Nanomed Res J 9(1):9-19, Winter 2024

RESEARCH ARTICLE

Synthesis of silver nanoparticles using *Phyllanthus emblica* leaf extract: Characterization, antioxidant, anti-inflammatory and antileishmanial activity against *L. donovani*

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ARTICLE INFO

Article History:
Received 09 Jan 2024
Accepted 14 Mar 2024
Published 01 Apr 2024

Keywords:
Phyllanthus emblica,
Nanoparticles,
Antioxidant,
Anti-inflammatory,
Anti-leishmanial activity

ABSTRACT

Objective(s): The objective of the study was to examine the antioxidant potential, anti-inflammatory, and anti-leishmanial activity of silver nanoparticles (AgNPs) synthesized from the extract of *Phyllanthus emblica* leaves.

Methods: UV–Vis spectroscopy, FTIR, FESEM, and Zeta potential were used to examine the green synthesized nanoparticles. A DPPH free radical scavenging assay was used to study the antioxidant activity. Anti-inflammatory activity was conducted to observe the inhibition of protein denaturation. The MTT assay was used to evaluate the anti-leishmanial activity against *Leishmania donovani.*

Results: The UV–Vis spectroscopy study at the band of 440 nm confirmed the fabrication of nanoparticles. FTIR confirmed the ingredients in *P. emblica* leaf extract which is responsible for capping and reducing the AgNPs. FESEM reported the AgNPs synthesized in the size range of 40–50 nm. The results showed a simple and feasible approach for obtaining aqueous monodispersive AgNPs. Furthermore, the biological potential of the biosynthesized AgNPs was examined. Concerning this, the dose-dependent antioxidant potential of AgNPs was identified to be comparable to standard ascorbic acid. This also applies to the anti-inflammatory properties. The study findings indicate that all concentrations of AgNPs exhibit anti-leishmanial action. After being exposed for 72 hours, the concentration of 100 µg/mL of AgNPs exhibited the most potent anti-leishmanial activity, achieving 100% effectiveness. Further, the IC50 content of AgNPs on *L. donovani* after 24, 48, and 72 hours was calculated to be 45.88, 36.86, and 24.81 µg/mL, respectively.

Conclusion: The results stated that the synthesized AgNPs using *P. emblica* leaves have the most potent *in vitro* antioxidant, anti-inflammatory and anti-leishmanial activity. Further investigation into its potential biomedical applications is needed.

How to cite this article
Sharma S, Kumar S, Pai K, Kumar R. Synthesis of silver nanoparticles using Phyllanthus emblica leaf extract: Characterization, antioxidant, anti-inflammatory and antileishmanial activity against L. donovani. Nanomed Res J, 2024; 9(1): 9-19. DOI: 10.22034/nmrj.2024.01.002

INTRODUCTION

Nanomedicine defines the application of nanotechnology in medicine and pursues several goals, including the therapeutic use of nanoparticles (NPs) [1,2]. Recently, scientists have been more and more interested in NPs [3]. Because of their exceptional qualities—such as their high surface area to volume ratio and their mechanical, chemical, optical, and magnetic properties [4], NPs are particularly promising in emerging industries like food, medicine, agriculture and genetics. The multifunctional therapeutic properties of noble metal NPs in biological applications, have attracted

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much interest [5]. In terms of environmental friendliness and biomedical relevance, green synthesis, avoiding the use of toxic chemicals during synthesis, has several advantages [6,7]. The green NPs are encouraged due to the availability of plant extracts, animal proteins, agricultural wastes, dyes, bacteria, fungi and small viruses in nanoparticles [8]. The secondary metabolites found in plants act as stabilizers and capping agents and can reduce metal ions to metal nanoparticles, synthesizing the necessary NPs for the beneficial properties as previously noted [9]. Over the past decade, the research in the field of green nanotechnology has increased due to environmental concerns [10]. The properties of NPs are mostly dependent on their shape, which increases their application potential but requires precise and consistent manufacturing [11]. Although studies show that less concentrations of $AgNO_3$ have greater therapeutic promise, higher catalytic activity, greater biocompatibility and chemical stability, higher concentrations of silver are dangerous [12].

Silver is inactive in its metallic form, but it reacts with skin moisture and wound fluid to become ionized [13]. AgNPs have been used in medicines to reduce burns and wound infections [14]. Due to the special properties of silver, scientists now pay special attention to biological effects such as anti-leishmanial [15], antibacterial, anti-cancer, antiviral, antifungal and anti-inflammatory activities. Leishmaniasis is not a single disease, but a group of diseases that can cause a wide variety of clinical signs, from skin ulcers that heal on their own to the most severe visceral infections and occasionally even death [16]. It is an infectious disease caused by the protozoa *Leishmania*, an obligate intracellular parasite of mammalian macrophages [17]. About 88 countries worldwide are affected by leishmaniasis, which occurs mainly in tropical and subtropical regions. These areas are home to nearly 350 million people. Annually, there are 1.5 to 2 million new cases, resulting in a total of around 12 million individuals globally being impacted [18,19]. There are around 500,000 incidences of visceral leishmaniasis (VL) and 1.5 million incidences of cutaneous leishmaniasis (CL) each year [20]. In Bangladesh, Nepal, Brazil, Sudan, and India, 90% of VL cases have been recorded; in Afghanistan, Brazil, Peru, Iran, Syria, and Saudi Arabia, 90% of cases of CL have been reported [21,22]. The Indian states most severely affected by VL are Assam, Bihar, eastern Uttar Pradesh and West Bengal, where drug resistance and recurrence of leishmaniasis are increasing. A more recent survey found an alarming number of 1,000,000 cases, of which 10,000 were resistant to pentamidine, antimonials and amphotericin B [23-26]. Bihar is the worst affected region in India, accounting for more than 90% of the total cases. The pharmacological arsenal against *Leishmania* still needs to be improved, despite the clinical use of miltefosine and amphotericin B [27].

This study aimed to synthesize AgNPs through a green method without using hazardous compounds. Here we present the distinctive properties of AgNPs synthesized using *Phyllanthus emblica* (commonly known as "Amla") leaf in hot water as a capping and reducing agent. The characterization of synthesized AgNPs were done by UV–Vis, zeta potential, scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR).The synthesis of AgNPs using *P. emblica* leaf extract have not been studied for their anti-leishmanial activity. Therefore, the antioxidant, anti-inflammatory and anti-leishmanial activities of the AgNPs were evaluated against the promastigotes of *L. donovani* (the cusative agent of Indian Kala-azar).

MATERIALS AND METHODS

Plant Extraction Procedure

The freshly collected leaves of *P. emblica* were collected from the premises of Bundelkhand University in Jhansi, Uttar Pradesh, India. Subsequently, the leaves underwent meticulous cleansing utilising a combination of tap water and distilled water. The aqueous extract of the leaves was produced by mixing 5 g of dried leaves with 50 ml of milli Q water in a 100 millilitre flask. The flask was wrapped with aluminium foil and the mixture was boiled at a temperature of 70°C for 15 minutes. The extract was filtered using Whatman no. 1 and subsequently stored at a temperature of 20°C for nanoparticle synthesis.

Biosynthesis of silver nanoparticles

The synthesis of AgNPs was performed using a ratio of 1:4 aqueous leaf extract to 1mM $AgNO_3$ solution (Sigma-Aldrich). For production, the mixture is placed in water bath at 70°C in the dark. The mixture was observed (hourly) for color change (from light lime to dark brown) and analysed using a UV-Vis spectrophotometer in the range of 300–700 nm.

Characterization of NPs

UV-Vis Spectrophotometer

UV-Vis spectroscopy is a valuable tool for characterising stability, optical qualities, and reaction factors such as temperature, pH, and time. A UV-Vis spectrophotometer (PerkinElmer, Germany) at wavelengths of 300–700 nm was used to characterize the AgNPs using *P. emblica* leaves extract. The absorption peak from 350 to 500 nm [28] indicates the reduction of silver ions. The results show the existence of silver ions and a reduction in the analysed substances.

Fourier Transform Infrared Spectroscopy (FTIR)

The AgNPs solution was centrifuged at 10,000 rpm for 30 min to perform FTIR measurements. Functional groups in the extract of leaf that may be responsible for the formation of AgNPs were identified with FTIR (PerkinElmer Spectrum Version 10.03.06) and the FTIR spectrum was examined. It might aid in the stabilization, capping, and reduction of AgNPs. The FTIR range of 400–4000 cm-1 was achieved using a spectroscopic array.

Zeta Potential Analysis

The surface charge and formulation stability are often determined using zeta potential studies. By measuring the velocity of the NPs, this analysis helps to examine the colloidal stability of AgNPs produced by green synthesis. The velocity of the NPs as they move toward the electrodes is measured under the electric field [29].

Scanning Electron Microscopy (SEM)

The dimensions, shape, and arrangement of the synthesised NPs were examined using scanning electron microscopy. The dried samples were placed on dual conductive tape attached to the sample container and left at room temperature. The samples were coated with a layer of platinum-gold to increase conductivity. Samples were analyzed under a voltage of 80 kV.

DPPH assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was performed to evaluate the free radical scavenging activity of the synthesized AgNPs [30]. The AgNPs were exposed to a 3 mL of 0.1 mM DPPH dissolved in methanol. After vigorously agitating the tubes, place them at ambient temperature for duration of 30 minutes in a lightless environment. A UV-visible spectrophotometer was used to measure the absorbance at a wavelength of 517 nm. In the experiment, distilled water served as the control, while ascorbic acid was used as the reference.

The percent free radical scavenging activity of AgNPs and the positive control ascorbic acid was determined with the following formula:

Free radical scavenging activity (%) = [(Ac−As)/Ac×100]

Where, Ac = Absorbance of control at 517 nm; As = Absorbance of the sample. Using a graph prism, the IC50 value, which represents the concentration of the sample required to scavenge 50% of the DPPH free radical, was determined.

Anti-inflammatory activity

The anti-inflammatory potential of AgNPs was studied as described by Padmanabhan et al (2012) [31] using the protein denaturation method. The potent nonsteroidal anti-inflammatory drug diclofenac sodium is used as the standard. The reaction mixture, consisting of 2 ml of AgNPs, 2.8 ml of phosphate-buffer saline (PBS, pH 6.4), and 2 ml of egg albumin (fresh chicken egg (1 mM)), was incubated at 27°C for 15 minutes. The mixture was heated in a water bath for 10 minutes at 70°C for denaturation. After cooling the sample, absorbance was measured at 660 nm. Each test was performed three times. Percent inhibition of protein denaturation was determined using the following formula:

% inhibition= ((Ac-As)/Ac)×100

Where, As = Absorbance of sample; Ac = Absorbance of control.

Parasite culture and cell viability analysis

Promastigotes of *L. donovani* were routinely cultured at 25±1°C in RPMI 1640 media supplemented with fetal calf serum (10% v/v), penicillin (100 U/ml) and streptomycin (100 g/ml). The log phase promastigotes were seeded (1×10^6 cells/mL) on 96-well microtiter plates with different concentrations of AgNPs and incubated at 25±1°C for 24, 48, and 72 h. Each plate was filled with 100 µl of MTT solution (5 mg/mL) and incubated for 4 h at 25±1°C. 100 µl DMSO was added to each well to finally solubilize the formed formazan,.

Absorbance was measured at 570 nm, with DMSO (0.5%) as control and miltefosine as reference.

The following formula calculated the percentage of cell viability [32]:

% Cell viability = (OD of cells with AgNPs / OD of cells without AgNPs) × 100

Statistical analysis

The findings from the research are presented as the mean±SD. The statistical disparities between groups were determined using Student's t-test. A P value was considered statistically significant if it was below 0.05.

RESULTS

Characterization of AgNPs

The aqueous extract of *P. emblica* leaves was used for the synthesis of AgNPs at a temperature of 70°C. With AgNPs synthesis, the initial colorless reaction mixture gradually turned dark brown after 30 min (Figure 1). Using leaf extract, the reduction of Ag ions to metal was confirmed by UV absorption spectrum value at 440 nm. *P. emblica* leaf extract was used to confirm the green synthesis of AgNPs using the surface plasmon resonance band at 440 nm. AgNPs synthesis was observed by UV-visible spectroscopy at different time intervals; the highest reduction and aggregation of AgNPs was observed after 6 h, as shown by the absorption intensity (Figure 2).

Biomolecules for effective capping and stabilization of metal NPs production by *P. emblica* leaf extract were determined by FTIR measurements. FTIR spectrum of *P. emblica* leaf extract is shown in Figure 3. Spectral analysis

Figure 1. Colour changes in the *P. emblica* leaves extract after addition of silver nitrate (1 mM aqueous solution of $AgNO_3$) for nanoparticles synthesis

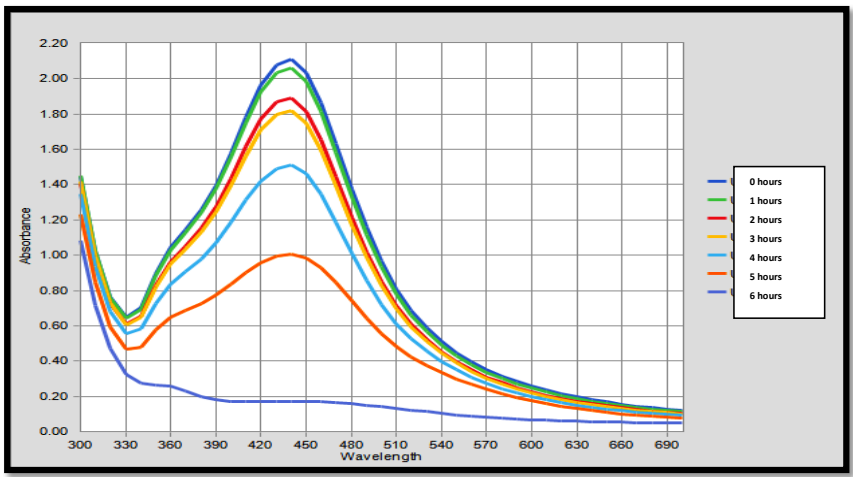

Figure 2. UV-Visible spectra of *P. emblica* fresh leaf silver nanoparticles from 0 to 6 hr.

Table 1. FTIR peak value represent the functional groups present in *P. emblica* fresh leaf silver nanoparticles

Peak Value	Functional Group
3851	O-H
3741	O-H
3405	N-H / O-H
2924	C-H / N-H
2345	O=C=O
2113	N=C=S
1597	N-O
1384	C-H / O-H
1063	S=O
673	C=C / C-Br

revealed the number of functional groups acting as limiting agents or stabilizers and responsible for stabilizing the NPs. Extraction mediated AgNPs-based FTIR measurement showed different absorption peaks at 3851, 3741, 3405, 2924, 2345, 2113, 1597, 1384, 1063 and 673 cm−1 with different functional groups O-H, O-H, N-H/O-H, C-H/ N-H, O=C=O, N=C=S, N-O, C-H/O-H, S=O and C=C/C-Br respectively (Table1).

The zeta potential is an important index to understand the surface charge and stability of NPs produced in colloidal systems. AgNPs were found to be stable at -15.2 mV through zeta potential study and size analysis revealed a size of 83.03 nm of AgNPs, confirmed by dynamic light scattering (DLS) (Figure 4).

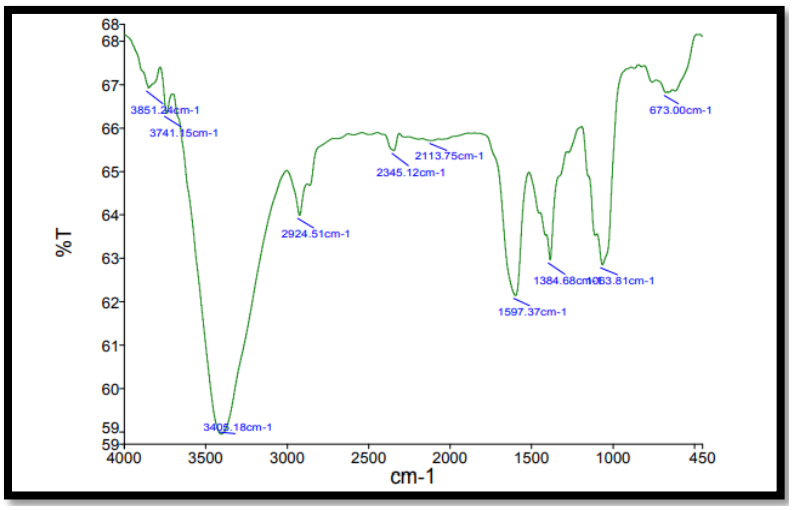

Figure 3. FTIR Spectra of *P. emblica* fresh leaf silver nanoparticles.

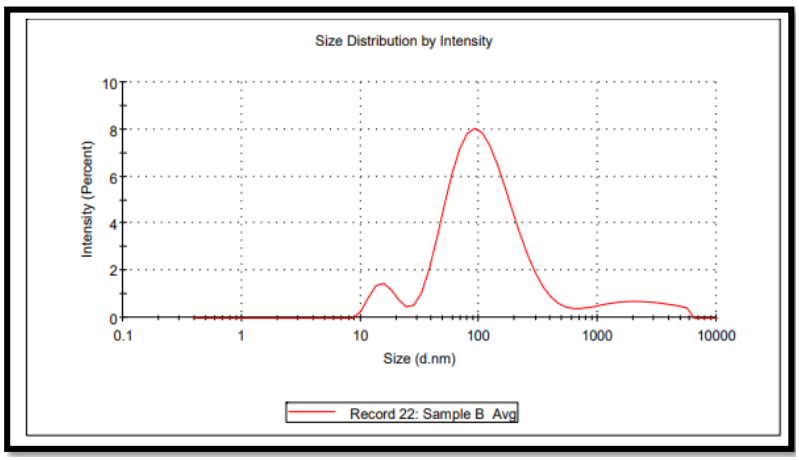

Figure 4. Zeta potential spectra of *P. emblica* fresh leaf silver nanoparticles

FESEM provides visible sample surface morphology. When electrons reflect off the sample surface, an image appears. High-resolution images of NPs surfaces provide us with useful details about their size, shape, topography, composition, conductivity, and other characteristics.The synthesized NPs were visible with FESEM. The shape of the NPs is round and oval. A small number of individual particles, with an average size of 40 to 50 nm, were also identified, but the majority of NPs were aggregated (Figure 5).

In vitro antioxidant activity of AgNPs

The antioxidant ability of the AgNPs was evaluated by testing them against ascorbic acid. Figure 6 illustrates the dose-dependent DPPH scavenging potential of the fabricated AgNPs. Compared with ascorbic acid with IC50 value of 80.09 µg/mL, the synthesized AgNPs have an IC50 value of 147.22 µg/mL.

In vitro Anti-Inflammatory Activity of AgNPs

The anti-inflammatory potential of silver

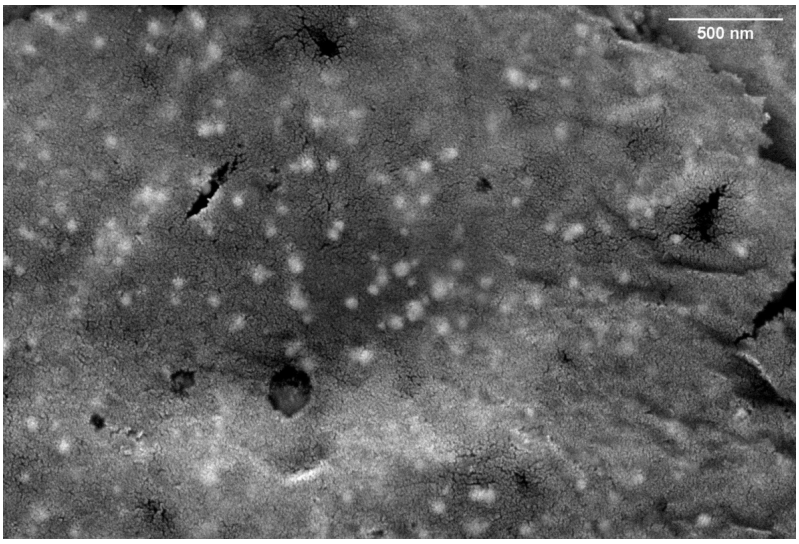

Figure 5. FESEM images of the biosynthesized silver nanoparticles (AgNPs)

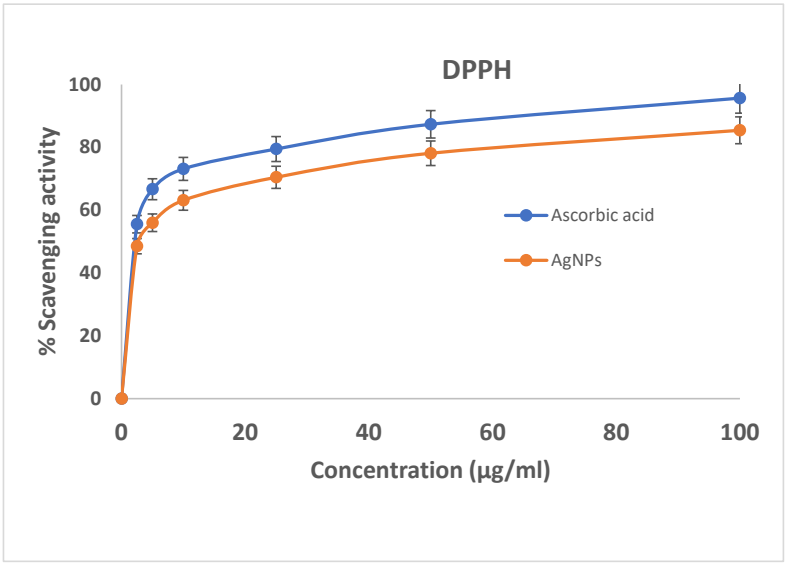

Figure 6. The scavenging activity on DPPH radical of various concentrations of synthesized AgNPs vs Ascorbic acid

NPs was evaluated using a protein denaturation assay and compared with the reference drug, Diclofenac sodium. With synthesized AgNPs, protein denaturation was observed (Figure 7). Conventional drugs showed the higher anti-inflammatory activity (91.67±1.90%), as compare to silver NPs (71.14± 0.88%).

Anti-promastigote activity of AgNPs

In vitro test was carried out to evaluate the effectiveness of different concentrations of AgNPs (2.5 to 100 µg/mL) after 24, 48 and 72 h of exposure to *L. donovani* promastigotes. After 24, 48 and 72 h on *L. donovani*, the IC50 content of AgNPs was determined to be 45.88, 36.86 and 24.81 µg/mL, respectively. At 100 µg/mL, a maximum anti-leishmanial effect (100%) was observed after an exposure time of 72h. The percentage of viable cells in *Leishmania* promastigotes subjected to different concentrations of AgNPs at various time intervals of incubation is shown in Figure 8.

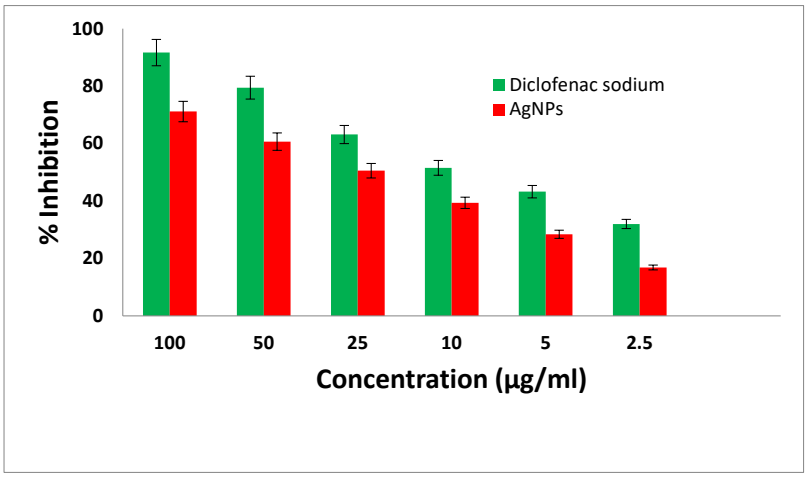

Figure 7. Anti-inflammatory activity of synthesized AgNPs vs Diclofenac sodium.

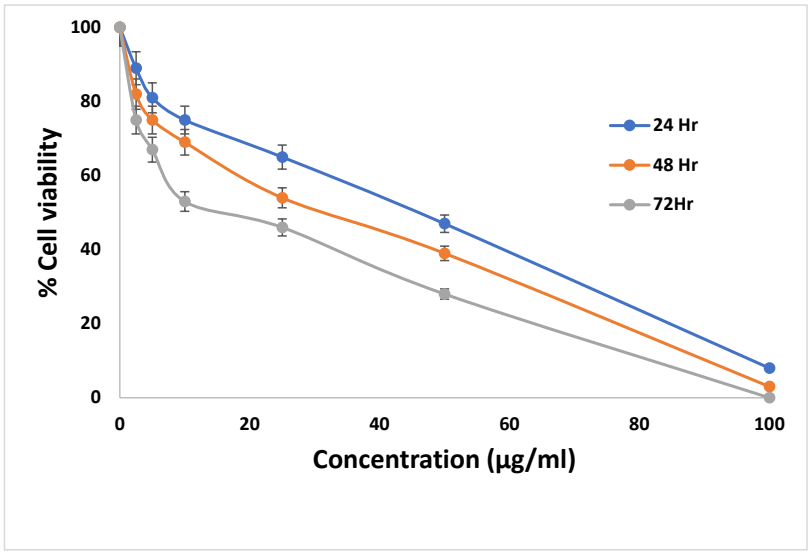

Figure 8. The effect of AgNPs on the viability of *L. donovani* promastigotes at different time of incubation i.e. 24, 48 and 72 hrs.

DISCUSSION

The synthesis process of "green" NPs, which is more expeditious than traditional chemical synthesis, is remarkable due to its environmentally conscious nature, cost-effectiveness, utility, and extensive range of applications. Biosynthesized NPs are being utilised in various applications such as cancer treatment, drug delivery, DNA analysis, gene therapy, antibacterial agents, biosensing, and response rate augmentation. This field is currently in its early stages of development. Nanoparticles find use in several fields such as electrical engineering, medical, chemistry, biology etc. The morphology and dimensions of colloidal metal particles have a pivotal role in a wide range of applications, such as the fabrication of magnetic and electronic devices, wound healing, the stimulation of antimicrobial genes, and the advancement of biocomposites. The electromagnetic and optical characteristics of noble metal NPs exhibit variations based on the size and morphology of the particles [33]. In contrast to physical and chemical techniques, the production of NPs using green and environment friendly technologies is characterised by its lack of toxicity, cost-effectiveness, and biocompatibility. Additionally, it necessitates a reduced amount of exertion and time. Algae, bacteria, fungus, and plants are commonly utilised as biological sources for synthesising AgNPs [34, 35]. The leaves of *P. emblica* in this study were analysed using the phytoreduction process to generate AgNPs. The process of converting silver into silver NPs involves the utilisation of *P. emblica* leaf extract as both a capping and reducing agent. A wide range of secondary metabolites, including tannins, glycosides, phenols, terpenoids, saponins, and flavonoids, have been identified in the quest for phytochemicals [36]. Furthermore, it has been found that aqueous extracts of *P. emblica* leaves possess significant quantities of flavonoids and phenols [36]. The presence of silver NPs was confirmed by the observed alteration in colour following the adding up of concentrated leaf extract to the $AgNO_3$ solution. After duration of 6 hours, the solution undergoes a transformation and assumes a dark brown colour.

An intense peak at 440 nm was found in the UV-Vis spectra, indicating the presence of silver NPs synthesised using green methods. FESEM was used to confirm the shape and surface area of AgNPs. The size of *P. emblica* AgNPs was evaluated via SEM examination and found to range from 40 to 50 nm. The FTIR spectra reveal the existence of many functional groups that can serve as both reducing and capping agents during the fabrication of AgNPs.

The time progression of the surface charge can be observed by employing the zeta potential methodology. The zeta potential method measures the temporal variation of surface charge. Metal nanoparticles exhibit a tendency to disperse and prevent clumping when their zeta potential is either positive or negative. Nevertheless, these particles aggregate and adhere to each other when the zeta potential is low due to the absence of any opposing force. The zeta potential and size of particles are crucial as they influence the stability of the particles, their dispersion in the body, and their uptake by cells [37,38]. This work utilised a zeta potential analyzer to determine the average size and size distribution of silver NPs that were produced using green fabrication methods. The zeta potential investigation showed that AgNPs remained stable at a value of -15.2 mV. Additionally, the size examination using dynamic light scattering (DLS) verified that the AgNPs had a size of 83.03 nm. The pH level of the dispersion and the concentration of electrolyte have a substantial influence on the zeta potential of the particles. AgNPs possess exceptional colloidal properties, exhibiting long-term stability and efficient dispersion, which can be attributed to their large negative potential values resulting from negative repulsion [39].

Unpaired free radicals refer to one or more electrons in a molecule that are not paired. Due of their ability to acquire electrons from other molecules, they exhibit a high level of instability. These free radicals accelerate the body's aberrant and unregulated oxidation process. This elevates the susceptibility to several ailments, such as cancer, inflammation, cardiovascular disease, hepatic illness, Alzheimer's disease, and Parkinson's disease. Additionally, it diminishes the efficacy of the antioxidant defence system and impairs cellular structure. Strengthening the natural antioxidant defence system of the body or consuming scientifically confirmed antioxidant supplements can minimise the risk of chronic disease and slow its course [40]. The DPPH assay results were quantified as the percentage of inhibition. A comparison was made between synthesised AgNPs and ascorbic acid, which served as the reference medication. The AgNPs and standard ascorbic acid exhibited inhibition rates of 95.67±0.7%

and 85.43±0.6%, respectively. The findings are corroborated by earlier studies [41] where the researchers investigated the antioxidant properties of AgNPs produced using *Rhizophora apiculata* leaf extract.

Inflammation has a substantial impact on various diseases [42]. Nonsteroidal anti-inflammatory medicines (NSAIDs) and steroids are the two primary categories of medications employed in the treatment of inflammation. Discovering alternative anti-inflammatory medications with comparable efficiency but without any adverse effects, such as gastrointestinal issues and leukopenia, is crucial [43,44]. The synthesised AgNPs were assessed for their anti-inflammatory properties using a protein denaturation assay. Both traditional diclofenac sodium and synthesised AgNPs exhibited anti-inflammatory effects *in vitro*, which were depending on the dosage. Based on earlier studies, AgNPs shown a protein denaturation inhibition rate of 71.65%, which is close to 94.24% of diclofenac, a commonly used anti-inflammatory medication [41].

Investigations in the field of plant sciences, particularly with the identification of bioactive plant components found in raw extracts of medicinal plants, have the capacity to provide groundbreaking, cost-effective medications that are safe for patients and demonstrate a satisfactory degree of efficacy. These considerations arise due to concerns regarding the adverse effects of the medications now employed for VL treatment, as well as the potential emergence of drug resistance resulting from parasite evolution. The initial proposal for the synthesis of environmentally friendly NPs was introduced by Raveendran et al. [45]. Therefore, the objective of the present study was to examine the leishmanicidal properties of artificially created AgNPs on parasites belonging to the *L. donovani* genus. The synthesis of NPs involved the selection of green leaves from *P. emblica*, which were then tested for their anti-leishmanial properties. Thus, it can be regarded as an anti-leishmanial agent in experimental models. Several research have investigated the anti-leishmanial characteristics of several metal NPs [46-48]. In addition, Zahir et al. (2015) discovered that the lowest cell viability ratio of *L. donovani* promastigotes was detected in produced AgNPs, as compared to aqueous *Euphorbia prostrate* leaf extract [49]. The anti-leishmanial effects of these NPs have been attributed to many processes, including the interaction of AgNPs with proteins and macromolecules including DNA, as well as the disruption of cell cycle proteins and mitochondrial enzymes [50]. However, the mechanism of the synthesised AgNPs has not been clarified in this study. Hence, additional investigation is required to examine the mechanism by which AgNPs act against *L. donovani* and to assess their effects *in vivo*.

CONCLUSION

Phyllanthus emblica leaf aqueous extract was found to be an efficient and green method to synthesize silver NPs. A better reducing and stabilizing agent is provided by the aqueous extract of *P. emblica*. UV-Vis spectroscopy, FTIR, SEM and zeta potential methods were used to characterize greenly synthesized AgNPs. The formation of AgNPs reveals the color change. The experimental results concluded that biogenic synthesised AgNPs had significant antioxidant (DPPH) and anti-inflammatory activity. Further, AgNPs possessed notable anti-leishmanial activity against the promastigotes of *L. donovani* in a dose-dependent manner. Therefore, it may be taken into account as a prospective agent for the future development of an anti-leishmanial drug.

CONFLICT OF INTEREST

The authors declare no conflict of interest in this study.

FUNDING

No funding involved

ETHICAL APPROVAL

None of the authors of this piece of work have conducted any research on humans or animals.

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