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GREEN SYNTHESIS, CHARACTERIZATION, OPTIMIZATION AND ANTI- LESHMANIAL INVESTIGATION OF SILVER NANOPARTICLES FROM CITRUS SINENSIS L. FRUIT PEELS

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Abstract
Plants have continuously served as a rich supply of beneficial items that have many applications in industries such as food, agriculture, industry, and medicine. The investigation utilized aqueous extracts obtained from the peels of Citrus sinensis to synthesize silver nanoparticles (AgNPs). The study discusses both qualitative and quantitative methods for the biological production of AgNPs, with a focus on the need to optimize the amount, shape, and factors of synthesis. The optimal reaction conditions for synthesizing AgNPs were determined to be temperatures ranging from 70°C to 90°C and a pH of 5-7. The paper offers a comprehensive review of the elements that affect the qualitative characteristics of AgNPs, investigating advancements in the methodologies used for synthesis, optimization, and characterization. Scanning electron microscopy (SEM) investigation showed the presence of a small number of tubular and regular structures made of silver nanoparticles (AgNPs). Transmission electron microscopy (TEM) verified that the majority of the nanoparticles were spherical in shape and varied in size from 20 nm to 50 nm. The XRD diffraction pattern indicated that the nanoparticles were uniformly distributed and stable. Additionally, EDX

examination revealed the presence of living components including carbon, potassium, calcium, oxygen, nitrogen, and chloride, in addition to silver. The silver nanoparticles (AgNPs) exhibited efficient bio-reduction at a wavelength of 420 nm, as evidenced by the results obtained from UV-Vis spectroscopy. Finally, the anti-leishmanial assay demonstrated that the silver nanoparticles had significant action against Leishmania tropica, surpassing the effectiveness of conventional medicines such as amphotericin B and miltefosine.

Keywords: *Citrus sinensis*, AgNPs, Optimization, Characterization, SEM, TEM, XRD, EDX and Anti-leishmanial

1. INTERODUCTION

Nanotechnology has greatly enhanced the progress of creating new materials, namely nanoparticles that have distinct physicochemical characteristics (Ali Syed et al., 2024). This has had a profound impact on different scientific fields. Out of these options, silver nanoparticles (AgNPs) have been thoroughly researched because of their strong ability to kill microorganisms, large surface area, and impressive optical characteristics (Ali Syed et al., 2024). Nevertheless, the traditional techniques for producing AgNPs usually require the use of hazardous substances and energy-demanding procedures, which give rise to environmental and safety issues (Laraib et al., 2023). Consequently, there has been an increasing fascination with green synthesis methods, which employ natural resources as environmentally benign substitutes for nanoparticle creation. The process of green synthesis of nanoparticles involves the use of biological entities, such as plant extracts, bacteria, and fungus, as agents to reduce and cap the nanoparticles (Khan et al., 2023; Muhammad et al., 2024). This technique is sustainable in nature. This technology is not only ecologically harmless but also economical and uncomplicated. Fruit peels have attracted interest among other plant-based materials because they include a high amount of bioactive chemicals such as flavonoids, phenolic, and terpenoids. These compounds have the ability to decrease metal ions and stabilize the nanoparticles that are formed. Using fruit peels not only helps reduce waste but also adds to the advancement of sustainable nanotechnology (Shah et al., 2023).

Citrus sinensis L., sometimes referred to as the sweet orange, is a widely acknowledged fruit renowned for its nutritional and therapeutic attributes (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2008). The peels of Citrus sinensis, which are commonly thrown away as agricultural waste, contain a high concentration of phytochemicals that have powerful reducing properties (Etebu & Nwauzoma, 2014). This makes them an excellent choice for the environmentally friendly production of silver nanoparticles. Utilizing Citrus sinensis peels for nanoparticle production is a novel waste management strategy that also yields AgNPs with improved bioactivity (Akarca & Sevik, 2021; Viuda-Martos, Ruiz-Navajas, Fernandez-Lopez, & Perez-Álvarez, 2008). The biological uses of silver nanoparticles, especially in the context of fighting infectious illnesses, are of significant interest due to their production. Leishmaniasis, a devastating tropical illness caused by protozoan parasites of the genus Leishmania, continues to be a significant public health problem, particularly in undeveloped areas (Desjeux, 2004; Murray, Berman, Davies, & Saravia, 2005). Current therapies for leishmaniasis are frequently hindered by drawbacks such as toxicity, exorbitant costs, and the emergence of drug resistance. Therefore, there is a pressing need for alternate treatment approaches (Reithinger et al., 2007; Torres-Guerrero, Quintanilla-Cedillo, Ruiz-Esmenjaud, & Arenas, 2017). Due to their distinctive physicochemical features, silver nanoparticles have shown promise as efficient treatments against a range of diseases, including Leishmania species (Alvar et al., 2012; Elmahallawy et al., 2014). This study specifically examines the process of creating silver nanoparticles using Citrus sinensis L. fruit peels in an environmentally friendly manner. The resulting nanoparticles are then thoroughly analysed to identify their physical and chemical characteristics. An investigation will be conducted to improve the effectiveness and durability of the nanoparticles by optimizing synthesis parameters such as pH, temperature, and reactant concentration. Furthermore, we will investigate the effectiveness of the silver nanoparticles created in combating leishmaniasis, thereby examining their potential as a new and innovative treatment for the disease. This discovery not only contributes to the progress of green nanotechnology but also tackles a crucial requirement in combating neglected tropical illnesses.

2. MATERIALS AND METHOD

2.1. Collection and Preparation of Citrus sinensis *Aqueous Peel Extract*

Citrus sinensis fruit peels were collected from trees in Hayatabad, Peshawar. Farrukh Hussain, a professor and former head of the Institute of Biological Sciences (IBS) at Sarhad University of Science and Information Technology in Peshawar, verified the fruit's identity. The citrus sinensis aqueous peel extract began with the peels being carefully washed and dried in the shade. After they dried, a grinder was used to crush them into a fine powder. Next, 500 millilitres of distilled water were combined with 25 grammes of powdered peel. Once the mixture had simmered for half an hour, it was strained through Whatman filter paper. Once the filtrate had cooled to room temperature, it was used for additional experiments.

2.2. Green Synthesis of Silver Nanoparticles (AgNPs)

The process of synthesizing silver nanoparticles (AgNPs) using environmentally friendly methods was carried out according to the procedure developed by Ahmad et al. (2016). To summarize, 10 mL of the aqueous peel extract was combined with 90 mL of a 1 mM silver nitrate solution. Subsequently, the concoction was transferred into a vigorously agitated water bath set at a temperature of 75°C for duration of 1 hour. The presence of a dark brown color suggests effective AgNP production. The nanoparticles were further purified using centrifugation at a speed of 12,000 revolutions per minute for duration of 10 minutes.

2.3. Optimization of Silver Nanoparticles (AgNPs)

The ideal parameters for AgNP production of AgNPs were identified through a sequence of experiments. In order to determine the optimal reaction temperature, the experiment was conducted at several temperatures ranging from 10°C to 100°C, with intervals of 10°C. In addition, the pH of the reaction mixture was modified within a range of 2 to 14 in order to determine the optimal pH conditions for the creation of nanoparticles. The substrate quantity was optimized by changing the volume from 5 mL to 50 mL in 5 mL increments, allowing the best amount for the reaction to be identified.

2.4. Characterization of Silver Nanoparticles (AgNPs)

According to their intended uses, several characterization procedures must be used to confirm the effective synthesis of silver nanoparticles (AgNPs). An uncomplicated approach to monitoring the synthesis of AgNPs is to watch the solution's color change from a yellowish hue to a brownish hue. In addition, the formation of nanoparticles can be verified by identifying distinct peaks in the visible region of the UV-Visible (UV-VIS) spectrum using a spectrophotometer. Additional characterization can be conducted by methods such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), and energy dispersive X-ray analysis (EDX) (Patil & Chougale, 2021).

2.5. Anti-leishmanial Activity

The evaluation of the effectiveness against leishmaniosis was conducted using the methodology described (Velez, Hendrickx, Robledo, & Agudelo, 2001). Using a colorimetric cell viability test, the antipromastigote effects of the Citrus sinensis extract were assessed. Harvested promastigotes in the logarithmic growth phase were transferred to 96-well tissue culture plates, with 100 µL of cells in each well. The cells in each well were cultured at a temperature of 25 °C for a duration of 48 hours with different doses of the alcoholic extract, ranging from 1 to 100 µg/mL. Following the incubation time, a solution of MTT (5 mg/mL) was introduced into each well, and the plates were then incubated at a temperature of 25 °C for a duration of four hours. As controls, promastigotes were cultivated in a complete medium without any medications as the positive control, while a blank culture was maintained in a complete medium without promastigotes or pharmaceuticals. The optical density of each well was quantified at a wavelength of 560 nm using an ELISA reader (BioTek-ELX800, USA) in order to assess the vitality of the cells.

3. REUSLTS

3.1. Optimization of Silver Nanoparticles (AgNPs)

The study concentrated on the eco-friendly production of silver nanoparticles (AgNPs) by the use of a water-based extract derived from the peels of Citrus sinensis (orange). These peels include a high concentration of natural compounds such as alkaloids, terpenoids, phenolic, proteins, enzymes, and nitrate reductases. These organic compounds facilitate the conversion of ionic silver into nanoparticles, offering an environmentally benign approach to synthesis. The research meticulously optimized crucial variables, including as temperature, pH, and substrate concentration, in order to maximize the formation of AgNPs. Temperature optimization experiments demonstrated that when the temperature was set at 30°C, there was no observable creation of nanoparticles that could be considered significant. Nevertheless, with the rise in temperature from 50°C to 90°C, the reaction mixture underwent a transformation from a yellowish colour to a dark brown hue, signifying the effective synthesis of AgNPs as a result of the presence of antioxidants in the peel extract. pH optimization experiments revealed that the formation of AgNPs did not occur within the acidic pH range of 2-5. During this period, the solution maintained a white and cloudy appearance. Conversely, when the pH values were between 6 and 10, the solution changed to a dark brown colour, with the fastest production and clumping of nanoparticles seen at a pH of 10. The substrate concentration was optimized by use a water bath, which allowed for meticulous regulation of the reaction conditions. The meticulous optimisation procedure enabled the efficient and regulated production of AgNPs with precise physicochemical characteristics, showcasing the promise of Citrus sinensis peels in environmentally friendly nanotechnology applications.

Table 1: Optimization of Silver Nanoparticle Synthesis: Temperature, pH, and Substrate Quantity

Temperature (°C)	Color Change	pH Value	Appearance	Substrate Quantity (ml)	Time (min)	Color Change After Heating (75°C)
10	No color change	2	White appearance	5	30	Slight color change
20	No color change	3	White appearance	10	60	Dense dark brown
30	Slightly changed to brown	4	White appearance	15	40	Dark brown
40	Slightly changed to brown	5	White appearance	20	50	Dark brown
50	Color changed to dark brown	6	Blackish brown	25	60	Dense dark brown
60	Color changed to dark brown	7	Dense blackish brown	30	60	No change
70	Color changed to dark brown	8	Dense blackish brown	35	60	No change
80	Color changed to dark brown	9	Dense blackish brown	40	60	No change
90	Color changed to dark brown	10	Dense blackish	45	60	No change

	brown			
		50	60	No change

3.2. Characterization of Synthesized Silver Nanoparticles (AgNPs)

SEM (Scanning Electron Microscopy)

The scanning electron microscope (SEM) pictures provide significant insights into the surface morphology and the process of silver nanoparticle (AgNP) creation utilising Citrus sinensis peel extract. When observed at a magnification of 1000x, the presence of tiny, dispersed particles on a smooth surface suggests that nanoparticle creation has been effective, although with some degree of aggregation. When seen at a magnification of 1200x, a surface that is rougher and contains nanoparticles that are denser and more aggregated may be detected. The variations observed indicate that the distribution and shape of nanoparticles are greatly influenced by synthesis conditions such as temperature and pH. This emphasises the need of fine-tuning these factors in order to achieve desired features of AgNPs.

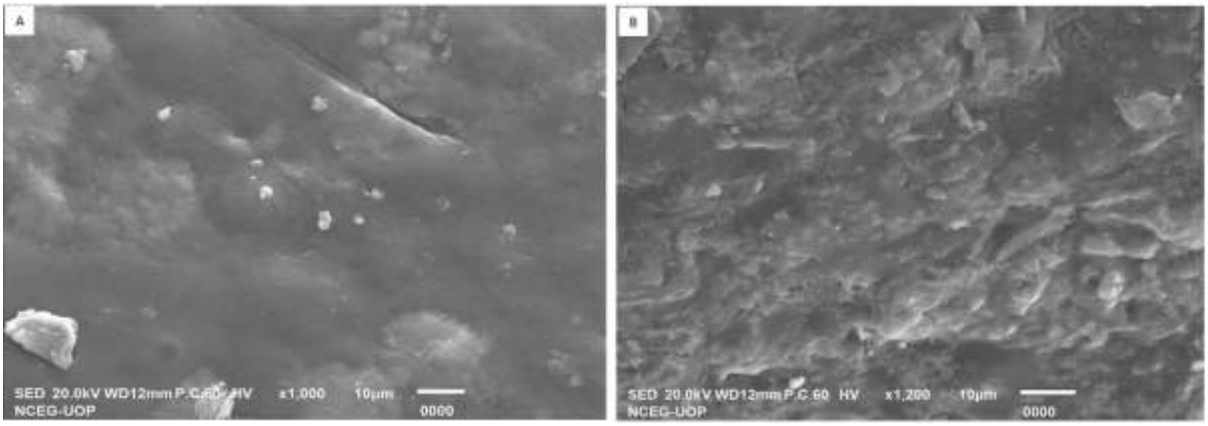

Figure 1: SEM images of synthesized silver nanoparticles. (A) 1000x magnification shows a smooth surface with scattered nanoparticles. (B) 1200x magnification reveals a rougher surface with more pronounced nanoparticle aggregation. Scale bar = 10 µm.

TEM (Transmission Electron Microscopy

The transmission electron microscopy (TEM) pictures depict the development of nanoparticle production, commencing with diminutive and evenly distributed particles measuring between 5 and 20 nanometres, as shown in the initial image. The nanoparticles exhibit growth, as seen by the ensuing photos, with their size expanding, and some even reaching dimensions of up to 200 nm. This increase in size is accompanied by a wider range of sizes and significant aggregation, when particles start to form clusters. The shift from dispersed to aggregated states indicates that precise regulation of synthesis conditions is crucial for preserving the optimal nanoparticle size and avoiding excessive agglomeration, which can adversely affect their functional characteristics in diverse applications.

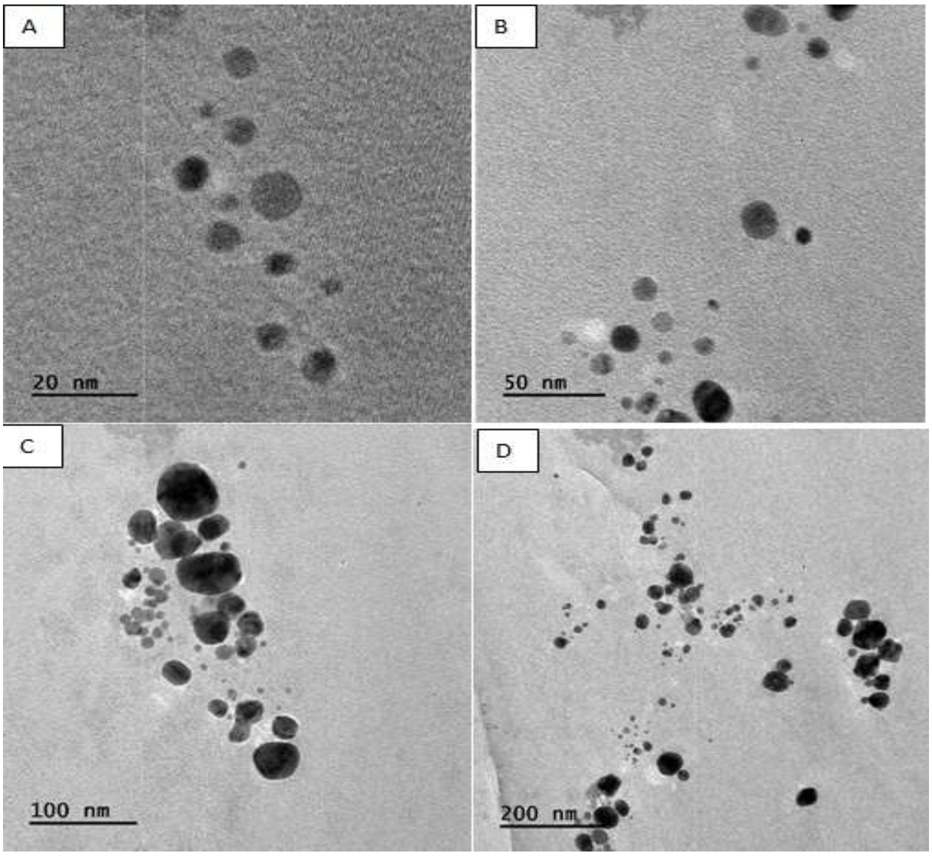

Figure 2: TEM images of silver nanoparticles at different magnifications. (A) 20 nm scale, showing small, well-dispersed nanoparticles. (B) 50 nm scale, with slightly larger particles and initial signs of aggregation. (C) 100 nm scale, displaying a mix of particle sizes with noticeable clustering. (D) 200 nm scale, revealing larger, aggregated nanoparticle clusters.

XRD (X-ray Diffraction Measurement)

The XRD patterns displayed offer valuable information on the crystalline structure of two distinct samples. The first pattern exhibits wide, faint peaks accompanied by considerable noise, indicating a limited level of crystallinity and the potential existence of amorphous substances or extremely tiny crystalline structures. On the other hand, the second pattern has more distinct and intense peaks, especially in the lower 2θ range, suggesting a greater level of crystallinity with well-defined crystallites. The disparities between the two patterns indicate that the second sample exhibits a higher degree of structural organisation, which is likely attributed to changes in synthesis conditions, such as temperature or pH, that influence the crystallisation process.

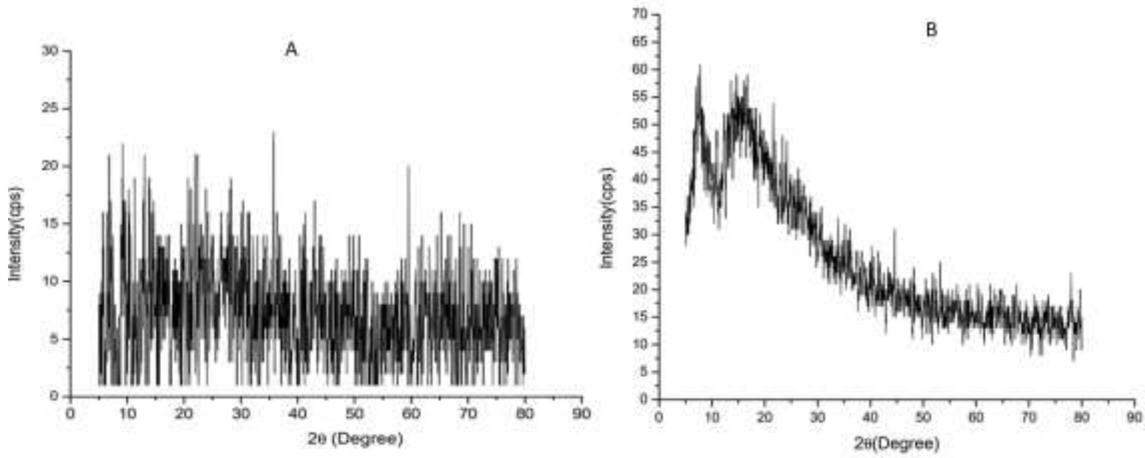

Figure 3: XRD patterns of silver nanoparticles. (A) XRD pattern showing broad, low-intensity peaks, indicating a lower degree of crystallinity and smaller crystallite sizes. (B) XRD pattern with sharper, higher-intensity peaks, suggesting a higher degree of crystallinity and larger, well-formed crystallites.

EDX (Energy Dispersive X-ray Analysis)

The EDX spectra provide information on the elemental makeup of two separate samples. The first spectrum has significant peaks corresponding to silver (Ag), as well as carbon (C), oxygen (O), and small quantities of potassium (K) and calcium (Ca). This indicates that the sample is most likely composed of silver, maybe in the form of silver nanoparticles that are stabilised by organic molecules. Conversely, the second spectrum is mostly composed of copper (Cu) and silicon (Si), as well as carbon and oxygen. This suggests that the material is predominantly made of copper and may be a component of a composite structure. The distinct constituents seen in each spectrum emphasise the diverse compositions and possible uses of the analysed materials.

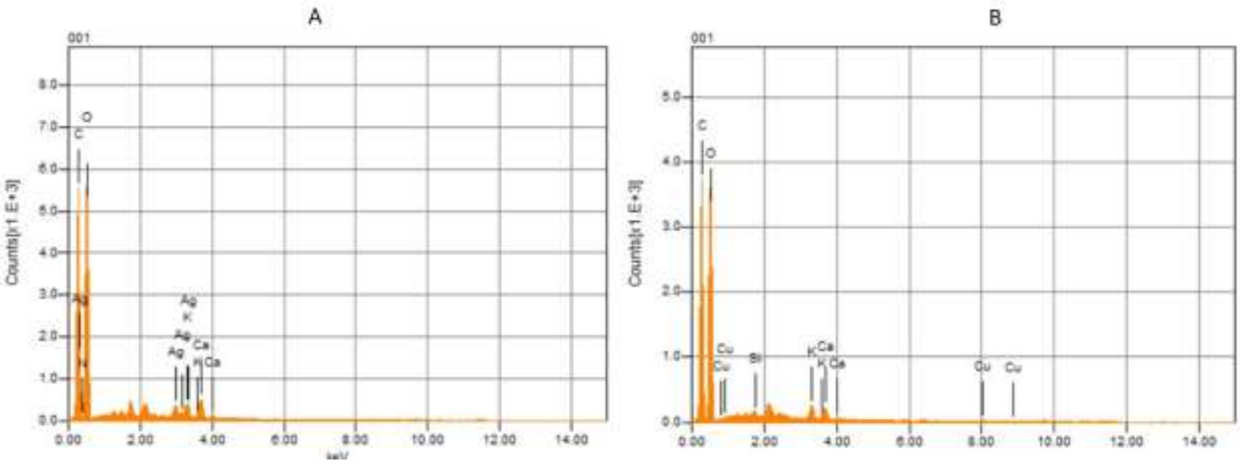

Figure 4: EDX spectra of silver nanoparticles. (A) Spectrum indicating the presence of silver (Ag) along with other elements such as carbon (C), oxygen (O), potassium (K), and calcium (Ca), suggesting a silver-based nanoparticle with some biological or organic components. (B) Spectrum showing copper (Cu) and silicon (Si) as major elements, along with carbon and oxygen, indicating a different material composition, possibly involving copper-based nanoparticles.

Ultraviolet-visible Spectroscopy

The absorption spectrum has a peak absorbance at around 420 nm, with a maximum absorbance of roughly 1.91. The observed peak is a distinct feature of the surface plasmon resonance (SPR) phenomenon in metallic nanoparticles, namely silver nanoparticles. These nanoparticles often display intense absorption in the visible spectrum, commonly occurring about 400-450 nm. The absorbance decreases on both sides of this peak, suggesting that the material has the most light absorption at this particular wavelength. This wavelength corresponds to the synchronised movement of electrons at the surface of the nanoparticle when exposed to light.

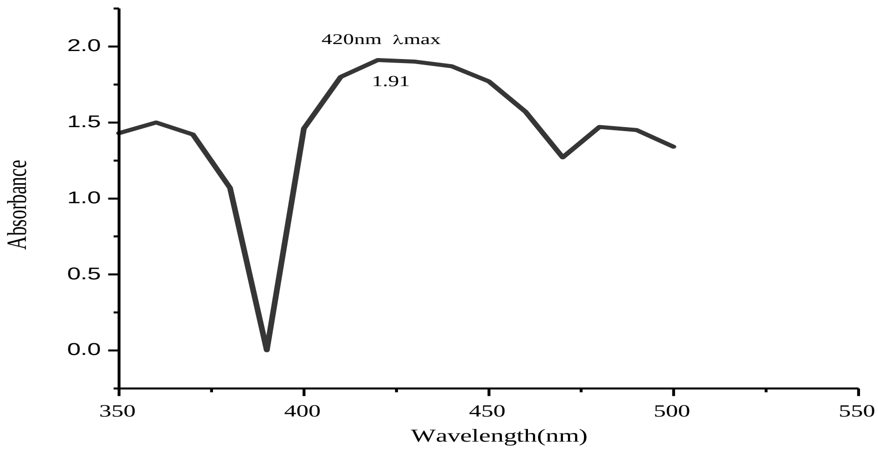

Figure 5: UV-Vis absorption spectrum of silver nanoparticles, showing a peak at 420 nm with a maximum absorbance of 1.91. This peak is characteristic of the surface plasmon resonance (SPR) of silver nanoparticles, indicating successful nanoparticle formation.

3.3. Anti-Leishmanial Bioassay

In the anti-Leishmania test conducted by (Ullah et al., 2018), a 96-well plate was used to perform serial dilutions of silver nanoparticles (AgNPs) and crude aqueous Citrus sinensis peel extract. At a concentration of 50 µg/mL, silver nanoparticles efficiently prevented the transformation of promastigotes into amastigotes in a comparative investigation on anti-leishmanial activity. The silver nanoparticles (AgNPs) exhibited an IC50 value of 36.2 ± 0.70 µg/mL. At concentrations lower than this, the material did not show any activity against Leishmania tropica. The activity of this substance is similar to that of amphotericin B and miltefosine, with IC50 values of about 41 ± 0.04 µg/mL and 42.6 ± 0.42 µg/mL, respectively. Conversely, the extract from the peel of Citrus sinensis was shown to be unsuccessful in inhibiting the growth of Leishmania tropica, even at a concentration of 100 µg/mL. This suggests that it is not suited for use as an agent to combat leishmaniasis.

Table 2: Antileishmanial Activity of Silver Nanoparticles Compared with Standard Drugs

Leishmanicidal activity	IC50(µg/ml) ± S.D.
Test compound	36.2 ± 0.70
Standard drugs	
Amphotericin B	3.41 ± 0.04µg
Miltefosin	42.6 ± 0.42µg

4. DISCUSSION

Currently, a range of chemical and biological techniques are being used to improve the production of silver nanoparticles, with green technologies becoming increasingly prominent due to their environmentally benign approach. The current investigation involved the synthesis of silver nanoparticles (AgNPs) utilizing peel extract derived from Citrus sinensis. Phytochemicals present in plants play a crucial role in reducing and bio-capping processes in plant-assisted approaches. The anti-leishmanial capabilities of these silver nanoparticles (AgNPs) were evaluated against Leishmania tropica, demonstrating that the nanoparticles exhibited superior efficacy compared to the aqueous extract alone. The results of this work are consistent with prior studies, indicating that the synthesized AgNPs have the potential to be turned into powerful antibiotics that can effectively prevent the development of microorganisms, which is a crucial factor in causing human diseases.

The silver nanoparticles (AgNPs) were produced by bio-reduction procedures with an aqueous extract derived from the leaves of Hibiscus Rosa-Sinensis (HRS). The reduction of Ag^+ ions to Ag^0 took place over a span of 30 minutes as part of the optimization process. The characterization of the AgNPs using a particle size analyzer, AFM, and SEM revealed that they were polydispersity, exhibiting a uniform size and shape. The average diameter of the particles was found to be between 25 and 30 nm, with a surface roughness of 52.8 nm. The XRD study verified that the AgNPs, which have an average size of 43 nm, have a crystalline structure. By contrast, our research yielded nanoparticles with sizes ranging from 20 to 50 nm. These nanoparticles had a crystalline structure and demonstrated effective reduction of silver ions.

The AgNPs in this work were characterized using a range of spectroscopic methods including SEM, TEM, UV-visible spectroscopy, XRD, and EDX. The findings indicated that the synthesized nanoparticles displayed a maximum value at 420 nm with an absorbance of 1.91. The silver nanoparticles were determined to be oval-shaped with diameters ranging from 35 to 45 nm, as validated by SEM and TEM pictures. These findings align with previous investigations, such as the work (Hashemi, Shirzadi-Ahodashti, Mortazavi-Derazkola, & Ebrahimzadeh, 2022). In addition, (Chan & Don, 2013) stated that the AgNPs showed efficacy against cancer cell lines and were also used as catalysts.

In a subsequent study conducted by (Chan & Don, 2013), they synthesized silver nanoparticles (AgNPs) using Pseudomonas sanguineus. The researchers optimized many parameters such as the concentration of $AgNO_3$, incubation temperature, and agitation speed. They employed the OFAT (one-factor-at-a-time) approach followed by the Box-Behnken technique under response surface methodology (RSM). The researchers discovered that the concentration of $AgNO_3$ played a vital role in the experiment. They observed that particles as tiny as 7.58 nm were formed when the temperature was set at 40°C and the rotation speed at 230 rpm. These particles were found to be 8.6 times smaller than the unoptimised AgNPs. The antimicrobial tests revealed that, when the circumstances were ideal, these AgNPs exhibited considerably higher biocidal efficacy, with the experimental results closely matching the predictions of the model.

Physicochemical variables are essential for controlling catalysis, protein dynamics, molecular mechanics, and reaction kinetics in the production of sustainable Nano products. This study also assessed the impact of temperature, pH, and concentration of $AgNO_3$, plant extract, incubation duration, and absorption of Ag salt on the synthesis and possible biological characteristics of the nanoparticles. The morphological analysis revealed that the nanoparticles produced using a water-based extract derived from the leaves of Mentha longifolia varied in size, ranging from 10 to 100 nm (Javed & Nadhman, 2020). The oxidative silver nanoparticles exhibited significant efficacy against Leishmania, as a concentration of 10 µg/mL resulted in the destruction of 66% of the cells. In contrast to our results, the silver nanoparticles synthesized in this investigation exhibited enhanced anti-leishmanial efficacy, necessitating far lower dosages compared to traditional therapies. This study emphasizes the capacity of silver nanoparticles generated from plants for use in biological applications.

5. CONCLUSION

This work demonstrates the successful use of extracts from the peels of Citrus sinensis to facilitate the production of silver nanoparticles (AgNPs), by harnessing the natural phytochemicals such as phenols, flavonoids, polymethoxylated flavones, and alkaloids found in the peels. After being purified and characterised, the AgNPs that were produced by biosynthesis showed a peak absorbance at 420 nm. Additionally, they were discovered to have a spherical shape and were sensitive to changes in temperature. The reaction circumstances had an impact on the characteristics of the particles, with the most favourable synthesis taking place at temperatures ranging from 70 to

90°C and a pH range of 5 to 7. The AgNPs shown more efficacy than conventional medications like amphotericin B and miltefosine in anti-leishmanial tests. This study highlights the benefits of using photosynthesised AgNPs as a straightforward, eco-friendly, and efficient substitute for traditional chemical and physical synthesis techniques. To further investigate the biological effects of AgNPs, it is advisable to utilise animal models. Additionally, it is recommended to analyse their electrical properties to advance technology, evaluate their potential against cancer cell lines, study their anti-inflammatory and anti-diabetic effects, and explore potential commercial applications.

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