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GREEN SYNTHESIS OF SILVER NANOPARTICLES USING LEAF EXTRACT OF *AZADIRECHTA INDICA*: CHARACTERISATION AND ACTIVITY AGAINST *TRYPANOSOMA BRUCEI BRUCIE*

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ABSTRACT

This study focused on the synthesis of silver nanoparticles (Ag-NPs) using *Azadirachta indica* leaf extract and explored its anti-trypanosomal potential against *Trypanosoma brucie brucie*. The synthesized Ag-NPs were characterized using ultraviolet-visible spectrophotometry (UV-Vis), Fourier–transform infrared (FTIR) spectroscopy, transmission electron microscopy (TEM), and energy-dispersive spectroscopy (EDS). *in vitro* anti-trypanosomal activity of the synthesized Ag-NPs was carried out using Roswell Park Memorial Institute (RMPI) culture medium containing trypanosomes at various concentrations. Their efficacy in inhibiting parasite growth and viability was assessed microscopically. The phytochemical analysis of *A. indica* leaf extracts reveals the presence of alkaloids, flavonoids, phenols, saponins, steroids, and tannins. The UV-Vis absorption spectra show maximum absorption at 430 nm for Ag-NPs. The FTIR revealed the presence of functional groups (OH, C=O, N=H, CO) responsible for reducing and stabilizing the Ag^{+} ions. The TEM result showed that synthesized NPs are spherical with a size range of 5-35 nm, with an average diameter of 13.75 nm. EDX spectra showed prominent peaks for Ag at around 3000 and 8000 keV. The *in vitro* anti-trypanosomal activity revealed that the synthesized NPs exhibited significant anti-trypanosomal activity against *T. b. brucie* with IC50 of 10.096 µg/ml p value ≤ 0.005 The study concludes that *A. indica* leaf extract can be used in a green and cost-effective method for the synthesis of Ag-NPs having promising potential in biomedical nanotechnology and the treatment of African trypanosomiasis.

Keywords: Green synthesis, Silver nanoparticles, *Azardirechta indica*, Anti-trypanosomal activity

INTRODUCTION

Nanotechnology has gained global attention as an active field that involves manipulating matter on a scale smaller than a micrometer. Among the various types of nanoparticles, those made of metal are particularly fascinating and hold great promise as innovative antimicrobial agents (Della Pepa *et al.*, 2017). Silver nanoparticles (Ag-NPs) are small metallic compounds that have gained significant interest due to their distinct characteristics and wide range of uses in domains such as catalysis, bio-sensing, target drug delivery, and antibacterial activity (Irshad *et al.*, 2023). Silver nanoparticles are highly intriguing because of their distinctive physicochemical characteristics, including morphological structure, size distribution, surface charge, catalytic function, conductivity, thermal stability, and antibacterial action (Crisan *et al.*, 2021) In addition, their significant biomedical applications such as anticancer (Hublikar *et al.*, 2023), antibacterial (Wahab *et al.*, 2023), mosquitocidal activity (Panneerselvam *et al.*, 2011), antifungal (Xu *et al.*, 2020), anti-inflammatory (Abdelhafez *et al.*, 2020), anti-viral (Galdiero *et al.*, 2011) have put them in the limelight in medicine. Nevertheless, the traditional approaches for producing these nanoparticles frequently entail the use of harmful substances, substantial energy utilization, and potential environmental risks (Dhir *et al.*, 2023). Consequently, there is an increasing interest in developing environmentally friendly and sustainable means of producing these nanoparticles by utilizing natural sources including plant extracts, microbes, and biomolecules.

Azadirachta indica, sometimes known as neem, is a medicinal plant found in tropical and subtropical climates that has the potential to be used as a natural source for nanoparticle fabrication. For centuries, traditional medicine has utilized it due to its anti-inflammatory, antimalarial, antipyretic, antifungal antidiabetic, and attributes (Hawadak *et al.*, 2022). *A. indica* contains a range of phytochemicals such as terpenoids, flavonoids, alkaloids, and polyphenols. These compounds can be used as reducing and stabilizing agents during the production of nanoparticles (Aglin *et al.*, 2019).

African trypanosomiasis is a parasitic infection caused by single-celled protozoa of the genus *trypanosoma*. The primary mode of transmission is by the bite of infected tsetse flies. Human African trypanosomiasis is caused by two subspecies of *Trypanosoma brucei: T. b. gambiense* and *T. b. rhodesiense*. On the other hand, animal African trypanosomiasis is caused by *T. b. brucei*, *T. congolense, T. vivax, T. evansi,* and *T. equiperdum (*Cayla *et al.*, 2019). Both human and animal trypanosomiases have a detrimental impact on the overall economy of Africa by compromising the health of both humans and animals (WHO, 2019).

The high cost associated with starting and maintaining tsetse control programs, along with the lack of available vaccine due to the parasite's antigenic variability, have resulted in the extensive tsetse-infested regions of sub-Saharan Africa relying heavily on the use of anti-trypanosomal drugs (Tauheed, 2017). The few available drugs for the prevention and treatment of African trypanosomiasis currently suffer from several drawbacks including resistance, toxicity, restriction to parenteral administration, lack of efficacy in some cases, and unaffordable prices (Delespaux *et al.*, 2018). Based on these setbacks, the search for alternative compounds for the treatment of trypanosomiasis remains an urgent and important task (Lun *et al.*, 2018). A possible source of such compounds lies in using natural products that demonstrate potent trypanocidal effects. The historical interest in herbal products, particularly in their raw form derived from plants with recognized medical qualities, dates back to ancient

civilizations (Aremu *et al.,* 2017). Many effective drugs used to treat protozoal infections in humans have natural origins. For example, quinine, an alkaloid obtained from Cinchona sp. (Rubiaceae), and artemisinin, a sesquiterpene lactone derived from Artemisia annua (Asteraceae), are used to treat malaria. Emetine, an alkaloid found in Cephaelis ipecacuanha (Rubiaceae), is used to treat amoebiasis (Akuru *et al.,* 2018). The compound emetine, derived from the plant Cephaelis ipecacuanha (Rubiaceae), is utilized for Herbal therapies show potential in treating trypanosomiasis, with certain herbs like *A. indica* proving to be effective trypanocides (Zongo *et al.,* 2019).

Azadirachta indica, a member of the *Meliaceae* family, is a small to moderate-sized tree that typically retains its leaf throughout the year. It can reach heights of up to 15 meters, with exceptional species reaching 30 meters. The tree's crown is circular and expansive, measuring between 10 and 20 meters in diameter. The therapeutic properties encompass analgesic, antioxidant, anti-inflammatory, antibacterial, and antiviral characteristics (Baby *et al.,* 2022). Many scientists have documented the synthesis of silver nanoparticles (Ag-NPs) using *A. indica* and explored their antimicrobial properties. However, there is a lack of clarity in the literature on their potential against trypanosomes. In this study, we utilized an aqueous extract of *A. indica* to synthesize Ag-NPs using $AgNO_3$ as a precursor. We then employed multiple techniques to analyze and evaluate its properties, specifically focusing on its anti-trypanosomal activity through *in vitro* testing.

MATERIALS AND METHODS

Plant identification and extraction

Fresh leaves of *A. indica* were collected from Sabon Gari T/wada, Kaduna state. The specimens were deposited at the herbarium of the Department of Biological Science Kaduna State University (KASU) Kaduna and identified by a taxonomist and voucher number KASU/BSH/1392 was assigned. The leaves were thoroughly washed with tap water and subsequently, with distilled water and air dried at room temperature for 2 weeks. The dried sample was pulverized into powder using a laboratory pestle and mortar. The powdered sample was weighed and stored in an airtight container until needed for analysis. (Shaba *et al.,* 2012; Umar *et al.,* 2013). Exactly 100 g of the powdered leaves were weighed and transferred to a 250 ml volumetric flask and extracted with 150 ml of distilled water at 60 ^{0}C for 15 min and allowed to further extract for 1 hour. The extract was then filtered using Whitman No.1 filter paper.

Phytochemical Analysis

Chemical tests of the *A. indica* aqueous leaf extract were carried out using standard methods as described by Trease and Evans (1989); Sofowora (1993) and Sayeed (2007).

Biosynthesis of Ag-NPs

The biosynthesis of Ag-NPs was carried out according to the method described by Adamu *et al*. (2021), with slight modification. Exactly 100 ml of 0.25 mM of each of $AgNO_3$ was measured using a measuring cylinder and mixed with 100 ml of the *A. indica* extract in a 500 ml beaker, it was then placed on a magnetic stirrer equipped with a magnetic rod and heated at 60 ^{0}C for 90 minutes with continuous stirring. The colour of the solution changed from yellow to dark brown indicating the formation of the Ag-NPs. The nanoparticles were recovered from the solution using a centrifuge, which was centrifuged at 10,000 rpm for 15 minutes to separate the nanoparticles from the supernatant. The recovered nanoparticles were washed three times with distilled water by and finally with ethanol to remove any impurities. It was then dried at 80 ^{0}C for 2 hours and stored in an air-tight container for further use.

Characterization of Green Synthesized Ag-NPs

The presence of Ag-NPs was verified 90 minutes after synthesis by analyzing its optical characteristics using UV–vis spectroscopy (PerkinElmer, Lambda 365) within the wavelength range of 280 to 500 nm. The phytochemical molecules responsible for the reduction of AgNO3 to Ag-NPs, as well as those involved in the stability of Ag-NPs, were determined using Fourier transform infrared spectroscopy (Perkin-Elmer Universal ATR 100) in the range of 4000–400 cm-1. The surface morphology of synthesized Ag-NPs was explored using transmission microscopy (TEM). The purity of the Ag-NPs was determined by EDX spectroscopy.

Anti-trypanosomal Activity of Ag-NPs

In vitro assessment of anti-trypanosomal activities of the synthesized Ag-NPs of *A. indica* leaf extract was carried out using the method described (Bulus and Addau 2013). Prior to conducting the assay, the solution containing the produced Ag-NPs and *A. indica* leaf extracts was prepared by dissolving them in 10% Dimethyl Sulphur Oxide (DMSO). A stock solution was prepared by dissolving one milligram of both Ag-NPs and *A. indica* leaf extract in one milliliter of the culture medium, resulting in a concentration of 1 mg/ml. The Ag/Cu NPs and *A. indica* leaf extract were each evaluated in triplicate in a 96-well microtiter plate (Costar, USA). Serial dilutions were performed using the culture medium stock media to get concentrations ranging from 500 µg/ml to 8.625 µg/ml. Fifty microliters of the prepared medium, consisting of both Ag-NPs and *A. indica* leaf extract, were placed into a well of the microtiter plate using a micropipette. Then, 50 µl containing about 106 trypanosomes per well were added, resulting in a total volume of 100 µl. Diminaze and DMSO were used as positive and negative controls, respectively, in separate wells for testing. The plates were placed in a 5CG-Desicator and incubated at a temperature of 37oC for a duration of 12 hours. During this incubation, the plates were exposed to an atmosphere containing 5% CO_2, as outlined in the method reported by Bulus et al. (2012). Observations were made under light microscopy at a magnification of 400X at four different time intervals: 3, 6, 9, and 12 hours after incubation. The "rapid matching" method was used, which involves counting the number of trypanosomes per field. Logarithm values of the trypanosomes number were obtained by comparing them with a table that was converted to logarithmic values. This allowed for the determination of the absolute number of trypanosomes per milliliter of blood.

Statistical Analysis

The data collected in this study has been compiled and presented as the mean value plus or minus the standard deviation. The data analysis was conducted utilizing the Statistical Package for Social Sciences (SPSS), specifically version 20.0. To assess the statistical significance, a one-way ANOVA was conducted followed by Duncan's multiple comparison test to compare the findings obtained from different concentrations. A P value < 0.05 was considered significant.

RESULTS AND DISCUSSION

Phytochemical Analysis

A.indica is a rich source of phytochemicals, which are biologically active compounds that have various medicinal

properties (Priya *et al.*, 2020). Some of the phytochemicals found in *A. indica* are alkaloids, saponins, flavonoids, terpenoids, phlobatannins, tannins, and steroids (Table 1) and these biomolecules have been reported to effectively reduce and stabilize metal ions to metal NPs (Chopra *et al.*, 2023).

Table 1: Phytochemicals present in the *A. indica* leaf extract

Phytochemical tested	Presence
Alkaloids	+++
Tannins	+
Saponins	++
Carbohydrate	++
Terpenoids	++
Steroids	++
Flavonoids	+++
Plobatanins	-

Keys:
+ indicates the presence of phytochemicals
++ indicates moderate presence
++++ indicates a high presence

UV–Vis Spectroscopy Analysis

The formation and stability of Ag-NPs in distilled water are confirmed using UV-vis spectrophotometry as presented Figure 1. The reaction mixtures showed a gradual change in color from the initial room temperature to 60^0 C, from light yellow to dark brown and the colour intensified after 90 minutes. The color change is due to the excitation of the Surface Plasmon Resonance phenomenon (SPR) which is the collective oscillation of electrons in response to light in the synthesized NPs (Pawar *et al.*, 2022). The SPR depends on the type of metal, the dielectric constant of the surrounding medium, and the inter-particle interactions**.**

The absorption spectrum of the synthesized Ag-NPs (Figure 1) was confirmed by the presence of characteristic absorption peaks between 400 nm and 430 nm which indicated the formation of Ag-NPs. This result is similar to the findings of Danazuni *et al*., (2024), who reported the maximum absorption peak for Ag-NPs at 420 nm. It is well known that Ag-NPs possess characteristic absorption peaks between 400 nm and 450 nm. If the peak rises above this range (400 nm to 450 nm), it indicates aggregation or precipitation resulting in particles with large size. On the other hand, the peak shift below this range (400 nm and 450 nm) indicates that the nanoparticle solution contains other participants (impurities, organic species, solvent, etc. (Perera *et al.*, 2020). The shift in absorption peak may be highly indicative of size, morphology, quantity, and nanoparticle growth (Joudeh and Linke 2022).

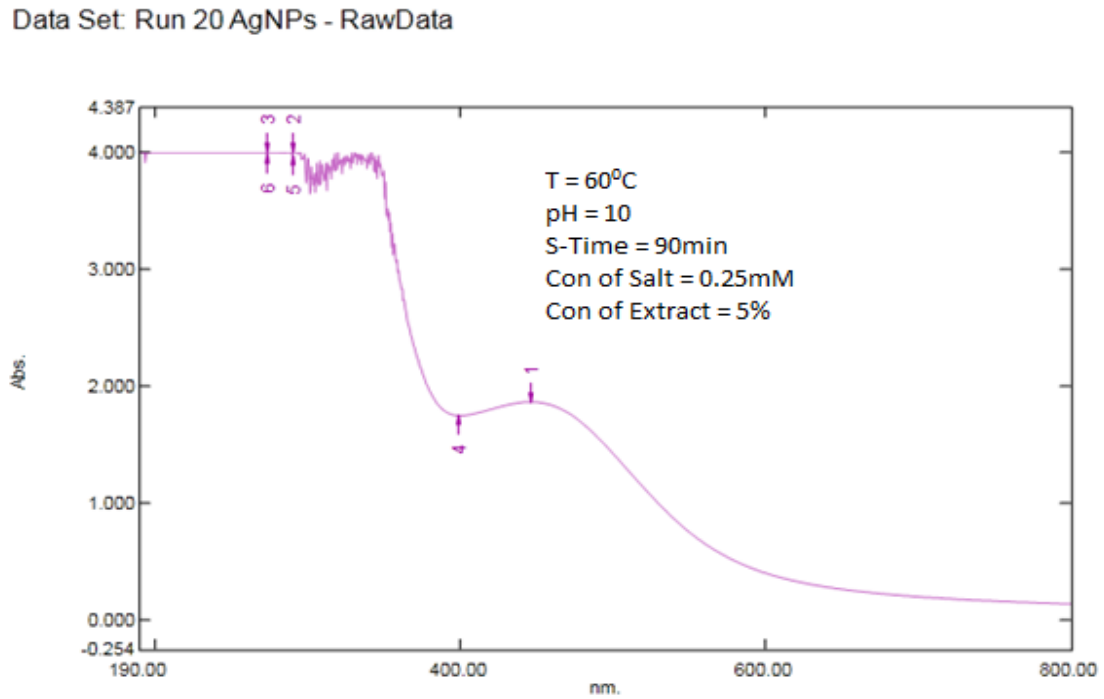

Figure 1: UV-Vis absorption spectra of Ag-NPs

FTIR Analysis

Fourier transform infrared spectroscopy (FT-IR) is employed to determine and provide an approximate identification of the potential biomolecules present in the *A. indica* leaf extract. The FTIR spectrum in Figure 2 (A) of the *A. indica* leaf extract has several absorption peaks, indicating its intricate composition. The intense and wide peak observed at 3446 cm-1 can be ascribed to the presence of hydrogen-bonded O-H groups in alcohols, phenols, and the N-H group in amides. The band at **2921 cm^{-1}** represents C-H stretching vibrations

typical of alkanes while the peak at **1638 cm^{-1}** suggests C=C stretching vibrations, indicating unsaturated compounds like alkenes or aromatics (Ahmed *et al.,* 2016). The peak at 1406 cm^{-1} is typically associated with the bending vibrations of C-H bonds. This suggests the presence of aliphatic hydrocarbons or other organic compounds containing carbon-hydrogen bonds (Rathore and Devra, 2022). The peak at 1068 cm-1 is commonly associated with the stretching vibrations of C-O bonds, suggesting the presence of alcohols, ethers, or esters in the extract. The process of synthesizing Ag-NPs utilizing an aqueous leaf extract of *A. indica* has been documented to involve these specific functional groups (Chand *et al.,* 2019; Shah, 2018; Vanlalveni *et al.,* 2021). The interaction of the functional groups present in the *A. indica* extract with Ag ions leads to the formation of nanoparticles. The shift in the FTIR peaks in the nanoparticles' spectrum figure 2 (B) is indicative of this interaction (Vanlalveni *et al.,* 2021).

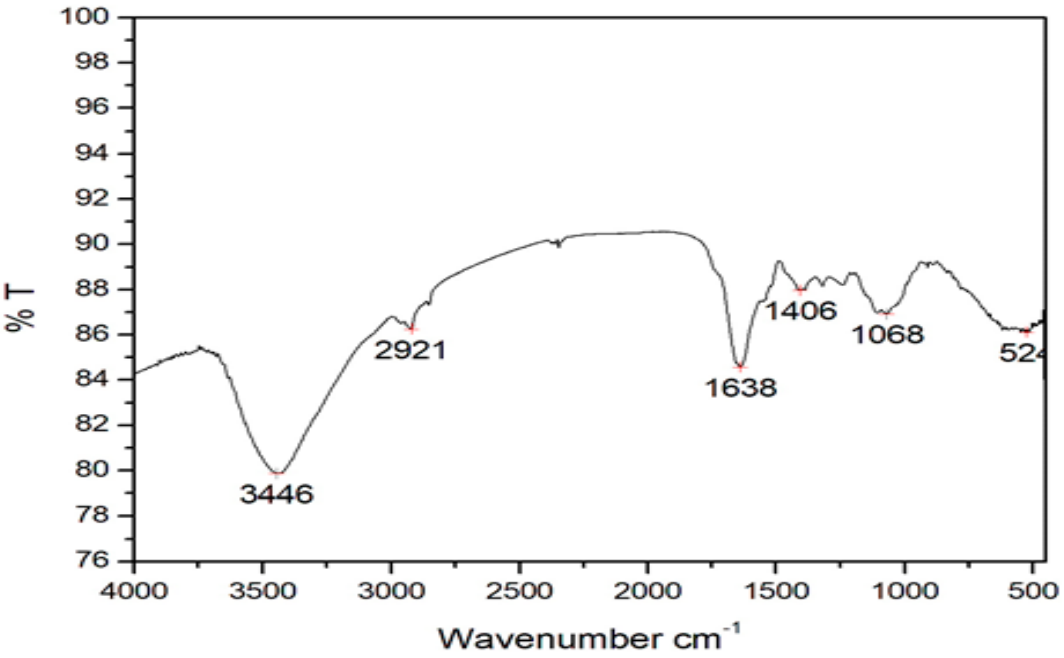

Figure 2 (a): FTIR spectral representation of *A. indica* leaf extract

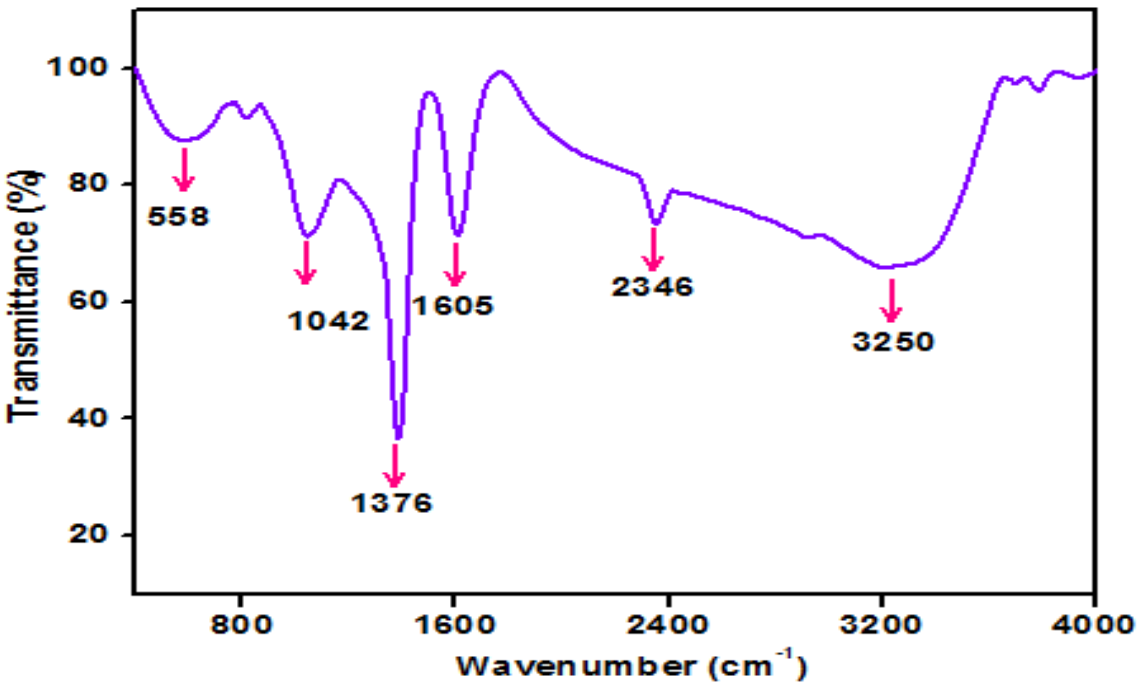

Figure 2 (b): FTIR spectral representation of Synthesized Ag-NPs using *A. indica* leaf extract

TEM Analysis

Also, the TEM analysis of the produced Ag-NPs, as shown in Plate 4, indicates that the Ag-NPs are spherical, which aligns with recent studies on green synthesized Ag-NPs utilizing plant extracts (Poudel *et al.,* 2022; Thangamani and Bhuvaneshwari, 2022). The morphology of the nanoparticles (NPs) can impact their optical, catalytic, and antibacterial abilities (Said *et al.,* 2023). The Ag-NPs have a size

distribution ranging from 5 to 35 nm, with a mean diameter of 13.75 nm. The plate also indicates that the Ag-NPs exhibit a high degree of crystallinity, as evidenced by the selective area electron diffraction (SAED) patterns (Plate 4c). The Scanning Electron Diffraction (SAED) patterns exhibit concentric rings with intermediate dots, showing that the Nanoparticles (NPs) possess a face-centered cubic (fcc) structure, which is the prevalent crystal structure of Ag. The degree of crystallinity of the nanoparticles can significantly impact their electrical, thermal, and mechanical characteristics (Thangamani and Bhuvaneshwari, 2022).

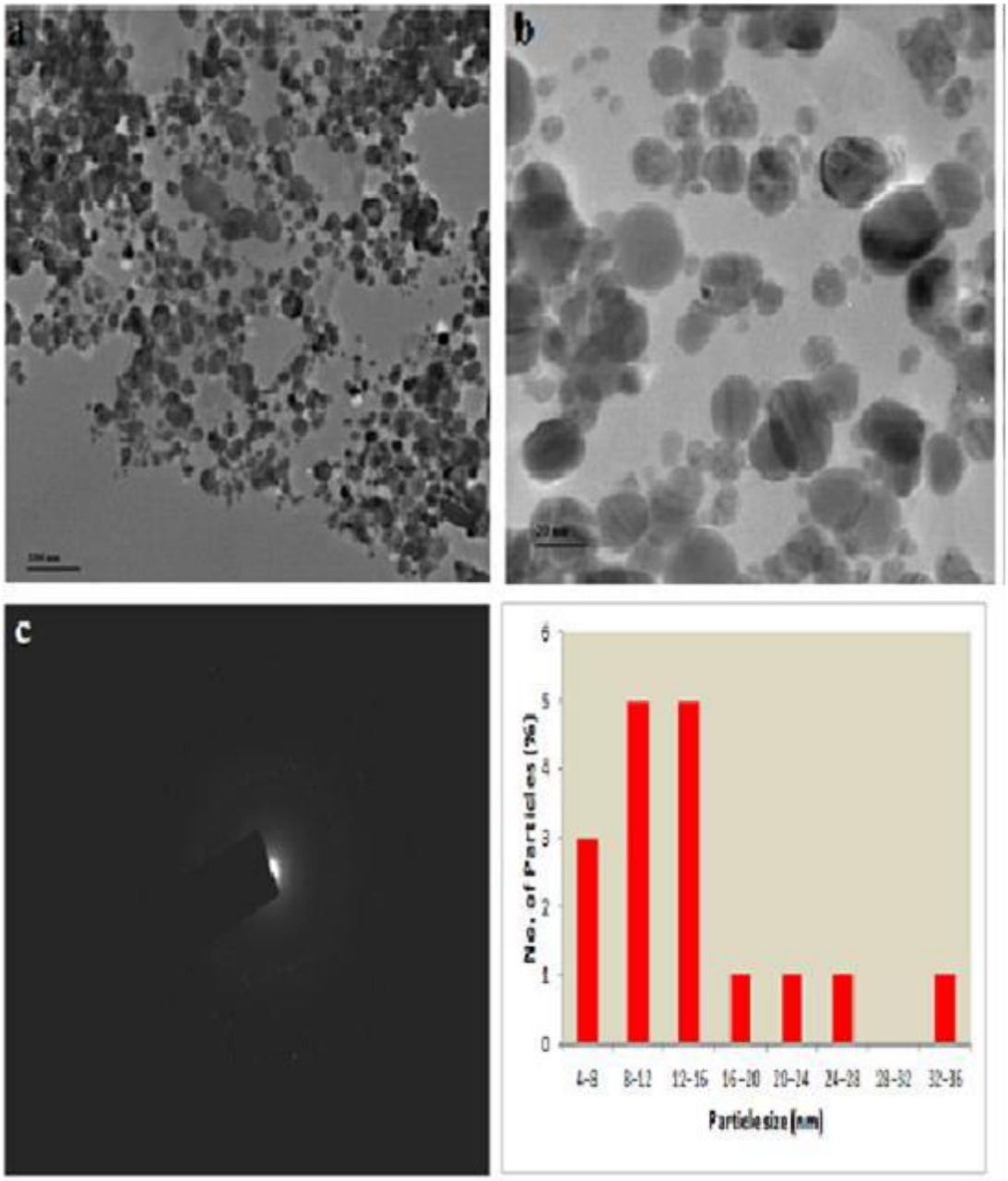

Plate 1: Enlarged TEM micrograph a) 100 nm scale b) 20 nm scale c) SAED pattern and d) Particle size distribution histogram of synthesized Ag-NPs using *A. indica* leaf extract

EDX Analysis

The energy-dispersive X-ray (EDX) is a technique that can detect the elemental composition of a sample based on the characteristic X-rays emitted by the atoms when they are irradiated by an electron beam (Gondwal and Joshi nee Pant, 2018).) Figure 3 shows the energy-dispersive EDX spectra of silver nanoparticles (Ag-NPs) synthesized from *A. indica* leaf extract. The peaks in the spectra indicate the presence and relative abundance of different elements in the NPs. The first spectrum shows prominent peaks for silver (Ag) at around 3000 and 8000 keV. These peaks confirm the formation of Ag-NPs from the leaf extract (Fatima and Wahid, 2022). The spectrum also shows some minor peaks for other elements, such as carbon, oxygen (O), nitrogen (N), and sulfur (S). These elements might be present as impurities, contaminants, or organic residues from the leaf extract or the synthesis process.

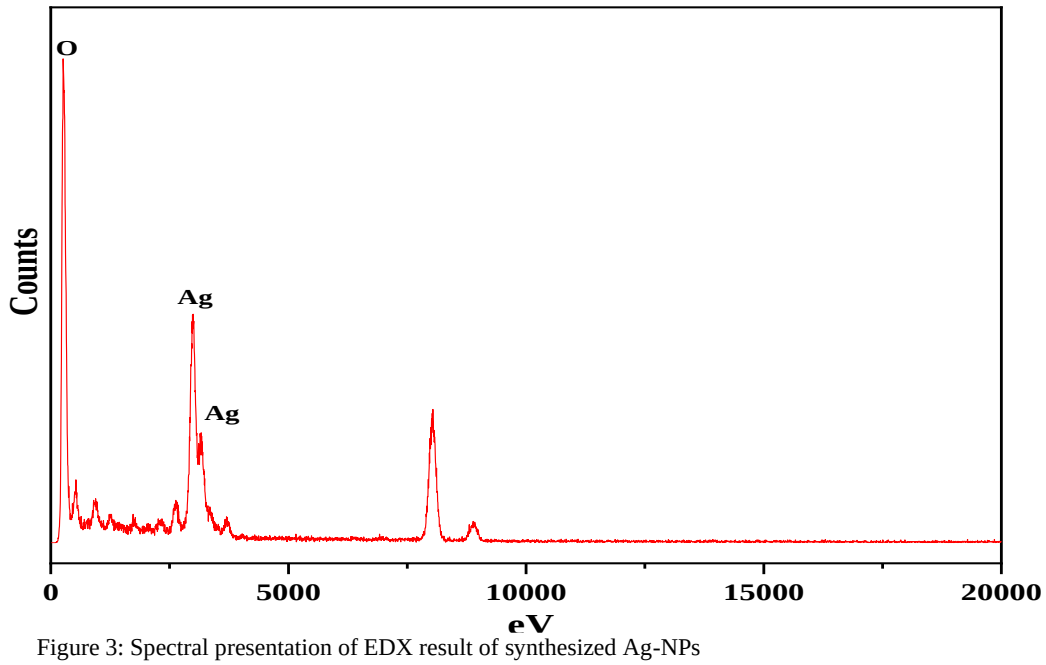

Figure 3: Spectral presentation of EDX result of synthesized Ag-NPs

Anti-trypanosomal activity of synthesized Ag-NPs

The AIL-Ag-NPs exhibited significant concentration-dependent activity against *Trypanosoma* sp. a decreasing number of surviving trypanosomes was seen with increasing concentrations of the synthesized AIL-Ag-NPs Between 500 and 250 µg /ml concentrations of the AIL-Ag-NPs, 100 percent trypanosome mortality was recorded, while the mean counts of surviving trypanosomes at lower concentrations of the AIL-Ag-NPs were significantly lower than those of the control.

The minimum trypanocidal concentration of the AIL-Ag-NPs against the trypanosomes was 250 µg /ml and that of the AIL-CE was above 500 µg /ml as motile trypanosomes were still detected at that concentration; while that of the Dimineazine aceturate was 125 µg /ml. The values for the median lethal concentrations for the AIL-Ag-NPs and AIL-CE of *A indica in vitro* were 10.096, 14.431, and 30.749 µg /ml, while that for the standard drug, Diminazine aceturate, was 7.889 µg /ml. Thus, based on LC_{50} the order of trypanocidal activity was Diminazine aceturate > >> AIL-Ag-NPs >>> AIL-CE (Table 2). These findings are in agreement with the results of A group of Bajwa *et al.*, 2022 who also studied the effect of Ag-NPs against *T. brucei gambience*, *T. cruzi*, *T. evansi*, and *T. congolense*. The NPs showed great efficacy against *T. brucei gambience* and *T. cruzi*, while growth was inhibited by 50 % in studies that targeted *T. evansi* and *T. congolense*.

Table 2: Effect of graded concentrations of *A. indica* synthesized Ag and Cu nanoparticles on trypanosome population, *in vitro*

Conc. (µg /ml)	No. trypanosomes/field		
	AIL-Ag-NPs	**AIL-CE**	**Diminazine**
500	0.00 ± 0.00^{a}	6.17 ± 1.17^{a}	0.00 ± 0.00^{a}
250	0.00 ± 0.00^{a}	11.0 ± 2.00^{b}	0.00 ± 0.00^{a}
125	0.00 ± 0.00^{a}	12.2 ± 3.37^{bc}	0.00 ± 0.00^{a}
62.5	0.33 ± 0.52^{a}	11.5 ± 1.05^{bc}	0.00 ± 0.00^{a}
31.25	1.17 ± 0.75^{a}	13.5 ± 1.52^{c}	1.17 ± 0.75^{a}
16.125	5.17 ± 2.04^{b}	16.0 ± 1.67^{d}	2.00 ± 1.09^{b}
8.625	15.0 ± 1.26^{b}	20.8 ± 1.60^{d}	10.50 ± 1.64^{c}
Control	23.8 ± 1.84^{c}	23.8 ± 1.84^{e}	23.8 ± 1.84^{d}
LC_{50} values (µg/ml)	**10.096**	**30.749**	**7.889**

Values are given as mean ± standard deviation. Values with different superscripts in each column, have statistical significance ($p < 0.05$). AIL-Ag-NPs: *A. indica* leaf silver nanoparticles; AIL-; AIL-CE: *A. indica* leaf crude aqueous extract; DA: Diminazine aceturate: IC50: median lethal concentration

CONCLUSION

The Ag-NPs produced from the leaf extract of *A. indica* exhibited significant anti-trypanosomal action against *T. b. brucie*. The aqueous leaf extract of *A. indica* exhibited a limited impact on *T. b. brucie*. The aqueous extract of *A. indica* leaf exhibited remarkable efficiency in synthesizing silver nanoparticles. These nanoparticles exhibited significant anti-trypanosomal activity, suggesting their potential use in controlling African trypanosomiasis and other biomedical applications.

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