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Antifungal Susceptibility Testing of Dermatophytes

Dr. JK Surekha, Dr. Chandana Konda*, Dr. I. Jahnavi, Dr. D. Sudha Madhuri, Dr. K. Nagamani
Department of Microbiology, Gandhi hospital, Musheerabad, Secunderabad, Telangana 500003, India

Original Research Article

***Corresponding author**
Dr. Chandana Konda

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Abstract: There is an increased prevalence of dermatophytes over the past few decades. Although recent antifungals have a high success rate, treatment failure may occur in 20% cases due to antifungal drug resistance. This study was done to determine antifungal susceptibility of identified dermatophytes to azole group of antifungal drugs. A total of 100 skin, hair and nail samples from clinically suspected cases of dermatophytosis were cultured on Sabouraud dextrose agar and Dermatophyte test media. Positive cases were further processed by subculturing on Potato Dextrose Agar and antifungal susceptibility against 3 azoles, fluconazole, itraconazole and ketoconazole was done by microbroth dilution method according to CLSI guidelines M38A2. Out of 100 clinically suspected cases of Dermatophytosis, Mean MIC (Minimum inhibitory concentration) of Fluconazole was 16.08µg/ml, Itraconazole was 0.239µg/ml and Ketoconazole was 0.339µg/ml. To conclude, in this study, Itraconazole with lowest MIC was most sensitive and most potent against dermatophytes Fluconazole with highest MIC was least sensitive and most resistant. Ketoconazole had intermediate MIC and sensitivity.

Keywords: Dermatophytes, Azole Antifungals, Resistance, Microbroth dilution, CSLI, MIC.

INTRODUCTION

The dermatophytes are a group of closely related fungi that invade the keratinized tissues of skin and its appendages including hair and nails, and cause an infection, dermatophytosis, commonly referred to as ringworm or tinea [1].

They are included in three fungal genera viz: Epidermophyton, Microsporum and Trichophyton [2].

These fungi colonize in the keratin tissues from where they obtain nutrition and cause inflammation as the host responds to metabolic by-products. Dermatophytes are also associated with secondary bacterial infections leading to systemic skin infections [3]. According to WHO, the prevalence rate of superficial mycotic infection worldwide has been found to be 20-25% [4].

Dermatophytoses generally respond well to topical antifungal therapy, although for extensive infections, systemic medication is required[5]. Griseofulvin was the only approved systemic antifungal agent, initially. However, at present new agents both topical (clotrimazole, naftifine, ciclopirox olamine) and systemic (Itraconazole and fluconazole, ketoconazole, terbinafine) have been introduced into clinical practice during last 5–10 years for effectively treating dermatophytic conditions.The increased use of antifungal drugs, often for prolonged periods, has led to acquired antifungal resistance among previously susceptible strains or species and to the increased incidence of infections with less common species [6]. With an increasing variety of drugs available for the treatment of dermatophytoses, the need for a reference method for the testing of the antifungal susceptibilities of dermatophytes has become apparent. In vitro, antifungal susceptibility testing could therefore, prove helpful in the better management of the dermatophytosis because effective antifungal agents can be selected by this method by determining minimum inhibitory concentrations (MIC's) of these agents. Broth macro- and micro-dilution methods, agar dilution and disc diffusion methods are routinely used for this purpose. Clinical and Laboratory Standards Institute (CLSI) had developed the standard broth micro dilution M38-A2 method for antifungal susceptibility of some filamentous fungi, including the dermatophytes in 2008 [7].

The present study was conducted to do antifungal susceptibility of identified cases dermatophytes by determining MIC's by microbroth dilution method according to CLSI standards M38-A2. This was beneficial for investigation of in vitro resistance of dermatophytic species and management of cases unresponsive to treatment.

MATERIALS AND METHODS

A total of 100 skin, hair and nail samples from clinically suspected cases of dermatophytosis were cultured on Sabouraud dextrose agar and Dermatophyte test media. Positive cases were further processed by subculturing on Potato Dextrose Agar and antifungal susceptibility against 3 azoles, fluconazole, itraconazole and ketoconazole was done by microbroth dilution method according to CLSI guidelines M38A2. This procedure was carried out after approval from the institutional ethics committee.

Antifungal susceptibility testing of dermatophytes by microbroth dilution method

The procedure followed is according to the CLSI M38-A2 [7] document entitled Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: Approved standard – Second Edition', intended for testing common filamentous fungi or moulds, including the dermatophytes.

Requirements

- Potato dextrose agar
- Antifungal drugs – fluconazole, itraconazole, ketoconazole.
- RPMI – 1640 broth with L-Glutamine without sodium bicarbonate, with phenol red indicator and MOPS buffer (3-morpholinopropane-1-sulfonic acid)
- Sterile distilled water
- Sterile tubes
- Dimethyl sulfoxide (DMSO)
- Falcon tubes
- 96 well flat bottomed, sterile disposable microtitre plates.

All chemicals and drugs- fluconazole and ketoconazole (both powder form) were obtained from Himedia laboratories. Itraconazole tablets were purchased from pharmacy, ground to powder and used for susceptibility testing.

Preparation of inoculum

Cultures of dermatophyte species (7–8 days old) grown on PDA slants at 30°C were used to prepare inoculums. A sterile swab was rolled over the growth on the slant that contained conidia and hyphal fragments. The swab was then emulsified in 5 ml of sterile saline (0.9%) taken in a sterile tube. The heavy particles were allowed to settle down for 10–15 min. The upper clear suspension was transferred to a fresh tube, and a drop of Tween 20 was added to the suspension. Its optical density was adjusted to 0.5 McFarland standards which matched the final cell density between 0.4×10^4 to 5×10^4 colony forming units per ml. which was used in the assay. 100µl of this inoculum was added to 5 ml of RPMI 1640 medium (1:50 dilution) in a falcon tube.

Preparation of antifungal drug stock solution:

The antifungal drugs used in the assay were fluconazole, itraconazole and ketoconazole.
The drug concentration ranges were as follows:
Fluconazole - 0.125 to 64 µg/ml
Itraconazole - 0.0313 to 16 µg/ml
Ketoconazole - 0.0313 to 16 µg/ml
Weight of drug powder required to be added to solvent was determined using the formula:

$$\textbf{Weight (mg)} = \frac{\text{Max MIC or highest conc of drug(µg/ml) X 100}}{1000}$$

Volume of solvent (DMSO) required to dissolve drug powder (ml) =

$$\frac{\text{weight of powder in mg x potency of drug (assuming 100\% potency or 1000µg/mg)}}{\text{highest conc of drug in µg/ml.}}$$

96 well, flat bottomed microtitre plate used for antifungal susceptibility testing. This amount of drug dissolved in the solvent DMSO was the stock solution which was prepared in a falcon tube.

Amount of stock solution required to be added to 1st well (V2) of microtitre plate

C1V1 = C2V2
C1= concentration required
V1= volume required (volume of micro titre plate well being 200µl)
C2= 100 times the highest desired concentration.

$$V2 = \frac{C1V1}{C2}$$

Test procedure

Sterile, flat bottomed, disposable, multiwell microdilution plates (96 wells) were used to perform the tests. 3 rows of wells were utilised for 3 drugs for each isolate. 198µl of pure RPMI broth was taken into the first well of each of the 3 rows using micropipette. 100 µl of RPMI was taken into the remaining wells from 2 to 10 using micropipette. 2µl of stock solution was

added to the first well of all 3 rows. 100µl of the solution in first wells was transferred to 2nd, 100µl from 2nd well was transferred to 3rd and so on till 10th well. 100µl was then discarded from the 10th well. 100µl of inoculum prepared earlier by diluting in RPMI was added to all wells.

For each test plate, two drug-free controls were included, one with the medium alone, 200µl of pure RPMI (sterile control) in 11th well and the other with 200µl of RPMI + inoculum (growth control) in the 12th well.

All the wells were sealed with sealers and the microdilution plates were incubated at 25°C and were read visually after 4, 7, and 10 days of incubation.

Candida krusei ATCC 6258 was taken as quality control reference strain as approved by CLSI and its susceptibility to the 3 drugs was tested in 3 rows for the set of isolates done on a particular day. Plate containing this strain was incubated at 25°C and read visually at 48 hours.

Reading and interpretation of MIC's

Endpoint determination readings were performed visually based on comparison of the growth in wells containing the drug with that of the growth control.

MIC was taken in the first well where turbidity was reduced to atleast 80%.

RESULTS

Table-1: Anti Fungal Susceptibility of Dermatophytic Isolates

Drugs	**Fluconazole**		**Itraconazole**		**Ketoconazole**	
Isolates	**MIC range (µg/ml)**	**Mean MIC (µg/ml)**	**MIC range (µg/ml)**	**Mean MIC (µg/ml)**	**MIC range (µg/ml)**	**Mean MIC(µg/m)**
T.mentagrophytes (n=8)	4-64	23.5	0.0625-0.5	0.179	0.0625-0.5	0.257
T.rubrum (n=3)	4-64	33.33	0.03125-1	0.385	0.25-0.5	0.416
T.verrucosum(n=8)	16-64	38	0.03125-1	0.417	0.0625 - 1	0.4375
T.tonsurans(n=17)	1-64	16.05	0.03125-1	0.229	0.0625-1	0.393
T.violaceum (n=1)	0.125	0.125	0.03125	0.03125	0.0625	0.0625
M.gypseum (n=1)	0.5	0.5	0.125	0.125	0.25	0.25
E.floccosum (n=2)	0.25 – 2	1.125	0.125-0.5	0.3125	0.125-1	0.5625

Antifungal susceptibility was done for all 40 isolates of dermatophytes.

In case of fluconazole, highest MIC 80 was shown by T.verrucosum with 38 µg/ml, followed by T.rubrum with 33µg/ml and lowest of 0.125 µg/ml was shown by T.violaceum.MIC 80 of T.mentagrophytes was 23.5µg/ml, T.tonsurans 16.05 µg/ml, M.gypseum 0.5 µg/ml and E.floccosum 1.125 µg/ml.

In case of itraconazole, highest MIC 80 was shown by T.verrucosum with 0.417µg/ml followed by T.rubrum with 0.385 µg/ml and lowest of 0.03125 µg/ml was shown by T.violaceum.MIC 80 of T.mentagrophytes was 0.179 µg/ml,T.tonsurans 0.229 µg/ml, M.gypseum 0.125 µg/ml and E.floccosum 0.3125 µg/ml.

In case of ketoconazole, highest MIC 80 was shown by E.floccosum with 0.5625 µg/ml followed by T.verrucosum with 0.4375 µg/ml and lowest of 0.0625 µg/ml was shown by T.violaceum. MIC 80 of T.mentagrophytes was 0.257, T.rubrum was 0.416 µg/ml, T.tonsurans 0.393 µg/ml and M.gypseum 0.25 µg/ml.

Requirements to Perform Antifungal Susceptibility Testing

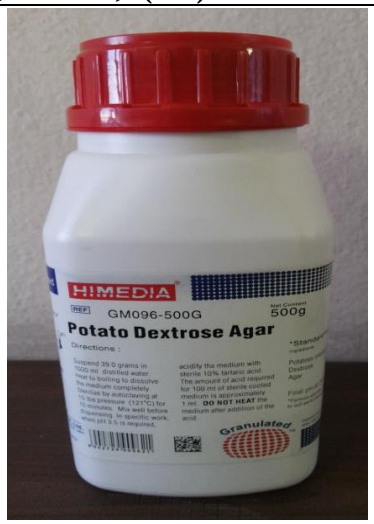

Fig 1: Potato Dextrose Agar (PDA)

Fig-2: Growth on PDA

Fig-3: **Dimethyl Sulphoxide**

Fig-4: RPMI medium

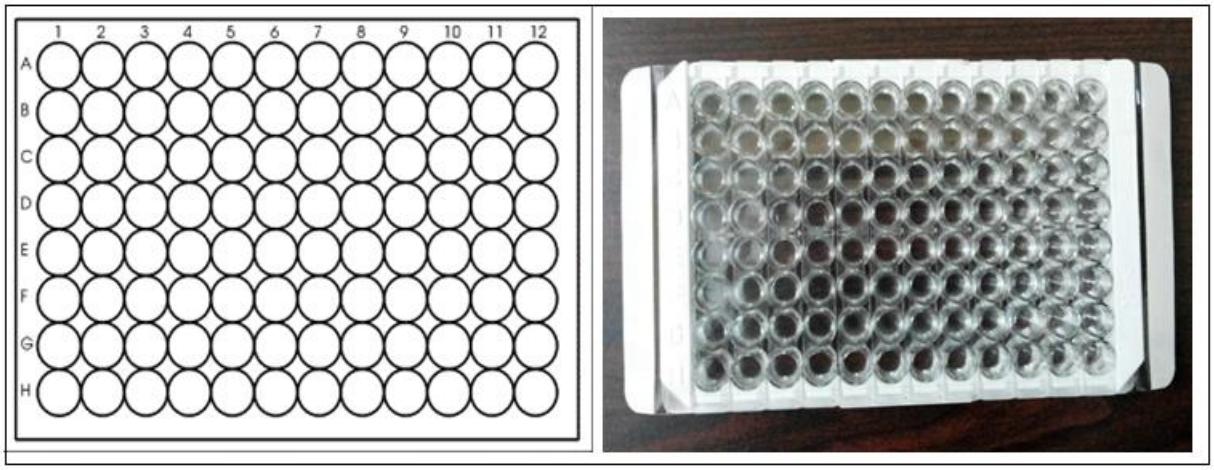

Fig-5: 96 Well Microtitre Plate

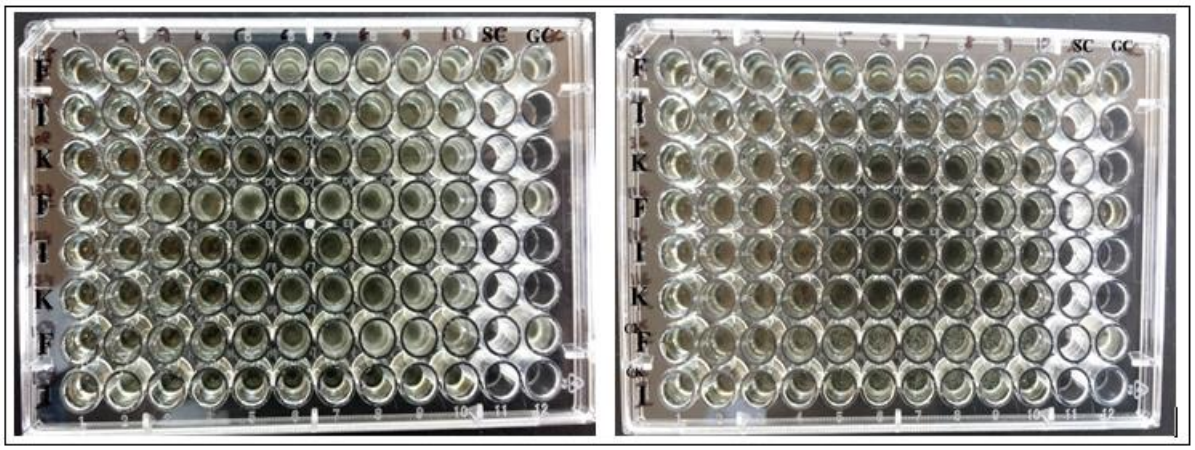

Fig-6: Antifungal susceptibility of dermatophytes done by micro broth dilution method against three drugs fluconazole, itraconazole and ketoconazole.

Candida krusei 6258 (CK) was used as control strain .It showed resistance to fluconazole.

DISCUSSION

Dermatophytes are molds affecting keratinized tissue causing superficial mycoses in humans and animals commonly known as ringworm infection.

In the present study, 100 clinically suspected cases of dermatophytosis were studied at Gandhi Hospital, Musheerabad during a period of one and half years. Out of these cases, 40 cases were culture positive. The study was undertaken to find out antifungal susceptibility of these identified dermatophytes.

Antifungal Susceptibility Testing

The introduction of wide range of new antifungal agents and recovery of clinical isolates exhibiting resistance to antifungal agents makes testing of the susceptibility of dermatophytes to these agents more important particularly for surveillance of resistant strains, in epidemiological studies. It might help clinicians for better management of the disease caused by them by selecting appropriate therapeutic options for checking further spread [3].

The antifungal susceptibility was done by broth dilution method with references to CLSI document M38-A2. The drugs evaluated in this study were fluconazole, ketoconazole and itraconazole. Candida krusei ATCC 6258 was used as the control strain in performing the antifungal susceptibility testings. The antifungal susceptibility testing for dermatophytes was done by Hanafy *et al* [8] 2012, Shalini gupta *et al* [9] 2015 and Dr.Stuti Kansra *et al* [10] 2016 with various antifungal drugs.

Antifungal Susceptibility against T.Mentagrophytes

T.mentagrophytes was found to be more susceptible to Itraconazole with MIC of 0.179µg/ml and to ketoconazole with MIC of 0.257µg/ml. It has less susceptibility to fluconazole with high MIC of 23.5µg/ml. In a study by Araujo C.R *et al* [11], T.mentagrophytes was equally susceptible to Itraconazole and ketoconazole with MIC of 0.25µg/ml. It was less susceptible to fluconazole with MIC of 16µg/ml.

Antifungal Susceptibility against T. Rubrum

T.rubrum was found to be more susceptible to Itraconazole with MIC of 0.385 µg/ml followed by ketoconazole with MIC of 0.416 µg/ml. It has less susceptibility to fluconazole with high MIC of 33.3µg/ml. This correlates with a study by Afshari *et al* [12], 2016 in which T.rubrum was susceptible to Itraconazole with MIC 0.25µg/ml , followed by ketoconazole with MIC 1 µg/ml and resistant to fluconazole with MIC of 4 µg/ml.

Antifungal Susceptibility against T. Verrucosum

T.verrucosum was found to be more susceptible to Itraconazole with MIC of 0.417 µg/ml followed by ketoconazole with MIC of 0.4375 µg/ml. It has less susceptibility to fluconazole with high MIC of 38µg/ml. In a study by Afshari et al 2016, T. verrucosum was found to be more susceptible to Itraconazole with MIC of 0.25 µg/ml followed by ketoconazole with MIC 0.5 µg/ml .It was found to be resistant to fluconazole with MIC 64 µg/ml.This is comparable to our study.

Antifungal Susceptibility against T. Tonsurans

T.tonsurans was found to be more susceptible to Itraconazole with MIC of 0.229 µg/ml followed by ketoconazole with MIC of 0.393 µg/ml. It has less susceptibility to fluconazole with high MIC of 16.05 µg/ml. Afshari et al showed T.tonsurans to be more susceptible to Itraconazole with MIC of 0.25 µg/ml followed by Ketoconazole with MIC of 1 µg/ml and resistant to fluconazole with MIC of 32 µg/ml.

Antifungal Susceptibility against T. Violaceum

Single isolate of T.violaceum was found to be more susceptible to Itraconazole with MIC of 0.03125 µg/ml followed by ketoconazole with MIC of 0.0625 µg/ml. It has less susceptibility to fluconazole with MIC of 0.125µg/ml. In a study by Indira G et al, 2014, T.violaceum was found to be more susceptible to Itraconazole with MIC 0.48 µg/ml followed by Ketoconazole with MIC 0.96 µg/ml and resistant to fluconazole with MIC of 5.12 µg/ml.

Antifungal Susceptibility against M. Gypseum

Single isolate of M.gypseum was found to be more susceptible to Itraconazole with MIC of 0.125 µg/ml followed by ketoconazole with MIC of 0.25 µg/ml. It has less susceptibility to fluconazole with a relatively high MIC of 0.5 µg/ml. Afshari *et al*, 2016 showed M.gypseum to be more sensitive to itraconazole with MIC of 0.0313 µg/ml followed by ketoconazole with MIC of 0.25 µg/ml. Least sensitive was to fluconazole with a high MIC of 64µg/ml. This study is comparable to our study.

Antifungal Susceptibility Against E. Floccosum

E.floccosum was found to be more susceptible to Itraconazole with MIC of 0.3125 µg/ml followed by ketoconazole with MIC of 0.5625 µg/ml. It has less susceptibility to fluconazole with high MIC of 1.125µg/ml. This is comparable to a study by Indira G, 2014 in which Itraconazole showed a MIC of 0.24µg/ml followed by ketoconazole with a MIC of 0.96µg/ml for E.floccosum. Fluconazole which was most resistant showed a MIC of 5.12µg/ml.

Table-2: observations of antifungal susceptibility by microbroth dilution method in various studies.

Author	Year	Place	Mean MIC		
			Fluconazole	Itraconazole	Ketoconazole
Fernandez-Torres *et al*	2001	London	20.8	0.235	1.577
Santos and Hamdan [13]	2005	Brazil	64	0.25	1
Araujo C.R *et al*	2009	Brazil	21.33	0.33	1.5
Indira G *et al*	2014	Warangal	8.172	1.176	0.816
Afshari *et al*	2016	Iran	37.14	0.397	0.89
Present study	2016	Hyderabad	16.08	0.239	0.339

The MIC ranges for all the 40 isolates of dermatophytes tested for antifungal susceptibility showed that itraconazole had the lowest MIC range of 0.03125 -1 µg/ml followed by ketoconazole at a MIC range of 0.0625-1µg/ml. The highest MIC range of 0.125-64 µg/ml was recorded for Fluconazole. The mean MIC of fluconazole was 16.08µg/ml followed by ketoconazole with MIC of 0.339µg/ml and itraconazole with MIC of 0.239 µg/ml. All the other authors, Fernandez-Torres et al, Santos and Hamdan, Araujo C.R et al and Afshari et al showed that Itraconazole had a lower MIC than Ketoconazole which is comparable to our study. Only the study by Indira G et al showed that ketoconazole had a lower MIC than itraconazole.

In our study we observed that itraconazole had the lowest MIC among the 3 azoles tested for antifungal susceptibility. Hence it is more sensitive and more potent against dermatophytes. Fluconazole had the highest MIC, and is least sensitive and more resistant among the three drugs tested. Ketoconazole showed intermediate sensitivity.

CONCLUSION

This study was done over a period of one and half years on 100 clinically suspected cases of dermatophytosis. Antifungal susceptibility by microbroth dilution method was done for all the 40 dermatophytes isolated. Itraconazole with lowest MIC was more sensitive and more potent against dermatophytes. Fluconazole with highest MIC is least sensitive and most resistant against dermatophytes tested. Ketoconazole showed intermediate sensitivity.

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REFERENCES

1. Ansari HQF, Patel MB, Siddiqui JAW. Epidemiology and In-Vitro Antifungal Susceptibility Testing of Dermatophytes in Hyderabad, India. International Journal of Advanced Research. 2014; 2(2): 553-560.
2. Indira G. In vitro antifungal susceptibility testing of 5 antifungal agents against dermatophytic species by CLSI (M38-A) micro dilution method. Clinical Microbiology: Open Access. 2014 Apr 30.
3. Bhatia VK, Sharma PC. Determination of minimum inhibitory concentrations of itraconazole, terbinafine and ketoconazole against dermatophyte species by broth microdilution method. Indian J Med Microbiol. 2015;33:533-7.
4. Lakshmanan A, Ganesh Kumar P, Mohan SR, Hemamalini M, Madhavan R. Epidemiological and clinical pattern of dermatomycoses in rural India. Indian J Med Microbiol. 2015;33, Suppl S1: 134-6.
5. Fernández-Torres B, Carrillo-Muñoz A, Ortoneda M, Pujol I, Pastor FJ, Guarro J. Interlaboratory evaluation of the Etest ® for antifungal susceptibility testing of dermatophytes. Med Mycol. 2003;41:125-30.
6. Matnani G, Roy I, Gandham N, Mandal A, Ujagare M, Jadhav SV. Identification and antifungal susceptibility testing of fungal infections in clinical samples of suspected superficial fungal infections. International Journal of Medical and Clinical Research. 2012 Jan 1;3(7):215.
7. Wayne PA. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi: approved standard. CLSI Document M38-A2. 2008;22(16).
8. Hanafy AMM, El-Adly AA, Alsarani AAQ, Ashfaq M and Ahmed Karam El-Din AZ. Epidemiology of cutaneous mycosis in the Medina region of Saudi Arabia correlated with studying the effect of light-induced gold nanoparticles on the growth of dermatophytes in vitro. African Journal of Microbiology Research. 2012; 6(37): 6668-6677.
9. Gupta S, Agarwal RK, Mittal G, Roy S, Khan F, Agarwal A. 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.
10. Kansra S, Devi P, Sidhu S. Prevalence of Dermatophytoses and Their Antifungal Susceptibility in a Tertiary Care Hospital of North India. 2016; 5(3):2277 – 817.
11. Araújo CR, Miranda KC, Fernandes OFL, Soares AJ, Silva MRR. In vitro susceptibility testing of dermatophytes isolated in Goiania, Brazil, against five antifungal agents by broth microdilution method. Rev. Inst. Med. trop. S. Paulo. 2009; 51(1): 9-12.
12. Afshari MA, Shams-Ghahfarokhi M, Razzaghi-Abyaneh M. Antifungal susceptibility and virulence factors of clinically isolated

dermatophytes in Tehran, Iran. Iranian Journal of Microbiology. 2016;8 (1):36-46.

13. Santos DA, Hamdan JS. Evaluation of broth microdilution antifungal susceptibility testing conditions for Trichophyton rubrum. J Clin Microbiol. 2005; 43:1917-20.