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Research Article

Nano-Ointment Formulation for Broad-Spectrum Antimicrobial Applications Using the Bacterial Red Pigment Prodigiosin

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ABSTRACT

The emergence of multidrug-resistant pathogens necessitates the exploration of novel bioactive compounds and nanotechnology-based formulations for effective antimicrobial therapies. Prodigiosin, a red pigment produced by certain bacteria, exhibits diverse biological properties, including antimicrobial activity. This study aimed to synthesize silver nanoparticles (AgNPs) using prodigiosin as a biogenic reducing and stabilizing agent, and to formulate a prodigiosin-based nano-ointment. The synthesized nanoparticles were identified by color change and by UV-Vis spectroscopy. The formulated nano-ointment exhibited significant antibacterial activity against Staphylococcus aureus and Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis and antifungal activity against different strains of Candida. Compared to control formulations, the prodigiosin-AgNP nano-ointment demonstrated enhanced and broad-spectrum antimicrobial efficacy. Prodigiosin acts as a promising biogenic agent for the eco-friendly synthesis of silver nanoparticles. The developed nano-ointment shows potent broad-spectrum antimicrobial activity, highlighting its potential as a novel therapeutic formulation against bacterial and fungal infections.

1. Introduction

The global emergence of antibiotic resistance among microorganisms has prompted researchers to seek solutions through the development of novel antibiotics and alternative strategies to address this pressing issue. Consequently, there has been a growing interest in the use of nanotechnology (Sharma et al., 2009) which is experiencing rapid growth, defined by the manipulation of particle sizes at the nanoscale, which endows these particles with unique properties and significant value that larger particles cannot achieve (Willems, 2005). Notably, nanoparticles exhibit a surface-to-volume ratio that is 35-40% higher than that of their larger counterparts, which contributes to their remarkable characteristics, including enhanced surface reactivity (Affan et al., 2009). Among the various types of metal nanoparticles, silver nanoparticles are prominently used in a wide array of fields such as food safety, healthcare, and medical applications, especially in diagnostics, drug delivery, and industrial contexts. Their exceptional properties allow for multiple applications, including antibacterial and anti-inflammatory functions, coatings for medical devices, orthopedic uses, and as agents with anticancer capabilities (Zhang et al., 2016).

The synthesis of nanoparticles can be achieved through a variety of methods, notably physical, chemical, and biological techniques. The physical and chemical methods are often associated with high costs and potential harm to human health and the environment (Gurunathan et al., 2015). Conversely, nanoparticles synthesized through biological methods exhibit advantages such as simplicity, uniformity, high yield, stability, and eco-friendliness. These methods utilize biological reducing agents, including bacteria, fungi, yeasts, and plants. As a result, nanoparticles produced through this environmentally sustainable approach are ideal for applications in the biomedical field (Pechyan et al., 2024).

Prodigiosin is a water-insoluble, non-diffusible red pigment characterized by a 4-methoxy-α,α-bipyrrole ring structure connected to a third pyrrole unit via a methane bridge. Initially identified as a secondary metabolite produced by Serratia marcescens, it was later found to be synthesized by various Gram-positive and Gram-negative bacteria and noted for its broad bioactivity including antimicrobial properties,

In the present study, an attempt was made to synthesize silver nanoparticles using prodigiosin and also to formulated

nano-ointment to harness the synergistic activity of both prodigiosin and silver nanoparticles for potential biomedical applications.

1. Materials and Methods

2.1. Chemicals

Silver nitrate ($AgNO_3$) was purchased from Sigma-Aldrich (USA). Ethanol, chloroform, methanol, hydrochloric acid, stearyl alcohol (25 g), white petrolatum (25 g), propylene glycol (12 g), sodium lauryl sulfate (1 g), and other analytical grade (AR) chemicals were obtained from HiMedia Laboratories (India). Prodigiosin was generously provided by the Department of Biotechnology, Chaitanya Deemed to be University, Hyderabad, India. All chemicals used were of analytical grade (AR).

2.2. Synthesis of Silver nano particles using Prodigiosin

The synthesis of silver nano particles was conducted with prodigiosin, following the methodology described by Alaa and Hassan, (2020) with slight modification. To briefly outline the procedure, a 2mM silver nitrate solution was prepared in 100ml of distilled water in individual flasks. Increasing concentrations of prodigiosin 1%, 2%, 3%, 4%, and 5% were subsequently introduced seperately. The flasks were then placed in a boiling water bath for one hour, and any alterations in color were carefully observed.

2.3. Identification of silver nano particles

2.3.1. UV-Vis spectroscopy analysis

The formation of silver nanoparticles was identified initially by change in color. Ultraviolet-visible (UV-Vis) spectral analysis was done by using ELICO SL-159 Spectrophotometer in the range of 350-470 nm. The reduction of pure silver ions synthesized by using prodigiosin was monitored by measuring the UV-Vis spectrum of the reaction mixture.

2.4. Preparation of Ointment Base

The ointment base was prepared as per the Indian Pharmacopoeia (2018). The following ingredients were weighed: stearyl alcohol (25 g), white petrolatum (25 g), propylene glycol (12 g), sodium lauryl sulfate (1 g), and purified water (q.s. to 100 g). The components were melted and mixed at 70 °C, followed by gradual cooling under continuous stirring to obtain a homogenous base.

2.4.1. Formulation of Prodigiosin-AgNP Ointment

The dried prodigiosin-AgNP nanocomposite was incorporated into the ointment base at a concentration of 1–5% (w/w). The formulation was homogenized using a mechanical stirrer until uniform dispersion was achieved. The prepared ointment was filled into sterile containers for further antimicrobial studies.

2.5. Antibacterial and antifungal activity of prodigiosin by agar well diffusion method.

The antimicrobial efficacy of prodigiosin nanocomposite against clinical isolates, including both bacteria and Candida species, was assessed using the agar well diffusion technique, as outlined by Karayildirim et al. (2024), with minor modifications. Initially, culture suspensions were inoculated onto Nutrient agar for bacterial strains and Sabouraud agar for fungal strains, utilizing a sterile L-shaped glass rod for even distribution. Following the drying of the agar surfaces, 8 mm wells were created using a sterilized borer. Subsequently, varying concentrations of prodigiosin specifically 12.5 mg/ml, 25 mg/ml, and 50 mg/ml dissolved in DMSO, were introduced into the wells and allowed to diffuse at ambient temperature for a duration of 2 hours. Ampicillin at a concentration of 50 µg/mL served as the positive control for bacteria,and flucanozole for fungi while, DMSO acted as the negative control. The inoculated plates were then incubated at 37 °C for 48 hours for bacterial cultures and at 27 °C for fungal cultures, after which the diameters of the inhibition zones were measured in milli meters.Antigenicity and allergenicity of the identified conserved regions were determined as follows. VaxiJen tool was used to identify immunoprotective sequences based on identifying such sequences from bacterial, viral, tumour, fungi and parasite antigens and calculating their auto-cross covariance values [11].

The threshold for viral models was 0.4 (default value); sequences that scored equal to or below 0.4 were considered non-antigenic, and those that scored above were considered antigenic. The Immune Epitope Database (IEDB) tools were used to find possible T-cell and B-cell epitopes in conserved protein sequences. From the input sequence, it identifies the string most likely to have immunogenicity and assigns a score of likelihood [12]. The NetCTL 1.2 tool was used to identify CTL epitopes in the amino acid sequences of SARS-CoV-2. It supports 12 MHC class 1 supertypes [13]. The allergenicity of the amino acid sequences was identified using the AllerTOP tool. Only those sequences with no allergenic effects were used for further analysis [14]. The conserved antigenic and non-allergenic epitopes were then mapped onto the respective crystal structure of the proteins as follows. Experimentally determined structures of non-structural proteins (NSPs) and structural proteins of SARS-CoV-2 were retrieved from the RSCB Protein Data Bank (PDB) [15]. The structure of conserved peptide sequences was visualised in PyMOL v2.4.1 [16]. The structures of NSP-4 and NSP-6 were predicted using the Alphafold2 v2.1.1, which uses a machine learning approach for modelling even without homologous structures [17]. The best model was selected from the five predicted structures based on the predicted local distance difference test (PLDDT) score. Further, the model was evaluated using the PROCHECK [18] and ERRAT [19] modules of the SAVES v6.0 server to validate the stereochemistry and overall quality factors. The loop regions of the predicted models were refined using the ModLoop server [18] and validated using the SAVES server. Modelled structures were analysed using PyMOL.

2. Results and Discussion

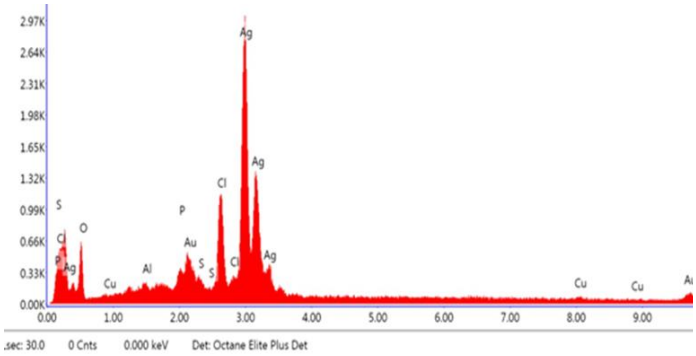
Silver nanoparticles were synthesized by reacting varying concentrations of prodigiosin with 2mM AgNO3. The formation of AgNPs was indicated by a noticeable color change from colourless to a brownish-yellow hue (Fig-4). Such a shift in color can be attributed to the excitation of surface plasmon resonance (SPR) of silver nanoparticles, a phenomenon commonly used as a preliminary indicator of nanoparticle formation. Akilandeswari et al. (2014) reported that the gradual transition of the medium from pale yellow to dark brown serves as a reliable confirmation of AgNP synthesis in the presence of biological reducing agents, including microbial pigments such
In our study, the strong peak at 420 nm with minimal broadening indicates that prodigiosin not only facilitates the reduction of Ag^+ ions but also stabilizes the nanoparticles,

thereby preventing aggregation. These findings are consistent with earlier reports where biologically synthesized AgNPs exhibited absorption maxima within the 410-430 nm range. For instance, Krithika et al. (2014) and El-Batal et al. (2016) observed similar SPR peaks during prodigiosin-mediated AgNP synthesis, attributing this to the unique electron-donating groups of the pigment that promote rapid nucleation and stabilization of nanoparticles. Moreover, the correlation between prodigiosin concentration and peak intensity in our spectra further confirms that higher pigment levels enhance the rate of Ag^+ ion reduction, resulting in the generation of a larger number of nanoparticles with smaller diameters (Safekordi et al., 2011). Thus, UV-Vis spectral analysis strongly validates the role of prodigiosin as both a reducing and stabilizing agent in the green synthesis of AgNPs, providing preliminary confirmation of nanoparticle formation before further physicochemical characterization.

4.1. Energy-Dispersive X-ray Spectroscopy (EDX) Analysis

The elemental composition of the synthesized silver nanoparticles was confirmed using Energy-Dispersive X-ray Spectroscopy (EDX), and the corresponding spectrum is presented in Figure 3. A strong signal for silver (Ag) was observed at approximately 3 keV, which is a characteristic peak for metallic silver nanoparticles, thereby validating the successful synthesis of AgNPs (Fig-1). The high intensity of the Ag peak indicates that silver is the major constituent element of the sample.

Fig 1. EDS Image of Silver nanoparticles



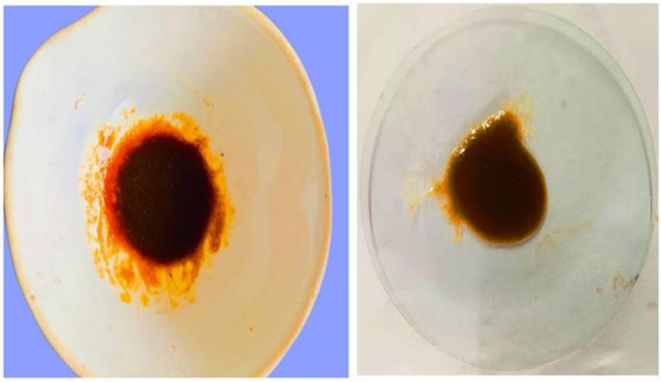
In addition to silver, weaker signals corresponding to oxygen (O), chlorine (Cl), sulfur (S), and phosphorus (P) were also detected. These elements are likely derived from the prodigiosin pigment and other biomolecules that acted as reducing and stabilizing agents during nanoparticle synthesis. Their presence supports the role of prodigiosin not only in the bioreduction of Ag^+ ions but also in the capping and stabilization of the nanoparticles, preventing aggregation.

Minor peaks of copper (Cu) and gold (Au) were also detected in the spectrum. These signals are attributed to the copper grid and thin gold coating commonly used during SEM/EDX sample preparation rather than being intrinsic to the nanoparticles. Similarly, the presence of aluminum (Al) may be due to background contamination from the substrate or instrument. Overall, the EDX spectrum clearly demonstrates that silver is the predominant element, with additional peaks confirming the presence of capping agents originating from the prodigiosin pigment. These findings are in agreement with earlier reports on biologically synthesized silver nanoparticles, where biomolecules contribute to both reduction and stabilization (El-Batal et al., 2016; Krithika et al., 2014). The combination of UV–Vis and EDX analyses thus provides strong evidence for the successful synthesis and stabilization of prodigiosin-mediated AgNPs.

4.2. Preparation of prodigiosin silver nanocomposite ointment

The prodigiosin-based nano-formulated ointment was successfully prepared and is depicted in Figure 2. The formulation exhibited a characteristic reddish-brown coloration, which can be attributed to the natural pigmentation of prodigiosin, a tripyrrole red pigment known for its stability and bioactivity (Darshan & Manonmani, 2015). The consistency of the ointment was smooth and homogenous, without visible aggregation or phase separation, suggesting successful incorporation of the prodigiosin–silver nanocomposite into the ointment base. The uniform dispersion of nanoparticles within the ointment matrix is a critical factor influencing its bioavailability and antimicrobial activity. Nanostructured formulations generally provide enhanced surface area, better penetration, and controlled release of bioactive molecules at the site of infection (Giri et al., 2014). The absence of clumps or crystalline deposits in the prepared formulation indicates that the nanoparticles remained well-stabilized, which may be due to the dual role of prodigiosin as both a bioactive agent and a stabilizing moiety during silver nanoparticle synthesis.From a pharmaceutical perspective, the ointment displayed desirable physicochemical properties, including spreadability and adhesion, which are essential for topical applications. These properties directly influence patient compliance and therapeutic efficiency, as ointments with appropriate viscosity ensure prolonged contact with the application site, thereby maximizing antibacterial efficacy (Mura et al., 2018).The reddish-brown coloration also serves as an indirect confirmation of nanoparticle–pigment interaction, since prodigiosin–metal nanocomposites typically exhibit visible spectral shifts due to surface plasmon resonance (SPR) phenomena of silver nanoparticles (Rai et al., 2012). Such interactions not only stabilize the formulation but also synergistically enhance antimicrobial action. The successful preparation of a stable prodigiosin nano-formulated ointment highlights its potential as a novel antimicrobial topical agent. By combining the intrinsic bioactivity of prodigiosin with the well-established antibacterial properties of silver nanoparticles, this formulation offers a promising strategy to combat multidrug-resistant pathogens.

Fig.2. Ointment formulation using prodigiosin nanocomposite



4.3. Antibacterial Activity of Prodigiosin-Silver Nanocomposite Ointment

The antibacterial activity of prodigiosin silver nanocomposite ointment was evaluated against *Escherichia coli, Pseudomonas*

aeruginosa, Bacillus subtilis, and *Staphylococcus aureus* at concentrations of 50, 100, and 200 mg/ml (Fig-3 and Fig-4). The inhibitory effect was measured in terms of the zone of inhibition (mm), and results are summarized in Table 1.

At 50 mg/ml, the nanocomposite exhibited mild to moderate inhibition, with zones of 7 mm for E. coli and 8 mm for S. aureus, while B. subtilis showed a comparatively higher inhibition of 18 mm. Notably, P. aeruginosa was resistant at this concentration, indicating its inherent resistance mechanisms such as efflux pumps and reduced membrane permeability, which often limit the efficacy of antimicrobial agents (Poole, 2011). At 100 mg/ml, a marked increase in antibacterial activity was observed across all tested bacteria. The zone of inhibition increased to 15 mm in E. coli and S. aureus, 24 mm in B. subtilis, and 10 mm in P. aeruginosa. The increased inhibition at this concentration suggests a dose-dependent effect of the nanocomposite ointment, which aligns with earlier reports where silver-based nanomaterials demonstrated concentration-dependent bactericidal activity (Rai et al., 2012).

At the highest tested concentration, 200 mg/ml, the nanocomposite showed significant antibacterial activity, with zones of inhibition of 21 mm, 19 mm, 32 mm, and 26 mm against *E. coli, P. aeruginosa, B. subtilis*, and *S. aureus*, respectively. Interestingly, the effect of the nanocomposite at 200 mg/ml was greater than the control ointment (200 mg/ml) against E. coli (21 mm vs. 12 mm), B. subtilis (32 mm vs. 30 mm), and S. aureus (26 mm vs. 21 mm). In the case of *P. aeruginosa*, the activity of the nanocomposite (19 mm) was nearly equivalent to that of the control (18 mm).

These results clearly indicate that the prodigiosin silver nanocomposite formulation enhances antibacterial activity compared to conventional ointment, particularly against Gram-positive bacteria (B. subtilis and S. aureus). The superior activity can be attributed to the synergistic effect of prodigiosin and silver nanoparticles. Prodigiosin, a tripyrrole red pigment, is well known for its broad-spectrum antimicrobial activity (Darshan & Manonmani, 2015), while silver nanoparticles disrupt bacterial membranes, induce oxidative stress, and interfere with DNA replication (Morones-Ramirez et al., 2013). The combination likely potentiates antimicrobial efficacy through multiple mechanisms of action.

Table 1. Antibacterial activity of prodigiosin nanocomposite ointment

s.no	Name of the bacteria	concentration of prodigiosin silver nanocomposite ointment			Control Ointment 200mg/ml
		50mg/ml	100mg/ml	200mg/ml	
		Zone of inhibition in man			
1	*Escherichia coli*	7	15	21	12
2	*Pseudomonas. aeruginosa*	-	10	19	18
3	*Bacillus subtilis*	18	24	32	30
4	*Staphylococcus aureus*	8	15	26	21

Moreover, the higher inhibition zones against Gram-positive bacteria compared to Gram-negative bacteria may be due to differences in cell wall architecture. Gram-negative bacteria such as E. coli and P. aeruginosa possess an outer membrane with lipopolysaccharides, which limits the penetration of antimicrobial agents. In contrast, Gram-positive bacteria, with a thicker peptidoglycan layer but lacking the protective outer membrane, are more susceptible to silver nanoparticle-mediated damage (Feng et al., 2000).

The results are consistent with previous studies where nanocomposite formulations incorporating natural pigments or secondary metabolites showed enhanced antimicrobial efficacy compared to individual agents (Liao et al., 2019). Therefore, the prodigiosin silver nanocomposite ointment demonstrates promising potential as an effective topical antibacterial agent, especially for combating Gram-positive bacterial infections.

Fig.3. Antibacterial activity of prodigiosin silver nanocomposite ointment

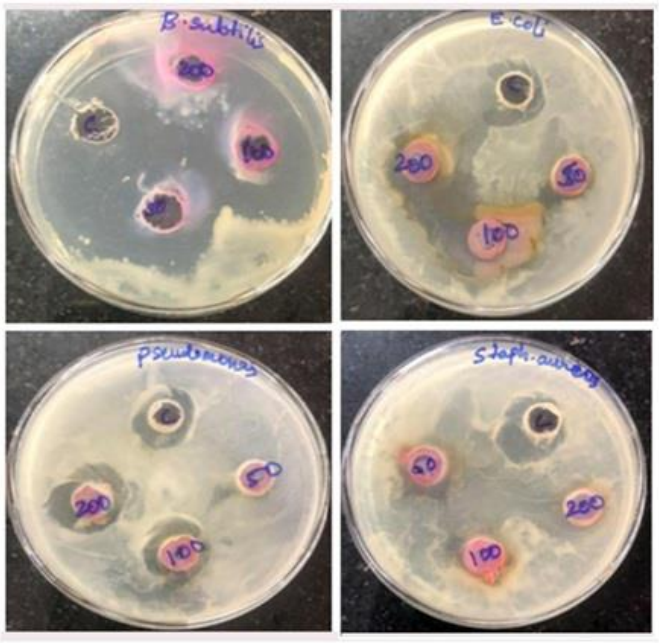
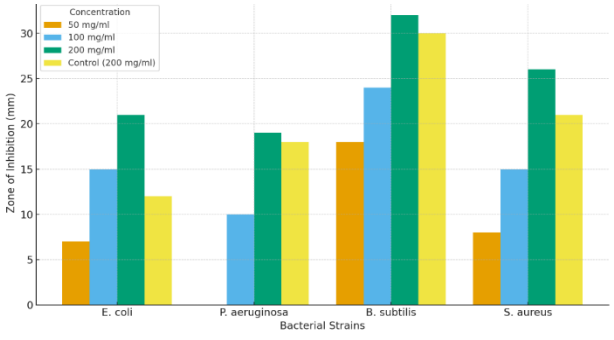


Fig.4. Antibacterial activity of Prodigiosin silver nanocomposite ointment



4.4. Antifungal activity of prodigiosin silver nanocomposite ointment pike Protein:

The antifungal activity of prodigiosin silver nanocomposite ointment was assessed against four pathogenic fungi-*Candida albicans, Candida tropicalis, Candida krusei,* and *Candida vitilis*-at concentrations of 50, 100, and 200 mg/ml. The results, presented in Table 2, Fig-5 and Fig-6. indicate that the formulation displayed dose-dependent antifungal activity, with higher concentrations showing increased zones of inhibition. At the lowest concentration (50 mg/ml), the

formulation exhibited modest inhibition, with zones of 8 mm for C. albicans, 17 mm for C. krusei, and 14 mm for C. vitilis. No activity was observed against C. tropicalis at this concentration, suggesting relative resistance of this strain at lower doses. At 100 mg/ml, inhibition zones increased considerably, with C. albicans showing 12 mm, C. tropicalis 14 mm, C. krusei 25 mm, and C. vitilis 16 mm. This trend suggests that the nanocomposite formulation exerts stronger fungicidal activity as the concentration increases, consistent with the typical dose-dependent effects of nanoparticle-based antifungal systems (Monteiro et al., 2012). At the highest tested concentration (200 mg/ml), significant antifungal activity was observed. The formulation produced inhibition zones of 22 mm, 32 mm, 28 mm, and 28 mm against *C. albicans, C. tropicalis, C. krusei*, and *C. vitilis*, respectively. Remarkably, the nanocomposite demonstrated equivalent or near-equivalent efficacy to the control ointment (200 mg/ml) in all tested fungi. In the case of C. tropicalis, C. krusei, and C. vitilis, the nanocomposite activity was comparable to the control (32 mm and 28 mm), whereas for C. albicans it was slightly lower (22 mm vs. 26 mm).

Table 2. Antifungal activity of prodigiosin silver nanocomposite ointment

s.no	Name of the fungi	concentration of prodigiosin silver nanocomposite ointment			Control Ointment 200mg/ml
		50mg/ml	100mg/ml	200mg/ml	
		Zone of inhibition in man			
1	*Candida albicans*	8	12	22	26
2	*Candida tropicalis*	-	14	32	32
3	*Candida krusei*	17	25	28	28
4	*Candida vitilis*	14	16	28	28

The pronounced antifungal effect may be attributed to the synergistic interaction between prodigiosin and silver nanoparticles. Prodigiosin is known to exert antifungal activity by inducing oxidative stress and disrupting membrane integrity in fungal cells (Suryawanshi et al., 2017; Kumar et al., 2020), while silver nanoparticles interact with fungal cell membranes, generate reactive oxygen species (ROS), and cause leakage of cellular components (Lara et al., 2010; Lunavath et al., 2013). Together, these mechanisms enhance the antifungal potency of the nanocomposite formulation.

Interestingly, *C. krusei* and *C. tropicalis*, which are often resistant to conventional antifungal drugs like fluconazole, displayed marked sensitivity to the nanocomposite ointment at higher concentrations. This observation underscores the therapeutic potential of the formulation against drug-resistant fungal pathogens (Pfaller & Diekema, 2007; Porika et al., 2024)). Overall, the results demonstrate that the prodigiosin silver nanocomposite ointment possesses broad-spectrum antifungal activity, with efficacy comparable to standard control ointment. This highlights its potential as an alternative topical antifungal agent, especially in treating infections caused by resistant Candida species.

Fig.5. Antifungal activity of Prodigiosin silver nanocomposite ointment

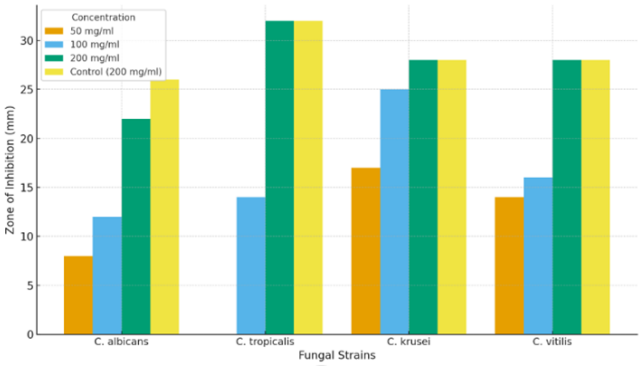
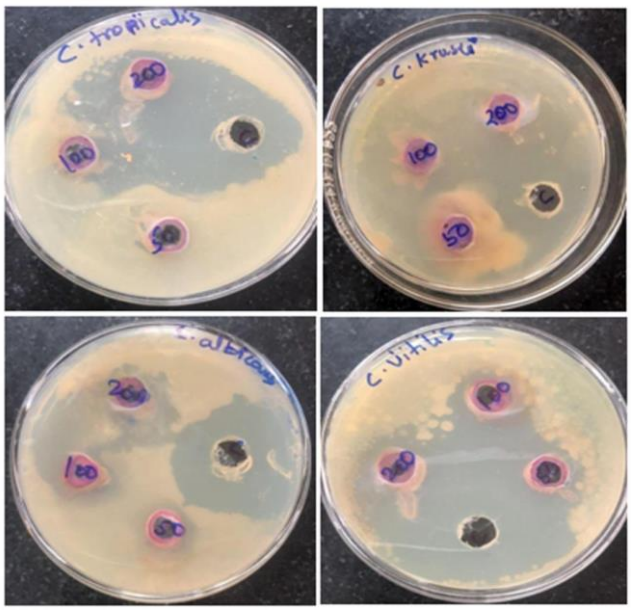


Fig.6 Antifungal activity of Prodigiosin silver nanocomposite ointment



Competing interests:

The authors declare that they have no competing interests

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