REMEDIATION OF ONYCHOMYCOSIS USING BIONANOTECHNOLOGY

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ABSTRACT

Onychomycosis is a common chronic fungal infection of the nails that causes discoloration and/or thickening of the nail plate. It is challenging to treat and is associated with high recurrence rate and require lengthy duration of treatment, which poses a risk of adverse effects and drug interactions. This study aims to investigate the in vitro antifungal activity of garlic extract, black pepper extract, chitosan, and their green-synthesized zinc oxide nanoparticles (ZnO NPs) against fungal strains isolated from patients with onychomycosis. The study further aims to develop these formulations as potential safe and effective alternatives to commercial antifungal drugs, addressing the limitations of current therapies. Their antifungal activity was assessed in vitro by agar diffusion method against isolated fungi that causing onychomycosis infection. The results revealed that the nano-formulations exhibited superior antifungal activity compared to their crude extracts. Garlic and black pepper extracts possess good antifungal activity and chitosan has low antifungal activity. While garlic-ZnONPs, Black pepper-ZnONPs and nano chitosan showed comparably strong antifungal activity toward isolated fungi that causing onychomycosis infection. The antifungal effect varied according to fungal species. The maximum activity was noted for that garlic-ZnONPs against *Candida albicans* (35 mm) and *T. rubrum* (35 mm). Nano Zn-black pepper showed good antifungal potential against *Candida albicans* (32 mm), *Rhodotorula muciliginosa* (32 mm) and *A.niger* (30 mm). Nano chitosan showed high effect on *Candida albicans* (33 mm) and *T.rubrum* (35 mm). Cells structural damage that showed on TEM cause fungal cells damage. The bioinformatics analysis confirmed the obtained results. In conclusion, nanoparticles improve drug targeting and enhances the drug profile and permeation. Future respective are required for detailed mechanism of action with more advanced investigations about the antifungal activity are important.

Keywords: Onychomycosis, Antifungal, nanoparticles.

INTRODUCTION

Onychomycosis is a prevalent superficial fungal infection affecting the nails, with a substantial global burden ***(Bermudez et al., 2023)***. While dermatophytes are the primary etiological agents, non-dermatophyte molds and yeasts are increasingly recognized as causative pathogens ***(Mohamed et al., 2024)***. This condition represents a considerable public health challenge due to its chronicity, high relapse rates, and difficulties in ensuring patient adherence to prolonged treatment regimens ***(Yousefian et al., 2024).*** Clinically, it is characterized by nail discoloration, thickening of the nail plate, and onycholysis ***(Leung et al., 2019)***. In advanced cases, the infection can progress to nail fragmentation and inflammatory changes in the periungual tissue, often accompanied by pain and discomfort ***(Falotico & Lipner, 2022)***.

Treatment of onychomycosis remains a considerable clinical challenge due to the protective barrier of the thickened nail plate, biofilm formation by fungal pathogens, and the increasing prevalence of antifungal resistance ***(Axler & Lipner, 2024)***. Current FDA-approved therapies encompass both oral and topical antifungal agents ***(Sabry et al., 2023).*** Oral antifungals, however, are frequently associated with systemic adverse effects, including drug–drug interactions, hepatotoxicity, and an increased risk of congestive heart failure, which limit their widespread use ***(Yousefian et al., 2024).***

In recent years, nanotechnology has emerged as a transformative tool across diverse disciplines, including environmental science, engineering, and medicine ***(Malik et al., 2023)***. There is an increasing emphasis on eco-friendly synthesis methods for nanoparticle production to mitigate the environmental and health hazards associated with traditional approaches ***(Abuzeid et al., 2023)***. Given the therapeutic challenges posed by onychomycosis, nanotechnology-based strategies are being investigated as promising alternatives ***(Aggarwal et al., 2020)***. Bio-nanotechnology, in particular, offers significant advantages over conventional physical and chemical synthesis methods, which often rely on toxic reagents, require high energy inputs, and produce hazardous by-products ***(Tiwari et al., 2017)***. In contrast, biologically mediated metallic nanoparticles are non-toxic, cost-effective, and environmentally benign, making them highly attractive for therapeutic and biomedical applications where safety and biocompatibility are critical ***(Xing et al., 2021; Keshwania et al., 2023)***.

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This study aims to evaluate the antifungal potential of selected plant extracts, along with their green-synthesized zinc nanoparticle formulations, as potential alternative therapies for onychomycosis. Garlic (*Allium sativum*), black pepper (*Piper nigrum*), and chitosan are promising natural agents due to their antifungal properties, supported by several recent studies ***(Magryś et al., 2021; Sultana et al., 2022; N.S. et al., 2024).*** The aim of this study includes assessing the in vitro antifungal activity of garlic and black pepper extracts, their corresponding green-synthesized zinc nanoparticle formulations, as well as chitosan and nano-chitosan nanoparticle formulations, against clinical fungal isolates obtained from infected nails.

METHODOLOGY

Nail Specimens Collection

According to ***Matthapan et al. (2024),*** nail samples (scrapings and clippings) were originally collected from 195 patients with clinically suspected onychomycosis at Souad Kafafi Hospital, which serves 6th October City and surrounding areas in Giza Governorate, Egypt. In the present study, clinical fungal isolates were obtained from anonymized, routine diagnostic laboratory samples, with no patient-identifiable information or direct patient contact involved.

The initial diagnosis was made by a dermatologist based on characteristic nail changes suggestive of fungal infection. Confirmation was performed at the Mycology Unit of the Dermatology and Venereology Department through direct microscopic examination using potassium hydroxide (KOH) preparation and fungal culture.

Inclusion and exclusion criteria were clearly defined. Inclusion criteria comprised patients presenting with clinical signs of onychomycosis, confirmed by a positive potassium hydroxide (KOH) microscopic examination. Patients who had received any topical or systemic antifungal treatment within a recent period prior to enrollment were excluded to eliminate the potential influence of prior therapy on diagnostic results.

Conventional Diagnostic Tools

A- Potassium Hydroxide Testing

Based on ***Gupta et al. (2022),*** direct potassium hydroxide (KOH) testing is a simple, quick, and inexpensive technique integral to dermatological practice for identifying fungal elements. It

involves retrieving the specimen from the nail bed and underneath the nail plate, then dissolving it in KOH (purchased from ADWIC -El Nasr Pharmaceutical Chemical Co., Egypt).

B- Cultivation of nail samples: According to ***(Lim et al., 2021)*** nail scrapings from each specimen were inoculated in Mycobiotic agar with cycloheximide and on Sabouraud's dextrose agar media with Garamycin (obtained from Condalab, Madrid, Spain) in three to four different sites and then incubated at 25^{o}c for 2 weeks. Isolated fungi were investigated by subjected to the direct microscopic examination of agar cultures by light microscope (Olympus) and digital camera (Olympus) using software (Camedia) at The Mycology Unit of Misr University for Science and Technology (MUST). And an image analysis system, soft imaging system GmbH software (analysis pro ® ver. 3.0) at Regional Center for Mycology and Biotechnology AL-Azhar University also was used. Characterization was done for isolates on the mentioned media by using keys manual according to***, (Taha, 2011***) and (***De Hoog et al., 2019)***.

Characterization of the isolates was performed after 3 days for yeast, 7 days for nondermatophytes, and 15 days for dermatophytes *(Marcos-Tejedor et al., 2021)***.** According to ***Acharya and Hare, 2022*** the characterization of yeast isolates was performed on chromogenic agar and on Corn Meal agar (purchased from OXOID, UK). Characterization of moulds into groups and genera of fungi was done according to microscopic examination with lactophenol cotton blue ((purchased from HIMEDIA, India)**.**

Preparation of antifungal extracts by traditional medicines

A-*Allium sativum* (Garlic) and *Pepper nigrum* (Black pepper): According to *(Primananda et al., 2021) and (Elkhawas et al., 2023)* a total of 40 grams of locally sourced Egyptian garlic were thoroughly crushed using a blender to obtain fresh garlic juice, while 40 grams of commercially available black pepper powder were acquired from the local market and finely ground to ensure uniform particle size. Each sample was divided into two equal portions (20 grams each). The first portion was subjected to aqueous extraction by mixing 20 g of the sample with 200 mL of distilled water at a 1:10 (w/v) ratio. The mixture was stirred at ambient temperature for 24 to 48 hours without heat application to preserve thermolabile compounds such as allicin in garlic and volatile alkaloids in black pepper. The extract was then filtered using Whatman No. 1 filter paper.

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The second portion (20g) underwent successive solvent extraction using solvents of increasing polarity: benzene, chloroform, ethyl acetate, and ethanol. In each step, the sample was mixed with 200 mL of the respective solvent (1:10 w/v ratio), stirred for 24 to 48 hours at room temperature, and filtered. The filtrates were then evaporated to dryness to obtain concentrated residues for biological evaluation as reported by *(Hajji Nabih et al., 2023)*

B-Chitosan: Chitosan was prepared by dissolving 0.5g of purified chitosan (CS) powder (high-molecular-weight, 82.4% deacetylated) in 50mL of 10% citric acid (purchased from NSCO scientific company, Giza, Egypt) by stirring using magnetic stirrer for about twenty-four hours.

Green Synthesis of nano particles- extracts

1. Green Synthesis of nano zinc –plants extract:

Zinc nitrate (ZnNO3) obtained from (NSCO) was used as a precursor for synthesizing zinc nanoparticles. Five mL of 1 mM zinc nitrate solution was added to 100 mL of plants extract (garlic or black pepper), and the mixture was shaken at 150 rpm and 30°C for 72 hours. The extracts served as reducing and stabilizing agents. Zinc nanoparticles were formed and then centrifuged at 5000 rpm for 10 minutes to obtain the pellet for further study *(Bouqellah et al., 2019)***.**

2. Green synthesis of nano chitosan:

Chitosan nanoparticles were prepared using the ionotropic gelation method. Two grams of chitosan (from Alpha Chemika) was dissolved in a 10% citric acid solution (NSCO) under magnetic stirring at room temperature for 24 hours. Sodium tripolyphosphate (from Alpha Chemika) was dissolved in water at 1% concentration, also under magnetic stirring. Then, 10 mL of the tripolyphosphate solution was added dropwise to 100 mL of chitosan solution and stirred at 3000 rpm for 30 minutes ***(El-Mohamedy et al., 2019)***.

Assessment of antifungal activity

1-Assessment of antifungal activity of synthetic antifungal agents on isolated fungi collection

Commercially available disks preloaded with fluconazole 25 µg, itraconazole 50 µg, voriconazole1ug and caspofungin 5 µg were used (Liofilchem, Italy) ***(Procop, 2022)*.** In sterile Petri dish, 1 ml from tested fungi suspension was poured in plates containing Sabouraud dextrose agar with garamycin at 45°C. After solidification of the plate, the four disks were put

in solid media. The seeded plates were incubated at 28-30°C, radial growth of colony was measured daily during one week after inoculation. The plates were examined for zone of inhibition and measured after (24-48 hours for yeast and yeast like fungi and 5-7 days for mold fungi) *(Samadi et al., 2019).*

2-Assessment of antifungal activity of natural extracts

Antifungal activity of the extracts of *Allium sativum*, *Piper nigrum*, chitosan was tested against *A.niger , P.chrysogenum,* and *C.albicans* using an agar well assay. In sterile Petri dish, 1 ml from tested fungi suspension on Sabouraud dextrose agar with garamycin. After solidification of the plate, wells made in solid media and filled with 1ml of each extract (garlic, pepper and chitosan) ***(Procop, 2022).*** Negative controls comprised 1 ml of distilled water, 1 ml of ethanol, and 1 ml of 1% citric acid, each tested in parallel for the corresponding extract. The seeded plates were incubated at 28-30°c, radial growth of colony was measured daily during one week after inoculation. The plates were examined for zones of inhibition and measured after (24-48 hours for yeast and yeast like fungi and 5-7 days for mold fungi 10-15 days for dermatophytes). The radial growth of colonies was measured at two points along the diameter of the plate and the mean of two measures was taken as the main diameter of the colony in control set ***(Al-Ogaidi et al., 2024).***

3-Assessment of antifungal activity of nano-bio extracts

To determine the effect of nano- extracts on fungal growth, different concentrations of nano-extracts (garlic, pepper and chitosan) were tested against isolated fungal strains in vitro. In sterile Petri dish, 1 ml from tested fungi suspension on Sabouraud dextrose agar with garamycin. After solidification of the plate, wells made in solid media and filled with 1ml of nano-extracts solution of different concentrations. Inoculated Petri plates were incubated at 25° C 2°C for 4-7-15 days.

According to ***Ogaidi & Al-Temeemy (2024)*** antifungal activity of the extracts of ZnONPs garlic extracts, ZnONPs -black pepper extracts (*Piper nigrum*) and chitosan nano particles were tested against *A. niger, P.chrysogenum* and *C.albicans* using an agar well assay method ***(Frei et al., 2022)***. The positive nanoparticle control consisted of ZnO nanoparticles, which were

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prepared and included to benchmark known antifungal activity, while itraconazole served as the pharmaceutical control, being used as the standard antifungal drug.

Characterization of nanoparticles (Nano chitosan, ZnONP-garlic and ZnONP -black pepper)

1-By optical properties were investigated using UV-Vis-DRS, with measurements recorded in air at room temperature within the 200-800 nm range for (ZnONP-g) and (ZnONP-bp***) (El-Shahaby et al., 2013; Bawazeer et al., 2022)***, and between 200-400 nm for chitosan (CNPs), using a UV-1800 SHIMADZU UV spectrophotometer at 25 °C and a 90° scattering angle.

2-By Transmission Electron Microscopy (TEM) as described **by *Zhang et al., (2020),*** twenty microliters of diluted samples were placed on a 200-mesh copper grid for 10 minutes. The grid was then stained with a drop of 3% phosphotungstic acid (PTA) and dried for 3 minutes. The coated grid was analyzed using a TEM microscope (Philips CM 12) at 120 kV.

3- By surface structure was analyzed using Fourier-transform infrared (FT-IR) spectroscopy (Shimadzu FTIR-8400 S) to investigate the functional groups in garlic extract, black pepper extract, ZnO NPs, and (CNPs) within the 400-4000 cm−1 range ***(Al-Dhabaan et al., 2017; Yang et al., 2020)****.*

4-The crystalline structure of the nanoparticles was analyzed using an X-ray diffractometer (XRD) with Cu Kα radiation at 25 °C (Bruker D8), to characterize ZnO NPs and CNPs nanoparticles ***(Kebede Urge et al., 2023; Yang et al., 2020).***

Nano particles mode of action on ultrastructure on *Candida albicans*

According to ***Desouky et al., (2025)*** transmission electron microscope was used to examine morphological changes of fungal cells before and after treatment with ZnO NPs and Nano chitosan.

Cytotoxicity Assay

The cytotoxic effects of the tested compounds were assessed using the MTT assay normal human melanocyte cells (HFB4). HFB4 cells were cultured in RPMI-1640 medium supplemented with 10% inactivated fetal bovine serum and 50 µg/ml gentamycin, maintained at 37°C in a humidified 5% CO_2 incubator. Cells were seeded in 96-well plates at a density of 5×10^4 cells/well and incubated for 24 hours. Each compound was tested at six concentrations in

triplicate, alongside vehicle controls (0.5% DMSO). After 24 hours of exposure, the MTT assay was performed by adding MTT reagent, followed by DMSO solubilization, and absorbance was read at 590 nm. Percentage viability was calculated and CC50 values were determined using dose-response curves generated by GraphPad Prism software. In addition, morphological changes were monitored microscopically following crystal violet staining to detect cytopathic effects at higher concentrations.

Statistical analysis

For statistical analysis, one-way analysis of variance (ANOVA) was performed to determine the significant differences between treatments using **IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA)**. Mean values and standard deviations were calculated, and a significance level of $p < 0.05$ was considered statistically significant.

Sample Size Justification

The sample size in this study was not determined through a formal statistical power calculation. Instead, it was based on the availability and feasibility of collecting clinical fungal isolates during the study period. This approach aligns with previous ***(Manandhar et al., 2019)*** in vitro antifungal studies that used a comparable number of isolates for preliminary screening purposes. Although limited, the sample size was sufficient to observe measurable antifungal activity and to conduct comparative analyses among the tested extracts.

Bioinformatics analysis

Sankey Plot analysis was performed using the R Statistical Software (Version R.4.4.3) with the (networkD3), (dplyr), and (tidyr) packages. heat map was performed using the Pakeg ggplot2 Statistical Software.

Ethical Considerations

Ethical approval was not required for this study, as the fungal isolates were obtained from anonymized, archived samples collected during routine diagnostic procedures. No patient-identifiable information was accessed or used, and no direct patient contact or clinical intervention was involved. This approach is in full compliance with the institutional guidelines concerning the use of anonymous laboratory materials for research purposes.

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RESULTS

The mycological examination in the present study revealed 25 negative cultures and the isolation of 170 fungal isolates belonging to nine species within seven genera of fungi.

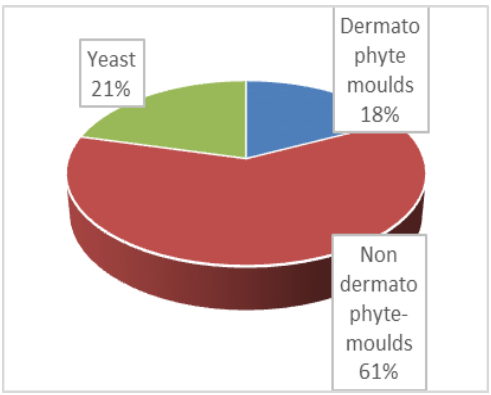

Figure 1: Fungal isolates groups in onychomycosis

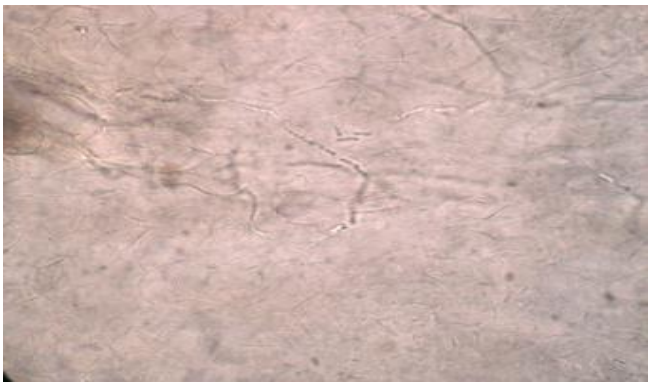

Figure 2: Direct KOH test for nail specimen showing arthrospores

Antifungal activity of *Allium sativum* (garlic)extracts

The finding of the successive extraction revealed that only the aqueous extract of garlic exhibited antifungal activity, whereas its other extracts showed no significant antifungal effects. The antifungal effects of garlic extract on the known pathogenic fungi species are shown in figure (3) and show the inhibition of fungal growth. Results obtained showed that aqueous extract of *Allium sativum* (garlic) had increase antifungal effect on *Penicillium chrysogynum*, *Rhodotroula muciliginosa*, *T. mentagrophytes*, *A. flavus* and *Candida albicans.*

Antifungal activity of *Piper nigrum* (black pepper) extracts

The only the ethanolic extract of black pepper demonstrated antifungal activity, while the remaining extracts were inactive against fungal growth. Figure (4) shows the antifungal properties of *Piper nigrum* extract on isolated pathogenic fungi species, as well as the inhibition of fungal growth. The results revealed that extracts of *Piper nigrum* (black pepper) had a stronger antifungal effect on *T.rubrum*, *Rhodotroula muciliginosa*, *Penicillium chrysogynum*, and *Candida albicans*. Black pepper extract has the strongest antifungal effect on *T.rubrum*. It was found that 80 % of *Piper nigrum* extract effectively inhibits *Rhodotroula muciliginosa*.

Antifungal activity of chitosan

The inhibitory effect of different concentrations of chitosan against pathogenic fungi which isolated from onychomycosis was investigated and the results are in figure (5). Results obtained showed that extract of chitosan had increase antifungal effect on *T.rubrum*, *Rhodotroula muciliginosa* , *Penicillium chrysogynum* and *Candida albicans*.

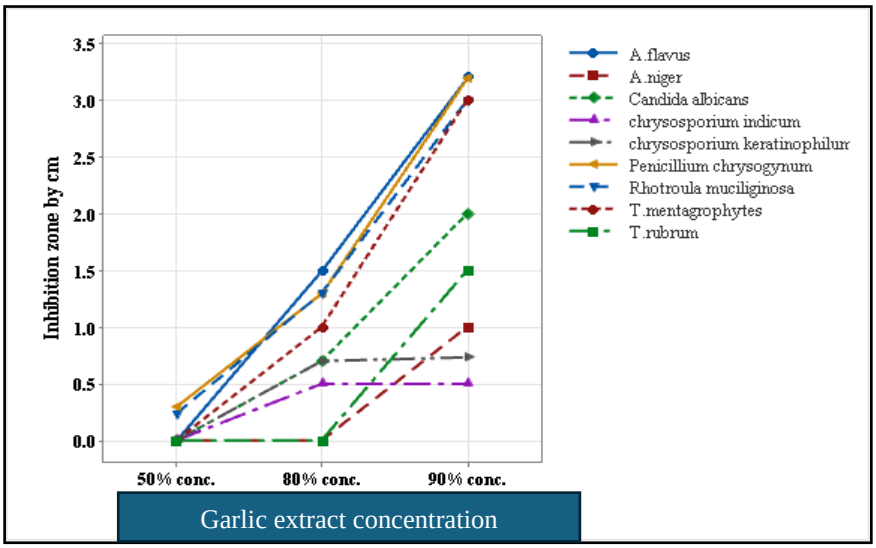

Figure 3: Inhibition zones of fungi treated with different concentrations of garlic extract.

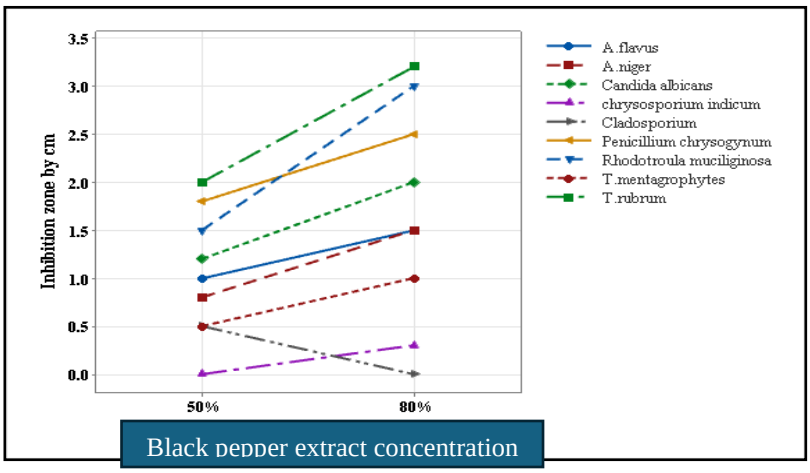

Figure 4: Inhibition zones of fungi treated with different concentrations of black pepper extract.

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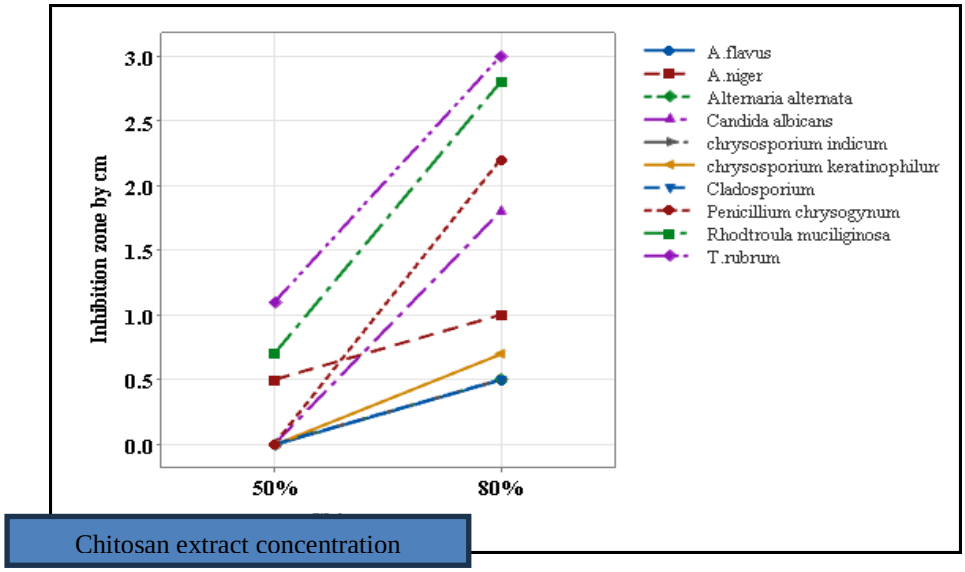

Figure 5: Inhibition zones of fungi treated with different concentrations of chitosan

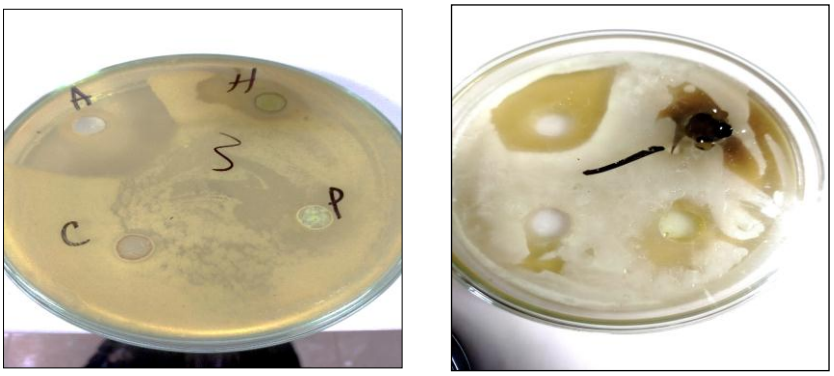

Figure 6: showing antifungal activity of garlic, black pepper and chitosan on *candida albicans* by well diffusion assay

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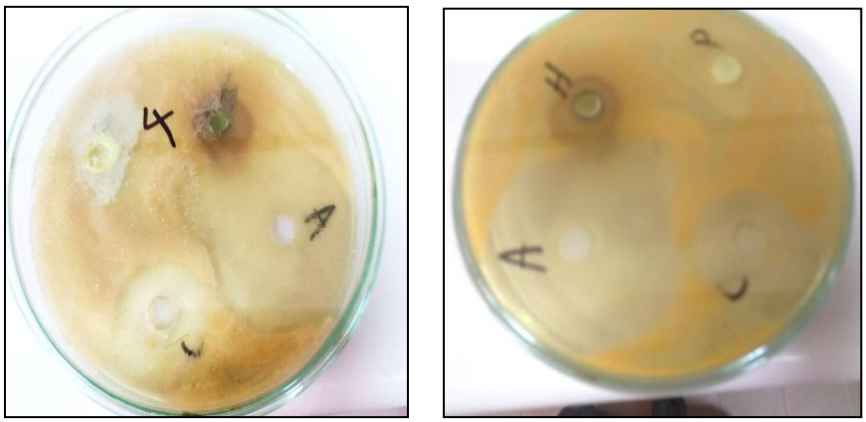

Figure 7: Showing antifungal activity of garlic, black pepper and chitosan on *Rhodotorula muciliginosa* by well diffusion assay.

Comparison of antifungal activity between ZnONP-formulated garlic and black pepper extracts, Nano-chitosan, and commercial antifungals

As shown in figure (٨) ZnONP-garlic, ZnONP-black pepper, and Nano chitosan had strong antifungal effects against *Candida albicans*. Itraconazole was less effective than these nano-based extracts. Additionally, ZnONP-black pepper was more effective against *Rhodotorula muciliginosa* compared to the other nano-extracts, while Itraconazole showed the least impact.

ZnONP-black pepper extract and itraconazole showed strong antifungal activity against *Penicillium chrysogenum*, while nano chitosan was less effective. Both Itraconazole (**commercial antifungals)** and ZnONP-black pepper extract exhibited the highest antifungal activity against *Aspergill niger*, with ZnONP-garlic extract and nano chitosan also showing good effects, but less potent than the former two.

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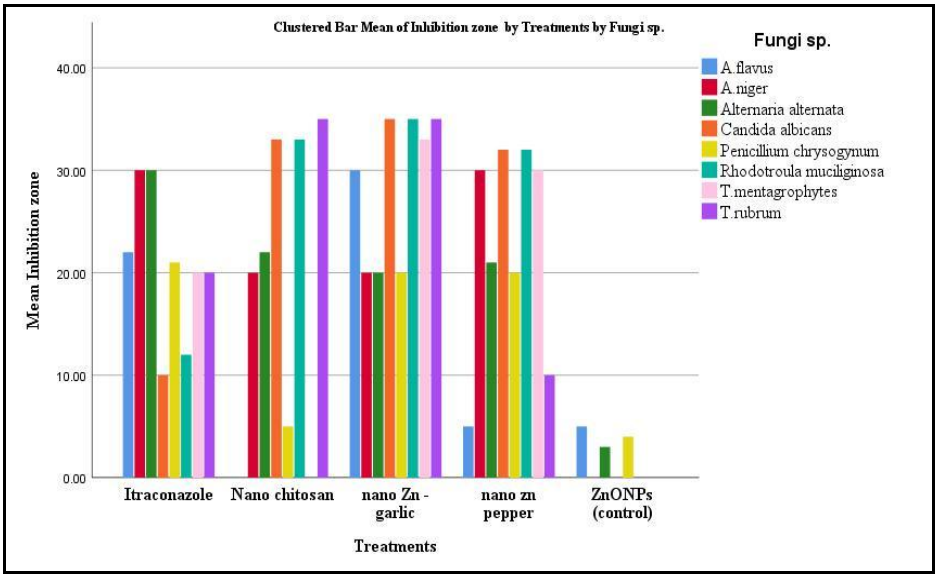

Figure 8: Clustered bar graph illustrating the mean inhibition zone (in mm) for Itraconazole, nano chitosan, ZnONP -garlic, ZnONP -pepper, and zinc oxide nanoparticles against eight fungal species. This graph demonstrates the relative effectiveness of each treatment in inhibiting fungal growth.

However, ZnONP -garlic extract and itraconazole shown strong antifungal effectiveness against *A. flavus*. Nano chitosan had no effect on *A. flavus*. Nano chitosan and ZnONP -garlic extract were the most effective antifungals against *T. rubrum*, with ZnONP--black pepper extract and itraconazole having a lesser effect. ZnONP-garlic extract, ZnONP-black

pepper extract, and itraconazole all show effective antifungal activity against *T. mentagrophytes*. Nano chitosan had no effect on *T. mentagrophytes*. *Alternaria alternata* responded strongly to ZnONP-garlic extract, ZnONP-black pepper extract, and nano chitosan. Itraconazole was the most effective antifungal against *Alternaria alternata.*

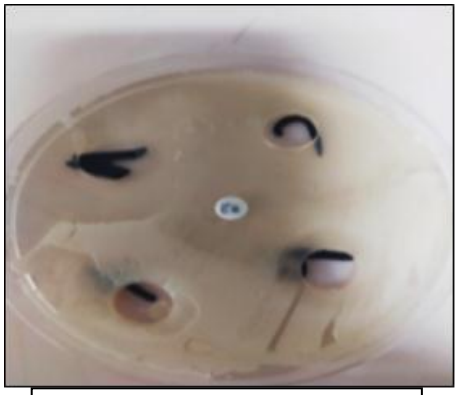

Figure .9: Showing antifungal activity of nano Zn garlic, nano Zn black pepper and nano chitosan on *Candida albicans* by well diffusion assay.

Figure .10: showing antifungal activity of nano Zn garlic, nano Zn black pepper and nano chitosan on *A.niger* by well diffusion assay.

Figure .11: Showing antifungal activity of nano Zn garlic, nano Zn black pepper and nano chitosan on *Rhodotroula muciliginosa* by well diffusion assay.

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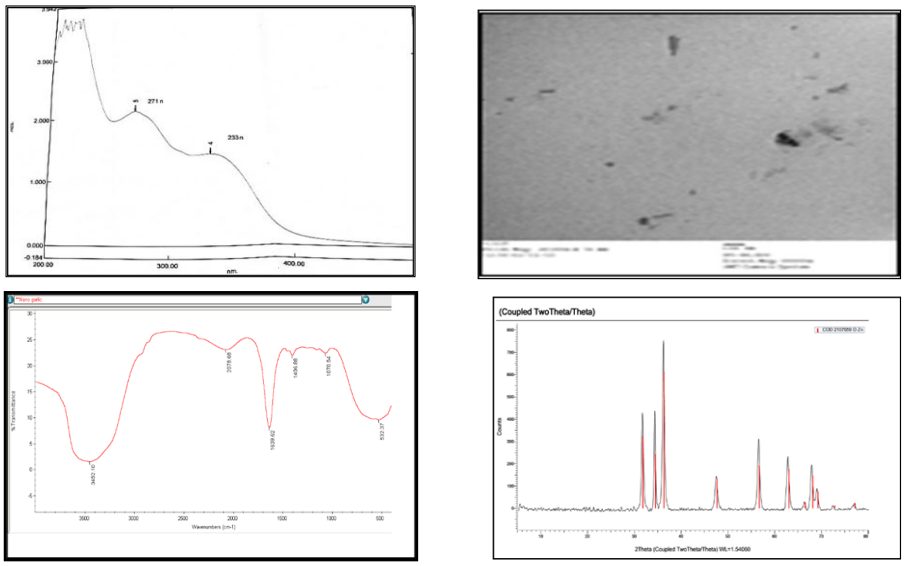
Characterization of nano particles

A-Characterization of ZnONP -garlic extract and ZnONP -black pepper extract

(a) **(b)**



(c) **(d)**

Figure 12 (a): Transmission electron microscopy (TEM) images showing zinc (ZnO-NPs) loaded on garlic extract. Image shows a high magnification of highlighting the spherical morphology. These images confirm the nanoscale size and morphology of the synthesized particles. **Figure12 (b):** UV characterization of Zn nanoparticles loaded on garlic. This figure presents the ultraviolet (UV) absorption spectrum of zinc nanoparticles (Zn NPs) loaded on garlic, showing characteristic peaks at 233 nm and 271 nm, indicating the successful loading of Zn NPs onto the garlic matrix. **Figure 12 (c):** FTIR spectrum of Zn nanoparticles loaded on garlic, revealing the presence of functional groups associated with garlic (e.g., O-H, C-O) and a peak at 532 cm^{-1} indicative of Zn-O stretching, confirming the loading of Zn nanoparticles. **Figure 12 (d):** XRD data of nano Zn loaded on garlic extract, revealing the crystalline nature of the synthesized nanoparticles.

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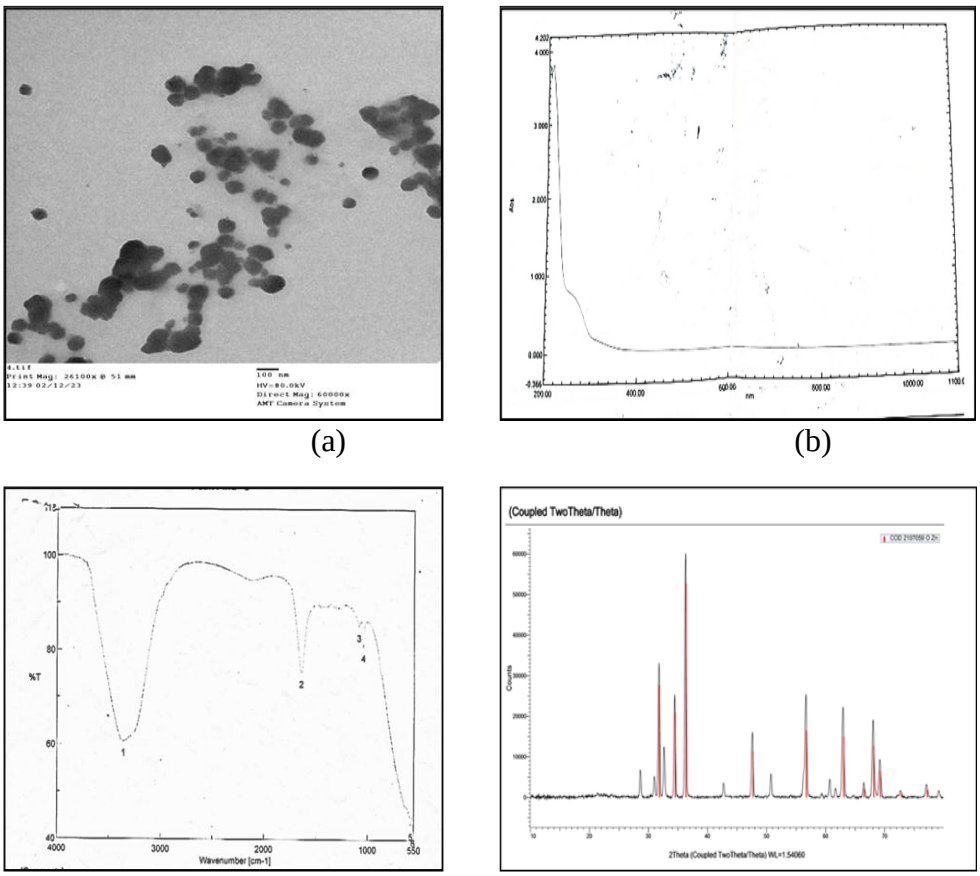

Figure 13 (a): Transmission electron microscopy (TEM) image of zinc oxide nanoparticles loaded on black pepper. The image reveals spherical shape and aggregation tendency of the nanoparticles and confirming their nanoscale size. **Figure 13 (b):** UV-Vis spectrum of Zn nanoparticles loaded on black pepper. The graph shows a strong UV absorption peak, confirming the presence of Zn nanoparticles, and a flat line in the visible region. This data supports the loading of Zn nanoparticles onto the black pepper. **Figure13 (c):** FTIR spectrum of ZnONP -black pepper, revealing the presence of functional groups associated with black pepper and a peak at 550 cm^{-1} indicative of Zn-O stretching, confirming the loading of Zn nanoparticles. **Figure 13 (d):** XRD data of ZnONP loaded on black pepper extract, revealing the crystalline nature of the synthesized nanoparticles.

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B- Characterization of Nano-chitosan

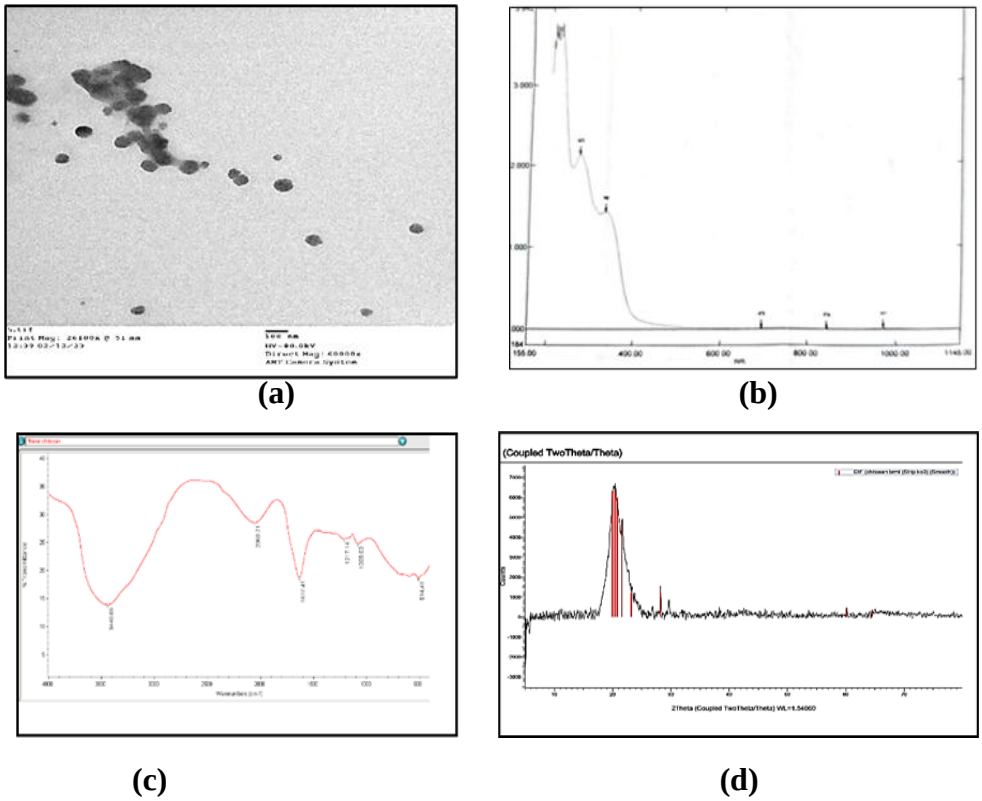

Figure 14 (a): Transmission electron microscopy (TEM) image of nano-chitosan particles showing spherical morphology with slight aggregation. The particle size appears to be in the nanometer range, indicating successful nanoscale formulation. **Figure 14 (b):** FTIR spectrum of nano-chitosan showing characteristic peaks confirming the presence of functional groups and structural integrity. **Figure 14 (c):** FTIR spectrum of nano-chitosan showing characteristic peaks corresponding to functional groups such as –OH, $–NH_2$, and –C=O. The spectrum confirms the structural integrity of chitosan at the nanoscale. **Figure 14 (d):** XRD pattern of nano-chitosan indicating a broad peak around 20°, confirming its semi-crystalline nature.

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Ultrastructure effect of ZnONP –garlic , ZnONP – pepper combinations and nano chitosan

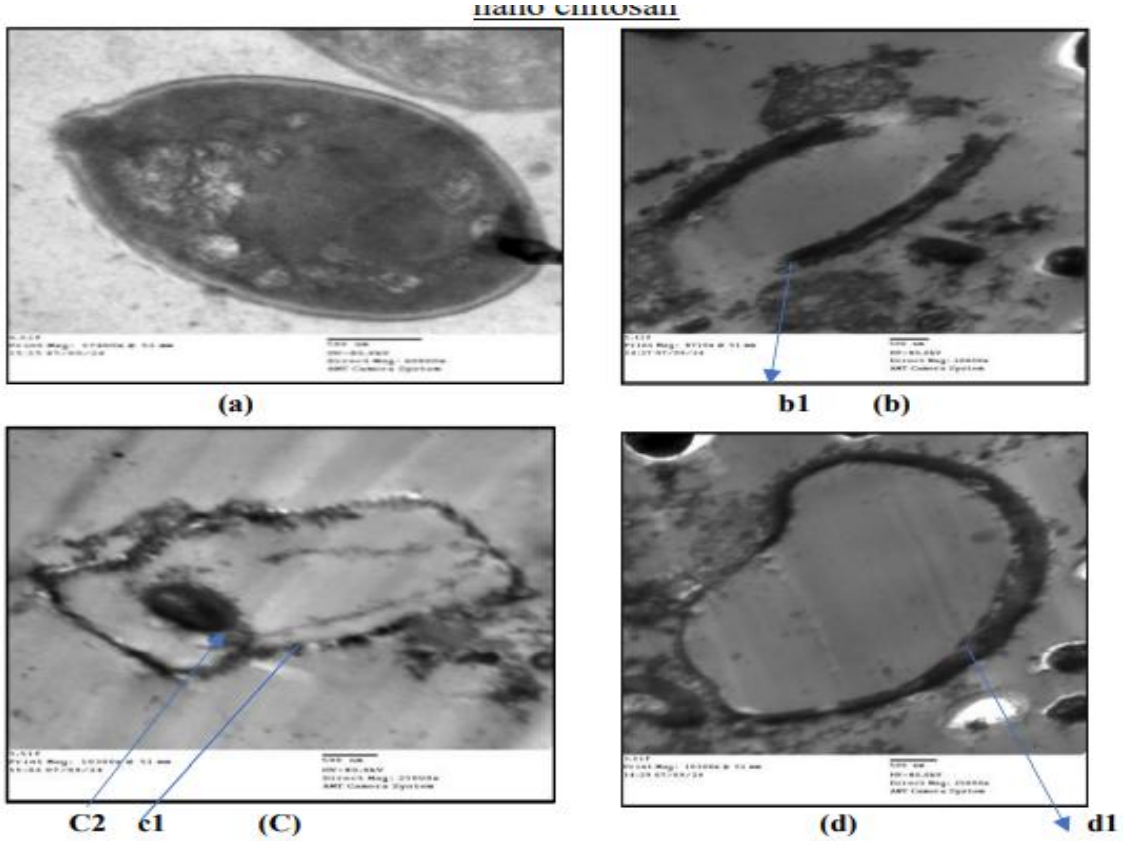

Figure 15 (a): control for *Candida albicans* cells on Transmition electron microscope. **Figure 15 (b):** Transmission electron microscopy image showing the impact of nano zinc loaded on garlic extract on *Candida albicans* cells. (b1) The observed cellular deformation provides visual evidence of the antifungal properties of the tested treatment. **Figure 15 (c):** TEM image showing nano Zn interaction with black pepper extract components. (c1) Cell wall disruption, (c2) Complex structure reveals nano Zn accumulation and a dark inclusion, suggesting nanoparticle binding within the extract. **Figure 15 (d):** TEM image of a *Candida albicans* ghost cell, characterized by a hollow structure and damaged cell wall, demonstrating the complete deformation induced by nano chitosan treatment.

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Bioinformatics analysis

The Sankey diagram is an effective tool for visually exploring the relationships between fungal species and their growth responses to various treatments. As shown on Figure 16 the width of the arrows represents the magnitude of these responses, making it easy to compare different species and treatments at a glance.

The data on Links helps identify species most responsive to treatments, aiding in pinpointing effective methods for controlling fungal growth.

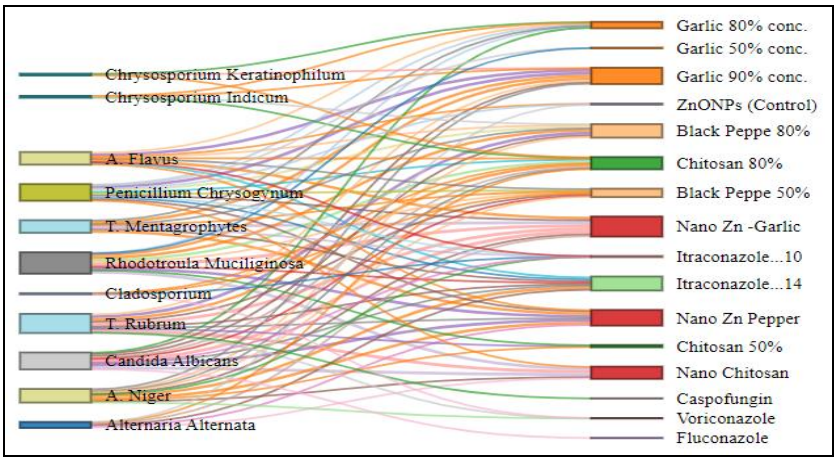

Figure 16: Sankey diagram showing the interaction between different isolated fungal species and various antifungal nano-formulations. This diagram shows that nano-formulations, particularly ZnONP -Garlic, ZnONP -Pepper, and Nano Chitosan, demonstrate broader and more effective antifungal activity compared to many traditional and natural treatments.

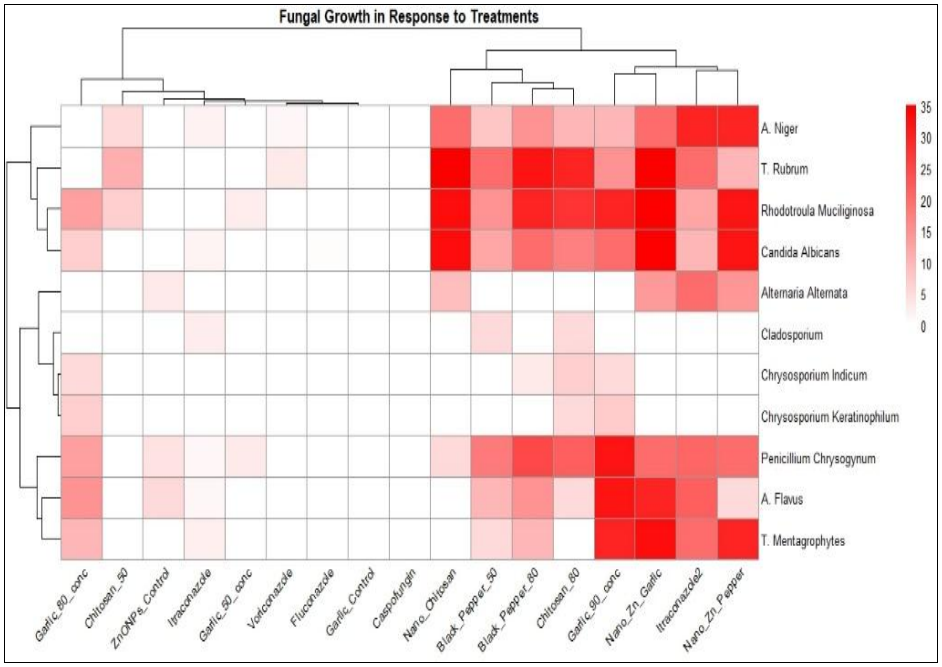

Figure 17: Heatmap showing the fungal growth response to various treatments. Red intensity indicates higher fungal growth, with *A. niger* and *T. rubrum* showing strong resistance to certain treatments such as ZnONP -Pepper and Itraconazole.

Statistical analysis

Nano-formulations exhibited higher antifungal activity compared to their respective plant extracts in most cases. For instance, *Rhotroula muciliginosa* showed a mean inhibition zone of 25.85 ± 12.18 mm for the nano-formulation, compared to 11.38 ± 12.36 mm for the plant extract. Similarly, *Candida albicans* and *Alternaria alternata* demonstrated significantly higher inhibition zones with nano-formulations ($p < 0.05$).

However, no significant differences were observed in *Penicillium chrysogynum*, *A.flavus*, and *T.mentagrophytes* between the plant extract and nano-formulations ($p > 0.05$).These results indicate that the incorporation of zinc nanoparticles enhances the antifungal efficacy of the tested plant extracts against several fungal pathogens.

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Table 1. One-way ANOVA results showing the statistical differences in antifungal activity (inhibition zone diameters, mm) between plant extracts and their nano-formulations against eight fungal species.

FUNGUS	F-STATISTIC	P-VALUE	SIGNIFICANCE
Rhotroula muciliginosa	8.68	0.0072	Significant
Candida albicans	18.00	0.0003	Significant
Penicillium chrysogynum	0.69	0.4153	Not Significant
A.niger	54.26	0.0000	Significant
A.flavus	0.12	0.7373	Not Significant
T.rubrum	21.42	0.0001	Significant
T.mentagrophytes	2.85	0.1047	Not Significant
Alternaria alternata	18.04	0.0003	Significant

Table 2. Mean inhibition zones (mm) ± standard deviation of plant extracts and their nano-formulations against different fungal species.

FUNGUS	PLANT EXTRACT (MM)	NANO EXTRACT (MM)
Rhotroula muciliginosa	11.38 ± 12.36	25.85 ± 12.18
Candida albicans	6.75 ± 8.58	25.38 ± 12.78
Penicillium chrysogynum	12.00 ± 13.19	15.54 ± 7.62
A.niger	2.50 ± 4.52	23.08 ± 8.64
A.flavus	11.75 ± 13.81	13.54 ± 12.53
T.rubrum	3.75 ± 7.11	23.08 ± 12.74
T.mentagrophytes	10.00 ± 12.82	19.15 ± 14.16
Alternaria alternata	0.00 ± 0.00	13.38 ± 10.90

The one-way ANOVA demonstrated a highly significant difference among the treatments ($F = 36.06$, $p < 0.0001$), confirming that the type of treatment had a marked effect on the inhibition of fungal growth. As illustrated in Figure X, nano-formulations generally exhibited greater antifungal activity compared to plant extracts, as evidenced by larger inhibition zones across the tested fungal species.

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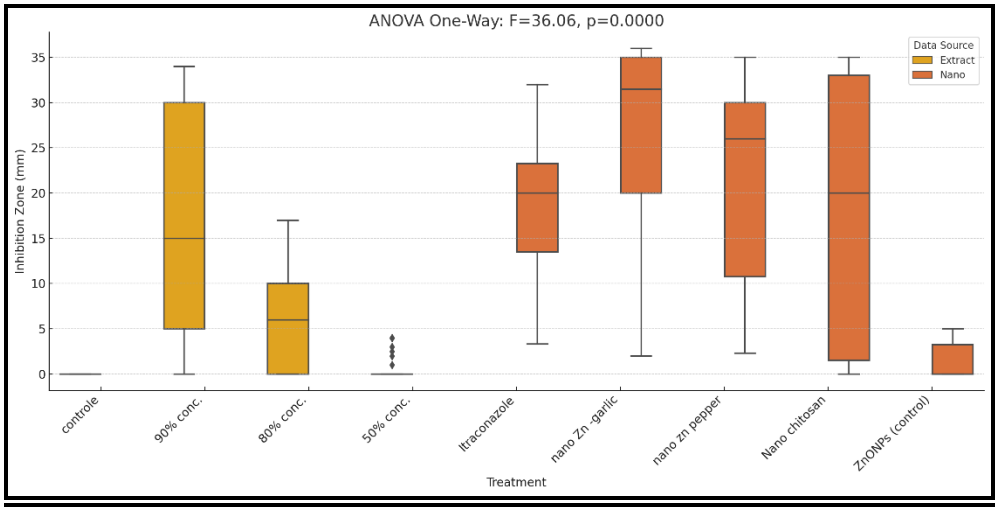

Figure 18. One-way ANOVA analysis of the inhibition zones (mm) of different fungal species under various treatments. The boxplot compares the antifungal activity of conventional plant extracts and nano-formulations against all tested fungi. A highly significant difference was observed among treatments ($F = 36.06$, $p < 0.0001$). Nano-formulations generally exhibited superior inhibitory effects compared to plant extracts.

The overall one-way ANOVA ($F = 36.06$, $p < 0.0001$) revealed a highly significant difference among treatments when all fungal species were considered collectively. Further species-specific ANOVA analyses (Table 1) showed that nano-formulations exhibited significantly higher antifungal activity against *Rhotroula muciliginosa*, *Candida albicans*, *A.niger*, *T.rubrum*, and *Alternaria alternata* ($p < 0.05$). In contrast, no significant differences were observed for *Penicillium chrysogynum*, *A.flavus*, and *T.mentagrophytes* ($p > 0.05$). The mean inhibition zones (±SD) for each treatment and fungal species are presented in Table 2.

Cytotoxicity of Zn- nanoparticles and chitosan nanoparticles

Garlic and black pepper ZnO nanoparticles exhibited weak cytotoxic activity across all tested concentrations, with CC50 values exceeding 1000 µg/ml, indicating low toxicity. Minimal inhibition was observed below 125 µg/ml for garlic ZnO and below 250 µg/ml for black pepper ZnO nanoparticles, with no notable effect at lower concentrations (Figures 19a and 19b). Similarly, chitosan nanoparticles demonstrated very low cytotoxicity, with CC50 > 1000 µg/ml, and negligible inhibition at or below 62.5 µg/ml (Figure 19c). Microscopic

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observation revealed slight morphological alterations (cell rounding, shrinkage, and partial detachment) at higher doses, while lower concentrations showed intact cell morphology, supporting the overall low cytotoxicity profile of the tested nanoparticles.

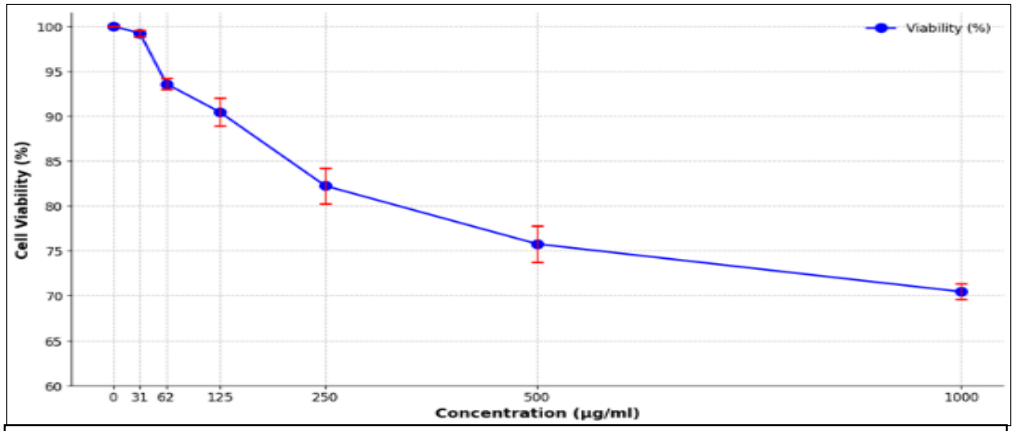

Figure 19(a): cell viability under the effect garlic-ZnO nanoparticles at different concentrations.**:**

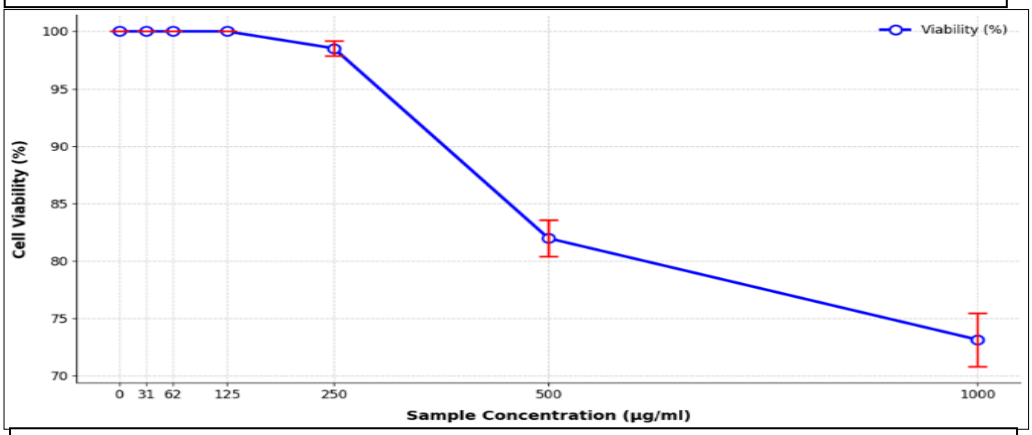

Figure 19 (b): cell viability under the effect black pepper-ZnO nanoparticles at different concentrations.

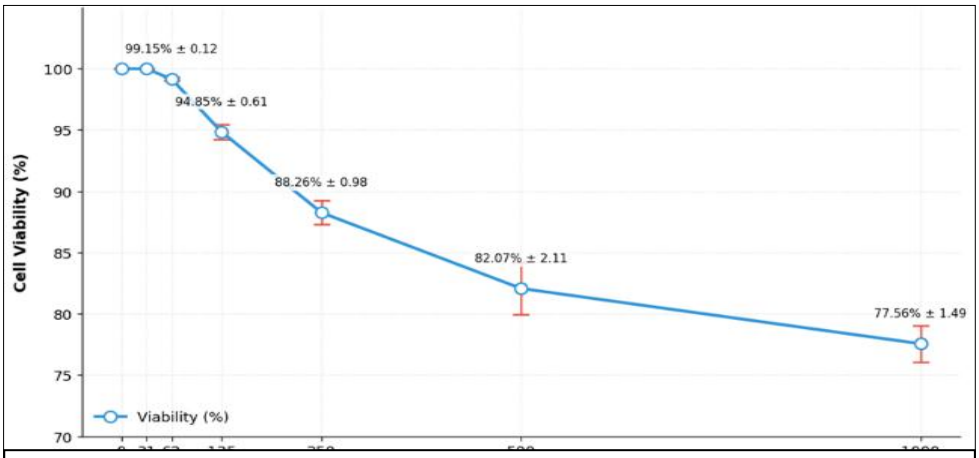

Figure 19 (c): cell viability under the effect chitosan nanoparticles at different concentrations.

DISCUSSION

In the present study, the most frequently isolated fungal groups were molds (٧٩.٠٣%), followed by yeasts (20.97%). The most common isolated species was *Aspergillus niger* (20%). These results were concided with a study in Egypt by ***(Elnagar & Shrief, 2021),*** which explains that non-dermatophyte molds are a common cause of onychomycosis and contribute to treatment failure. Previous nail damage caused by dermatophytes, trauma, or other nail diseases increases the likelihood of infection by non-dermatophyte molds. The widespread use of broad-spectrum antibiotics, diabetes mellitus, corticosteroids, and other immunosuppressive drugs has contributed to the rise in nail infections caused by non-dermatophyte molds ***(Motamedi et al., 2016).***

In Egypt, a study by ***Bedaiwy et al. (2017)*** classified fungal isolates causing onychomycosis into yeasts (56%), dermatophytes (36%), and non-dermatophytes (8%). Another study in Ethiopia showed that 25.8% of the isolates were dermatophytes, 61.1% were non-dermatophyte molds, and 13.1% were yeasts ***(Bitew & Wolde, 2019).***

The study in Nepal ***(Jha et al., 2021)*** identified dermatophytes as the most common cause of onychomycosis, with *Trichophyton rubrum* being the leading pathogen. Dermatophytes were also the most common species, making up 72.73% of the total, according to ***(Abdo et al., 2016).***

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In India***, (Bedaiwy et al., 2017)*** also reported *T. rubrum* as the most common dermatophyte associated with onychomycosis.

Herbal and natural antifungal remedies are a less expensive, safer, and more widely available alternative treatment for onychomycosis ***(Talele et al.,2023)***.

Results obtained in the present study showed that the aqueous extract of *Allium sativum* (garlic) was the most powerful antifungal effect. Garlic extract has antifungal effects on *Penicillium chrysogynum, T. mentagrophytes, Rhodotroula muciliginosa, A. flavus* and *Candida albicans.*

Garlic (*Allium sativum*) contains various biologically active components that play a significant role in the treatment of fungal infections. It contains sulfur compounds like allicin, ajoene, allylmethyltrisulfide, diallyltrisulfide, diallyldisulphide and others which exhibit various biological properties like antimicrobial effects ***(Tudu et al., 2022).***

(Aala et al., 2011) investigate the effects of Garlic (*Allium sativum*) and pure allicin on the growth of hypha in *T. rubrum* using electron microscopy. SEM surveys showed hypha treated with garlic extract exhibiting degradation and dissolution of cytoplasm components, demolition of cell wall and cell membrane, and hypha appeared to break. Garlic extracts and pure allicin could be used as alternatives in treatment of dermatophytosis. ***(Ezeorba et al., 2022)*** reported *Allium sativum* extract can result in irreversible ultrastructural changes and loss of structural integrity in fungal cells**.**

So that, ***(Bashir Kutawa et al., 2018)*** revealed that garlic extract showed antifungal activity against the test organism. Moreover, the ethanolic extract showed inhibitory activity among the tested fungi. Together, the antifungal and antioxidant activities support that *A. sativum* is a potential alternative treatment in onychomycosis ***(Pârvu et al., 2019).***

(Chen, 2021) reported that the *A. sativum* extract is effective against *Rhodotorula muciliginosa* cultured from the toenails of a patient with onychomycosis.

Nanotechnology has gained significant attention from scientists for developing plant-based natural nanoparticles (NPs), valued for their safety, effectiveness, and eco-friendly properties ***(Bawazeer et al., 2022).***

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Despite numerous attempts to overcome the solid nail barrier, challenges in nail delivery persist. Recently, attention has turned to nanoparticles as innovative drug delivery systems. These nanoparticles have shown great potential as effective antifungal agents in the treatment of onychomycosis ***(Dhamoon et al., 2019).***

Researchers have also reported the biological synthesis of zinc oxide nanoparticles (ZnO-NPs) using the plant petal extract of *Rosa indica L.* (rose) ***(Tiwari et al., 2017).***

The present results showed that aqueous nano-Zn oxide loaded on garlic extract effectively inhibited the growth of *Rhodotorula muciliginosa*, *Candida albicans*, *Penicillium chrysogynum, A. niger, A. flavus, T. rubrum, T. mentagrophytes,* and *Alternaria alternata*.

The study by ***(Abdelmoteleb et al., 2024)*** successfully synthesized zinc oxide nanoparticles (ZnO NPs) using garlic plant extracts, showing antifungal activity and potential for various applications. ***(Fulekar, 2020)*** also discussed the synthesis of ZnO nanoparticles using garlic skin, highlighting their uses in biotechnology, biomedicine, catalysis, sensors, and water remediation, with the green method being eco-friendly, cost-effective, and efficient.

In the current study *piper nigrum* (black pepper) extract has the most powerful antifungal effect on *T. rubrum* and also, has good inhibition on *Rhodotroula muciliginosa, Penicillium chrysogynum* and *Candida albicans*.

The bioactive compounds in *piper nigrum* including piperine, essential oils, phenolic compounds and flavonoids, contribute to its pharmacological properties including antimicrobial effects (***Author & Tekade, 2024).***

*(**Silva et al., 2014)*** explained the significant antifungal activity of essential oil components arises from their hydrophobicity and could include attack on cell wall synthesis and retraction of cytoplasm in hyphae, which affects fungal growth and the morphogenesis, resulting in death of mycelium.

The antifungal activity of many spices, such as black pepper extracts, against *Candida albicans* was shown in a compatible result by ***Bravo-Chaucanés et al. (2022),*** which could be effective in the treatment of oral candidiasis. Additionally, the aqueous extract of black pepper seeds totally inhibits the growth of *Penicillium citrinum* and exhibits moderate action in

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inhibiting the mycelial growth of *Aspergillus niger* and *Aspergillus flavus* ***(Daigham & Mahfouz, 2020).***

The present study demonstrated that zinc oxide nanoparticles synthesized with black pepper extract showed increased antifungal activity against *T. rubrum, Rhodotroula muciliginosa, Penicillium chrysogenum,* and *Candida albicans*. Furthermore, (***Ck & Bandari, 2024)*** reported that piperine demonstrated potent antimicrobial properties against oral infections, such as *Candida albicans*, when paired with zinc oxide. Similarly, tests of the antifungal activity of black pepper extract and its gold nanoparticles (AuNPs) revealed that they were effective against *A. solani, A. niger, A. flavus,* and *C. albicans*.

Black pepper (BP) extract and its gold nanoparticles (AuNPs) had modest antifungal efficacy, according to research by ***Wawzeer et al. (2022).*** The main component of BP, piperine, is responsible for the antimicrobial impact, indicating that it has antimicrobial potential.

Results obtained in the present study showed that extract of chitosan had increase antifungal effect on *T. rubrum, Rhodotroula muciliginosa, Penicillium chrysogynum* and *Candida albicans*.

Numerous researchers have been very interested in chitosan since its antifungal effects were proven. In addition to the chitosan formulation, previous studies showed that the kind of fungus also affected the chitosan's efficacy in addition to its formulation ***(Xing et al., 2022).***

Chitosan is an adaptable substance with a wide range of biotechnological uses. According to ***Lopez-Moya et al. (2019),*** chitosan enhances the formation of intracellular oxygen species (ROS) and permeabilizes their high-fluidity plasma membrane. According to the study by ***Abo El-Ela et al. (2024)***, chitosan particles that are nanosized can stick to fungal cell membranes more firmly and compromise their integrity because of their higher surface area. Chitosan inhibits DNA/RNA synthesis and causes fungal cell death by blocking nutrition channels and interfering with cellular functions. By interacting with negatively charged membranes, chitosan nanoparticles' positive charge increases their antifungal effectiveness. According to research by ***Ing et al. (2012),*** chitosan nanoparticles are more effectively absorbed by cells than bulk chitosan, which leads to higher antifungal effects since they may penetrate fungal cells and interfere with vital processes.

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According to ***Shih et al. (2019),*** chitosan may have an antifungal effect on *Candida albicans* via suppressing the expression of the SAGA complex gene, which lessens the cell surface's resistance to chitosan. According to the study by ***Almeida et al. (2023)***, chitosan may be able to stop the growth of human pathogenic dermatophytes like *Microsporum canis*.

The findings of the current study revealed that chitosan nanoparticles exhibited significant antifungal activity against *T. rubrum, Rhodotroula muciliginosa, Candida albicans, Alternaria alternata,* and *A. niger*. ***(Vitali et al., 2022)*** found that chitosan nanoparticles loaded with carvacrol were particularly effective against planktonic *Candida* forms, with *C. tropicalis* and *C. krusei* being the most susceptible.

According to the current study's findings, chitosan nanoparticles had strong antifungal activity against *Alternaria alternata, Candida albicans, Rhodotroula muciliginosa, T. rubrum,* and *A. niger*. Carvacrol-loaded chitosan nanoparticles were found to be very efficient against planktonic *Candida* forms, with *C. tropicalis* and *C. krusei* being the most vulnerable ***(Vitali et al., 2022).***

The study found that Itraconazole, Fluconazole, Voriconazole, and Caspofungin had a good inhibitory impact against *T. rubrum* and *T. mentagrophytes.* Commercial antifungal compounds have been discovered and developed over many years and are essential for treating a wide range of fungal infections that affect the body locally and systemically (***Mandal, 2024)***. Itraconazole and Voriconazole were found to be more effective against *Rhodotorula muciliginosa* than Fluconazole and Caspofungin, but the effects were similar.

Evans (1999) described how Itraconazole is used to treat onychomycosis, a fungal nail disease, in Iceland. The clinical effectiveness and microbiological susceptibility to Itraconazole for the fungus causing dermatophytosis in India were confirmed by a real-world investigation conducted by ***(Handa et al., 2023)***.

(Maskan Bermudez et al., 2023) stated that therapies with Terbinafine and Itraconazole are limited, and the resistance to Terbinafine is starting to develop worldwide. Itraconazole tested showed good in vitro activity against clinical isolates of *T. rubrum* and Fluconazole is poor activity against *T. rubrum* ***(Azambuja et al., 2014).***

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In the current study aqueous garlic extract has a good effect more than Itraconazole. Also, aqueous garlic extract alone is potentially as good as antifungal compounds against dermatophytes, better than synthetic drug Fluconazole and almost as good as Ketoconazole (***Aala et al., 2011).***

Zinc oxide nanoparticles can bind to microbial cells and subsequently enter them, producing structural alterations in the cell membrane such as increased permeability and cell death (***Ck & Bandari, 2024***).

According to the study, when zinc oxide nanoparticles (ZnONPs) are applied to fungi, a hydrogen bond between the oxygen atom in ZnONPs and the hydroxyl groups in the fungal cell wall causes hydrogen peroxide ($H2O_2$) to be formed. This method inhibits the growth of fungi. By disrupting the respiratory chain and halting cell division once within the cells, ZnONPs further inhibit proliferation. The fungal cell wall develops pores as a result of reactive oxygen species (ROS), allowing cellular organelles to leak out. Moreover, ZnONPs increase the number of nucleic acids and carbohydrates in fungal hyphal cells, causing structural deformation ***(Tiwari et al., 2017).***

(Abdelmoteleb et al., 2024) describe how plant phytochemicals, such as flavonoids and tannins, contribute to the environmentally benign production of ZnO-NPs, according to. These chemicals use the plant's capacity to bioaccumulate metal ions to decrease Zn^{2+} ions to ZnO-NPs and stabilize them by preventing agglomeration. The presence of carboxyl and amine groups on the cell surface of biosynthesized ZnO NPs, as well as the high affinity of ZnO ions for these groups, may be responsible for their antibacterial effect.

ZnO NPs accumulate on *C. albicans* cells, causing morphological changes as membrane ru pture, cell wall pitting, and hyphal degradation, as seen by SEM imaging ***(Djearamane et al., 2 022)***. They have dose dependent antifungal action, and cell death is most likely caused by a damaged cell membrane.

Allium sativum was the most effective than *Pepper nigrum* and chitosan while *Pepper nigrum* showed marked inhibition against *Trichophyton rubrum, Rhodotroula muciliginosa, Penicillium chrysogynum*. Also, chitosan extracts show good effects on *T. rubrum, Rhodotroula muciliginosa, Penicillium chrysogynum* and *Candida albicans*.

The cytotoxicity results suggest that the tested ZnO and chitosan nanoparticles possess a favorable safety profile, especially at lower concentrations. The high CC50 values (>1000 µg/ml) across all compounds indicate minimal toxicity toward human normal melanocytes (HFB4) and support their potential applicability in biomedical contexts. The weak cytopathic effects observed microscopically further validate their biocompatibility. These findings are consistent with previous reports indicating the low toxicity of biosynthesized nanoparticles when applied at appropriate doses. Nevertheless, further in vivo toxicity testing is warranted to confirm their safety beyond the cellular level.

The bioinformatics analysis confirmed the findings. To sum up, nanoparticles enhance drug profile, increase drug penetration, and improve therapeutic targeting.

The results of the one-way ANOVA ($F = 36.06$, $p < 0.0001$) clearly demonstrate the significant influence of treatment type on fungal inhibition. The superior performance of nano-formulations over conventional plant extracts can be attributed to the enhanced bioavailability, improved surface area, and unique physicochemical properties of nanoparticles, which are known to facilitate better interaction with microbial cell walls. These findings are consistent with previous studies as ***(Elayaperumal et al., 2025)*** reporting the enhanced antifungal efficacy of nanomaterials against various fungal pathogens. Moreover, the observed variability among fungal species suggests differential susceptibility, likely due to variations in cell wall composition and resistance mechanisms. Post-hoc analyses (e.g., Tukey's HSD test) are recommended to identify specific pairwise differences between treatments.

The overall one-way ANOVA ($F = 36.06$, $p < 0.0001$) revealed a highly significant difference among treatments when all fungal species were considered collectively. Further species-specific ANOVA analyses (Table 1) showed that nano-formulations exhibited significantly higher antifungal activity against *Rhotroula muciliginosa, Candida albicans, A.niger, T.rubrum,* and *Alternaria alternata* ($p < 0.05$). In contrast, no significant differences were observed for *Penicillium chrysogynum, A.flavus*, and *T.mentagrophytes* ($p > 0.05$). The mean inhibition zones (±SD) for each treatment and fungal species are presented in Table 2.

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CONCLUSION

This study demonstrated promising in vitro antifungal activity of nano-formulated garlic and black pepper extracts, as well as nano chitosan, against clinical isolates from onychomycosis cases. However, several limitations should be considered. The experiments were conducted under in vitro conditions using the Hfb4 cell line, which does not fully mimic the structure and physiology of human nail tissue. Moreover, quantitative analysis of the key active compounds — allicin in garlic extract and piperine in black pepper extract — was not performed at this stage, and this has been acknowledged in the limitations. Despite these considerations, the results provide a solid foundation for future in vivo and clinical investigations aimed at validating the therapeutic potential of these Nano formulations.

RECOMMENDATION

This investigation suggests further elucidating the antifungal mechanisms of zinc nanoparticles and nano chitosan, particularly in the context of loading zinc nanoparticles onto plant extracts. It also proposes determining the key active constituents responsible for this activity and examining whether their combined application results in a synergistic outcome. It also recommends looking into non-therapeutic uses, creating sophisticated delivery methods, and broadening the application to include other kinds of fungi. The study also highlights the significance of evaluating the stability of the nano-formulations and doing a thorough safety and in vivo efficacy assessment. Lastly, it suggests combining therapeutic and diagnostic methods into a single system (Theranostics) to improve fungal infection detection and focused treatment

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معالجة فطريات تينيا الأظافر باستخدام تقنية النانو الحيوية

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المستخلص

يُعد داء فطريات الأظافر عدوى فطرية شائعة ومزمنة تصيب الأظافر، وغالبًا ما تتميز بتغير لون صفيحة الظفر وزيادة سُمكها. لا يزال هذا المرض يُشكّل تحديًا علاجيًا بسبب ارتفاع معدل تكراره وطول مدة العلاج، مما قد يؤدي إلى آثار جانبية وتفاعلات دوائية. هدفت هذه الدراسة إلى استقصاء الفعالية المضادة للفطريات لمستخلصات نباتية طبية و/أو جسيمات نانوية مُخلّقة خضراء كبدائل علاجية آمنة مقارنة بالعقاقير التقليدية. تم تقييم النشاط المضاد للفطريات معمليًا باستخدام طريقة الانتشار في الوسط الغذائي ضد عزلات فطرية سريرية مسببة لداء فطريات الأظافر. أظهرت مستخلصات الثوم والفلفل الأسود نشاطًا مضادًا للفطريات ملحوظًا، بينما أظهر الكيتوسان نشاطًا أقل. وعلى النقيض من ذلك، أظهرت جسيمات نانو أكسيد الزنك المخلقة باستخدام الثوم والفلفل الأسود، وكذلك نانو الكيتوسان، فعالية قوية ضد الفطريات المعزولة. وقد تباينت الفعالية المضادة للفطريات حسب نوع الفطر. سُجلت أعلى فعالية لجسيمات نانو اكسيد الزنك–الثوم ضد الكانديدا البيكانز وترايكوفايتون روبرام بقطر تثبيط بلغ ٣٥ مم. كما أظهرت جسيمات نانو اكسيد الزنك–الفلفل الأسود فعالية جيدة ضد كانديدا البيكانز (٣٢ مم)، وروردوارولا ميسيليجينوزا (٣٢ مم)، واسبريجلاس نايجر (٣٠ مم). بينما أظهر نانو الكيتوسان فعالية عالية

ضد كانديدا البيكانز (٣٣ مم) و ترايكوفايتون روبرام (٣٥ مم). أظهر الفحص بالمجهر الإلكتروني النافذ وجود أضرار هيكلية بخلايا الفطريات، مما يؤكد آلية عمل الجسيمات النانوية. كما دعمت تحليلات المعلومات الحيوية (التحليل الاحصائي الحيوي) هذه النتائج. في الختام، تساهم الجسيمات النانوية المُخلّقة حيويًا في تحسين استهداف الدواء وتعزيز فعاليته، مما يجعلها بديلًا آمنًا وواعدًا للعلاج التقليدي. ويوصى بإجراء دراسات مستقبلية للكشف بشكل أعمق عن آلية العمل وفعالية هذه الجسيمات ضد الفطريات.

الكلمات المفتاحية: فطريات الأظافر، مضاد للفطريات، الجسيمات النانوية.