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Exploring the therapeutic potential of silver nanocomposition of *Catharanthus roseus* leaves extract for antimicrobial and antiviral activities: A pilot study

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ABSTRACT

Silver nanoparticles (AgNPs) synthesized from natural sources offer promising solutions for combating microbial and viral infections. *Catharanthus roseus* (Periwinkle), renowned for its diverse pharmacological properties, provides a sustainable and eco-friendly method for producing AgNPs with significant antimicrobial and antiviral effects. This study explores the cytotoxic potential of AgNPs derived from *C. roseus* and their antibacterial, antifungal, and anti-HIV activities, highlighting the novelty of employing a green synthesis approach. AgNPs from *C. roseus* leaf extract (AgNP-CR) were synthesized and characterized using spectroscopic and microscopic techniques to determine their physicochemical properties. The antibacterial activity of AgNP-CR was assessed against clinically relevant bacterial strains, and antifungal activity was evaluated against common fungal pathogens. Additionally, anti-HIV activity was investigated through in vitro assays using HIV-infected cells. Results demonstrated significant antibacterial activity of AgNP-CR against both gram-positive and gram-negative bacteria. Furthermore, AgNP-CR exhibited antifungal activity against pathogenic *Aspergillus* species. Importantly, AgNP-CR showed promising anti-HIV activity by inhibiting viral replication and cytopathic effects in infected cells. Cytotoxicity assays were also conducted to ensure the safety profile of the nanoparticles. Overall, this pilot study underscores the therapeutic potential of AgNPs synthesized from *C. roseus* in addressing bacterial, fungal, and viral infections. Further research is warranted to elucidate their mechanisms of action and optimize formulations for clinical applications.

1. Introduction

Nanotechnology, the manipulation of matter on an atomic and molecular scale, has revolutionized various fields, including medicine, pharmacology, and anti-pathogenic drug delivery. In healthcare, nanoparticles have garnered considerable attention for their role in transforming treatment modalities (Davis et al., 2008). Particularly in cancer therapy, nanoparticles serve as potent tools, facilitating targeted drug delivery and enhancing therapeutic effectiveness while minimizing side effects (Gavas et al., 2021; Mundekkad & Cho, 2022). Amid the increasing threat of antimicrobial resistance and the persistent challenge posed by infectious diseases, the quest for novel therapeutic agents with broad-spectrum efficacy has intensified. Silver nanoparticles (AgNPs) have emerged as promising candidates due to their exceptional antimicrobial properties and potential antiviral activity (Bruna et al., 2021; Luceri et al., 2023; Naumenko et al., 2023; Ratan et al., 2021; Yin et al., 2020). Utilizing natural sources to synthesize AgNPs represents an environmentally sustainable approach that aligns with the principles of green chemistry (Osman et al., 2024; Sharma et al., 2009).

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In recent years, the synthesis of AgNPs using phytoextracts has garnered significant attention as a green and cost-effective approach. These nanoparticles inherit the bioactive properties of the plant extract, thus presenting a multifaceted strategy for combating microbial infections and viral diseases. Despite extensive research on the antimicrobial properties of AgNPs, there remains a notable gap in understanding the efficacy of plant-mediated synthesis methods, particularly those involving *Catharanthus roseus* (L.) G. Don, recognized for its diverse pharmacological properties. *C. roseus*, commonly known as Periwinkle, is distinguished by its rich phytochemical composition and has long been esteemed in traditional medicine for its medicinal virtues, containing bioactive compounds such as alkaloids, flavonoids, terpenoids, and phenolic molecules (Lee et al., 2020; Pham et al., 2020).

While previous studies have investigated the antibacterial and antifungal activities of AgNPs, research on their antiviral effects, particularly against HIV, remains limited. This study aims to address these gaps by utilizing a green synthesis approach with *C. roseus* leaf extract to produce AgNPs and evaluating their comprehensive antimicrobial and antiviral potential. This approach offers a sustainable and effective solution for combating a wide range of pathogens. The silver nanoparticles derived from *C. roseus* leaf extracts used in this research were prepared in our previous study (Joshi & Aithal, 2024). *C. roseus* leaves were selected due to their rich content of bioactive compounds known for various pharmacological properties (Bansal et al., 2023). The aqueous extract was chosen for its eco-friendly and sustainable nature, preserving water-soluble phytochemicals crucial for nanoparticle synthesis (Bhardwaj et al., 2020). Specific bacteria (both gram-positive and gram-negative) and fungi (*Aspergillus* species) were selected based on their clinical relevance and susceptibility to silver nanoparticles, facilitating a comprehensive assessment of the antibacterial and antifungal efficacy of the synthesized AgNP-CR composition. Furthermore, we aimed to evaluate the potential of AgNP-CR on TZM-bl cells as antiviral agents against HIV, a persistent global health threat. TZM-bl cells were chosen for their high susceptibility to HIV infection and their ability to express β-galactosidase and luciferase upon viral entry, enabling precise measurement of viral replication and cytopathic effects (Gaikwad et al., 2023). The cytotoxic concentration (CC_{50}) of AgNP-CR on TZM-bl cells was determined to be 0.08 mg/ml, whereas its anti-HIV-1 activity was observed at a much lower concentration of 0.02 mg/ml (EC_{50}), indicating a favorable safety profile and a promising therapeutic window for antiviral applications.

By exploring the multifunctional properties of the silver nanoparticle-based composition AgNP-CR, our study aims to contribute to the expanding knowledge in the fields of nanotechnology, antimicrobial research, and virology. The insights gained from this research hold promise for developing novel therapeutic strategies to tackle challenges associated with antimicrobial resistance and viral infections. Furthermore, the use of AgNP-CR highlights the significance of leveraging nature's pharmacopeia for innovative solutions in combating infectious diseases.

2. Materials and methods

2.1. Chemicals and equipment

The study utilized high-purity silver nitrate ($AgNO_3$) as the precursor for nanoparticle synthesis, sourced from HiMedia, India. Antimicrobial and antifungal assessments were conducted using nutrient agar and potato dextrose agar (Gibco, USA) media, along with sterile filter paper discs. Additional reagents included cell culture media and supplements such as DMEM (Gibco, USA), FBS (Moregate, Australia), HEPES (Gibco, USA), antibiotics (Sigma, USA), and other standard reagents essential for microbial and cell culture maintenance. Key equipment used included a UV-VIS spectrophotometer for nanoparticle characterization, an autoclave for sterilization, incubators for microbial growth, and microscopy tools for visual inspection. Antiviral studies were performed in Biosafety Cabinets (MicroFilt, India) for culture handling, CO_2 incubators (Thermo Fisher Scientific, USA) for maintaining cell cultures, and a Victor 4 Luminometer (Perkin Elmer, USA) for luminescence-based assays. Additionally, an ELISA plate reader (Thermo Fisher Scientific, USA) was utilized for p24 ELISA to quantify HIV-1 viral replication.

2.2. Preparation of C. roseus leaf extract

The collection of plant material was conducted following the requisite permissions and adhering strictly to international and institutional guidelines and legislation. Fresh and young leaves of *C. roseus* were collected following specific agronomical practices to ensure the quality and consistency of the plant material. The plants were cultivated under controlled conditions in the Botanical Garden of Dnyanopasak College, Parbhani, Maharashtra, India, using organic farming methods to avoid contamination with pesticides or synthetic fertilizers **(Figure S1-supplementary file)**. The leaves were harvested during the flowering stage in July to ensure optimal phytochemical content and were immediately processed to preserve their bioactive compounds. Voucher specimens were formally identified and deposited at the Western Regional Centre, Botanical Survey of India (BSI), Pune, India (authentication number SUPACARO2), by Prof. V. N. Naik, a distinguished taxonomist at Babasaheb Ambedkar Marathwada University, Maharashtra, India. We are grateful for his generous gift of the *C. roseus* leaf extract used in this study. Briefly, the collected leaves were thoroughly cleansed under tap water, followed by rinsing with distilled water to eliminate any dust particles. The cleaned leaves were then carefully shade-dried and pulverized to a fine consistency. The aqueous extract was prepared according to established protocols. Specifically, 1 g of the dried leaf powder was dissolved in 100ml of warm distilled water. This mixture was incubated at 60 °C on a rotary shaker for various time intervals: 15, 30, 45, 60, and 80 minutes. After the designated incubation period, the mixture was allowed to settle for 10 minutes to facilitate sedimentation. The supernatant was then meticulously filtered through Whatman filter paper no. 1 with a pore size of 25µm to obtain clear extracts. These filtered extracts were subsequently cooled and utilized as stock solutions for further investigations, as described in our previous study and elsewhere (Joshi & Aithal, 2024; Sulaiman et al., 2013).

2.3. Preparation and synthesis of silver nanocomposition – AgNP-CR

The silver nanocomposition of *C. roseus* extracts was prepared and characterized previously by our research group (Joshi & Aithal, 2024). Briefly, a 1mM aqueous solution of silver nitrate ($AgNO_3$) was meticulously prepared following the method outlined by Sulaiman et al. (2013), using distilled water at room temperature. Specifically, 0.169 g of silver nitrate was accurately weighed and dissolved in 1000 ml of distilled water, ensuring thorough mixing until a clear solution of $AgNO_3$ was achieved. Subsequently, the synthesis of silver nanoparticles (AgNPs) using extracts from periwinkle leaves was initiated. The particle size was determined using Scanning Electron Microscopy (SEM), and the size of the silver nanoparticles ranged from 23.56 nm to 361.2 nm. Extracts corresponding to different extraction times (10, 20, 30, 40, 50, 60, 70, and 80 minutes) were separately aliquoted into labeled screw-cap tubes. Then, 9 ml of the prepared $AgNO_3$ aqueous solution was added to each tube, creating a reaction mixture. The reaction tubes were carefully

shielded from light to prevent photoreactivation and were left to incubate at room temperature. After thirty minutes, a discernible color change was observed in the reaction solutions within the tubes, ranging from a pale-yellow hue to a deep brown coloration. These color changes served as indicators of the successful synthesis of silver nanoparticles. The observed results, including the color changes and reaction outcomes, were meticulously recorded. The synthesized colored solutions were then utilized for subsequent experimental analyses as per the methodology described earlier (Appidi et al., 2008; Joshi & Aithal, 2024).

2.4. Microorganisms

For the investigation of the antimicrobial potential of the prepared AgNP-CR, five microorganisms were selected. These included bacterial species, specifically *Escherichia coli* (MTCC 739/Y429) and *Bacillus subtilis* (MTCC 441/JCM), as well as fungal species, namely *Aspergillus niger* (MTCC 281/CEP) and *Aspergillus fumigatus* (MTCC 343/CEP). These microorganisms were obtained from the culture collection of the Microbiology Research Centre at DSM College, Parbhani, sourced from the Microbial Type Culture Collection (MTCC) in India, and were used to investigate the antimicrobial potential of the prepared silver nanoparticles (AgNPs). Additionally, one primary isolate of HIV-1 (VB028) was employed in this study, conducted at ICMR-National AIDS Research Institute, Pune.

2.5. Culture method

For the cultivation of bacterial species, *E. coli* and *B. subtilis*, and fungal species, *A. niger* and *A. fumigatus*, standard culture techniques were employed (Cavalieri, 2005). Bacterial strains were cultured on nutrient agar plates and incubated at 37 °C for 24 hours. Fungal strains were cultured on potato dextrose agar plates and incubated at 30 °C for 48 hours. Based on standard practices for antimicrobial assays, bacterial strains (*E. coli* and *B. subtilis*) were used at a concentration of approximately 1 x 10^5 CFU/ml, and fungal strains (*A. niger* and *A. fumigatus*) were used at a concentration of approximately 1 x 10^4 spores/ml. These concentrations ensure the reliability and reproducibility of the antimicrobial assessments. Additionally, the primary isolate of HIV-1VB028 (R5, Subtype C) was propagated in human peripheral blood mononuclear cells (PBMCs) using established protocols (Gaikwad et al., 2023). TZM-bl cells (a modified HeLa cell line) were procured from the National Institute of Health (NIH) HIV Reagent Program and maintained in DMEM (Gibco, USA) containing 10% FBS (Moregate, Australia) and supplemented with HEPES (Gibco, USA) and antibiotics (Sigma, USA) at 37 °C in a 5% CO_2 humidified chamber. The infection with HIV-1VB028 was allowed to proceed for a designated period under controlled laboratory conditions. The culture of bacterial and fungal species, as well as the antibacterial and/or antifungal studies, were conducted at the Department of Microbiology, Dyanopasak Shikshan Mandal, Parbhani. The anti-HIV assays were performed at the Division of Virology, ICMR-National AIDS Research Institute, Pune. Overall, the utilization of these diverse microorganisms enabled a comprehensive assessment of the antimicrobial potential of the prepared AgNP-CR composition against a range of pathogens, including bacteria, fungi, and viruses.

2.6. Antibacterial assessment and bioassay of silver nanoparticles

The antimicrobial efficacy of silver nanoparticles synthesized from *C. roseus* was meticulously evaluated against the gram-positive bacterium *B. subtilis* and the gram-negative bacterium *E. coli*. To confirm the presence and assess the antimicrobial activity of the nanoparticles, we adopted the widely employed agar disc diffusion method. Seeded agar plates were prepared using active cultures of *E. coli* and *B. subtilis* in 50% nutrient agar medium, following established protocols (Baker et al., 2005; Sahoo et al., 2023). Sterile filter paper discs impregnated with the synthesized silver nanoparticles were carefully positioned onto the surface of the agar plates inoculated with the respective microbial cultures. After a specified incubation period, typically 24 hours, the plates were thoroughly examined for the presence of discernible zones of inhibition surrounding the discs. These zones served as visual indicators of the antimicrobial activity of the nanoparticles against the tested microorganisms. The diameter of the zones of inhibition was measured using calibrated instruments and compared to control discs containing known antimicrobial agents (gentamicin 10 µg, HiMedia, India) to gauge the relative efficacy of the silver nanoparticles. Additionally, to further evaluate the antibacterial activity, seeded agar plating was conducted and allowed to solidify. Subsequently, sterile filter paper discs were immersed in tubes containing the synthesized silver nanoparticles. These discs were carefully placed onto the seeded agar plates inoculated with *E. coli* and *B. subtilis*, following the method outlined earlier (Kim et al., 2007). Each concentration of the extract, corresponding to different time intervals up to 1 hour (10, 20, 30, 40, 50, 60, 70, and 80 minutes), was meticulously applied to the agar plates. Following application, all plates were incubated at 37 °C for 24 hours to allow for bacterial growth and the manifestation of potential antibacterial activity. After the incubation period, zones of inhibition surrounding the discs were visually observed, measured, and recorded in accordance with the protocol outlined previously (Thakkar et al., 2010).

The results from both the agar disc diffusion assay and the seeded agar plating provided invaluable insights into the antimicrobial potency of the silver nanoparticles synthesized from *C. roseus* against *E. coli* and *B. subtilis*. These findings highlight the potential applications of these nanoparticles in combating microbial infections, thus contributing significantly to the field of antimicrobial research and fostering further exploration of their therapeutic utility.

2.7. Antifungal assessment and bioassay of silver nanoparticles

The antifungal activity of silver nanoparticles synthesized from *C. roseus* was evaluated against *A. niger* and *A. fumigatus*, adhering to established protocols (Bidaud et al., 2021). Agar plates were prepared by pouring agar seeded with *A. niger* and *A. fumigatus* into petri dishes, allowing it to solidify according to the methodology described earlier (Gajbhiye et al., 2009). Filter paper discs were immersed in the respective solutions containing the synthesized silver nanoparticles and were meticulously positioned onto the seeded agar plates to ensure uniform distribution across the surface. The agar plates were then incubated at 37 °C for 24 hours to facilitate fungal growth and the potential manifestation of antifungal activity exerted by the silver nanoparticles. Following the incubation period, zones of inhibition surrounding the discs were carefully examined, measured, and recorded following established protocols (Krishnaraj et al., 2012; Monteiro et al., 2012). Ketoconazole (50 µg - HiMedia, India) discs were used for antimicrobial susceptibility testing of fungal cultures.

These evaluations provided critical insights into the antifungal efficacy of the synthesized silver nanoparticles against *A. niger* and *A. fumigatus*. The results from both the initial antifungal assessment and the bioassay underscored the potential therapeutic applications of the synthesized silver nanoparticles in combating fungal infections, thereby contributing significantly to understanding their effectiveness against fungal pathogens.

2.8. Antiviral assessment of AgNP-CR

The silver nanocomposition prepared from the leaf extract of *C. roseus*, developed by the Department of Microbiology, Dyanopasak Shikshan Mandal, Parbhani, was evaluated for its anti-HIV-1 activity at the Indian Council of Medical Research - National AIDS Research Institute, Pune (ICMR-NARI), following the standard protocol outlined by Gaikwad et al. (2023). The biological activity of AgNP-CR was assessed using high-throughput cell-based mechanistic studies, as previously described (Jadaun et al., 2022; Jadaun et al., 2023).

2.9. Cytotoxicity assay by MTT

The cytotoxic effect of the pure extract of *C. roseus* (CR-Pure) and AgNP-CR was evaluated in the TZM-bl cell line following established methodologies (Rakshit et al., 2024). Briefly, 1×10^5 adherent TZM-bl cells/well were seeded onto a 96-well plate and incubated for 24 hours with 5% CO_2 at 37 °C. The phyto-extract dilutions and their nanocomposition counterpart were applied to the cell-seeded plates in a dose-dependent manner and incubated for 48 hours. After the incubation period, the treated cells were assessed by adding 20 µl of 5 mg/ml MTT to all wells and further incubating for 3 hours to allow for MTT metabolism. The supernatant was then replaced with 150 µl of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. Following a final hour of incubation, the optical density (OD) values were recorded at 550 nm and 630 nm using a multimode plate reader. Cell viability was determined by comparing the absorbance of untreated and treated cells. The percentage of cell viability was calculated using the formula:

$$\text{Percentage of cell viability} = \frac{\text{Absorbance of treatment} - \text{Absorbance of blank}}{\text{Absorbance of control} - \text{Absorbance of blank}} \times 100\%$$

The CC_{50} was determined as the concentration at which 50% of the cells remained viable in the presence of CR-Pure or AgNP-CR, based on three independent assays.

2.10. Cell associated assay

Based on the CC_{50} values, a range of non-cytotoxic concentrations of CR-Pure and AgNP-CR were utilized, and the anti-HIV-1 activity was evaluated following established methods (Mutalik et al., 2023). Briefly, TZM-bl cells (1×10^4 cells/well) were initially infected with the HIV-1VB028 virus for 2 hours at 37 °C in a 5% CO_2 incubator, followed by treatment with various dilutions of the extracts. Additionally, 5 mg/ml DEAE-dextran was added to facilitate viral internalization. After a 48-hour incubation period, luciferase activity was measured using the BriteliteTM Plus reagent on a luminometer (Perkin Elmer, USA). The standard nucleoside reverse transcriptase inhibitor drug Azidothymidine (AZT) was used as a positive control at a known concentration of 0.49 µM/ml.

2.11. Statistical analysis of in vitro assays

The mean values from a minimum of three replicates were used for each experiment. The final results were calculated and presented as percentage inhibition graphs after subtracting the blank and comparing them with the respective controls from at least three independent experiments. Error bars indicate the standard deviation of the mean from three assays for each experiment. A p-value of < 0.05 was considered statistically significant for all assays.

3. Results and discussion

The synthesis and evaluation of silver nanoparticles (AgNPs) derived from *C. roseus* extract have significant implications for antimicrobial, antiviral, and antifungal applications (Mukunthan et al., 2011; Salleh et al., 2020). This study presents a comprehensive exploration of the synthesis process, followed by an extensive assessment of the biological activities of AgNP-CR against various pathogens.

3.1. Synthesis of silver nanoparticles (AgNP-CR)

The synthesis of silver nanoparticles (AgNPs) via the reduction of an aqueous solution of silver nitrate represents a well-established method in nanotechnology (Šileikaitė et al., 2006). In this investigation, we focused on the production of a unique composition termed AgNP-CR, derived from the extract of *C. roseus* leaves, and explored its antimicrobial potential against various pathogens. The formation of AgNP-CR was visually indicated by a distinct transition in color, initially manifesting as a pale-yellow hue, which gradually transformed into a deep reddish color within the screw-cap tubes **(Figure 1)**.

A B

Figure 1. Visual indication of AgNP-CR formation
(A) Four samples of pale-yellow aqueous leaf extract of *C. roseus* without $AgNO_3$ (control)
(B) Colour change from pale yellow to dark reddish-brown after adding 9 ml of $AgNO_3$ and heating for 5 minutes

The color transformation was meticulously observed and documented in the screw-cap tubes containing 9 ml of silver nitrate ($AgNO_3$) solution and 1 ml of plant extract before and after a brief heating period of 5 minutes **(Figure 1)**. Notably, the color change was monitored across different time intervals corresponding to the extraction process of the plant extract, ranging from 10 to 80 minutes (i.e., 10, 20, 30, 40, 50, 60, 70, and 80 minutes). This systematic comparison facilitated the observation of potential variations in the color change corresponding to different extraction durations.

The emergence of distinct color profiles at varying time intervals provided preliminary insights into the kinetics of nanoparticle formation, potentially correlating with the phytochemical composition and reductive potential of the *C. roseus* leaf extract. Furthermore, these visual cues served as initial indicators of successful nanoparti-

cle synthesis, paving the way for further characterization and evaluation of the antimicrobial properties of the AgNP-CR composition against a spectrum of microbial pathogens.

The successful synthesis of AgNPs from *C. roseus* extract, as evidenced by the color change from pale-yellow to dark reddish, represents a significant achievement. This method utilizes the reducing properties of plant extracts to convert silver ions into nanoparticles, offering an environmentally friendly approach to nanoparticle synthesis. The observed color change across different extraction times highlights the influence of synthesis duration on nanoparticle characteristics, emphasizing the importance of optimizing synthesis parameters to achieve desired nanoparticle properties.

3.2. Antimicrobial activity of AgNP-CR against gram-positive and gram-negative bacteria

In this investigation, the efficacy of AgNP-CR as antimicrobial agents was evaluated against selected bacterial strains using both agar plate and liquid medium assays, compared with the leaf extract alone (CR-Pure) and $AgNO_3$ only (Figure 2, Figure S2-supplementary file). Blank controls were utilized to ensure the validity of the results, providing a baseline for comparison and confirming that any observed inhibitory effects were attributable to the AgNP-CR and not to other variables. The findings revealed that among the bacteria tested, the gram-positive *B. subtilis* exhibited significant susceptibility to AgNP-CR compared to the gram-negative *E. coli* (Figure 2). Moreover, the inhibitory effect on bacterial growth was influenced by the duration of AgNP-CR synthesis, with *B. subtilis* being more effectively inhibited with increasing synthesis times compared to *E. coli* (Figure 3). Notably, AgNP-CR synthesized after one hour exhibited heightened efficacy in inhibiting a higher concentration of bacteria, as depicted in Figure 3.

Silver nanoparticles have demonstrated remarkable antimicrobial properties against bacteria, with minimum inhibitory concentrations (MIC) of 0.04 mg/ml for *E. coli* and 0.03 mg/ml for *B. subtilis*. This efficacy is attributed to their high surface-area-to-volume ratio and distinctive chemical and physical characteristics. Typically ranging in size from 1 to 100 nm, AgNPs exhibit an augmented surface-area-to-volume ratio as particle size decreases. Previous studies have shown that AgNPs within the size range of 10-100 nm exert potent antimicrobial effects against both gram-positive and gram-negative bacteria, similar to the strains used in this study (Morones et al., 2005). The small particle size enables AgNPs to adhere to bacterial cell walls and penetrate bacterial cells more readily, thereby enhancing their antimicrobial potency. The antimicrobial efficacy of AgNPs has been particularly noteworthy against multidrug-resistant bacteria, such as multidrug-resistant *E. coli*, as reported previously (Kar et al., 2016; Paredes et al., 2014).

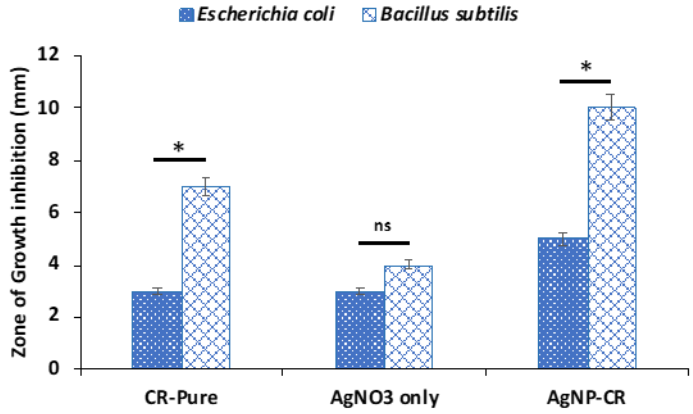


Figure 2. Antibacterial activity of nanoparticles synthesized using *C. roseus* extracts (AgNP-CR) against bacterial strains

Comparative analysis of bacterial growth inhibition on nutrient agar plates treated with CR-Pure, $AgNO_3$, and AgNP-CR demonstrates the efficacy of the synthesized nanoparticles in inhibiting the growth of both gram-negative and gram-positive bacteria. Standard deviations were calculated from three independent assays and are represented as error bars.

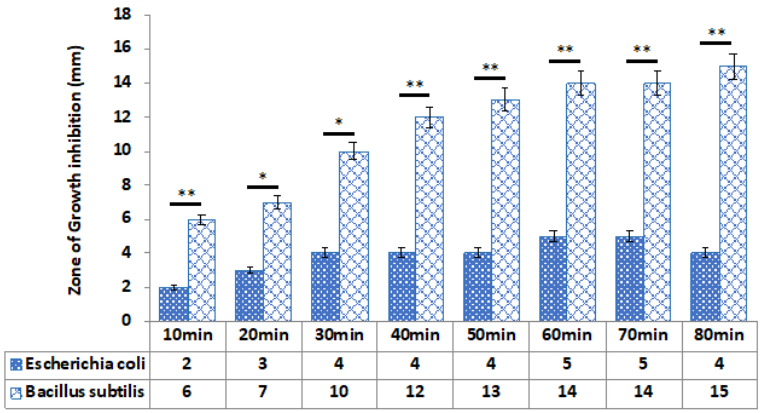


Figure 3. Effect of time interval on the synthesis of silver nanoparticles (AgNP-CR) and its antibacterial activity on *E. coli* and *B. subtilis* over 24 hours

The figure illustrates the relationship between nanoparticle synthesis duration and antibacterial efficacy, highlighting how varying synthesis times impact the antimicrobial properties of AgNP-CR against the two bacterial strains. Standard deviations were calculated from three independent assays and represented as error bars.

3.3. Antifungal activity of AgNP-CR on Aspergillus spp.

In this study, the antifungal potential of AgNP-CR against *Aspergillus* species was assessed using the agar well diffusion method. The results revealed significant zones of inhibition against both fungal strains, with no discernible difference in efficacy between the two strains at a concentration of 0.0625 mg/ml. As depicted in Figure 4, a notable increase in the inhibition zone (4 mm) was observed with green AgNP-CR compared to CR-Pure and $AgNO_3$, which exhibited smaller zones of inhibition (2 mm). These findings underscore the effectiveness of the synthesized silver nanoparticles as antifungal agents against phytopathogens. Interestingly, a distinct pattern of inhibition was observed concerning the effect of synthesis time on the antifungal activity of AgNP-CR on potato dextrose agar (PDA) medium, contrasting with the pattern observed in the bacterial study. As illustrated in Figure 5, the antifungal activity of AgNP-CR against the phytopathogens peaked at a synthesis time of 40 minutes, with an inhibition zone measuring 4 mm. However, beyond this optimal synthesis duration, the inhibitory action of AgNP-CR declined significantly, reaching its lowest point at 80 minutes. These results highlight the dynamic nature of AgNP-CR and its consequent impact on antifungal efficacy.

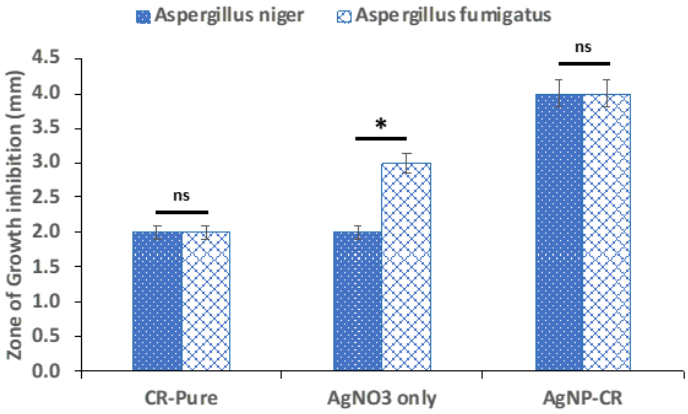
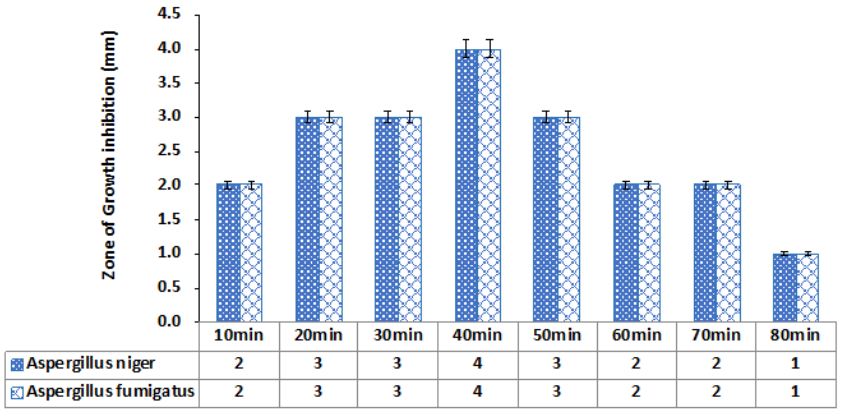


Figure 4. Antifungal activity of AgNP-CR synthesized using phytoextracts of *C. roseus* on potato dextrose agar plates against *Aspergillus* spp
The figure shows the zones of inhibition formed around the discs impregnated with AgNP-CR, indicating the nanoparticles' effectiveness in inhibiting the growth of *A. niger* and *A. fumigatus*. Standard deviations were calculated from three independent assays and are represented as error bars.



	10min	20min	30min	40min	50min	60min	70min	80min
Aspergillus niger	2	3	3	4	3	2	2	1
Aspergillus fumigatus	2	3	3	4	3	2	2	1

Figure 5. Effect of synthesis time interval on the antifungal activity of silver nanoparticles using *C. roseus* extract (AgNP-CR) against *Aspergillus* spp. on potato dextrose agar (PDA) medium incubated at room temperature for 4 days
The figure demonstrates how different synthesis durations influence the antifungal efficacy of AgNP-CR, with consistent inhibition observed for both *A. niger* and *A. fumigatus* strains. Standard deviations were calculated from three independent assays and are represented as error bars.

Furthermore, the biosynthesized AgNP-CR exhibited promising antifungal activity against the tested fungal strains, effectively inhibiting the growth of *A. niger* and *A. fumigatus*. These fungi pose significant threats to agriculture and human health by causing agricultural damage and skin-related diseases, respectively. Collectively, these findings highlight the substantial antifungal activity of AgNP-CR, demonstrating potent effects against the tested fungi in vitro. The primary mechanisms underlying the antifungal action of AgNPs include the disruption of fungal cell walls and membranes, interference with protein function, generation of reactive oxygen species (ROS), and disruption of protein structures (Jian et al., 2022). However, further research is warranted to explore the applicability of AgNP-CR as antifungal agents in field settings and to elucidate their mechanisms of action in greater detail.

3.4. In vitro cytotoxicity of AgNP-CR on TZM-bl cells

The cytotoxic effects of the *C. roseus* phyto-extract and its silver nanocomposition (AgNP-CR) on TZM-bl cells were initially evaluated using the MTT quantitative colorimetric assay. A dose-response relationship ranging from 5.0 to 0.005 mg/ml was examined to assess the impact of *C. roseus* and AgNP-CR on cellular viability, as depicted in Figures 6A and 6B. From three independent replicates, the concentrations at which 50% of cells remained viable (CC_{50} values) were determined to be 3.43 mg/ml for *C. roseus* and 0.08 mg/ml for AgNP-CR. The cytotoxicity of AgNP alone (Figure S3-supplementary file) and the FDA-approved standard drug Azidothymidine (data not shown) were also determined for experimental validation.

3.5. CR extracts and AgNP-CR mediated inhibition of HIV-1 replication

TZM-bl cells were used to screen the anti-HIV-1 activity. Based on the results of the cell viability assay, optimal concentrations of 0.5 mg/ml for CR extracts and 0.08 mg/ml for AgNP-CR were selected as the initial testing concentrations for the cell-based anti-HIV-1 assay.

In the cell-associated assay, the half maximal effective concentration (EC_{50}) values of the *C. roseus* extract and its metal nanocomposition, AgNP-CR, against HIV-1VB028 (R5, Subtype C) were determined. The *C. roseus* extract exhibited an EC_{50} value of 0.16 mg/ml, whereas AgNP-CR inhibited 50% of HIV-1 virus replication at a much lower concentration of 0.02 mg/ml. A dose-dependent inhibition of HIV-1 replication was observed for both the *C. roseus* extract and AgNP-CR in TZM-bl cells, as illustrated in Figures 7A and 7B. While the *C. roseus* extract demonstrated significant inhibition of replicating cell-associated virus across the concentration range of 0.50 to 0.005 mg/ml (Figure 7A), AgNP-CR exhibited better inhibitory effects within the concentration range of 0.080 to 0.0006 mg/ml (Figure 7B). The EC_{50} values were compared with the standard drug AZT (0.49 µM, data not shown) and AgNP alone (Figure S3B-supplementary file).

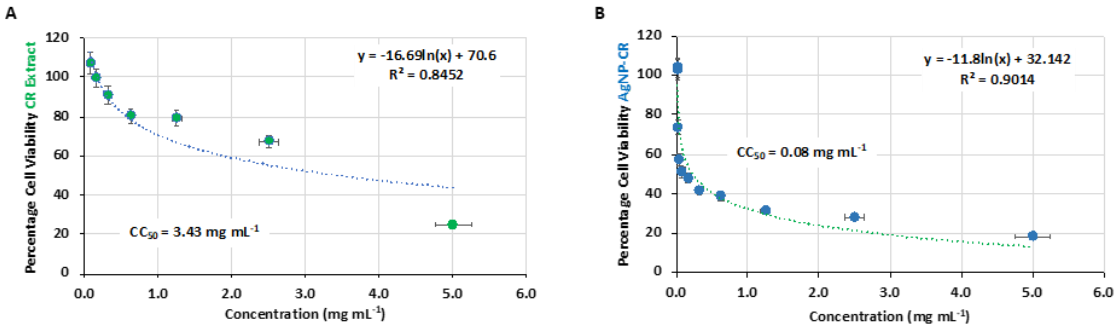


Figure 6. Determination of cell viability of *C. roseus* leaf extract and its silver nanoparticle composition

The figure depicts the impact of varying concentrations of (A) CR-extract and (B) AgNP-CR on TZM-bl cells examined through the MTT assay. The cell viability curves indicate the cytotoxic effects, with calculated CC_{50} values derived from three independent experiments, demonstrating the concentration at which 50% of the cells remain viable. Standard deviations were calculated and are represented as error bars.

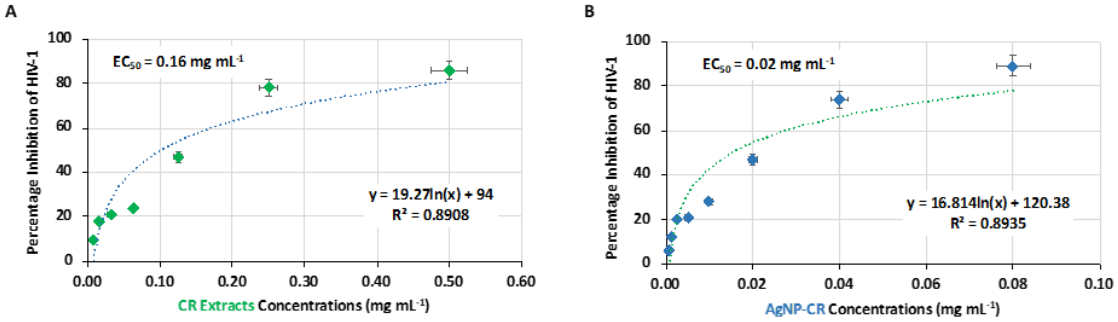


Figure 7. Anti-HIV-1 activity of silver nanoparticle composition of *C. roseus* leaf extract

The figure illustrates the percentage inhibition of HIV-1 replication by (A) CR-extract and (B) AgNP-CR in HIV-1VB028 (R5 - Subtype C) infected TZM-bl cells through Cell-Associated assays. The graphs show the average effective concentrations (EC_{50} values) required to inhibit 50% of viral infections, derived from three independent experiments, highlighting the potential of AgNP-CR as an anti-HIV agent. Standard deviations were calculated and are represented as error bars.

The antiviral assessment of AgNP-CR against HIV-1 underscores its potential in combating viral infections. The cytotoxicity assay using TZM-bl cells revealed low cytotoxicity for AgNP-CR, indicating a favorable safety profile for potential therapeutic applications. Subsequent evaluation of anti-HIV-1 activity demonstrated dose-dependent inhibition of viral replication by both the *C. roseus* extract and AgNP-CR. Notably, AgNP-CR exhibited superior efficacy in inhibiting HIV-1 replication compared to the plant extract alone, highlighting the enhanced antiviral activity conferred by the nanoparticle composition.

4. Conclusions

The synthesis of silver nanoparticles (AgNPs) from *C. roseus* (periwinkle) demonstrates significant therapeutic potential. The study revealed that AgNP-CR exhibits antimicrobial effects against both gram-positive (*B. subtilis*) and gram-negative (*E. coli*) bacteria, as well as fungal pathogens such as *A. niger* and *A. fumigatus*. Additionally, AgNP-CR showed promising anti-HIV activity, indicating potential as an antiviral agent against HIV infections. The primary mechanism of action appears to involve the disruption of microbial cell membranes, which inhibits microbial growth and viral replication. The utilization of *C. roseus* for nanoparticle synthesis not only aligns with sustainable and eco-friendly practices in nanomedicine but also leverages the plant's rich phytochemical profile. The findings underscore the multifaceted therapeutic properties of AgNP-CR, suggesting its utility in developing novel antimicrobial and antiviral agents. However, several limitations need to be addressed in future research. The precise mechanisms underlying the observed antimicrobial and antiviral activities require further elucidation. Additionally, the synthesis protocols must be optimized to enhance therapeutic efficacy and consistency. While the study highlights the superior antibacterial activity of AgNP-CR, its relatively lower antifungal activity warrants further investigation to fully understand its clinical applicability.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Statement of ethics

No animal or human specimens were used in this study, and ethical approval was waived under protocol number of NARI/EC/Approval/2022/662.

Availability of data and materials

The original contributions presented in this study are included in the article and supplementary materials. Further inquiries can be directed to the corresponding author(s).

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Supplementary File

The supplementary file accompanying this article is available at https://ijpbp.com/index.php/ijpbp/libraryFiles/downloadPublic/21.

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