Evaluation of the Use of Bioactive Dye for Rapid Anti-dermatophytes Susceptibility Testing

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Background and study aim: Fungal infections affecting the epidermis, hair, and nails are mostly caused by dermatophytes. They can infect immunocompromised and immune-competent patients as well. The study aims to evaluate the use of Resazurin in anti-dermatophytes susceptibility testing.

Patients and Methods: Patients with dermatophytic infection, attending dermatology outpatient clinic in Ain Shams University Hospital were examined and 50 clinical specimens were collected. All clinical specimens were cultured on Sabarouds dextrose agar (SDA). To improve sporulation, isolates from SDA were subcultured on Potato Dextrose Agar (PDA) and incubated at 28°C for 7 days. The colonies were collected in sterile saline, to adjust fungal suspension to 0.5 McFarland. The antifungal susceptibility was made using two methods: disc diffusion (DD) and broth microdilution (BMD) with and without Resazurin.

Results: Antifungal susceptibility results were available after 24 hours by broth microdilution with bioactive dye (Resazurin). While antifungal susceptibility by broth microdilution method without bioactive dye results was available after 48-72 hours and disc diffusion results were available after an incubation period of 7 to 15 days. In addition, using bioactive dye (Resazurin) method was easier in visual interpretation. There were a perfect agreements between the antifungal susceptibility testing of Itraconazole (kappa 1.00), Terbinafine (kappa 0.947) and Fluconazole (kappa 0.878) by disk diffusion method and Broth micro-dilution method without and with bioactive dye (Resazurin).

Conclusion: Usage of bioactive dyes such as Resazurin can help in providing rapid and accurate antifungal susceptibility results for better patient care.

INTRODUCTION

The frequency of fungal infections has risen dramatically, particularly among persons whose immune systems have been weakened by age, HIV infection, organ reception, or cancer treatment **[1].** Dermatophytes, which are responsible for the bulk of fungal infections affecting the hair, nails, and skin can infect immunocompetent persons as well **[2].** Many antifungal medicines are used to treat dermatophytosis **[3].** Resistance to antifungal medicines has developed as a result of the concomitant growth in fungal infections and increased usage of antifungal treatments, typically for lengthy periods **[4].**

Antifungal susceptibility tests in vitro are currently mostly utilized for epidemiological studies. Antifungal susceptibility testing is proposed to help doctors choose the best antifungal medications for a specific fungal illness. To establish the antifungal activity against isolates, it is also necessary to test dermatophytes utilizing a quick, standardized, simple, and repeatable in-vitro method **[4]**. Due to the long incubation period of susceptibility plates, the antifungal susceptibility results take a lot of time. This delay has an impact on patient treatment and, as a result, the prognosis. Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a nontoxic, cell-permeable, and redox-sensitive phenoxazine dye. Because it

is irreversibly reduced to the pink-colored and highly fluorescent resorufin (7-Hydroxy-3H-phenoxazin-3-one), Resazurin is utilised in microbiological, cellular, and enzymatic tests. Resorufin can be identified by looking at its pink color or by fluorimetry **[5].** This study aims to evaluate the use of bioactive dye like Resazurin in antifungal susceptibility testing for obtaining rapid and accurate results.

PATIENTS AND METHODS

Study design: A cohort study

Study settings: Dermatology outpatient clinic of Ain Shams University Hospital

Collection of specimens: Nail, hair, and skin scrapings were collected in sterile disposable 9 mm petri dishes.

Patients

Study patients: Patients suffering from dermatophytic infection of nail, hair, and skin.

Sample size: All patients suffering from dermatophytic infection of nail, hair, and skin attending dermatology outpatient clinic of Ain Shams University Hospital in the period from September and October 2019.

All Patients are examined for dermatophytic lesions in hair, skin, and nails.

Clinical signs of the lesions were classified as **Tinea capitis;** black dots or scales and easily epilated hair, **Tinea circinata;** circinate lesions with scales and active edge on skin, **Onychomycosis;** onycholysis, dystrophy, subungual hyperkeratosis, discoloration and paronychia of nails.

Methods

Culture: All specimens were cultured on sabaroud dextrose agar (SDA) (Conda®, Spain) are incubated at 28°C until growth was detected as plates were examined daily for three weeks. Dermatophytes isolates recovered from SDA were subcultured on PDA (Micro Master® - India) and incubated at 28ºC for 7 days to provoke sporulation. A scotch tape technique was done for identification of genus and species of a dermatophytes. Germ tube test was done for all candida to identify C. albicans from C. non albicans species. Then antifungal susceptibility was done **[6].**

- **Antifungal susceptibility using Disk diffusion (DD) method :**

The Dermatophytes isolates were harvested from PDA in sterile saline and the suspension was adjusted to 0.5 McFarland. Mueller-Hinton agar plates (Conda® - Spain) were uniformly streaked with sterile swabs from inoculum suspension made from colonies of PDA. Fluconazole (25ug/disk), Itraconazole (10ug/disk), and Terbinafine (2ug/disk) antifungal discs (MUST®- Italy) were applied on Mueller-Hinton agar plates that were incubated for 15 days at 28°C **[7].** The diameter of zones of inhibition for each antifungal drug was assessed as growth progressed. The Clinical Laboratory Standards Institute (CLSI) 2017 recommendations were followed for interpreting zone diameters **[8].**

- **Antifungal susceptibility using Broth microdilution (BMD) method without Resazurin:**

The solution of antifungal drugs (Fluconazole, Itraconazole, and Terbinafine) was made by mixing the drug powder (HIMEDIA, India) with Muller Hinton Broth (TPC®-India). One hundred microliters of these drugs suspensions were transferred to the wells of 96- well round bottom microtiter plate (CITOTEST, Haimen, China), then two-fold serial dilutions of the antifungal drugs were made to reach six dilutions of each drug. The concentration of drugs was double the required strength i.e. 4-128 µg/ml for Fluconazole (FLC), 0.125-4.0 µg/ml for Itraconazole (ITR), and 0.03-1 µg/ml for Terbinafine (TER). Then the wells were inoculated with 100 µl of the dermatophytes isolates inoculum suspension, which is equivalent to 0.5 McFarland, resulting in a final drug dilution of 2.0-64.0 µg/ml for FLC, 0.062-2.0 µg/ml for ITR, and 0.015-0.50 µg/ml for TER. For 5 days, the plates were incubated at 28°C and monitored every day. The turbidity of the broth was used to calculate the minimum inhibitory concentration (MIC) for each antifungal drug **[9].**

- **Antifungal susceptibility using Broth microdilution (BMD/Resazurin) method with Resazurin 0.01% (Oxford, UK):**

On the same previous six dilutions of the medicines, 50 µl of Muller Hinton broth and 50 µl of Resazurin dye 0.01% (Oxford, UK) were added. The wells were inoculated with 100 µl of dermatophytes isolates inoculum suspension that

is equivalent to 0.5 McFarland. The plates were incubated at 28°C for 5 days and the color change was observed visually every day. The color shift of Resazurin from purple to pink was used to determine the minimum inhibitory concentration (MIC) for each antifungal agent **[5].**

Statistical analysis

The collected data were computerized and statistically analyzed using the Statistical Package for Social Sciences (SPSS 24 Inc. Chicago, IL, USA).

The kappa statistic is used to test interrater reliability. The Kappa result was interpreted as follows: values less than or equal zero indicating no agreement and range of (0.01–0.20) none to slight agreement, range of (0.21–0.40) as fair agreement, range of (0.41– 0.60) as moderate agreement, range of (0.61–0.80) as substantial agreement, and range of (0.81–1.00) as almost perfect agreement.

P- value was used to determine level of significance as follows: P>0.05: Non-significant (NS), P< 0.05: Significant (S), and P<0.01: Highly significant (HS).

RESULTS

As regards the demographic characteristics, Out of fifty five patients, age ranged from 3 - 18 years, there was a statistical significance between young age of patients and incidence of dermatophytic infection (P = 0.019). Nineteen patients (34%) gave history of previous systemic antifungal intake, thirty patients (54.5%) had positive risk factor for fungal infections.

The 55 patients suffering from dermatophytic infection were examined clinically and classified into tinea capitis 38/55 (69.09%), tinea circinata 12/55 (21.1%), and *onchymycosis* 5/55 (9.09%).

Identification of different dermatophytes species from 50 patients was done on PDA. The five patients with *onchymycosis* grew Candida only. According to type of dermatophyte, out of 50 patients, 35 (70%) had Trichophyton infection (T. tonsurans 24 (48%), T. mentegrophytes 9 (18%), and T. rubrum 2(4%)), 14 (28%) had Microsporum infection (M. canis 10(20%), M. audonii 2(4%), and M. gypsum 1(2%)) and 1 (2%) only had Epidermophyton infection (E. floccosum) (table 1).

In tinea capitis 21/38 (55.3%) patients were T. tonsurans, 10/38 (26.3%) patients were M. canis, and 7/38 (18.4%) patients were T. mentegrophytes. **In tinea circinata** 3/12 (25%) patients were T. tonsurans, 3/12 (25%) patients were M. audonii, 2/12 (16.7%) patients were T. mentegrophytes, 2/12 (16.7%) patients were T. rubrum, 1/12 (8.3%) patient was M. gypsum and 1/12 (8.3%) patient was E. floccosum. **All onychomycosis** five cases revealed candida species. All candida species were C. albicans by germ tube test (table 1).

-In Trichophyton isolates (T. tonsurans, T. mentegrophytes, T. rubrum) 4/35(11.4%) were sensitive to fluconazole by DD, BMD, and (BMD/Resazurin), (8/35) 22.8% were sensitive by DD whereas (9/35) 25.7% were sensitive by BMD, BMD/Resazurin to Itraconazole, (13/35) 37.1 % were sensitive by DD and (9/35) 25.7% were sensitive by BMD, BMD/Resazurin to Terbinafine.

-In Microsporum isolates (1/14) 7 % were sensitive to fluconazole by DD which were resistant by BMD and BMD/Rezasurin. Microsporum isolates were resistant to Itraconazole and Terbinafine by the three methods.

-Epidermophyton isolate was sensitive to Itraconazole by DD, BMD, and BMD/Resazurin Generally, the antifungal susceptibility patterns by DD method for dermatophytes was found to be 10% of isolates were sensitive to fluconazole and 90% were resistant to fluconazole, 20% of isolates were sensitive to itraconazole and 80% were resistant to itraconazole, and 26% of isolates were sensitive to terbinafine and 74% were resistant to terbinafine.

-Antifungal susceptibility results were detected after 24 hours with Resazurin in the form of color change from purple to pink compared to broth microdilution method without Resazurin, as results were detected after 72 hours and disc diffusion results were detected after an incubation period of 7 to 15 days. In addition, the Resazurin method was easier in visual interpretation.

-There was a perfect agreement between the antifungal susceptibility testing by DD method and BMD without and with Resazurin method with Kappa= 1 for itraconazole (table 2), Kappa= 0.947 for terbinafine (table 3), and Kappa= 0.878 for fluconazole (table 4).

-The sensitivity of DD method in comparison to BMD, and BMD/ Resazurin methods for fluconazole was 80%, for itraconazole was 100% and for terbinafine was 92.3%. Specificity for fluconazole was 97.7%, for itraconazole was 100% and for terbinafine was 97.4%. The positive predictive value for fluconazole was 80%, for itraconazole was 100% and for terbinafine was 92.3%. The negative predictive value for fluconazole was 97.9%, for itraconazole was 100% and for terbinafine was 97.4% (table 5).

Table 1: Types of dermatophyte species among cases

		Diagnosis			
		Tinea capitis		Tinea circinate	
Strains of organism	Trichophyton tonsurans	21	55.3%	3	25%
	Microsporum canis	10	26.3%	0	0%
	Trichophyton mentegrophytes %)	7	18.4%	2	16.7%
	Microsporum audonii	0	0%	3	25%
	Trichophyton rubrum	0	0%	2	16.7%
	Microsporum gypsum	0	0%	1	8.3%
	Epidermophyton floccosum	0	0%	1	8.3%

Table 2: Agreement between Disk diffusion method and Broth micro-dilution sensitivity method for Itraconazole.

		DD Itraconazole				Kappa	P	Sig
		S		R				
		N	%	N	%			
MIC Itraconazole	S	10	100.0%	0	.0%	1.00	0.0001	HS
	R	0	.0%	40	100.0%			

*kappa statistics

Table 3: Agreement between Disk diffusion method and Broth micro-dilution sensitivity method for terbinafine.

		DD Terbinafine				Kappa	P	Sig
		S		R				
		N	%	N	%			
MIC Terbinafine	S	12	92.3%	0	.0%	0.947	0.0001	HS
	R	1	7.7%	37	100.0%			

*kappa statistics

Table 4: Agreement between Disk diffusion method and Broth micro-dilution sensitivity method for fluconazole

		DD Fluconazole				kappa	P	Sig
		S		R				
		N	%	N	%			
MIC Fluconazole	S	4	80.0%	0	0%	0.878	0.0001	HS
	R	1	20.0%	45	100.0%			

*kappa statistics

Table 5: Sensitivity and specificity of Disk diffusion method in relation to Broth micro-dilution (with and without Resazurin) sensitivity method

	Sensitivity	**Specificity**	**PPV**	**NPV**
Fluconazole	80%	97.7%	80%	97.99
Itraconazole	100%	100%	100%	100%
Terbinafine	92.3%	97.4%	92.3%	97.4%

PPV= Positive predictive value
NPV= Negative predictive value.

DISCUSSION

There was a statistically significant difference between dermatophytic infections and young age in the current study. This was in line with the findings of **Abdel-Rahman et al. [10]** who studied 50 patients with dermatophytic infections and discovered that tinea infection is mostly a disease of children, with an average age of onset of 5 to 10 years.

In the current study, positive risk factors for developing dermatophytic infections were found in 60% of patients. In another study, it was found that 65% of patients had positive risk factors for getting dermatophytic infections, which could explain the role of personal hygiene, immunosuppression, and environmental elements in developing the dermatophytic infections **[11].**

In our study, it was found that 34% of patients had a history of previous systemic antifungal intake, they were found more resistant to antifungal therapy, later in the study. In another study, researchers investigated the mechanisms of resistance to antifungal drugs and concluded that as the use of different classes of antifungal drugs increases, it is expected that the fungal species will increase resistance to these drugs **[12].**

In the current study, it was found that the most common dermatophyte species was Trichophyton tonsurans (48%) followed by Microsporum canis (20%), Trichophyton mentegrophytes (18%), M. audonii (6%), Trichophyton rubrum (4%), Microsporum gypsum (2%) and Epidermophyton floccosum (2%). On the contrary, **Gupta et al. [13]** in India found that the most common dermatophyte species was Trichophyton rubrum (46.5%), followed by Trichophyton mentegrophytes (39.6%), Microsporum gypsum (6.8%), Trichophyton tonsurans (3.4%), Microsporum oudonii (1.7%) and Microsporum ferrugineum (1.7%), this can be explained by different environmental factors and fungal population as well **[13].**

In the current study, the most common organism to cause tinea capitis was Trichophyton tonsurans (55.3%). This agree with **Nasir et al. [14]** who studied 391 children with suspected tinea capitis and found that Trichophyton *tonsurans* accounts for most of cases of infection (90%), followed by Microsporum canis (1.1%) then Trichophyton mentegrophytes (0.7%).

In our study, in patients suffering from tinea circinata, both M. audonii and T. tonsurans were equally recovered (25%) of cases for each. In another study, it was found that T.*tonsurans* is the most common cause of tinea capitis (95.8%), and associated tinea circinata were more likely to occur in people with an anthropophilic tinea capitis infection **[15].** T. tonsurans was found to be the main cause of tinea capitis and tinea circinata, in other study **[16].**

In the current work, the antifungal susceptibility patterns were studied by DD method for dermatophytes and it was found that 10% of isolates were sensitive to fluconazole and 90% were resistant to fluconazole, 20% of isolates were sensitive to itraconazole and 80% were resistant to itraconazole, and 26% of isolates were sensitive to terbinafine and 74% were resistant to terbinafine. This contradicts the findings of other study in which both DD and BMD methods were performed on 58 clinical isolates of dermatophytes using four antifungals (fluconazole, itraconazole, terbinafine, and griseofulvin). They showed only six strains resistant to fluconazole, five resistant to terbinafine, and five, four, and three strains were found intermediate sensitive to fluconazole, itraconazole, and griseofulvin respectively. All other strains in their study were sensitive to all the four antifungal agents. The high antifungal medication resistance in our isolates might be explained by a long history of systemic antifungal use **[13].**

In the current research, the antifungal susceptibility patterns were studied by BMD method for dermatophytes, it was found that 10% of isolates were sensitive to fluconazole and 90% were resistant, 20% of isolates were sensitive to itraconazole and 80% were resistant, and 26% of isolates were sensitive to terbinafine and 74% were resistant. This agree with the findings of other study in which fifty clinical isolates of Trichophyton spp were cultured. Broth microdilution method was used for antifungals susceptibilities testing of dermatophytes, by using five antifungals (fluconazole, ketoconazole, griseofulvin, itraconazole, and terbinafine). They found that terbinafine was the most potent active drug as observed in the current study **[17].** In another study, they found

Fluconazole was found to be the least effective drug as the results in the current study **[13].**

Resazurin was employed as a color indicator for metabolic activity of dermatophytes in the current work in the BMD technique. Resazurin is a purple-blue non-toxic dye that is converted to pink and fluorescent resorufin in the presence of a living organism due to its metabolic activity. The use of the Resazurin dye test in antifungal susceptibility testing has the advantages of ease of endpoint determination, and cheap cost **[18].**

In the current study, it was found that results of Resazurin MIC in BMD method were similar to that of MIC BMD without Resazurin for all samples. However, it was more rapid as it started to change its color after 12 hours and complete change was achieved after 24 hours of incubation, while MIC without Resazurin gave results in 48-72 hours proving that BMD with Resazurin provide faster results to the physicians which can alter the treatment plans of patients.

In another study, the use of Resazurin for estimating the abundance of contaminant-degrading micro-organisms studied on 24 clinical isolates and it was found that the color change of Resazurin from purple to pink occurred after overnight incubation **[19].** Another one compared a photometric approach utilising Resazurin dye against the standardized methods of antifungal susceptibility testing of yeasts on 101 clinical isolates and found that Resazurin is a suitable cell viability indicator in antifungal susceptibility testing **[20].**

Other studies were made to evaluate the agreement between BMD and DD methods for antifungal susceptibility testing of dermatophytes **[21, 22].** They used itraconazole, fluconazole and voriconazole against dermatophytes. They obtained inconsistent results, with little or no association between broth micro dilution and disc diffusion, and they rationalized their findings by using dermasel agar medium, which is not suitable for antifungal susceptibility testing. The kind and size of inoculum, the composition of the medium, the temperature and duration of incubation, and disc strength are all said to impact the results of broth microdilution and disc diffusion **[7].**

In the current research, the DD method was highly sensitive and specific compared to the BMD method which is used as the standard method for antifungal susceptibility testing. Compared to BMD results, the sensitivity of itraconazole, terbinafine, and fluconazole were 100%, 92.3%, and 80%, respectively and the specificity of itraconazole, fluconazole, and terbinafine were 100%, 97.7%, and 97.4%, respectively by disk diffusion method. These findings agree with another study in which researchers found that sensitivity of fluconazole, itraconazole, and terbinafine were 98.1%, 96.6%, and 94.3% respectively and specificity of fluconazole, itraconazole, and terbinafine were 87.5%, 75%, and 72.7% respectively using DD method against BMD method **[13].**

In Conclusion, in the current study, there was a perfect agreement between the antifungal susceptibility testing by disk diffusion method and Broth micro-dilution without and with Resazurin method with Kappa= 1 for itraconazole, Kappa= 0.947 for terbinafine, and Kappa= 0.878 for fluconazole. Using broth microdilution method supplemented with bioactive dyes such as Resazurin can provide accurate antifungal susceptibility results within 24 hours which can help in better patient care.

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Conflict of interest: None.

Ethical considerations: All procedures performed in this study were with the ethical standards of the Declaration of Helsinki and approved by the ethical committee of faculty of medicine, Ain Shams University.

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HIGHLIGHTS

- This research work gives hope to use bioactive dye like Resazurin in antifungal susceptibility testing to obtain rapid and accurate results.
- In this research I compared results of antifungal susceptibility testing by disk diffusion, broth microdilution with and without Resazurin
- There was a perfect agreement between the antifungal susceptibility testing of Itraconazole (kappa=1.00), Terbinafine (kappa =0.947) and Fluconazole (kappa=0.878) by disk diffusion method and Broth micro-dilution method without and with Resazurin.
- Broth micro-dilution method with Resazurin gave results in 24 hours.

REFERENCES

1. Hawkins D and Smidt A. Superficial fungal infections in children. *Pediatr Clin. North Am.* 2014; 61:443.
2. Chinelli P, Sofiatti A, Nunes R and Martins J. Dermatophyte agents in the city of São Paulo, from 1992 to 2002 Revista do Instituto de Medicina Tropical de São Paulo., 2003; 45: 259-263.
3. Pakshir K, Bahaedinie L and Rezaei Z. In vitro activity of six antifungal drugs against clinically important dermatophytes *IJMM*., 2009; 2(4):158-163.
4. Jain N, Sharma M and Saxena V. Identification and antifungal susceptibility testing of fungal infections in clinical samples of suspected superficial fungal infection *Indian J. Dermatol. Venerol. Leprol*., 2008; 74(3): 274 75.
5. Markantonatou AM, Samaras K, Zachrou E, Vyzantiadis TA. Comparison of Four Methods for the in vitro Susceptibility Testing of Dermatophytes. *Front Microbiol*. 2020; Jul 14; 11:1593. doi: 10.3389/fmicb.2020.01593. PMID: 32760372; PMCID: PMC7371995.
6. Kannan P, Janaki C and Selvi G. Identification and antifungal susceptibility testing of fungal infections in clinical samples of suspected superficial fungal infections Indian *J. Med. Microbiol*.2006; 24(3): 212 15.
7. Fernández-Torres B, Cabañes FJ, Carrillo-Muñoz AJ, Esteban A, Inza I, Abarca L, et al., Collaborative evaluation of optimal antifungal susceptibility testing condition for dermatophytes. *J Clin Microbiol*, 2002; 40: 3999-4003.
8. CLSI. M61: Performance standards of Antifungal susceptibility testing of filamentous fungi, first edition, November, 2017.
9. CLSI. M38: Reference Method for Broth Dilution Antifungal susceptibility testing, third edition, November, 2017.
10. Abdel-Rahman S, Farrand N, Schuenemann E , Schuenemann E, Stering T K., Preuett B, et al. The prevalence of infections with Trichophyton tonsurans in school children: the CAPITIS study. *Ped*., 2010;125 (5):966-73.
11. Cafarchia C, camarda A, Coccioli C, Figueredo L. A., Circella E., Danesi P. et al. Epidemiology and risk factors for dermatophytoses in rabbit farms. *Med Mycol*., 2010; 48, 975–980.
12. Ghannoum M and Rice L. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin Microbiol Rev*., 1999; 12(4): 501–517.
13. Gupta S, Kumar R, Mittal G, Roy S, Khan F and Agarwal A et al. Comparison of Broth Micro Dilution and Disk Diffusion Method for Susceptibility Testing of Dermatophytes. *Int J Curr Microbiol App Sci*., 2015; 4(5): 24-33.
14. Nasir S, Ralph N, O'Neill C, Cunney R, Lenane P, O'Donnell B. Trends in Tinea Capitis in an Irish Pediatric Population and a Comparison of Scalp Brushings Versus Scalp Scrapings as Methods of Investigation. *Pediatr Dermatol*., 2014; (5*)*:622-3.
15. Hryncewicz-Gwózdz A, Beck-Jendroschek V, Brasch J, Kalinowska K and Jagielski T. Tinea capitis and tinea corporis with a severe inflammatory response due to Trichophyton tonsurans. *Acta Derm Venereol*., 2011; 91(6):708-10.
16. Ravenscroft J, Goodfield M and Evans E. Tricophyton tonsurans Tinea capitis and tinea corporis: treatment and follow up of four affected family members. *Pediatr. Dermatol*., 2000; 17(5): 407-409.
17. Santos D and Hamdan J. Evaluation of broth microdilution antifungal susceptibility testing conditions for Trichophyton rubrum. *J. Clin. Microbiol*. 2005; 43: 1917-1920.
18. Tizzard, A, Bergsma J and Lloyd-Jones G. A resazurin-based biosensor for organic pollutants. *Biosens Bio electron*. 2006; 22:759–763.
19. Mania D, Hilpert K, Ruden S, Fischer R, Takeshita N. Screening for antifungal peptides and their modes of action in aspergillus nidulans, *J. Applied and Envir. Microbiol*. 2010; 7102–7108.76-21.
20. Tiballi R, Zarins T, Revankar S and Kauffman C. Use of a Calorimetric system for yeast susceptibility testing. *J Clin Microbiol*. 1995; 33:915-7.
21. Singh J, Zaman M and Gupta K. Evaluation of micro dilution and disk diffusion methods for antifungal susceptibility testing of dermatophytes. *Med. Mycol*. 2007; 45: 595-602.
22. Méndez CC, Serrano MC, Valverde A, Pemán J, Almeida C, Martín-Mazuelos E. Comparison of E-test disk diffusion and a modified CLSI broth micro dilution (M 38-A) method for in-vitro testing of itraconazole, fluconazole and voriconazole against dermatophytes. *Med. Mycol*., 2008; 46(2): 119 23.