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Biosynthesis of Silver Nanoparticles by *Bacillus Subtilis* and Their Anti-Leishmanial Activity in Vitro

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ABSTRACT

One of the most prevalent parasite infections in the Middle East is cutaneous leishmaniasis (CL). It is the first time in this work that in vitro anti-leishmanial effects of *B. subtilis*/AgNPs were examined on *L. tropica* promastigote, and their efficacies were compared with pentostam. These nanoparticles are described to confirm using the techniques: UV-–visible Spectroscopy (UV-Vis), where showed an absorption peak at 428 nm, and A scanning electron microscope, where the results showed that the sizes ranged between 41.5 - 68 nm at an average of 44.2 ± 1.13 nm, XRD was confirmed the presence of cubic crystal system has been identified to be due to silver metalfor. As for the FTIR analysis, several absorption peaks appeared, but a strong band shown at 3484.27 cm^{-1} can be assigned to the primary amine N-H stretching that is present in the bacterial supernatant. Five NP concentrations were used, and each concentration of AgNPs displayed remarkable anti-leishmanial activity against *L. tropica* promastigotes in vitro. However, a concentration of (200 µg/ml) displayed a superior inhibitory impact on the growth of parasites compared to the rest of the other concentrations, where the rate of inhibition was the percentage 59.47 ± 2.7 after 24 hours. At 48 h and 72 h, the average percentage of parasite inhibition was 67.72±1.95 and 72.79 ± 0.45, respectively. The outcomes were remarkably similar to the reference medicine (pentostam). As for the lowest inhibition among the rest of the concentrations, it was the concentration (800 µg/ml), where the average percentage of inhibition reached 59.47 ± 2.7, 67.72 ± 1.95, and 72.79 ± 0.45 after 24, 48, and 72 hours, respectively.

Keywords: Anti-Leishmanial Activities, Biological Synthesis, *B. Subtilis*, Silver Nanoparticles

1 Introduction

THE word "nanotechnology" was first used in 1974 by the late Norio Taniguchi (University of Tokyo) to refer to the ability to precisely create materials at the scale of nanometers [1]. In the past ten years, researchers have discovered that bacteria, viruses, fungi, and parasites can all be treated with nanoparticles (NPs) [2]. When certain materials are shrunk down to the nanoscale, their properties alter. One of their most crucial properties is that while being quite small, their extensive surface area makes it easier for them to interact with other molecules [3]. For the prevention, detection, diagnosis, and treatment of infectious illnesses, these Nanoscale structures have been widely exploited [4]. These NPs' chemical, mechanical, and optical characteristics are clearly described. One of these NPs' characteristics is their cytotoxicity, which varies depending on the NPs' size, shape, charge, purity, and stability [5]. They have had remarkable outcomes when treating parasite infections [6]. Depending on the necessary size, form, or material composition, many synthesis techniques for NPs have been devised [7]. One of these techniques is biosynthesis by microorganisms, bacteria [8], and yeast [9].

Bacteria are one of the greatest biological sources because of their extraordinary ability to reduce ions of heavy metal and produce nanoparticles. Where a few types of bacteria have gained the capacity to use particular protection systems against pressures like the toxicity of metals or heavy metal ions [10], it was found that some of them, including *Pseudomonas stutzeri* and *P. aeruginosa*, could grow and thrive in environments with excessive levels of metal ions [11]. In addition, there is the possibility of manipulating their genetic information and the ease of dealing with them [12]. One of these bacteria that role as a reducing agent is *Bacillus subtilis*. When grown on standard Nutrient agar, this bacteria forms an irregular, opaque white or slightly yellow circular colony with rough edges [13, 14]. It has earned the moniker "a bacterium for all seasons" because of the extraordinary diversity of alternate states that one bacteria can display. This organism has developed to adjust to a variety of environmental challenges [15]. Bacillus species and strains produce more than 70 distinct antibiotics, as well as a huge number of other compounds having antibacterial properties. One of the most significant species in the Bacillus genus is *B. subtilis*, which is a common bacterium that can be found in soil, water, and the air [16]. It inhibits the growth of some harmful fungi and bacteria, and some of its metabolites exhibit antifungal and/or antibacterial activity (against a range of microorganisms that cause plant disease). This is probably due to ' competition with these microbes for nutrients, growth sites on the plant, and direct colonization and attachment of fungal pathogens [17, 18]. Globally, this encourages the isolation and selection of new *B. subtilis* strains that display an even larger spectrum of action against pathogens in lab tests. The biosynthesis of AgNPs using some Bacilli Species was reported in some works of literature [19-21]. In this research, *B. subtilis* was used for the synthesis of AgNPs. The produced AgNPs were examined using UV, FTIR, SEM, and XRD. Additionally, the anti-leishmanial effectiveness of these AgNPs against Cutaneous Leishmaniasis (*L. troipca*) in vitro.

2 Materials and Methods

2.1 Bacillus Subtilis Culture

In accordance with the manufacturing company's guidelines, the media was prepared, and its constituents were dissolved with distilled water (D.W). Then, a burner was used to melt completely .The media was put in an autoclave for 15 minutes at 121 °C to sterilize it. The media was left to cool in the water bath and distributed in sterilized Petri dishes inside a laminar hood. To guarantee sterility, at 37°C for 24 hours, the medium was incubated [22]. After Nutrient agar was inoculated with *B. subtilis* at 37°C for 24 hrs, it was incubated, which was identified based on morphological and biochemical characteristics according to [23, 24], as is shown in Figure 1.

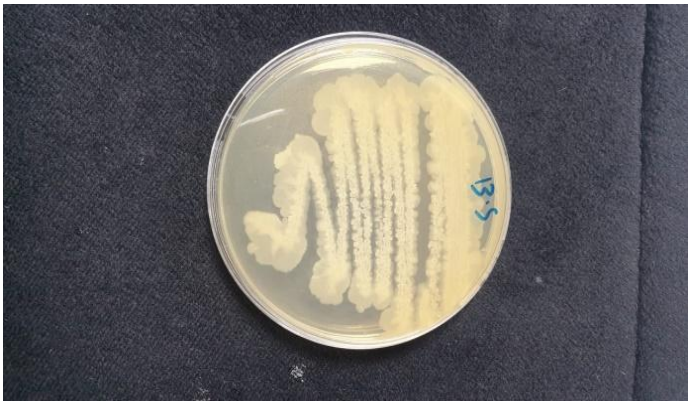


Fig. 1. Growth *Bacillus subtilis* on nutrient agar.

2.2 Preparation of AgNo3

Silver nitrate (AgNO3) and other chemicals were purchased from Sigma-Aldrich, and in all experiments, deionized water was used. To prepare 10 mM of silver nitrate, we take 1.6987 of silver nitrate and dissolve it in 1000 ml of deionized water, with continuous stirring for 30 minutes. Cover the glass flask with aluminum foil and store it under laboratory conditions until use.

2.3 Preparation of Cell Free Supernatant of Bacillus Subtilis

Colonies are taken from this isolate and grown in a liquid medium, either Brain Heart Broth or Nutrient Broth, then placed in a shaking incubator for 24 hrs. to ensure rapid multiplication of bacteria. For 10 minutes, the *B. subtilis* culture was centrifuged at 4500 rpm following a 24-hour incubation period in order to get the cell-free supernatant. *B. subtilis* cells that precipitated at the bottom of the tube were removed following centrifugation, and cell-free supernatants were collected and filtered by syringe filter to use in the biosynthesis of AgNPs, according to the method [25], with a few changes.

2.4 Biosynthesis of AgNPs using Cell-Free Supernatant

B. subtilis culture supernatant was used to create nanoparticles by employing a modified form of the process outlined in a previous publication [26]. AgNO3 was added with a concentration of 10 mM to the cell-free supernatant, which was put in a flask, and this step was prepared in a dark environment to prevent AgNO3 oxidation. The final product was incubated in a shaking incubator at 150 rpm for 24 hours at 37 °C. The reaction mixture was centrifuged at 10000 rpm for 10 minutes after the color change was noticed during incubation. The supernatant was discarded and replaced with deionized distilled water. The reaction mixture was then centrifuged three more times at the same speed and duration to remove any remaining supernatant. The pellet deposit at the bottom of the tube, which

represents a collection of nanoparticles, was then dried in an oven at 40 °C for 18 to 24 hours. Carefully gathered and placed in a sample vial for further analysis was the dry powder.

2.5 Examination and Characterization of Bio Synthesized AgNPs

The prepared Ag/*B. subtilis* were characterized by the biosynthesis of AgNPs was initially identified by a shift in the solution's color.

- **UV – visible spectroscopy:** As a function of wavelength, a UV-vis, a spectrophotometer from 200 to 800 nm with a resolution of 1 nm was employed to analyze the spectral properties of silver nanoparticles.
- **Scanning electron microscope:** Scanner electron microscopy (SEM) was used to measure the extracellularly produced silver nanoparticles' size and surface shape. The mixture of colloidal AgNPs was dropped onto a glass slide to create samples for SEM investigation, which were then left to dry. The dried specimen was placed on a holder for specimen and examined using a microscope [27].
- **FT-IR spectroscopy:** Using FTIR, the bioreduced (AgNPs) that the microbial extract had produced were identified to identify the potential molecular compounds of reduced Ag ions and covering the particles. By employing FTIR spectroscopy, functional groups and potential partnerships in the biogenesis of silver nanoparticles can be identified [28].
- **X-Ray diffraction (XRD) analysis:** The XRD examination supports the nanoparticle structure, and the development of a narrow peak with Bragg's angle suggests the crystalline nature of nanoparticles. Stabilization of the nanoparticles occurs by some covering agents, which are confirmed by the sharp peaks. The presence, crystalline nature, and size of nanoparticles were determined by use of the Debye-Scherr equation ($D = 0.94 \times \lambda/\beta \times \cos\theta$), whereas D is nanoparticle size, β is crest width at average height, θ is Bragg angle and λ is wavelength (nm) [26].

2.6 In Vitro Experiments

2.6.1 Leishmania parasite culture

The molecularly diagnosed parasite was obtained from the Research Center/University of Baghdad, then cultured using RPMI-1640 medium prepared from American Bio Innovations (USA) in the form of a package of 500 ml where the promastigote was grown in the laboratory as in Figure 2.

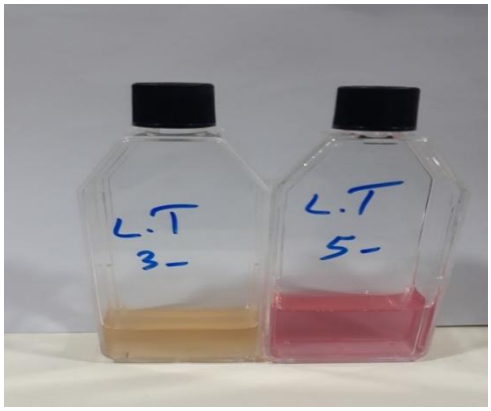


Fig. 2. *Leishmania* parasite in culture flasks before and after growth.

The growth of the parasite during culture goes through four phases, starting with the dormant phase, which lasts during the first and second days after transplantation, followed by the logarithmic phase, which extends between the third and fifth days. Then, the homeostasis phase begins from the sixth to the eighth day, during which reproduction stabilizes and during which the number of parasites reaches its maximum. Then, the decline and dissolution phase begin on the ninth day and continues until the end of the Parasite culture [29].

2.6.2 MTT test for cell cytotoxicity

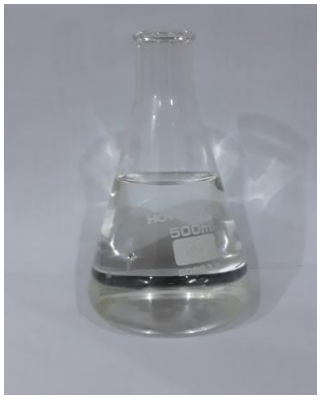
Parasites were resuspended by add (5 ml of RPMI 1640 medium supplemented with 10% FBS) and before being treated with the commercial medication Pentostam and 100 µl of NPs (100, 200, 400, 600, and 800 µg/mL), 10 ml of *L. tropica* promastigotes were prepared previously and grown in 96-well plates in 100 µL of optimized media. For 24, 48, or 72 hours, the treated parasites were allowed to continue growing, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) was added for each concentration at the conclusion of each incubation time at a temperature of 26 °C. Next, 96-well plates were kept in the dark for 3h before adding 50 µL of dimethyl sulfoxide (DMSO) and also shook the 96-well plates in the dark for 15 min to dissolve formazan crystals. After each treatment, optical density (OD) at 630 nm was measured using an ELISA device. Each experiment was repeated thrice.

3 Results and Discussion

3.1 Biosynthesized (AgNPs)

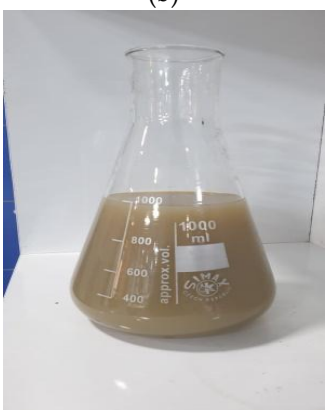
Bacillus subtilis showed their ability for extracellular biosynthesis of AgNPs by using cell-free supernatant after adding (AgNO3) in 10 mM concentration, where change can be seen in the color of the reaction mixture from yellow to brown after incubation of mixtures for 24hrs at 37°C in shaking incubator (150 rpm), This serves as a marker for production of the AgNPs by the mixture of *B. subtilis* Figure

3.



(a)

(b)



(c)

Fig. 3. (a) silver nitrate (AgNO3) (b) the original color (yellow) is *B. subtilis* supernatant without silver nitrate (c) color change (brown) of supernatant after adding (10 mM) of AgNO3 and incubate the reaction mixture at 37° C for 24 hrs. in a shaking incubator (150 rpm).

When the color of the aqueous precursor-bacterial enzymatic extracts specimens began to shift from yellow to light brown as a result of the surface-plasmon resonance phenomena, the beginning of the creation of AgNPs was noticed [30]. Considering that just the culture supernatant was employed, this was the first indication of the extracellular creation of AgNPs. In the extracellular approach, the microbial cell secretes reductases that are employed in the bio-reduction of metal ions into the corresponding NPs [31]. The biological method was applied due to its reproducibility and flexibility of implementation. The reduction of Ag + ions and subsequent production of AgNPs may be caused by peptides or proteins. It has been shown that the alpha-amylase enzyme can mediate the manufacture of silver nanoparticles, and the type of Bacillus employed in this work is well-recognized for producing this form of the enzyme [32]. One of the most favored biogenic methods is the bacterial manufacture of AgNPs since it is simple, eco-friendly, inexpensive, and biocompatible [33]. Depending on the type of microorganism utilized for the synthesis, the physical properties of AgNPs produced by biosynthesis differ [21]. Some works of literature reported that several Bacilli, including *B. licheniformis, B. subtilis, B. cereus, B. brevis, B. amyloliquefa ciens, B. megaterium, B. marisflavi, B. flexus, B. thuringiensis, Lysinibacillus sphaericus, B. pumilus, and B. methylotropicus*, were used in the biosynthesis of AgNPs [19-21, 34]. In an area that is still in its infancy, the use of biological systems for manufacturing, researchers have been keeping an eye on microorganisms as prospective eco-friendly nano-factories [35].

3.2 Examination and Characterization of Bio-AgNs

3.2.1 UV –visible spectroscopy

The UV–visible spectrum of the AgNP-containing solutions produced with *B. subtillis* supernatant is presented in Figure 4. At a wavelength of (428) nm, the surface plasmon resonance peak for silver reduced by *B. subtillis* was found.

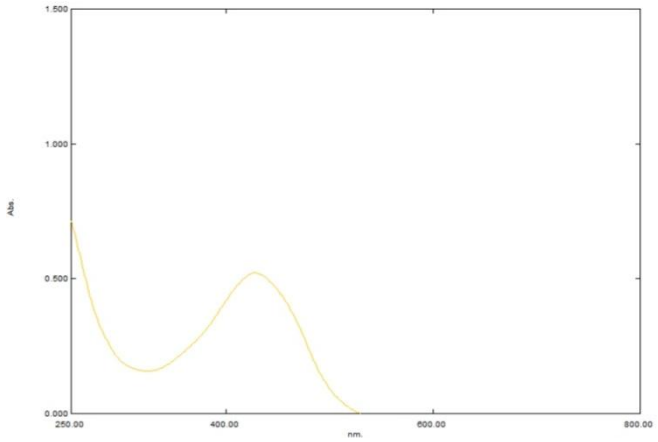


Fig. 4. UV-Vis. spectrum of the produced AgNPs by *B. subtilis*.

The first step in examining the formation of AgNPs in the aqueous solution is UV-Vis. spectroscopy. AgNPs have free electrons, and these coupled vibrations of the AgNPs' electrons in resonance with the incident light's oscillating electric field give birth to an SPR absorption band [36]. The absorbance peaks for AgNPs biosynthesized by several Bacilli (*Bacillus megaterium, Lysinibacillus sphaericus, Bacillus flexus, Bacillus thuringiensis, Bacillus brevis, and Bacillus strain*

CS11) were ranged from 390 to 450 nm [34]. According to earlier reports, these peaks roughly match the plasmon resolution of silver nanoparticles that have been used *B. subtillis*, such as [37], were observed peaks between 420–430 nm wavelengths [28] between 400 and 470 nm [27] at (428) nm. Wavelength [30] at 414 nm.

3.2.2 SEM analysis

Scanning electron microscopy was used to analyze the morphology of AgNPs prepared by bacteria regarding the crystallite size utilized. The SEM images of the biosynthesized Ag nanoparticles demonstrated that particles were spherical in form with a size diameter of 41.5 to 68 nm at an average of 44.2 ± 1.13 nm, which was less than 100 according to Figure 5.

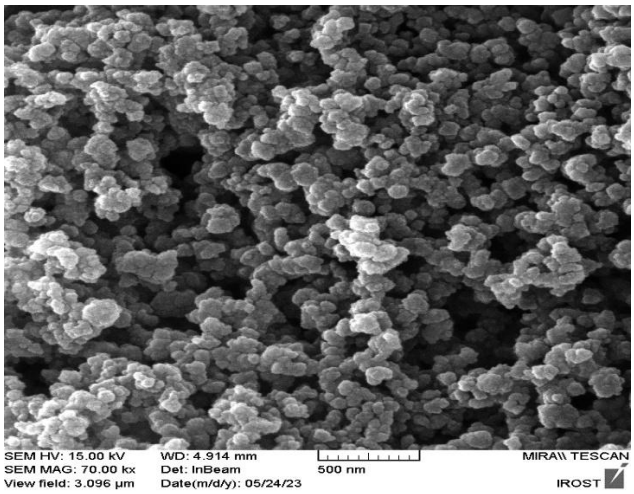


Fig. 5. SEM images of AgNPs synthesized by culture supernatant of *B. subtillis* and 1 mM AgNO3 solution.

The size distribution study proved the existence of AgNPs as the average size of nanoparticles achieved inside the range of distinguishing nanoparticles and corroborated the UV-Vis spectroscopy results. Studies also reveal that nanoparticles synthesized using similar microbial species can differ in size and shape, such as [27] under SEM, it was discovered that the particles were spherical, with an average diameter of around 80 ± 0.18 nm [30] NPs for *B. subtilis* 58.54 nm.

3.2.3 FTIR analysis

To identify any possible interactions between Ag and bioactive compounds that might be responsible for the synthesis and stability of AgNPs as a covering agent, FTIR measurement was used, Where FTIR analysis of prepared AgNPs gives various absorption peaks that are attributed to various biomolecule functional groups. Such biomolecules' main absorption bands typically showed up at wave numbers 1668.41, 1413.06, and 648.63 cm^{-1}, which are assigned for (C=C) of alkene (O-H) of alcohol and acetylenic C-H bend of alkynes, respectively. In addition, the vibrations of aliphatic C–H appeared at about 2932.65 cm^{-1}. The band at 3484.27 cm^{-1} corresponds to amine N-H stretching, and a peak at 924.03 cm^{-1} can be assigned to O-H bend of carboxylic acids Figure 6. According to a study, amides, alkanes, alkenes, amines, hydroxyl, carboxylate, and carbonyl groups found in biomolecules generated by microbes can be exploited for biosynthesis and capping the nanoparticle [38].

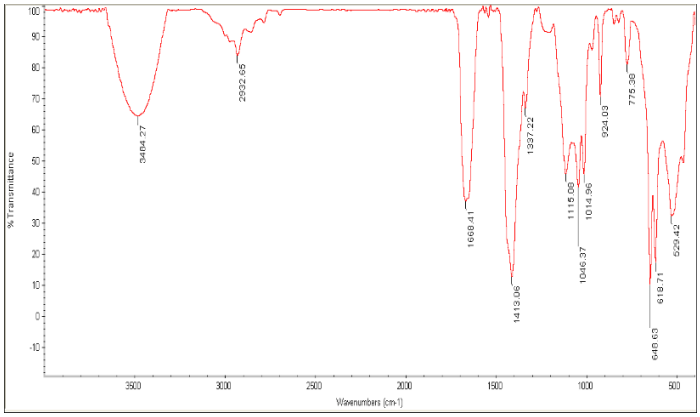


Fig. 6. FTIR spectrum of AgNPs.

El-Batal and others [39] propose that free amine groups, cysteine residues in proteins, and electrical attraction processes of negatively charged carboxyl groups in enzymes all play a role in interactions between proteins and nanoparticles. This evidence suggests that silver nanoparticle formation and stabilization in aqueous media can occur when molecules of protein are liberated from inside the bacterial cell [28].

3.2.4 XRD analysis

X-ray diffraction was used to obtain data regarding the structure of the crystalline material. The (XRD) pattern of the prepared sample of silver nanoparticles is depicted in Figure 7. Data was taken for the 2θ range of 10 to 80 degrees with a step of 0.0202 degrees. Five main peaks at 2θ values of 28.24°, 33.01, 46.12°, 55.26, and 58.06°in the experimental diffractogram, and for all of the silver-related diffraction peaks, the crystallite size was determined using the Debye-Scherrer equation. So, XRD confirmed the presence of face-centered cubic silver crystals.

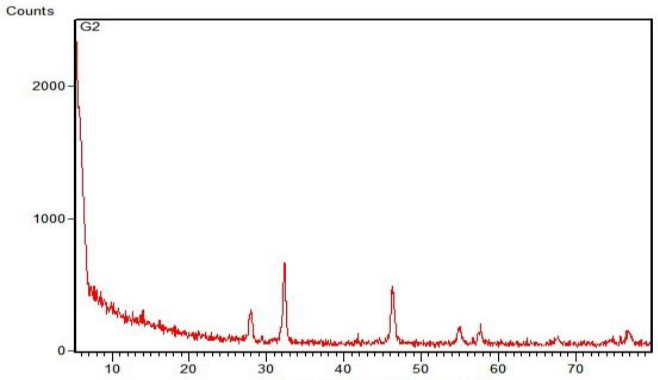


Fig. 7. X-ray diffraction patterns of biosynthesized AgNPs.

3.3 Anti-Leishmanial Activity of B. Subtilis AgNPs

Using optical density (OD) after MTT assay, the cytotoxicity impact of the nanoparticles against L. tropica promastigotes was examined, after 24, 48, and 72 hours of incubation, the mean percentage of growth inhibition of promastigotes by different concentrations of Ag/*B. subtilis* NPs was displayed in Figure 8. Following a 24-hour incubation period, the results showed a significant ($P \leq 0.05$) increase in the promastigote inhibition of Nano Silver at concentrations of 100, 200, and 400 µg/mL, which resulted in inhibition growth (53.63 ± 5.17, 59.47 ± 2.7, 57.23 ± 5.13%) in comparison to other used concentrations. However, there was no significant ($P \leq 0.05$) difference with the standard treatment pentostam (60.75 ± 4.83%).

At 48 hrs. of promastigotes exposure to tested materials, the maximum inhibition percentage was observed at 200 µg/ml with no significant ($P \leq 0.05$) differences with 100 µg/ml (64.33 ± 3.44), 400 µg/ml (66.06 ± 4.19) and the activity of pentecostal (68.54 ± 3.15). Simultaneously, the concentrations of 600 and 800 µg/ml of silver nanoparticles did not exhibit any significant ($P \leq 0.05$) differences. The minimum inhibition was recorded at 800 µg/ml (47.4 ± 0.86) with no significant difference with 600 µg/ml (55.92±4.04%). At 72 hrs. of promastigotes exposure to the treatments, the maximum inhibition was recorded at 200 µg/ml (72.79 ± 0.45%) with no alterations with 100, 200, 400 µg/ml and pentostam (70.23 ± 0.61, 71.43 ± 3.52, 64.01 ± 0.67 and 73.6 ± 2.33%) respectively. Also, the minimum inhibition was recorded at 800 µg/ml (57.53 ± 0.64) with no significant difference with 600 µg/ml (64.01 ± 0.67%). Additionally, the findings showed a proportionate link between the time of exposure and the promastigote's growth inhibition percentage across all Nano Silver concentrations and Pentecostal standard treatment.

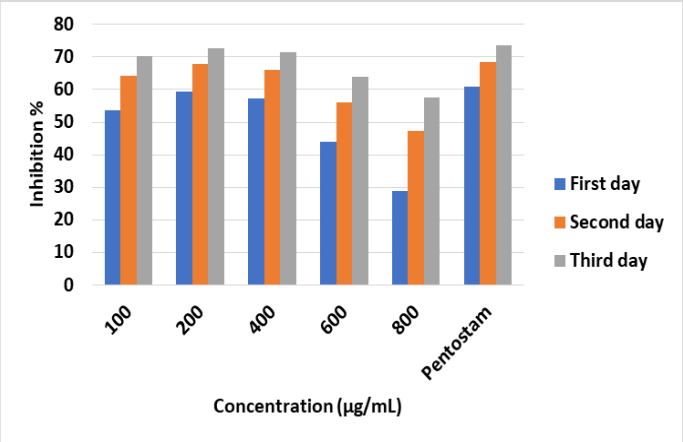


Fig. 8. Anti-leishmanial effects of Bio-AgNPs and Pentecostal on *L. Tropica* promastigotes.

The creation of nanoparticles with leishmanicidal characteristics and other significant applications in the health sector is gaining considerable attention as a result of nanobiotechnology. Silver nanoparticles, among the many nanomaterials, have a wide range of antimicrobial characteristics. In order to combat different growing microbial resistance, AgNPs have been used as an alternate therapy agent [40]. Due to its metabolic activity, the microbial source that produces AgNPs has a strong interest in the precipitation of nanoparticles [41]. There is no research on the investigation of silver nanoparticles' anti-leishmanial properties in the literature that biosynthesized by Bacteria, in particular *B. subtilis*, but We synthesized silver nanoparticles mediated by *B. subtilis* bacteria and evaluated its effectiveness in killing leishmania parasites Compared with standard treatment pentostam, This study indicates the anti-leishmania ability of nanoparticles after 24, 48, 72 hours of evaluation, indicating that the vital number of parasites is decreasing as time progresses and Nanoparticles inhibit parasite growth in a concentration-dependent manner. There are many studies on the biosynthesis of AgNPs from other microorganisms (fungi) and their anti-leishmanial effect such as [42-44]. Also, the examination of the antimicrobial properties of *B. subtilis* AgNPs against various pathogens has been the subject of numerous investigations in the literature [27, 30, 37]. All These works demonstrated that biologically manufactured AgNPs from *B. subtilis* have promising antibacterial properties against pathogenic and multidrug-resistant bacteria.

In our study, the application of AgNPs as an anti-leishmanial agent was recently demonstrated, and extremely concentrated, non-hazardous nano-sized silver particles may be made easily and affordably [45, 46]. Silver is an ancient antibiotic that has found many new uses due to its unique properties on the nanoscale [47]. O_2 and other molecules in biological systems can readily oxidize the surface of nanosilver, releasing Ag^+, a recognized toxin. Therefore, Ag^+ release is intimately linked to the toxicity of Nanosilver. Empirical evidence suggests that nanosilver can penetrate the cell and become internalized due to its small size. Thus, Nano silver often acts as a source of Ag^+ inside the cell. One of the primary methods of toxicity is the production of reactive oxygen species, which leads to oxidative stress and damage to cellular components such as DNA damage, the activation of antioxidant enzymes, the depletion of antioxidant molecules (e.g., glutathione), binding and disabling of proteins, and the cell membrane was damaged [48]. Silver is also an efficient trypanothione/ trypanothione reductase (TR) inhibitor [49, 50], a similar mechanism of action with antimony, the first-line drug against leishmaniasis in most endemic countries [49], where it was shown that, in order to have a significant effect on parasite redox state and viability, TR activity must be reduced by at least 90% [51, 52], It has been proven that antimonials, among the drugs currently in use to treat leishmaniasis, interfere with the trypanothione metabolism

and inhibit TR, reinforcing the idea that targeting this protein (enzyme) is a concrete option for the treatment of these diseases [53]. The anti-leishmanial and cytotoxic ability of Ag NPs in this study can also be due to their small size; they reach smaller than 50 nm, which is 500 times smaller than a parasite. This makes it easier for them to enter into the cells, leading to the destruction of membrane and intracellular organelles where Nano silver interacts with nucleic acids, lipid molecules, and proteins in the biological system. It is worth mentioning Nanosilver was able to induce an anti-proliferative effect on the parasites at metal concentrations lower than those used with antimony.

4 Conclusion

These results point to a green and environmentally benign strategy for producing biogenic AgNPs by *Bacillus subtilis*. In the supernatant of a Bacillus subtilis culture, silver nanoparticles were reduced. The supernatant of the bacterium B. subtilis acted as a stabilizing and reducing agent. The effectiveness of silver bio-nanoparticles against leishmaniasis shows a commitment to their usage as a potent therapy to treat Cutaneous leishmaniasis, especially for patients who cannot be given pentostam, who suffer from heart disease, have a weak immune system, or whom organ transplant recipients. Reports of widespread drug resistance and unsuccessful treatment have also been presented in recent years.

Conflict of Interest: The author declares no conflict of interest.

Financing: The study was performed without external funding.

Ethical consideration: The study was approved by University of Al-Qadisiyah, Iraq

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