Research highlight

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Copper oxide nanoparticles: an antidermatophytic agent for *Trichophyton* spp.

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Abstract: Copper oxide (CuO) is one of the most important transition metal oxides due to its unique properties. It is used in various technological applications such as high critical temperature, superconductors, gas sensors, in photoconductive applications and so on. Recently, it has been used as an antimicrobial agent against various pathogenic bacteria. In the present investigation, we studied the structural and antidermatophytic properties of CuO nanoparticles (NPs) synthesized by a precipitation technique. Copper sulfate was used as a precursor and sodium hydroxide as a reducing agent. Scanning electron microscopy (SEM) showed flower-shaped CuO NPs and X-ray diffraction (XRD) pattern showed the crystalline nature of CuO NPs. These NPs were evaluated against two prevalent species of dermatophytes, i.e. *Trichophyton rubrum* and *T. mentagrophytes* by using the broth microdilution technique. Further, the NPs activity was also compared with synthetic sertaconazole. Although better antidermatophytic activity was exhibited with sertaconazole as compared to NPs, being synthetic, sertaconazole may not be preferred, as it shows different adverse effects. *Trichophyton mentagrophytes* is more susceptible to NPs than *T. rubrum*. A phylogenetic approach was applied for predicting differences in susceptibility of pathogens.

Keywords: antimicrobial agent; broth microdilution method; SEM; *Trichophyton*; XRD.

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1 Introduction

Nanoparticles (NPs) have been extensively studied over the last decade due to their characteristic physical, chemical, electronic, electrical, mechanical, magnetic, thermal, dielectric, optical and biological properties [1, 2]. Therefore, they may be considered as building blocks of the next generation of technology with applications in many industrial sectors. In particular, NPs are also receiving increasing attention in a large variety of applications in medical science [3, 4]. The oxides of transition metals are a group of important semiconductors, which have applications in magnetic storage media, solar energy transformation, electronics, gas sensors and catalysis [5–8]. Metal oxide NPs are of great interest because of their unique optical, electronic and magnetic properties. Apart from NPs of the other transition metal oxides, copper oxide (CuO) NPs are of special interest because of their efficiency as nanofluids in heat transfer application. For example, it has been reported that a 4% addition of CuO improves the thermal conductivity of water by 20% [9].

The prevalence of superficial dermatophytic infections have increased to an alarming level in the last few years; such that skin mycoses now affects more than 20% world's population [10]. Dermatophytes are a group of keratinophilic fungi that can infect keratinized tissues of humans and animals such as skin, hair and nails, causing dermatophytosis [11]. The predominant agent of dermatophytoses is *Trichophyton* followed by *Epidermophyton* and *Microsporum*. Within the genus *Trichophyton*, *T. rubrum* is the most dominant etiological agent accounting for more than 65% of infections followed by *T. mentagrophytes*, *T. verrucosum* and *T. tonsurans* [12]. It is difficult to control dermatophytes because they have developed resistance to many conventional antidermatophytic agents having an azole group. To overcome this resistance, numerous methods have been employed to control or prevent the growth of fungal cells by the involvement of synthetic as well as herbal antifungal agents [13, 14]. Recently, nanomaterials

have attracted attention due to their unique physical and chemical properties which differ significantly from their conventional counterparts [15, 16]. Among them, metal and metal oxide nanomaterials are among the most highly produced nanomaterials; their available applications include catalysis, sensors, and environmental remediation and personal care products [17].

In the present investigation, the CuO NPs were synthesized using a chemical method and the product was structurally characterized with the aid of a scanning electron microscope and an X-ray diffractometer. Further, these NPs were tested against two predominant dermatophytic species, i.e. *T. mentagrophytes* and *T. rubrum* using the Clinical and Laboratory Standards Institute (CLSI) recommended broth microdilution antifungal susceptibility method [18].

2 Materials and methods

2.1 Synthesis of CuO NPs

The four-step preparation scheme for CuO NPs was carried out by modification of a method described by of Dang et al. [19], which starts with dissolving copper (II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 0.01 M), salt, in deionized water to obtain a blue solution. Next, polyethylene glycol 6000, (PEG 6000, 0.02 M) was dissolved in water and added to the aqueous solution containing the copper salt while vigorously stirring. In this step, the solution changed from blue to white. In the third step, ascorbic acid (0.02 M) and sodium hydroxide (NaOH, 0.1 M) were dissolved in water and added to the synthesis solution. Color change occurred in the aqueous phase from white to yellow. Finally, a solution of sodium borohydride ($NaBH_4$, 0.1 M) in deionized water was prepared and added to the solution under continuous rapid stirring. An instant color change occurred in the aqueous phase from yellow to blackish brown. The appearance of this dark color indicates the onset of the reduction reaction. The source of electrons for the reaction was BH_4^-. The mixture was further stirred rapidly for around 10 min in ambient atmosphere, to allow the reaction to complete. Stable aqueous dispersions of CuO NPs (50 mg/ml) were used as stock solution by dispersing 50 mg/ml of CuO NPs in double distilled water through ultrasonication. All the chemicals utilized were of analytical grade and procured from Himedia, India.

2.2 Characterization studies

The average size of NPs was determined using photon correlation spectroscopy (Brookhaven Instruments, USA). The morphology of CuO NPs was characterized using scanning electron microscopy (SEM) (Zeiss Ultra SEM, Germany), operated at an accelerating voltage of 5 kV. For SEM, a small amount of synthesized NP powder was placed on a silicon grid and kept desiccating for 72 h, allowing the moisture to evaporate. Further, the crystalline nature of prepared CuO NPs was observed using a powder X-ray diffractometer (Rigaku-Smartlab X-ray Diffractometer, Japan) using CuKα radiation, at 40 keV in the two theta ranges of 10°–80°. In addition, to find out the impact of CuO NPs on the mycelium of dominant *T. rubrum*, SEM studies were also performed at the National Centre of Experimental Mineralogy and Petrology, University of Allahabad, Allahabad, India using an electron probe microanalyzer (JXA8100, JEOL, Japan).

2.3 Selection of test pathogens

Different symptoms generated by dermatophytes were seen in some patients (Figure 1); they are all caused by *Trichophyton*, *Microsporum* and *Epidermatophyton*, but since *Trichophyton* genus is most prevalent regarding dermatophytoses, we selected it for further studies.

Two species of *Trichophyton* genus were selected for this study (Figure 2). Both of the species were procured

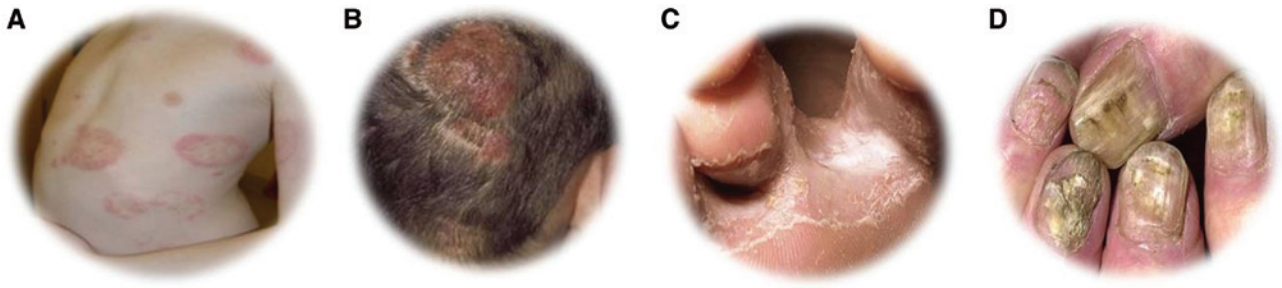

Figure 1: Different forms of dermatophytoses: (A) *Tinea corporis*, (B) *Tinea capitis*, (C) *Tinea pedis*, (D) *Tinea unguium*.

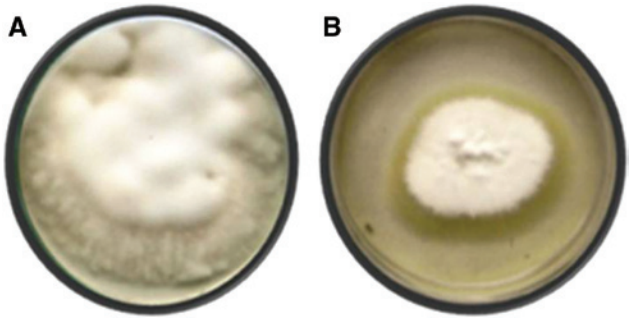

Figure 2: Cultures of dermatophytes on Sabouraud dextrose agar (SDA) medium: (A) *Trichophyton mentagrophytes,* (B) *Trichophyton rubrum*.

from the Microbial Type Culture Collection (MTCC), Chandigarh, India. *Trichophyton rubrum* (MTCC 296) and *T. mentagrophytes* (MTCC7687) were tested *in vitro* to evaluate the antidermatophytic activity of CuO NPs. These strains were recultured on Sabouraud dextrose agar medium, supplemented with 0.05% streptomycin, in Petri dishes and incubated at 30°C for 7 days. The isolates were again cultured in Petri dishes and incubated for another 5–7 days at 32°C.

2.4 Antidermatophytic susceptibility testing assay

A broth microdilution protocol was performed according to CLSI guidelines in flat bottom 96-well microtiter plates. Here, RPMI 1640 medium (with L-glutamine but without sodium bicarbonate, GIBCO BRL, Life Technologies, Woerden, The Netherlands) buffered to pH 7.0 with 0.165 M 3-*N*-morpholinopropanesulfonic acid (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as assay medium for viability of dermal pathogens. Seven-day-old cultures of each pathogen were collected with an inoculating needle and suspended in sterile saline containing 0.1% of Tween 80. After allowing heavier particles to settle down, cells (with spores) were transferred to 5 ml of RPMI1640, followed by preparation of final inoculum density.

The turbidity of the full growing fungal suspension was compared and matched with the turbidity of 0.5 McFarland units. The McFarland 0.5 standard corresponds approximately to a homogeneous suspension of 1.5×10^6 cells/ml. An amount of 100 µl of medium was transferred to each well, followed by 90 µl and 80 µl of medium in each well of Column 3 and Column 4, respectively. Then, 10 µl and 20 µl of CuO NPs suspension was added in each well of Column 3 and Column 4, respectively. Serial dilutions were performed from Column 4 to Column 11, to obtain the final NPs concentrations, which varied from 2.5 mg/ml (4th well) to 0.020 mg/ml (11th well). Fungal inoculum of 100 µl was added to each well except all wells of Column 2 and Column 3, making the final volume of 200 µl. NPs free well (Column 12) containing medium and inoculum served as a growth control. Column 1 which contained medium, inoculum and formaldehyde, served as a negative control. Column 2 served as medium control and Column 3 served as the drug control containing 200 µl medium and 190 µl medium with 10 µl of drug, respectively (Figure 3).

2.5 Quantification assay for growth of pathogens

After inoculation, the microtiter plates were kept in a wet chamber and incubated for growth of pathogens at 37°C inside a B.O.D. incubator. The optical density ($OD_{530\ nm}$) was recorded spectrophotometrically periodically at 24 h, 48 h and 72 h (3 times). After the first 24 h of incubation inside the plate reader, incubation of the microtiter plates was continued under the same conditions in same incubator. The changes in ODs over concentration of NPs were used to generate growth inhibition curves at each concentration.

2.6 Determination of minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations (MICs) were determined visually and spectrophotometrically after the abovementioned incubation periods for each species according to the CLSI guidelines. For CuO NPs, the MIC was determined as the lowest NP concentration showing

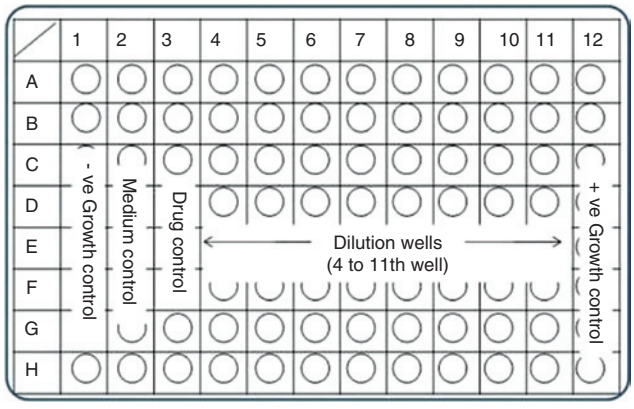

Figure 3: Ninety-six well microtiter plate with designed broth microdilution protocol.

absence of growth visually or 80% growth inhibition compared with the growth in the drug-free well. The 50% growth inhibition compared to the growth in the drug-free well was determined as IC_{50}.

3 Results and discussion

3.1 Characterization studies

The morphology of CuO NPs was examined under SEM (at 100 KX) (Figure 4A), and demonstrated that a bulk quantity of bunches of nanorods were present. These particles were closely packed with radiating structures in nanometric scale. The picture confirms the formation of NPs and substantiates approximate rod-shaped or flower-shaped NPs when aggregated in clusters. The X-ray diffraction (XRD) pattern of the powder was studied with the diffraction angle 20°–80° (Figure 4B). No other characteristic impurities peaks were present, which also confirmed that the product obtained was in the pure phase. When samples were compared, XRD spectra showed strong diffraction peaks at 35.9, 39.1 2θ which corresponds to (002), (111) crystal planes and indexed crystalline structure of CuO NPs. However, the average particle size of CuO NPs was found to be 160 nm by a Zetasizer.

3.2 Antifungal susceptibility assay

The antifungal activity of NPs of CuO was quantitatively assessed by observing OD at 530 nm (OD_{530}, Figure 4) for determining the MICs as well as IC_{50} values after incubation of 24 h, 48 h and 72 h, but since good growth inhibition was observed after 48 h, this was taken as standard in the present investigation (Figure 5). The colloidal suspension of CuO NPs showed broad spectrum antifungal activity. The CuO NPs generated very good values of antidermatophytic activity in the form of MICs (mg/ml) (Table 1) against both of the pathogens, i.e. *T. mentagrophytes* (MIC 1.78, IC_{50} 0.65 mg/ml) *T. rubrum*, (MIC 2.19, IC_{50} 1.08 mg/ml). Sertaconazole, used as synthetic standard, showed inhibitory effects on both tested dermatophytes (0.733 mg/ml, 0.867 mg/ml, against *T. mentagrophytes* and *T. rubrum*, respectively); however, due to a high incidence of disease reoccurrence and other side effects such as itching, burning, etc., this cannot be used as a permanent solution as compared to CuO NPs, which might be proven to be more acceptable topically and safe (Figure 6). A scanning electron micrograph exhibited morphological deformity, ultimately lysis in the mycelium of *T. rubrum*-treated (4 h) CuO NPs. By contrast, untreated *T. rubrum* mycelium was found to be normal with microconidia (Figure 7A and B). The study also suggests that the dermatophytic population was decreased with an increased treatment concentration. Based on these results, it can be concluded that these synthesized CuO NPs had significant antifungal actions on both of the species, which may be attributed to the greater abundance of amines and carboxyl groups on their cell surface and greater affinity of copper ions toward these groups [20]. CuO NPs show efficient action due to their extremely large surface area, which provides better contact with pathogens. Copper ions released subsequently may bind with DNA molecules and lead to disorder of the helical structure by cross-linking within and between the nucleic acid strands, resulting in the lysis of dermatophytic cells

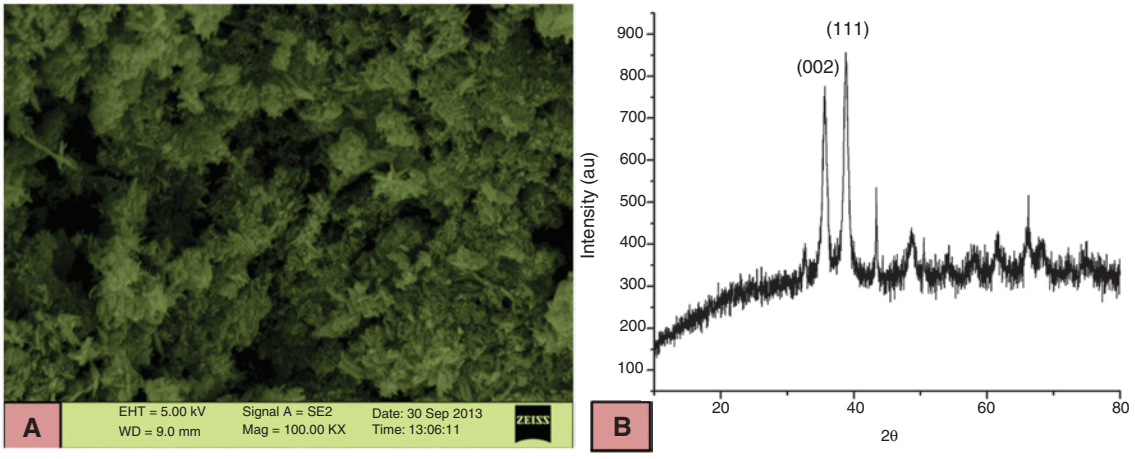

Figure 4: (A) Scanning electron microscopy (SEM) micrograph of CuO nanoparticles (NPs) at 100.00 KX; (B) X-ray diffraction pattern of CuO NPs.

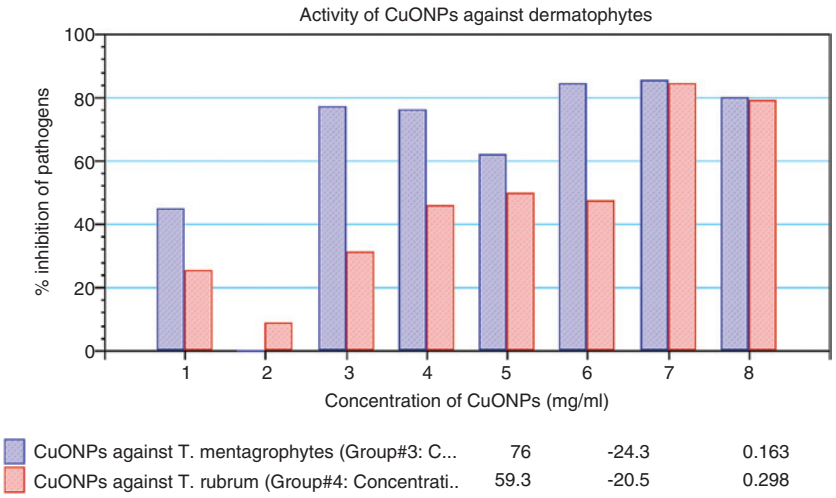

Figure 5: Graphical representation of antifungal susceptibility of dermatophytes against CuO nanoparticles (NPs).

Table 1: Antidermatophytic activities of CuO nanoparticles (NPs) and sertaconazole against dermatophytes (mg/ml).

Pathogen	CuO NPs	Sertaconazole
	MIC	MIC
Trichophyton mentagrophytes	1.78	0.733
Trichophyton rubrum	2.19	0.867

MIC, minimum inhibitory concentration.

[21, 22]. It has been also reported that copper ions penetrate inside cells and also disrupt biochemical processes [23]. The exact mechanism behind this is not known and needs to be studied further.

The greater the size of the NPs (more than 30 nm) the safer they are for human health, as they will not enter the bloodstream through skin when applied with a lotion or a cream-based product. No study has reported that NPs >30 nm can penetrate human skin, whereas chemical ingredients of sunscreen, which are molecular in size and thus significantly smaller than NPs, are designed to be absorbed into the skin and therefore can get into the blood. The biggest concern with NPs in cosmetics is the threat of inhalation when they are used in powders and sprays; this is not a matter of concern with CuO NPs as they will be dispersed in cream or lotion [24].

With the view to reducing the cost of experimentation for analyzing the variability in MICs against CuO NPs, different dermatophytes were exposed to phylogenetic analysis. To explain the susceptibility of pathogens, these species were analyzed using ClustalW and bootstrapping NJ plotting using MEGA 4 (Version 4.0.1). Broad phylogenetic trees of various dermatophytic strains were found homologous in the *BlastX* search of chitin synthase gene

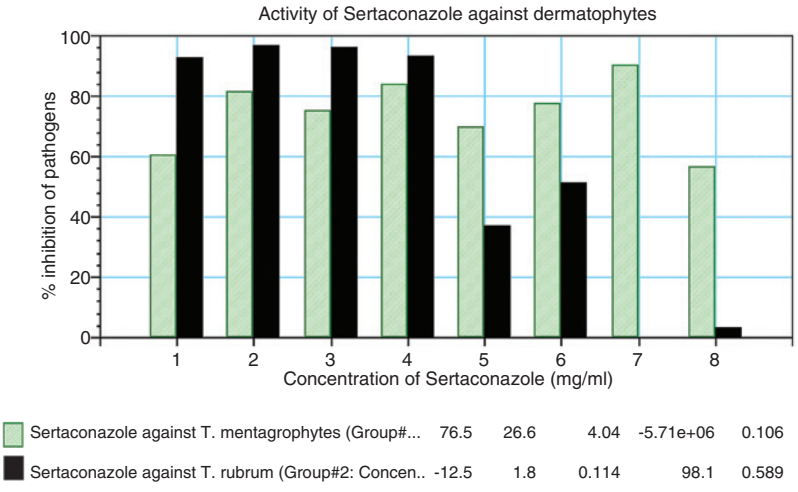

Figure 6: Graphical representation of antifungal susceptibility of dermatophytes against sertaconazole.

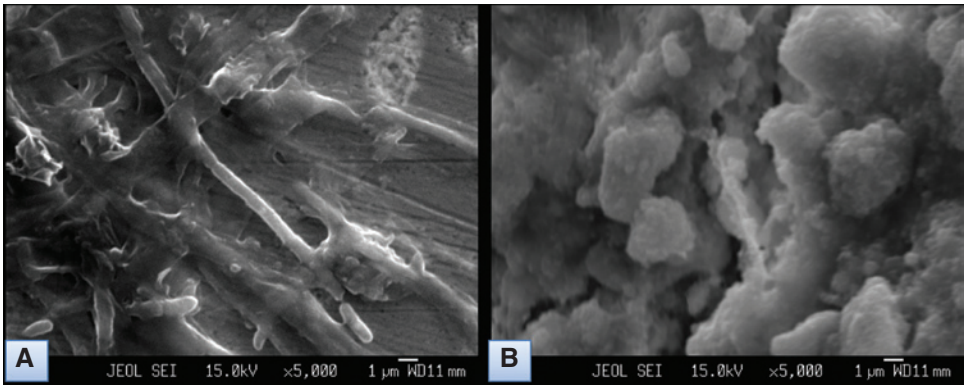

Figure 7: Scanning electron micrograph of: (A) normal, (B) lysed mycelium of *Trichophyton rubrum*.

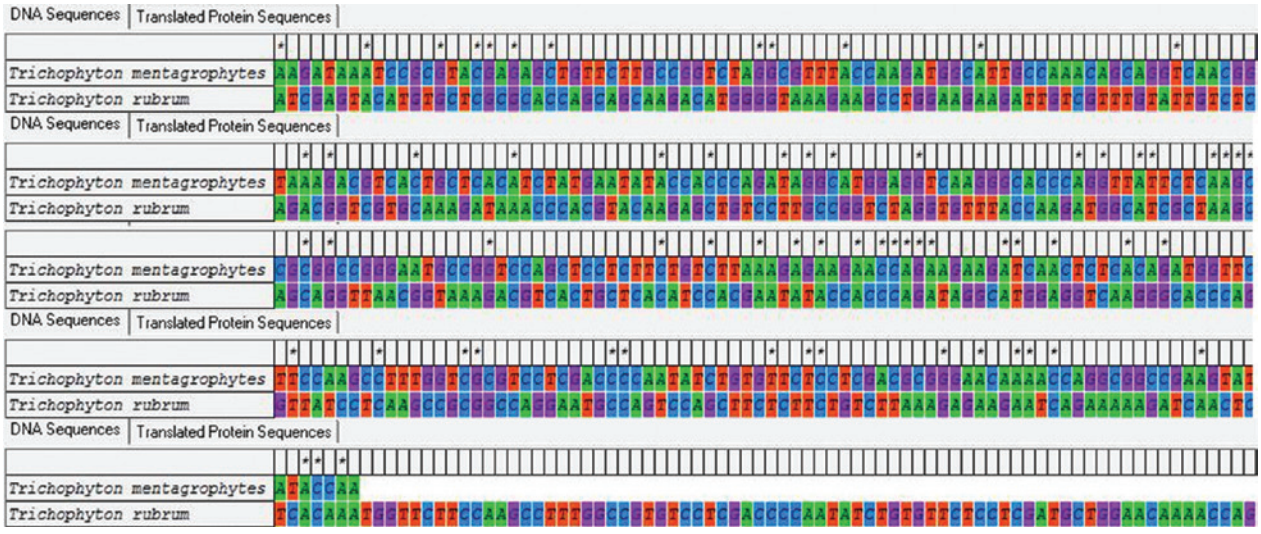

Figure 8: Alignment of the chitin synthase gene 1 (*chs* gene 1) of *Trichophyton mentagrophytes* and *Trichophyton rubrum* by the bootstrapped ClustalW program in MEGA 4 and its phylogenetic tree constructed using the sequences of (*chs* gene 1) by the bootstrapped N-J plot used for the antifungal susceptibility test (sequences of the strains were obtained from NCBI database).

(*chs gene 1*). The gene alignment and protein sequences of *chs gene 1* extracted from NCBI blast have shown homology in sequences (Figures 8 and 9) and greater confidence levels in the 1000 bootstrapped N-J plot. From the phylogenetic analysis it was evident that *T. mentagrophytes* and *T. rubrum* are far apart in the alignment tree, which may be a factor responsible for their sensitivity against NPs. In the phylogenetic tree the two pathogens, i.e. *T. rubrum* and *T. mentagrophytes*, lie far apart, yet the NPs were active against them. It is concluded that pathogens aligned between these two would also be susceptible to CuO NPs according to their alignment in the tree, thereby reducing the cost of experimentation. Moreover, *T. rubrum* is aligned in the bottom of the tree, showing its less evolved characters during the course of evolution; it is thus less susceptible to NPs.

4 Conclusions

Turbidimetry is a rapid, accurate and quantitative method for monitoring the growth of dermatophytic fungi which is based on the CLSI recommended broth microdilution method. The study has opened a wide channel for exploring the sophisticated nanotechnological tools in developing novel antifungal agents. The treatment with chemically synthesized CuO resulted in lysis of dermatophytic cells. The prediction of the susceptibility of the pathogenic dermatophytes towards active NPs based on their phylogenetic position is a novel approach. Further, the present findings strongly support the potential of the CuO NPs as a useful cosmeceutical and can be used in developing effective formulations against dermatophytoses, after successful topical testing in collaboration of

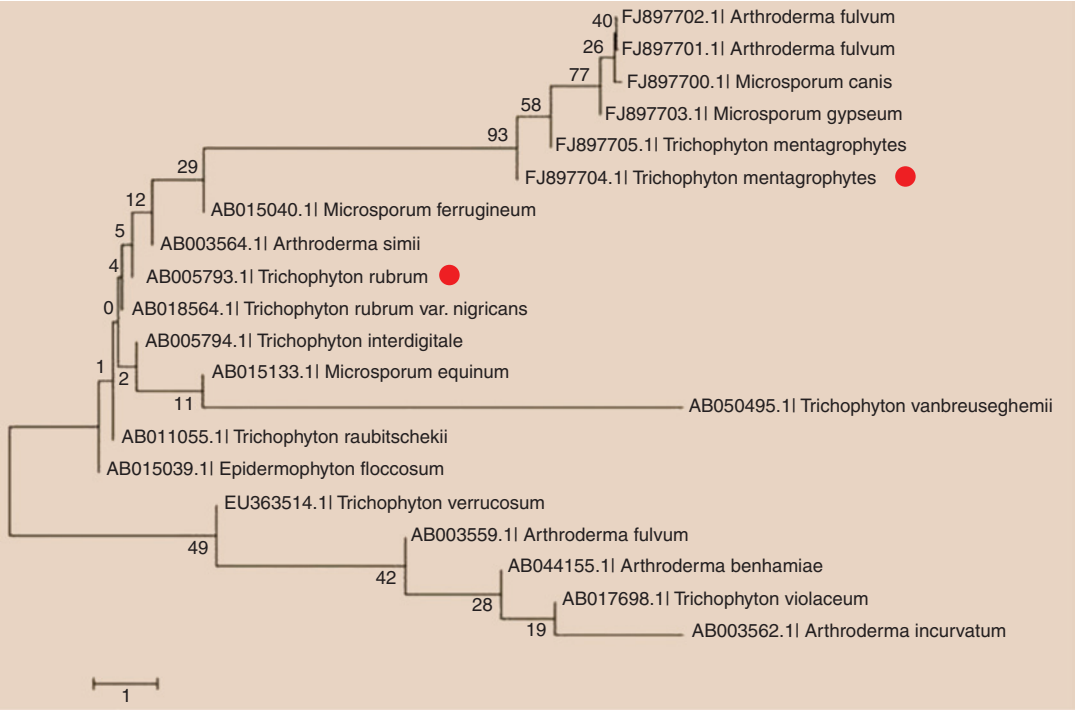

Figure 9: Molecular phylogenetic tree constructed using the sequences of chitin synthase gene 1 (*chs* gene 1) of available strains of the some pathogenic fungi (ClustalW 1.6 alignment). The number of branch points represents the percentage of 1000 bootstrapped datasheets showing specific internal branches.

Dr. A.K. Bajaj, former head of the dermatology department of Moti Lal Nehru Medical College, Allahabad, India, which is in progress.

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