Study of Trailer Growth in *Candida albicans* Associated with Azoles –Drug Resistance.

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Abstract:
Background:*Candida albicans* is considered as one of the normal flora that habitat human body and has a virulence capability to cause an opportunistic mycoses,especially in immune-compromised individuals.
Objective:To evaluate the emergence of resistance to azole antifungals in *C.albicans* associated with trailing growth phenomena.
Methods: Different isolates of *C.albicans* were identified and tested against azole agents using disk diffusion and microdilution methods to test the antifungals susceptibility profile and determine the resistant isolates depending on the presence of trailer growth phenomena in media after 24-72 hours of incubation .
Results:The antifungals susceptibility profile showed the emergence of trailing growth in *C.albicans* grown in Sabouraud dextrose agar after 24-48 hours of incubation when tested with azole agents(flucocanozole and ketoconazole) and most of the sensitive and intermediate isolates revealed a trailing growth phenomena.
Conclusion:The present study proved the capability of*C.albicans* to resist the azole antifungals even the sensitive isolates when tested toward antifungal drugs .
Keywords:*Candida albicans* ; Trailer growth ; Azole antifungals,Drug resistance.

Introduction

Candida albicans is a dimorphic fungus that exists as a commensal of humans. It colonizes mucosal surfaces of the oral and vaginal cavities and the digestive tract and is able to cause a variety of infection, depending on the nature of the underlying host defect (1). The past decade has witnessed a significant increase in the prevalence of resistance toantibacterial and antifungal agents. Resistance to these agents has important implications for morbidity, mortality and health care cost in the community (2). The increase of predisposing factors for infection with *Candida* spp., especially in the immunocompromised patient and appearance of mutant strains capable to resist the antifungal therapy in addition to the little attention to the clinical significance of antifungal cross – resistance,. For these reasons, this study was conduct.

The azoles groups of antifungal agents have been find to consist of the imidazole and the triazoles. (3).Traditionally agents used in this group include clotrimazole, miconazole, fluconazole, itraconazole, and ketoconazole.. All of these agents interact with enzyme involved in the synthesis of ergosterol from sequalen (4). At high concentrations, the azoles may also interact directly with lipids in the membranes and emergence of resistance to azole is an increasing problem (5).

Resistance has been defined as persistence or progression of an infection despite appropriate antimicrobial therapy. The *in vitro*resistance of an isolate might be describe as either primary or

secondary(6). Organism that is resistant to an agent prior to exposure was described as having primary or intrinsic resistance ,Secondary resistance develops in response to exposure to an antimicrobial agent . Both primary and secondary resistances to antifungal agents have been observed (9).

As described previousely, clinical isolates that are resistant to one azole are frequently cross-resistant to other azole drugs and can be cross-resistant to polyenes as well. With the current understanding of molecular mechanisms, predictions can be made about the molecular mechanisms that are associated with cross-resistance. Overxpression of the *CDR* genes is a common mechanism of resistance, and it appears that the *CDR*

Genes render a cell resistant to many different azoles while over expression of *MDR1* appears to be specific for fluconazole and is not associated with cross-resistance (10). It is not clear if specific point mutations in *ERG11* will always be associated with cross-resistance. Similarly, there has been insufficient analysis to determine if alterations in other enzymes in the ergosterol pathway will result in cross-resistance (10).

In a previous study, Odds (11) noticed that resistance of a *Candida* spp. to one azole derivative implies cross-resistance to the other azole antifungal agents.Trailing growth is one of the tool expressed by *C.albicans* to resist the antifungal agents when treated with high concentrations of azole agents after 24 hours of growth which make a difficulty in determination of minimal inhibitory concentrations(MICs).Different studies referred to trailing growth in Candida species that associated with azole agents especially flucoconazole and ketoconazole(12,13).Marr et al.(14) established the trailing growth as one feature of the resistance exhibited by *C.albicans* .For these reasons, thepresent study aimed to evaluate the relationship between the trailing growth phenomena of *C.albicans* and the antifungal susceptibility profile toward selected antifungal agents.

Materials and Methods:

Collection,isolation and identification of *Candida albicans*: A total of 120 swabs were obtained from patients suffering from candidiasis. Specimens were taken using sterile swabs, and then transported to the laboratory for diagnosis.The collected specimens are inoculated and streaked onto sabouraud's dextroseagar,incubated at 37 C for 48 hrs.A portion of colonies are taken from the culture and placed on a slide with Gram stain, teased apart and covered with a cover slip and tested under the light microscope to determine the morphology of the yeast cells .After this, a single colony were seeded on CHROMagar Candida and incubated at 37ºC for 48 h. The CHROMagar Candida allows selective yeast isolation, identifying colonies of *C.albicans* by morphology and color reaction. The isolates were identified according to the manufacturer's instructions, which differentiated *C. albicans* as green colonies, *C. tropicalis* as metallic blue colonies, *C. krusei* colonies as showing pink color and rough aspect, and the other species as developing colonies from white to mauve .

The API- Yeast- IDENT system is used for the identification of the yeasts isolates. The tests are carried out as follows : A suitable amount of fungal growth is emulsified into 2 ml of sterile 0.85 % sodium chloride using a sterile wooden application stick. The emulsion

is then inoculated into the cupules of different testes as soon as possible. The strip is placed within distilled water, and then incubated at 37C˚ for (18 – 24) hours. Readings of the results are then carried out according to the color changes and compared to the standards supplied by the manufacturer. Forty isolates of Candida albicans were isolated and identified based on morphological (Gram s staining), cultural(CHROMagar morphotyping), biochemical(Api20 testing) after collecting from patients suffering from candidiasis.

Antifungal Susceptibility Test:

Forty isolates belong to the species of *C. albicans* were tested for antifungal susceptibility. Tube containing 5 ml of sabouraud's dextrose broth was inoculated with two loopfuls of a yeast colony to be tested from (1-2 days SDA) old cultures. Tubes were incubated overnight at 37 C˚, then diluted by 1:100 with sterilized distilled water then shacked vigorously. The suspension was adjusted into 10^5 cell/ml compared with MacFarland standard solution (0.5) (14). The quality control for antifungal susceptibility test testing was performed by using *Candida albicans* ATCC 10231as reference strain. Emmons modification sabouraud's agar (ESDA) (2% dextrose) was used for in vitro susceptibility test. The pH of the medium was adjusted to 6.8-7.0. The antifungal: fluconazole ,itraconazole ,clotrimazole and ketoconazole were prepared in an initial concentration of 10000µg/ml by dissolving 50mg of the agent in 5ml of dimethyl sulphoxide (DMSO) in a clean sterile screw-capped glass vials. The stock solution was kept at -20C˚for further use .

Batches of small discs number 100 of absorbent paper (6.5 mm diameter) were dispensed in screw-capped vials, sterilized by autoclaving, and left in the electric oven for 30 minutes at 40 ˚C to be dried. Final concentration of the (1000 µg/ml) was prepared from the original stock solutions of each antifungal. One ml of final concentration was added to each vial containing 100 discs to obtain a concentration of 10 µg /disc. These discs were stored in wet condition in screw-capped vials tightly screwed and kept at -20 ˚C until used. These were prepared according to Okeke and Gugnani(15).

1- Disc Diffusion Method:

This methods was carried out according to (20), add 0.2 ml of yeast inocula suspension prepared in steps B. on the surface of the (ESDA) plates, the inocula were evenly spread with sterilized L-shaped spreader. The inverted inoculated plates were left on the bench for one hour. The antifungal discs (10µg /disc) were placed on the surface of the medium and left in the refrigerator for 1-3 hours prediffusion. At the same time, growth controls without antifungal discs of all strains on ESDA were prepared. The inoculated plates incubated at 30˚C and the assay was recorded after 24-48 hours of incubation. The inhibition zones of were expressed as diameter of the clear zones around the antifungal discs measured in millimeters. Duplicate plates were used. The diameter of inhibition zone for individual antifungal agent was measured according to standard values of CLSI,M44-A2(16).

2-Determination of Minimal Inhibitory Concentrations (MICs)

The minimal inhibition concentrations (MICs) of the test agents were established using agar dilution method, described by McGinnis(14) :Two ml from the stock agent solution

(10000µg/ml) was pipetted into 18ml of sabouraud's dextrose broth to obtain a final consternation of (1000µg/ml). This was the working agent solution and numbered as a tube No.1. A serial of antifungal agents solution were prepared from the original working drug solution. It was added 3 ml from each delution of SDB to two tubes. Each on of these tubes contains 27ml of SDA media preserved in water bath of 52-50 ˚C and mix it well. Then after that. Pour each 30 ml of those tubes in a glass plate and leave it to cool. A yeast inoculums of 0.05 ml was poured on the surface of each Petri dish prepared previously. The inoculated plates were left undisturbed to permit the inocula to be absorbed in to the medium surface. The inoculated plates were incubated at 37 C˚ until the macroscopic growth appeared on the control plates.
the MIC for azoles drug was recorded as the lowest consternation that reduced (80%) of yeast growth.

Two groups of *C. albicans* were tested. The first group consisted of previously defined five clinical isolates for which nystatin MICs was relatively high (≥ 64µg/ml). The second group consists of five clinical isolate for which fluconazole was relatively high (≥ 8µg/ml) according to standard values of CLSI,M27-A2(16).

Statistical Analysis: Chi – square test and analysis of variance were used for statistical analysis of the data (17).

Results and Discussion:

Isolation and Identification:

Phenotypic characteristics of *Candida* species and other yeasts:

Microscopic examination of yeast isolates appeared mostly spherical to oval or elongated oval or cylindrical and positive for Gram's stain .The presence of clusters is relatively sensitive and highly specific for *C. albicans*, and application of these results could provide useful preliminary information for guiding diagnosis(18).The suspected colonies of *Candida* sp. on the Sabouraud's dextrose agar are characterized by cream colored, and smooth, at 30 C◦ (Figure 1) and developed to wrinkle whitish creamy colonies after further incubation for 7 days and odor of yeast like.These characteristics were identical to those described by Bodey(19),and on chromagar media , the results showed green colonies in chromogenic medium, indicating *C. albicans*.

Moreover, *C. albicans* colonies can grow sharing a variation of green color, ranging from light-green to dark green, depending on growth density and incubation period often with the periphery of the colonies having a color distinctly different from that of the rest of the colony (18). However, Mesa *et al.* (19) observed that among 55 strains identified as *C. albicans* tested by CHROMagar medium.

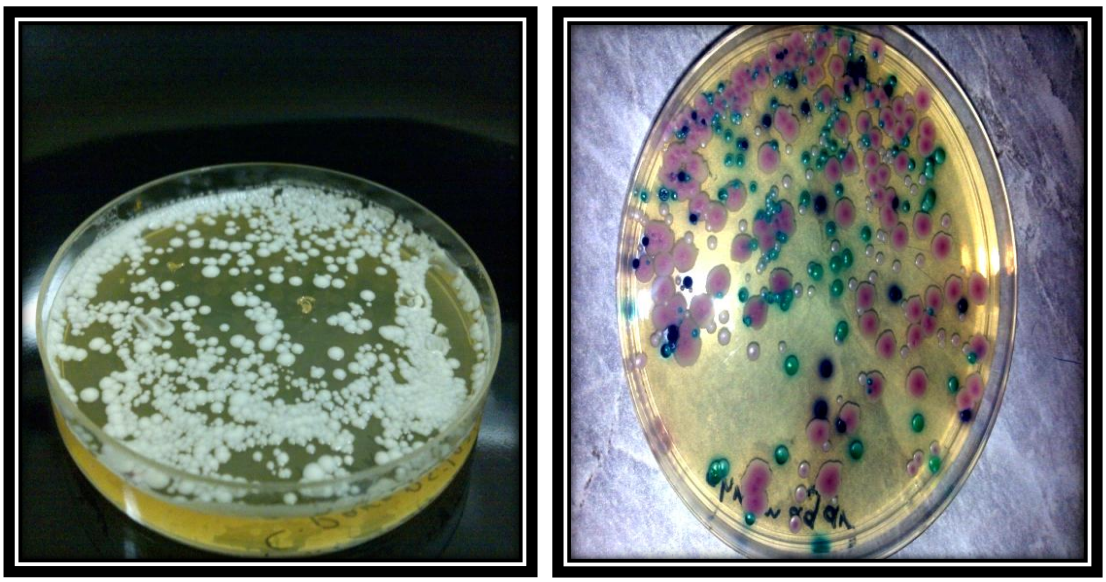


Figure (1): *Candida albicans* isolates:A)on Sabouraud's dextrose agar showing round, smooth and creamy colonies.B)on **CHROMagar Candida showing green color colonies .**

To more confirmation, the identification of the isolates was done by the use of API *Candida* kit because it gives results that are more accurate in the diagnosis of the species (Figure 2).

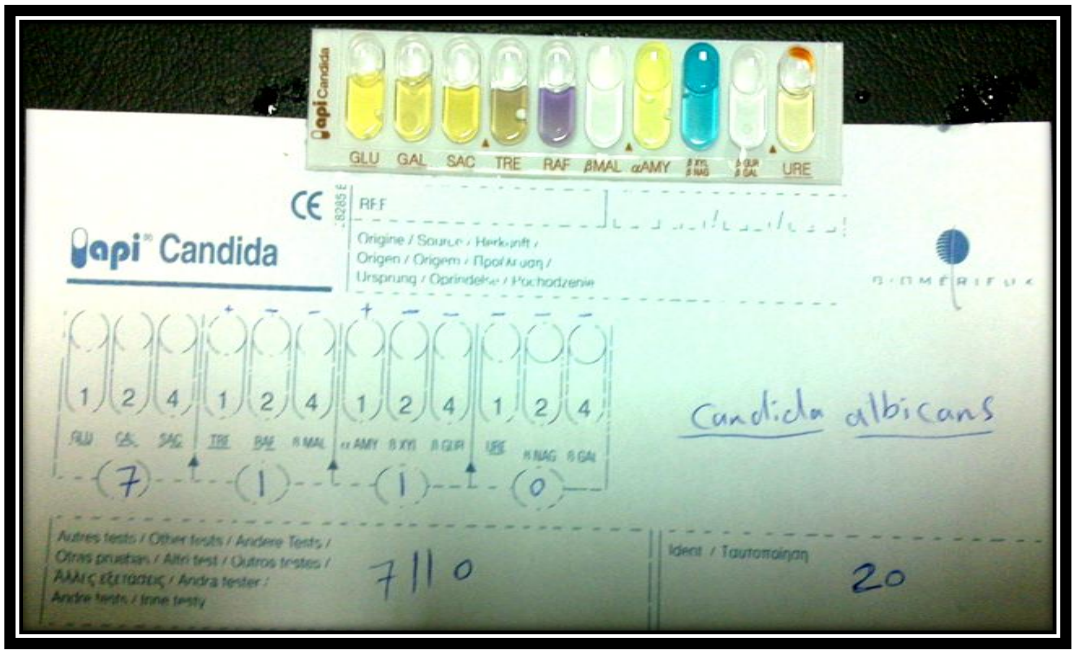


Figure (2): A standard Profile Identification (Api) Candida System..

Antifungal susceptibility test :

The results showed that the *in vitro* susceptibility of the isolates of *Candida* towards available antifungal agents. Data were reported as MIC ranged and as the concentration of each antifungal agent necessary to inhibit 50% (MIC_{50}) and 90% (MIC_{90}) of isolates(Table 1).

The interpretation of fluconazole and itraconazole susceptibility tests is often complicated by the occurrence of growth. This phenomenon will influence the outcome of the test depending on whether the incubation period is 24 or 48 hr. Although the CLSI method currently recommends a 48 hrs incubation period, there is mounting evidence that this time period may lead to an overestimation of MIC_s and that 24 hrs results correlate better with clinical outcome (20). Using the CLSI reference broth microdilution method with spectrophotometric reading, important differences due to growth were observed between results obtained at 24 hrs and those obtained at 48 hr especially with *C. albicans*.In the present study, it is considered that the incubation time for 24 hours since the readings after 48 hrs were unacceptably high, a fact that has been reported and a similar solution by reading the results at 24 hrs. Many investigators have suggested that determination of MIC_s after 24 hrs of incubation results in better match with *in vitro* response and avoids the incorrectly high MIC_s readings. The emergence of azole drug-resistant strains due to the acquisition of resistance by previously susceptible strains(21).

Table (1): Susceptibility pattern of *Candida albicans* against antifungals used in the study.

Yeast isolates	Fluconazole			Itraconazole			Ketoconazole			Total
	S <8 µg/ml	SDD 16-32	R >64 µg/ml	S <0.125 µg/ml	SDD 0.25-0.5	R >1 µg/ml	S <1 µg/ml	SDD 1-2 µg/ml	R >2 µg/ml	
C.albicans	11	2	0	9	4	0	7	6	0	13
***C.albicans* ATCC 10231**	2	2	0	3	1	0	2	2	0	4

S : Susceptible , R : Resistant, SDD: Susceptible dose dependant.

The results reaveled that C.albicans was sensitive to azole agents after 24 hrs.while when incubated continuously for 48 hrs,it noticed the appearance the zone of growth inhibition and the growth rate was weak and gave moderate resistance to the tested antifungals,this phenomena is called trailing growth.Table (2) shows the percent of resistance to azoles (10 mg/l.) which reached 38.9% with zone of growth inhibition ≤ 26 mm, in addition to that ,the percent of sensitive isolates were 5.6% with zone of growth inhibition ≥ 29 mm.Also, the present study proved that most isolates gave trailing growth in the presence of of fluconazole at percent of 55.6% and the fig .(2) showed this phenomena in the presence of tested antifungals with significance differences($p \leq 0.05$).

Table(2):Antifungal susceptibility percent of *C.albicans* against some antifungal agents(N=36).

antifungal	Concentration (mg/l.)	No.of trailer growth isolates	%	No. of resistant isolates	%	No. of sensitive isolates	%
Fluconazole	10	20	55.6	14	38.9	2	5.7
Itraconazle	10	22	61.1	9	25	5	13.9
Ketoconazole	10	14	38.9	10	27.8	12	33.3
Clotrimazole	10	3	8.3	2	5.6	31	86.1
X^2 tab.=12.59				X^2 cal.=64.09		significant at p≤ 0.05	

Table(3),shows the minimal inhibitory concentrations(MICs) values for fluconazole(8 , 16, 32, 64 ug/ml) ,where the most growth inhibition of C. albicans was after 24 hrs of incubation.The sensitive isolates appeared at concentrations(64, 32, 16, 8, ug/ml) ,where as the resistant isolates appeared at concentrations(0.25, 0.5, 1, 2 ug/ml).

Regarding the results of incubation of C.albicans with fluconazole(16, 32, 64 ug/ml) after 48 hrs ,the resistance increased ,while at concentrations(8, 16, 32, 64 ug/ml) the isolates were sensitive for fucoconazle and resistance at 0.25 and 0.5 ug/ml.It was noticed that trailing growth takeplace when MICs≤8 ug/ml after 24 hrs of incubation. while after 48 hrs of continuous incubation,the tailing growth takeplace when MICs ≤ 64 ug/ml for fluconazole.The statistical analysis showed a significance differences (p≤ 0.05) among tested isolates(22).

Table(3):Number and percent of trailer growth isolates of C.albicans treated with fluconazole after 24-48 hrs of incubation(n=30).

MIC(ug/ml)	Incubation after 24 hrs.						Incubation after 48 hrs.					
	R	%	T	%	S	%	R	%	T	%	S	%
64	6	20	3	10	21	70	14	46.7	15	50	1	3.3
32	7	23.3	5	16.7	18	60	16	53.3	14	46.7	0	0
16	9	30	4	13.3	17	56.7	17	56.7	12	40	1	3.3
8	9	30	6	20	15	50	18	60	11	36.7	1	3.3
4	10	33.3	6	20	14	46.7	21	70	9	30	0	0
2	12	40	8	26.7	10	33.3	21	70	9	30	0	0
1	15	50	7	23.3	8	26.7	22	73.3	8	26.7	0	0
0.5	13	43.3	8	26.7	9	30	24	80	6	20	0	0

0.25	16	53.3	8	26.7	6	20	25	83.3	5	16.7	0	0
0.125	15	50	8	26.7	7	23.3	26	86.7	4	13.3	0	0
S:sensitive; T:traiter growth ;R:resistant X^2 tab.=28.87 X^2 cal.=34.14 significant (p≤ 0.05)						X^2 tab.=28.87 X^2 cal.=27.52;Non significant (p≥ 0.05)						

In respect with ketoconazole , the results showed that MICs values(2,4,8,16 ug/ml) were the most inhibition values after 24 hrs of incubation for sensitive isolates of C. albicans, while the resistant isolates gave the MICs values 0.03,0.06,0.125,0.25 ug/ml(Table 4). These resistance were increased after 48 hrs of incubation C.albicans with ketoconazole at concentrations 0.5,1,2,4 ug/ml.This may be due to the phenomena of trailing growth which takeplace with continuous incubation of isolates at high concentrations of this antifungal in culture media(23).In addition to that ,trailing growth takeplace when MICs ≤ 2 ug/ml after 24 hrs of incubation, while MICs were ≤ 8 ug/ml after 48 hrs with significance differences(p≤0.05).

Table(4):Number and percent of trailer growth isolates of C.albicans treated with ketoconazole after 24-48 hrs of incubation(n=30).

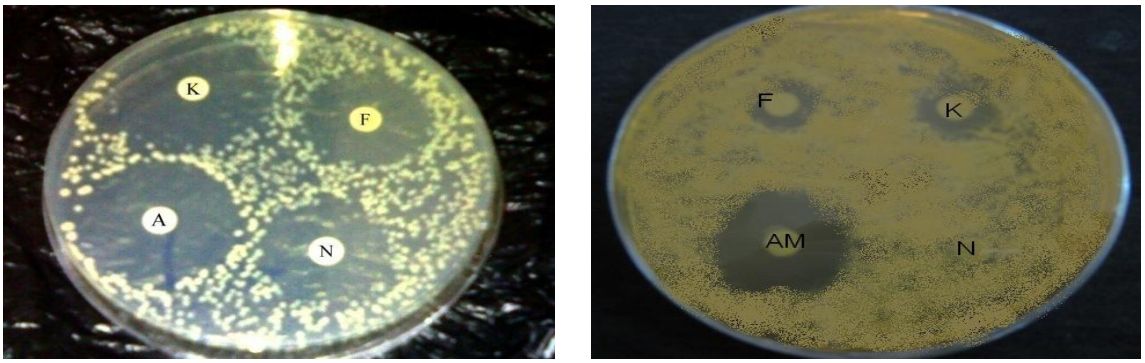
MIC(ug/ml)	Incubation after 24 hrs.						Incubation after 48 hrs.					
	R	%	T	%	S	%	R	%	T	%	S	%
16	3	10	0	0	27	90	4	13.3	1	3.3	25	83.3
8	2	6.7	0	0	28	93.3	5	16.7	2	6.7	23	76.6
4	4	13.3	2	6.7	24	80	8	26.7	6	20	16	53.3
2	7	23.3	3	10	20	66.7	10	33.3	7	23.3	13	43.3
1	10	33.3	6	20	14	46.7	11	36.7	13	43.3	6	20
0.5	10	33.3	8	26.7	12	40	12	40	11	36.7	7	23.3
0.25	11	36.7	9	30	10	33.3	14	46.7	12	40	4	13.3
0.125	12	40	10	33.3	8	26.6	15	50	12	40	3	10
0.06	12	40	9	30	9	30	15	50	11	36.7	4	13.3
0.03	11	36.7	8	26.7	11	36.6	16	53.3	12	40	2	6.7
S:sensitive; T:traiter growth ;R:resistant X^2 tab.=28.87 X^2 cal.=95.73 significant (p≤ 0.05)						X^2 tab.=28.87 X^2 cal.=78.03; significant (p≤ 0.05)						

Table (5),Figure(3) shows the results o C.albicans growth in media at different concentrations of MIC which revealed trailing growth previously and grown again on Sabouraud dextrose agar for 24,48 and 96 hrs of incubation at 37 C where the rates of growth were varied in the presence of antifungal agents.

Locally , there are no studies refer to test the ability of incomplete inhibition isolates or trailer growth in the presence of azole agents.This may due to the difficulty of determination the end point of growth for tested isolates, where the appearance of pseudo or false resistance may don't related with growth changes, but its related with another conditions that originated genetically to resist antifungal agents.(21).These chanes are related to some genes that encoded to proteins and enzymes regarding to their resistance to antifungals and how dimensh it such as efflux pump systems which then determine the ability of microorganisms to grow again or not.In addition to ability the fungus to continuous biosynthesis of cell membrane through the over-expression or high regulation of these genes in the presence or absence of antifungals(**24**,25).

Table (5):Grade of trailing growth of C.albicans that subculture on SDA media grown at different MICs and incubation time.

antifungal	MICs(mg/ml)	Growth after 24 hrs.	Growth after 48 hrs.	Growth after 72 hrs.
Fluconazole	64	+	+1	+1
	16	+1	+1	+4
	32	+	+1	+3
	0.125	+2	+3	+4
	64	_	+1	+3
	32	_	+1	+3
Ketoconazole	4	_	+1	+3
	0.25	+	+1	+4
	4	_	+1	+3
	8	_	_	+
	0.03	+	+3	+4
	8	_	_	+
(-):No growth; (+): very weak growth;(+1):weak growth;(+2):moderate growth; (+3):heavy growth:(+4) : very heavy growth				



1) 2)

Figure (3): zone of growth inhibition of *C. albicans* toward four antifungal agents:1) sensitive ; 2) resistant(Trailing growth). (A= amphotericin B, N=nystatin as control ; F=fluconazole, K= ketoconazole as test).

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