15 *Egypt. J. Bot.*, **Vol**. **54,** No.2 pp. 247- 262 (2014)

Effect of Fluconazole and Terbinafine on the Activities of Lanosterol 14 α-Demethylase and Squalene Epoxidase in Ergosterol Biosynthetic Pathway

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THE ACTIVITIES of the ergosterol biosynthetic enzymes, lanosterol 14α-demethylase and squalene epoxidase were assayed by measuring the ergosterol content of *C. albicans* cell membrane under different antifungal drug treatments as compared to control. The ergosterol was measured using the spectrophotometric sterol quantification method. Fluconazole (lanosterol 14 α-demethylase inhibitor) and terbinafine (squalene epoxidase inhibitor) inactivate both enzymes and stopped the ergosterol biosynthesis completely in susceptible isolates of *C. albicans*. Partial inhibition was observed in resistant isolates of *C. albicans*. Combined mixture of fluconazole (FLU) with terbinafine (TRB) acted synergistically on the ergosterol biosynthetic enzymes leasing to disappearance of the 4-peaked curve characteristic to ergosterol.

Keywords: *Candida albicans*, Lanosterol 14α-demethylase, Squalene epoxidase, Antifungal drugs, Ergosterol.

Ergosterol is the predominant sterol in fungal plasma membranes; it is important for membrane integrity, for activity of many membrane-bound enzymes and it serves as a bioregulator of fungal membrane fluidity and integrity (Ghannoum and Rice., 1999).

Ergosterol was isolated by multi level extraction associated with saponification and analyzed by reverse phase high performance liquid chromatography (Lavová *et al.* 2013).

Commonly used antifungal drugs inhibit sterol biosynthesis (azoles, allylamines and morpholines), directly interact with the cell membrane (polyenes) or target cell wall biosynthesis (echinocandins) (Casalinuovo *et al*., 2004). The annual death rate due to candidiasis was steady between 1950 and about 1970. Since 1970, this rate increased significantly in association with several changes in medical practice, including more widespread use of therapies

that depress the immune system, the frequent and often indiscriminate use of broad-spectrum antibacterial agents, the common use of indwelling intravenous devices, and the advent of chronic immunosuppressive viral infections such as AIDS. These developments and the associated increase in fungal infections (Beck-Sagué and Jarvis, 1993) intensified the search for new, safer, and more efficient agents to combat serious fungal infections.

The antifungal drugs currently available for the treatment of invasive mycoses can be divided into 4 different classes on the basis of their mechanisms of action: (1) alteration of membrane function (amphotericin B); (2) inhibition of DNA or RNA synthesis (flucytosine); (3) inhibition of ergosterol biosynthesis (terbinafine, fluconazole, itraconazole, and the newer agents voriconazole, posaconazole, and ravuconazole)); and (4) inhibition of glucan synthesis (echinocandins: caspofungin, micafungin, and anidulafungin) (Perea and Patterson, 2002).

The antifungal drugs used are belonging to ten different major groups of substances (Zhang *et al*., 2007). These are allylamines, benzofurans, echinocandins, hydroxypyridones, imidazoles, morpholines, polyenes, pyrimidines, thiocarbamates and triazoles (Polak, 1988; Bohme & Karthaus, 1999). Most therapies, designed to treat fungal infections, target the ergosterol biosynthesis pathway or its end product, ergosterol, a membrane sterol that is unique to fungi. It is the main sterol, and thus is necessary for growth and normal membrane function of fungal cell (Lupetti *et al*., 2002).

Clinical resistance is classically defined as persistence or progression of an infection despite the administration of appropriate antimicrobial treatment. The prediction of the clinical outcome for a patient with a mycotic infection is often a difficult question-and one in which many factors intervene. The antifungal susceptibility of the fungal isolate is only one of the elements that contribute to clinical resistance; other factors include the pharmacokinetics of the antifungal drug used, host factors, the site of infection, and the fungal pathogen itself. In general, fungi can be intrinsically resistant to antifungal drugs (primary resistance) or can develop resistance in response to exposure to the drug during treatment (secondary resistance) (Perea and Patterson, 2002).

The newer antifungals have been effective in the treatment of systemic fungal infections and offer a potent alternative to potentially toxic amphotericin B therapy (Lewis & Klepser, 1999). It is well documented, however, that several pathogenic yeasts have either intrinsic or acquired resistance to the azole antifungal drugs (Rex *et al*., 1995). It is advisable, therefore, to determine the antifungal susceptibility patterns of microbial isolates, which may assist in making appropriate decisions regarding the best therapeutic option (Pfaller *et al*., 1997).

Infections by *Candida albicans* and related fungal pathogens pose a serious health problem for immune compromised patients. Azole drugs, the most common agents used to combat infections, target the sterol biosynthetic pathway (Gallo-Ebert *et al.,* 2013). *Candida* infections can be spread to vulnerable

people with depressed immune systems who are in the hospital, where the fungus is commonly found on the hands of care givers and where indwelling catheters can allow an infection to take hold. The number of available drugs for the treatment of systemic fungal infections is limited. The antifungals known nowadays can be classified into azolic and polienics. The azolics are elected in the first instance for treating these diseases and are generally fungistatics, while the latter are fungicides. In recent years, the terbinafine, amphotericin B and azoles mainly ketoconazole, fluconazole and itraconazole have been the drugs of choice in therapy (Goodman and Gilman, 1996).

Fluconazole is a member of azoles family that targets essential enzyme lanosterol 14α-demethylase (Erg11) (Song, *et al*., 2004). Terbinafine is a member of allylamines family it has specific mechanism on squalene epoxidase (Graminha *et al*., 2004). Inhibition of both enzymes which are essential in ergosterol biosynthesis in *C. albicans* is considered as a mode of control of the serious candidiasis disease. Hence, the objective of this study was focused at studying the disorders induced in ergosterol biosynthetic enzymes under antifungal treatments using *C. albicans* isolates screened from immunocompromized childrens suffering from Candidiasis.

Material and Methods

Candida albicans

Isolates were screened from blood, urine and sputum of immunocompromized children hospitalized in Abo El-Reesh pediatric Hospital, Cairo University. They all suffer from candidiasis. *C. albicans* isolates were distinguished into white and opaque isolates and identified by germ tube method and API (Naguib, 2013).

Determination of MIC90

Mic90 values were determined by Mohsen *et al*. (2013) in the previous paper.

Ergosterol assay by spectrophotometric method

The enzyme activities were assayed by quantitative determination of membrane ergosterol , spectrophotometrically, in antifungal treated *C. albicans* isolates as compared with control. The method of Arthington-Skaggs *et al*. (1999) was adopted.

A single *C. albicans* colony from an overnight Sabouraud dextrose agar (SDA) plate culture was used to inoculate 50 ml of Sabouraud dextrose broth containing 0, 0.25, 0.5, 1xMIC_{90} µg/ml of each antifungal (Breivik and Owades, 1977). The cultures were incubated for 16 hr with shaking (150 rpm) at 35◦C. The stationary-phase cells were harvested by centrifugation at 2700 rpm (HERAEUS LABOFUGE 200 centrifuge) for 5 min and washed once with sterile distilled water. The net wet weight of the cell pellets was determined. Three milliliters of 25% alcoholic potassium hydroxide solution (25g of KOH and 35 ml of sterile distilled water,

brought to 100 ml with 100% ethanol), was added to the pellets and vortex mixed for 1 min. Cell suspensions were transferred to a clean 16 mm borosilicate glass screw-cap tube and were incubated in an 85°C water bath for 1hr. Following incubation, tubes were allowed to cool to room temperature. Sterols were then extracted by addition of a mixture of 1 ml of sterile distilled water and 3 ml of n-heptane followed by vigorous vortex mixing for 3 min. The n-heptane layer was transferred to a clean borosilicate glass screw-cap tube and stored at –20°C for as long as 24 hr prior to analysis, a 20 ml aliquot of sterol extract was diluted five fold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm with a Gilford Response Spectrophotometer (SHIMADZU UV-1650PC).

The presence of ergosterol and the late sterol intermediate 24(28) Dehydroergosterol [24(28) DHE)] in the extracted sample resulted in a characteristic four-peaked curve. The absence of detectable ergosterol in extracts was indicated by a flat line. Any dose-dependent activity lead to decrease in the height of the absorbance peaks which corresponded to decreased ergosterol concentration due to inhibition in both lanosterol 14α-demethylase and squalene epoxidase activities. Ergosterol content was calculated as a percentage of the wet weight of the cell by the following equations:

1: % ergosterol + %24(28) DHE = $[(A_{281.5}/290) \times F]$ / pellet weight

2: % 24(28) DHE = $[(A_{230}/518) \times F]$ / pellet weight,

% ergosterol = 1 – 2

Where F is the factor for dilution in ethanol and 290 and 518 are the E values (in percentages per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively.

Statistical analysis

Duncan test was executed to show homogeneity among the means at ($P<0.05$). Statistical analyses were performed using SPSS software (version 15; SPSS, Chicago, IL).

Results

Ergosterol percentage in the membrane of *C. albicans* isolates treated with FLU.

The data in Table 1 clearly indicate that the wet weight decreased as a function of fluconazole concentration in all isolates, but was more drastically in the susceptible isolates.

The data in Table 2 evidently indicated that FLU treatment completely inhibited ergosterol biosynthesis in the susceptible isolates (white & opaque). In resistant isolates, the white form of *C. albicans* showed a partial inhibition at the low concentration of FLU ($0.25xMIC_{90}$), then complete inhibition in ergosterol biosynthesis at the higher concentrations was observed. Opaque resistant isolate showed a dose-dependent inhibition in ergosterol biosynthesis due to partial inactivation of lanosterol 14 α-demethylase. This indicated fungistatic effect of fluconazole.

TABLE 1. Effect of different fluconazole concentrations on the wet weight of *C. albicans* isolates .

FLU conc.	Wet weight			
	Resistant		Susceptible	
	White	Opaque	White	Opaque
Control	0.4861±0.10^{b}	0.5746±0.13^{b}	0.4261±0.07^{c}	0.6136±0.15^{c}
0.25xMIC$_{90}$	0.3356±0.13^{b}	0.2894±0.16^{a}	0.3123±0.05^{b}	0.3092±0.19^{b}
0.5xMIC$_{90}$	0.1430±0.09^{a}	0.2535±0.17^{a}	0.1757±0.05^{a}	0.2423±0.04^{ab}
1xMIC$_{90}$	0.0834±0.02^{a}	0.2293±0.09^{a}	0.1435±0.06^{a}	0.0128±0.00^{b}
Values of MIC$_{90}$ (µg ml^{-1})	200	76	15	6.5

TABLE 2. Percentage of ergosterol in *C. albicans* isolates treated with FLU .

FLU conc.	% of ergosterol			
	Resistant isolate		Susceptible isolate	
	White	Opaque	White	Opaque
Control	0.0102±0.01^{a}	0.0118±0.00^{c}	0.0184±0.00	0.0183±0.01
0.25xMIC$_{90}$	0.0082±0.00^{a}	0.0033±0.00^{ab}	--	--
0.5xMIC$_{90}$	--	0.0045±0.00^{b}	--	--
1xMIC$_{90}$	--	0.0016±0.00^{a}	--	--

Mean±SD, n=3. Across the same column, means with the same letter(s) are not significantly different at *P>0.05*, whereas those with different letters are significantly different at *P<0.05*.

Table 3 reveals that the wet weight decreased with increased TRB concentrations with more prominent reduction in the susceptible forms.

TABLE 3. Effect of different terbinafine concentrations on the wet weight of *C. albicans* isolates .

TRB conc.	Wet weight			
	Resistant		Susceptible	
	White	Opaque	White	Opaque
Control	0.5795±0.05^{b}	0.5364±0.04^{b}	0.3649±0.17^{b}	0.7519±0.00^{c}
0.25xMIC$_{90}$	0.2433±0.06^{a}	0.4299±0.08^{b}	0.1217±0.02^{a}	0.1287±0.03^{b}
0.5xMIC$_{90}$	0.2346±0.10^{a}	0.2854±0.06^{a}	0.1512±0.01^{a}	0.0994±0.01^{b}
1xMIC$_{90}$	0.1835±0.02^{a}	0.1891±0.03^{a}	0.0306±0.01^{a}	0.0478±0.02^{a}
Values of MIC$_{90}$ (µg ml^{-1})	450	56	5	0.6

In the FLU-susceptible opaque isolate, Fig. 1 Reveals that curve a (control; 0.0 FLU) gave the four-peaked curve characteristic for ergosterol biosynthesis. When concentration of FLU increased, the peaks disappeared; indicating inhibition of ergosterol biosynthesis due to inhibition in lanosterol 14α-

demethylase activity. At concentration 0.25xMIC_{90} (curve b), there was a minor peak; then all peaks were totally disappeared in treatment 0.5xMIC_{90} (curve c). The flat line d in treatment with 1xMIC_{90}, indicated undetectable ergosterol content.

In case of FLU-susceptible white isolate, Fig. 2 indicated that curve a (control; 0.0 FLU) gave the four-peaked curve typical to ergosterol biosynthesis. The peaks disappeared completely (flat lines b, c, and d) at all concentrations of FLU, indicating the blockage in ergosterol biosynthesis.

In case of FLU-resistant opaque isolate, Fig. 3 revealed that curves (a, b, c and d) gave the typical four-peaked curves characteristic to the ergosterol biosynthesis. This indicated that FLU at all concentrations did not affect ergosterol biosynthesis as well as the lanosterol 14α-demethylase activity.

In case of FLU-resistant white *C. albicans* isolate, Fig. 4 showed that the control (curve a, 0.0 FLU) and 0.25xMIC_{90} treatment (curve b) showed the four-peaked curves typically relevant to ergosterol biosynthesis. As concentration of FLU increased, the peaks disappeared. In treatment with 0.5xMIC_{90} two minor peaks were evident (curve c), then all peaks were totally abolished in a FLU concentration of 1xMIC_{90} which resulted in a flat line d. This dose-dependent decrease indicated partial inhibition of lanosterol 14α-demethylase.

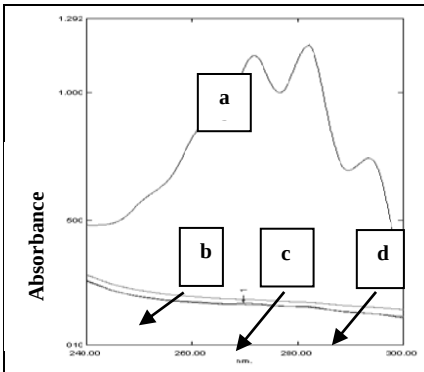

Fig.1. UV spectrophotometric ergosterol profiles of representative FLU-susceptible opaque *C. albicans* isolate.
Isolate was grown for 16 h in Sabouraud dextrose broth containing 0.0 of FLU (curve a), 0.25xMIC_{90} (curve b), 0.5xMIC_{90} (curve c) or 1xMIC_{90} (curve d) µg/ml FLU. Sterols were extracted from cell membranes, and spectral profiles between 240 and 300 nm were determined.

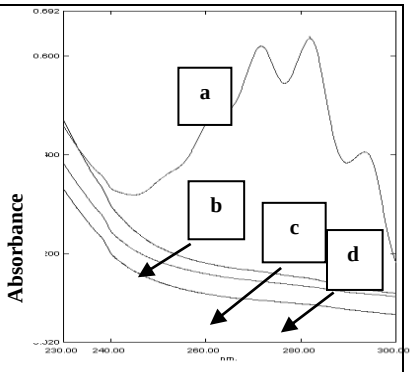

Fig.2. UV spectrophotometric ergosterol profiles of representative FLU-susceptible white *C. albicans* isolate.
Isolate was grown for 16 h in Sabouraud dextrose broth containing 0.0 of FLU (curve a), 0.25xMIC_{90} (curve b), 0.5xMIC_{90} (curve c) or 1xMIC_{90} (curve d) µg/ml of FLU, sterols were extracted from cell membranes, and spectral profiles between 240 and 300 nm were determined .

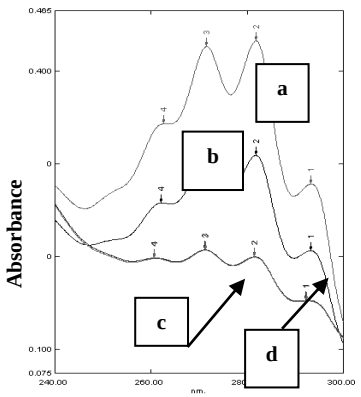

Fig. 3. UV spectrophotometric ergosterol profiles of representative FLU-resistant opaque *C. albicans* isolate. Isolate was grown for 16 h in Sabouraud dextrose broth containing 0.0 of FLU (curve a), 0.25xMIC$_{90}$ (curve b), 0.5xMIC$_{90}$ (curve c) or 1xMIC$_{90}$ (curve d) µg/ml FLU. Sterols were extracted from cell membranes, and spectral profiles between 240 and 300 nm were determined.

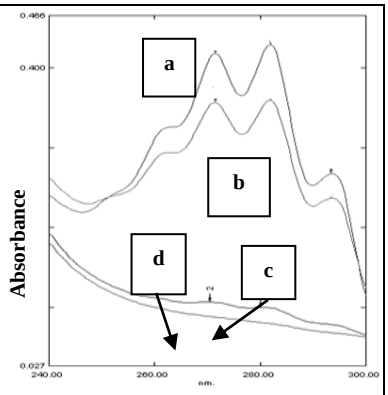

Fig. 4. UV spectrophotometric ergosterol profiles of representative FLU-resistant white *C. albicans* isolate. Isolate was grown for 16 h in Sabouraud dextrose broth containing 0.0 of FLU (curve a), 0.25xMIC$_{90}$ (curve b), 0.5xMIC$_{90}$ (curve c) or 1xMIC$_{90}$ (curve d) µg/ml of FLU, sterols were extracted from cell memberanes, and spectral profiles between 240 and 300 nm were determined.

Ergosterol percentage in the membrane of C. albicans isolates treated with the squalene inhibitror TRB

The data in Table 4 showed that TRB efficiently inhibit the ergosterol biosynthesis in *C. albicans* susceptible-isolates either white or opaque. In case of resistant isolates, the ergosterol biosynthesis appeared to be slightly affected by TRB at all concentrations. It is worth noting that TRB treatment, at 0.25 MIC$_{90}$, of resistant white isolate increased the ergosterol biosynthesis indicating its high resistance to the TRB antifungal drug.

TABLE 4. Percentage of ergosterol in *C. albicans* isolates treated with TRB .

TRB conc.	% of ergosterol			
	Resistant		Susceptible	
	White	Opaque	White	Opaque
Control	0.0256±0.00^{b}	0.0464±0.06^{a}	0.016967±0.02	0.0122±0.00
0.25xMIC$_{90}$	0.0313±0.00^{c}	0.0109±0.01^{a}	--	--
0.5xMIC$_{90}$	0.0192±0.00^{a}	0.0102±0.01^{a}	--	--
1xMIC$_{90}$	0.0183±0.00^{a}	0.0100±0.01^{a}	--	--

Mean±SD, n=3. Across the same column, means with the same letter are not significantly different at *P>0.05*, whereas those with different letters are significantly different at *P<0.05*.

In case of TRB-susceptible opaque isolate (Fig. 5) shows that the control; 0.0 TRB (curve a) displayed the four-peaked curve characteristic to ergosterol biosynthesis. As the concentration of TRB increased, the peaks disappeared. In treatment with 0.25xMIC_{90} (curve b) three minor peaks were evident, but with 0.5xMIC_{90} treatment (curve c) only one minor peak was apparent, then all peaks completely disappeared with concentration of 1xMIC_{90} (curve d). These results indicated that applying TRB with the mentioned doses resulted in high disturbances in squalene epoxidase activity which reflected decrease in ergosterol concentrations.

In comparing the susceptibilities of the isolate opaque-susceptible to either FLU or TRB it was found that at all FLU concentrations used, flat-lines corresponded to block in ergosterol content were evident (Fig. 1). While, in case of treatment with TRB; disturbances in ergosterol was dose dependent (Fig. 5). These results suggested that FLU is more effective than TRB against *C. albicans*.

In case of TRB-susceptible white isolate (Fig. 6) showed that only the control (curve a; 0.0 TRB) displayed the typical four-peaked profile for ergosterol biosynthesis. As the concentration of TRB increased, two minor peaks were observed as shown in curves b, c, and d for the treatments 0.25xMIC_{90}, 0.5xMIC_{90}, and 1xMIC_{90}, respectively. This indicated high disturbance and decrement in ergosterol content at all TRB concentrations compared to the control.

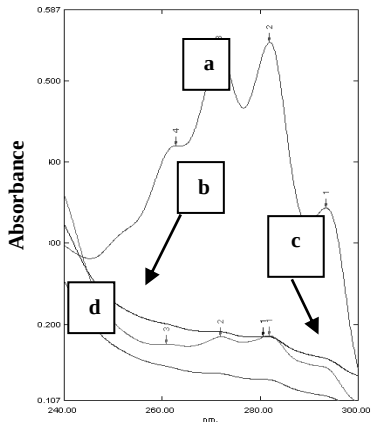

Fig. 5. UV spectrophotometric ergosterol profiles of representative TRB-susceptible opaque *C. albicans* isoate. Isolate was grown for 16 h in Sabouraud dextrose broth containing 0.0 of TRB (curve a), 0.25xMIC_{90} (curve b), 0.5xMIC_{90} (curve c) or 1xMIC_{90} (curve d) µg/ml TRB. Sterols were extracted from cell membranes, and spectral profiles between 240 and 300 nm were determined.

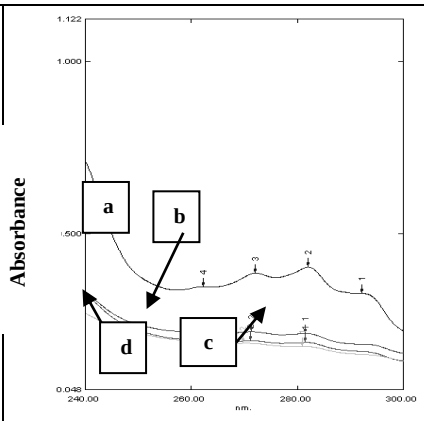

Fig. 6. UV spectrophotometric ergosterol profiles of representative TRB-susceptible white *C. albicans* isolate. Isolate was grown for 16 h in Sabouraud dextrose broth containing 0.0 of TRB (curve a), 0.25xMIC_{90} (curve b), 0.5xMIC_{90} (curve c) or 1xMIC_{90} (curve d) µg/ml of TRB, sterols were extracted from cell memberanes, and spectral profiles between 240 and 300 nm were determined.

In TRB-resistant opaque *C. albicans* isolate (Fig. 7) it was found that treatments with TRB, except 1xMIC_{90}, resulted in ergosterol biosynthesis characteristic four-peaked curves. As the concentration of TRB increased the height of peaks decreased. At 1xMIC_{90} treatment (curve d), only two peaks were displayed. This suggests the corruption in ergosterol biosynthesis as a result of partial inhibition in squalene epoxidase at highest concentration of TRB (1xMIC_{90}).

In case of TRB-resistant white *C. albicans* isolate (Fig. 8) revealed that all concentrations of TRB had no effect on ergosterol biosynthesis and typical four-peaked curves characteristic to ergosterol content were detected indicating that squalene epoxidase activity in this starin was not inhibited by TRB.

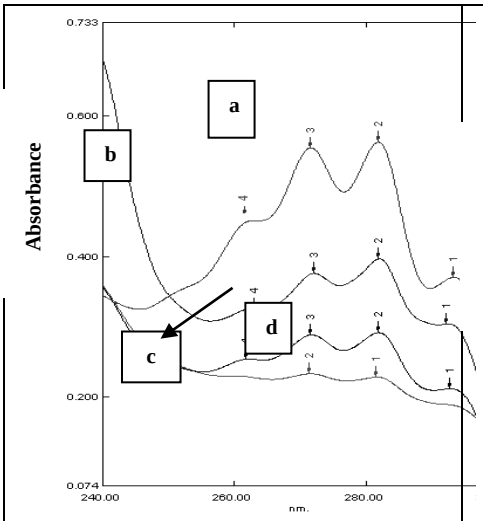

Fig. 7. UV spectrophotometric ergosterol profiles of representative TRB-resistant opaque *C. albicans* isolate. Isolate was grown for 16 h in Sabouraud dextrose broth containing 0.0 of TRB (curve a), 0.25xMIC_{90} (curve b), 0.5xMIC_{90} (curve c) or 1xMIC_{90} (curve d) µg/ml TRB. Sterols were extracted from cell membranes, and spectral profiles between 240 and 300 nm were determined.

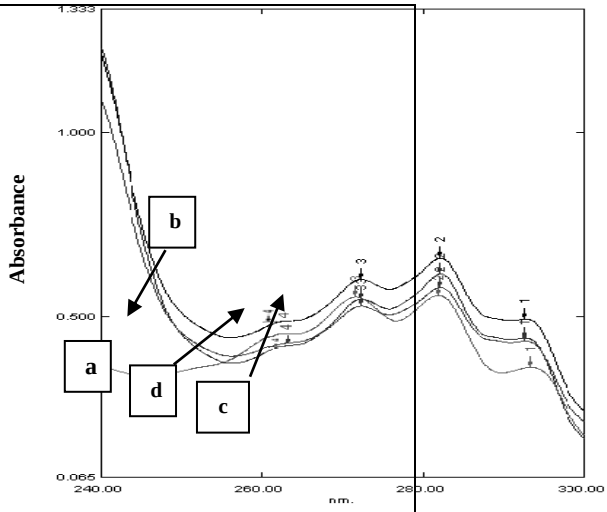

Fig. 8. UV spectrophotometric ergosterol profiles of representative TRB-resistant white *C. albicans* isolate. Isolate was grown for 16 h in Sabouraud dextrose broth containing 0.0 of TRB (curve a), 0.25xMIC_{90} (curve b), 0.5xMIC_{90} (curve c) or 1xMIC_{90} (curve d) µg/ml of TRB, sterols were extracted from cell membranes, and spectral profiles between 240 and 300 nm were determined.

Discussion

Ergosterol, a membrane sterol, unique to fungi, is necessary for growth and normal membrane function in fungal cells. Besides its role in memberane fluidity, asymmetry and integrity, it contributes to proper function in membrane bound enzymes (Lupetti *et al*., 2002 and Lopes *et al*., 2013)). Fluconazole (azole compound) targets the ergosterol biosynthesis enzyme (Erg11) lanosterol 14α-demethylase and are widely applied class of antifungal agents because of their wide spectrum of activity (Onyewu, *et al*., 2003). Terbinafine is an antifungal belonging to the allylamine class of synthetic antifungal agents (Petranyi, *et al*., 1984). It inhibits another biosynthesis enzyme (Erg1) squalene epoxidase.

A unique and ergosterol indicative spectral absorption four-peaked curve was produced between 240-300 nm. The sterol quantitation method (SQM) based on measuring of ergosterol and dehydroergosterol (late sterol pathway intermediate) contents were used (Arthington-Skaggs *et al*., 1999; Aijaz *et al*., 2010 and Phin, 2012).

In the present study, SQM was used to evaluate the effect of antifungal drugs (FLU and TRB) on *C. albicans* isolates. This method discriminates between the activity of lanosterol 14α-demethylase and squalene epoxidase under different treatments. It was found that a typical 4-peaked curve was detected in absence of antifungals (controls). FLU and TRB inhibited the ergosterol biosynthesis due to inhibition of ergosterol biosynthetic enzymes in susceptible isolates of *C. albicans* (white and opaque forms). TRB partially inhibited ergosterol biosynthetic enzyme; squalene epoxidase in the resistant white and opaque isolates in a dose-dependent manner. FLU inhibited the ergosterol biosynthetic enzyme, lanosterol 14α-demethylase in resistant white isolates, while, it partially inhibited the enzyme activity in the opaque resistant isolates.

Arthington-Skaggs *et al*. (1999) used SQM to test 38 isolates of susceptible, dose-dependent susceptible and resistant *C. albicans* against FLU. 18 susceptible isolates showed significant reduction in ergosterol content, in dose-dependent manner. They concluded that SQM discriminated between resistant, highly resistant and susceptible isolates indicating that it may predict clinical outcome more accurately.

Azole drugs inhibit ergosterol biosynthesis in fungal cells by binding to their cytochrome P-450 sterol 14α-demethylase (Erg11) (Vanden Bossche *et al*., 1983; Song *et al*., 2004 and Warrilow *et al*., 2012). Voriconazole is 1.6 and 160-fold more active than FLU in inhibiting ergosterol P-450 dependent 14 α - demethylase in *C. albicans* and *Aspergillus fumigatus* lysate, respectively. Three general mechanisms of azole resistance have been described by *C. albicans*: i] alteration of the target enzyme 14α-demythelase (Lamb *et al*., 1997; White, 1997; Sanglard *et al*., 1998), ii] decreasing alteration in the drug uptake or efflux (Parkinson, 1996; Sanglard *et al*., 1995), iii] deficiency of sterol desaturase which suppresses the accumulation of toxic intermediates (Geber *et al*., 1995; Kelly *et al*., 1997).

Onyewu *et al*. (2003) claimed that azoles target ergosterol biosynthetic enzyme lanosterol 14α-demethylase and are a widely applied class of antifungal agents because of their broad therapeutic window, wide spectrum of activity and low toxicity. They established that drugs targeting other enzymes in the ergosterol biosynthetic pathway (terbinafine and fenpropimorph) also exhibit dramatic synergistic antifungal activity against wild-type *C. albicans* when used in combination.

C. albicans, growth inhibition with TRB appears to result from the ergosterol deficiency resulting from the non competitive inhibition in squalene epoxidase (Erg1), an essential enzyme in ergosterol biosynthesis. The fungal death is caused by the accumulation of high levels of intracellular squalene, probably in combination with ergosterol deficiency as a result of terbinafine-induced inhibition of squalene peroxidase. He also reported that ergosterol deficiency is not the primary cause of death (the azoles inhibit ergosterol biosynthesis that result in ergosterol deficiency but are primary fungistatic in action) this suggested that squalene accumulation rather than ergosterol deficiency is the key to the fungicidal action of terbinafine (Ryder, 1992).

Terbinafine acts by blocking fungal ergosterol biosynthesis via inhibition of squalene epoxidase (Ryder *et al*., 1984; Graminha *et al*., 2004 and Shahnaz *et al*., 2013). It was reported that the antifungal activity of terbinafine is based on the inhibition of fungal ergosterol at the point of squalene epoxidation resulting in accumulation of intracellular squalene which is fungicidal to dermatophytic yeasts (Ryder and Troke, 1981; Paltauf *et al*., 1982; Ryder, 1985; Ryder and Dupont, 1985; Ryder, 1992). It is suggested that inhibition of ergosterol biosynthesis may render the *C. albicans* memberanes susceptible to further damage, indicating direct damage of squalene epoxidase with inhibitors such as terbinafine and noftafine (Georgopapadakou and Bertasso, 1992).

Allylamine and azoles are synthetic antifungal agents which inhibits ergosterol biosynthesis. Allylamines inhibit ergosterol biosynthetic pathway at the level of squalene epoxidase causing the accumulation of squalene (Melloni *et al*., 1974; Bianchi *et al*., 1977; Georgopoulos *et al*., 1981; Paltauf *et al*., 1982; Ryder and Troke, 1981; Morita and Nozawa, 1985; Ryder *et al*., 1986) These compounds are highly fungicidal against dermatophytes (Ganzinger *et al*., 1986; Goodfield *et al*., 1989). The azole antifungal inhibits ergosterol biosynthesis at the level of C14 demethylase and is clinically useful as broad-spectrum antifungal agents (Berg *et al*., 1986; Fromtling, 1988). Terbinafine have little or no activity against *C. albicans* in yeast form, although filamentous form is susceptible (Schaude *et al*., 1987).

Docking studies followed by molecular dynamic simulation and quantum interaction energy calculations resulted in identification of the terbinafine-

squalene mode of action. The terbinafine lipophitic moiety is located vertically inside the squalene epoxidase binding pocket with the test - butyl group oriented toward its center. Such a position result in conformational changes in squalene epoxidase and prevents the natural substrate from being able to bind to the enzyme's active site (Nowosielski *et al*., 2011)

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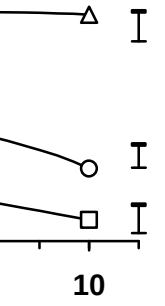
(*Received* 18/11/2013;
accepted 3/12/2013)



تأثير الفلوكونازول والتيربينافين على أنشطة الانوستيرول 14 الفا دى ميثيليز و الاسكوالين ايبوكسيد فى المسار التصنيعى للايرجوستيرول

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لقد تم دراسة أنشطة إنزيمات التصنيع الحيوى للايرجوستيرول واللانوستيرول 14 الفا دى ميثيليز والاسكوالين ايبوكسيدو ذلك بقياس محتوى الإرجوستيرول الموجود فى الغشاء الخلوى فى الكانديدا البيكانز تحت تاثير المعالجة بمضادات الفطريات المختلفة بالمقارنة مع المحكم. و قد تم قياس الإرجوستيرول باستخدام الطريقة الكمية الطيفية للستيرول. و قد تبين ان الفلوكونازول و التيربينافين قد ثبطا نشاط كلا من الانزيمين وتوقف التصنيع الحيوي للارجوستيرول تماما في العزلات الحساسة, بينما سببا تثبيطا جزئيا في العزلات المقاومة من الكانديدا البيكانز. و كان الخليط المشترك من الفلوكونازول والتربينافين قد عضد بعضهما البعض على انزيمات التصنيع الحيوى للايرجوستيرول.