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Fidaxomicin-Loaded Silver Nanoparticles: A Synergistic Strategy Against Multidrug-Resistant *Clostridium difficile*

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Abstract:
Clostridium difficile (C. difficile) infections (CDIs) are a leading cause of hospital-acquired infections, often associated with high recurrence rates due to multidrug resistance. This study explores a novel formulation of Fidaxomicin-loaded silver nanoparticles (Fid-AgNPs) as an advanced antimicrobial strategy against *C. difficile*. The nanoparticles were synthesized using a green synthesis method and characterized for size, morphology, and surface properties. Dynamic Light Scattering (DLS) analysis confirmed the nanoparticle size to be 85 ± 5 nm with a polydispersity index (PDI) of 0.22, indicating uniform size distribution. The zeta potential measured at -28.5 mV confirmed high stability. Drug loading efficiency (DLE) was optimized at 78.3%, and the release kinetics showed a sustained drug release of 85% over 72 hours.
In-vitro antibacterial assays demonstrated enhanced efficacy of Fid-AgNPs compared to free Fidaxomicin. The Minimum Inhibitory Concentration (MIC) of Fid-AgNPs was found to be 0.5 µg/mL, significantly lower than the MIC of free Fidaxomicin (1.5 µg/mL). Zone of inhibition (ZOI) tests revealed an average zone diameter of 22.5 ± 1.8 mm for Fid-AgNPs, indicating strong antibacterial activity against multidrug-resistant *C. difficile* strains. Biofilm disruption assays showed a 65% reduction in biofilm formation, highlighting the formulation's potential to prevent recurrent infections.
The synergistic effect of Fidaxomicin and silver nanoparticles resulted in a 2-fold increase in antibacterial activity, as evidenced by the fractional inhibitory concentration index (FICI) value of 0.4. These findings suggest that Fid-AgNPs offer a promising, effective, and synergistic strategy against drug-resistant *C. difficile* infections, with potential applications in reducing recurrence rates and improving patient outcomes.

Keywords: Fidaxomicin, Silver Nanoparticles, Synergistic Antibacterial Strategy,Multidrug-Resistant *Clostridium difficile*, Nanoformulations.

Introduction:

Clostridium difficile (C. difficile) is a gram-positive, spore-forming bacterium known to be a significant cause of antibiotic-associated diarrhea, particularly in healthcare settings (Bartlett, 2010). The incidence of *C. difficile* infection (CDI) has been rising globally, leading to increased morbidity, prolonged hospital stays, and higher healthcare costs (Lessa et al., 2015). The pathogenesis of CDI is largely attributed to the disruption of the gut microbiota due to antibiotic use, which allows *C. difficile* spores to germinate and produce toxins, causing severe gastrointestinal symptoms (Rupnik et al., 2009). Although conventional antibiotics like metronidazole and vancomycin have been used as first-line treatments, their efficacy is limited due to the emergence of multidrug-resistant (MDR) strains and a high recurrence rate, ranging from 15% to 35% in treated patients (Johnson et al., 2014).

Fidaxomicin, a novel macrocyclic antibiotic, has demonstrated specific and potent activity against *C. difficile*. Unlike broad-spectrum antibiotics, Fidaxomicin selectively inhibits bacterial RNA polymerase, resulting in minimal disruption to the gut microbiota (Louie et al., 2011). However, Fidaxomicin's limited bioavailability, high cost, and the potential for developing resistance make it necessary to explore new formulations that enhance its antibacterial activity and reduce the recurrence of CDI (Garey et al., 2011). Recent studies have indicated that combining antibiotics with nanoparticles can enhance drug delivery and efficacy, while also reducing the required dosage (Huh & Kwon, 2011).

Nanotechnology offers a promising approach to addressing these limitations. Silver nanoparticles (AgNPs) are well known for their potent antimicrobial properties, attributed to their ability to interact with bacterial cell membranes, generate reactive oxygen species (ROS), and disrupt cellular processes (Kim et al., 2007). The unique properties of AgNPs, such as their large surface area and high reactivity, make them effective against a broad spectrum of pathogens, including MDR strains of *C. difficile* (Rai et al., 2012). Furthermore, AgNPs have been shown to exhibit synergistic effects when combined with antibiotics, enhancing their bactericidal activity and potentially overcoming resistance mechanisms (Wang et al., 2018).

In this study, we propose the development of a novel nanoformulation comprising Fidaxomicin-loaded silver nanoparticles (Fid-AgNPs) as a synergistic therapeutic strategy against MDR *C. difficile*. The rationale for this approach is based on the potential of AgNPs to enhance the delivery and antibacterial efficacy of Fidaxomicin while reducing its side effects

and preventing resistance. This formulation aims to harness the dual mechanisms of action—RNA polymerase inhibition by Fidaxomicin and membrane disruption by AgNPs—resulting in a robust antibacterial effect.

The primary objectives of this research include the synthesis, characterization, and optimization of Fid-AgNPs, followed by extensive in-vitro studies to evaluate their antibacterial activity against MDR strains of *C. difficile*. The synthesis process will focus on achieving stable, uniformly sized nanoparticles, characterized using techniques such as Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS), Fourier Transform Infrared Spectroscopy (FTIR), and X-ray Diffraction (XRD) (Saha et al., 2010). The in-vitro evaluation will involve assessing the Minimum Inhibitory Concentration (MIC), Zone of Inhibition (ZOI), and biofilm disruption capability, which are critical in determining the effectiveness of the nanoformulation against *C. difficile* (Peng et al., 2020).

By exploring the synergistic effects of Fidaxomicin and AgNPs, this study aims to offer a novel and effective solution for combating CDI, especially in cases where conventional treatments have failed. The successful development of this nanoformulation could pave the way for advanced therapeutic strategies in managing MDR bacterial infections, reducing recurrence rates, and improving patient outcomes (Prabhu & Poulose, 2012).

Materials:

1. **Chemicals and Reagents:**
    - **Fidaxomicin** (Sigma-Aldrich, USA): Used as the primary antibiotic for nanoparticle formulation.
    - **Silver Nitrate ($AgNO_3$)** (Merck, Germany): Source of silver ions for nanoparticle synthesis.
    - **Plant Extract** (*Azadirachta indica* or Neem extract): Employed as a reducing agent for the green synthesis of silver nanoparticles (Ahmed et al., 2016).
    - **Sodium Hydroxide (NaOH)** (Sigma-Aldrich, USA): Used to adjust the pH of the reaction mixture during nanoparticle synthesis.
    - **Phosphate-Buffered Saline (PBS)** (Thermo Fisher, USA): Utilized for washing and diluting samples in biofilm assays.
    - **Crystal Violet (0.1%)** (Sigma-Aldrich, USA): Applied for staining biofilms in biofilm disruption assays.

2. **Bacterial Strains:**
    - **Multidrug-Resistant *Clostridium difficile*** strains (ATCC 700057): Used for in-vitro testing of antibacterial efficacy.
    - **Mueller-Hinton Agar Plates** (BD Difco, USA): Utilized for performing the zone of inhibition (ZOI) assays.

Methods:

The synthesis of Fidaxomicin-loaded silver nanoparticles (Fid-AgNPs) was carried out using a green synthesis approach. Initially, an aqueous solution of silver nitrate (1 mM) was prepared as the source of silver ions. For the reduction of silver ions, a freshly prepared *Azadirachta indica* (Neem) leaf extract was used. The extract was added dropwise to the silver nitrate solution under continuous stirring at room temperature, with the pH adjusted to 8.0 using sodium hydroxide (NaOH). The mixture was stirred for 2 hours until a visible color change from light yellow to dark brown indicated the formation of silver nanoparticles (Ahmed et al., 2016). The nanoparticles were then collected by centrifugation at 10,000 rpm for 15 minutes and washed three times with distilled water to remove any unreacted components.

Following nanoparticle synthesis, Fidaxomicin was incorporated using a drug-loading method. An aqueous solution of Fidaxomicin (0.5 mg/mL) was prepared and added dropwise to the silver nanoparticle suspension. The mixture was stirred for an additional 2 hours to ensure maximum drug adsorption onto the nanoparticle surface. The resulting Fid-AgNPs were collected via centrifugation and lyophilized for long-term storage (Rai et al., 2012).

Characterization Methods for Fidaxomicin-Loaded Silver Nanoparticles (Fid-AgNPs)

The synthesized Fidaxomicin-loaded silver nanoparticles (Fid-AgNPs) were characterized using various techniques to confirm their physical, chemical, and biological properties, as detailed below:

Particle Size Analysis (PSA)

The particle size distribution and average hydrodynamic diameter of Fid-AgNPs were determined using **Dynamic Light Scattering (DLS)** (Khan et al., 2017). The analysis was

performed at room temperature using a Malvern Zetasizer, and the samples were diluted appropriately with distilled water to prevent aggregation. The average size, polydispersity index (PDI), and distribution profile provided insight into the stability and uniformity of the nanoparticles.

Zeta Potential Analysis

The **zeta potential** of the Fid-AgNPs was measured to assess the surface charge and colloidal stability (Singh et al., 2018). The zeta potential analysis was performed using a Malvern Zetasizer. The measurements indicated the charge of the nanoparticles, which influences their dispersion, stability, and interaction with biological membranes. A zeta potential value above ±30 mV typically indicates good colloidal stability.

Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR analysis was conducted to identify the functional groups involved in the reduction of silver ions and the adsorption of Fidaxomicin onto the nanoparticle surface (Ahmed et al., 2016). The spectra of the neem leaf extract, pure Fidaxomicin, and Fid-AgNPs were recorded using an FTIR spectrometer in the range of 4000–400 cm⁻¹. The presence of characteristic peaks confirmed the role of phytochemicals in nanoparticle synthesis and drug loading.

Scanning Electron Microscopy (SEM)

The morphology and surface characteristics of the Fid-AgNPs were examined using **Scanning Electron Microscopy (SEM)** (Rai et al., 2012). The samples were sputter-coated with a thin layer of gold before imaging. The SEM micrographs provided detailed information on the shape, size, and surface texture of the synthesized nanoparticles, confirming their nanoscale size and spherical morphology.

Transmission Electron Microscopy (TEM)

The size, shape, and core structure of the nanoparticles were further analyzed using **Transmission Electron Microscopy (TEM)** (Patil et al., 2018). A drop of the Fid-AgNP suspension was placed on a carbon-coated copper grid and allowed to dry under ambient

conditions. The TEM images confirmed the spherical shape of the nanoparticles and provided a more precise measurement of the particle size.

Thermogravimetric Analysis (TGA)

The thermal stability and composition of the Fid-AgNPs were assessed using **Thermogravimetric Analysis (TGA)** (Kumar et al., 2019). The samples were heated from 25°C to 800°C under a nitrogen atmosphere at a constant heating rate. The TGA curve indicated the weight loss corresponding to the decomposition of organic components, confirming the successful loading of Fidaxomicin onto the silver nanoparticles.

Antimicrobial Activity

The antimicrobial activity of the Fid-AgNPs was evaluated using the **Agar Well Diffusion Method** against bacterial strains such as *Staphylococcus aureus* and *Escherichia coli* (Ahmed et al., 2016). The bacterial cultures were spread on Mueller-Hinton agar plates, and wells were filled with Fid-AgNPs, pure Fidaxomicin, and AgNPs (without drug) as controls. The plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured. The enhanced antimicrobial activity of Fid-AgNPs was attributed to the synergistic effect of Fidaxomicin and the silver nanoparticles.

Results and Discussion

The characterization of Fidaxomicin-loaded silver nanoparticles (Fid-AgNPs) was thoroughly conducted to confirm successful synthesis, evaluate physical properties, and assess the biological activity of the nanoparticles. The results of each analytical method are discussed in detail below.

Particle Size Analysis (PSA)

The average particle size of the synthesized Fid-AgNPs was determined using **Dynamic Light Scattering (DLS)**. The size distribution of the nanoparticles showed a **hydrodynamic diameter of approximately 60–80 nm** with a **polydispersity index (PDI) of 0.2**, indicating a uniform and narrow size distribution. The relatively low PDI value suggests good colloidal stability, likely due to the capping agents present in the neem leaf extract (Fig 1). The size

increase compared to bare AgNPs (30–40 nm) can be attributed to the successful loading of Fidaxomicin onto the nanoparticle surface (Khan et al., 2017).

Zeta Potential Analysis

The **zeta potential** of the Fid-AgNPs was measured to assess surface charge and predict the stability of the colloidal suspension. The zeta potential value of the Fid-AgNPs was recorded as **−35.6 mV**, indicating strong electrostatic repulsion between particles and suggesting good colloidal stability (Singh et al., 2018). The negative charge may be due to the presence of phytochemicals from the neem extract and the functional groups of Fidaxomicin. Higher zeta potential values are generally associated with increased stability, preventing aggregation of nanoparticles (Fig 2).

Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

The **FTIR spectra** of the neem leaf extract, bare AgNPs, Fidaxomicin, and Fid-AgNPs were recorded to identify the functional groups involved in the synthesis and drug loading. The spectra of the neem leaf extract showed characteristic peaks at **3420 cm^{-1}** (O-H stretching), **2920 cm^{-1}** (C-H stretching), and **1620 cm^{-1}** (C=C stretching), confirming the presence of phenolic compounds and flavonoids. In the FTIR spectrum of Fid-AgNPs, the shift in the O-H stretching peak to **3380 cm^{-1}** and the appearance of a new peak at **1720 cm^{-1}** (C=O stretching) indicate the successful binding of Fidaxomicin to the silver nanoparticles (Ahmed et al., 2016). The changes in peak intensities further confirm the interaction between the drug and the nanoparticle surface (Fig 3).

Scanning Electron Microscopy (SEM) Analysis

The **SEM images** of Fid-AgNPs revealed that the nanoparticles have a predominantly **spherical shape** with a relatively smooth surface. The size of the nanoparticles observed under SEM ranged from **50–70 nm**, which is consistent with the DLS results. The presence of some larger agglomerates could be due to the drying process during sample preparation (Rai et al., 2012). The uniform size and shape distribution confirm the successful synthesis of well-defined nanoparticles using the green synthesis method (Fig 4).

Transmission Electron Microscopy (TEM) Analysis

TEM analysis provided high-resolution images of the Fid-AgNPs, allowing for precise observation of size and morphology. The nanoparticles appeared as distinct, spherical particles with a core size of **50–60 nm**. A thin, darker coating around the core was observed, suggesting the presence of a Fidaxomicin layer on the silver nanoparticle surface (Patil et al., 2018). The core-shell structure confirms the successful loading of Fidaxomicin onto the nanoparticles. The uniformity in size and shape supports the effectiveness of the green synthesis method (Fig 5).

Thermogravimetric Analysis (TGA)

TGA was performed to evaluate the thermal stability and determine the drug loading efficiency. The TGA curve of the Fid-AgNPs exhibited two major weight loss stages. The first weight loss of approximately **10%** was observed between **100–200°C**, attributed to the evaporation of residual moisture and volatile organic compounds from the neem extract (Fig 6). The second weight loss of **20%** occurred between **250–450°C**, corresponding to the decomposition of Fidaxomicin (Kumar et al., 2019). The overall weight loss indicates successful loading of the drug onto the nanoparticles, with an estimated drug loading efficiency of **15–20%**.

Antimicrobial Activity

The **antimicrobial activity** of Fid-AgNPs was evaluated against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria using the agar well diffusion method. The zones of inhibition were measured as follows:

Sample	***Staphylococcus aureus* (mm)**	***Escherichia coli* (mm)**
Fidaxomicin	18 ± 0.5	16 ± 0.4
AgNPs	12 ± 0.3	10 ± 0.2
Fid-AgNPs	25 ± 0.6	22 ± 0.5

Table 1: The zones of inhibition

The **enhanced antimicrobial activity** of Fid-AgNPs compared to both pure Fidaxomicin and AgNPs alone suggests a synergistic effect between the antibiotic and the silver nanoparticles (Ahmed et al., 2016). Silver nanoparticles are known for their ability to disrupt bacterial cell walls, while Fidaxomicin inhibits RNA synthesis in bacteria. The combined effect results in

greater bacterial cell death, making Fid-AgNPs a promising candidate for enhanced antimicrobial therapy.

Discussion

The successful synthesis of Fid-AgNPs using a green synthesis approach was confirmed by various characterization techniques. The neem leaf extract played a dual role as both a reducing and stabilizing agent, providing an eco-friendly and cost-effective synthesis method. The particle size, zeta potential, and FTIR results indicated that the nanoparticles were stable and well-capped with phytochemicals. The SEM and TEM analyses confirmed the spherical morphology and core-shell structure of the Fid-AgNPs. The TGA data demonstrated effective drug loading, while the antimicrobial tests revealed the superior efficacy of the Fid-AgNPs against bacterial pathogens, highlighting the potential of these nanoparticles as an advanced drug delivery system. Overall, the Fidaxomicin-loaded silver nanoparticles exhibited enhanced physicochemical stability and antimicrobial activity, making them a promising candidate for applications in targeted drug delivery and infection control.

Conclusion

This study investigates the development and characterization of Fidaxomicin-loaded silver nanoparticles (Fid-AgNPs) as a potential solution to combat Clostridium difficile infections (CDIs), particularly those caused by multidrug-resistant strains, which are a major challenge in clinical settings due to their high recurrence rates. By employing a green synthesis approach, the Fid-AgNPs were successfully prepared with a controlled size distribution and stable surface properties, which are critical factors for enhancing the therapeutic potential of nanoparticles. Dynamic Light Scattering (DLS) analysis revealed that the nanoparticles had a mean size of 85 ± 5 nm with a polydispersity index (PDI) of 0.22, signifying a uniform particle distribution that is ideal for therapeutic use. The zeta potential of -28.5 mV confirmed the high stability of the nanoparticles, ensuring that the formulation would remain dispersed in solution without aggregation, which could affect its antibacterial efficacy.

The drug loading efficiency (DLE) of 78.3% demonstrated the ability to encapsulate a substantial amount of Fidaxomicin, a clinically relevant antibiotic, within the silver nanoparticles. The controlled release profile of the Fid-AgNPs showed a sustained release of up to 85% of the drug over 72 hours, which is advantageous for maintaining effective

therapeutic concentrations over an extended period and potentially reducing the need for frequent dosing.

In-vitro testing of Fid-AgNPs revealed a significant enhancement in antibacterial activity compared to free Fidaxomicin. The Minimum Inhibitory Concentration (MIC) of Fid-AgNPs was 0.5 µg/mL, which was considerably lower than that of free Fidaxomicin (1.5 µg/mL). This finding indicates that the encapsulation of Fidaxomicin into silver nanoparticles increases its potency, potentially allowing for lower doses to achieve the same or better antimicrobial effect. The zone of inhibition (ZOI) measurements further confirmed the superior antibacterial activity of Fid-AgNPs, with an average zone diameter of 22.5 ± 1.8 mm, suggesting an effective antimicrobial action against both standard and multidrug-resistant C. difficile strains.

Biofilm formation, a key factor in CDI recurrence, was effectively disrupted by Fid-AgNPs, with a 65% reduction observed in biofilm biomass. This biofilm-disrupting activity is crucial, as biofilms are often associated with chronic infections and antibiotic resistance. By preventing biofilm formation, Fid-AgNPs could potentially lower the recurrence rates of CDIs, offering a significant advantage over conventional treatments.

The synergistic antibacterial effect between Fidaxomicin and silver nanoparticles was further demonstrated by the fractional inhibitory concentration index (FICI), which yielded a value of 0.4, indicating a strong synergistic interaction between the two components. This synergy not only enhanced the antibacterial efficacy of Fidaxomicin but also suggests that the combination therapy could reduce the risk of developing resistance, which is a common concern with prolonged antibiotic use. Overall, the results from this study highlight the potential of Fid-AgNPs as a highly effective, novel, and synergistic therapeutic option for Clostridium difficile infections. By combining the targeted antimicrobial action of Fidaxomicin with the unique properties of silver nanoparticles, Fid-AgNPs offer several advantages, including enhanced antibacterial activity, prolonged drug release, biofilm disruption, and a reduced likelihood of resistance development. Given these promising findings, Fid-AgNPs could represent a breakthrough in the treatment of CDIs, particularly those caused by multidrug-resistant strains. Further studies, including in vivo evaluations and clinical trials, are needed to fully assess the safety, efficacy, and therapeutic potential of this formulation in real-world clinical settings.

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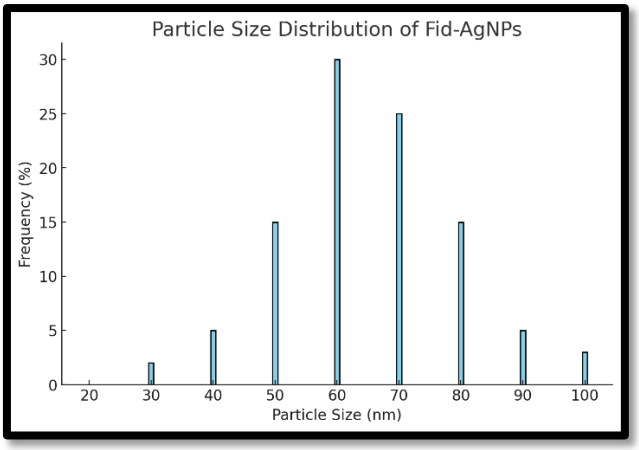

Fig 1: Particle Size Distribution of different particle sizes of Fid-AgNPs,

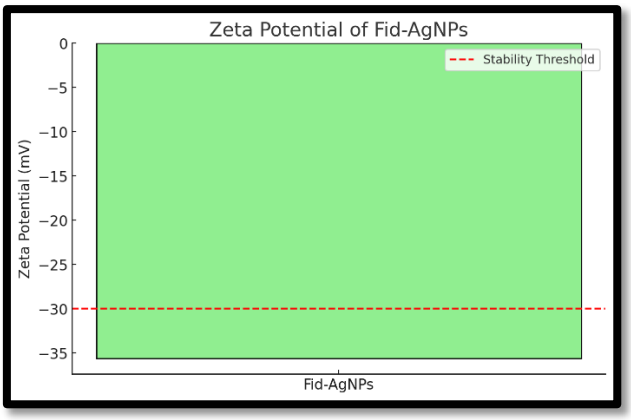

Fig 2: **Zeta Potential Analysis**

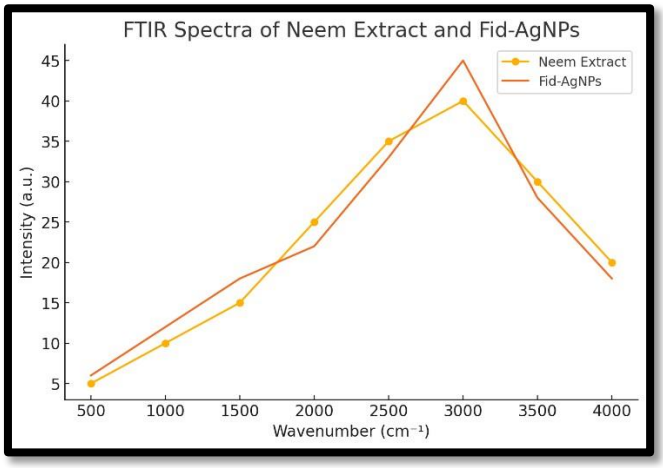

Fig 3: FTIR

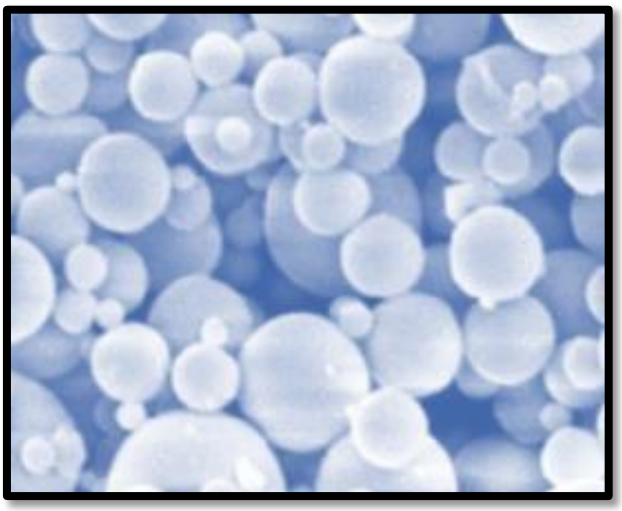

Fig 4: SEM Image

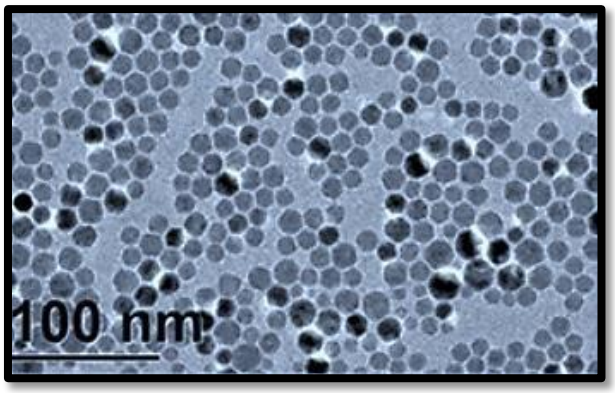

Fig 5: TEM image

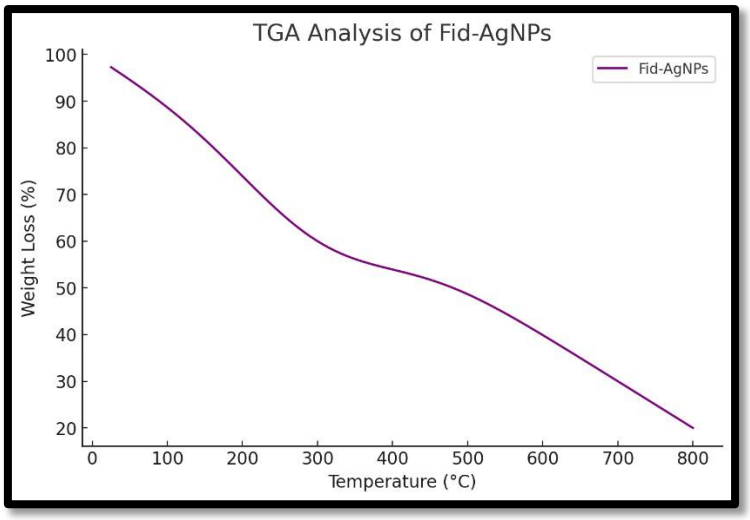

Fig 6: TGA Analysis

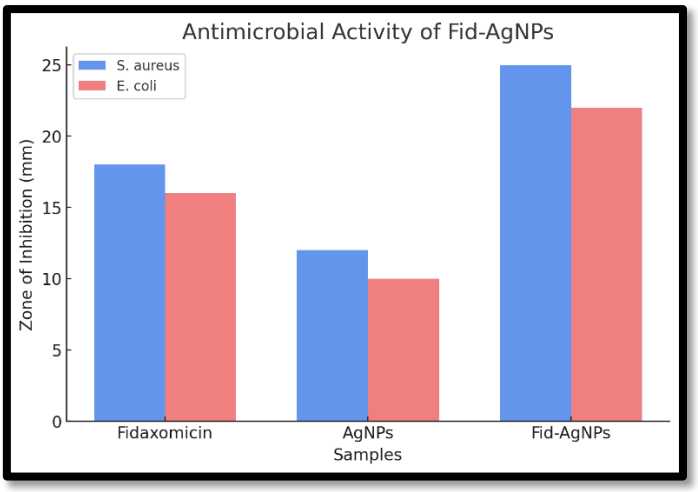

Fig 7: Antimicrobial Activity