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Green synthesis of silver nanoparticles using *Azadirachta indica* and *Ocimum sanctum* and assessment of its antimicrobial activity against isolates and pure culture of *Escherichia coli*

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Abstract
Nowadays plant-mediated green synthesis of nanoparticles has been increasingly gaining popularity due to its eco-friendly nature and cost-effectiveness. In this present study, synthesis of silver nanoparticles was done using leaf extracts of *Azadirachta indica* and *Ocimum sanctum* when added with 1mM silver nitrate solution at different conditions. For synthesizing silver nanoparticles, the plant leaf extracts act as both reducing as well as capping agents due to the presence of flavonoids and terpenoids. Various techniques were used to characterize synthesized silver nanoparticles such as UV-Visible spectrophotometer, Zeta-potential method, and Size distribution by intensity. The absorption spectrum of the silver nano solution prepared by using *Azadirachta indica* and *Ocimum sanctum* leaf extracts showed a surface plasmon absorption band with a maximum of 430 nm and 420 nm respectively. The zeta value of silver nanoparticles synthesized from *Azadirachta indica* and *Ocimum sanctum leaf* extract was -8.87 mV and -12.3 mV, size of distribution intensity was 112.3 nm and 156.9 nm respectively. In this study, the silver nanoparticle's antimicrobial activity was compared both on isolates and pure culture of *Escherichia coli* using agar well diffusion method and also compared the antimicrobial resistance of antibiotics on isolates and pure culture of *Escherichia coli*. Results confirmed this green synthesis protocol as simple, rapid, eco-friendly, non-toxic, and an alternative conventional physical and chemical methods and showed better antimicrobial activity to *Escherichia coli*.

Keywords: Green synthesis, silver nanoparticles, Uv-visible spectroscopy, antimicrobial activity, gram-negative bacteria

1. Introduction
Nanotechnology is the study and application of tiny objects that may be used across different fields such as chemistry, biology, physics, and engineering [1]. Nano means a billionth or 10^{-9} units. its size ranges from 1 to 100 nm because it is too small in size and it occupies a position in various fields of nanoscience and nanotechnology [2]. Nanoparticles play an indispensable role in drug delivery, diagnostics, imaging, sensing, gene delivery, artificial implants, and tissue engineering [3]. Nanoparticles can be synthesized through different methods: chemical, physical and biological methods. But those physical and chemical methods are often extremely expensive and non-environmentally friendly due to the use of toxic, combustible, and hazardous chemicals, which may pose potential environmental and biological risks and high energy requirements [4]. The other drawbacks are low production rate, structural particle deformation, and inhibition of particle growth. Recently, green synthesis has gained importance over other physical and chemical methods, since, it offers environmentally friendly, cheap, biocompatible, shape and size-controlled nanoparticles. Various synthesis methods are available in green synthesis routes based on using plants, bacteria, and fungi [5]. With their antioxidant or reducing properties, they are usually responsible for the reduction of metal compounds into their respective nanoparticles.
Nowadays, the use of medicinal plants has been increased by traditional medical practitioners for treating various diseases. Among the all-medicinal plants, *Azadirachta indica* (commonly known as neem) a species of the family *Meliaceae*, each part of this tree has been used as a household remedy for antiquity, treatment against viral, bacterial and fungal infections. Silver nanoparticles can be formed at low concentrations of their leaf extract without using any extraneous chemical or physical technique [6].

Ocimum sanctum belongs to the labiates family, is a medicinal plant, contains eugenol the active constituent (l-hydroxy-2-methoxy-4-allylbenzene), which is mainly responsible for therapeutic potentials of the central nervous system, cardiovascular system, reproductive system, gastric system, blood biochemistry, immune system, and urinary system and also significant in various ailments in modern medicine [7]. The enzymes present in leaf extract combines with silver ions to form an enzyme-substrate complex with a charge transfer between quercetin and Ag+ resulting in the formation of protein-capped silver nanoparticles.

Nowadays antibiotics are extensively used in the medical field; their prolonged exposure enables the bacteria to develop resistance. This may be the result of bacteria's self-defense mechanism due to which mutation in genes might occur, which facilitates the formation of enzymes that inactivate antibiotics [8]. Thus, antibiotic resistance in bacteria has become a major issue of concern. To overcome this challenge, scientists have reported several new approaches; the antimicrobial activity of NPs being one of them. Amongst various NPs, silver is the most potent antibacterial agent. Besides, AgNPs are used as nanocarriers for drugs and antibiotics which help in enhancing the activity of antibiotics against resistant microbes [9]. The antibacterial activity of AgNPs depends upon their size and shape [10]. AgNPs generate reactive oxygen species (ROS) that are responsible for the oxidation of cellular components i.e., cellular DNA and protein. AgNPs binds to the membrane of the cells and alter their permeability by changing the membrane potential [11]. Once it enters the cell, these AgNPs get converted into silver ions and start interacting with cellular biomolecules that cause damage to the cells. It hinders DNA replication by binding with DNA.

In the present study, we report the green synthesis of AgNPs by using *Azadirachta indica* and *Ocimum sanctum* leaf extract and also evaluation of their antimicrobial action against both isolates and pure culture of *Escherichia coli* and also compare the antimicrobial activity with certain antibiotics by disc diffusion method.

2. Materials and Methods

2.1 Collection of plant leaves and preparation of leaf extract

Fresh leaves of *Azadirachta indica* (Fig.1) and *Ocimum sanctum* (Fig.2) leaves were collected from different places in and around the College of Veterinary Science, Rajendranagar, and transported to the laboratory, Department of Veterinary Public Health and Epidemiology. The leaves were washed several times with water to remove any foreign particles including dust and followed to shade dry. For *Azadirachta indica*, 20 g leaves were crushed to a fine powder using a mortar and pestle, and that powder was added to 100 ml of double-distilled water, boiled for 10 min, cooled, and filtered through Whatman No.1 filter paper for further use. Similarly, 20 g of finely chopped leaves of *Ocimum sanctum* was added to 100 ml of double-distilled water, stirred at 60 ^{0}C for 1h. The extract obtained was filtered through Whatman No.1 filter paper and the filtrate was collected, stored till further use

Fig 1: *Azadirachta indica* leaves

Fig 2: *Ocimum sanctum* leaves

2.2 Synthesis of Silver Nanoparticles

One hundred milliliters of 1 mM silver nitrate solution were prepared and every 10 ml of silver nitrate solution was transferred into 5 test tubes. Then 1, 2, 3, 4, and 5 ml of *Azadirachta indica* leaf extract was added to five test tubes later incubated in the dark chamber to avoid photo activation till the colorless solution changed to brown color (Fig.3). Similarly, 5 ml of 1mM silver nitrate solution was transferred into 5 test tubes and added 1, 2, 3, 4, and 5 ml of *Ocimum sanctum* leaf extract, later incubated in the dark chamber to avoid photo activation till pale yellow solution changes to deep mustard yellow color (Fig.4).

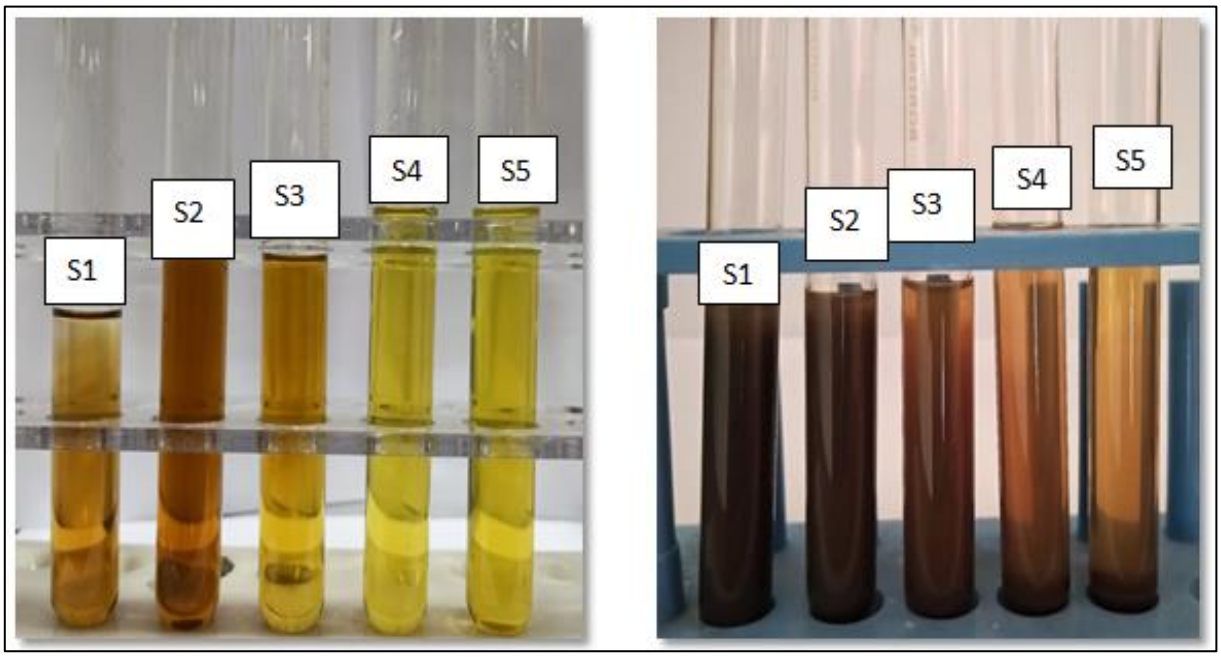

Fig 3: to $AgNO_3$ and after an incubation period, the color turns into brown color

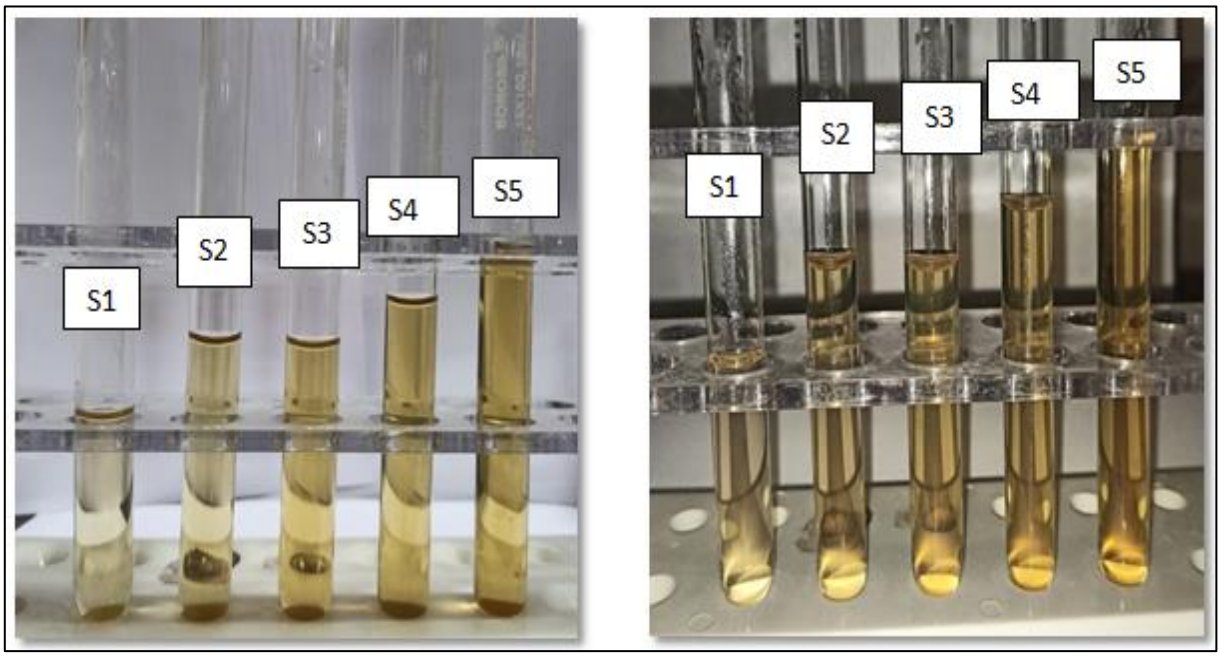

Fig 4: Addition of *Ocimum sanctum* leaf extract to $AgNO_3$ and after an incubation period, the color turns into dark yellow color

2.3 Characterization of synthesized silver nanoparticles

2.3.1 UV-Visible spectrophotometer

The silver nanoparticles were confirmed by measuring the wavelength of the reaction mixture in the UV-vis spectrum of the Thermo fisher spectrophotometer at a resolution of 1 nm (from 300 to 600 nm) in a 2 ml quartz cuvette with 1 cm path length. One milliliter of the sample was pipette into a test tube and subsequently analyzed at room temperature.

2.3.2 Zeta potential and Size distribution by intensity

The prepared sample was dispersed in deionized water followed by ultra-sonication. Afterward, the solution was filtered and centrifuged for 15 min at 25°c at 5000 rpm and the supernatant was collected. The supernatant was diluted 4 to 5 times and the particle distribution in liquid was studied in a computer-controlled particle size analyzer (ZETA sizer nano series, Malvern instrument Nano Zs).

2.4 Antimicrobial analysis

2.4.1 Isolation of *Escherichia coli* from poultry samples

For *Escherichia coli* isolation, A total of 150 poultry samples (50 each from chicken fecal, cloacal, and egg swabs) were aseptically collected from college farms. The isolation of *Escherichia coli* was carried out by using BPW broth for the enrichment and EMB agar media. The presumptive *Escherichia coli* colonies were subjected to Gram staining and various biochemical tests (Table.1)

2.4.2 Pure strains

The pure strain of *Escherichia coli* was obtained from MTCC at Chandigarh, India. All the cultures were maintained at 4^0c in nutrient broth and sub cultured in nutrient agar at regular intervals of days

Table 1: Biochemical tests for *Escherichia coli*

S. No	Tests	*Escherichia coli*
1	Gram staining test	Negative (-ve)
2	Motility test	Motile
3	Indole test	positive
4	Methyl red test	positive
5	Voges – Proskauer test	Negative
6	Simmons citrate	Negative
7	TSI	Acid butt (Y), Alkaline slant (Y), H_2S (-), Gas production

Antimicrobial susceptibility of the isolates was done by the disc diffusion assay with Muller-Hinton (MH) agar and following CLSI recommendations. MH broth was inoculated with five colonies of the isolate and tubes were incubated at 37 ^{0}C for 2-8 h until achieving turbidity equivalent to 0.5 on the Mac Farland scale. After turbidity adjustment, a sterile swab was introduced, pressed against the tube well to remove any excess liquid, and then seeded on the surface of a petri dish containing MH agar, for *Escherichia coli* rotating at least twice. Using sterile forceps 4 discs (Table.2) impregnated with antimicrobials and 50 µl each of AgNPs of *Azadirachta indica* and *Ocimum sanctum*, $AgNO_3$ solution, *Azadirachta indica*, and *Ocimum sanctum* pure leaf extracts and Distilled water were placed at equal distances from each other on the surface of an inoculated agar plate. Subsequently, the plate was inverted and incubated at 37 ^{0}C for 24 h. Disc readings were performed after incubation and the diameter of inhibition halos was measured with the aid of a ruler. The interpretation was made as per the zone size interpretation chart provided by the manufacturer of discs. The antibacterial activity against pure culture and isolates of *Escherichia coli* from poultry samples was compared.

Table 2: Antibiotics used in the antibiotic resistance/susceptible test

S. No	Antibiotics	Abbreviations	Concentration (µg/unit)
1.	Chloramphenicol	C	30
2.	Ampicillin	AMP	10
3.	Ciprofloxacin	CIP	5
4.	Tetracycline	TE	30

3. Results

3.1 UV- Visible Spectroscopy

The silver nanoparticles were characterized by UV- visible spectroscopy, for structural characterization of silver nanoparticles. The absorption spectrum of the brown silver nano solution prepared by using *Azadirachta indica* leaf extract showed a surface plasmon absorption band with a maximum of 430 nm, (Fig.5) indicating the presence of silver nanoparticles The absorption spectrum of the deep mustard yellow silver nano solution prepared by using *Ocimum sanctum* leaf extract showed a surface plasmon absorption band with a maximum of 420 nm (Fig.6), indicating the presence of silver nanoparticles.

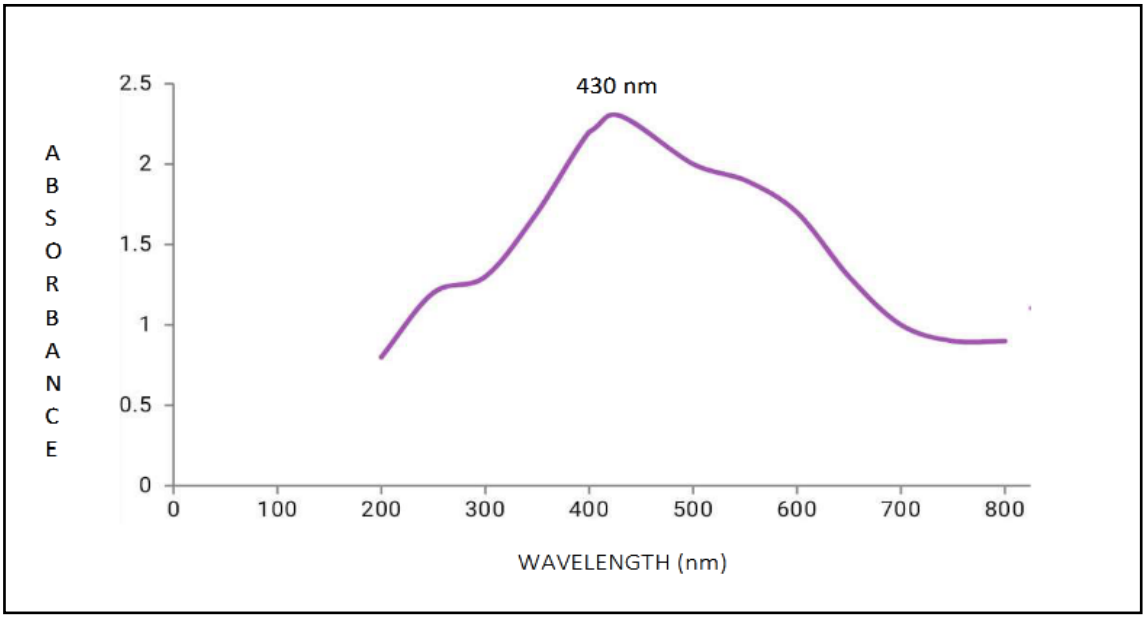

Fig 5: UV- Visible absorption peak for AgNPs prepared with *Azadirachta indica* showing a peak at 430 nm, sample (10:3)

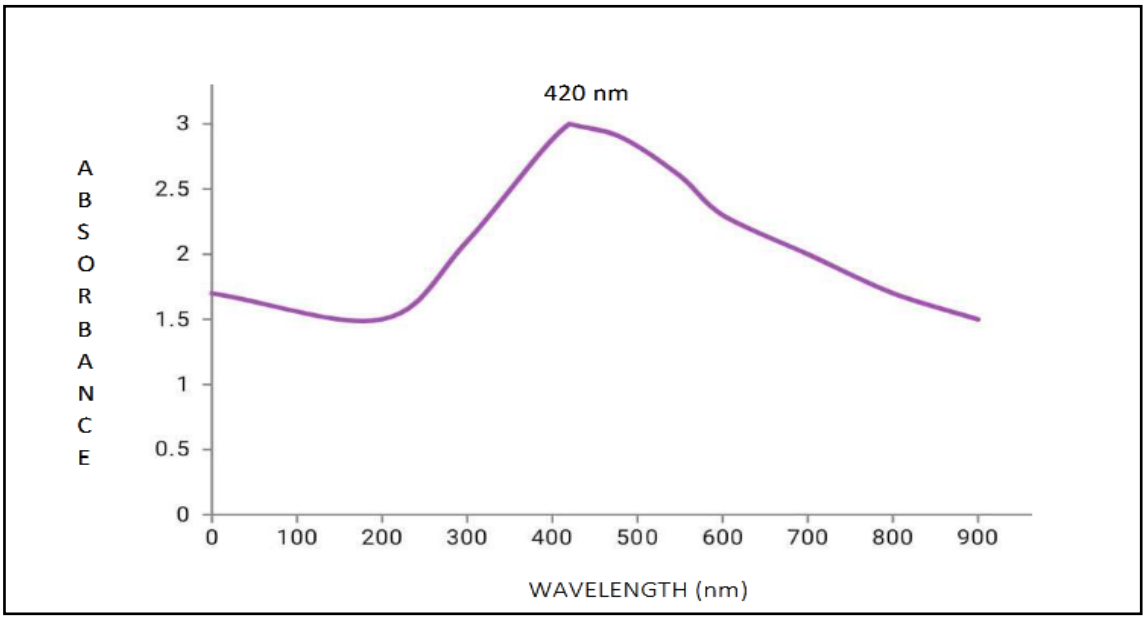

Fig 6: UV-Visible absorption peak for AgNPs prepared with *Ocimum sanctum* showing a peak at 420 nm, sample (5:5)

3.2 Zeta potential and size distribution by intensity

Zeta potential measures the potential stability of the nanoparticles in the colloidal suspension. The zeta potential values of silver nanoparticles synthesized from *Azadirachta indica* and *Ocimum sanctum* leaf extract were -8.87 mV and -12.30 mV respectively (Fig.7 and Fig.8). The DLS measures size of the silver nanoparticles was 112.3 nm (Fig.9) and 156.9 nm (Fig.10) for AgNPs with *Azadirachta indica* and *Ocimum sanctum* respectively. These zeta potential values indicate the good stability of silver nanoparticles synthesized.

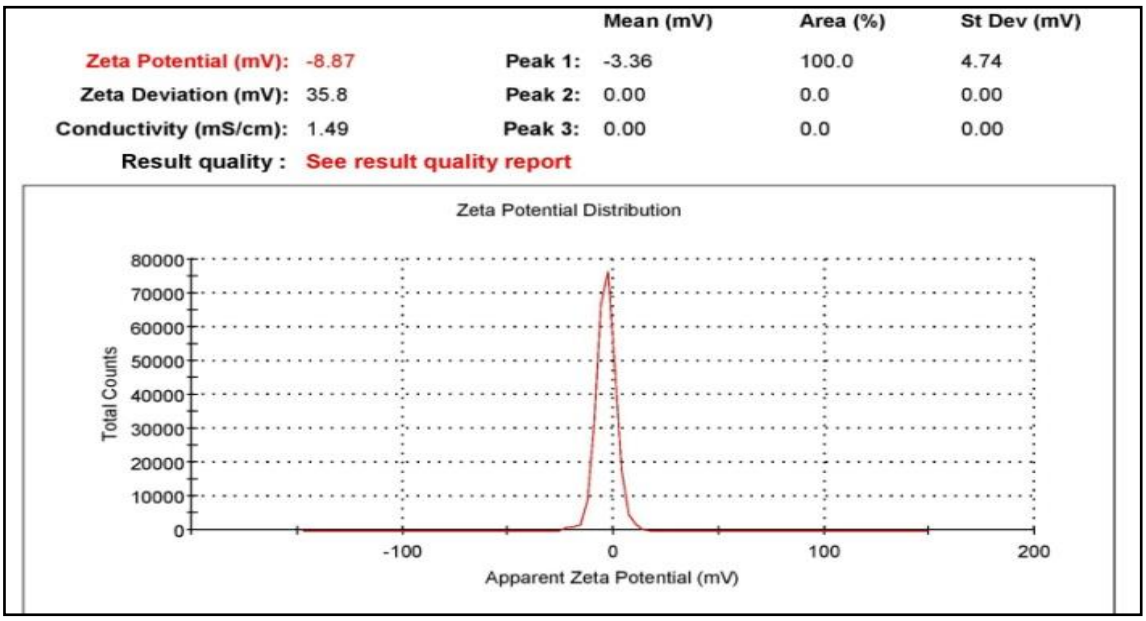

Fig 7: Zeta potential distribution of AgNPs prepared with *Azadirachta indica* leaf extract

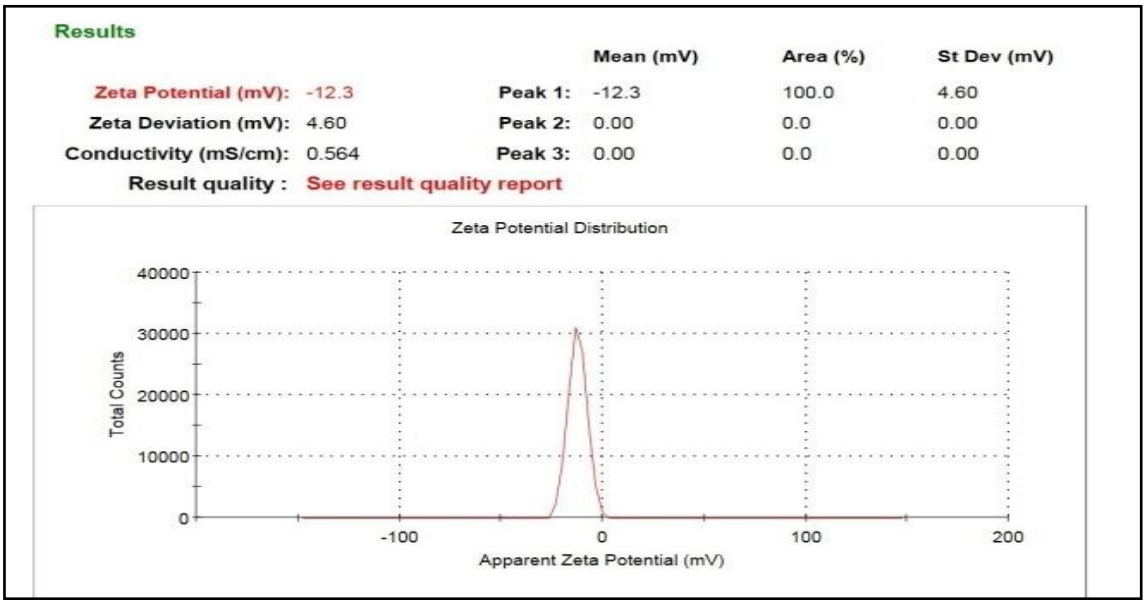

Fig 8: Zeta potential distribution of AgNPs prepared with *Ocimum sanctum* leaf extract

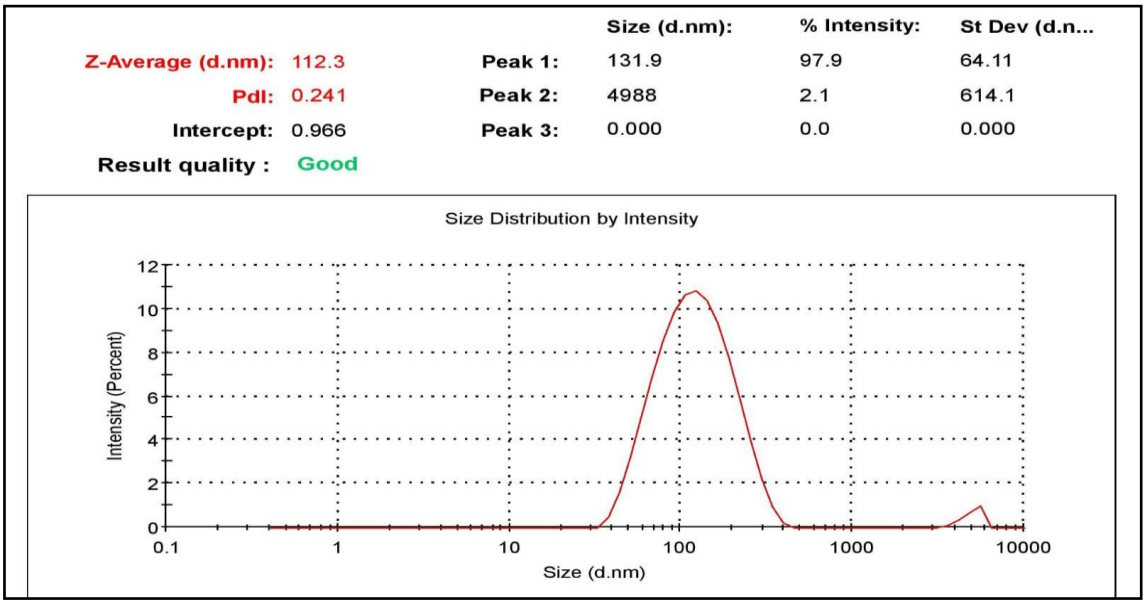

Fig 9: Size distribution by intensity (DLS) of AgNPs prepared with *Azadirachta indica* leaf extract

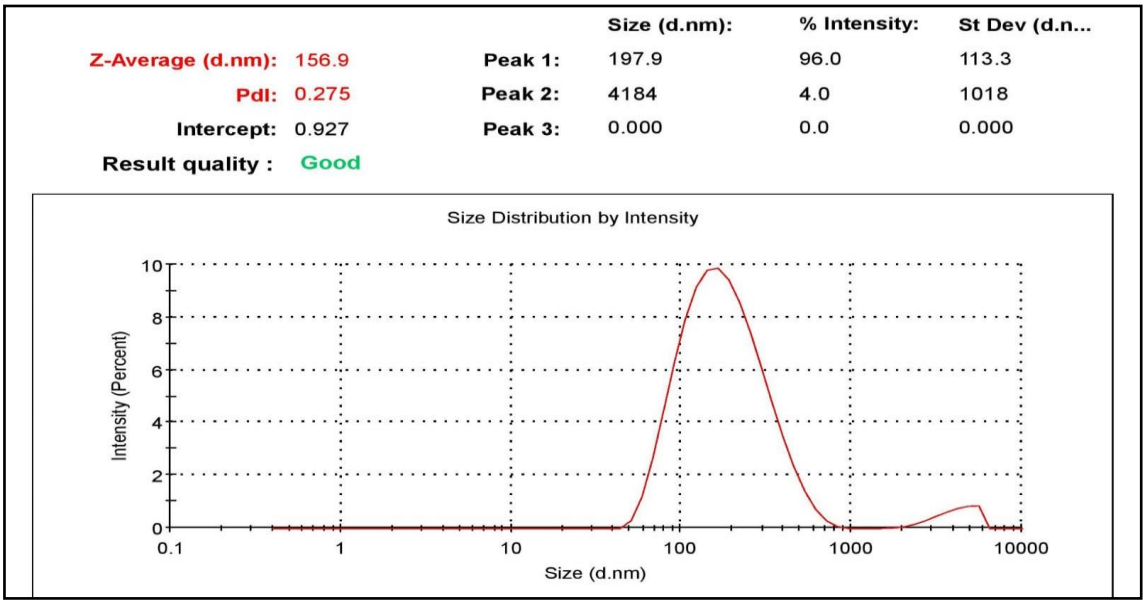

Fig 10: Size distribution by intensity (DLS) of AgNPs prepared with *Ocimum sanctum* leaf extract

3.3 Isolation and Identification of *Escherichia coli* by a cultural method

Out of 150 poultry samples (50 each of fecal samples, egg and cloacal swabs) 95 (63.33%) samples were positive for *Escherichia coli* by a cultural method. The isolation of *Escherichia coli* was carried out by using BPW broth for the enrichment and EMB agar media. *Escherichia coli* often produces a metallic green sheen on Eosin methylene blue agar (Fig.11). The presumptive colonies were subjected to gram staining (Fig.12) and biochemical tests (Fig.13) for confirmation.

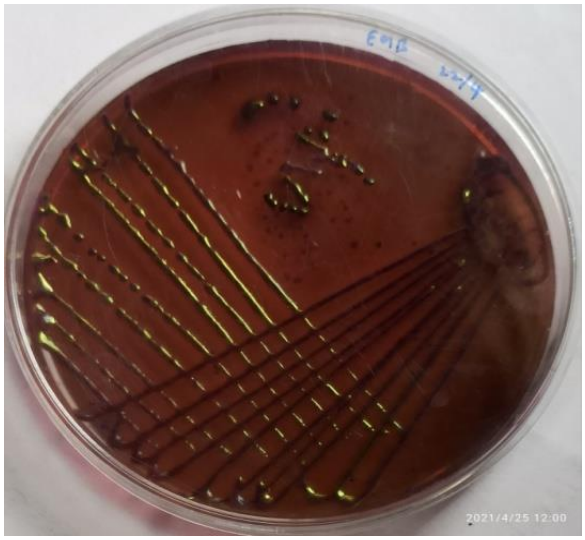

Fig 11: Plate showing the growth of *Escherichia coli* on EMB media

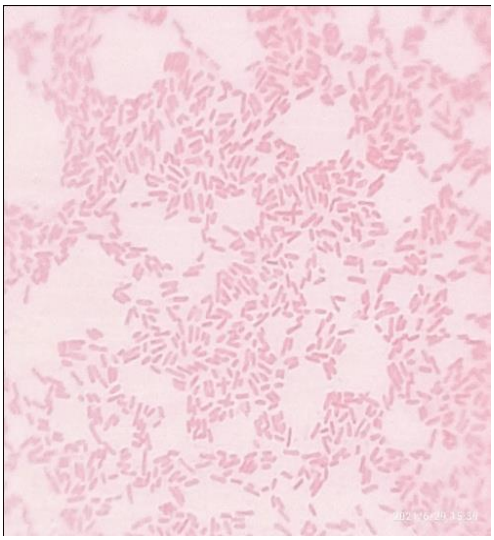

Fig 12: Gram staining for *Escherichia coli*

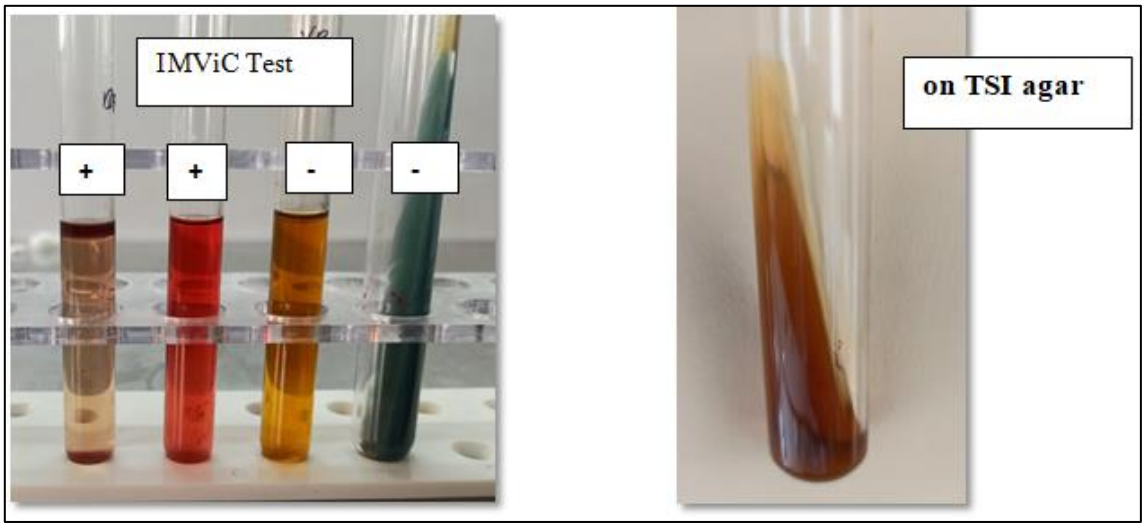

Fig 13: Results of Biochemical Tests for *Escherichia coli* (++--)

3.4 Antibacterial Activity against *Escherichia coli*

The antibiotic activity of leaf extracts of *Azadirachta indica* and *Ocimum sanctum*, Pure silver nitrate ($AgNO_3$) solution, and silver nanoparticles (AgNPs) prepared with *Azadirachta Indica*, *Ocimum sanctum* leaf extracts and Antibiotics on isolates and pure culture of *Escherichia coli* was present in the Table.3, Fig.14-17. Distilled water has been taken as control which showed no mean of ZOI against *Escherichia coli.*

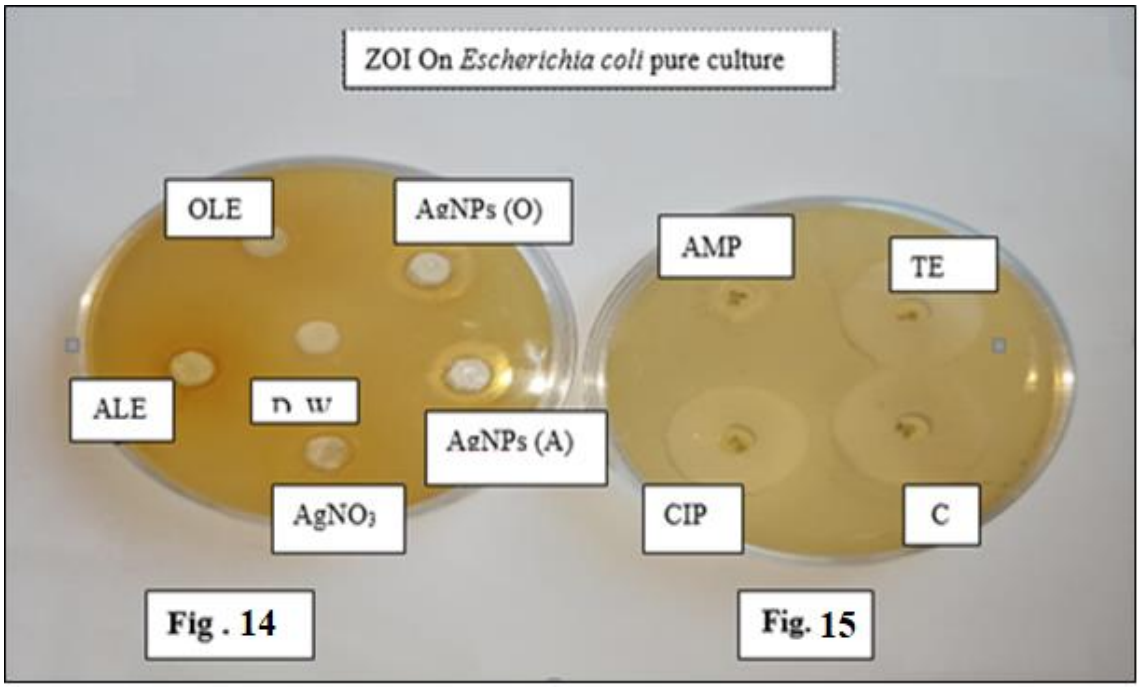

Fig 14: ZOI of *Azadirachta indica* leaf extract, *Ocimum sanctum* leaf extract, $AgNO_3$ solution, AgNPs prepared from *Azadirachta indica*, and *Ocimum sanctum* leaf extracts for a pure culture of *Escherichia coli*.

Fig 15: ZOI of Antibiotics for a pure culture of *Escherichia coli*

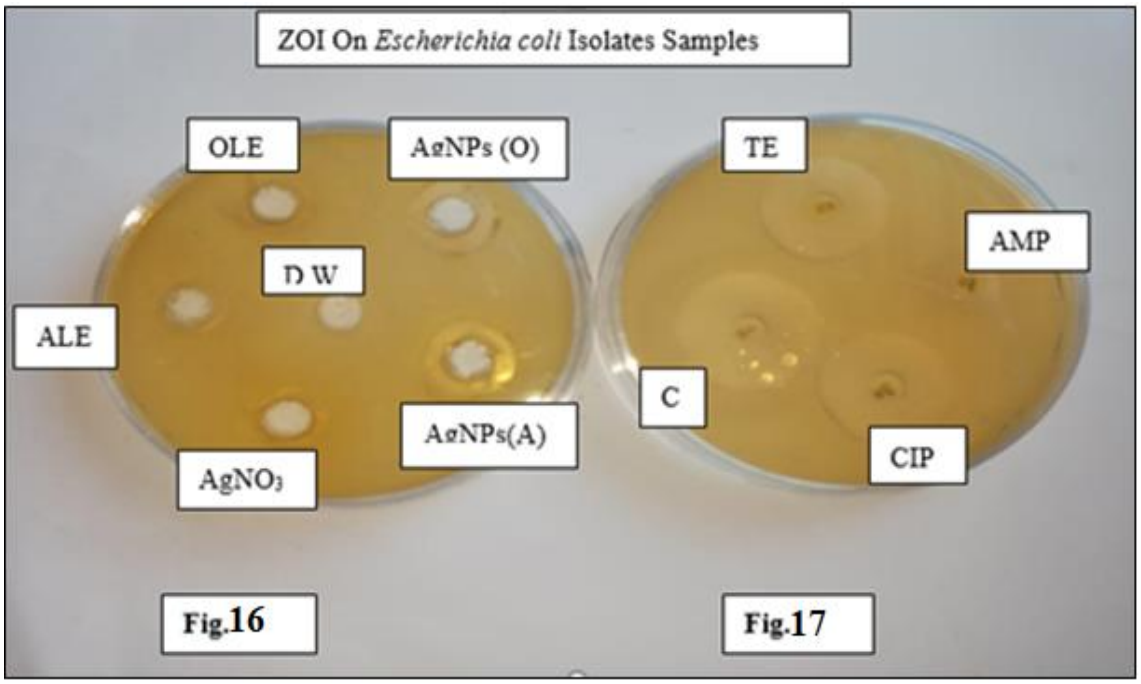

Fig 16: ZOI of *Azadirachta indica* leaf extract, *Ocimum sanctum* leaf extract, $AgNO_3$ solution, AgNPs prepared from *Azadirachta indica*, and *Ocimum sanctum* leaf extracts for isolates of *Escherichia coli*.

Fig 17: ZOI of Antibiotics for Isolates of *Escherichia coli*

$AgNO_3$: Silver nitrate solution**;** ALE: *Azadirachta indica* leaf extract**;** OLE: *Ocimum sanctum* leaf extract

AgNPs (O): Silver Nanoparticles prepared with *Ocimum sanctum*

AgNPs (A): Silver Nanoparticles prepared with *Azadirachta indica*

A: Ampicilin**;** CIP: Ciprofloxacin**;** C: Chloramphenicol**;** TE: Tetracycline

Table 3: The antibacterial activity of Antibiotics, Silver nitrate solution, and Silver nanoparticles on Pure cultures and Isolates of *Escherichia coli* from Poultry Samples

S. No	Antibiotic/ material	Zone of inhibition (mm)	
		On pure culture	On isolates (Mean±SE)
1	$AgNO_3$Solution	10	9.04±0.23
2	*Azadirachta indica leaf extract*	4	3.02±0.46
3	*Ocimum sanctum* Leaf extract	2	1.02±0.08
4	*Azadirachta indica* AgNPs	16	14.01±0.19
5	*Ocimum sanctum* AgNPs	15	13.02±0.21
6	Chloramphenicol	24	23.16±0.20
7	Ciprofloxacin	21	19.44±0.18
8	Tetracycline	15	14.06±0.28
9	Ampicillin	13	11.13±0.21

4. Discussion

4.1 Physical examination of silver nano solution

When the *Azadirachta indica* leaf extract was added to the colorless silver nitrate solution, the color changed from the colorless solution changes to the brown color in the present study. The color change of silver nitrate solution into colorless to dark brown color indicates that silver nanoparticles were formed. The color was changed into dark brown color for silver nano solution prepared with *Azadirachta indica* leaf extract [12, 13]. The color of silver nitrate solution turns yellowish to reddish-brown color [14]; whereas reddish yellow to brown color observed [15, 16]; the color changed from transparent to brown color for silver nanoparticles prepared by using *Azadirachta indica* leaf extract [17]. In the present study, the color of *Ocimum sanctum* leaf extract was pale yellow, and when it was added to the silver nitrate solution pale yellow solution changed to dark yellow color. The color change of silver nitrate solution into pale yellow to dark yellow color indicates that silver nanoparticles were formed. the color changes from transparent to pale yellow, yellow, reddish, and finally wine-red color for the silver nanoparticles at different time intervals prepared with *Ocimum sanctum* leaf extract [18]. The color of silver nitrate solution changed from pale yellow to dark yellow [19]; whereas pale yellow to reddish-brown color [20, 21]; The color of silver nitrate changed from transparent to dark yellow color for the silver nanoparticles prepared by using *Ocimum sanctum* leaf extract [22, 23]. The appearance of the final colors confirms the reduction of silver nitrate into silver nanoparticles due to the excitation of free electrons in the reaction mixture [24].

4.2 Characterization of silver nanoparticles

4.2.1 UV-Visible Spectroscopy

The structural characterization of silver nanoparticles was characterized by UV-Vis's spectroscopy. The formation of AgNPs will be confirmed by UV-Visible spectral study, which is an authentic technique to monitor the progress of the reaction during the reduction of silver ions. The absorption spectrum of the silver nano solution prepared by using *Azadirachta indica* and *Ocimum sanctum* showed a surface plasmon absorption band with a maximum of 430 nm and 420 nm indicating the presence of silver nanoparticles. The occurrence of a peak at 430 and 420 nm is due to the phenomenon of surface plasmon resonance, which occurs due to the excitation of the surface plasmon present on the outer surface of the silver nanoparticles which gets excited to the applied electromagnetic field [25]. The UV absorption peak of silver nanoparticles ranges from 400 nm to 450 nm [26]. The UV-Visible spectral peak at 430 nm for the Ag nanoparticles prepared with *Azadirachta indica* leaf extract [12], was similar to the spectral peak at 430 nm in the present study. UV-Vis spectral peak at 450 nm for the AgNPs prepared by using *Azadirachta indica* leaf extract [16], which was higher than the spectral peak in the present study. Whereas UV-Vis spectral peak of 436 - 446 nm range for the AgNPs prepared by using *Azadirachta indica* leaf extract [14], which was slightly higher than the peak observed in the present study (430 nm), whereas UV absorption peaks of silver nanoparticles prepared by using *Azadirachta indica* observed at 421 nm [27] and 420 nm [15] respectively were less than the present study (430 nm). The UV-Vis spectral peak at 420 nm was prepared by using *Ocimum sanctum* leaf extract [28], which was similar to the peak (420) observed in the present study. The UV-Vis spectral peak at 430 nm (18, 21) and UV-Vis's spectra peak at 431 nm [20] for the AgNPs prepared by using *Ocimum sanctum*, which was higher than the present study. The UV-visible spectral peak was at 406 nm [23], whereas the peak at 413 nm [22] was very low than the present study.

4.2.2 Zeta potential

Zeta potential measures the potential stability of the nanoparticles in the colloidal suspension. The zeta potential of silver nanoparticles synthesized from *Azadirachta indica* and *Ocimum sanctum* was -8.87 mV and -12.30 mV respectively. These zeta potential values indicate the good stability of silver nanoparticles synthesized. The zeta potential value for green synthesized (*Azadirachta indica* leaf extract) silver nanoparticles ranged from -19.6 to -22.8 mV [29], which was a lesser value than the value obtained in the present study, whereas higher zeta potential value of (+34.6mV) AgNPs [15]. The zeta potential value was used to know the stability of AgNPs synthesized by using *Ocimum sanctum* leaf extract and observed zeta potential value of -55.0 mV [23], which was a lesser value than the value (-12.30 mV) obtained in the present study.

4.2.3 Anti-bacterial activity against pure culture and isolates of *Escherichia coli*

The antibacterial activity was recorded as the mean of the zone of inhibition (Mean±SE). Distilled water has been taken as control which showed no mean of ZOI against *Escherichia coli*. The mean of ZOI with *Azadirachta indica* leaf extract was 4 mm and 3.02±0.46 mm for pure culture and isolates of *Escherichia coli* from poultry samples respectively in the present study, whereas no ZOI [14]. The ZOI for *Azadirachta indica* leaf extract was 8±0.015 mm [30], which was higher than the mean of the zone of inhibition observed in the present study. The mean of ZOI against pure $AgNO_3$ solution was 10 mm and 9.04±0.23 mm respectively for pure culture and isolates of *Escherichia coli* from poultry samples in the present study, which was similar to the zone of inhibition [30].

The mean of ZOI against AgNPs prepared by using *Azadirachta indica* leaf extract was 16 mm and 14.01±0.19 mm respectively for pure culture and isolates of *Escherichia coli* from poultry samples, which was almost similar to the ZOI (16.67 mm) [12], whereas ZOI (12±0.007mm) observed on *Escherichia coli* [30]. The mean of ZOI of AgNPs prepared using *Ocimum sanctum* leaf extract was 15 and 13.02±0.21mm respectively in the present study, which was almost similar to the ZOI [30], whereas the mean of ZOI (11±0.81 mm) against *Escherichia coli* [13] lesser than the present study. The mean of ZOI against Chloramphenicol, Ampicillin, Tetracycline, Ciprofloxacin, AgNPs with *Azadirachta indica* and AgNPs with *Ocimum sanctum* were 24 mm, 13 mm, 15 mm, 21 mm, 16 mm, and 15 mm respectively against Escherichia coli pure cultures, whereas the mean of ZOI against Escherichia coli isolates from poultry samples was 23±0.20 mm, 11±0.21 mm, 14±0.28 mm, 19±0.18 mm, 14±0.19 mm and 13±0.21 mm respectively. The mean of ZOI of Chloramphenicol (26.0±0.57 mm) on *Escherichia coli* [13] was higher than the mean of ZOI (23±0.2 mm) in the present study and the mean of ZOI (7.66±1.69 mm) on *Escherichia coli* was less than the mean of ZOI of Ampicillin in the present study (11±0.21 mm). The mean of ZOI for AgNPs prepared with *Azadirachta indica* leaf extract was slightly higher (16 mm and 14.06±0.19 mm) than the mean of ZOI for Ag nanoparticles prepared with *Ocimum sanctum* leaf extract (15 mm and 13.02±0.21 mm) for pure cultures and isolates from Poultry samples respectively. The mean of ZOI of AgNPs prepared with *Azadirachta indica* is almost similar to the ZOI obtained with Tetracycline both for pure cultures and isolates obtained from poultry samples, but higher than the ZOI obtained for Ampicillin and lower than Chloramphenicol and Ciprofloxacin. The mean of ZOI for Ag nanoparticles prepared with *Ocimum sanctum* was higher than the mean width of zone of inhibition obtained with Ampicillin for pure cultures and isolates obtained from poultry samples, and less than the mean of ZOI obtained from Chloramphenicol, Ciprofloxacin, and Tetracycline.

5. Conclusion

Biosynthesis of metal nanoparticles has been proposed as a simple, cost-effective, high yield, and environment-friendly way of fabricating these materials. AgNPs prepared with both *Azadirachta indica* and *Ocimum sanctum* leaf extract were almost equally efficient against *Escherichia coli* and the AgNPs antibacterial activity is slightly similar to most of the antibiotics studied in this work.

6. Acknowledgments

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