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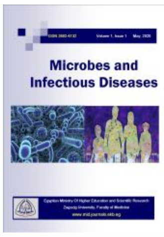

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Original article

Biosynthesis of silver nanoparticles using cow dung extract and evaluation of their antibacterial potentials

Manoja Das*[1], Diptikanta Acharya[1], Malati Hembrom[1] and Ipsita Nayak[1]

1- Department of Biotechnology, GIET University, Gunupur-765022, Odisha, India

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ABSTRACT

Background: Cow dung, also called cow pies or cow manure, is the excrement left behind by bovine animals, such as buffalo, yak, and cows.
Methods: The work aims to green synthesize the silver nanoparticles accomplished by aqueous extract from cow dung. To convert $AgNO_3$ into silver nanoforms, After mixing dung extract with 100 mM silver nitrate ($AgNO_3$), the mixture was incubated for 24 hours at 27 ± 2º C. The first indication that the green synthesis of nanoforms had occurred was the combination solution's hue changing from slightly yellow to brown after incubation. The biogenesis of silver nanoforms was further validated by transmission electron microscopy (TEM), UV-VIS spectrophotometer analysis, and X-ray diffraction (XRD). The functionality groups of nanoforms are ascertained using Fourier transform infrared (FTIR) spectroscopy. **Results**: The nanoforms had the absorption maxima (ʎ max) at 442 nm. The TEM photography indicated that the biosynthesised nanoforms were nearly rounded structure-functional groups. It revealed that the nanoparticles' average size was 39 nm. The ideal mixture of raw material (AgNO3) and the extract was found to be 9:1. The alkaline solution (pH 8.5) favours the conversation process. Moreover, the antibacterial properties of the generated nanoparticles against three pathogens were examined using the agar well diffusion experiment, namely *Escherichia coli, Bacillus subtilis*, and *Staphylococcus aureus*. **Conclusion**: The synthesised nanoforms had the efficacy to inhibit all the studied microorganisms, which is attributed to the therapeutic use of the nanoforms.

Introduction

Nanometals are being used today for various purposes and are one of the promising areas for the study. However, nanoparticles are prepared from silver, gold, platinum, palladium, etc. Silver nanoparticles are emphasised more due to their wide application in electrical, optical, sensor, pharmaceutical, etc [1-4]. It is known that silver nanoparticles possess larvicidal, antifungal, and antibacterial properties. Preparing silver nanoforms is essential in this context due to their societal demand. There are three ways to create nanometals: chemically, physically, and biologically [5]. Many researchers have tried producing silver nanoparticles via biosynthesis because of the drawbacks of physical and chemical approaches. This process is considered eco-friendly because organic substances like plants and microorganisms and their products are used for nanometals' biosynthesis. At the time of biosynthesis, no hazardous wastes are released. Extensive research has been conducted on the utilisation of many plant

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* *Corresponding author:* Manoja Das
E-mail address: *manojadas@giet.edu*

components, including leaves, stems, roots, bark, flowers, fruits, and fruit segments, as well as fungus, bacteria, algae, mushrooms, and animal cell cultures, in the production of nanometals [6-10]. It is also reported that green synthesis by the extracellular method has more advantages due to the low cost incurred in downstream processing at the nanometals' purification time. The biochemical compounds in plants, microorganisms, or animal cells act as catalysing agents for converting compounds into their forms.

This work used an aqueous extract of cow dung as a catalytic mediator to produce silver nanoparticles from silver ions. Cow dung is the undigested faecal matter of bovine animals. It is rich in organic matter and minerals. Generally, it is used for manure and fuel purposes. It contains beneficial minerals such as phosphorus, potassium, and nitrogen that support the growth of soil microorganisms [11]. Cow dung has been utilized to make pots, prepare skin tonics for psoriasis and dermatitis, and produce biogas for green energy uses, etc. It also has antibacterial and antifungal properties. People have been treated for heat rashes and boils by combining neem leaf extract with cow dung extracts [12]. In villages of India, cow dung is useful to the walls and floors of houses as a part of the cleaning process. It is also used in Hindu rituals. It also has mosquito-repellent properties. Recently, textile paints have been prepared from cow dung, with eco-friendly, antibacterial, and antifungal properties. The current work was designed to create silver nanoparticles using cow dung extract since humans use cow dung for multiple purposes. The effectiveness of the produced nanoforms against bacteria, including *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*, was investigated. According to the study, cow dung extract may be used to create silver nanoparticles, and because of their antibacterial qualities, the created silver nanoforms are significant for therapeutic use.

Materials and Methods

Preparation of extracts: Silver nanoparticles are synthesised from the silver nitrate ($AgNO_3$) using cow dung extract as a catalyst. Cow dung was collected from a cow shed near the university and immediately brought to the laboratory for further use. Cow dungs are sun-dried and crushed into powder forms. 500 ml of distilled water and 100 g of powdered cow dung were boiled for 20 minutes, and the mixture was then filtered through muslin fabric. After collecting the supernatant, the mixture was centrifuged for 15 minutes at 3000 rpm. The liquid extract was stored in a coloured container for future experiments.

Preparation of the silver nanoparticles: The commercially available powdered form of silver nitrate (AgNO3) has been utilised to produce silver nanoforms. In laboratory, a standard concentration of 100 mM silver nitrate was prepared using distilled water, and the silver nitrate as a raw material to make nanoforms. To produce silver nanoparticles, 90 mm of AgNO3 solution and 10 mm of cow dung extract were combined (at a ratio of 9:1) in a conical flask, which was then left to incubate for 48 hours at 27 ± 2^0 C in the dark. During the incubation, the mixtures were regularly checked at an interval of 1hr, wherein a change in the colour of the solution was marked. The reaction was understood to be complete when the solution's peak color developed.

Ascertain the synthesis of silver nanoparticles: The mixture's initial colour was a delicate golden hue, which became brown during incubation. When a reaction is catalysed, the colour of the solution changes. The formation of silver nanoparticles was confirmed using a UV-VIS spectrophotometer. . The absorption spectra of the nanoforms produced were examined in a monochromatic wavelength range of 300 to 600 nm. The maximum absorption spectra of monochromatic light (*ʎ max*) were recorded. Further, the morphological shapes of the nanoforms were ascertained by examining the particles using high-resolution transmission electron microscopy (TEM). The nano forms were collected from the mixture by centrifuging at a high-speed centrifuge (15000 rpm) for 15 minutes. The assembled pallets were cleaned with distilled water, ethyl alcohol (75 %), and dimethyl sulfoxide (DMSO, 5 %) to eliminate any organic substances contaminating the surface. The nanoforms were carefully dried for 30 minutes at 70^o C in a vacuum oven. . TEM photographs were taken at different angles, and the picture showing the nano forms was recorded. X-ray diffraction (XRD, Model 13010) was used to examine the synthesised nanomaterials to determine the underlying crystal structure of the material. The XRD data and the microscopic observations are carefully correlated for the nanomaterials.

Standardization of synthesis of silver nanoparticles

Mixture of cow dung extract and $AgNO_3$ solution: As previously mentioned, cow dung extract was made and kept in an airtight-coloured bottle. To standardise the mixture of $AgNO_3$ and cow dung extract to prepare nano forms, the ratio of the cow dung extract to the silver nitrate solution at 100 mM concentration was mottled such as 8:1, 8:2, 9:1, 9:2, and 10:1 in separate containers respectively and then incubated in the dark at 27 ± 2^o C for 24 hr. The mixture solution that had maximum conversion with stabilised silver nanoforms is considered the ideal mixture, and the same mixture was used in future experiments.

Standardization of bioconversion period: In a glass container, a perfect combination of extract and silver nitrate solution was made, and it was incubated at 27 ± 2 C for 24 hrs. The blend solution's colour changed gradually over an hour until the final colour was reached. This progress was visually watched. The attainment of climax colour is indicated if no further reaction occurs in the mixture and recorded as the standard time for converting the silver solution to silver nanoforms.

Standardization of medium of the mixture: In catalytic processes, the reaction medium is crucial. The medium in which a reaction occurs affects the reaction rate by utilising energy transported from the reacting particles and modifying their potential power. Thus, to ascertain the suitable PH of the medium to biosynthesise the nanoforms, separate containers with a pH range of 4 to 10 were upheld, and all were incubated for twenty-four hours at room temperature. The container showing maximum conversion was recorded. The solution's colour change considers the synthesis of nano forms per the standard time and the solution's absorption as determined by a UV-visible spectrophotometer. The pH of the mixture showing maximum conversion in the least incubation period is considered a suitable pH for synthesising the nanoparticles.

Standardizing cow dung: Fodders are very important for domestic animals. Cows are given fodder to maintain good health and for milking purposes. In this study, four types of breeds are chosen for the collection of cow dung: Binjharpuri, Khariar, Ghumusari, and Kalahandi. The cow dung was collected for the experiment only in the morning. The extraction of bioactive compounds and the preparation of nanomaterials are prepared as described earlier. The time required for the biosynthesis of silver nanomaterials is recorded. The composition of cow dung is also affected by the food given to the cow. Therefore, to find suitable cow dung to biosynthesise the silver nanoparticles, they are collected from the cows fed with different fodders such as pasture, grain, and silage.

Efficacy of antimicrobial activity study: Silver nanoparticles generally show antimicrobial properties. The potential of synthesized nanoparticles against investigated microorganisms, including *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*, was determined using the agar disc diffusion technique. A pure strain of microorganisms was received from the Institute of Microbial Technology (IMT), Chandigarh, India, and the microbe subculture was maintained in the laboratory using the suggested growth medium. Agar plates of muller-hinton medium were prepared in separate petri dishes, and the pure strains of cultured microorganisms were transferred to the individuals' plates. The saturated paper discs of synthesised silver nanoforms were added to the muller-hinton agar plates following standard procedure. After that, the agar plates were incubated in a BOD incubator for 48 hrs at 370C. It was recorded the nanoparticles' zone of inhibition (ZOI) against each microorganism by the synthesized nanoparticles. The reference drug that was utilized a broad-spectrum antibiotic gentamicin.

Results

The cow dung extract and the 100 mM silver nitrate solution were combined in a specific ratio and incubated. The mixture solution had a light-yellow hue. After incubation, the transformation of the colour of the solution was initiated after 2 hrs of incubation and reached the climax colour, i.e., brown, after 6 hrs of incubation (**Fig. l**). Catalysers cause a reduction process that changes the colour of the mixed solution. The change of colour is the preliminary evidence of the formation of nanoforms. The absorption spectra of the produced nanoforms displayed a full bell-shaped curve (**Fig. 2**), with the absorption maxima (λ max) at 442 nm. A high-resolution transmission electron microscope (TEM) is frequently used to characterise the size and shape of nanoparticles. TEM can well examine the surface morphology of the nanoparticles because the instrument detects scattered electrons from the surface of the particles. The TEM of the biosynthesised nano forms shows in a 200 nm scale that they are nearly globular in shape (**Fig 3**). The synthetic nanoparticles' x-ray reflectance vs x-ray intensity XRD pattern graph is shown (**Fig. 4**). The silver nanoforms have a comparatively equal shape. The most popular type of infrared spectroscopy is Fourier transform infrared (FTIR), which operates on the tenet that some infrared (IR) light enters the sample and is absorbed while some of it goes through. In this technique, the radiations that pass through the sample are recorded. This analysis is usually performed to identify and classify the possible biomolecules that can be trustworthy for capping, leading to the capable stabilisation of nanoparticles. The FTIR analysis of the synthesised silver nano forms shows absorption peaks at 3318.32, 1642.19, 432.80, and 417.51 wavelengths.cm^{-1} (**Fig. 5**). The absorption spectra of the $AgNO_3$ solution and extract mixture of ratio 9:1 shows a completely bell-shaped curve having a height peak as compared to other mixtures (**Fig. 6**). Alternatively, the proportion of mixture of $AgNO_3$ solution and extract of 8:1, 8:2, 9:2, and 10:1 developed the flatten curve as compared to the mixture of ratio 9:1. Thus, the suitable combination of $AgNO_3$ and extract is 9:1. The same combination was used for all the experimental studies. The medium suitable for the bioconversion process was established by maintaining the mixture of pH from pH 4 to 10 in different containers. Absorption spectra of the solution after incubation were recorded (**Fig.7**). The alkaline medium (pH 8.5) favours the biosynthesis of the nanoparticles.

The synthesised silver nanomaterials were studied for their potential against studied microorganisms such as *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus* by the diffusion of agar wells (**Fig 8**). During the study, cow dung extract was used as a control, and a broad-spectrum antibiotic called gentamicin served as the standard drug. The maximum inhibitory zone was recorded for *Staphylococcus aureus* (5.2 cm), whereas the most minor inhibitory site was found in *Bacillus subtilis* (2.4 cm). However, all three studied microorganisms received the antagonistic effect to the silver nanoparticles that were synthesised. Because it was in its pure form, the antibiotic gentamicin was more effective in the inhibition zone.

Discussion

Several publications are now available on the biosynthesis of silver nanoparticles using silver nitrate as the raw material. [13-15]. The secondary metabolites present in the plants catalyse the conversion process. In this investigation, an extract of cow dung was used as a catalyst for the conversion process. Cow dung contains nitrogen (3%), phosphorus (2%), and potassium (1%), i.e. 3-2-1 NPK, cellulose, hemicellulose, manganese, calcium, zinc, and trace elements [16]. However, the real composition of the cow dung is determined by the cow's feeding. Cow dung increases the count of beneficial microorganisms when added to the soil. The absorption spectrum represents the incident radiation absorbed by the material over a spectrum of frequencies of electromagnetic radiation. It is the reflection of the atomic and molecular composition of the materials. A single peak with a maximum at 442 nm in the produced nanoparticle solution supported the existence of identical particle types. Usually, when the solution contains different particles, it shows more than one peak due to variations in the absorption capacity of the particles. The biosynthesised particles observed the single peak in the examination with UV VIS spectrophotometer indicates the presence of similar types of particles in the examined solution [17]. In an X-ray diffraction (XRD) examination, x-rays are scattered when they impact nanoparticles because the atom's nucleus's electrons rotate. Generally, XRD provides data regarding the nanoparticles' crystalline structure, nature, and lattice parameters. Using the broadening of the strongest peak from an XRD measurement of the nanoparticles, Scherer's equation is used to determine the lattice parameter. It has been reported that secondary metabolites present in plants can serve as catalysing agents for the reduction process [18]. The cow dung contains semi-digested plant materials and microorganisms. Silver ions (Ag+) in the fluid are converted to silver nanoforms (Ag^o) by phytochemicals found in semi-digested plant parts, metabolites of microbes, or both.

It is established that when functional groups of the biomolecules are present, shifting peaks arise. This is possible due to the binding or biomolecules being capped onto nanoforms. Usually, the non-metals are produced by physical or chemical methods or both to generate the micro and macro forms. However, the nanoparticles produced through green synthesis have fewer propensities to combine due to the coating of the biomolecules, which stabilises the nanoforms.

Earlier reports also indicate that the alkaline environment is quite suitable for the reduction reaction during the biogenesis of nanoparticles made of both gold and silver [19]. The acidic environment develops a highly protonated solution, which affects the biomolecules and reduces the catalysing capacity of the cow dung extract. The slightly alkaline environment favours the stability of the nanoforms. Moreover, because the biomolecules were not capping the nanoforms, they were unstable when they developed in an acidic environment. As a result, the nanoforms combined themselves and formed the microforms. Also, non-stabilized forms result due to the nanoforms nucleating in an acidic environment. Therefore, in an acidic environment, the absorption spectra of the nanoforms show a flattened curve due to the formation of non-stabilized nanoparticles. In contrast, in an alkaline environment, nanoforms show a definite peak, indicating the formation of stabilised nanoparticles [20]. The antibacterial characteristics of gold and silver nanoparticles were well established [21-23]. It is found that nanoparticles interfere with cellular transportation, disrupt the cell wall, or affect the normal metabolism of the microorganisms; as a result, the organisms have been killed [24,25]. According to the study, the produced nanoparticles effectively prevent the growth of all three microorganisms.

Figure 1. Mixture of silver nitrate and cow dung extract A. Before conversion B. After conversion (0.5 mM, 1mM, 2 mM, 5 mM, 10 mM respectively).

Figure 2. The absorption spectra of synthesized nanoparticles having absorption maxima (*λmax*) at 441.5 nm.

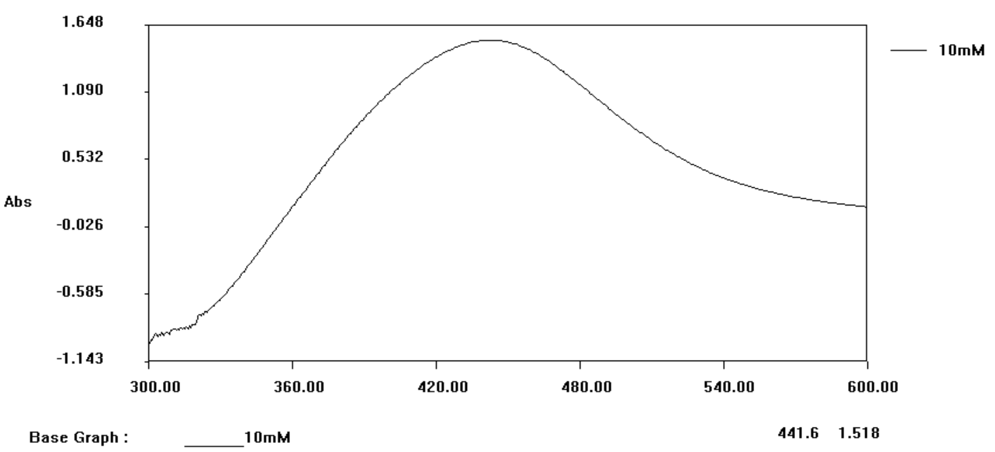


Figure 3. Scanning electron micrograph of synthesized nanoparticles. A. in 200 nm scale B.50 nm scale.

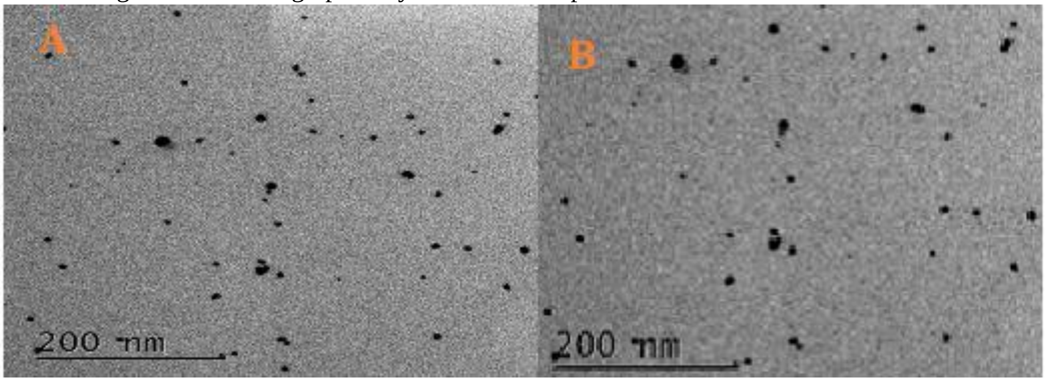


Figure 4. The x-ray diffraction (XRD) graph of synthesized nanoparticles, 2θ angle vs intensity.

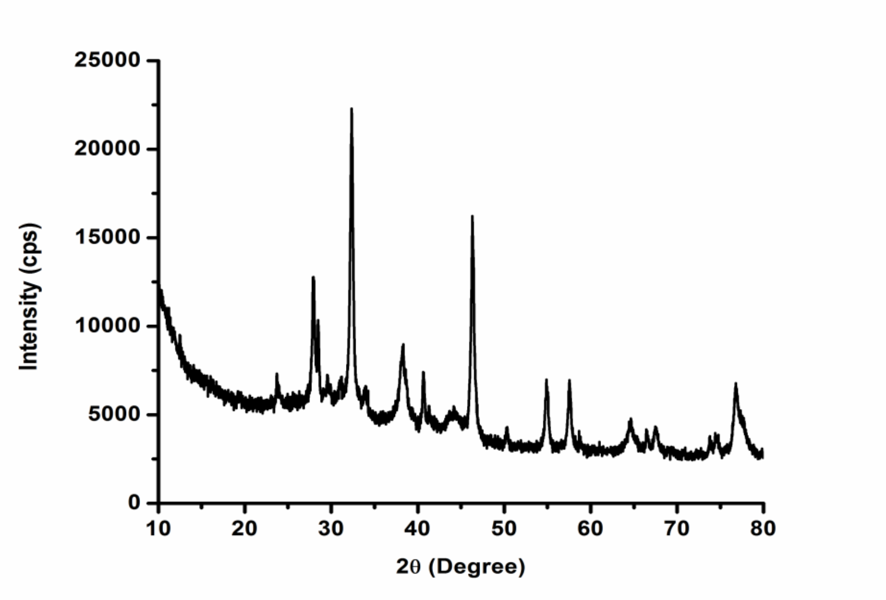


Figure 5. Fourier transmission infrared spectroscopy (FTIR) of synthesized nanoparticles.

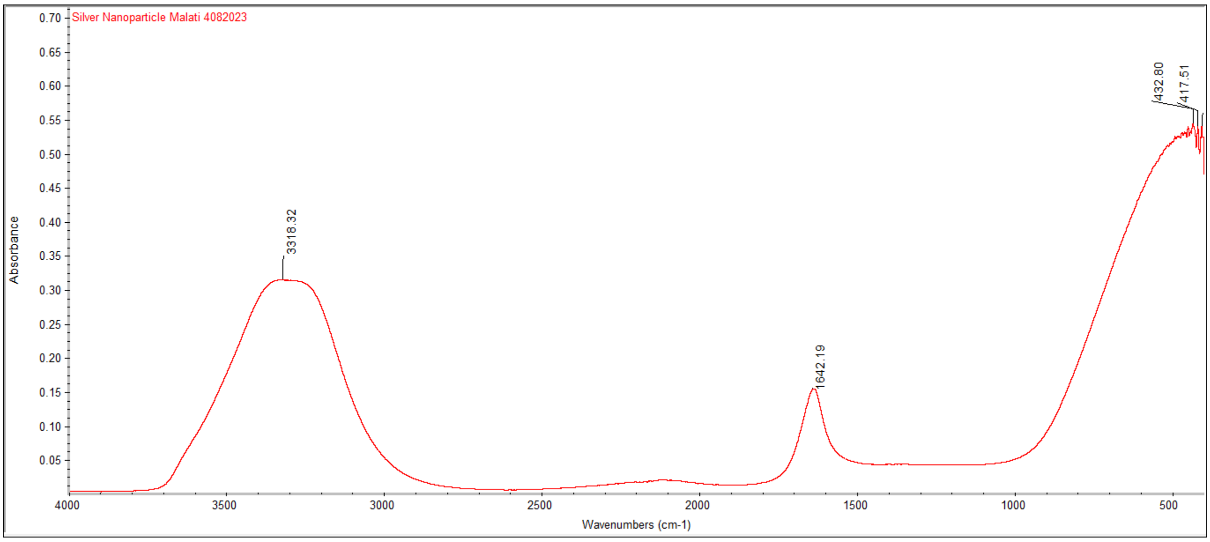


Figure 6. Absorption spectrum of green synthesized nanoparticles in the mixture of $AgNO_3$ and cow dung extract. (A. 8:1; B. 8:2; C. 9:1; D. 9:2; E. 10:1)

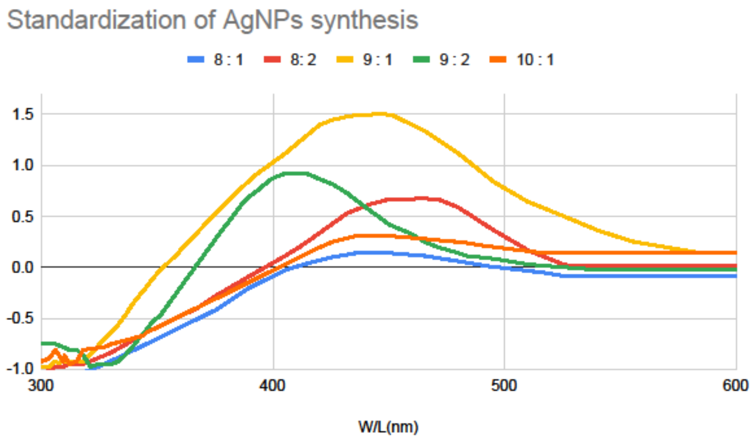


Figure 7. Absorption spectrum of synthesized silver nanoparticles in different medium (pH 4.0; pH 5.5; pH 7.0; pH 8.5; pH 10)

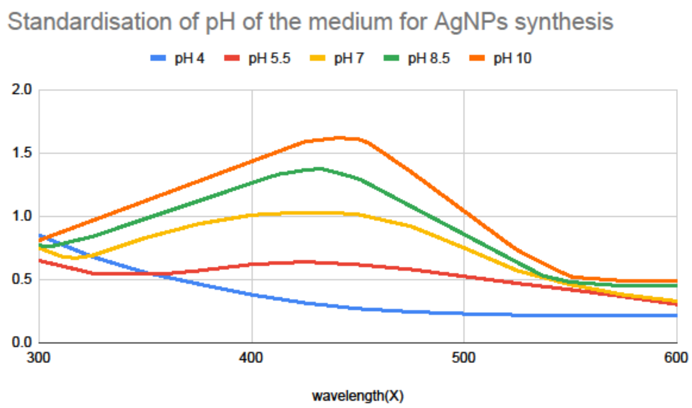
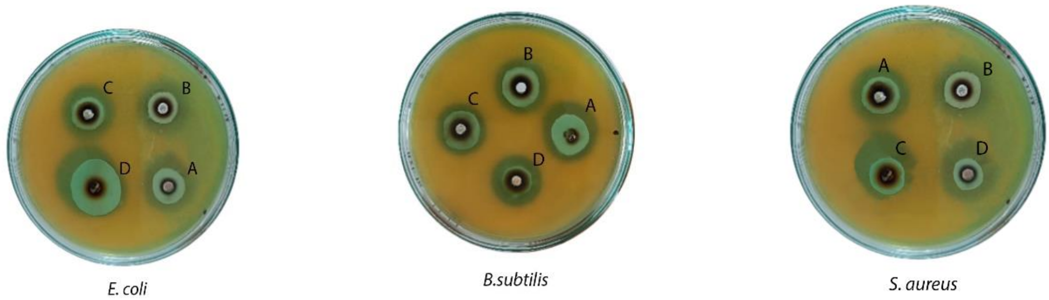


Figure 8. Zone of Inhibition developed by the synthesized silver nano particles against three microorganisms such as *Escherichia coli, Bacillus subtilis and Streptococcus aureus*.



Conclusion

Nanotechnology is a rapidly expanding subject include the cross-disciplinary study. Though nanoparticles are being used in different field, still biological activities of nanoparticles are less understood. Now, one of the important use of nano particles is clinical domain. The nanoparticles are utilized as antimicrobial agent and also as antibacterial coating material to avoid infections on implanted devices and related medication materials. In the long run, they are taken into consideration for the healing of wounds. These would be active in the creation of antibacterial drugs, bacterial infection control, and microbiological diagnostics. Due to the widespread application of silver nanoparticles across several industries like pharmaceuticals, electronics, and others, the demand for silver nanoparticles in the market is very high. Therefore, producing AgNP at cost-effective and with rapid processing to develop is the call of the day. Many researchers are trying to figure out a practical and environmentally beneficial way to produce silver nanoparticles. In this study, we have used cow dung extract to produce silver nanoparticles. As cow dung is relatively cheap and easily available in rural areas, by considering it, the aqueous extract of cow dung was utilised for the biosynthesis of silver nanoparticles. The mixture of $AgNO_3$ and extract in a ratio of 9:1 was good for manufacturing. The total time required for the bioconversion process is 6 hr. Alkaline medium favours the conversion process. The synthesised nanoforms are analysed with TEM and FTIR, and it is found that the biosynthesised metals are spherical, stabilised, and capped with biomolecules. The biosynthesised silver nanoparticles have efficacy opposing the development of the three investigated microbes, such as *Escherichia coli, Staphylococcus aureus,* and *Bacillus subtilis*. The process followed for preparing silver nanoparticles was safe and eco-friendly since no dangerous compounds were released during or after the production process. Here, we have utilised a cheaper material as the catalysing agent. Because the nanomaterial has antibacterial characteristics that explain its therapeutic significance, this procedure can be included in the environmentally friendly silver nanoparticle production database.

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Conflict of interest

` All authors affirm no conflict of interest in the work.

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