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Antifungal efficacy of Citrusfusion mediated silver nanoparticles in *Candida* species

A. Harine[1†], S. Ranjani[1†] and S. Hemalatha[1*]

Abstract

Background *Candida* species are commensal fungi that can become opportunistic pathogens under specific host and environmental conditions. The emergence of multidrug-resistant *Candida* strains poses a significant challenge. Nanotechnology represents a cutting-edge field offering precise and targeted delivery systems for combating fungal infections, leveraging the unique properties of plant-derived bioactive compounds. This investigation employed a biogenic approach utilizing polyherbal leaf extracts from *Citrus limon* and *Citrus medica*, known for their abundant Citral content.

Results *Citrus* sp. extracts were used to synthesize Citrusfusion silver nanoparticles (CitAgNPs) through a green synthesis method. Characterization of CitAgNPs was carried out using advanced analytical methods ensuring the quality, uniformity, size, and charge. The synthesized CitAgNPs exhibited non toxic effect when tested on *Vigna radiata* and *Danio rerio*, highlighting their potential for sustainability and safe therapeutic use. Antifungal assays demonstrated the potent efficacy of CitAgNPs in various *Candida* strains, with low MIC and MFC. CitAgNPs exhibited remarkable biofilm inhibition capabilities and elucidated specific mechanisms of action in *Candida* species, surpassing the performance of fluconazole.

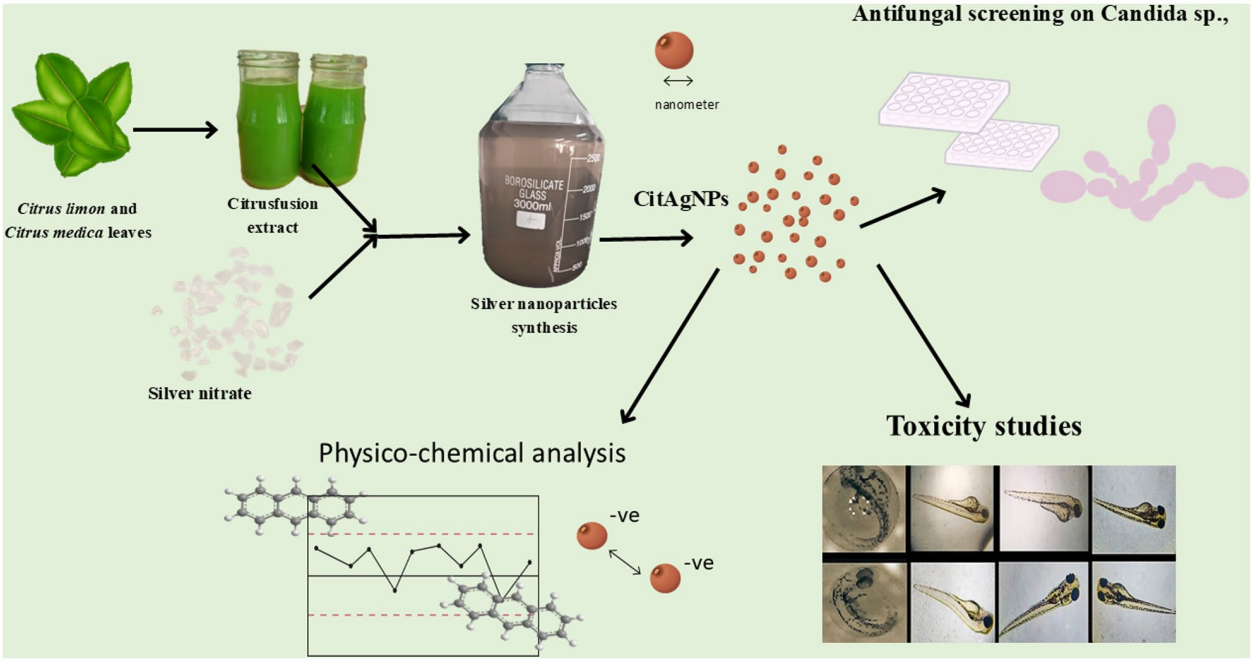
Conclusion This study underscores the immense potential of nanotechnology-driven approaches harnessing *Citrus* leaf extract for synthesizing highly effective antifungal nanoparticles. The fusion of biogenic nanoparticles with *Citrus* bioactive compounds presents a sustainable strategy for addressing the escalating challenge of azole-resistant *Candida* infections. The research outcomes suggest that CitAgNPs have promising applications in inhibiting *Candida* biofilms, offering potential solutions for infections caused by diaper rashes and onychomycosis, providing safe and effective alternatives to antifungal therapies.

†A. Harine and S. Ranjani: equal contribution.

*Correspondence:
S. Hemalatha
hemalatha.sls@bsauniv.ac.in

Full list of author information is available at the end of the article

Graphical abstract



Keywords *Candida* strains, Citrusfusion nanoparticles, Azole-resistant, Nanotechnology, Biofilm inhibition

Background

Candida species are commensal organisms of the human microbiota but can shift to opportunistic pathogens, causing substantial infections in moist body sites such as the groins, underarms, skin folds, nail beds, and interdigital spaces [1]. Notable species include *Candida albicans, Candida tropicalis, and Candida metapsilosis* (from *Candida parapsilosis* and *Candida orthopsilosis* complex), with *C. albicans* predominating as a leading cause of fungal infections in critically ill or immunocompromised patients. *C. tropicalis*, recognized as the second most virulent *Candida* species, normally colonizes skin and nails but can instigate skin infections [2, 3]. A pivotal challenge posed by *Candida* species is their propensity to develop robust biofilms, which confer protection against antifungal agents and the immune system, thus complicating treatment efforts. These biofilms are intricate assemblies of microorganisms adhering to surfaces [4]. *Candida* also manifests dimorphism, characterized by the transition from a yeast-like to an invasive, filamentous form in response to environmental cues, enabling tissue invasion. Overgrowth of *Candida* can lead to superinfections, particularly in immunocompromised individuals, where infections become highly refractory to antifungal therapy. *Candida*-associated morbidity and mortality are significant concerns, affecting both immunocompromised and healthy individuals, influenced by factors such as variations in skin microbiota, antibiotic usage, underlying conditions, and immune dysregulation [5]. Fluconazole resistance remains a critical challenge in the management of *Candida* infections, particularly with emerging species like *Candida albicans* and *Candida tropicalis*, which pose significant global health threats. While some resistance mechanism observed in *C. albicans* are shared with non-albicans species, there are also distinct differences. Research efforts are urgently needed to elucidate the complex mechanisms underlying fluconazole resistance, particularly in emerging *Candida* species, to inform targeted therapeutic interventions and mitigate the growing impact of antifungal resistance in clinical settings [6].

Nanotechnology holds immense potential in medicine, particularly in the development of advanced drug delivery systems and targeted therapies. Green synthesis, utilizing plants for the synthesis of nanoparticles, aligns with the principles of green nanotechnology by offering sustainable and eco-friendly methods to produce nanomaterials with biomedical applications. In the field of nanomedicine, nanoparticles play a crucial role as carriers for drug delivery due to their unique properties, including high surface area, tuneable size and shape, and ability to encapsulate or attach therapeutic agents [7]. Particularly, silver nanoparticles have high potential as a microbicidal agent by breaking the microbial

cell wall and affecting its function [8]. Green synthesis methods, which involve utilizing plant extracts or phytochemicals as reducing and stabilizing agents, offer several advantages over conventional synthesis approaches. These methods are generally free from toxic chemicals, use renewable plant-based inputs, and operate at lower temperatures, reducing energy consumption and environmental impact [9]. Plants possess a rich repository of bioactive compounds such as flavonoids, alkaloids, and terpenoids, which can effectively reduce metal ions and facilitate the formation of nanoparticles.

The exploration of *Citrus* species namely *Citrus limon* (lemon) and *Citrus medica* (citron) for their bioactive compounds has gained significant attention in biomedical research, particularly in synthesising nanoparticles and the development of novel antifungal agents. *Citrus limon*, commonly referred to as lemon, is recognized for its rich content of alkaloids, which have demonstrated potent antibacterial properties. Lemon leaves, in particular, contain a repository of alkaloids that exhibit promising medicinal potential. Furthermore, flavonoids found in lemon have broad-spectrum biological activities, including antibacterial [10], antifungal, and antiviral effects. *Citrus medica*, or citron, is another *Citrus* species rich in bioactive compounds with therapeutic properties [11]. Phytochemical analysis of citron leaves has revealed the presence of flavonoids, alkaloids, terpenoids, and other constituents. *Citrus medica* holds promise for various medicinal applications due to its diverse chemical composition and traditional use in folk medicine. The synergy of both lemon and citron plant leaf extracts contributes significantly to silver nanoparticle synthesis, specifically in the form of polyherbal extract, serving as an effective reducing agent for silver ions and as a stabilizing agent in the formation of Citrusfusion silver nanoparticles (CitAgNPs). These synthesized nanoparticles undergo rigorous evaluation for toxicity and are designed to target infection-causing *candida* strains. Their activity is assessed through broth microdilution assays to identify their Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) and antibiofilm assays and the mechanism of action against *Candida* strains gauge their efficacy against candidal infections. This study was necessary to explore the potential of Citrusfusion extract in synthesizing CitAgNPs for combating *Candida* infections, addressing the need for effective and safe alternatives to conventional antifungal treatments.

Methods

Preparation of plant extract

The preparation of a Citrusfusion extract involved obtaining leaves from *Citrus limon* and *Citrus medica* for green synthesis of nanoparticles, leveraging their bioactive compounds. Fresh leaves were carefully harvested, sterilized, and ground into a fine paste. This paste was then macerated in autoclaved distilled water overnight, utilizing a specific weight-to-volume ratio. Subsequently, the macerated extract underwent triple filtration using centrifugation and Whatman filter paper to eliminate fibres and small leaf sediments, ensuring a pure extract. The *Citrus limon* and *Citrus medica* extracts were mixed into Citrusfusion extract with an equal 1:1 ratio. The clarified extract was then stored at 4 °C for preservation.

The phytochemical analysis of *Citrus limon* and *Citrus medica* leaf extracts involved a comprehensive assessment of their bioactive constituents, including phenols, tannins, flavonoids, saponins, anthocyanins, alkaloids, steroids, and carotenoids. The evaluation followed established protocols as outlined by [12], ensuring meticulous identification and characterization of these phytochemicals.

Citrus limon (lemon) and *Citrus medica* (citron) used in study was identified and authenticated by Dr. D. Narasimhan, Botanist, Chennai. A specimen of the plant was deposited at School of Life Sciences, BSACIST, Chennai (Accession number: SLS-BSAU-23,008).

Synthesis of nanoparticles (CitAgNPs)

The Citrusfusion extract was employed in the synthesis of nanoparticles, as described by [13]. A 1 mM solution of silver nitrate (Sisco Research Labs Pvt. Ltd (SRL), based in India) was mixed with the extract in a 1:5 ratio, resulting in a noticeable change in colour within 24 hours. This colour change was characteristic of the reduction of silver ions to silver nanoparticles and the synthesised nanoparticles settled down, facilitated by the bioactive compounds present in the Citrusfusion extract [13]. Following the synthesis process, the mixture underwent centrifugation using a refrigerated cooling centrifuge, specifically the Eppendorf 5810 R, to separate and remove excess silver ions and other sediments. Subsequently, the silver nanoparticles were suspended in dimethyl sulfoxide (DMSO) (Sisco Research Labs Pvt. Ltd (SRL), based in India) and sonicated in a cold environment until the nanoparticles were fully dissolved in the solvent for further characterization and antifungal screening. The entire synthesis process involving colour change, centrifugation, and sonication was conducted under controlled conditions to ensure the formation of stable and uniform nanoparticles. The resulting nanoparticles were termed Citrusfusion nanoparticles (CitAgNPs).

Characterization of nanoparticles (CitAgNPs)

The dark reddish-brown mixture containing Citrusfusion silver nanoparticles underwent UV–visible spectroscopy analysis across the wavelength range of 200 nm to 800 nm to confirm the successful synthesis of nanoparticles.

UV–visible spectroscopy (JASCO Model V730 UV-Vis Spectrometer) is essential for characterizing the optical properties of nanoparticles, particularly their plasmonic behaviour [14]. FTIR (Fourier Transform Infrared) spectroscopy (JASCO Model 6300 FTIR Spectrometer). The analysis was conducted in the ATR mode with a resolution of 4 cm^{-1}. Spectrum was obtained by averaging 32 accumulations to ensure high signal-to-noise ratio. The wavenumber range analyzed extended from 4000 to 400 cm^{-1} to identify secondary metabolites, and chemical bonds present in the *Citrus limon* and *Citrus medica* leaf extract mediated CitAgNPs. FTIR is instrumental in elucidating molecular structures and functional groups within the samples and the molecular groups that act as capping agents can be identified [12].

Further characterization of the CitAgNPs was carried out by using FESEM (Field Emission Scanning Electron Microscopy and EDAX (Energy Dispersive X-ray Analysis) (Carl Zeiss SIGMA HV SEM equipped with a Bruker Quantax 200-Z10 Energy Dispersive X-ray Spectroscopy (EDS) Detector) to examine their size, shape, morphology, and elemental composition. FESEM offers high-resolution imaging and elemental morphology. To assess the crystalline structure of the CitAgNPs, XRD (X-ray Diffraction) analysis was performed. XRD (Rigaku Ultima IV) was utilized to identify the crystal phases and determine the crystallinity of nanoparticles, aiding in understanding their structural properties [15]. Particle size, surface charge, polydispersity index, and hydrodynamic size of the CitAgNPs were analyzed using a particle size analyzer with the Dynamic Light Scattering (DLS) principle combined with a Zeta analyzer (Malvern Instruments Ltd.). This analysis provides crucial information about the physical, and chemical properties and stability of the nanoparticles in solution [16].

Toxicity assessment of CitAgNPs

Toxicity assessments of the Citrusfusion nanoparticles were conducted using plant and animal models to evaluate their safety profile. Plant toxicity testing aimed to ensure the nanoparticles compatibility with environmental sustainability. *Vigna radiata* (mung bean) was selected as the plant model [17], Different concentrations of CitAgNPs (1, 5, and 10 µg/ml) were applied, and seedling germination was monitored for 5 days. Measurements of plant size and weight were recorded to assess any potential adverse effects.

Further, toxicity evaluation was performed using *Danio rerio* (zebrafish) embryos as a model organism to infer potential impacts on human health [18]. Wild type *Danio rerio* (zebrafish) embryos were procured from Tarun Fish Farm, Manimangalam, Chennai 601 301. The CitAgNPs of varying concentrations were added to the medium containing embryos, and the hatching process and subsequent growth of the hatchlings were observed over four days using a compound microscope. This approach enabled the detection of any developmental abnormalities or physiological changes associated with exposure to the Citrusfusion nanoparticles. These toxicity studies contribute vital information regarding the safety and biocompatibility of the Citrusfusion nanoparticles, addressing key concerns from both environmental and human health perspectives [12, 19].

Antifungal screening of CitAgNPs

Candida strains including *Candida albicans* ATCC 10231, *Candida albicans, Candida tropicalis,* and *Candida metapsilosis* were utilized in this study following appropriate ethical clearance. Clinical strains namely *Candida albicans, Candida tropicalis,* and *Candida metapsilosis* was isolated from patient samples and were obtained from Tagore Medical College and Hospital, Chennai after proper ethical approval from BSACIST (Ref. no. BSAU: REG-OFF: 2016/02SLS). The antifungal efficacy of CitAgNPs was assessed using several standardized assays. The antifungal efficacy of CitAgNPs was assessed through the agar well diffusion method. Each well was loaded with A. 25 µL of silver nitrate solution, B. CitAgNPs 12.5 µg/mL, C. CitAgNPs 25 µg/mL, D. CitAgNPs 50 µg/mL, E. CitAgNPs 100 µg/mL, F. 25 µL of fluconazole (1 mg/mL) as a reference antifungal agent. The inoculated agar plates were incubated at 37 °C for 24 hours, after which the inhibition zone diameters were measured [20].

Further, Micro broth dilution antifungal susceptibility testing following CLSI M27-A3 guidelines to determine the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of CitAgNPs against *Candida* strains [21]. Serial dilutions of CitAgNPs (ranging from 100 to 0.78 µg/ml) were prepared and added to 96-well plates containing Yeast Peptone Dextrose broth (YPD broth) and fungal inoculum. Fluconazole a standard antifungal agent, served as a positive control at 25 µg/ml concentration. The plates were then incubated at 37 °C for 48 hours, and optical density readings were taken at 12-hour intervals using a multimode spectrophotometer to construct time-kill curves. After 24 hours, the MIC was visually determined as the lowest concentration of CitAgNPs that inhibited fungal growth based on turbidity. To determine the MFC, the contents of the wells corresponding to MIC values were sub-cultured onto agar plates and further incubated at 37 °C for 24 hours. The concentration showing the least colony formation, or no colony formation indicated the MFC value, representing the concentration at which CitAgNPs exerted fungicidal activity.

Additionally, an anti-biofilm assay was conducted using both the 96-well microplate method [22] and the tube

assay. *Candida sp*. cultures were incubated for 48 hours to facilitate biofilm development. After staining with crystal violet and subsequent destaining, absorbance was recorded at 517 nm using a spectrophotometer, following the protocol described by [19, 23]. This assay provided insights into the ability of CitAgNPs to inhibit *Candida* biofilm formation, a critical factor in fungal pathogenesis and treatment resistance.

Mechanism of action of CitAgNPs on Candida species

In vitro assays were conducted to assess the mechanism of action of CitAgNPs (Citrusfusion silver nanoparticles) on fungal cell integrity and oxidative stress levels, tailored to the specific MIC concentrations for each fungal isolate. The assay for leakage of total sugars (Anthrone method) and proteins (Lowry method) provided insights into the disruption caused by CitAgNPs to fungal cell membranes. MIC concentrations were prepared and incubated with fungal cells (10^6 CFU/ml) for 6 hours at 37 °C. Subsequent centrifugation of culture samples allowed for analysis of the supernatant, which was frozen for later assessment of protein and sugar levels. This approach elucidates the mechanisms by which CitAgNPs induce cellular leakage, potentially compromising fungal cell function [24, 25]. Measurement of malondialdehyde (MDA) levels was performed to evaluate lipid peroxidation and oxidative stress induced by CitAgNPs treatment. Fungal cells cultured in YPD broth were treated with CitAgNPs. The Thio-barbituric acid (TBA) method was employed by mixing treated cell cultures with 10% SDS and TBA solution, followed by incubation and centrifugation. The absorbance of the supernatant at 530 nm allowed for precise determination of MDA levels, indicating cellular oxidative damage.

The assessment of glutathione reductase (GSH) levels provided insights into cellular antioxidant responses to CitAgNPs exposure [26]. After a 12-hour incubation period, fungal cells were harvested, washed, and lysed. GSH levels were quantified using Ellman's reagent, reacting with GSH to produce a yellow product measurable at 412 nm. This assay illuminates the role of GSH in combating oxidative stress induced by CitAgNPs.

Estimation of catalase (CAT) and superoxide dismutase (SOD) activities were conducted to evaluate antioxidant enzyme responses [27, 28]. Catalase enzyme activity was assessed by measuring the reduction of hydrogen peroxide (H2O2), while SOD activity was analysed through measurement of absorbance at 560 nm following incubation with a reaction mixture containing specific components. These *in vitro* assays provide a comprehensive understanding of the biochemical and cellular responses of *Candida* isolates to CitAgNPs exposure, crucial for elucidating the mechanisms of action underlying the antifungal properties of CitAgNPs

Statistical analysis

The experiments described were conducted in triplicate, and statistical analysis was carried out using Microsoft Excel. Error bars depicting means with standard errors were utilized to represent data obtained from experimental assays. To assess statistical significance for each in vitro experiment, a Student's t-test was performed. The results of the t-test are denoted by asterisks (*), with significance levels indicated as * for $P<0.05$, ** for $P<0.01$, and *** for $P<0.001$.

Results

Synthesis and characterisation of CitAgNPs

The phytochemical composition of *Citrus limon* and *Citrus medica* leaves, as identified through the Harborne method, underscores the rich diversity of bioactive constituents present in these botanical extracts. Phenols, tannins, alkaloids, flavonoids, saponins, and steroids are among the key phytochemicals identified in both *Citrus limon* and *Citrus medica* leaf extracts (Table 1).

During synthesis observed colour change from pale light yellow to reddish brown signifies successful nanoparticle synthesis. Visible precipitation indicates nanoparticle formation and settling, suggesting the plant's bioactive compounds act as both reducing and capping agents. The synthesised Citrusfusion nanoparticles (CitAgNPs) were characterized using UV–visible spectroscopy. The resonance peak associated with the surface plasmon of CitAgNPs was detected between wavelengths spanning from 350 to 500 nm. (Fig. 1a), confirming the biosynthesis of silver nanoparticles at 400 nm highest peak was confirmed. Previous studies have reported a similar SPR peak formation in this range, with peak intensity correlating positively with UV radiation exposure duration. The FT–IR analysis of CitAgNPs (Table 2) revealed the presence of various functional groups, including alkanes, arenes, amine salts, alcohol, Nitro compound, carboxylic acids, alkynes, alkenes, aromatics, C–Br, and C–I bonds (Fig. 1b). These functional groups play significant roles in the bioactivity and chemical properties of the nanoparticles. The synthesized silver nanoparticles exhibited an average size of 21.4 nm

Table 1 Phytochemicals present in Citrusfusion (*Citrus limon* and *Citrus medica* leaf) extract

S.no	Phytocompounds	Present/ Absent
1.	Phenols	Present
2.	Tannins	Present
3.	Flavonoids	Present
4.	Saponins	Present
5.	Anthocyanins	Absent
6.	Alkaloids	Present
7.	Steroids	Present
8.	Carotenoids	Absent

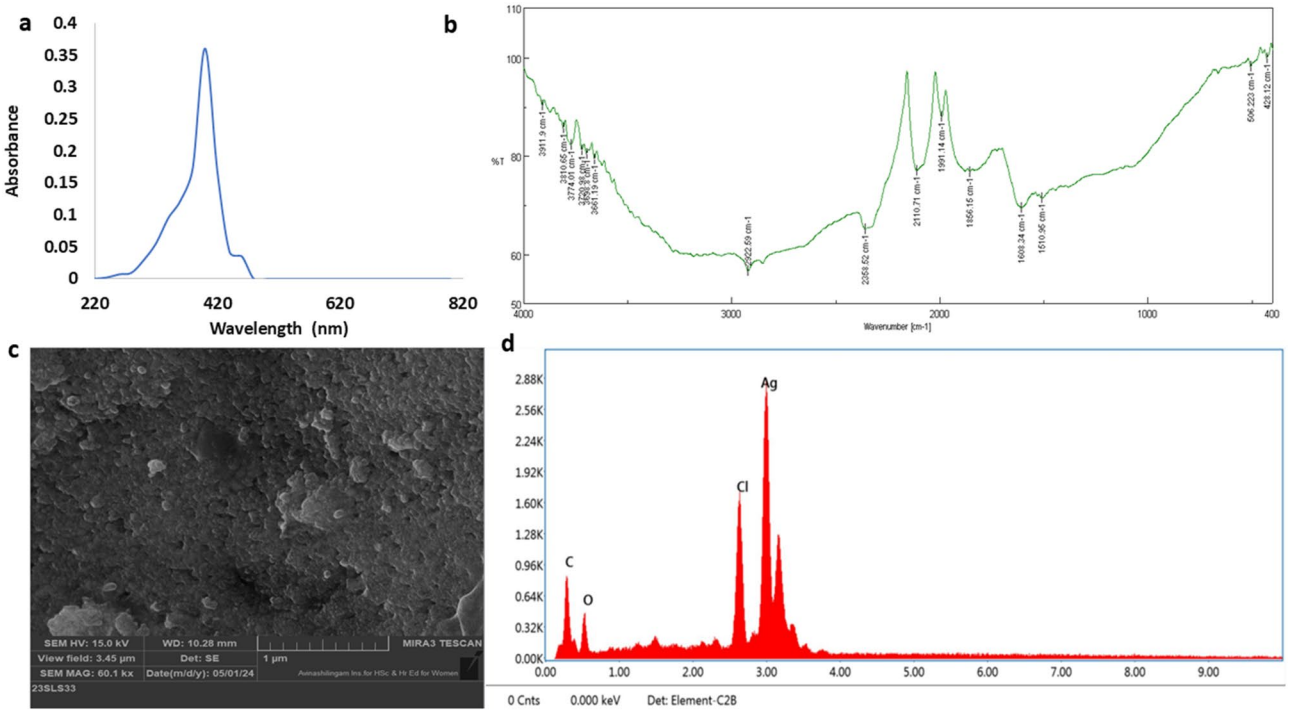


Fig. 1 Physicochemical characterization and Elemental composition analysis of CitAgNps: (**a**) Uv–Vis absorption spectrum of CitAgNps (**b**) FT–IR spectrum of CitAgNps (**c**) FESEM microscopic image of CitAgNps (**d**) EDAX spectrum of CitAgNps

Table 2 FTIR analysis Peak wavelength and functional group present in CitAgNps

S.no	Wavenumber cm^{-1}	Compounds	Stretching
1.	3698.8	Alcohol	O-H bending
2.	3661.19	Carboxylic acids	O–H stretching
3.	2922.59	Amine salt	N–H Stretching
4.	2922.59	Alkane	C–H Stretching
5.	2110.71	Alkyne	C≡C stretching
6.	1991.14	Allene	C≡C≡C Stretching
7.	1738.51	Arene	C≡C Stretching
8.	1608.34	Alkene	C=C stretching
9.	1510.96	Nitro compound	N–O Stretching
10.	506.223	Halo compound	C–I stretching

in FESEM analysis, falling within the typical nanoscale range of 1–100 nm, as observed at 100 kx magnification. The nanoparticles displayed a spherical morphology (Fig. 1c). The EDAX analysis revealed a predominant presence of silver, characterized by a strong elemental peak at 3 keV indicative of surface plasmon resonance. Silver comprised 66.37% by weight percentage and 25.03% by atomic percentage. Other detected elements include carbon (10.35% wt, 35.07% at), oxygen (9.45% wt, 24.04% at), and chlorine (13.82% wt, 15.86%) (Fig. 1d). X-ray diffraction (XRD) analysis confirmed the crystalline structure of CitAgNPs by analysing its sharp peaks, exhibiting characteristic 2θ values indicative of a face-centred cubic (fcc) lattice with reflections corresponding to the (111), (200), (220), and (311) planes (Fig. 2a) as per JCPDS file No. 04–0783. Similar XRD patterns have been observed in prior studies of silver nanoparticles. Additional peaks in the XRD spectrum are attributed to the presence of aqueous extracts from *Citrus limon* and *Citrus medica*, crucial for bioreduction and capping processes in CitAgNP synthesis [29]. Dynamic Light Scattering (DLS) was employed to analyze the surface charge, polydispersity index (PDI), and hydrodynamic size of CitAgNPs [20]. The average hydrodynamic size of the CitAgNPs was determined as 107.07 nanometers (Fig. 2b). The calculated PDI of 0.27 suggests a relatively narrow size distribution. A PDI value above 0.2 indicates polydispersity, where particles vary in size. The surface charge of the CitAgNPs was measured at − 22.89 mV (Fig. 2c), indicating a negative charge distribution that promotes repulsion between particles, thereby preventing aggregation. This inherent stability allows for prolonged storage of the CitAgNPs.

Toxicity assessment of CitAgNPs

CitAgNPs significantly influenced *Vigna radiata* seedling growth and germination at different concentrations over 5 days. It was observed that at all the concentrations there were no toxic effects, particularly at a concentration of 5 µg/ml, CitAgNPs significantly increased shoot length compared to the control with a maximum height of 10 cm,13 cm and 15 cm (Fig. 3a). and root length ranging from 3.5–3.8 cm observed under nanoparticles

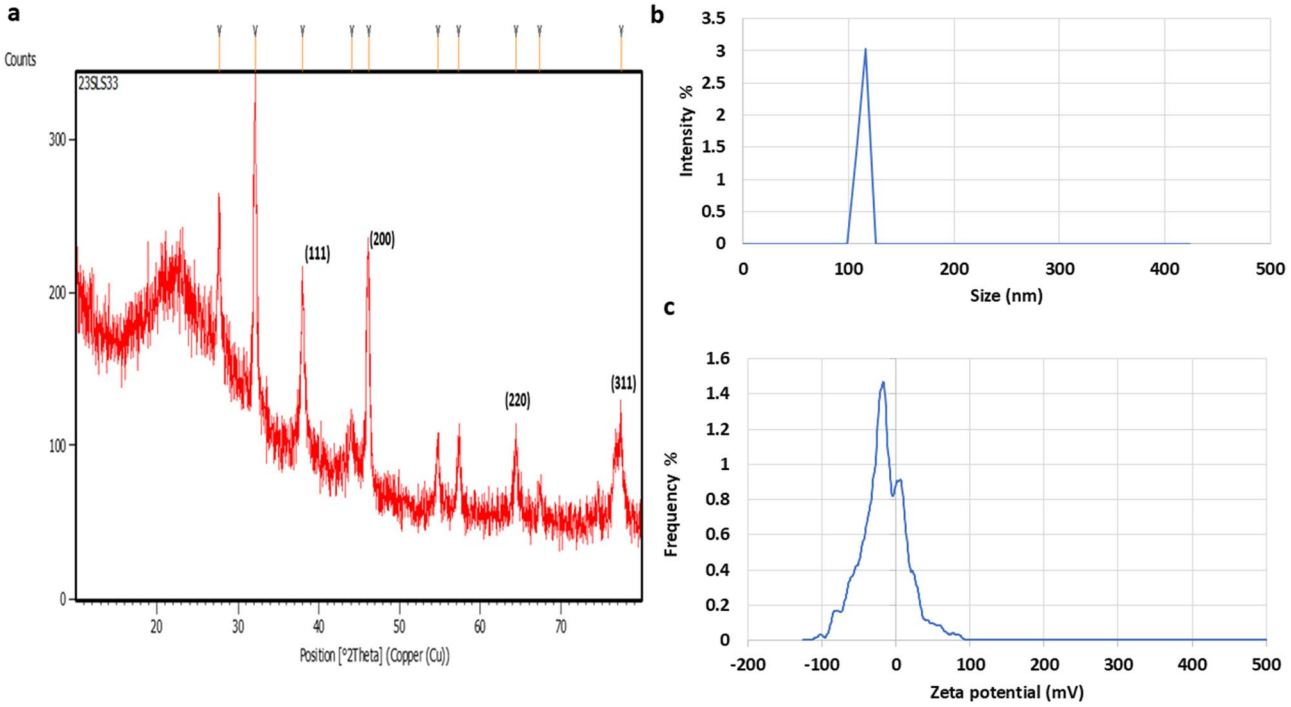


Fig. 2 Physicochemical characterization of CitAgNPs: (**a**) X-Ray Diffraction of CitAgNPs (**b**) Particle size distribution analysis using Dynamic Light Scattering (DLS) under Brownian movement (**c**) Zeta potential of Cit AgNPs

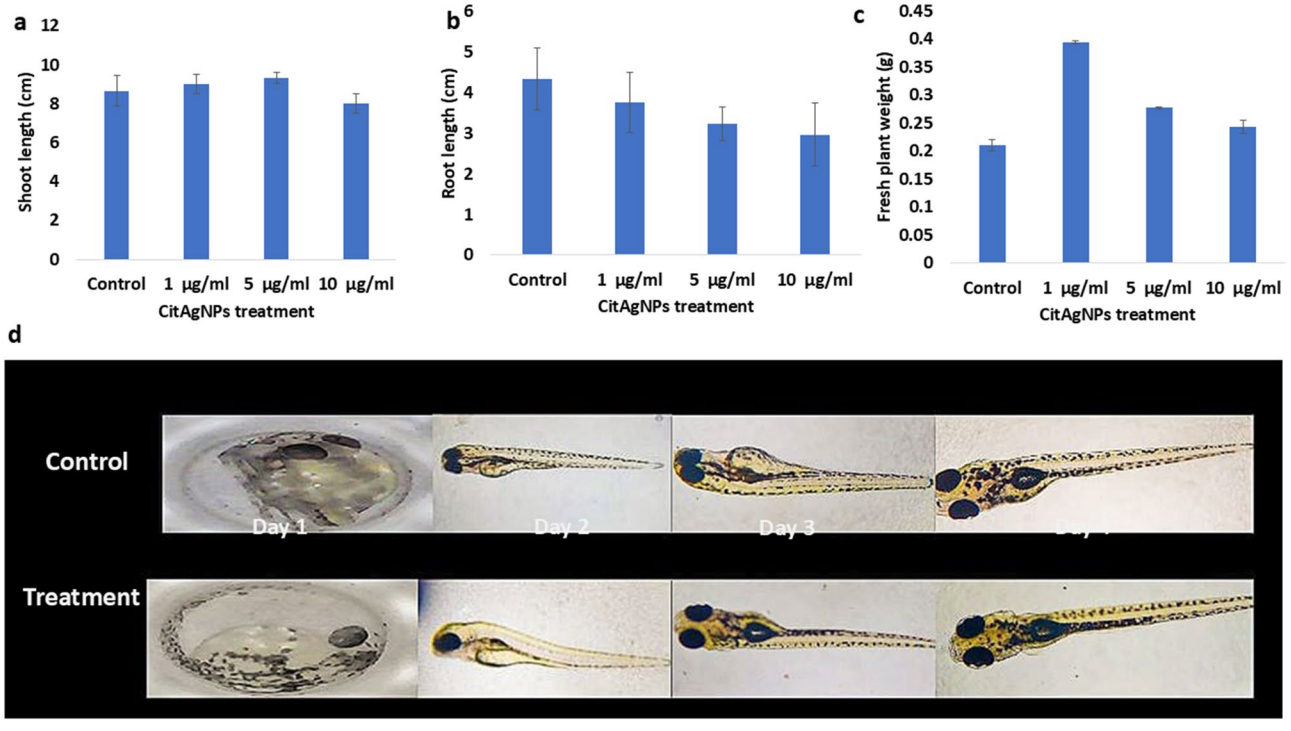


Fig. 3 Toxicity assessment of CitAgNPs: Effect of CitAgNPs in *Vigna radiata* seedlings on comparing with control (**a**) Shoot length (**b**) Root (**c**) Fresh weight. Effect of CitAgNPs in *Danio rerio*: (**d**) Toxicity studies on *Danio rerio* for period of four days comparing with control and maximum concentration of 50 µg/ml CitAgNPs

treatment versus average of 9.5 cm shoot length and 3.6 – 4.2 cm root length in the control nearly equal to treatment (Fig. 3b). CitAgNPs did not adversely affect seedling weight resulting with a maximum weight of 0.35, 0.39, and 0.40 grams while in control the average of 0.32 grams was noted (Fig. 3c), suggesting a lack of toxicity and even had the potential to promote plant growth. Furthermore, toxicity was assessed using *Danio rerio* (zebrafish) hatchlings exposed to CitAgNPs at a maximum concentration of 50 µg/ml. The hatchlings were closely monitored over 4 days. On the first day, the eggs successfully hatched, and by the second day, appendage development was evident, on the third day their nerves were visible. By the fourth day, the hatchlings appeared healthy with well-defined morphological features with eyes and heart (Fig. 3d).

Screening antifungal property of CitAgNPs

Measurement of zone of inhibition, MIC, MFC and time-kill kinetics of CitAgNPs

The antifungal potential of CitAgNPs in various *Candida albicans* strains, including both ATCC 10231, and pathogenic strains, *Candida albicans, Candida tropicalis, Candida metapsilosis,* was investigated through an agar well diffusion assay. Each wells were loaded with A. 25 µL of silver nitrate solution, B. CitAgNPs 12.5 µg/mL, C. CitAgNPs 25 µg/mL, D. CitAgNPs 50 µg/mL, E. CitAgNPs 100 µg/mL, F. 25 µL of fluconazole (1 mg/mL) as a reference antifungal agent. After incubation the zone of inhibition in *Candida albicans* strains ATCC 10231 was measured as 10, 12, 14, 16, 10 mm for 25 µL of silver nitrate solution, 12.5 µg/mL, 25 µg/mL, 50 µg/mL µL, 100 µg/mL of CitAgNPs, and 25 µL of fluconazole (1 mg/mL) respectively. The zone of inhibition in pathogenic *Candida albicans* was measured as 12, 13, 16, 17, 19 mm for 25 µL of silver nitrate solution, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL of CitAgNPs respectively and no zone for fluconazole. The zone of inhibition in pathogenic *Candida tropicalis* was measured as 11, 12, 14, 15, 16 mm for 25 µL of silver nitrate solution, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL of CitAgNPs respectively and no zone for fluconazole. The zone of inhibition in pathogenic *Candida metapsilosis* was measured as 12, 13, 14, 15, 17 mm for 25 µL of silver nitrate solution, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL of CitAgNPs respectively and no zone for fluconazole (Fig. 4).

Micro broth dilution assay was carried out by following CLSI M27-A3 guidelines. This study was designed for determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentrations (MFC) of CitAgNPs, coupled with assessments of time-kill kinetics. The findings of this study revealed significant antifungal activity of CitAgNPs, characterized by notably low MIC values across the tested *Candida* strains. Specifically, CitAgNPs demonstrated a MIC of 3.125 µg/ml against both *Candida albicans* and *Candida metapsilosis*, while exhibiting an even lower MIC of 0.78 µg/ml in *Candida tropicalis*. Furthermore, the MIC against *Candida albicans* ATCC 10231 was found to be as low as 0.39 µg/ml. The study evaluated the Minimum Fungicidal Concentrations (MFC) of CitAgNPs, which are critical for understanding the concentration required to achieve fungicidal activity [30]. The MFC values further highlighted the potent antifungal properties of CitAgNPs, with the lowest MFC observed against *C.albicans* ATCC 10231 (0.78 µg/ml), followed by *C. albicans* (3.125 µg/ml), *C. metapsilosis* (25 µg/ml), and *C. tropicalis* (6.25 µg/ml).

Based on the observed MFC/MIC ratios, CitAgNPs derived from Citrusfusion extract exhibit fungicidal properties, with MFC/MIC ratios ≤ 4 across all strains. A time-kill curve assay was conducted to assess the concentration-dependent and time-dependent killing kinetics of CitAgNPs at their respective MIC in *Candida* strains over 24, 36, and 48 hours (Fig. 5 a,b,c). The growth curves exhibited a significant decrease in growth percentage at 24 hours of CitAgNPs treatment by showing 83%, 82.8%, 79%, 73.7% compared to the control, after 48 hours of incubation the growth has decreased up to 10%-15% from 24hour growth it is noted there is a fluctuation in the growth curve in fluconazole treated cells that the growth percentage increased up to 12% at 48 hrs. These results underscore the potent antifungal activity and time-dependent effectiveness of CitAgNPs against *Candida* species.

Antibiofilm activity

Candida biofilms, prevalent in topical regions, pose significant challenges in infection management due to their complex, multi-layered structure and increased resistance to conventional antifungal therapies.

Biofilm assay was performed in tube method for *Candida* sp. and observed for formation of biofilm formation over the walls of the test tube in control, fluconazole and CitAgNPs treated tubes (Fig. 6a). The results of tube method showed blue colouration over the walls of the tubes showing the formation of biofilm. In *Candida albicans* ATCC 10231, *Candida albicans, Candida tropicalis*, and *Candida metapsilosis* the control and fluconazole tubes were blue in colour showing the formation of biofilm, whereas the intensity of the blue colour was very less or nil in CitAgNPs treated tubes. This shows the antibiofilm efficacy of CitAgNPs in tested *Candida* sp.

The biofilm inhibitory effects of nanoparticles are evaluated by microtitre plate method where CitAgNPs treatment (spherical-shaped silver nanoparticles) demonstrated substantial inhibition of biofilm formation across various *Candida strains*, including *Candida*

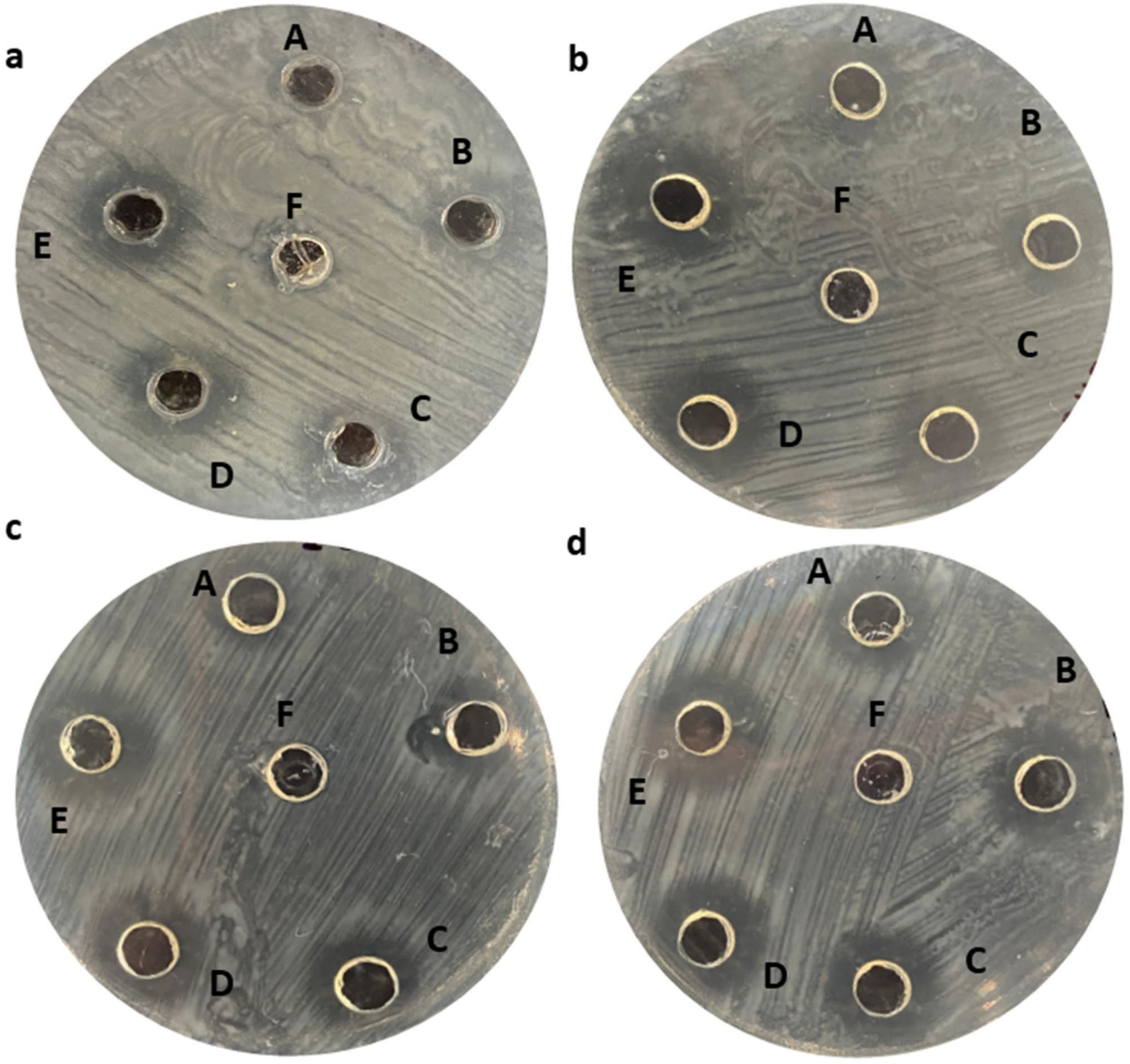


Fig. 4 Antibacterial potential of CitAgNPs in *Candida* strains: (**a**) *Candida albicans* ATCC 10231, (**b**) *Candida albicans*, (**c**) *Candida tropicalis*, (**d**) *Candida metapsilosis* Whereas the wells denote A. 25 µL of silver nitrate solution, B. CitAgNPs 12.5 µg/mL, C. CitAgNPs 25 µg/mL, D. CitAgNPs 50 µg/mL, E. CitAgNPs 100 µg/mL, F. 25 µL of fluconazole (1 mg/mL)

albicans ATCC 10231 (64%), *Candida albicans* (46%), *Candida tropicalis* (52%), and *Candida metapsilosis* (48%) (Fig. 6b). The fluconazole treatment showed the biofilm formation of 51%, 65%, 53%, 62% respectively in *Candida albicans* ATCC 10231, *Candida albicans, Candida tropicalis*, and *Candida metapsilosis* respectively.

Mechanism of action of CitAgNPs on Candida species

Protein and sugar leakage from fungal cells was assessed post-CitAgNPs treatment using the Minimum inhibitory concentrations. Our findings for leakage of sugar using the Anthrone method revealed elevated sugar concentrations in treated samples 4.5 and 4.8 µg/ml compared to control 2.2 and 2.3 µg/ml, indicating increased fungal cell permeability due to nanoparticle exposure and impact over the cell wall (Fig. 7a). Protein leakage from treated fungal strains was quantified using the Lowry method. This assay relies on cell wall disruption as of how sugar leaked, resulting in intracellular protein release into the surrounding medium of 5.1 µg/ml has observed in all the species and in control 3.2 µg/ml upon CitAgNPs treatment (Fig. 7b).

Elevated MDA levels are indicative of oxidative stress and can serve as a marker for assessing cellular

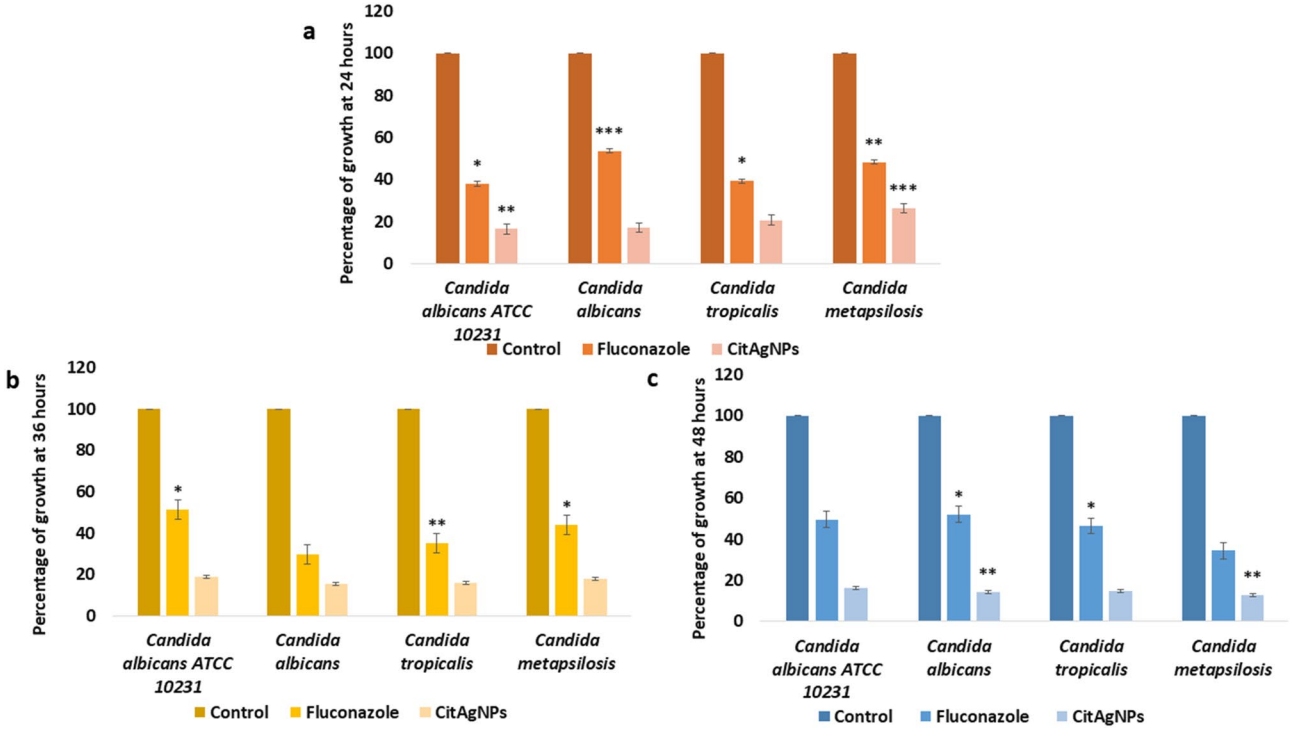


Fig. 5 Screening of antifungal activity of CitAgNPs in *Candida* strains: (**a**) Growth curve of *Candida* strains after 24 hrs of treatment with CitAgNPs when compared to control and fluconazole treated strains (**b**) Growth curve of *Candida* strains after 36 hrs of treatment with CitAgNPs when compared to control and fluconazole treated strains (**c**) Growth curve of *Candida* strains after 48 hrs of treatment with CitAgNPs when compared to control and fluconazole treated strains

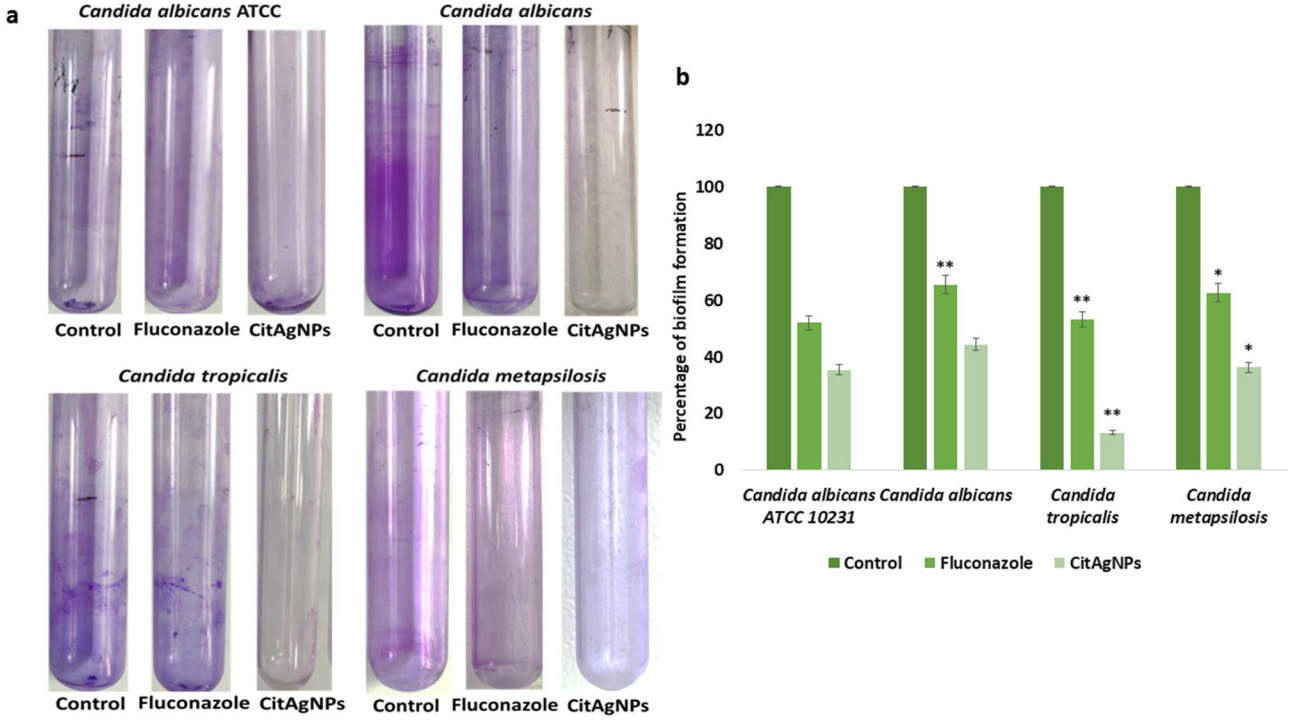


Fig. 6 Antibiofilm potential of CitAgNPs in *Candida* sp. (**a**) Tube assay depicting the biofilm formation in control, fluconazole, and CitAgNPs treated tubes. (**b**) Percentage of biofilm formation of *Candida* strains upon treatment with CitAgNPs when compared to control and fluconazole treated strains. Error bars represented means ± standard deviation. *Denotes T-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

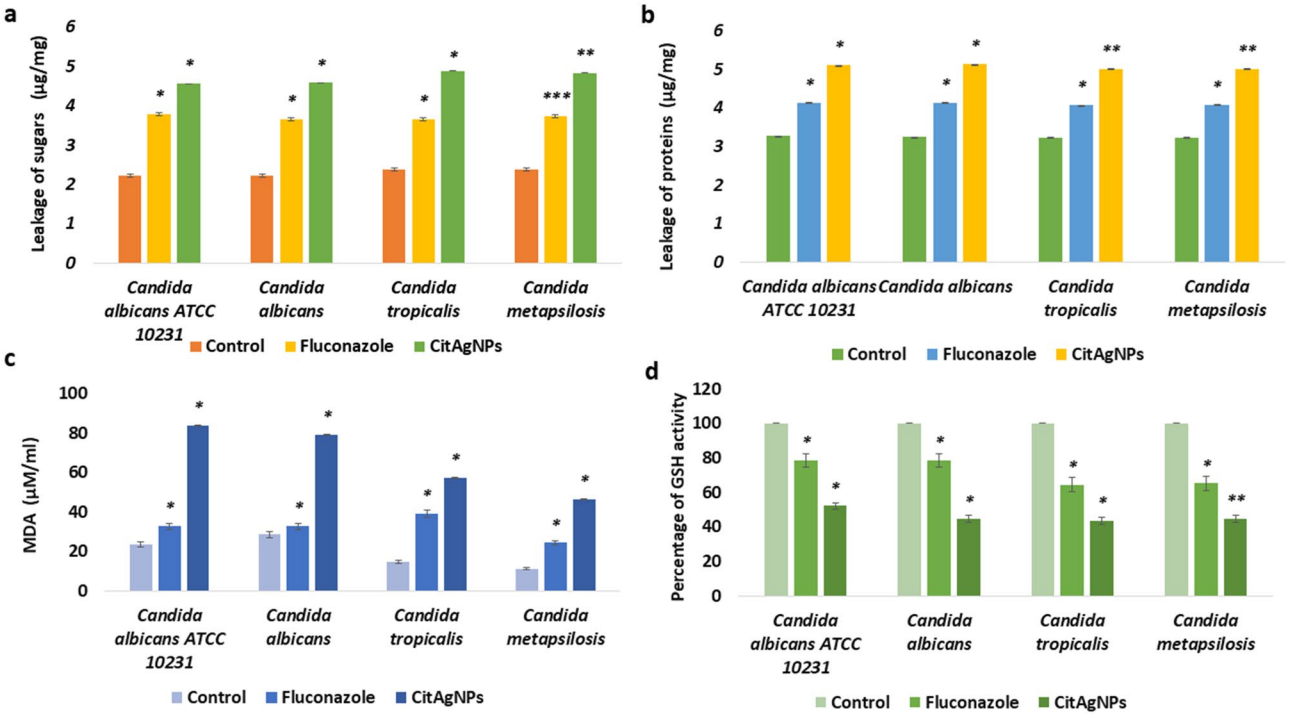


Fig. 7 Mechanism of action of CitAgNPs in *Candida* strains: (**a**) Leakage of sugars (**b**). Leakage of proteins upon treatment with CitAgNPs (**c**) Analysis of ROS production in *Candida* treated with CitAgNPs by MDA release (**d**) Percentage activity of GSH upon treatment with CitAgNPs. Error bars represented means ± standard deviation. * Denotes T-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

damage caused by oxidative processes CitAgNPs The findings showed that MDA levels were notably elevated in *Candida* strains treated with CitAgNPs compared to untreated control cells. Specifically, MDA levels were 40% higher in CitAgNPs-treated cells than those treated with the antifungal drug fluconazole or the control group (Fig. 7c). The impact of CitAgNPs on antioxidant levels in fungal cells was investigated through measurements of glutathione (GSH) levels. The results revealed a significant reduction in GSH levels in both fluconazole-treated and CitAgNPs-treated cells compared to the control. Specifically, fluconazole treatment led to a 20% decrease in GSH levels in *Candida albicans* ATCC 10231, *Candida albicans* which are fluconazole-sensitive strains, *Candida tropicalis*, and *Candida metapsilosis*. Meanwhile, CitAgNPs treatment resulted in more pronounced reductions (ranging from 45% to 66%) across these *Candida* strains (Fig. 7d). The dissolution of hydrogen peroxide (H2O2) catalyzed by CitAgNPs leads to the production of free radicals (hydroxyl -OH and/or superoxide -O2). Monitoring the activity of ROS-related enzymes, particularly superoxide dismutase (SOD) and catalase (CAT), provides insight into ROS formation and its impact on the antioxidant defence system. The findings from treating *Candida* strains with fluconazole and CitAgNPs revealed decreased catalase activity compared to untreated cells. Specifically, catalase levels were reduced following treatment with Fluconazole (77 U/ml) and CitAgNPs (45, 60, 50, 55 U/ml) across *C. albicans* ATCC 10231, *C. albicans*, *C. tropicalis*, and *C. metapsilosis*, respectively, compared to the control. Similarly, superoxide dismutase levels were diminished after treatment with CitAgNPs (23, 38, 18, 15 U/ml) which shows higher concentration comparing other studies [31] and fluconazole (40, 45, 44, 38 µmol /ml) (Fig. 8a) across the same four *Candida* strains compared to the control (100 U/ml). The reduction in these antioxidant enzymes (SOD, CAT, and GSH) leads to ROS accumulation, contributing to oxidative stress and cell viability loss. Decreased levels of these enzymes following CitAgNPs with 40–63% and fluconazole treatment (Fig. 8b) suggest compromised antioxidant defence mechanisms, leading to ROS accumulation and subsequent cellular damage [32].

Discussion

In phytochemical analysis it is found that both the *Citrus* leaf extracts are rich in Flavonoids, Phenols, Saponins, Steroids. Flavonoids, classified as polyphenolic compounds, exhibit notable antifungal activity, impacting fungal growth through various mechanisms such as plasma membrane disruption and induction of mitochondrial dysfunction. Additionally, flavonoids can inhibit microbial enzymes and interfere with microbial metabolism, thereby impeding fungal proliferation as mentioned in the early analysis of flavonoids as antibiotics [33]. Phenolic compounds derived from natural

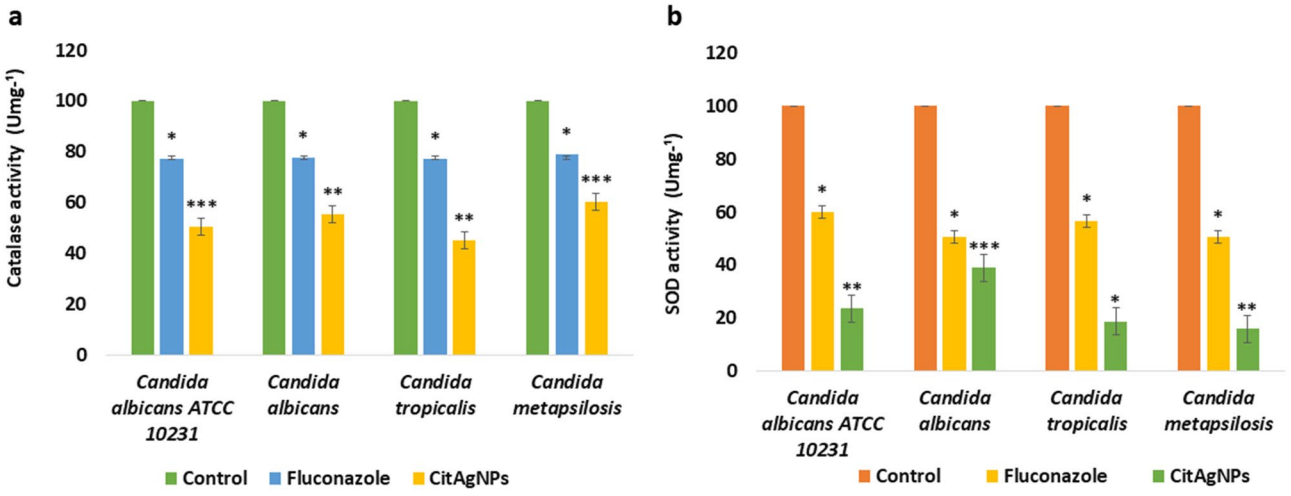


Fig. 8 Mechanism of action of CitAgNPs inducing oxidative stress in *Candida* strains: (**a**) Activity of catalase upon treatment with CitAgNPs (**b**) Activity of SOD activity upon treatment with CitAgNPs. Error bars represented means ± standard deviation. *Denotes T-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

sources, including *Citrus* leaves, possess significant antifungal properties, making them promising candidates for combating fungal infections [34]. Saponins and steroids, present in *Citrus* species, play vital roles in inhibiting biofilm formation—a critical factor in microbial pathogenesis and resistance [35]. Saponins disrupt microbial membranes, while steroids exhibit biofilm-inhibiting properties, collectively contributing to antimicrobial efficacy. These phytochemicals serve as crucial agents in silver nanoparticle (CitAgNP) synthesis. Flavonoids and phenolic compounds act as reducing agents, facilitating the conversion of Ag + ions to AgNPs during nanoparticle synthesis. Additionally, they function as capping agents, ensuring the stability and dispersibility of CitAgNPs by preventing agglomeration. Saponins and steroids, with their surface-active properties, aid in stabilizing CitAgNPs and preventing aggregation, enhancing the overall efficacy and application potential of these nanoparticles. The observed colour change and visible precipitation in the synthesis process of CitAgNPs indicate successful nanoparticle formation, likely facilitated by bioactive compounds in citrus extracts acting as reducing and capping agents. UV-visible spectroscopy confirmed the presence of silver nanoparticles, with a characteristic surface plasmon resonance peak between 350 to 500 nm, validating the biosynthesis process. These findings highlight an effective green synthesis approach using plant-derived materials for nanoparticle fabrication. Previous studies have reported a similar SPR peak formation in this range, with peak intensity correlating positively with UV radiation exposure duration [36]. The optical properties of silver nanoparticles, particularly SPR, are closely linked to their morphology, offering insights into nanoparticle size and shape. SPR analysis serves as a valuable tool for assessing the structural characteristics of synthesized nanoparticles. FTIR footprints showed the presence of functional groups as follows the wavenumber 3698.8 cm^{-1} corresponds to alcohol showing O–H bending, 3661.19 cm^{-1} corresponds to carboxylic acids by showing the O–H stretching, 2922.59 cm^{-1} corresponds to amine salt with N–H stretching, 2922.59 cm^{-1} corresponds to alkane with C–H stretching, 2110.71 cm^{-1} showed the presence of alkyne with C≡C stretching, 1991.14 cm^{-1} corresponds to allene with C≡C≡C stretching, 1738.51 cm^{-1} corresponds to arene with C≡C stretching, 1608.34 cm^{-1} corresponds to alkene with C=C stretching, 1510.96 cm^{-1} indicates the presence of nitro compound by showing N–O Stretching, 506.223 cm^{-1} corresponds to halo compound with C–I stretching (https://www.chem.ucla.edu/%7Ebacher/General/30BL/IR/ir.html). These functional groups play significant roles in the bioactivity and chemical properties of the nanoparticles. Alkanes contribute to hydrophobic interactions, while carboxylic acids can be involved in stabilizing the nanoparticles. Alkynes and alkenes provide structural integrity, and aromatic compounds may contribute to antioxidant properties. The presence of halogen bonds [C–Br and C–I) could influence reactivity and interactions with biological systems [37]. The spherical morphology and nanoscale size (21.4 nm] of the synthesized silver nanoparticles which is observed in FESEM analysis are advantageous for antifungal activity. This specific size and shape optimize surface area-to-volume ratio, enhancing interactions with fungal membranes and cellular structures [38]. The EDAX analysis of the sample highlighted a significant presence of silver, as evidenced by a distinct elemental peak at 3 keV, suggesting surface plasmon resonance. Silver constituted 66.37% by weight and 25.03% by atomic percentage. Other elements identified were carbon, oxygen, and chlorine, likely originating

from phenols and alkaloids in a Citrusfusion extract. Phenolic compounds, rich in carbon, oxygen, and hydrogen, are well-known contributors, while the presence of chlorine points to alkaloid compounds. This chemical composition underscores the complex organic nature of the sample. The X-ray diffraction (XRD) analysis of CitAgNPs revealed a face-centered cubic (fcc) crystalline structure, as indicated by sharp peaks at characteristic 2θ values corresponding to the respective planes. This confirms the typical structure of silver nanoparticles (AgNPs) and aligns with (JCPDS file No. 04–0783). The presence of additional peaks attributed to *Citrus* extracts suggests a biologically mediated synthesis route.

The Dynamic Light Scattering (DLS) data showed an average hydrodynamic size of 107.07 nanometres and a low polydispersity index (PDI) of 0.27, indicating a relatively uniform size distribution crucial for effective biological interactions. The surface charge of − 22.89 mV enhances stability by preventing particle aggregation. These properties are significant for the potency of CitAgNPs against fungi. The controlled size distribution and stable surface charge likely optimize interactions with fungal membranes, enhancing antifungal efficacy. Comparisons with prior studies highlight consistent nanoparticle characteristics critical for biological applications, including antifungal activity.

The results of the toxicity assessments on both *Vigna radiata* (mung bean) seedlings and *Danio rerio* (zebrafish) hatchlings indicate that CitAgNPs (citrate-coated silver nanoparticles) exhibit minimal to no toxicity across tested concentrations. In the case of mung bean seedlings, exposure to CitAgNPs at concentrations of 1, 5, and 10 µg/ml did not result in observable toxic effects; instead, a stimulatory effect on growth was noted, particularly at 5 µg/ml where significant increases in shoot length, root length and seedling weight were observed compared to the control. This suggests that CitAgNPs, at certain concentrations, may have the potential to promote plant growth without adverse consequences.

Similarly, in zebrafish hatchlings exposed to a maximum concentration of 50 µg/ml of CitAgNPs the hatchlings displayed normal developmental milestones, including hatching, appendage development, and the emergence of vital structures like nerves, eyes, and heart, indicating the absence of developmental toxicity. These findings collectively suggest that CitAgNPs exhibit efficacy in promoting plant growth and pose minimal risk for application. Further studies exploring the underlying mechanisms of these effects would be beneficial for harnessing the beneficial properties of CitAgNPs while ensuring environmental safety.

The agar well diffusion assay confirmed the antifungal activity of CitAgNPs in dose dependent manner. The clear zone of inhibition increases with increase in concentration. The mechanism of action of CitAgNPs may vary depends on the composition and consistency of medium. As the growth medium is solid the ability of nanoparticles to diffuse and its interaction may vary from one to another organism. The large surface area of the nanoparticles allows to interact with more number of organism which impedes the growth and forms the clear zone around the wells. The observed Minimum Inhibitory Concentration (MIC) values of CitAgNPs against *Candida* strains in this study demonstrate a notably higher potency compared to previous reports. Previous research has documented MIC values ranging from 32 to 4 µg/ml for various nanoparticles against *Candida* species [39]. In contrast, our study found much lower MIC values for CitAgNPs, with values as low as 0.39 µg/ml to 0.78 µg/ml against *Candida* strains. These findings highlight the exceptional antifungal efficacy of CitAgNPs, indicating a potential superiority over other nanoparticle formulations previously investigated. This enhanced efficacy could be attributed to specific properties of CitAgNPs, such as their particle size, surface charge, or the unique mechanism of silver ion release, which may synergistically enhance their antifungal activity. The Minimum Fungicidal Concentrations (MFC) of CitAgNPs were notably lower across various *Candida* species, with the lowest MFC recorded against *C. albicans* ATCC 10231 at 0.78 µg/ml. This is significantly lower than values reported in earlier studies, where MFC values ranged from 8 to 64 µg/ml [39]. The observed MFC/MIC ratios ≤ 4 for all strains indicate effective fungicidal activity, as the concentration required to kill fungal cells (MFC) is within fourfold of the concentration that inhibits growth (MIC). These results underscore the superior fungicidal properties of CitAgNPs derived from Citrusfusion extract, providing a promising alternative for combating *Candida* infections at relatively lower concentrations superior to prior values from other studies [40]. The time-kill curve assay results for CitAgNPs against *Candida* strains, as depicted in Fig. 5 a,b,c, reveal a pronounced concentration-dependent and time-dependent antifungal effect. The growth inhibition observed at 24 hours (ranging from 79% to 83%) underscores the rapid onset of CitAgNPs activity compared to conventional agents like fluconazole. Notably, at 48 hours, a further decrease in growth (10%-15% from 24-hour levels) signifies sustained efficacy. These findings align with prior research on silver nanoparticles (AgNPs) and their antimicrobial potential against *Candida*, but uniquely highlight the heightened and prolonged effect of CitAgNPs over time, enhancing their appeal for combating Candida infections.The time-kill kinetics of CitAgNPs demonstrate efficient fungicidal activity, with a rapid initial reduction in growth percentage within the first 24 hours, followed by sustained

suppression over 48 hours. The biofilm inhibitory effects of CitAgNPs (spherical-shaped silver nanoparticles) against *Candida* strains, demonstrated significant inhibition rates as evidenced by this study. The observed inhibition percentages (ranging from 46% to 64%) indicate a potent antibiofilm activity of CitAgNPs against these *Candida* species. The observed inhibitory effects of CitAgNPs are attributed to their ability interfere with certain gene regulation and inhibiting the translation process of amino acids, disrupting the production of exopolysaccharide layers essential for biofilm formation. This disruption likely interferes with cell-cell communication mechanisms, further impeding biofilm development. The unique properties of CitAgNPs, such as their shape and surface chemistry, play a crucial role in their antibiofilm activity. While in previous studies with aloe vera AgNPs [39] and grapevine AgNPs [41] showed similar results respectively, CitAgNPs have controlled their biofilm formation more effectively. The ability of CitAgNPs to inhibit biofilm formation highlights their potential as alternative or adjunctive therapies for managing *Candida* infections, particularly those involving biofilm-associated pathogens. The elevated sugar concentrations observed in treated samples (4.5 and 4.8 µg/ml) compared to controls (2.2 and 2.3 µg/ml) indicate increased cell permeability due to nanoparticles exposure. This leakage is indicative of compromised cell wall integrity, potentially caused by the physical interaction of CitAgNPs with the fungal surface. The quantified increase in protein leakage (5.1 µg/ml in treated vs. 3.2 µg/ml in control) further supports the notion of significant cell wall damage induced by CitAgNPs. This damage likely involves nanoparticle penetration through the cell wall, leading to the release of intracellular proteins. Overall, these findings highlight the efficacy of CitAgNPs against *Candida* strains by disrupting fungal cell wall integrity, which may involve nanoparticle-induced pitting and structural alterations. The significant 40% increase in MDA levels compared to fluconazole-treated or control cells suggests that CitAgNPs induce oxidative stress leading to lipid peroxidation in the fungal membranes [42]. This oxidative damage could disrupt cellular integrity, contributing to Candida's susceptibility to CitAgNPs. The observed decrease in GSH levels following CitAgNPs treatment suggests heightened ROS production, overwhelming the cellular antioxidant defence mechanisms and contributing to oxidative stress and cellular damage. The observed reduction in catalase activity post-treatment with both CitAgNPs and fluconazole across various *Candida* strains signifies compromised antioxidant defense mechanisms. Catalase, responsible for degrading hydrogen peroxide into water and oxygen, plays a crucial role in neutralizing reactive oxygen species (ROS) generated within cells. The decline in catalase levels following treatment indicates an inability to efficiently detoxify hydrogen peroxide, contributing to ROS accumulation and oxidative stress. Observed diminished superoxide dismutase (SOD) levels post-treatment imply decreased ability to convert superoxide radicals to hydrogen peroxide, further exacerbating ROS accumulation. The cumulative effect of reduced SOD and catalase activity underscores an imbalance in ROS homeostasis, ultimately leading to increased oxidative stress within *Candida* cells. The specific mechanism by which CitAgNPs induce oxidative stress involves potential cleavage of the cell wall and subsequent entry into the cell, where the released silver ions may interact with cellular components, disrupting redox balance. This disruption likely leads to the observed decrease in SOD and catalase activities, as well as compromised glutathione (GSH) levels, another critical antioxidant defense component. Based on the data presented, a hypothesis can be formulated that CitAgNPs exert antifungal activity by inducing oxidative stress through disruption of cellular redox homeostasis, leading to diminished antioxidant enzyme activities and accumulation of ROS. This oxidative stress cascade likely contributes to the observed lethality in *Candida* strains treated with CitAgNPs.

Conclusions

Our research underscores the exceptional antifungal efficacy of Citrusfusion silver nanoparticles (CitAgNPs) against Candida strains, particularly by targeting their biofilm-forming ability—a critical factor contributing to antifungal resistance and persistence. CitAgNPs demonstrated significant biofilm inhibition, effectively disrupting the resistance mechanism and preventing further fungal growth. This capability highlights CitAgNPs as a promising alternative to conventional antifungal treatments. A key application identified in this study is the potential integration of CitAgNPs into biomedical devices, such as catheters and diapers, which are prone to Candida biofilm formation. Incorporating CitAgNPs into these surfaces could mitigate biofilm-related infections, enhancing device safety and reducing healthcare-associated risks. Additionally, the ability of CitAgNPs to generate reactive oxygen species (ROS) further complements their antifungal mechanism, providing a robust and multi-functional approach to fungal inhibition. Our findings also reveal the potential of CitAgNPs for the development of innovative topical formulations. Their low toxicity, bioactivity, and ability to penetrate fungal cell walls make them ideal for addressing superficial fungal infections, particularly onychomycosis (nail infections). The ROS-generating properties of CitAgNPs, coupled with their antifungal efficiency, position them as ideal candidates for nail serums. Such formulations could provide targeted treatment, reducing fungal load while preserving the integrity of the nail and surrounding

tissue. These results establish CitAgNPs as a versatile and safe therapeutic option for managing resistant Candida infections, advancing antifungal therapy with novel, application-specific solutions. However, trials should be carried out to validate the efficacy of CitAgNPs real-time applications. After proper validation, based on the need and requirements tailormade products can be developed for the benefit of the society.

Abbreviations

CitAgNPs	Citrusfusion silver nanoparticles
FTIR	Fourier Transform Infrared Spectroscopy
FESEM	Field Emission Scanning Electron Microscopy
EDAX	Energy Dispersive X-ray Analysis
MIC	Minimum Inhibitory Concentration
MFC	Minimum Fungicidal Concentration

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Author contribution
SH: Conceptualization, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, original draft, Writing, review & editing. HA: Formal Analysis, Investigation, Methodology, Visualization, original draft, Writing. SR: Formal Analysis, Investigation, Methodology, Visualization, original draft, Writing

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Data availability
Data will be available on request

Declarations

Ethics approval and consent to participate
Clinical strains namely *Candida albicans, Candida tropicalis,* and *Candida metapsilosis* was isolated from patient samples and were obtained from Tagore Medical College and Hospital, Chennai after proper ethical approval from BSACIST (Ref. no. BSAU: REG-OFF: 2016/02SLS). The toxicity study of CitAgNPs in the embryos of *Danio rerio* was approved by institutional ethics committee of Sri Lakshmi Narayana Institute of Medical Sciences, Puducherry (NO. IEC/C-P/11/2023) in accordance with the institute guidelines and regulations. Wild type *Danio rerio* (zebrafish) embryos were procured from Tarun Fish Farm, Manimangalam, Chennai 601 301. Informed consent was obtained from the farm owners to carry out this research work.

Trial registration
Clinical trial number: Not applicable

Consent for publication
All authors read and approved the manuscript for publication

Competing interests
The authors declare no competing interests.

Author details
[1]School of Life Sciences, B. S. Abdur Rahman Crescent Institute of Science and Technology, Vandalur, Chennai, Tamil Nadu 600048, India

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