

# To Study The Antifungal Susceptibility Pattern, Molecular Profiling And Its Sequencing Analysis Of *Trichophyton Rubrum* Isolated From Human Sample: A Cross Sectional Case Study From Uttar Pradesh, India

Manoj Kumar Maurya<sup>1</sup>, Geeta Sharma<sup>2</sup>, Anil Kumar<sup>3</sup>, Anupam Das<sup>4</sup>, Sarita Maurya<sup>5</sup>, R. Sujatha<sup>6\*</sup>

<sup>1</sup>Ph.D Scholar, Department of Microbiology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India. Email: manoj0914@gmail.com

<sup>2</sup>Associate Professor, Department of Dermatology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India. Email: drgeeta54321@gmail.com

<sup>3</sup>Assistant Professor, Department of Biotechnology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India. Email: anil.biotech@gmail.com

<sup>4</sup>Professor, Department of Microbiology, Ram Mangham Lohia Institute of Medical Sciences, Lucknow, India. Email : anuj.cool1410@gmail.com

<sup>5</sup>Research Scholar, Department of Biotechnology, Allahabad University, Allahabad, India. Email : sarita.biotech2@gmail.com

<sup>6\*</sup>Professor and Head of Department of Microbiology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India. Email: drsujatha152@gmail.com

\*Corresponding Author: R. Sujatha

\*Professor and Head of Department of Microbiology, Rama Medical College Hospital and Research Centre, Mandhana, Kanpur, Uttar Pradesh, India. Email: [drsujatha152@gmail.com](mailto:drsujatha152@gmail.com)

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## Abstract

**Introduction:** There has been a sudden, unexplained surge in dermatophytoses in India since past 5 years. *T. rubrum* being the most common etiological agent isolated from clinically diagnosed lesions of cutaneous dermatophytosis. It has a significantly higher capacity of transmission than other anthropophilic dermatophytes.

**Aim and Objective:** To study the Antifungal Susceptibility Pattern, Molecular Profiling and its Sequencing Analysis of *Trichophyton rubrum* isolated from human Sample.

**Material and Methods:** This was a prospective study carried out in the Department of Microbiology at Rama Medical College Hospital and Research Centre Mandhana, Kanpur for a period of 1 year i.e, March 2017 to March 2018. A total of 375 clinically suspected cases of superficial fungal infection were collected with proper informed consent. Dermatophytosis was confirmed in cases by direct microscopy and culture methods. Antifungal drug susceptibility profiles, MIC of isolates were 50-90 determined using the broth microdilution method according to the CLSI guidelines. The DNA isolation was done using the Qiagen DNA extraction kit followed by the PCR. The sequencing methods were used for the genetic analysis of virulence gene for the confirmation.

**Results:** Out of the total of 375 clinically suspected cases of superficial fungal infection 31 isolates of *Trichophyton rubrum* were studied. The ratio of Male were more 27 (87%) as compared to the Female 4 (12.9%). The maximum number of isolates was found in the Skin 28 (90.3%) followed by the Nail 3 (9.6%). The ITZ was most sensitive toward *T. rubrum* than KTZ, TBF, and FCZ. The molecular characterization for the detection of 18s gene was performed which was confirmed by the Sequencing Analysis.

**Conclusion:** Antifungal resistance against *T. rubrum* is increasing over the last few decades due to genetic diversity in the fungal genome. The molecular identification is a gold standard technique for the identification of *T. rubrum* allowing important factor in providing fungal infection prevention and treatment approaches.

**Keywords:** *Trichophyton rubrum*, Antifungal, Molecular Profiling, Sequencing

## INTRODUCTION

Dermatophytes are a group of closely related filamentous fungi able to damage and utilize keratin found in the skin, hair, and nails [1]. They represent around 39 closely related species in three genera, namely *Trichophyton*, *Microsporium*, and *Epidermophyton*. Infection of keratinized tissue caused by these fungi is called dermatophytosis, which

is among the most common public health problems in hot and humid tropical countries like India. Although these infections are superficial, these fungi can also behave in an invasive manner, causing deeper and disseminated infection, especially in immunocompromised patients [2].

*T. rubrum* is introduced as a predominant mold among all dermatophytes followed by *T. mentagrophytes* and suggested as the most significant transmissible fungus among all dermatophytes [3]. It is one of the most devastating dermatophytosis-causing etiologic microbes usually manifested as tinea pedis and tinea unguium in humans since the early first century worldwide [4,5].

Owing to several factors such as indiscriminate, inadequate and irregular use of various drugs, antifungal drug resistance has emerged as a major public health concern in recent times where anti-mycotic drug resistance emerging as a serious issue in India along with changing trends in the clinico-epidemiological profile, thereby leading to ineffective treatment needing multiple drugs over longer treatment durations [6].

Molecular sequencing studies revealed that *T. rubrum* has higher clonal conserved gene content, low variability, and recombination [7]. Moreover, low virulence is one of the significant factors for enhancing the global transmission of *T. rubrum* [8]. Conventional methods including KOH microscopy and culture are the first line of strategy to diagnose *T. rubrum*. The culture method offers microbial colonies which are further utilized for more precise identification and characterization by employing molecular analysis using PCR and sequencing tools [9]. Microbes including *T. rubrum* evolve due to ecological and environmental effects which allow genetic modifications in their genome and make identification complex. The identification and characterization of *T. rubrum* by conventional means may not be enough to support immediate clinical management. Therefore, introducing new tools and techniques is required to discriminate between strain and genetic variation the ribosomal deoxyribonucleic acid (rDNA) internal transcribed spacer (ITS) barcoding gene in most clinical settings [10].

Therefore, the present study was undertaken to study the Antifungal Susceptibility Pattern, Molecular Profiling and its Sequencing Analysis of *Trichophyton rubrum* isolated from human which may help in early identifying and understanding antifungal drug response patterns in *T. rubrum* for the future management of this disease.

## MATERIAL AND METHODS

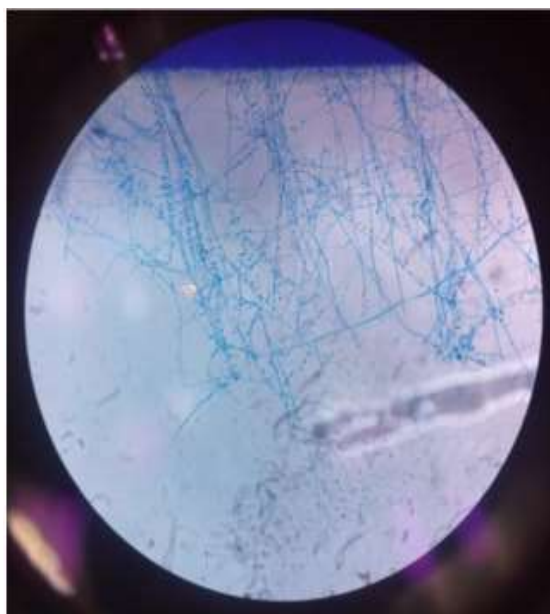
This was a prospective study carried out in the Department of Microbiology at Rama Medical College Hospital and Research Centre Mandhana, Kanpur for a period of 1 year i.e, March 2017 to March 2018. A total of 375 clinically suspected cases of superficial fungal infection were collected with proper informed consent. In the inclusion criteria the study included subjects of all age groups and any gender with clinically diagnosed dermatophytosis were studied. Any subjects with the history of use of antifungal agents for 4 weeks were excluded from the study.

## IDENTIFICATION AND SAMPLE PROCESSING

The Clinical Samples isolated from skin and nails were subjected to direct microscopic examination and conventional culture method via scalping using 10% KOH and 20% KOH for skin and nails respectively. *T. rubrum* was cultured in Saboured dextrose agar with chloramphenicol and cyclohexamide (Hi Media) at 25-30 °C for 2-4 weeks and follow-up at intervals during incubation to examine the progress of colony formation. Further, microscopic slide culture or microculture was prepared to morphologically characterize this dermatophyte using light microscopy according to the CLSI guidelines 2017 [11].



**Figure No. 1:** *T. rubrum* colony appearance: White to cream, suede-like to downy and red pigment on reverse



**Figure No. 2:** Microscopic illustration *T. rubrum* of (LPCB X400). Hyaline, septate, and branched hyphae on microconidia

#### Antifungal Susceptibility Pattern of *T. rubrum*

In vitro Antifungal susceptibility pattern of *T. rubrum* was determined by using broth micro dilution assay (BMA) protocol according to (CLSI-M38-3rd edition, 2017) standard guideline [11]. The reference strain of *T. rubrum* (ATCC 10218) was used as quality control. The antifungal potential reagent grade powder of Fluconazole and Itraconazole (Metro Chem API Pvt. Hyderabad, India), Ketoconazole (Arti drugs Ltd. Maharashtra, India), and Terbinafine (Shreeji Pharma International, Sarabhi, Vadodara, Gujarat, India) were used. All drugs were dissolved in RPMI-1640 medium supplemented with L-glutamine and without sodium bicarbonate (pH 7.0, adjusted with 0.165 M of morpholinepropanesulfonic acid) along with 1N NaOH (Hi Media). The final concentrations of antifungal agents were 0.5 to 64  $\mu\text{g/ml}$ , for fluconazole, whereas 0.0078  $\mu\text{g/ml}$  to 128  $\mu\text{g/ml}$  for Itraconazole, Ketoconazole, and Terbinafine. Spore suspensions of *T. rubrum* were prepared as protocol previously described by [12].

Briefly, cultures of fungal isolates (7-8 days old) were grown on SDA slants at 25°C and used to prepare inoculums. To obtain the final inoculums ( $2-6 \times 10^3$  CFU/mL) cell suspension was diluted in RPMI-1640 medium. Further, 100  $\mu\text{l}$  cell suspension was seeded in each well of 96 well plates and incubated with different antifungal stocks. After thorough mixing plates were incubated at 35 °C for 4-5 days.

The minimum inhibitory concentration (MIC) of the antifungal agent was defined as the point at which dermatophytes were inhibited by the lowest concentration of agents in comparison with the control. Further, MIC50, and MIC90 were determined against *T. rubrum*. All experiments were performed in duplicate to remove statistical biases.

### Molecular Identification of *T. rubrum*

The DNA was isolated using the Qiaamp DNA Blood Mini Kit (QIAGEN, Germany) as per the manufactures guidelines. The DNA was eluted in 60 µl elution buffer and preserve at -20 °C till PCR analysis. For amplification of the target gene, PCR was carried out in a 50 µL reaction mixture. The 18SrDNA region of fungal genomic DNA was amplified by using fungal primer sequences specific for DH1 were used . The primers were purchased from Eurofins Genomics India Pvt. Ltd., Bangalore, India.

Gene	Primer sequence	Length (bp)
18SrDNA	Forward- 5'-TGC ACT GGT CCG GCT GGG-3'	180bp
	Reverse 5'-CGG CGG TCC TAG AAA CCA AC-3'	

**Table No. 1 :** Primers for Gene Polymorphism [13]

The PCR cycling conditions used were one initial cycle of 3 min at 94°C, followed by 35 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 58 °C, and 1 min of extension at 72 °C, with a final extension of 7 min at 72 °C. The resulting PCR product of approximately 160-180 bp was subjected to 1 % agarose gel electrophoresis and visualized by Gel Doc™ EZ Gel Documentation System (Bio-Rad Laboratories Inc., Hercules, CA, USA). A 100-bp molecular marker (Fermentas Life Sciences, Waltham, MA, USA) was used to evaluate the PCR product of the sample[13].



**Figure No. 3:** The DNA Extraction kit **Figure No. 4:** The Reagents used for the DNA Extraction

### The Sequencing Analysis of *T. rubrum*

The resulting PCR product was packed and send to Applied Biosystems, Bangalore for sequencing analysis which was subjected to confirmatory molecular identification by Sanger sequencing the ITS region. The forward and reverse sequence of the sample was subjected to BioEdit software version 7.0.5 to distinguish similarities and differences between nucleotide sequences and revised manually to improve the alignment precision. The sequence data was incorporated into the Basic Local Alignment Search Tool (BLASTN) of NCBI and Centraalbureau Voor Schimmel cultures (CBS) for the identification and comparison of fungi.

### STATISTICAL ANALYSIS

The statistical analysis was done by *t*-test using SPSS 20 software in order to find the independence of the variables or whether they had similarities in their MIC values with  $P < 0.005$ .

### RESULTS

A total of 375 clinically suspected cases of superficial fungal infection were included in the study. Out of which 31 isolates of *Trichophyton rubrum* were observed after clinical identification with conventional diagnostic approaches such as KOH microscopy and culture method. *T. rubrum* isolates appeared as flat to slightly raised white to cream, pseudo-like to velvety [Figure 1]with a yellow-brown to wine- red pigment. The detailed microscopic morphological presentation was illustrated in [Figure 2] of *T. rubrum*.

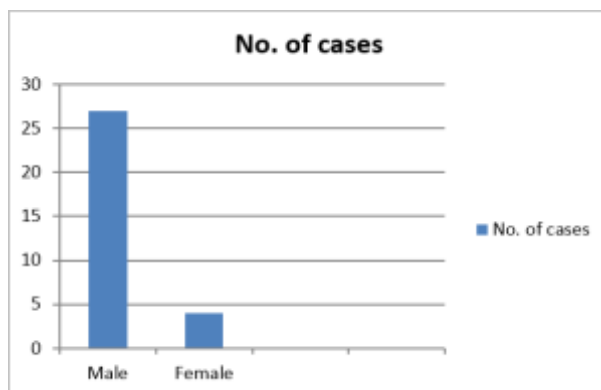
Type of Isolates	Total Number of Isolates
Clinical Isolates	375
<i>Trichophyton rubrum</i>	31

**Table No. 2:** The total number of Isolates

The ratio of Male were more 27 (87%) as compared to the Female 4 (12.9% ). The maximum number of isolates was found in the Skin 28 (90.3%) followed by the Nail 3 (9.6%). The ITZ was most sensitive toward *T. rubrum* than KTZ, TBF, and FCZ. The molecular characterization for the detection of 18SrDNA gene was performed which was confirmed by the Sequencing Analysis.

Gender	Total no. of Cases studies	Percentage
Male	27	87 %
Female	4	12.9%
<b>Total</b>	<b>31</b>	

**Table No. 3 :** Genderwise distribution of the *Trichophyton rubrum* cases.



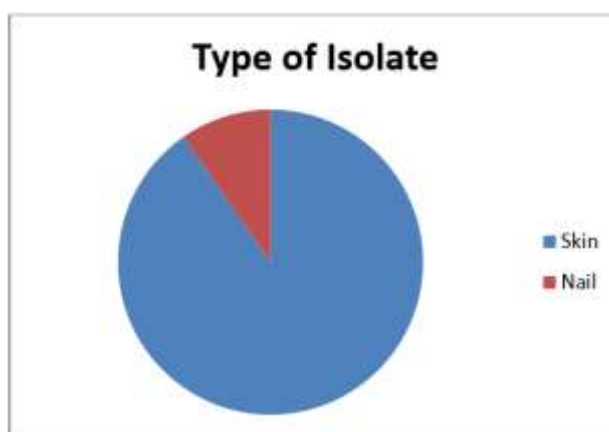
**Graph No. 1:** Graphical representation of Genderwise distribution

S. No.	Age (in years)	No. of Cases	Percentage
1.	0- 10	-	-
2.	11-20	1	3.2%
3.	21-30	16	51.6%
4.	31-40	7	22.5%
5.	41-50	2	6.4%
6.	51-60	2	6.4%
7.	≥61	3	9.6%

**Table No.4 :** Agewise distribution of *Trichophyton rubrum* patients from the study

Type of Isolate	Number of Isolate	Percentage
Skin	28	90.3%
Nail	3	9.6%

**Table No. 5:** The Type of Isolate



**Graph No. 2:** Graphical representation of the Type of Isolates

In our study, the antifungal susceptibility trend of various antifungal agents was evaluated against *T. rubrum* as depicted below in Table No. 6.

Antibiotics	Fungal species	MIC range (µg/ml)
Itraconazole	<i>T. rubrum</i>	0.0156-0.25
Ketoconazole		0.0156-0.5
Terbinafine		0.0312-16
Fluconazole		2-64

**Table No. 6:** In vitro Antifungal susceptibility of *T. rubrum* against different antifungal Drugs

Itraconazole was shown the highest antifungal potential (MIC range 0.0156-0.25 µg/ml) against *T. rubrum* followed by Ketoconazole (MIC range 0.0156-0.5 µg/ml), terbinafine (MIC range 0.0312-16 µg/ml), fluconazole (MIC range 2-64 µg/ml) respectively. Our finding demonstrated that Itraconazole might be used for the management of *T. rubrum* infection. Furthermore, our results confined that there was no significant difference recorded between susceptibility patterns of antifungal agents within studies regions.

#### Dermatophyte-specific PCR and Sequencing of *T. rubrum* genome

*T. rubrum* was identified by Conventional PCR using 18S ribosomal DNA region-specific primers which resulted in 180 bp of undigested PCR product conferring the selective and specific amplification of the 18S rDNA region of *T. rubrum*.



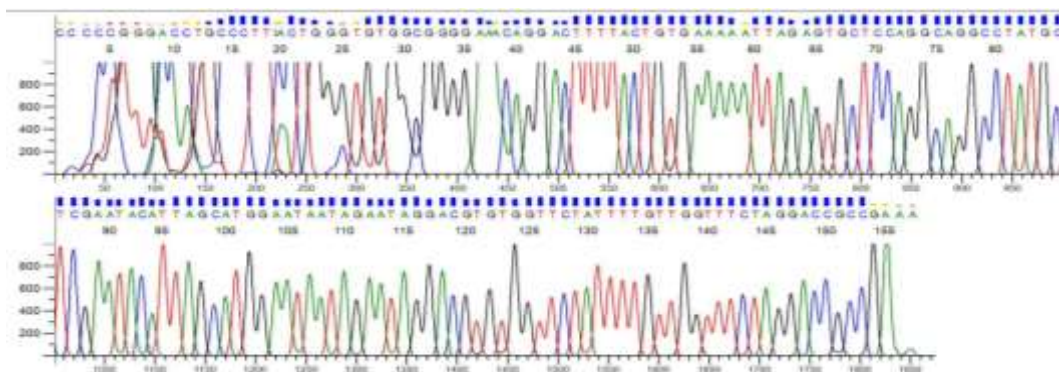
**Figure No. 5:** The DNA isolated visualized by Gel documentation system

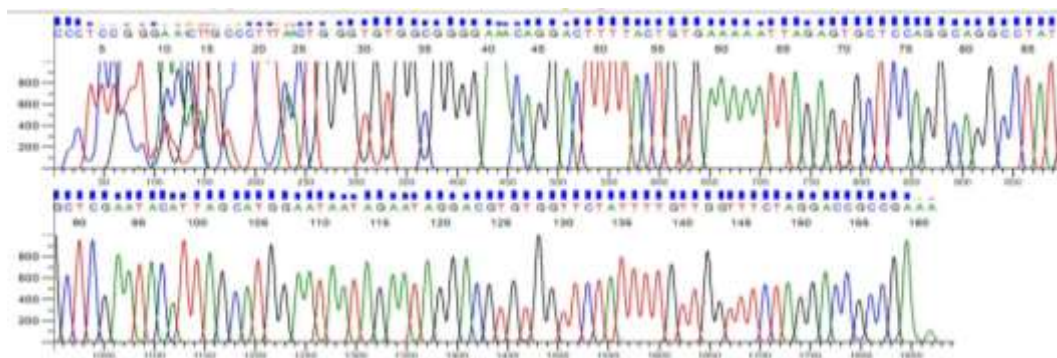


**Figure No. 6:** The DNA Amplification outcome of 18S rDNA region of *T. rubrum* using Agarose gel Electrophoresis.

Lane 1-10 and 12-14 was 18S rDNA region of *T. rubrum* amplified 180 bp region using dermatophyte specific primers. Lane 11 was 100 bp DNA marker. Lane 15 is the positive control for *T. rubrum* and Lane 16, 17 & 18 negative controls without template DNA

Further, *T. rubrum* species was validated through sequencing analysis using the NCBI Gen Bank database (GBD) by BLASTn. The sequence of representative fungal species *T. rubrum* was 100% identical when compared to the gene sequence of control *T. rubrum* strain ATCC 10218.





**Figure No. 7:** Sequencing analysis of *T. rubrum* isolate displaying 100% similarity and coverage to control strain.

## DISCUSSION

Millions of cutaneous fungal infections are observed in humans annually in which dermatophytosis ranks the first with 10-20% of the population been affected by dermatophytes worldwide [14].

Isolation and morphological identification of dermatophytes is a very common practice in medical science today. However, morphological identification and prevalence may vary according to ethnicity and geographical regions. Of note, superficial fungal infection (SFI) affects more than 25% population worldwide [15]. In this study, the most commonly isolated dermatophytosis-causing agent was *T. rubrum* which is predominantly distributed worldwide except in the African population [15].

Until recently, traditional approaches such as KOH and culture-based methods were routinely adopted for the diagnosis and identification of dermatophytes [16]. However, due to phenotypic complexity and variability [17] modern science demands new interventions to timely diagnose dermatophytes. In the present study the maximum number of isolates was found in Males 27 (87%) as compared to the Females 4 (12.9%). This study was in support with the study performed by the other author where the ratio of males (62.6%) was comparatively more than females (37.4%) [18]. This is expectedly due to more social interaction, nature of work, and occupation hazards. Besides, the lower incidence in the female population may be due to prevailing social stigma in the rural area of India and low reporting of women with OPD in the hospital.

It was also observed that the maximum number of cases for *T. rubrum* was found in the age group of 21-30 years of age followed by 31-40 years and the least in the age group below 20 years of age. This study was parallel to the study by other author where the similar results was observed [18]. In the present study, skin infections were found to be the commonest clinical presentation (90.3%) followed by nail infection (9.6%). Similar observation was been made by Patel P et al., and Kannan et al., in their study who reported dermatophytic skin infection to be more common compared to dermatophytic nail and hair infections [19,20]. The high prevalence of skin infection is probably due to severe itching associated with it inducing the patient to seek early medical advice as well as environmental conditions such as hot and humid weather characteristic of this study area and their nature of clothing which adds to increase in warmth of the skin, providing a suitable area for dermatophytes to grow on skin [21].

Molecular techniques offer an advanced technique to identify dermatophytes and are preferred over conventional approaches. In the present study the detection of 18s gene of *T. rubrum* was done. Therefore, the implementation of molecular analysis tools for genomic level identification is very important to distinguish between strain and species at the gene level.

Over the last few decades, the most commonly studied antifungal drugs are ITZ, FCZ, KTZ, and TBF. Response of antifungal agents plays a vital role in the management of dermatophytes. In this study in vitro antifungal sensitivity of different antifungal agents towards *T. rubrum* infection was recorded. We noted that Itraconazole (ITZ) showed the highest sensitivity against *T. rubrum*, while the sensitivity of Ketoconazole (KTZ), terbinafine (TBF), and fluconazole (FCZ) were recorded lower. Afshari et al.[22] also reported the highest sensitivity of ITZ against *T. rubrum* along with *E. floccosum*. Instead, a recent finding from china found TBF as a most sensitive antifungal drug for *T. rubrum* with MIC value (GM = 0.00688 lg/ml, MIC50 = 0.008 lg/ml, MIC90 = 0.015 lg/ml) (Jiang et al., 2021)[23]. However, data demonstrated that *T. rubrum* isolated from the different geographical areas of India was resistant to TBF (Ebert et al., 2020) [24].

Nowadays, PCR- based molecular techniques are higher sensitivity, specificity and fast results include diagnostic of superficial fungal infections. The PCR-based study was employed in this study to identify the isolated *T. rubrum* strain from that particular region of India and further validated by sequencing tool via comparing with control. So, our findings demonstrate that the genomic frequency of isolated *T. rubrum* strain has particularly higher in the Kanpur region of India. DNA sequences are very useful for this purpose and permit an accurate identification. The present study might be helpful for the management of *T. rubrum*-associated fungal infection in that particular region of India. Poor hygiene among low socio economic group, environment and climatic conditions plays a vital role in causing the infection. Early diagnosis and identification is the key for prevention and treatment of dermatophytosis among such population [25].

## CONCLUSION

The dermatophytosis pose a significant health problem in India where the climate is hot and humid. Hence, there is need for frequent monitoring and to create awareness regarding the adequate preventive measures in those regions. Over all the most effective antifungal agents against *T. rubrum* was Itraconazole followed by Ketoconazole. Which allow clinicians to improve the treatment outcome and cope with this disease via strategically tracking aggressiveness.

## ACKNOWLEDGEMENT

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## CONFLICTS OF INTEREST

The authors declare that there was no potential conflict of interest associated with this study.

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