Original Article

Comparative Evaluation of Antibacterial Activity of Silver and Gold Nanoparticles Synthesized Using Some Medicinal Plants' Leaves Ethanolic Extract

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Abstract

Nanotechnology is a rapidly emerging field with a wide range of biomedical science applications. Simultaneously, silver has been used as an antibacterial substance and disinfectant with few side effects. Antibacterial, antifungal, and antiviral activities are all present in silver nanoparticles**.**Antibiotic activity of gold nanoparticles (AuNPs) and ionic forms has been examined. Some organic Au (I & III) ion complexes have antimicrobial properties. AuNPs are antifungal, however the evidence for their antibacterial efficacy is mixed. In this study, the antibacterial activity of silver nitrate and gold chloride were examined either alone or in combination with plant ethanolic extract (*Lawsonia inermis, Olea europaea*) to produce LAgNPs, LAuNPs, OAgNPs, and OAuNPs. The effect was further tested against the antibacterial effect of two widely used antibiotis (ciprofloxacin "C", cephalexin "K"). The effect of a combination of the nanoparticle, ethanolic extract of either *L. inermis* or *O. europaea,* and one of the antibiotics) were examined against five of the gram negative microbial strains (*Klebsiella pneumoniae, Escherichia coli, Salmonella Typhimurium, Pseudomonas aeruginosa* and *Citrobacter freundii*). Silver nitrate showed a significant increase in the inhibition zone of *Pseudomonas aeruginosa* growth, while no effect against other bacteria. *L. inermis* extract only affect the growth of *Klebsiella pneumonia* and *Escherichia coli*, while LAgNPs inhibit the growth of *Salmonella typhimuriumm*, *Escherichia coli* and *Pseudomonas aeruginosa* significantly. The antibacterial effect of ciprofloxacin and cephalexin was enhanced when combined with LAgNPs apart from *Citrobacter freundii, Klebsiella pneumonia* and LAgNPs + K against *Escherichia coli* and LAgNPs + C against *Salmonella typhimurium*, while no changed was noticed against *Citrobacter freundii* with LAgNPs + C. for OAgNPs, no significant effect on the growth of *Klebsiella pneumonia* was noticed. When combined with ciprofloxacin, only *Citrobacter freundii* and *Salmonella typhimurium* showed an increase in the zone of inhibition compared to the antibiotic alone, while for cephalexin + OAgNPs, only *Pseudomonas aeruginosa* was affected than when using each of them independently. $AuCl_4$ significantly inhibit the expression of *Pseudomonas aeruginosa* and *Citrobacter freundii.* Only affect *Klebsiella pneumonia* and *Escherichia coli*. Combining the two together, LAuNPs significantly increase the zone of inhibition of only two of Bactria *Pseudomonas aeruginosa and* Escherichia coli. The bactericidal effect of Ciprofloxacin was enhanced when combined with LAuNPs only against *Klebsiella pneumonia* and *Pseudomonas aeruginosa, while* LAuNPs+ cephalexin inhibitory effect was only noticed against *Pseudomonas aeruginosa. For* O. europaea extract ethanolic extract, only *Escherichia coli* was affected, while both *Escherichia coli* and *Pseudomonas aeruginosa* zone of inhibition were increased when treated with OAuNPs. Using OAuNPs with Ciprofloxacin increased the growth inhibition for only *Escherichia coli* compared to the use of the antibiotic alone, While *Pseudomonas aeruginosa and Salmonella typhimurium* was the only type of bacteria affected using OAuNPs + cephalexin compared to the use of the antibiotic alone. To conclude, the synergistic effect of using the nanoparticles with the antibiotic was noticeable with only some of the bacterial types while there was no or only slight effect against other bacteria and the same applied on comparing the effect of the aqueous extract of the tested medicinal plants compared to the combination with silver nitrate or gold chloride nanoparticles.

Keywords: *Lawsonia Inermis, Olea Europaea*, Ethanolic Extract, Silver Nitrate ($AgNO_3$), Gold Chloride ($AuCl_4$), Nanoparticles.

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INTRODUCTION

Antimicrobial resistance is an important health issue all over the world. Antibiotic resistance and the multidrug-resistant bacteria become a global issue. Gram-negative ones, such as *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, and Gram-positive bacteria, like *Staphylococcus aureus*, are among the types of bacteria which developed the highest levels of resistance to antibiotics due to the high frequency of infection to human with these

types. The effort to developing new and effective antibiotics against these bacteria is increasing to overcome one of the most dangerous worldwide issue [1,2,3,4]. Nanoparticles (NPs) have attracted considerable interest in their development as potential antimicrobial drugs. Researchers have been investigating nanoparticles (NPs) in relation to their development as antibacterial drugs. It has been shown that biophysical interactions occur between NPs and bacteria including biosorption, NPs breakdown or aggregation, and cellular uptake [5]. The use of medicinal plants has been used in many cultures for thousands of years to treat a broad range of human diseases. Researchers have recently focused on safer phytomedicines and biologically active compounds isolated from plants that are. commonly used. Plants and the products from them are used to treat a variety of health problems both in traditional and allopathic medicine. Since ancient times, numerous plant species have been found to have pharmacological properties as they contain various phytochemicals [6,7,8]. The biological synthesis of nanoparticles from plant extracts, and testing their antimicrobial activity are under intensive investigations. Since the 1980s, a great deal of effort has been put into developing new drugs from natural products, because microorganisms have been resistant to the existing medications. Nature has provided many of the products being used today [9]. The present study is an attempt to test the antibacterial efficacy of silver and gold nanoparticles produced using the ethanolic leaf extract of two medicinal plants (*Lawsonia inermis* and *Olea europaea***)**, which have been used in traditional medicine against a number of bacterial isolates including: *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella Typhimurium*, *Pseudomonas aeruginosa* and *Citrobacter freundii.*

Materials and Methods

- **Chemicals and plant materials:**

Silver nitrate ($AgNO_3$), ACS, 99.9+% (metal basis) was supplied by Alfa Aesar, Thermo Fisher (Kandal) Germany (molecular mass= 169.87 g/ mol). Gold chloride ($AuCl_4$) was purchased from Sigma-Aldrich, USA (molecular mass= 393.83 g/ mol). Both chemicals were used without any further purification. In addition, two medicinal plants were used in this experiment including *Lawsonia inermis* (Henna) and *Olea europaea* (Olive). They were selected from gardens at Al-Diwaniyah city on the basis of medicinal characteristics, ease of availability and cost effectiveness. Fresh and healthy leaves were collected locally and washed thoroughly first with tap water followed by deionized water to remove all the dirt and undesired visible particles, cut into small pieces and dried at room temperature. After drying, leaves of each plant were grinded very well until it become as a fine powder, weighed and kept aside to be used later on for preparation of ethanolic extract [10].

- **Production of plant extracts:**

Ethanolic extracts were obtained from leaves of selected medicinal plants according to the procedure reported by [11]. Aqueous ethanol at the concentration of 50% (v/v) was used as solvent. The solvent -to-leaves powder ratio was 10 ml/ g, the time of extraction was two hours and the rate of stirring was 400 rpm. The extracted liquid was then separated from the solid materials by filtration through whatman paper No. 1 and kept for later use.

- **Synthesis of silver and gold nanoparticles (AgNPs and AuNPs):**

Stock solutions of 4 Mm silver nitrate and gold chloride were prepared in the lab by using deionized water [12]. Since the molar mass of silver nitrate is 169.87 g/mol, a 1 mol solution of $AgNO_3$ would be 169.87 g in 1 liter. For preparation of 200 ml of 4 Mm of $AgNO_3$solution, 0.136 g of $AgNO_3$ was taken. By the same way, the molar mass of gold chloride is 393.83 g/mol, so 200 ml of 4 Mm of $AuCl_4$solution was prepared by dissolving 0.315 g of $AuCl_4$ in 200 ml of deionized water. Each of freshly prepared stock solutions was then transmitted into 500 ml Erlenmeyer flask and heated at 60-70 °C using hot plate stirrer equipped with a magnetic bar, the stirring rate was 350 rpm to ensure complete dissolving and production of clear homogenous solution in each flask. Production of silver and gold nanoparticles was then done by taking 100 ml of 4 Mm of each stock solution and introduced in a conical flask. Then 6 ml of each plant Ethanolic extract (*L. inermis*, *O. europaea*) was taken and made to add drop by drop into each of silver nitrate and gold chloride solutions [13]. Each mixture (*L. inermis* based AgNPs, *O. europaea* based AgNPs, *L. inermis* based AuNPs, *O. europaea* based AuNPs) was gradually heated at varying temperature ranging from 30-95 °C for 30 minutes using thermal magnetic stirrer [14]. In the mean time, the colour changing of the mixture from light to yellowish brown to reddish brown to colloidal brown was monitored. All reactions were done in darkness to avoid photosensitization of silver nitrate and gold chloride. Complete reduction of $AgNO_3$ to Ag^+ ions and $AuCl_4$ to Au^+ ions as a result of ethanolic extract were approved by the colour changing from colorless to colloidal brown [10]. On the other hand, mixture of ciprofloxacin (C) (ciprodar® XL 1000/tab-DAD) and cephalexin (K) (cefex-500 cap., Bangalore-India) with ethanolic extract of *L. inermis*, *O. europaea* were also used for the reduction of $AgNO_3$ to AgNPs and $AuCl_4$ to AuNPs. Stock solutions of each tested antibiotic was prepared by dissolving 0.3 mg in 1 ml deionized water. The ratio of combination of each metal to each extract to each antibiotic was optimized at 1:1:0.5 (i.e., concentration of mixture would be 40%: 40%: 20%). This means that 20 ml of 4 Mm of silver nitrate solution was mixed with 20 ml of *L. inermis* extract followed by adding 10 ml of ciprofloxacin solution and so on for the other mixture [12]. Finally, all the colloidal mixtures were sealed, covered with aluminum foil and kept aside for complete bioreduction.

Formation of AgNPs and AuNPs were furthermore confirmed by spectrophotometric analysis.

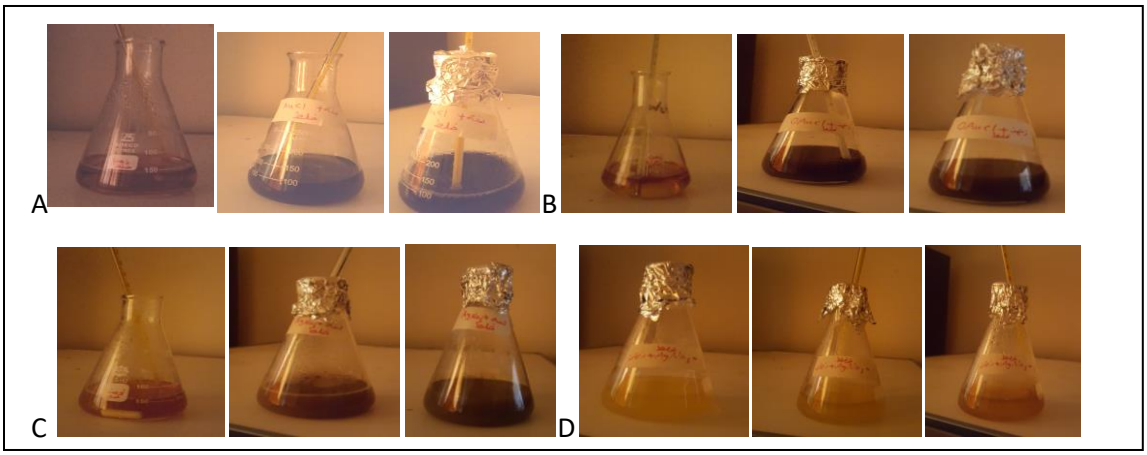

Figure 1: Colour change at varying temperatures (from left to right): 30 ˚C, 60 ˚C, 95 ˚C.
(A) *L. inermis* based AuNPs, (B) *O. europaea* based AuNPs, (C) *L. inermis* based AgNPs, (D) *O. europaea* based AgNPs

Selection of bacterial strains and diagnosis:

Five kinds of gram negative microbial strains {*Klebsiella pneumoniae* (*K. pneumoniae*), *Escherichia coli* (*E. coli*), *Salmonella Typhimurium* (*S. Typhimurium*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Citrobacter freundii* (*C. freundii*)} were isolated from different clinical samples and identified by using conventional biochemical tests and Vitek 2 system (Biomeraux, France) and cultivated in pure cultures at the lab of Microbiology, College of Vet. Medicine, University of AL-Qadisiyah, Iraq.

- **Chemical characterization of AgNPs and AuNPs:**

1- UV-visible spectroscopy:

The synthesis of AgNPs and AuNPs, was confirmed by scanning the absorption maxima of freshly prepared nanoparticles samples using UV–Vis spectroscopy on UV-1280 Shimadzu spectrophotometer. For all the reactions (*L. inermis* based AgNPs, *O. europaea* based AgNPs, *L. inermis* based AuNPs, *O. europaea* based AuNPs and control), the UV–Vis spectrum was recorded between 350 and 650 nm. Before UV–Vis analysis, all the solutions were diluted twofold in equal volume of deionized water. The final 2 mL of the silver and gold nanoparticles were subject to the final UV–Vis analysis using 1 cm path length quartz cuvettes at temperature range of 24-28 °C [(12)].

2- Fourier Transform Infrared Spectroscope (FTIR):

The FTIR analysis was determined with FTIR spectrometer, Nicolet iS50 (Thermo Fisher Scientific, Waltham, MA, USA). Each of freeze-dried silver and gold nanoparticles was mixed with KBr pellet (FTIR grade) and scanned on FTIR over the range of 4000-380 cm-1at a resolution of 4 cm-1. FTIR measurements were carried out for the identification of the possible functional molecules in the leaves extract responsible for the ion reduction and also the capping agents responsible for the stability of the biogenic nanoparticles solutions [(15)].

3- X-Ray diffraction analysis:

X-Ray diffraction (XRD) was performed to study the crystalline nature of AgNPs and AuNPs. For XRD analysis, dried form of the biogenic AgNPs and AuNPs were placed in the Shimadzu-Model, XRD 6000. The scanning mode at 40 kV with a current of 30 mA and Cu/Karadiation (in the 2q range of 2-80) was used for recording the diffraction pattern. The average particle size of the synthesized AgNPs and AuNPs was determined using the Debye-Scherrer equation.

$$D = \kappa\lambda/\beta \cos\theta D = \kappa\lambda/\beta \cos\theta$$

where D represents crystal size, κ represents shape factor (the ideal value of κ is 0.94), λ represents the X-ray wavelength (λ = 1.5418 A°), β represents the full width in radians at half maximum, and θ gives the Bragg's angle [(12)].

4- Scanning Electron Microscopy (SEM):

Scanning Electron Microscopic analysis was done by (JOEL-Model 6390). Thin films of the nanoparticles sample were prepared on a carbon coated gird, a very small amount of the specimen on the sample holder was placed and extra solution was removed using a blotting paper, then the film on the SEM allowed to dry under a mercury lamb for 5 minutes [(16)].

- **Antibacterial characterization of AgNPs and AuNPs:**

Antibacterial activity of biologically synthesized

nanoparticles besides synergistic efficacy of them with different antibiotics were determined according to agar well-diffusion method [16].The tested organisms were grown in nutrient broth (HIMEDIA Laboratories, Mumbai-India), several colonies of each of the tested bacteria were suspended with the help of sterile flamed loop into the tubes of nutrient broth. After mixing well, all the tubes were incubated at 37 ºC for 24 hours to produce bacterial suspensions discovered by the presence of turbidity. The turbidity of the cultural broth was compared with 0.5% McFarland Nephelometer standard by diluting with 0.9% NaCl solution to get 1.5 x 10^8 CFU/ml. the newly prepared and autoclaved Mueller-Hinton agar (HIMEDIA Laboratories, Mumbai-India) poured over on 60 sterile petri plates (3 plates/treatment) and it allowed to solidification. Agar wells of 8 mm diameter were made with the help of sterilized stainless steel cork borer (7 wells/ plate). Then the different bacterial suspensions swabbed uniformly into the individual plates using sterile cotton swabs. With the help of sterile micropipette, 50 µl of different prepared solutions (ethanolic plant extracts, nanoparticles solutions, antibiotics alone, antibiotics +nanoparticles) were loaded on cut wells of pre-sterilized medium of each plate. These plates were incubated at 37 ºC for 24 hours in inverted position to look for a circular area around the wells. The diameter of inhibition zone was measured in millimeter using measuring scale for each organism. The assay was performed in triplicate and tabulated.

- **Statistical analysis**

The values were given as mean ± SE and the data were analyzed by ANOVA test with least significant differences (LSD) at significant level of $P<0.05$ by using SPSS (Version 25.2021).

Results and Discussion

1- Antibacterial activity of AgNPs and AuNPs:

In this study, the efficacy of AgNPs, AuNPs and antibiotics in the terms of zones of inhibition (mm) was measured against five different bacteria (table 1, 2). The interactive effects of AgNPs or AuNPs with antibiotics were also investigated against those pathogenic strains. Different mixtures of ethanolic extract mediated nanoparticles, antibiotic+ethanolic extract mediated nanoparticles, antibiotic alone and ethanolic extract alone were loaded into the agar wells. Antibiotic alone and ethanolic extract alone were used as a control for the activity. For both types of AgNPs and AuNPs with their conjugates, the activity was performed on separate plates against each strain of tested bacteria (figure 2,3,4,5,6).

- **AgNPs and their conjugates:**

Although silver nitrate is an antibacterial agent and has been employed for this purpose, additional silver-based agents such as AgNPs should also be investigated. Antibacterial activity of silver nanoparticles has been demonstrated against a wide range of bacteria. The fact that Ag^+ is largely harmless to human and animal cells, as well as being particularly efficient against fungi and viruses, is a major advantage of using silver nitrate and silver compounds in general for antibacterial diseases. They can be manufactured in a variety of sizes, employed alone or in combination with other materials, are insoluble in water and can be removed after disinfection, and have a variety of additional qualities that make AgNPs appealing and interesting [17].

All the bacteria except *P. aeruginosa* was resistant to silver nitrate solution on plates related to LAgNPs and OAgNPs (zones of inhibition= 13 mm, 11.33 mm respectively). This data was compatible to what been previously mentioned about *E. coli* which developed resistance to aqueous silver nitrate and which survived exposure to greater concentrations than the 0.5 per cent solution [18]. Similarly, ethanolic extract of *L. inermis* showed limited antibacterial efficacy which was maximum against *E. coli* (13 mm) and *K. pneumonia* (11 mm) which agreed with inhibition zones gained by Raja and his colleagues in 2013 using *L. inermis* extract against a number of G^+ and G^- bacteria [19]. The antibacterial activity of *L. inermis* against bacterial isolates was also agreed with the data obtained by Habbal *et al* (2011) who showed a significant effect of this plant extract against *P. aeruginosa* [20].

In the case of LAgNPs, mild-moderated bactericidal activities were observed against all the bacterial strains except *K. pneumonia*, they were maximum against *E. coli* (25.33 mm), *P. aeruginosa* (16.33 mm), *C. freundii* (12 mm), and *S. typhimurium* (11 mm). Combination of antibiotics and nanoparticles resulted in significant increases in antibacterial activity (zone of inhibition) when compared with nanoparticle, ethanolic extract and silver nitrate, but to some extent, they were produced higher or lower efficacy in comparison to antibiotic alone, which was also agreed with Raja, *et al* (2013) who also noticed a reduction the antibiotics therapeutic dose when combined with the plant\nanoparticle extract [19].

Ciprofloxacin+LAgNPs conjugate showed activity against the entire tested microbial community which was maximum against *S. typhimurium* (39.66 mm), *P. aeruginosa* (39 mm), *E. coli* (38.66 mm), *C. freundii* (31.33 mm), and *K. pneumonia* (36.66 mm). Whereas ciprofloxacin alone showed bactericidal activity against *P. aeruginosa* (42 mm), *E. coli* (36 mm), *S. typhimurium* (33.33 mm), *C. freundii* (31 mm), and *K. pneumonia* (27.33 mm). overall antibacterial combination of cephalexin+LAgNPs revealed the maximum efficacy against *K. pneumonia*, *E. coli*, *S. typhimurium*, and *C. freundii* (26.66 mm, 25 mm, 24.66 mm, and 21 mm respectively). *P. aeruginosa* was resistant to cephalexin+LAgNPs conjugate, but it was sensitive to cephalexin alone in a similar way to that showed by all the other tested bacteria with maximum zone of inhibition; 30 mm against *E. coli*, 26.33 mm against *K. pneumonia*, 25.33 mm against *P. aeruginosa* and 19.66 mm against *C. freundii*.

Among the tested bacteria, only *E. coli* was sensitive to the antibacterial activity of ethanolic extract of *O. europaea* (zone of inhibition was 14 mm). *K. pneumonia* still resistant to *O. europaea* extract mediated silver nanoparticles just like to *L. inermis* extract mediated silver nanoparticles, while all the other used microbes exhibited efficacies of 27 mm, 14 mm, 13 mm, 12 mm against *E. coli*, *P. aeruginosa*, *S. typhimurium* and *C. freundii* respectively. Combination of OAgNPs and ciprofloxacin resulted in bactericidal effect against all the tested bacteria with maximum zone f inhibition; 43.66 mm against *S. typhimurium*, 37.66 mm against *E. coli*, 31.66 mm against *P. aeruginosa*, 30 mm against *K. pneumonia* and 29 mm against *C. freundii*. Ciprofloxacin alone exhibited antibacterial efficacy recorded as 40.33 mm, 38.66 mm, 37.33 mm, 29.33 mm and 25.33 mm against *P. aeruginosa*, *E. coli*, *S. typhimurium*, *K. pneumonia* and *C. freundii* respectively. OAgNPs+cephalexin conjugate could also revealed strong antibacterial activity in comparison to OAgNPs, its effect was maximum against *P. aeruginosa* (43.66 mm), *E. coli* (29.33 mm), *S. typhimurium* (27.33 mm), *K. pneumonia* (26 mm) and *C. freundii* (20.66 mm). On the same plates related to OAgNPs and their conjugates, all the tested microbes showed susceptibility in the range of 28 mm against *K. pneumonia* - 35 mm against *E. coli* for cephalexin.

Even while the reaction of Ag^+ ions with bacteria has been researched extensively for a long time, the inactivation process is unknown, and the reactivity of nanoparticles with these pathogens is even less so [21]. Silver nanoparticles have the capacity to penetrate bacterial cell walls, altering cell membrane structure and even causing cell death. Their effectiveness stems not only from their nanoscale size, but also from the enormous surface area to volume ratio [22]. By releasing silver ions, they can increase the permeability of cell membranes, allowing photogenerated single oxygen to react with DNA and hinder replication[23]. Ag^+ ions have been known to react with maltose transporter and fructose bisphosphate aldolase protein at a concentration of parts per billion, resulting in a decrease in expression [24, 25].

After Ag^+ has penetrated the cell wall, it has been discovered that it degrades many proteins and associates with bacterial DNA [26]. Silver is also engaged in catalytic oxidation events that result in R-S-S-R disulfides, which are formed when oxygen dissolved in the cell reacts with the hydrogen of the S-H thiol group [27]. The thiol bases in DNA are considered to be attacked by Ag^+ ions, which create dimers that impede DNA replication. In aqueous solutions, AgNP and silver metal release silver in the form of Ag^+ ions, which function as antibacterial agents in the same way as $AgNO_3$ does. As a result, the reactivity of these three species to bacteria is thought to be essentially same, however the pace of inactivation may differ significantly, most likely due to the rate of Ag^+ release. It's also been known for quite some time that Ag^+ ions form stable S-Ag bonds [28]. Silver enters the bacterial cell and intercalates between the purine and pyrimidine base pairs, disrupting H bonding between antiparallel strands and resulting in DNA denaturation and bacterial inactivation. Our findings show that silver in its ionic form, Ag^+, is an efficient antibacterial agent and that silver in its metallic onion form is mostly innocuous [29–31], as most researchers assume.

- **AuNPs and their conjugates:**

Due to their optical and electrical properties, which are highly dependent on their form and size, gold nanoparticles (AuNPs) have gotten a lot of interest. By changing the components and concentrations, AuNPs of various forms and sizes can be easily synthesized. AuNPs can be made spherical, rod-like, cage-like, and various morphologies in both experimental and industrial contexts. The majority of AuNPs investigations have looked at how they interact with diverse biomolecules such medicines, genes, peptides, and other targeted ligands. When compared to antibiotics or medications alone, AuNPs conjugated with antibiotics or pharmaceuticals have better antibacterial or antiviral action[32].

This study showed that gold chloride produced antibacterial efficacy against only two of the tested bacteria; *P. aeruginosa* and *C. freundii* on plates related to LAuNPs and OAuNPs and their conjugates in the range of 11-14 mm. *K. pneumonia* and *E. coli* showed sensitivity to the ethanolic extract of *L. inermis* (11mm and 14 mm respectively).

All the microbes were resistant to *L. inermis* extract mediated AuNPs except *E. coli* and *P. aeruginosa*, which showed efficacy around 19 mm. Ciprofloxacin and LAuNPs combination exhibited an increase in the zones of inhibition in comparison to gold chloride, *L. inermis* extract and LAuNPs alone, maximum inhibition zones were 40.33 mm, 40 mm, 35.3 mm, 34.66 mm, and 30.33 mm against *P. aeruginosa*, *C. freundii*, *S. typhimurium*, *E. coli*, and *K. pneumonia* respectively. Ciprofloxacin alone produced efficacy against all the tested strains, zones of inhibition were maximum against *C. freundii* (40 mm), *P. aeruginosa* (36 mm), *E. coli* (36 mm), *S. typhimurium* (35.66 mm) and *K. pneumonia* (28.66 mm). For all the five strains, maximum inhibition zones of cephalexin + *L. inermis* extract mediated AuNPs to *P. aeruginosa*, *S. typhimurium*, *E. coli*, *K. pneumonia* and *C. freundii* were 48.33 mm, 30.66 mm, 24.66 mm, 24.66 mm, and 22 mm respectively. Cephalexin alone had also showed bactericidal activity against all the tested bacteria, it was maximum against *E. coli* (38 mm), *P. aeruginosa* (30.66 mm), *C. freundii* (30.66 mm), *S. typhimurium* (30 mm) and *K. pneumonia* (27.33 mm). In plates related to OAuNPs and their conjugates, ethanolic extract of *O. europaea* was effective only against *E. coli* (zone of inhibition was 14 mm). Among the tested microbes, only *E. coli* (25 mm) and *P. aeruginosa* (19 mm) were sensitive to *O. europaea* extract mediated AuNPs. Antibacterial combination of ciprofloxacin and *O. europaea* mediated AuNPs showed the maximum efficacy in the range of 31-39 mm against the entire tested microbes, similar results showed by ciprofloxacin alone against all the bacterial

strains ranging from 30.33-36.66 mm. In the case of cephalexin +*O. europaea* extract mediated AuNPs, antibacterial activities were maximum with *P. aeruginosa* (44.33 mm),*S. typhimurium* (41.33 mm), *E. coli* (25.33 mm), *K. pneumonia* (24 mm), and *C. freundii* (22.33 mm), on the other hand, cephalexin alone produced inhibitory effect against the entire tested bacterial community in the range of 24.33-32.33 mm.

Our data were compatible with previous studies about the effect of combining gold nanoparticles with some well-known antibiotics to maximize its antibacterial effect. A study by Payne and his colleagues in (2016) investigated the antibacterial activities of kanamycin and AuNP-kanamycin conjugates against Gram-positive *Staphylococcus epidermidis* and Gram-negative *Enterobacter aerogenes*, concluding that the conjugate's minimum inhibitory concentration was significantly lower than free kanamycin [(33)]. Another study in 2017 (Bagga *et al*., 2017) found that 272-nm AuNP-levofloxacin conjugates were more effective than levofloxacin alone in killing *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* bacteria[(34)]. Gold has low toxicity to biological systems, whether bacteria, animal, or human, due to its elemental properties [(35)].

Because of their non-toxicity, versatility in surface modification, polyvalent effects, and photothermal effects, AuNPs could be valuable in the development of antibacterial techniques. AuNPs can potentially operate as antibiotic transporters or delivery vehicles, boosting the antibiotics' bactericidal activity[(36)]. AuNPs are antifungal, however the evidence for their antibacterial efficacy is mixed. AuNPs are either not bactericidal or just slightly bactericidal at high dosages. However, the bactericidal activity of co-existing molecules not totally eliminated from AuNPs, such as gold ions, surface coating agents, and compounds used in the synthesis, could explain why AuNPs appear to be bactericidal. AuNPs can potentially operate as antibiotic transporters or delivery vehicles, boosting the antibiotics' bactericidal activity[(37)]. Furthermore, conjugation of AuNPs stabilizes the particles, improving delivery efficacy. Bactericidal actions are better in condensed compounds with high delivery efficacy. AuNPs may interact with bacterial components such as lysosomes, ribosomes, and enzymes, changing the permeability of the cell membrane, causing electrolyte imbalance, enzyme inhibition, and protein deactivation, according to one theory [(38,39)].

The experimental data shows that when the two or three components react together within a dose range, a synergistic process is at work, and when they react alone against bacteria, it is additive.

Table 1: individual and combined efficacy of antibiotics and silver nanoparticles against selected bacteria (average ± standard deviation)

Zone of inhibition (mm)									
OAgNPs and their conjugates							LSD value		
$AgNO_3$	*O. europaea* extract	OAgNPs	OAgNPs + C	OAgNPs + K	C	K			
0±0	0±0	0±0	30±0	26± 0.57	29.33±0 .33	28±1	1.423		
0±0	14±0	27±0	37.66± 1.45	29.33± 0.33	38.66±0 .33	35.66± 0.66	1.723		
0±0	0±0	13±0	43± 1.73	27.33± 0.66	37.33±0 .33	32.33± 0.33	1.622		
11.33± 0.33	0±0	14±0	31.66± 0.33	43.66± 1.85	40.33±0 .33	29.66± 0.33	1.414		
0±0	0±0	12±0	29± 0.57	20.66± 0.33	25.33±0 .33	31.33± 0.66	2.014		

Values are mean n=3

Abbreviations: $AgNO_3$,silver nitrate; **LAgNPs**, *L. inermis* extract + silver nanoparticle; **LAgNPs + C**, *L. inermis* extract + silver nanoparticles + ciprofloxacin; **LAgNPs + K**, *L. inermis* extract + silver nanoparticles + cephalexin; **C**, ciprofloxacin; **K**, cephalexin; **OAgNPs**, *O. europaea* extract + silver nanoparticle; **OAgNPs + C**, *O. europaea* extract + silver nanoparticles + ciprofloxacin; **OAgNPs + K**, *O. europaea* extract + silver nanoparticles + cephalexin.

Test organisms	LAgNPs and their conjugates						
	$AgNO_3$	*L. inermis* extract	LAgNPs	LAgNPs + C	LAgNPs + K	C	K
Klebsiella pneumonia	0±0	11±0	0±0	26.66± 0.33	26.66± 0.33	27.33±0 .88	26.33±0 .88
Escherichia coli	0±0	13±0	25.33± 0.66	38.66± 1.2	25±0	36±1	28± 1.52
Salmonella typhimurium	0±0	0±0	11±0	39.66± 0.57	24.66± 0.57	33.33±4 .16	30±0
Pseudomonas aeruginosa	13±0	0±0	16.33± 0.33	39±1	0±0	42± 3.5	25.33±0 .33
Citrobacter freundii	0±0	0±0	12±0	31.33± 0.33	21± 0.57	31± 0.57	19.66±0 .33

Table 2: individual and combined efficacy of antibiotics and gold nanoparticles against selected bacteria (average ± standard deviation)

Zone of inhibition (mm)					
OAuNPs and their conjugates					LSD value
OAuNPs	OAuNPs + C	OAuNPs + K	C	K	
0±0	31± 0.57	24±0	30.33± 0.33	24.33±0 .33	1.423
25±0	39± 0.57	25.33± 0.33	36± 0.57	26.66±0 .66	1.723
0±0	34.33± 0.57	41.33± 1.15	33.66± 0.57	32.33±0 .57	1.622
19± 0.57	34.33± 0.33	44.33± 0.33	34±0	30±0	1.414
0±0	31± 0.57	22.33± 1.45	36.66± 0.88	26.66±0 .33	2.014

Values are mean n=3

Abbreviations: $AuCl_4$, gold chloride; **LAuNPs**, *L. inermis* extract + gold nanoparticle; **LAuNPs + C**, *L. inermis* extract + gold nanoparticles + ciprofloxacin; **LAuNPs + K**, *L. inermis* extract + gold nanoparticles + cephalexin; **C**, ciprofloxacin; **K**, cephalexin; **OAuNPs**, *O. europaea* extract + gold nanoparticle; **OAuNPs + C**, *O. europaea* extract + gold nanoparticles + ciprofloxacin; **OAuNPs + K**, *O. europaea* extract + gold nanoparticles + cephalexin.

Test organisms	LAuNPs and their conjugates									
	$AuCl_4$	*L. inermis* extract	LAuNPs	LAuNPs + C	LAuNPs + K	C	K	$AuCl_4$	*O. europaea* extract
Klebsiella pneumonia	0±0	11±0	0±0	30.33± 0.33	24.66± 0.33	28.66±0 .33	27.33± 0.33	0±0	0±0
Escherichia coli	0±0	14±0	19.66± 0.33	34.66± 0.33	24.66± 0.33	36±1	38±1	0±0	14±0
Salmonella typhimurium	0±0	0±0	0±0	35.33± 0.57	30.66± 1.52	35.66±0 .57	30±0	0±0	0±0
Pseudomonas aeruginosa	12±0	0±0	19±0	40.33± 0.33	48.33± 2.02	36±1	30.66± 0.33	12±0	0±0
Citrobacter freundii	11±0	0±0	0±0	40±0	22± 1.52	40± 0.66	30.66± 0.66	14±0	0±0

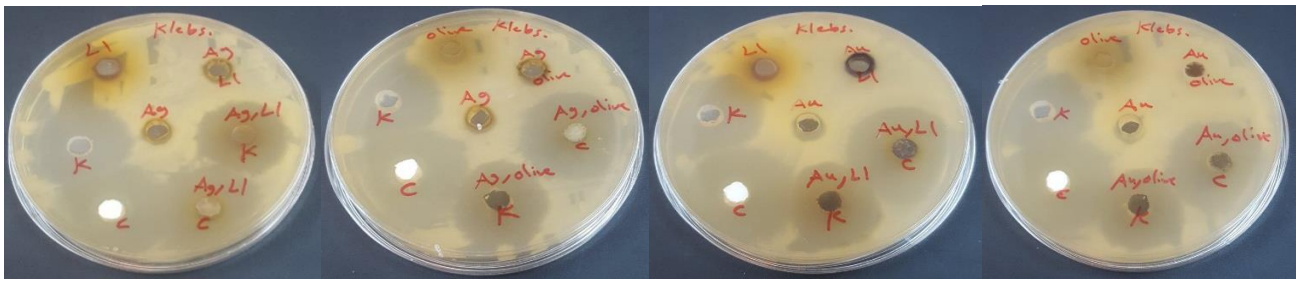

Figure 2: effect of AgNPs, AuNPs, and their conjugates on the growth of Klebsiella pneumonia

Figure 3: effect of AgNPs, AuNPs, and their conjugates on the growth of Escherichia coli

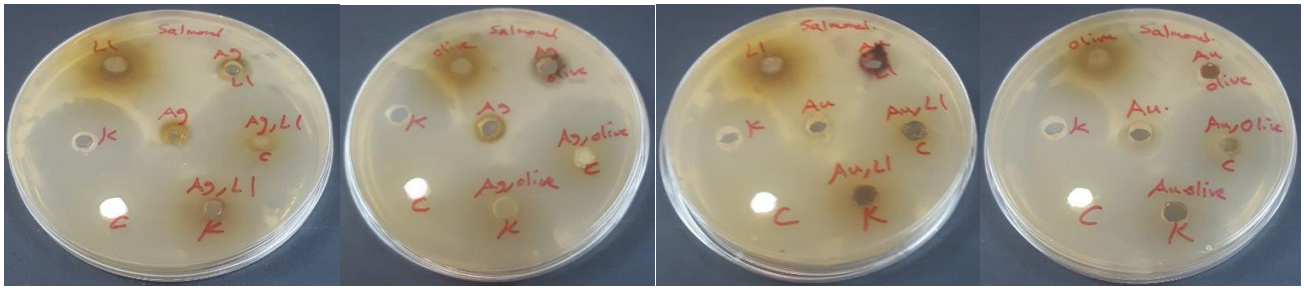

Figure 4: effect of AgNPs, AuNPs, and their conjugates on the growth of Salmonella typhimurium

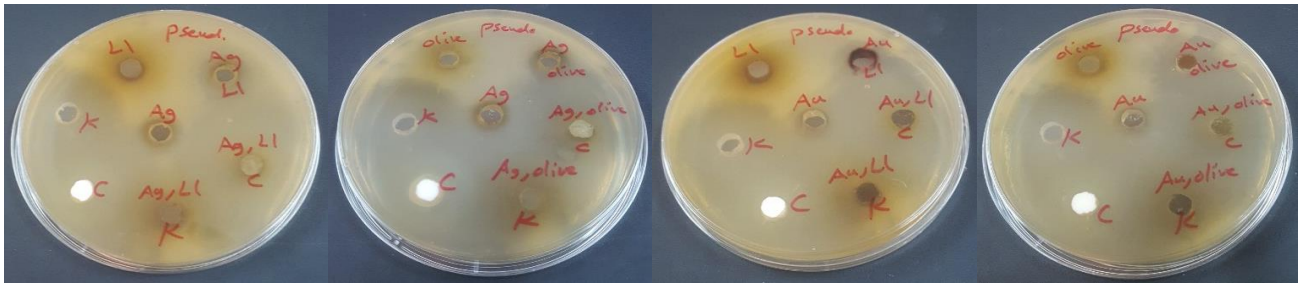

Figure 5: effect of AgNPs, AuNPs, and their conjugates on the growth of Pseudomonas aeruginosa

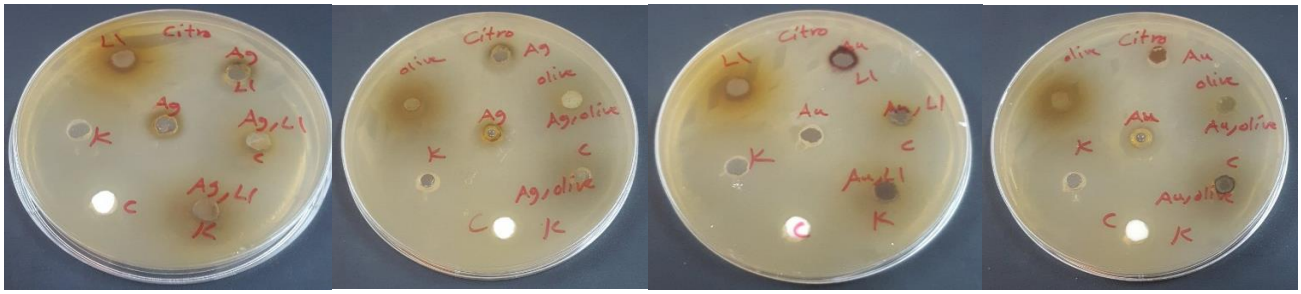

Figure 6: effect of AgNPs, AuNPs, and their conjugates on the growth of Citrobacter freundii

2- UV-visible spectroscopy:

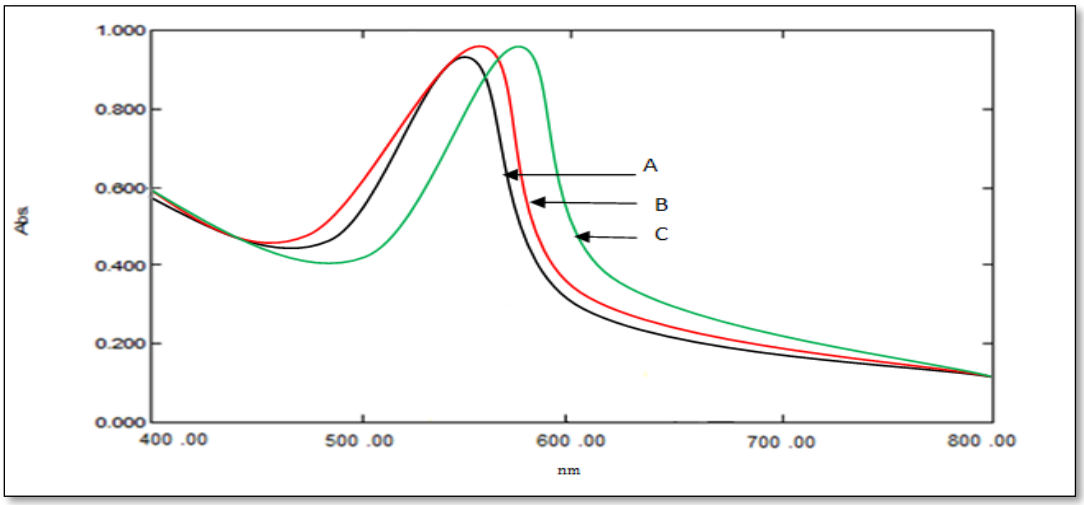

Figure 7: UV-Vis spectra of A. AuNPs. Only B. L. inermis mediated AuNPs C. O. europaea mediated AuNPs

Clear red shift in the λmax of AuNPs after reduction by *L. inermis* and *O. europaea* (λmax of AuNPs is 543 nm) agree with literatures [40,41].

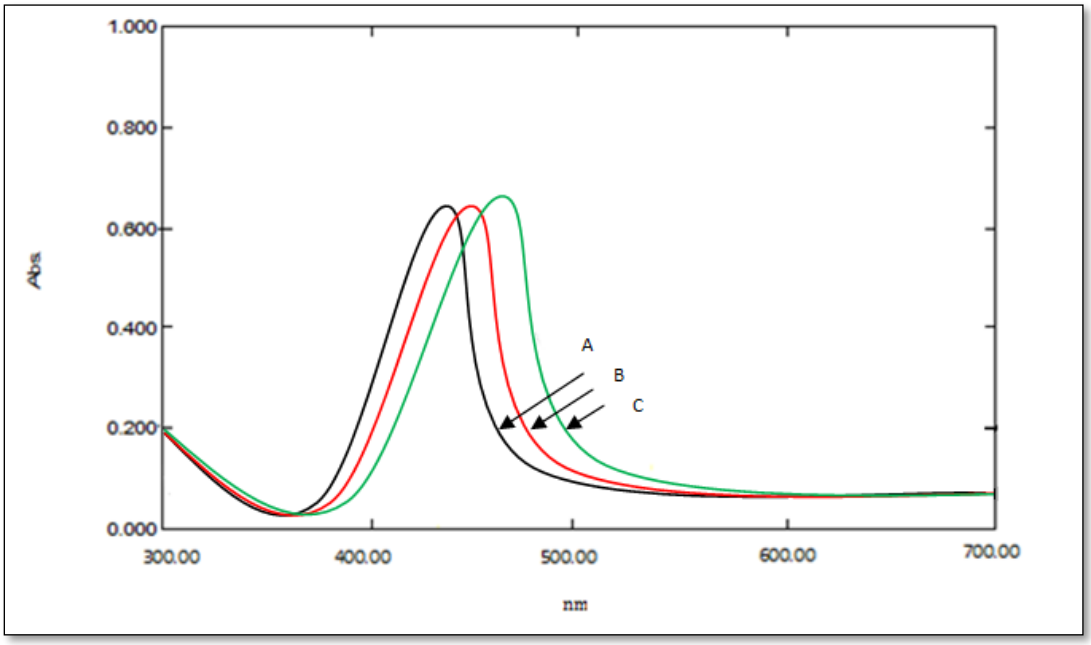

Figure 8: UV-Vis spectra of A. AgNPs. Only B. L. inermis mediated AgNPs C. O. europaea mediated AgNPs.

Clear red shift in the λmax of silver nitrate nanoparticles after reduction by *L. inermis* and *O. europaea* (λmax of AgNPs is 431 nm) agree with literatures[42,43].

3- Fourier Transform Infrared Spectroscope (FTIR):

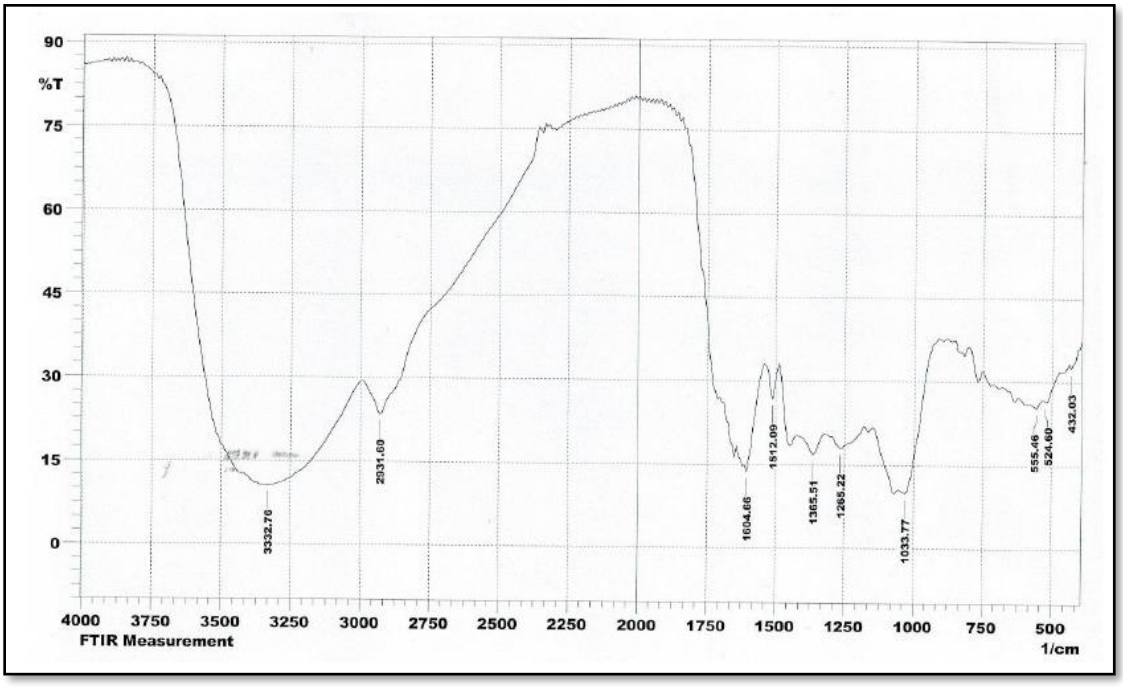

Figure 9: IR spectra for Lawsonia inermis ethanolic extract using FTIR

Three major functional groups which can be derived from the structure are phenols O-H and C=O and alkenes C=C [44].They are also known as flavonoids which consists of carbonyl group (C=O, Ketone), O-H group, and aromatic ring

[45]. Figure 9 shows the IR spectrum of *L. inermis*. The phenolic group (O-H) stretch appeared at 3332.76 cm-1. The aromatic C=C stretching frequency appeared at 1604.2 cm -1 [46].

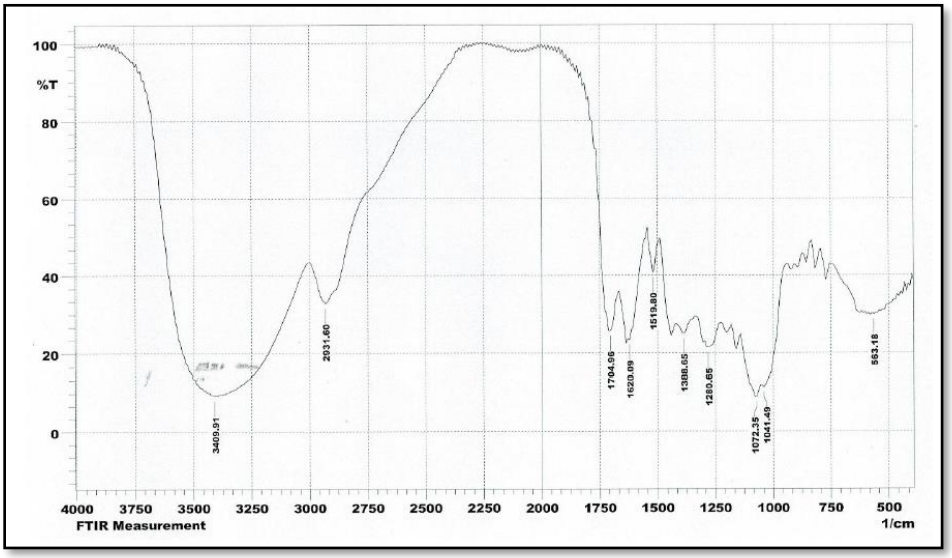

Figure 10: IR spectra for Olea europaea ethanolic extract using FTIR

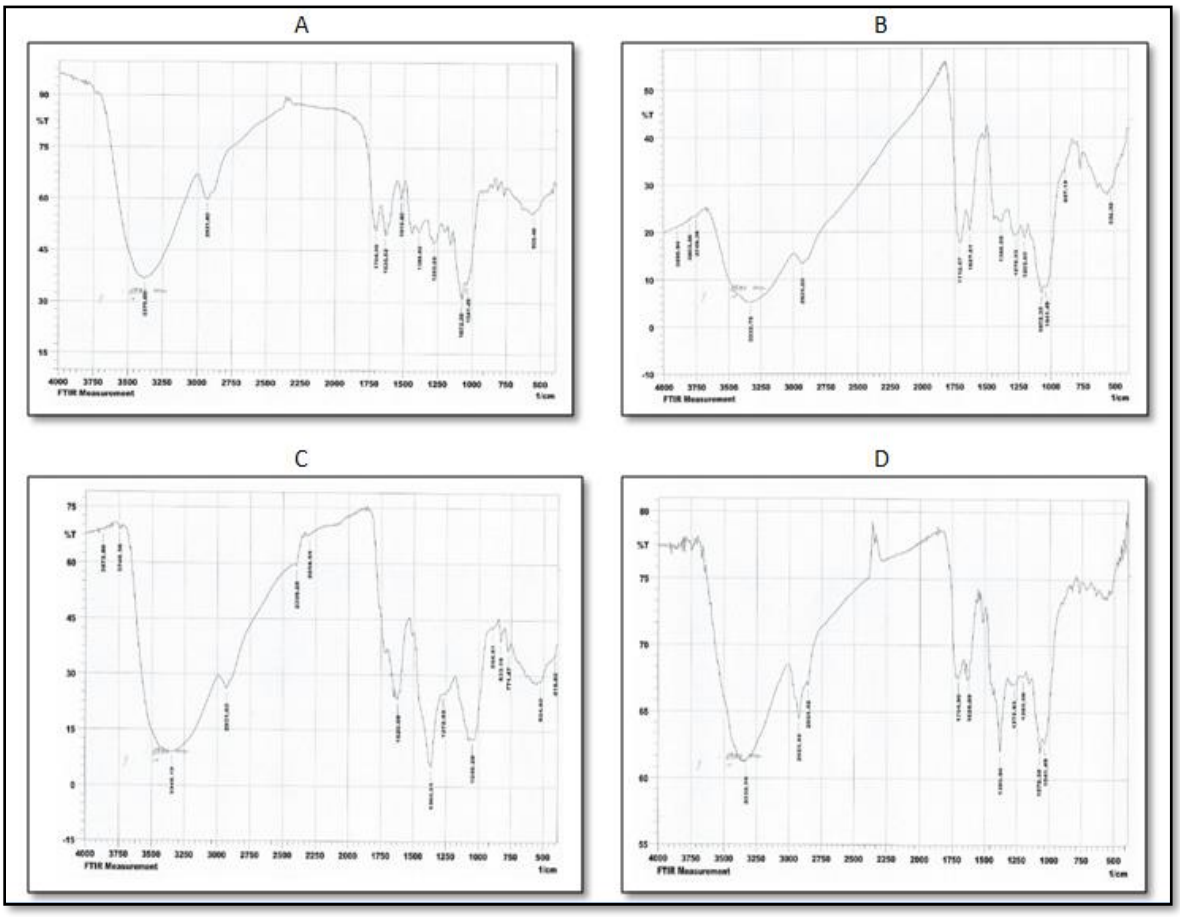

Figure 11: IR spectrum of; A. L. inermis mediated AuNPs, B. O. europaea mediated AuNPs, C. L. inermis mediated AgNPs D. O. europaea mediated AgNPs

A peak observed at 3409.91 cm-1 is due to the stretching of the N-H bond of amino groups and indicative of bonded hydroxyl (-OH) group. The strong absorption peak at 2931.6 cm-1 could be assigned to -CH stretching vibrations of -CH3 and -CH2 functional groups. The shoulder peak at 1704.96 cm-1 assigned for C=O group of carboxylic acids". "The peak IR bands observed at 3409.91 and 1704.96 cm-1 in OLE were characteristic of the O-H and C=O stretching modes for the OH and C=O groups possibly of oleuropein, apigenin7-glucoside and/or luteolin-7-glucoside present in the olive leaf " [47,48]. "The peak at 1620.09 cm-1 indicated the fingerprint region of CO, C-O and O-H groups, which exists as functional groups of *O. europaea* leaves extract. The absorption peaks at 1620.09 cm-1 could be attributed to the presence of C-O stretching in carboxyl coupled to the amide linkage in amide I. The band at 1519.8 cm-1 was characteristic of amide II arises as a result of the N-H stretching modes of vibration in the amide linkage. The band at 1388.65 cm-1 assigned to the methylene scissoring vibrations from the proteins". "The intense band at 1076 cm-1 can be assigned to the C-N stretching vibrations of aliphatic amines [47] or C-OH vibrations of the protein in the *O. europaea* leaf " [48].

"FTIR study of both *O. europaea* leaves extract and *L. inermis* leaves extract with gold and silver nanoparticles respectively indicated that the carboxyl (-C=O), hydroxyl (-OH) and amine (N-H) groups of leaves extract were mainly involved in reduction of nanoparticles. Proteins present in the extract can bind through either free amino or carboxyl groups in the proteins" [49].

4- X-Ray diffraction analysis:

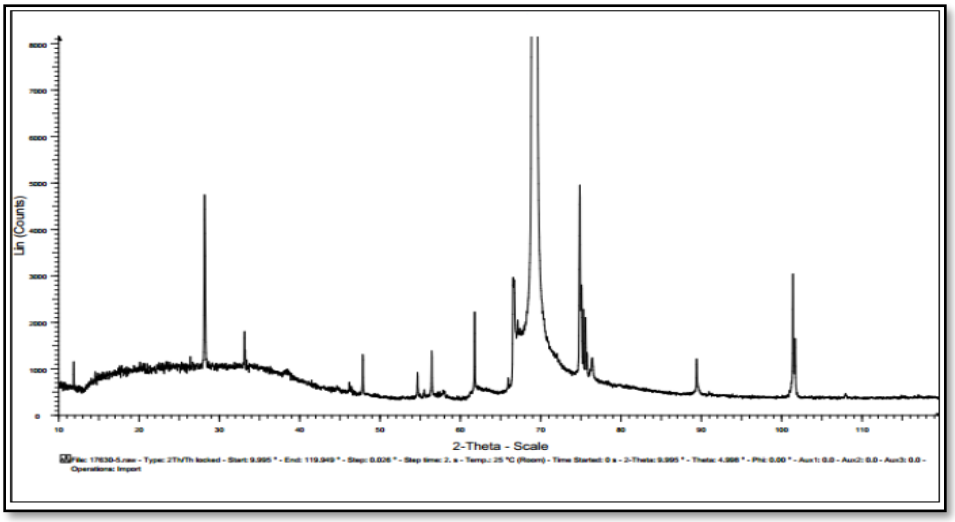

Figure 12: XRD of L. inermis mediated AuNPs

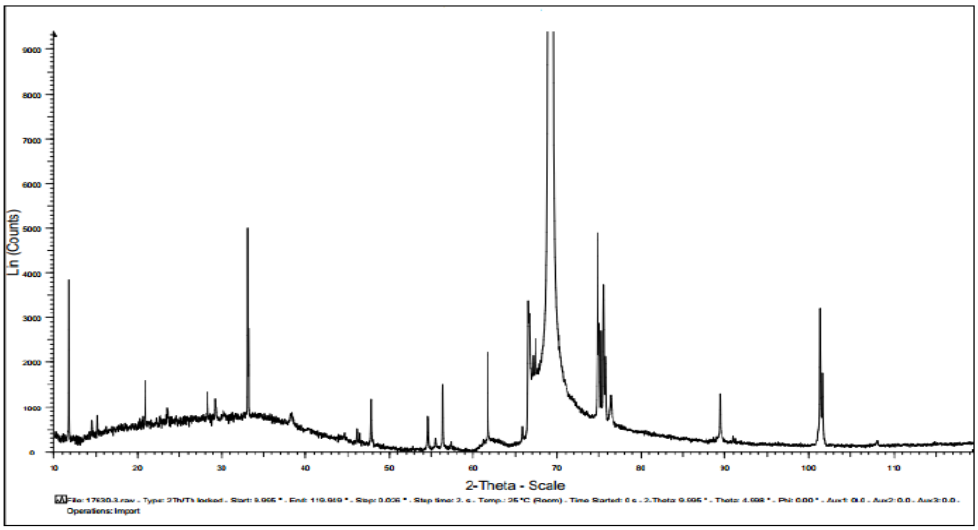

Figure 13: XRD of O. europaea mediated AuNPs

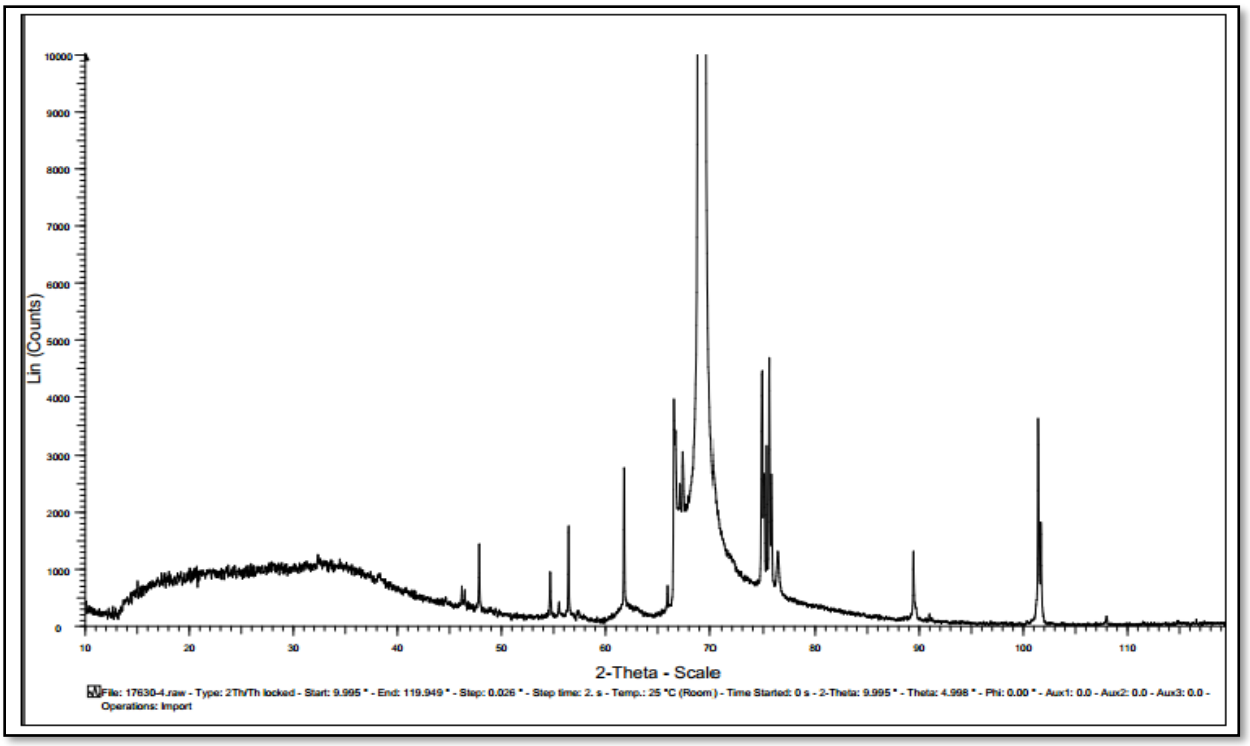

Figure 14: XRD of L. inermis mediated AgNPs

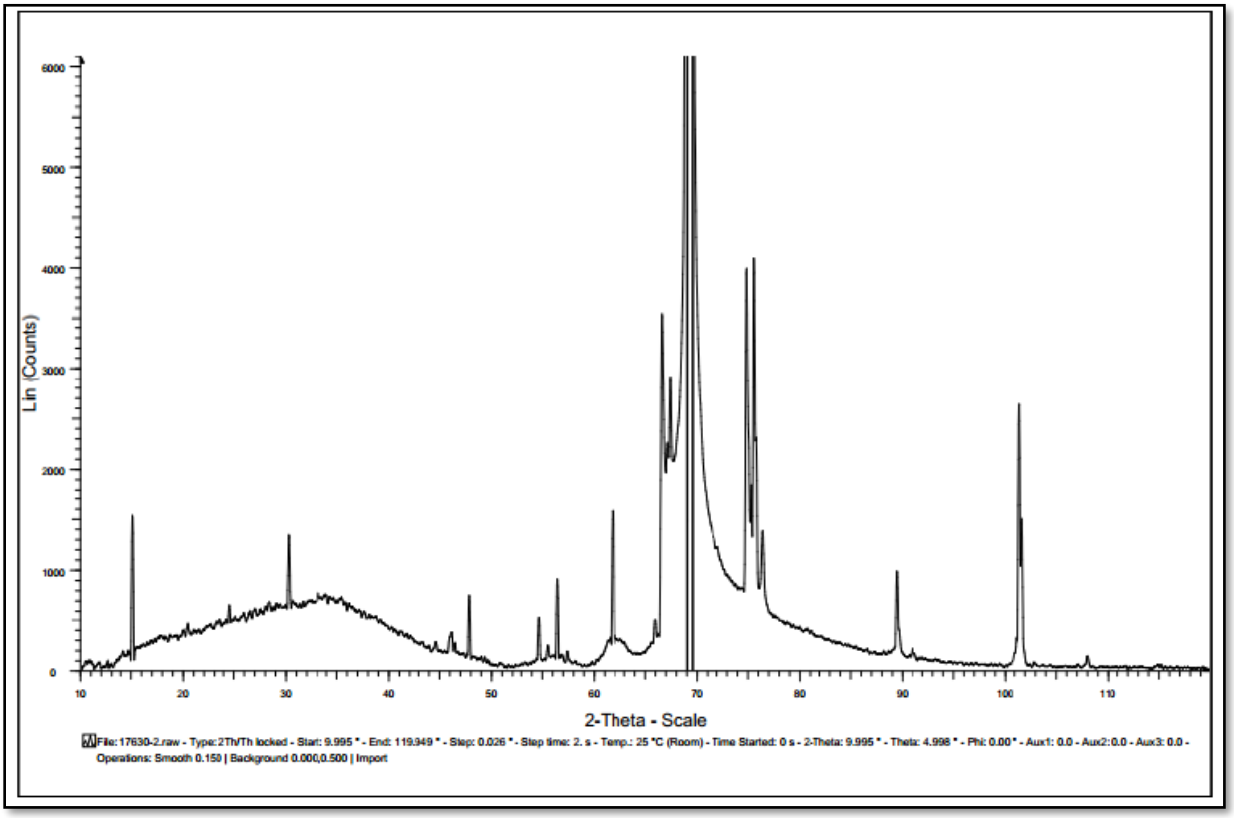

Figure 15: XRD of O. europaea mediated AgNPs

AgNPs in both extracts had a similar diffraction profile, and XRD peaks at 2θ of 32.18°, 69.72° and 77.40° could. The XRD pattern thus clearly illustrated that the Ag-NPs formed

in this study were crystalline in nature. The main crystalline phase was silver, and there were no obvious other phases as impurities were found in the XRD patterns (figure 14, 15). The diffraction peaks at $2\theta = 64.67°$, 69.88° and 77.45° obtained are identical with those reported for the standard gold metal.

5- Scanning Electron Microscopy (SEM):

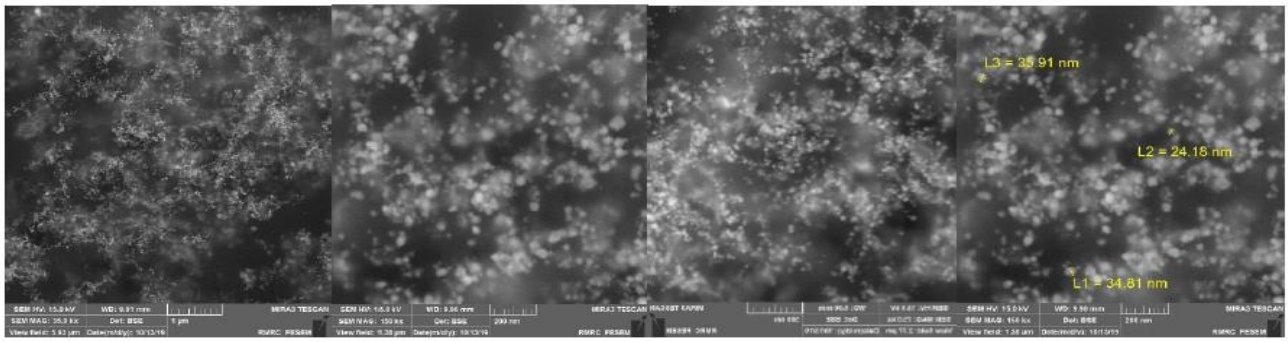

Figure 16: SEM of L. inermis mediated AuNPs

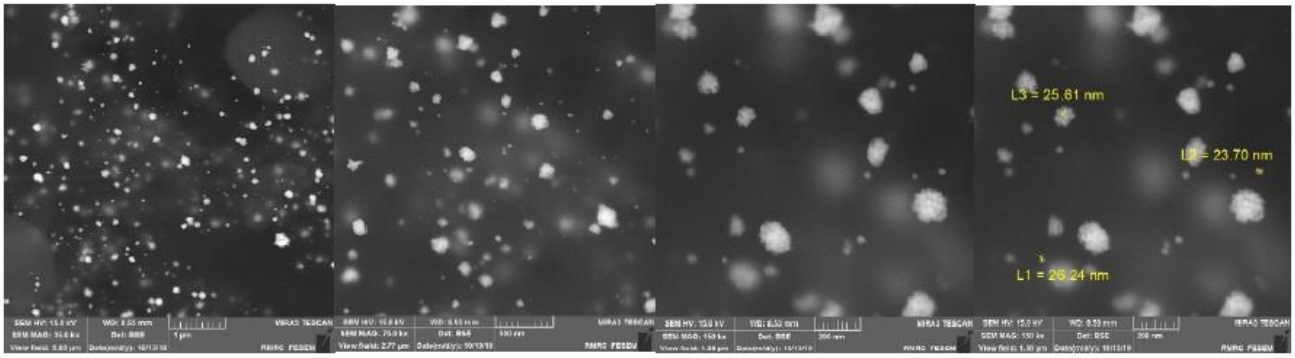

Figure 17: SEM of O. europaea mediated AuNPs

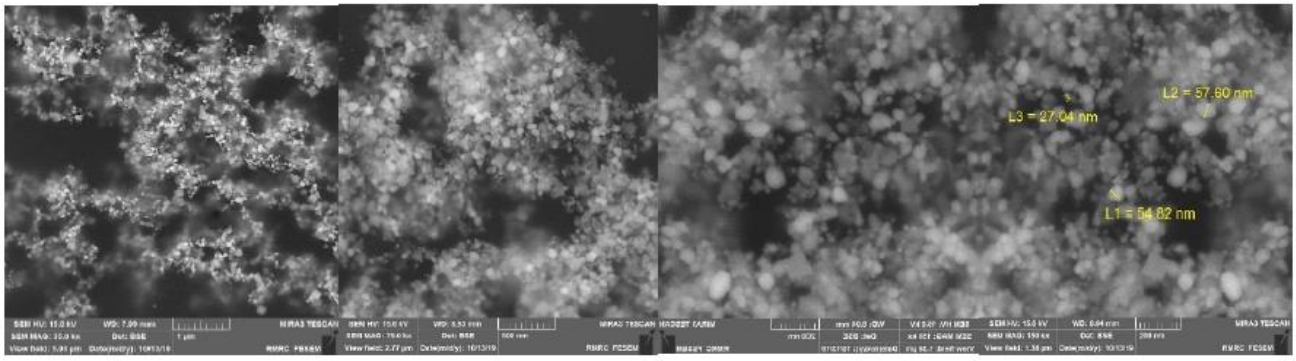

Figure 18: SEM of L. inermis mediated AgNPs

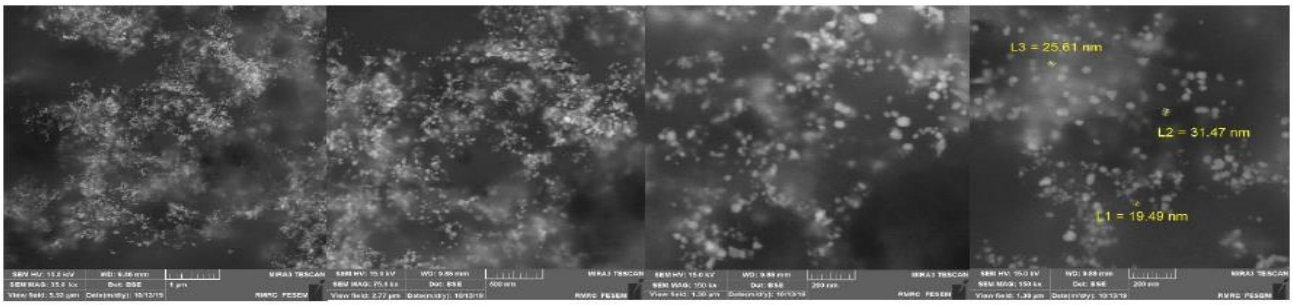

Figure 19: SEM of O. europaea mediated AgNPs

SEM is a sensitive technique in which electrons are used instead of light to form an image. So simply SEM is a type of electron microscope that produce images of a sample by scanning it with a focused beam of electrons. In the present research work scanning electron microscopy of AuNPs of *O. europaea* leaves and *L. inermis* leaves extract was carried out to find the particles shape and morphology. The SEM results revealed that the gold nanoparticles possessed spherical shape with particle size range of about 23-35 nm figure 16,17. It's clearly observed the aggregation of AuNPs in case of *L. inermis* extract compared to that of *O. europaea* extract. The SEM images of the silver nanoparticles are shown in figure 18, 19. The surface morphology of silver nanoparticles showed spherical nature. In the present study, the histogram of the particle size ranges from 19 to 57 nm.

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