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Green Synthesis of Silver Nanoparticles using *Lawsonia alba* Lam (Henna) Plant Extract and Its Antibacterial Activity and Toxicity

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Abstract Currently there is a growing need for green synthesis of nanoparticles using biological sources without using any harmful chemicals plays a very important role. Plants produce wide array of bioactive molecules or phytochemicals which are found to be useful for the treatment of various ailments. At present development of reliable green chemistry route to synthesis nanoparticles specifically in biology, medicine and water purification being carried out for wide applications. The effect of *Lawsonia alba* Lam henna extract after treatment with $AgNO_3$ and formation of AgNPs were characterized viz. UV-vis, SEM with EDX, XRD analysis. The antibacterial activity of henna extract and Extract after treatment with $AgNO_3$ was done against selected gram positive and gram negative bacterial strains and their zone of inhibition was measured & recorded. The toxicity of the three examined material were carried out using Microtox analyzer. The toxicity of silvers decreased in the following order: silver ions > bio reduced silver nanoparticles. The goal of our study to assessment the use of *Lawsonia alba* Lam (henna plant) extract after treatment with $AgNO_3$ to apply in many applications the most important one is water purification due to their antimicrobial activity against wide ranges of microbes that causes infectious diseases in humans.

Keywords Green synthesis, AgNPs, *Lawsonia alba* Lam henna extract, $AgNO_3$, Antibacterial Activity, Toxicity

Introduction

Nanotechnology is an important field which plays a role in solving many problems faced by humanity. Nanoscience has been used as a strategy and manipulation of particles with nano-size objects with least toxicity, effective & eco-friendly which possess new mode of action to cater rising emergency. It is widely agreed that now, it is possible to synthesize nanomaterials by using biological system with the help of plants, micro-organisms either by bottom-up or top-down strategies [1]. Nanotechnology provides a good platform to modify & develop the properties of synthesized nanoparticles which having promising applications as an anti-microbial agents as well as unique Chemical and Physical properties. Now-a-Days synthesized AgNPs offers numerous benefits on Pharmaceutical and Bio-Medical applications as they do not use any toxic chemicals for Synthesis [2].

Nanoparticles of metals and their compounds have attracted the interest of many communities over the years for many reasons. In particular, Ag and Cu nanoparticles, both show excellent antimicrobial properties [3,4]. In the biomedical field, Ag has been used widely in medical care. For medical devices, water purification, and antimicrobial uses [5]. In addition, Ag has effective antimicrobial effects against both gram positive bacteria and gram negative bacteria including aeruginosa [6].

Silver nanoparticles deposited or impregnated materials (metal, polymer, metal oxide, carbon, cellulose) have been widely investigated for their unique physico-chemical properties such as catalytic, electrical, optical and anti-microbial, due to unusual interfacial effects [3].

Henna, *Lawsonia alba* lam (*L. alba* lam) is a medical plant belonging to the family Lythracea. Powdered leaves of henna is commonly used for staining plam, hairs, hands and other body parts [7,8]. Henna is a natural product with small health risk potential [9-11]. Leaves of henna contain major phyto-chemicals such as glycosides, phtosterolm saponins, tannins and flavonoids. Flavonoids and glycosides are commonly known to posse antimicrobial activity [12]. Bacteria are most abundant group of organisms, and bacterial pathogens are the major source of disease and mortality to human populations, worldwide [13]. Treatment by antibiontics to face such pathogens is facing major challenges as bacteria evolve resistance to the synthesized antibiotics [14-16].

Recently, by taking into account the side effects of chemical antibiotics. The use of plant extracts as pharmaceutical purposes increased. The henna leaves are used in the treatments of wounds, ulcers, cough, bronchitis, rheumatagia, inflammations, dysentery, anaemia, fever, falling of hair and grayness hair [17-19]. The medicinal properties exhibited by this plant mainly due to its wide range of phyto-chemical compounds present in them. Theses includes 1,4-Naphquinone, 2-Hydroxy-1,4-Naphtha oquinone, Esculetin, Quinone, etc. [20-21].

In this study, *L. alba* lam (Henna) plant has been used as a biological source of synthesizing silver nanopartcles (AgNPs) using the aqueous extract of *L. alba* lam (Henna) showed colloidal grey color, then anti-bacterial activity were done by disc diffusion method against gram positive & gram negative bacterial strains which causes infectious diseases in humans and toxicity. The toxicity of the three examined material were carried out.

Materials and Methods

Materials

Silver nitrate ($AgNO_3$) was purchased from Acros Organics.

Plant Sample Collection

The leaves of *L. alba* lam (Henna) plant was collected freshly from local garden in Cairo, The plant was identified *L. alba* lam (Henna) at the Department of Botany, Cairo University, Science College, Egypt.

Preparation of *L. alba* lam (Henna) Extract

Leaves of *L. alba* lam (Henna) was collected and rinsed with tap water then by de-ionized water and allowed to dry in air. Then they were cut into small pieces, after that, 20 grams was taken in conical flask containing 200 ml distilled water and boiled them for 10 minutes. Then left them to cool, after that, they are filtered using whatmann No.1 filter paper and kept at 4^oC which be used for further work [22].

Biological Synthesis of AgNPs

Aqueous extract of plant (20 ml) was prepared is taken in conical flask and 180 mL of 1 mM of $AgNO_3$ was added and kept at room temperature for reduction and change of color was observed [22]. Entire process was carried out in darkness to avoid photo activation of $AgNO_3$ at room temperature.

Bacterial Cultures

Four bacterial strains were use for testing the activity in this study , two of them were gram positive strains namely *Enterococcus faecalis* NCTC 775/ATCC 19433 and *Bacillus subtillis* NCTC 10400/ATCC 6633 others were gram negative strains namely *Escherichia coli* NCTC 12241/ATCC 25422 and *Staphylcoccus aureus* NCTC 10788/ATCC 6538. These test organisms were maintained and stored at -20ºC.

Preparation of Inoculums

Disc of each strain was suspended in 5 ml of nutrient broth incubated for 24 hours at 37ºC.

Preparation of Media

Nutrient agar medium was prepared and adjust PH to 7 & sterilized by autoclaving at 121ºC for 15 min [23].

Antibacterial Assay

Petri plates containing 20 ml of autoclaved and solidified nutrient agar medium were inoclumed by 100 µl of prepared inoculum were then plated onto agar surface followed by impregnated disc after dried with the three

examined material as follow (a: $AgNO_3$, b: synthesized Ag-NPs by Henna extract, c: Henna extract), gently pressed down to ensure contact and incubation at 37°C for 24 hrs .after incubation time the inhibition zone around the discs were measured and recorded for each organism. The extracts were tested for their antimicrobial potential against the microorganisms using the standardized disc diffusion method described by [24,25]

Toxicity Test

1ml of each examined material a, b & c were added to 1 l of autoclaved distilled water and the test carried and results recorded according to [26]. Bioluminescent Tests, the Microtox Procedure employs the bioluminescent marine gram-negative bacterium *Vibrio fischeri* as test organism. The bacteria are exposed to a range of concentration of the three materials in suspension being tested. The reduction in intensity of light emitted from the bacteria is measured along with standard solutions and control samples. Toxicity inversely proportional to the intensity of the light emitted after contact with the toxic substances. The change in light output and concentration of the toxicant produce a dose/response relationship. The results are normalized and the EC50 (concentration producing a 50% reduction in light) is calculated using Microtox 500 Analyzer, and bioluminescence measurements were monitored at 0, 5 and 15 min of exposure. The effective concentrations causing 50% of bioluminescence inhibition were computed using the software for Microtox Omni Azur (AZUR environmental, 1998).

Characterization Techniques of Silver Nanoparticles

Characterization of synthesized AgNPs is very important to understand their Physical & Chemical properties as well as their applications. It is performed by using a variety of techniques such as UV-Vis Spectroscopy, Scanning Electron Microscopy (SEM), X-Ray Diffractometer (XRD) [27].

UV-Vis Spectroscopy

The bio-reduction of Ag+ in aqueous solution was detected using UV-vis Spectro-photometer (Shimadzu 3600 NIR, Kyoto, Japan) at room temperature with wave lengths of 200-800 at a resolution of 1 nm.

Scanning Electron Microscopy

Scanning electron microscope was used to characterize the surface of synthesized nanoparticles. The solution containing silver nanoparticles synthesized from *L. alba* lam leaf extract was inverted into powder using Lypophilizer equipment. Thin films were prepared on carbon coated grids. These images of bio synthesized silver nanoparticles were obtained in SEM (Inspect FEI Ltd, Holland) operated at 30 KV at different magnification level.

Energy-Dispersive X-Ray (EDX) Analysis

The synthesized silver nanoparticles using *L. alba* lam (Henna) aqueous extract subject to the energy dispersive spectrum to confirm the presence of silver and to detect other elementary compositions of the particle.

XRD Analysis

The bio-reduced silver nano particles was made into powder using Lypophilizer equipment and they are coated on XRD grid and analyzed for the formation of nanoparticles using X-Ray diffractometer (X-Pert Pro, Panalytical, Holland), X-Ray generator operated at a voltage of 5 KV and tube current of 50 mA with Cu kα1 radiation with λ of 1.5406.

Results and Discussion

Visual observation

The synthesis of silver nano particles in the solution of 1 mM $AgNO_3$ and aqueous extract of *L. alba* lam (Henna) plant sample was confirmed by change in color of the mixture from dark brown to colloidal grey which indicates the formation of AgNPs compared to the control (without treatment with 1mM $AgNO_3$) that remained dark brown Fig. (1). It is well known that silver nanoparticles exhibit dark grey colour in water due to extinction of surface Plasmon vibration in metal nano particles [28]. Control (without silver nitrate) shows no color change , the colour change in the aqueous extract with silver nitrate solution may be due to the presence of bioactive compounds in aqueous extract like Gallic and Lawsone acid responsible for the reduction of silver nitrate to silver nano particles. The

different type of various phyto-chemicals and antioxidants are responsible for the reduction of silver ions, similar type observations were reported by another authors [29-30].

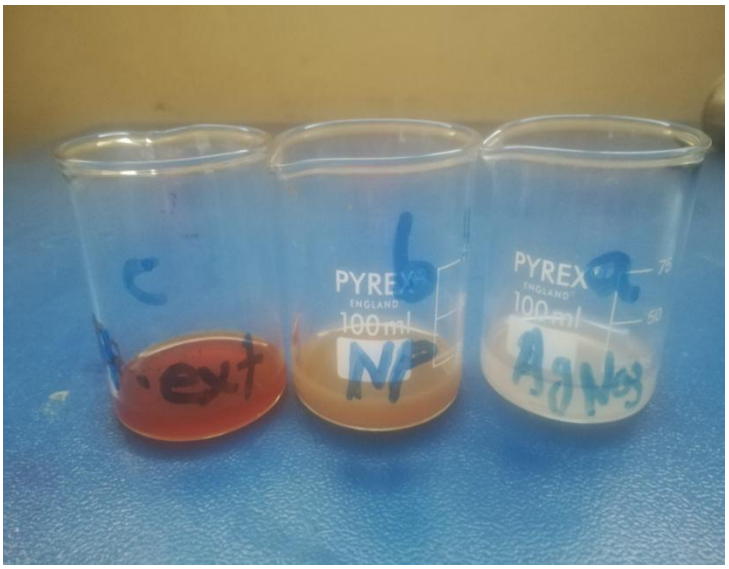

Figure 1: (a) $AgNo_3$ solution, (b) Henna extract after treatment with $AgNO_3$, (c) Alba lam leaves extract (control)

UV-vis Spectral Analysis

The silver nano particles (AgNPs) formed by reduction of silver nitrate using aqueous extract of *L. alba* lam leaf after 24 hours incubation samples were characterized by UV-visible spectroscopy, and the results confirmed by the biological AgNPs formation in reaction mixture. In UV-visible spectrum, a broad peak located between (420 nm – 500 nm) was observed relatively uniform as shown in Fig. (2a), Fig. (2b). this reveals that the formation of AgNPs occurs rapidly within 2 hours for completion of the reaction. Similar observations were reported in Geranium, leaf extract [28]. In this study, the synthesized AgNPs were shown characteristic peak at 470 nm in visible light regions.

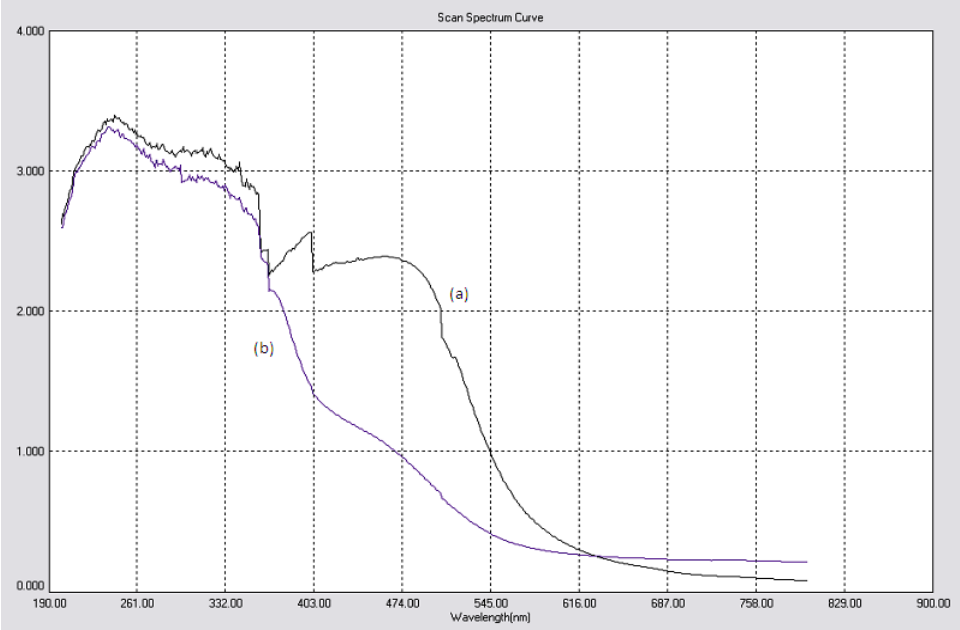

Figure 2: (a) Extract after treatment with $AgNO_3$, (b) Alba lam leaves extract (control)

SEM Analysis

SEM analysis shows AgNPs synthesized by *L. alba* lam (Henna) leaf extract. It was shown that relatively uniform and spherical AgNPs were formed with diameter of 14 to 65 nm, as shown in Fig. (3). The SEM image of silver nanoparticles was due to interaction of hydrogen bond and electrostatic interactions between the bioorganic capping molecules bound to the AgNPs. The larger silver nano particles may be due to the aggregation of the smaller ones, due to the SEM measurements.

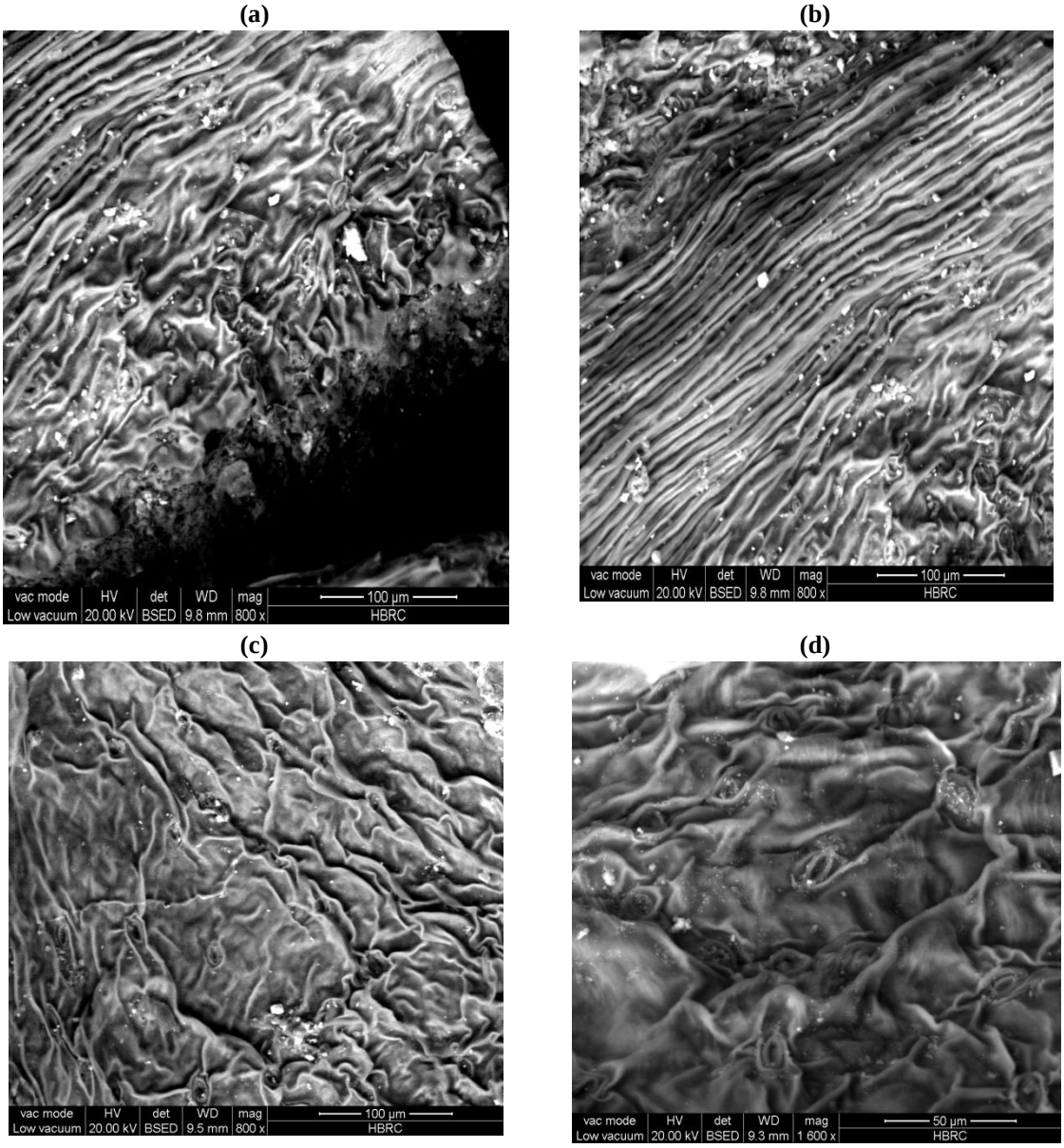

Figure 3: (a,b) SEM Results of Alba lam (henna) extract, (c,d) SEM Results of Alba lam henna extract after treatment with $AgNO_3$

EDX Analysis

EDX spectra recorded from the silver nano particles were shown in Fig. 4. From EDX spectra, it is observed that silver nanoparticles reduced by *L. alba* lam extract have the weight percentage of silver as 68% and 28.2%. The EDX spectrum spherical in shape with high aggregation of silver nano particles on the surface of the cell prepared with this bio reduction method using *L. alba* lam (Henna) shown maximum peaks around 3.4 KeV correspond to binding energies of silver ions. Some additional peaks belonging to bioorganic compounds present in the reaction

mixture. The EDX analysis reveals strong signals in the silver region that confirms the formation of silver nano particles using biological source. There were other spectrum peaks for Cl, Si, O and Ca suggesting that they are mixed precipitates in the plant extract [31].

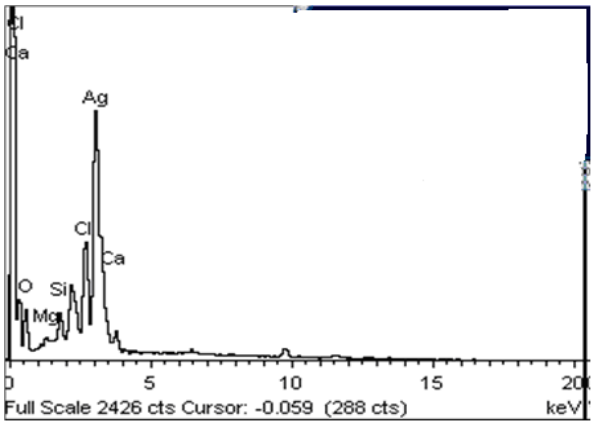

Figure 4: EDX Results of Synthesized AgNPs of L. alba Lam Henna

XRD Analysis

The XRD patterns obtained for the AgNPs synthesized using Henna extract is shown in Fig.5. The Braggs reflections were observed in the XRD pattern at 2θ = 32.1, 26.92, 45.41 and 37.92 These reflections clearly indicated the presence of (100), (52), (43) and (42) sets of lattice planes and further on the basis that they can be indexed as Face-centered-cubic (FCC) structure of silver [29] reported that the XRD pattern green synthesized silver nano particles showed number of Bragg's reflections that may be indexed on the basis of the face centered cubic structure of silver. Since, they present study clearly indicated the X-Ray diffraction pattern of biological synthesized silver nano particles formed crystalline.

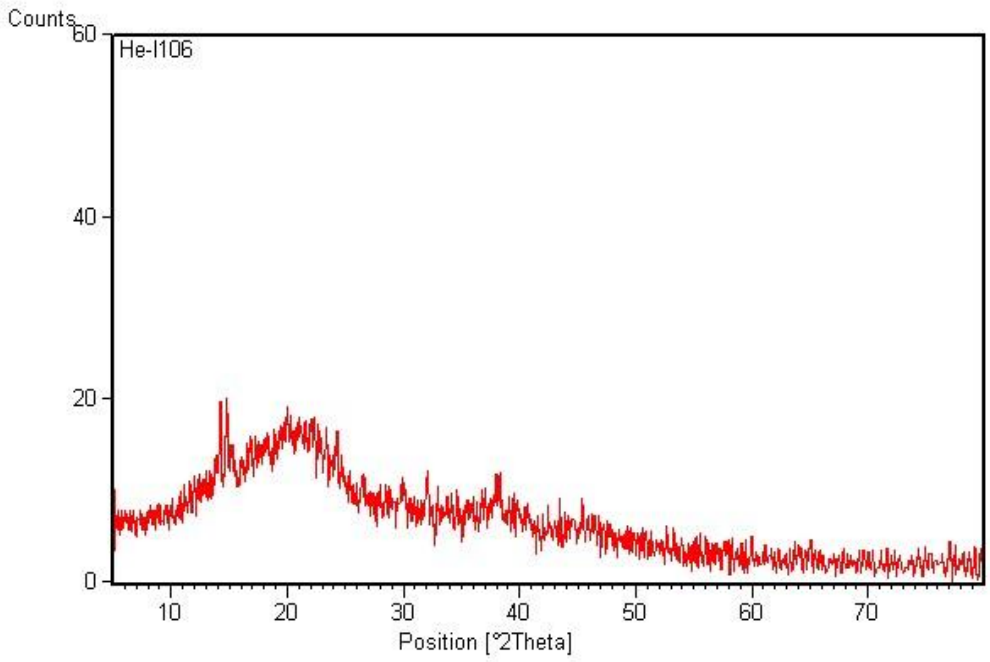

Figure 5: XRD Results of Synthesized AgNPs of L. alba Lam (Henna)

The Antibacterial Activity

The Ag-NPs showed antibacterial activity against two Gram Positive Bacterial Strains *Enterococcus faecalis* & *Bacillus subtillis* as shown in Fig 6(a,b) and two Gram Negative Bacterial Strains like *Escherichia coli* and *Staphylcoccus aureus* as shown in Fig. 7(a,b).

Table (1) showed the antimicrobial activity of the two Gram Positive Bacterial Strains the highest antibacterial effect in henna extract after treatment with $AgNO_3$, $AgNO_3$ and henna extract respectively were observed in *Enterococcus faecalis* with zone of inhibition (25, 20 & 15 mm) respectively and lowest antibacterial effect in henna extract after treatment with $AgNO_3$, $AgNO_3$ and henna extract respectively was observed in *Bacillus subtillis* with zone of inhibition (19, 16 & 9mm) respectively.

Table 1: Measurement of Zone of Inhibition (mm) of henna extract, $AgNO_3$ solution and Synthesized AgNPs by *L. alba* lam (Henna) Plant against Gram- Positive Bacterial Strains

	Inhibition zone in mm		
Bacterial strains	**Pure Plant Extract**	**Pure Silver Solution**	**Synthesized AgNps**
Enterococcus faecalis	15	20	25
Bacillus subtillis	9	16	19

Figure 6a: Antimicrobial activity of the Gram Positive Bacterial Strains "Enterococcus faecalis"
(a) pure silver solution
(b) Pure plant extract
(c) Synthesized AgNPs

Figure 6b: Antimicrobial activity of the Gram Positive Bacterial Strains "Bacillus subtillis"
(a)pure silver solution
(b) Pure plant extract
(c) Synthesized AgNPs

Table (2) and figures (7a) and (7b) showed the antimicrobial activity of the two Gram negative bacterial Strains the highest antibacterial effect in henna extract after treatment with $AgNO_3$, $AgNO_3$ and henna extract respectively were observed in *Escherichia coli* was found with zone of inhibition (30, 23 & 20 mm) and lowest antibacterial effect in Extract after treatment with $AgNO_3$, $AgNO_3$ and henna extract respectively were observed in *Staphylcoccus aureus*. Zone of inhibition (21, 19 & 18 mm) respectively.

Table 2: Measurement of Zone of Inhibition (mm) of henna extract, $AgNO_3$ and Synthesized AgNPs by *L. alba* lam (Henna) Plant against Gram- negative Bacterial Strains

	Inhibition zone in mm		
Bacterial strains	**Pure Plant Extract**	**Pure Silver Solution**	**Synthesized AgNps**
Escherichia coli	20	23	30
Staphylcoccus aureus	18	19	21

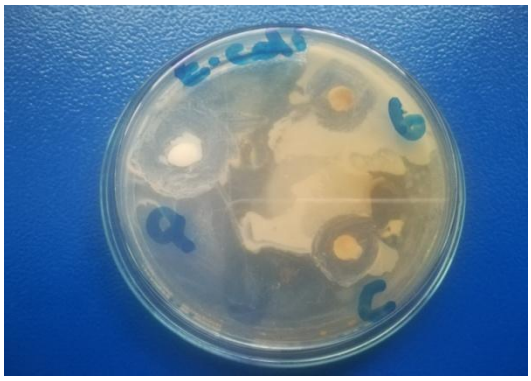

Figure 7a: Antimicrobial activity of the Gram Negative Bacterial Strains *"Escherichia coli"*
(a) pure silver solution
(b) Pure plant extract
(c) Synthesized AgNPs

Figure 7b: Antimicrobial activity of the Gram Negative Bacterial Strains *"Staphylcoccus aureus"*
(a) pure silver solution
(b) Pure plant extract
(c) Synthesized AgNPs

The action of silver nanoparticles on microbes to cause the microbicidal effect. The silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate and followed by the disruption of ATP production and DNA replication, thereby causing structural changes in the cell membrane and death of the cell [32].
Evidently report of some authors mentioned that there is the formation of "pits" on the cell surface when accumulating these nanoparticles on the cell surface and causes damage to the microbes [33,34].
According to [35,36] there is a general rule that any plant is considered active against fungi and bacteria when the zone of inhibition is greater than 6mm thus supporting our results, which are more than 6mm, at the three tested materials ($AgNO_3$, Henna extract & Extract after treatment with $AgNO_3$)
Regarding the mechanism of action of henna, it may be due to presence of mucilage mannite, tannic acid and gallic acid which are in the form of a mixture.
Antimicrobial activity is due to free hydroxyls that have ability to combine with proteins and carbohydrates in the bacterial cell wall and get attached to enzyme sites making them inactive [37].

The Toxicity

Effective concentration (EC) for three materials that result in 20% & 50% inhibition in test system that measure Bioluminescence of *Vibro Fischeri*. The EC_{50} & EC_{20} values were calculated after 30 min exposure time at 25 ºC

Table 3: Toxicity degree according to EC_{50}

Degree of toxicity	Extremely Toxic	Very Toxic	Toxic	Moderate Toxic	Non-Toxic
EC_{50} Value	0-19	20-39	40-59	60-79	80-≥100

EC_{20}: The effective concentration causing 20% luminescence inhibition EC_{50}: The effective concentration causing 50% luminescence inhibition Reference substances used: 3,5-Dichlorophenol , Zinc sulfate hyptahydrate, Potassium dichromate

Table 4: effective concentrations and toxicity degree

Sample	EC $_{50}$(%)	EC_{20} (%)	Toxicity degree	pH before adjustment	pH after adjustment
$AgNO_3$	80	32	Non Toxic	6	7.2
Henna extract	91	36.4	Non Toxic	7.2	7.2
Extract after treatment with $AgNO_3$	97	38.8	Non Toxic	6.5	7

The calculated EC_{50} after 5 and 15 minutes exposure time are quite similar. The slight decrease could mean that the nanoparticles need a short time to diffuse into the cells and interact with lipids, carbohydrates, proteins and DNA. Extract after treatment with $AgNO_3$ with EC50 ranging from 90 to 100 $mg \cdot l^{-1}$, can be classified as non toxic to aquatic micro-organisms according to the Commission Directive 93/67/EEC from the European Union for the assessment of risk to man and the environment of substances, also the toxicity of silver ions and silver nanoparticles on bacteria V. fischeri. According to the EC and, the toxicity of silvers decreased in the following order: silver ions > bio reduced silver nanoparticles [3].

Conclusion

The silver nanoparticles have been synthesized by *L. alba* lam (Henna) extracts, which is an economical, efficient and eco-friendly process and the characterization techniques viz., UV–Visible spectrophotometer, SEM, EDX, XRD have confirmed that the reduction of silver nitrate to silver nanoparticles. The zones of inhibition were formed in the antimicrobial screening test indicated that, the Ag-NPs synthesized in this process has the efficient antimicrobial activity against pathogenic bacteria and non toxic to aquatic micro-organisms.

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