16]. Same studies have also demonstrated an energy dependent drug efflux mechanism in *C. albicans* similar to the mechanism described for resistance of cancer cells to anticancer agents[17].

AIM OF WORK

The aim of the present study was the isolation of *C. albicans* causing vulvo- vaginal candidiasis (VVC) and skin candidiasis among different patients and determination of in vitro susceptibility to commonly used antifungal agents alone and with ascorbic acid and Elidel against multiple resistant *C. albicans*. The effectiveness of miconazole with ASC in treatment of vulvovaginal (VVC) and skin candidiasis was also clinically investigated.

MATERIALS & METHODS

Patients : One hundred patients were classified into 2 groups. First group was 60 women with VVC recruited from out patients Clinic of Obstetrics & Gynecology, Tanta University Hospital during the period of January 2003 to July 2003. The second group was 40 patients with skin and/or nail infection from Dermatology & Venereology department at the Cancer institute. Both groups were subjected to thorough clinical examinations (after an informed consent was obtained from all patients). The age group ranged between 22-47 years. The clinical diagnosis of VVC involved the following criteria: symptoms of pruritis and irritation in vulva, thick or paste-cottage cheese like vaginal discharge. Diabetic and pregnant women were excluded from our study.

Examination of cervix and vagina:

Inspection of cervix and vagina, under complete aseptic technique by Cusco's speculum was done. After visualization of the cervix, a sterile cotton swab was inserted in the posterior fornix to pick up the collected sample & discharge, and was inoculated and incubated in the Sabouraud broth at room temperature for culture and microscopical examination. Vaginal wet smear with 10% KOH was applied for all VVC patients.

Isolation and identification of *C. albicans* from the tested cases:

Vulvovaginal and skin samples were collected using sterile swabs for microscopical examination and culturing. The swab was immediately streaked into Sabouraud dextrose agar (oxoid) plate and incubated at 30°C for 1-3 days. Candidal growth was recorded using clonal morphology and microscopical characteristics.

The colonies were further identified as *C. albicans* by using both the conventional method[18] and the commercial multi test system API 20 (Biomereux, France). The local treatment after mixing it with ASC was applied for 7 days.

Antifungal agents and other chemicals: Fluconazole (Flu) was obtained from Pfizer, New York. Miconazole (Mico), tioconazole (Ti), nystatin (Nys), amphotericin B (AmB) and ASC were obtained from Sigma, USA. Clotrimazole (Clo) from Bayer, Germany, itraconazole (Itra) from Janssen, Belgium; Elidel a free medical sample obtained from Novartis, pharma S.A.E., Cairo, Egypt.

Antifungal susceptibility testing: Antifungal susceptibility testing of different *C. albicans* isolates were determined by agar diffusion method [19,20,21]. *C. albicans* isolates grown on exponential phase were applied as spots (10^5 CFU/sopt) by a multipoint inoculator onto the surface of Sabouraud dextrose agar (SDA) (oxoid) containing breakpoint concentration of one of the following antifungal agents (Flu, > 8 µg/ml), (Itra), (Ti) and (Nys) were 8 µg/ml while (Mico), (Clo) and (AmB) were (2 µg/ml). The plates were incubated for 24 hr at 30°C. Isolates that failed to produce visible growth on the agar plates were considered sensitive.

In-vitro synergy testing: Combination of ASC (2 mM) and Elidel cream (50 µg/ml) with different antifungals were tested for each *C. albicans* isolate as mentioned above. Each plate was inoculated with 10^5 CFU/ml, incubated at 30°C for 24 hrs and read as described for the in-vitro susceptibility testing.

MIC determination: The minimum inhibitory concentrations (MICs) of miconazole was determined by agar dilution technique[21] on SDA plates with and without ASC and Elidel using a multipoint inoculator device and an inoculum of 10^5 CFU/spot. The MIC recorded after 24 hrs of incubation at 30°C was defined as the lowest concentration which suppressed any visible growth [19].

Time-course viability studies: Time-course and effects of ASC action on the viability of fungal cells were determined according to Brajtburg *et al.* (1989) [22] as follows: *C. albicans* $ATCC_{32354}$ tested strain was grown in sabouraud medium, harvested in midlogarithmic phase of growth and dispersed at a density of about 10^5 cells/ml of medium. The cells were treated first for 2h with the ascorbic acid (2 mM), miconazole was then added, the tubes were incubated at 30°C with continuous agitation in orbital shaker. Samples wer drawn at 0, 2, 4, 6, 8, 12 and 24 hrs, diluted, then plated on Sabouraud dextrose agar plates. The plates were incubated at 30°C for 24 hrs and colony counts were determined. A proper control lacking miconazole and ASC was included.

Preparation ASC / antifungal combinations cream:

The ASC / antifungal combinations were prepared in cream form to facilitate their clinical application. Miconazole and tioconazole were selected for this study. The marketed miconazole cream (Daktarin (2%) & Gyno-Daktarin (2%), Minapharm, Egypt, under license of Janssen pharmaceutics, Belgium and tioconazole cream (Trosyd (1%w/w) cream Pfizer, Egypt) were employed. ASC was incorporated in these cream to

produce final concentration of 2 mM (the concentration producing the highest synergistic effect with these drugs in the solution form). This involved dissolving the required amount of ascorbic acid in the least amount of distilled water. This solution was incorporated into the antifungal cream by trituration in the mortar. The same amount of distilled water was added to the corresponding antifungal cream and used as control.

drug release from cream base:

The test cream (10 mg) was spread on a filter paper disc (5 mm diameter). These were mounted on the surface of seeded SDA, plates were incubated at 28°C for 24 hours. The inhibition zone diameters were then recorded. The values were compared with that obtained after incubation of similar filter paper discs inoculated with 10µl of the corresponding drug solution sufficient to produce the same amount of drug as in 10 mg of the creams.

Clinical investigations: the efficiency of miconazole / ASC cream in treatment of patients with vaginal or skin infection was evaluated by applying the cream by plastic applicator into the posterior fornix to the affected area once daily for one week. Clinical investigation was carried out after three days and at the end of the course of the treatment. At the same time vaginal and skin swabs were taken for microbiological exmaination as described above.

RESULTS

In this study, 100 clinical isolates of *C. albicans* from patients suffering from VVC and skin candidiasis wer tested for thier susceptibility to 7 different antifungal agents. Table (I) summarizes the in vitro susceptibility of the tested isolates. The data revealed that ticonazole, amphotricin B and miconazole were the most active drugs (the % of resistance ranged between 12.5-33.3%). On the other hand, the lowest activity was observed with nystatin and fluconazole (55-61%).

The addition of ascorbic acid to these agents decreased the incidence of resistance to a higher extent (Fig. 1). The ascorbic acid caused the higher reduction (12.7-78.1%) of resistance.

Miconazole was the drug most potentiated by ascorbic acid (Table II). So this combination was selected for in vivo study . Time course viability study of miconazole with ascorbic acid against *C. albicans* was shown in Fig.(2). Fungal cells treated with ASC and then exposed to miconazole yielded fewer colonies in subculture than did cells incubated without ASC. The log number of viable cells was decreased by value of 5.0 after 24 hr. (Fig.2).

The MICs of miconazole in combination with ascorbic acid or Elidel against the tested clinical isolates are shown in Table III. Generally, the presence of any of these compounds reduced the MICs of miconazole against all tested isolates. ASC was more effective than Elidel in reducing the MICs of miconazole (Table III). Only 25 out of 32 (78.1%) resistant isolates became sensitive by addition of ASC. On the contrary, 50% of tested resistant isolates became sensitive by addition of Elidel. The results of miconazole and tioconazole release from ASC and Elidel creams were indicated in Table(IV). ASC is more effective in potentiating the activity of antifungals than Elidel as indicated by the inhibition zone diameter.

The results of application of miconazole ascorbic acid cream on clinical cases are illustrated in Table (V). These revealed that after 3 days of the treatment, symptoms including itching, redness and discharge disappeared in 60, 50% in VVC and 55, 40% in cutaneous candidiasis respectivley. *Candida albicans* were not detected in 14 (70%)

and 12 (60%) of the treated cases respectively. After 7 days of the treatment nearly all cases (95-100%) were clinically and microbiologically cured (Table V).

Table I : Incidence of resistance of *Candida albicans* isolates to different antifungal agents among different groups of patients.

Candida albicans isolate (No)	No of resistant isolates (%) to						
	Flu	Itra	Mico	Clo	Ti	Nys	AmB
	No (%)						
Group I (VVC) (60)	36 (60)	34 (58.3)	20 (33.3)	23 (38.3)	8 (13.3)	33 (55.0)	15 (25.0)
Group II skin candidiasis (40)	25 (62.5)	24 (60.0)	12 (30.0)	16 (40.0)	5 (12.5)	22 (55.0)	11 (27.5)

Flu (fluconazole), Itra (itraconazole), Mico (Miconazole), Clo (Clotrimazole), Ti (Ticonazole), Nys (Nystatin), AmB (Amphotericin B).

Table II : Effect of ascorbic acid on activities of the different antifungal agents against the resistant *Candida albicans* isolates.

Antifungal agents	No. of resistant isolates		% reduction of * resistance
	ASC untreated	ASC treated	
Fluconazole	61	40	34.4
Itraconazole	59	48	18.6
Miconazole	32	7	78.1
Clotrimazole	39	10	74.3
Ticonazole	13	3	76.9
Nystatin	55	48	12.7
Amphotericin B	26	10	61.5

* number of sensitive isolates out of resistant treated.

Table III : Effect of ascorbic acid and Elidel on the MICs of miconazole for *Candida albicans*.

Drugs	Number of isolates with MIC (µg/ml)									
	0.125	0.25	0.5	1	2	4	8	16	32	64
Mico Alone	23	18	15	12	11	8	6	4	2	1
Mico + Asc	30	25	20	18	3	2	1	1	-	-
Mic + Elidel	24	20	21	19	7	5	2	1	1	-
Mico + Elidel + Asc	30	25	21	19	3	1	1	-	-	-

Table IV : Release of miconazole and tioconazole from ascorbic acid and Elidel cream.

Drugs	I. Z. diameter (mm)		% release
	Cream	Solution	
Elidel	10	9.7	97
Ascorbic acid	10	9.8	98
Miconazole	28	28	100
ticonazole	25	24.8	99.2
Miconazole + Elidel	39	38.8	99.4
Ticonazole + Elidel	36	35.6	98.8
Miconazole + Ascorbic acid	42	42	100
Ticonazole + Ascorbic acid	38	37.8	99.4
Miconazole + Elidel + Ascorbic acid	45	45	100
Ticonazole + Elidel + Ascorbic acid	43	42.8	99.5

I.Z.: inhibition zone diameter

Diameter of the disc: 10 mm

Table V : Clinical manifestations and microbiological examination of VVC and skin cases during the course of treatment with miconazole - ascorbic acid cream.

Finding after	Number of cases with			
	VVC		Skin candidiasis	
	+ ve (%)	– ve (%)	+ ve (%)	– ve (%)
Three days				
Itching	8 (40.0)	12 (60)	9 (45)	11 (55)
Redness	10 (50.0)	10 (50)	12 (60)	8 (40)
Discharge	10 (50.0)	10 (50)	--	--
Yeast Detection	6 (30.0)	14 (70)	8 (40)	12 (60)
After seven days				
Itching	1 (5)	10 (95)	1 (5)	19 (95)
Redness	1 (5)	19 (95)	1 (5)	19 (95)
Discharge	1 (5)	19 (95)	--	--
Yeast Detection	- (0)	20 (100)	--	20 (100)

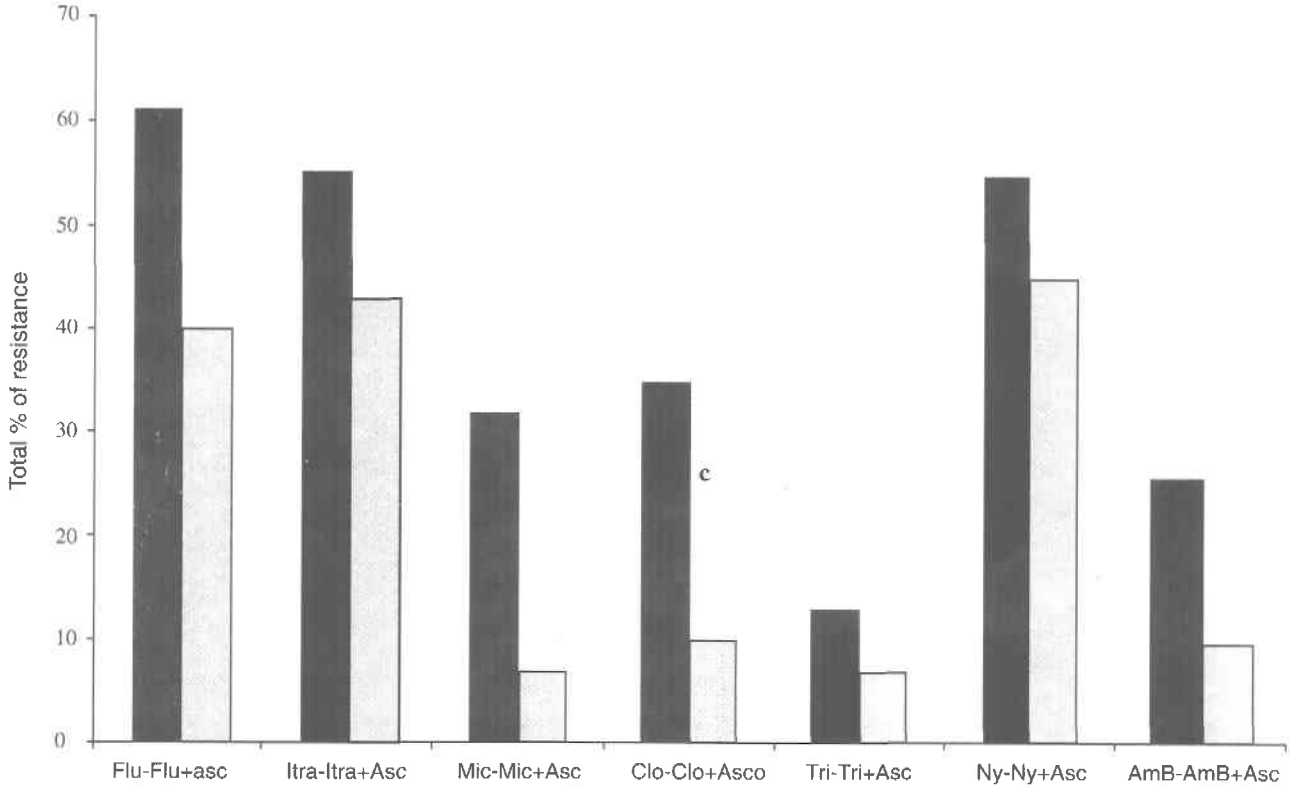

Fig. (1) : Incidence of resistance of Candida albicans without and after treatment with ascorbic acid

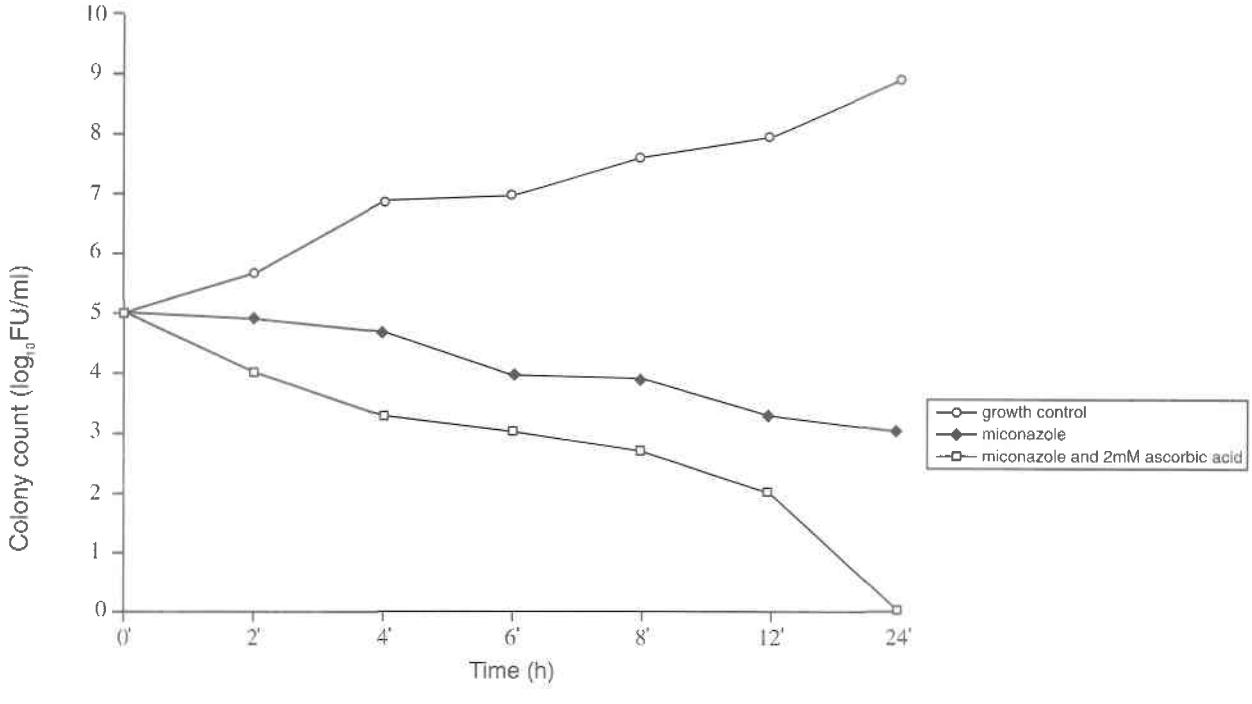

Fig.(2) : Time kill studies of miconazole alone and in combination with ascorbic acid aganist Candida albicans

N.B : Ascorbic acid alone as growth control.

DISCUSSION

The importance of candidiasis is attributed in part to the high incidence of candida as commensals, and their rapidity to spread and to cause infection when the natural resistance is altered by predisposing factors[23]. *Candida albicans* is the predominant causative agent of candidiasis of either the skin or mucosal surfaces [1]. The widespread and increased use of immunosuppressive therapy together with broad spectrum antifungal drugs lead to increased frequency and chronicity of muscosal and cutaneous infections. In the present study, the susceptibility of 100 *C. albicans* strains, isolated from vaginal and skin patients, to common antifungal agents was determined. The results revealed that fluconazole followed by itraconazole and nystatin had the highest percentage of resistance comparing with other drugs. These were in agreement with several investigators who reported high percentage of azole resistance [24,25,26,27,28]. Perea *et al.* (2001)[26] reported the high level fluconazole - resistant strains of *C. albicans* commonly display multiple mechanisms of resistance,including over expression of MDR and CDR efflux pumps as well as alteration in the target enzyme and over-expression of the genes encoding enzymes. the susceptibility of *c. albicans* to other azoles than fluconazole may probably do not over express CDR genes while strains which are resistant to fluconazole and other azoles are well known to over-express CDR genes. So it is not surprising that our isolates would be resistant to the new triazole as well. The emergence of azole resistance in candida emphasizes the urgent need for new antifungal agents or new treatment strategy by combing with other substances with antifungal agents to decrease the incidence of resistance. So, in this study the ability of ascorbic acid and Elidel to eliminate or decrease the resistance of *C. albicans* to different antifungal agents was examined. The ascorbic acid was selected for this study because it was reported to be genotoxic [29] and its ability to eliminate plasmids of Gram positive and negative bacteria [30,31]. On the other hand pimecrolimus (Elidel) is an ascomycin macrolactam derivative and a cell-selective inhibitor of inflammatory cytokines specifically developed to treat inflammatory skin diseases[32]. It is one of calcineurin inhibitors which has similar mechanism but weaker action than tacrolimus (FK 506). It exhibits drug synergy with ergosterol inhibitors and could extend the utility and efficacy of this therapeutic approach from fungistatic to fungicidal potential effect[33].

The results of combination of ascorbic acid with miconazole and tioconazole showed the higher ability for reduction of the percentage of miconazole resistance (to 78%) than for tioconazole to 76% and hence overcome multi-drug resistance. This is attributed to the fact that ascorbic acid may provide an acid media which potentiates the antifungal activity and acts as antioxidant or as pro-oxidant which potentiates the lethal action of drugs. The enhancement of antifungal activity of ketoconazole in acid media had previously been confirmed against aspergillus species as reported tby Elsanabary *et al.* 1995[34]. In addition the enhancement of anticandidal action of amphotericin B by ascorbic acid had previously been reported by Beggs *et al.* (1979), Pethig *et al.* (1983), Kapila and Modi (1986), Liebler *et al.* (1986) and Brajthurg *et al.* (1989) [22,35,36,37,38].

Results of in-vitro release of miconazole and tioconazole from its cream were indicated in Table (IV). These results revealed that the incorporation of ascorbic acid and Elidel with antifungal agents potentiated th effect of these antifungals when compared to each drug alone.

Application of miconazole ascorbic acid cream once daily showed marked effect in the treatment of skin and VVC within one week. In addition, it showed inhibitory effect *in vitro,* and eradicated *Candida* in the vagina and skin or at least inhibited its adherence. this might prevent yeast proliferation or minimized its ability to invade mucosal epithelium for establishment of infection[3].

Topical tacrolimus (FK 506) and pimecrolimus have recently been FDA-approved for the treatment of atopic dermatitis. Both agents act by binding with high affinity to the 12 kDa macrophilin and inhibit the phosphatase activity of the calcium-dependent serine/threonine phosphatase, calcineurin-tacrolimus and pimecrolimus inhibit the activation of a number of key effector cells involved in atopic dermatitis[39].

Similar studies - but with tacrolimus - have demonstrated the antifungal activity against *Malassezia furfur*, a lipophilic yeast and a member of the normal skin microflora, and this property is involved in the clinical efficacy in patients with atopic dermatitis[40]. In the present study, pimecrolimus showed no antifungal activity against *Candida albicans*. This was in agreement with Maesaki *et. al* 1998 [15] who reported similar results but with tacrolimus. However, the combination of pimecrolimus and azole antifungal agents showed synergic effect against azole resistanc to *C. albicans* strain. This has recently been reported by Maesaki *et al.* 1998 and Onyewu *et al.* 2003[15,33]. They explained the results by the suggestion that tacrolimus might cause a marked increase in the intracellular accumulation of azole in the candidal strain, thus tacrolimus and other calcineurin inhibitors as pimecrolimus may interact with the common drug efflux pump and inhibit azole efflux from the cell. In addition calcineurin inhibitors compete with cytotoxic drug for binding sites on MDR p-glycoprotein, can potentially reverse MDR phenotype [15].

The synergy between calcineurin inhibitors as pimecrolmus and azoles shown in this study might suggest that the latter agent could be topically used with ascorbate in patients with VVC and skin candidiasis specially those patients with high resistance to azole antifungal agents.

CONCLUSIONS

In summary, because ascorbate has little toxicity and can be increased by either oral or parentral administration, and because antifungal resistance is increasing, it may be worth while to explore the clinical potential of ascorbate alone and/or with pimecrolimus as an adjunct to antifungal drugs for treatment of multiple resistant fungal infections.

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