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Original Article

Enhanced Antifungal Efficacy of KZ-β/γ-CD Capped Silver Nanoparticles against *Trichophyton rubrum*

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ABSTRACT

Background: The present medications are inefficient in treating fungal infections, so there is a need to develop an efficient antifungal treatment with minimal side effects and rapid healing. Ketoconazole (KZ) is frequently prescribed for managing fungi-related skin infections However, it is a poorly soluble with low absorption, making it less effective. **Objectives:** The study aims to enhance the absorption and effectiveness of the antifungal medication KZ by complexing it with β and γ Cyclodextrins (CD) coated with silver nanoparticles (AgNps). **Materials and Methods:** KZ inclusion complexes with AgNps capped β- and γ-CD (β/γ-CD-KZ-AgNp) were prepared and analysed by UV-vis spectroscopy, FTIR, SEM and XRD. The antifungal efficacy of these complexes against *Trichophyton rubrum* was assessed by Minimum Inhibitory Concentration (MIC) and Disc-Diffusion assay. **Results:** The drug KZ was successfully loaded into the AgNp-capped β-CD and γ-CD inclusion complexes, as indicated by a UV peak at 293 nm. The FTIR spectra of the complexes (β/γ-CD-KZ-AgNp) showed peaks for aldehyde, CH stretching and-OH stretching for AgNp, CDs and KZ. The crystalline nature and AgNp coating were confirmed by the X-ray diffraction. Scanning electron microscope images revealed that the complexes were less agglomerated, spherical and embedded with AgNps. The minimum inhibitory concentration of the complexes against *Trichophyton rubrum* was much higher than that of the drug alone, with 0.5 µg/mL MIC for β-CD-KZ-AgNp and 1 µg/mL for γ-CD-KZ-AgNp in comparison to the control drug KZ (2 µg/mL). The complexes also demonstrated excellent antifungal activity against *T. rubrum*, with inhibition zone of 29.7 mm for β-CD-KZ-AgNp and 29.1 mm for γ-CD-KZ-AgNp compared to 14.74 mm for the drug alone. **Conclusion:** The inclusion complexes show great potential as a treatment against fungal infections.

Keywords: Ketoconazole, Silver Nano-particles, β and γ Cyclodextrin, inclusion complexes, Fungal Infection.

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INTRODUCTION

Fungal infections are a major source of medical disorders in both immune-compromised and non-immune compromised individuals, placing a great burden on global healthcare providers. These infections could be invasive, superficial, or both. 20% to 25% of the world's population is thought to be afflicted with superficial fungal infections and the frequency rises yearly.[1] While considering various microbial infections, fungal infections are especially significant because of their reported rapid rise.[2] Most of the saprophytic fungi that were prevalent in the past have evolved into opportunistic human infections, according to recent studies, particularly in individuals with immunological diseases.[3] *Trichophyton rubrum* is the most common etiological agent isolated from clinically diagnosed cutaneous dermatophytotic lesions and it has a substantially higher transmission capability in humans. Since the fungus feeds on keratin, these infections mostly affect keratinized layers like skin, hair and nails.[4] The available antifungal drugs are insufficient to treat dermatophytosis; therefore, it is necessary to develop more potent antifungal treatments with fewer limitations, less side effects and a broad spectrum of antifungal activity.

Complications in treating dermatophytosis include a lack of antifungal that are effective against dermatophytes and the drug resistance to the fungus[5] and drug undergoing first pass metabolism, poor solubility and permeability. Infections are treated with the azole drug Ketoconazole (KZ).[6,7] It has been associated to cutaneous irritation (free drug), large molecular weight (531.41 Da), low water solubility (0.04 mg/mL), high oral dose (200 mg/day), short elimination half-life (3.3 hr) and unfavorable effects on oral administration.[8,9] The biopharmaceutical classification system divides drugs into four groups depending on their solubility and permeability; ketoconazole is classified as a class II

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drug due to its high permeability and low solubility. In essence, a drug's solubility in aqueous media significantly impacts its pharmacokinetic and pharmacodynamic characteristics. This makes it challenging to produce drugs that are poorly soluble, particularly for oral, parenteral and topical forms.[10] As a result, many chemical and physical techniques were used to enhance drug solubility.

Cyclodextrins (CDs) have been a solubilizing compound in pharmaceutical formulations for many years.[11] Different hydrophobic substances can be incorporated in the cavities of CDs due to their structural properties, which include a hydrophilic outer surface and a hydrophobic interior cavity. It is possible to control the release of aromatic compounds due to the hydrophilic surface and hydrophobic cavity of β-Cyclodextrin (β-CD).[12,13] Along with β-CD, γ-CD has also gained captivating interest as topical antifungal formulations.[14,15] The present antifungals have limitations in terms of efficiency, efficacy, selectivity, toxicity, mechanisms of resistance and activity spectrum.[16]

Nanotechnology has drawn a lot of attention in recent decades[17] because of its great nano size, which enables it to quickly absorb the drug.[18] AgNPs have demonstrated exceptional antibacterial activity through innovations in nanotechnology, owing to their small size and huge specific surface area.[19,20] As a result, they are frequently employed in the antibacterial sector.[21] Nevertheless, AgNPs readily aggregate, significantly decreasing their antibacterial efficacy.[22] Thus, inclusion complexes improve efficacy by decreasing agglomeration.

This study aims to enhance antifungal efficacy against *Trichophyton rubrum* dermatophyte by inclusion complexing KZ into β- and γ-CD and capping AgNps with different ratios and their structural, morphological and functional features were evaluated, The *in vitro* antifungal activity against *Trichophyton rubrum* was studied using Disc-Diffusion assay and Minimum Inhibitory Concentration (MIC) tests. The combination of β-CD/KZ/AgNps has economically cheaper, high efficacy and antifungal qualities, making it ideal for treating fungal infections.

MATERIALS AND METHODS

Materials

Ketoconazole (KZ) (>95%), β and γ Cyclodextrins (CD) were purchased from M/s Yarrow Chem, Mumbai. Sabouraud Dextrose Agar (SDA) selection media plates, silver nitrate and other solvents were procured from Himedia Laboratories, Mumbai, India. *Trichophyton rubrum* (ATCC 28188) strain was a gift from Dr. Sanjana A.S from BGSIMS Repository, DMSO was supplied from Thermo Fisher Scientific and all the other chemical used in this study are of analytical grade.

Methods

Silver nanoparticle (Ag-Np) Synthesis

To synthesize AgNps, 2 mM silver nitrate solution was added to a 50 mL beaker, followed by 20 mM ice cold sodium bromide was added dropwise with constant stirring for 24 hr. After centrifugation, the precipitate was cleaned with deionized water, dried and separated to produce Silver Nanoparticles (AgNps). The particle size measurement and distribution of AgNp's was determined using the Anton Paar Litesizer 500 particle size analyser, which operates in the back scatter automatic mode.[23] The AgNps were dispersed in triple distilled water (1:50 ratio) and sonicated for 30 min before introducing the sample into the cuvettes for measurement.

Ketoconazole complexation with β and γ-CD

The kneading technique was utilized to complex Ketoconazole (KZ) with β and γ-CD. KZ and β-CD and KZ and γ-CD were combined with 100 mL of distilled water in a 250 mL beaker to create complexes at different molar ratios (1:1, 1:2 and 1:4). After that, the suspension was vigorously agitated in a shaker incubator for three days at 25±2°C. After the mixture had dried and been filtered through Whatman filter paper, the sediment was weighed.[24]

Doping of AgNPs on the KZ-β and γ-CD inclusion complexes

AgNp's were embedded on the β and γ CD-KZ inclusion complexes by adding 100 mg of AgNp to the inclusion complex in 30 mL of water. After adjusting the pH to 11, the mixture was agitated for 3 hr at 80°C. To obtain β-CD-KZ-AgNp and γ-CD-KZ-AgNp, the resultant opaque solution was separated by centrifugation for 15 min at 10,000 rpm and then dried at room temperature.[25]

Characterization of the Inclusion complexes

UV-vis spectroscopy

A 10 µg/mL drug solution was prepared in phosphate buffer with a pH of 7.4. The solution was scanned against phosphate buffer as a blank in a double beam UV spectrophotometer (T90+ UV-vis Spectrophotometer) at wavelengths ranging from 200 to 400 nm. The absorption maxima (λ_{max}) values were noted for drug from the respective plots of absorbance v/s wavelength. The same procedure employed for inclusion complex to note the drug presence in the inclusion complex.[26]

FT-IR Spectroscopy

The inclusion complexes weighing 50 mg were taken in a mortar and triturated with 150 mg of Potassium Bromide (KBr). The triturated samples were placed in a holder and scanned between 400 and 4000/cm by diffraction method. The spectrum of the complexes β-CD-KZ-AgNp and γ-CD-KZ-AgNp (1:1, 1:2,

1:4) was compared with pure KZ, β-CD, γ-CD, AgNps. The FT-IR spectra were measured using SCHIMADZU FT-IR spectrophotometer.[27,28]

Field Emission Scanning Electron Microscopy (FESEM)

The particle size, morphology and size distribution of inclusion complexes were assessed using Scanning Electron Microscopy (FESEM instrument from Thermo scientific- Apreo).[29] Before being placed on the stubs, the samples underwent a gold palladium coating. The coated films were examined using a microscope and images were taken at appropriate magnifications. The morphological examination was conducted using the S. IR Spirit-L software.

X-ray Diffraction

A Siemen D5000 X-ray diffractometer was used to perform crystallographic studies on the inclusion complexes. Plotting and measurement of 2θ values were performed using Cu-kα radiation at 40 kV and a scan rate of 5°/min.[30]

In vitro anti-fungal activity

Inoculum preparation

The glycerol stock of *Trichophyton rubrum* (ATCC 28188) was revived and streaked onto sterile Sabouraud's Dextrose Agar (SDA) plates and incubated for 10-12 days at 25°C. Following incubation, the culture was identified and the optical density of the fungal suspension was adjusted to ~5×10^6 spores/mL for the experiment.[31]

Minimum Inhibitory Concentration assay (MIC)

Stock solutions for the MIC studies were prepared in accordance with CLSI guidelines.[31] The 1:1, 1:2 and 1:4 ratios of inclusion complexes of β-CD-KZ-AgNp and γ-CD-KZ-AgNp were prepared in 1 mL of 0.9% trisodium citrate, while the control medication ketoconazole (32 mg/mL) was prepared in 0.1% DMSO. The master stock was further diluted to attain various concentrations of working solutions.

The MIC experiment was conducted using a 96-well microtiter plate with a total assay volume of 200 µL. Each well was supplemented with 50 µL of *T. rubrum* fungal culture containing approximately 5×10^6 spores/mL. Additionally, AgNps, β-CD, γ-CD, KZ, β-CD-AgNp, γ-CD-AgNp and various ratios (1:1, 1:2, 1:4) of β-CD-KZ-AgNp and γ-CD-KZ-AgNp inclusion complexes were added. The control groups included Broth Control (BC, broth only), Vehicle Control (VC, 0.1% DMSO in broth culture) and *T. rubrum* Culture Control (CC). The microtiter plate was incubated for 8-9 days at 26±1°C. To monitor fungal growth, the optical density at 520 nm was measured following incubation.

Disc Diffusion assay

The zone of inhibition was observed using the Disc Diffusion technique. KZ was administered to the control discs at a dose of 2 µg/mL. Test discs were prepared at a concentration of 32 µg/mL using a 1:1 ratio of both inclusion complexes. The discs were incubated at 25°C for duration of twelve days on SDA plates containing *T. rubrum* culture. The diameters and zone of inhibition were calculated.[29]

RESULTS

Characterization of prepared Silver Nanoparticles (AgNp)

AgNps were characterized using the method described by Saied *et al.*[32] The reaction mixture presented a color change from light yellow to brownish after 24 hr, indicating the formation of AgNps. The particle distribution curve showed two characteristic peaks, a small peak at 8.39 nm and a larger peak at 192 nm that accounted for 99.6% of the distribution, confirming the nanoparticle nature for the AgNps (Figure 1).

Biophysical Characterization of the inclusion complexes

UV-vis Spectroscopic analysis

The UV-vis spectra of KZ, β-CD, β-CD-KZ and β-CD-KZ-AgNp (at 1:4, 1:2 and 3:1 ratios) are shown in Figure 2a, while the spectra of γ-CD, γ-CD-KZ and γ-CD-KZ-AgNp (also at 1:1, 1:2 and 1:4) are shown in Figure 2b. Pure KZ typically presented a UV peak at 293 nm and the appearance of this peak in all the inclusion complexes, regardless of the composition and concentration, confirm the successful incorporation of KZ in the composites. The peaks were observed to be optimum at 1:1 and 1:2 ratios of inclusion complexes.

FTIR studies

FTIR spectra validated the formation of the inclusion complexes, providing insights into the molecular interactions and structural changes that occur inside them, allowing for a better understanding of their formation and properties. The FTIR spectra typically exhibit a large peak at 3236 cm^{-1} to indicate the O-H stretching. Additionally, a C=O band is observed at 1632 cm^{-1} and the aldehyde C-H stretching is observed at 2422 cm^{-1}. In addition, the stretching of C-C and C-N bonds is represented by bands at 1383 and 1120 cm^{-1}, respectively. Figure 3 depicts the FTIR bands corresponding to different ratios of AgNps, β-CD-KZ-AgNp (1:1, 1:2) and γ-CD-KZ-AgNp (1:1, 1:2). The β-CD-KZ-AgNp 1:1 and 1:2 samples exhibited typical peaks for β-CD at 3347, 1637, 1157 and 1026 cm-1, indicating -OH bending, C=O, H-O-H, asymmetric and C-O-C stretching vibrations. Since both β and γ-CD are structurally similar, the γ-CD-KZ-AgNp 1:1 and 1:2 samples also showed identical peaks.

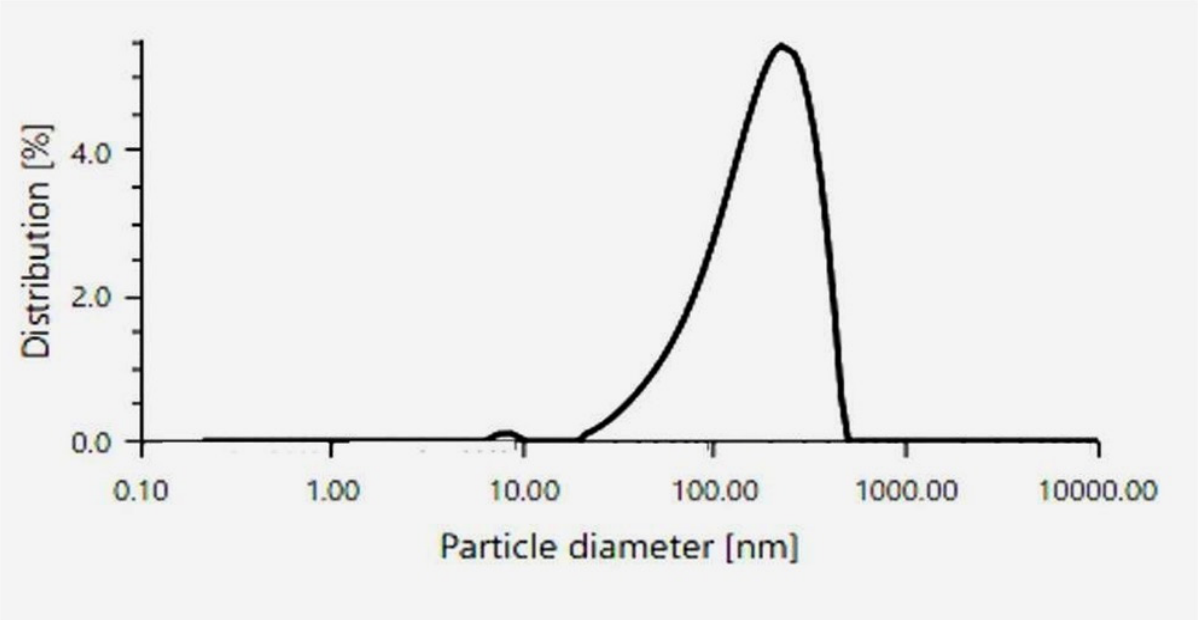

Figure 1: The Particle size distribution of Silver Nano particles measured in nanometres (nm) along the x-axis against the percentage distribution (%) on the y-axis.

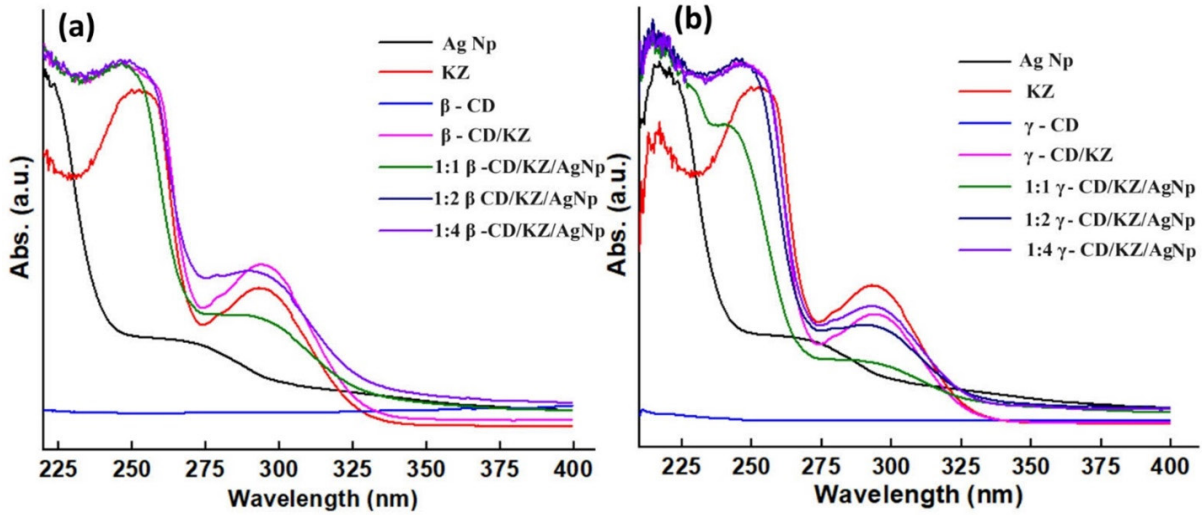

Figure 2: UV-Visible spectra of prepared inclusion complexes. (a) UV-Vis absorption spectra of AgNP's, KZ, β-CD, β-CD/KZ and β-CD/KZ/AgNp in varying ratios; (b) UV-Vis absorption spectra of Ag Np, KZ, γ-CD, γ-CD/KZ and γ-CD/KZ/AgNp in varying ratios, with both spectra measured over the wavelength range of 225-400 nm.

EDX and FESEM Analysis

The prepared composites' elemental composition was validated using Energy Dispersive X-ray Spectroscopy (EDX) and their surface structure was investigated using Field Emission Scanning Electron Microscopy (FESEM). Figure 4a, 4b and 4c displays the FESEM micrographs of AgNPs, β-CD-KZ-AgNp 1:1 and γ-CD-Keto-AgNp 1:1 and elemental composition inset tables. The EDX examination revealed the presence of Ag, C, O and N, but no other elements were found in the samples.

XRD analysis

The existence of AgNP's was confirmed by the XRD patterns in Figure 5. In the (111), (200), (220) and (311) planes, respectively, distinct Bragg reflections are produced for 2θ values of 38.53°, 44.67°, 65.08° and 78.06°, verifying the spherical structure of nanomaterials. The β- and γ-CDs were identified as the origins of the large peaks observed in the composites between 10°C and 25°C.

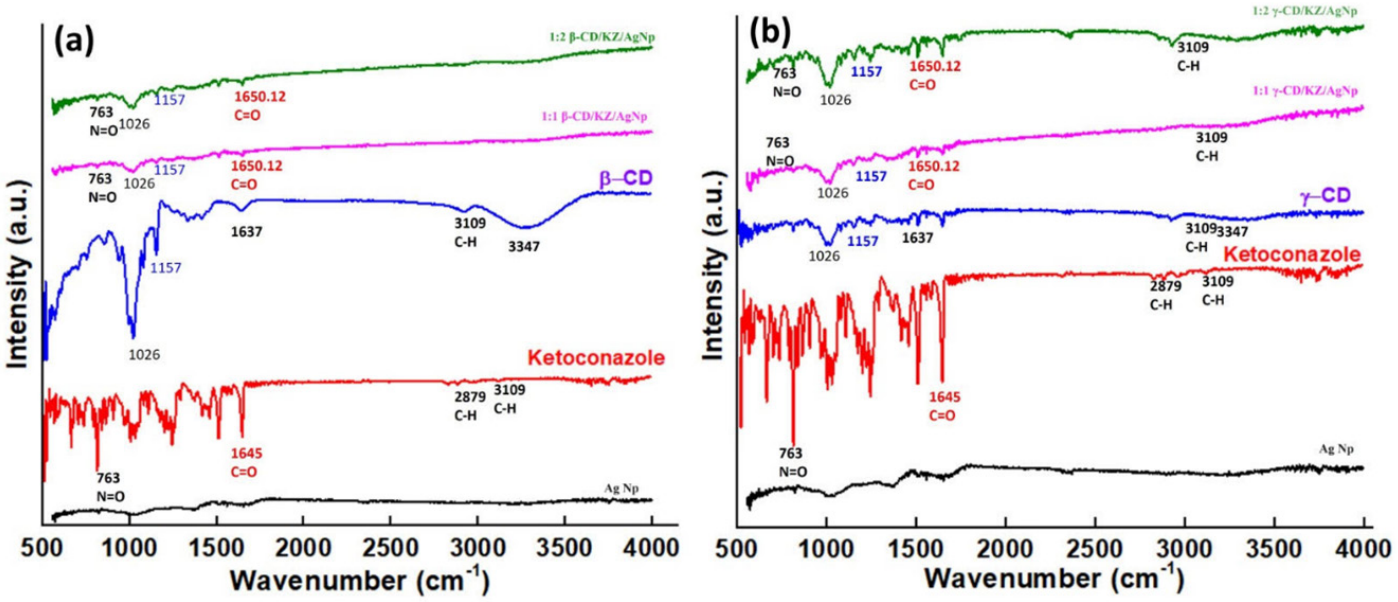

Figure 3: FTIR spectra of (a) FTIR spectra of AgNP, Ketoconazole, β-CD and β-CD/KZ/AgNp in 1:1 and 1:2 ratios; (b) FTIR spectra of AgNP, Ketoconazole, γ-CD and γ-CD/KZ/AgNp in 1:1 and 1:2 ratios, with spectra measured over the wavenumber range of 500-4000 cm^{-1}.

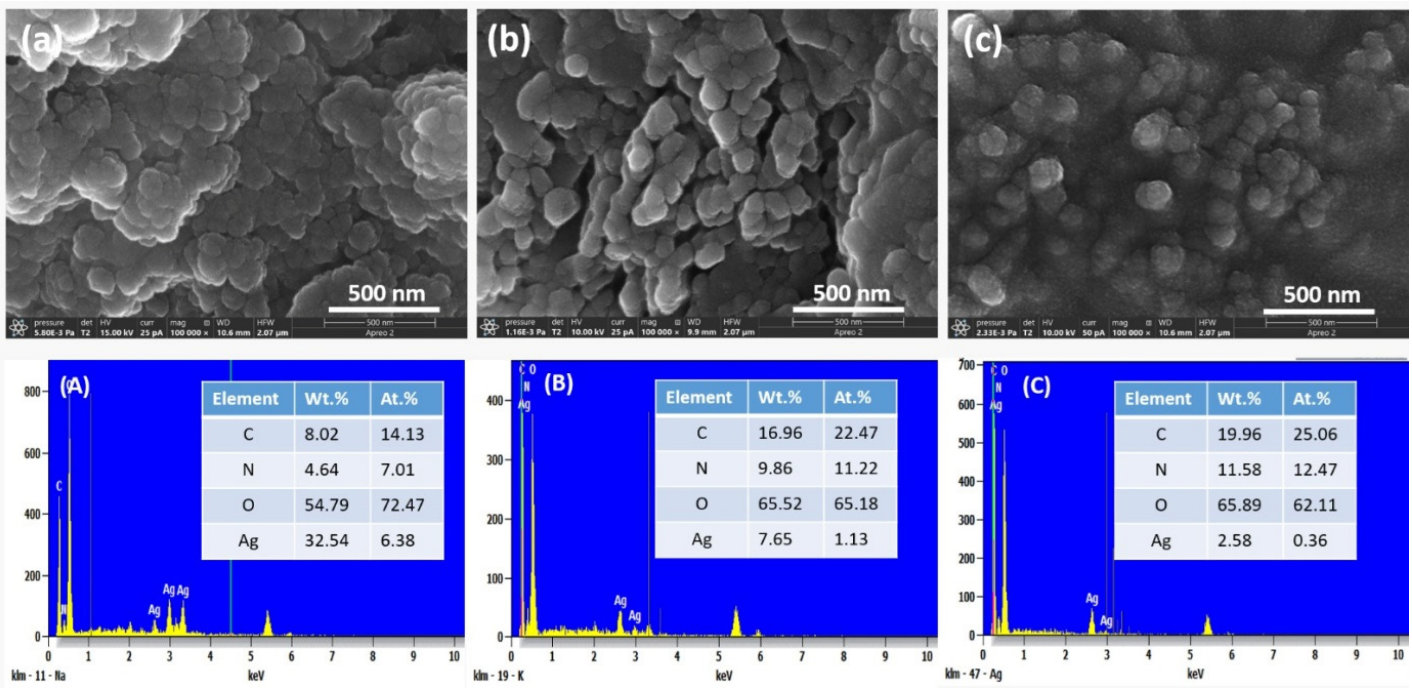

Figure 4: Scanning Electron Microscopy (SEM) images of samples (a), (b) and (c) at 500 nm scale along with their corresponding Energy Dispersive X-ray Spectroscopy (EDS) spectra (A), (B) and (C) showing elemental compositions in weight percent (Wt.%) and atomic percent (At.%).

In vitro antifungal activity

MIC assay

To ascertain the minimum concentration of inclusion complexes that are effective against *T. rubrum* susceptibility, MIC assays were conducted with inclusion complexes and individual compounds AgNp, KZ, β-CD and γ-CD. AgNps exhibited a MIC of 32 µg/mL whereas both β-CD and γ-CD exhibited MIC values greater than 32 µg/mL, suggesting limited antimicrobial activity. Nevertheless, the MIC for both β-CD-AgNp and γ-CD-AgNp decreased to 16 µg/mL when CDs were combined with AgNps, indicating a synergistic effect. Furthermore, formulations that combined KZ, cyclodextrins and AgNps exhibited excellent antimicrobial activity as compared to KZ alone (2 µg/mL). The β-CD-KZ-AgNp at a ratio of 1:1 and 1:4 had the strongest synergistic interaction, indicated by the lowest MIC of 0.5 µg/mL.

Similarly, γ-CD-KZ-AgNp at a ratio of 1:4, exhibited a MIC of 1 µg/mL and the results as shown in Table 1.

ZOI test

Zone of Inhibition (ZOI) values for the β-D and γ-CD inclusion complexes, as well as control, AgNP's and KZ, against *Trichophyton rubrum* are illustrated in Figures 6a and 6b, which were obtained using the disc diffusion method. No zone of inhibition was observed for control, β-CD, or γ-CD. A 4.1 mm ZOI was observed for AgNp while the complexes of β-CD-AgNp and β-CD-KZ-AgNp 1:1 exhibited a significant ZOI of 20.4 mm and 29.7 mm, respectively. The combination of γ-CD-Ag and γ-CD-KZ-AgNp 1:1 also showed ZOI measurements of 17.8 mm and 29.1 mm, respectively compared to the positive control, ketoconazole (2 µg/mL), which gave a mean Zone of Inhibition (ZOI) of 14.4 mm only.

Table 1: MIC of test compounds and β and γ-CD. Inclusion complexes against *Trichophyton rubrum*.

Test compound	MIC (µg/ml)
Ag Nps	32
KZ	2
β-CD	>32
γ-CD	>32
β-CD/Ag	16
γ-CD/Ag	16
β-CD/Ag/KZ 1:1	0.5
β-CD/Ag/KZ 1:2	2
β-CD/Ag/KZ 1:4	0.5
γ-CD/Ag/KZ 1:1	8
γ-CD/Ag/KZ 1:2	2
γ-CD/Ag/KZ 1:4	1

DISCUSSION

The synthesis and characterization of AgNps were primarily confirmed by the color change, UV-vis, FTIR, EDX, FESEM and XRD analysis. The color transition to brownish and particle size distribution together corroborates the nanoparticle formation.[32] The identification of optimal incorporation ratios (1:1 and 1:2) is crucial for maximizing the efficiency of the incorporated complexes in potential applications. The molecular interactions within the complexes were revealed by the FTIR spectra. The distinct peaks observed in the β-CD and γ-CD complexes for -OH bending, C=O stretching and additional functional groups suggest that the structural integrity of cyclodextrin is preserved after inclusion. The observed overlapped peaks between AgNp and β-CD-KZ indicate potential interactions or binding between the nanoparticles and the cyclodextrin complex, potentially impacting the ultimate product's stability and usefulness.[27,28]

Significant structural details regarding the composites were uncovered by XRD investigation. Crystalline nature of AgNps is confirmed by the presence of discrete Bragg reflections, while the broad peaks attributed to CDs suggest that the complexation process imparted an amorphous character. The transition

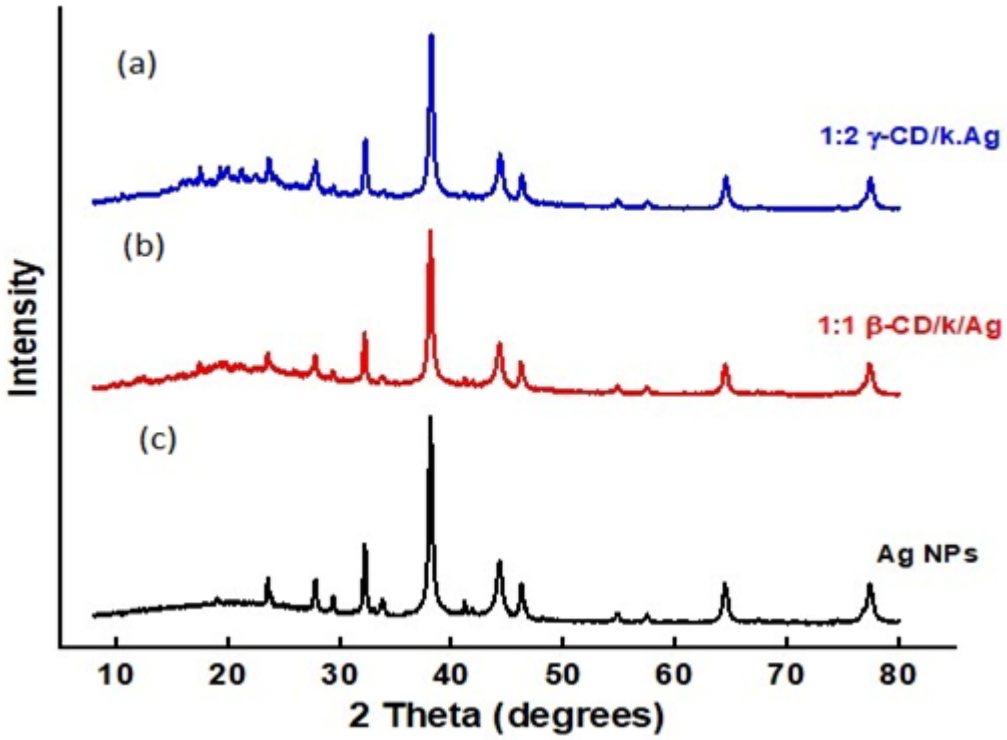

Figure 5: X-ray Diffraction (XRD) patterns of (a) 1:2 β-CD/KZ/AgNp, (b) 1:1 β-CD/KZ/AgNp and (c) AgNp sample.

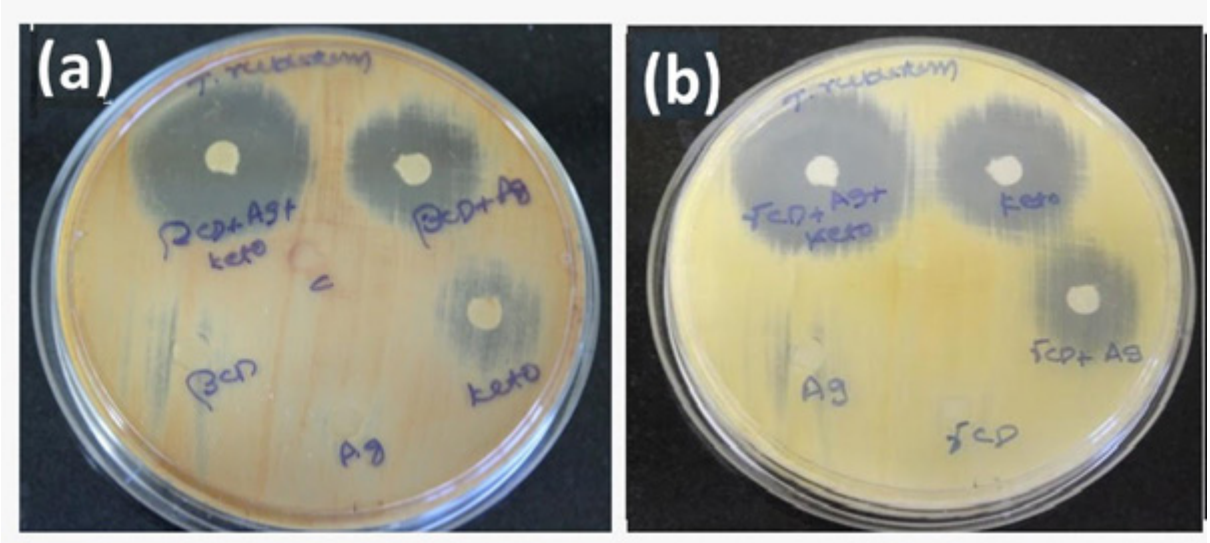

Figure 6: Antifungal activity of different treatments against *T. rubrum*: (a) β-CD-KZ-AgNp, KZ, AgNp, β-CD-AgNp and β-CD treatments; (b) γ-CD-KZ-AgNp, KZ, AgNp, γ -CD-AgNp and γ -CD treatments.

from crystalline to amorphous forms of the composites may have an effect on the bioavailability of active components and rate of dissolution, which is especially important for boosting antibacterial activity.[29] The weight percentage as well as atomic percentage of silver (Ag) decreased in the β- and γ-CD-Keto-AgNp 1:1 compared to AgNps in EDX data. This reduction may be attributed to the complexation process, which introduces additional carbon (C), oxygen (O) and nitrogen (N) into the samples. However, the concentration of carbon (C), nitrogen (N) and oxygen (O) was higher in the inclusion complexes compared to silver nanoparticles (Ag NPs).[30]

Strong proof of the increased antifungal efficacy of the prepared composites was given by the *in vitro* antifungal tests. A synergistic impact can be seen in the considerable decrease in MIC values for the β-CD-KZ-AgNp and γ-CD-KZ-AgNp complexes when compared to AgNps, β-CD and γ-CD alone. The lowest MIC of 0.5 µg/mL for β-CD-KZ-AgNp 1:1 and 1:4 indicate that these ratios are very efficient in stopping the growth of *T. rubrum*. These results are supported by the ZOI tests, which demonstrate that the complexes have noticeably wider zones of inhibition than either the individual components or the control. The combined effects of antimicrobial qualities of AgNps, incorporation of CDs and antifungal activity of KZ are responsible for this increase in antifungal activity.

To summarize, the addition of KZ to β- and γ-CD complexes containing AgNps greatly amplified their antifungal efficacy against *T. rubrum*. By utilizing the distinct qualities of each component, the combination produces a synergistic impact that may lead to the development of more potent antifungal medicines. This work lays a solid basis for future investigations into the uses of these complexes in the pharmaceutical and medical industries.

CONCLUSION

This study provides valuable insights into the potential use of silver nanoparticles and cyclodextrins as a novel approach for enhancing the antimicrobial activity of ketoconazole. The findings suggest that the combination of these materials could be a promising strategy for developing more effective treatments for fungal infections. Further research is needed to explore the mechanisms underlying the enhanced antimicrobial efficacy observed in this study and to optimize the formulation of these complexes for clinical applications. Overall, the findings of this work add to the growing body of knowledge about the use of nanotechnology in medicine, highlighting the potential of silver nanoparticles and cyclodextrins as novel weapons for fighting fungal infections.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

ABBREVIATIONS

KZ: Ketoconazole**; β-CD:** β-Cyclodextrin; **γ-CD:** γ-Cyclodextrin; **AgNps:** Silver Nano particles; **T. rubrum:** *Trichophyton Rubrum*.

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