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Antibacterial activity of Silver Nanoparticles on some Pathogenic Bacteria spp. isolated from Poultry Cages in Baghdad \Iraq.

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Abstract— In the recent decades, antimicrobial resistance (AMR), has been emerged as public health challenges. Since miss uses of antibiotics particularly in the developing countries. The threat of drug-resistant bacteria requires great efforts to develop highly effective and safe bactericide. This study aimed to create silver nano particles (AgNPs) by reduction of silver nitrate with sodium citrate salt. The project included preparation of silver nano particles and characterization of AgNPs; UV–vis, XRD and AFM. Besides, Fifty cloacal swabs were collected from broiler then, bacterial isolation and agar diffusion gel were done. The result of AgNPs characterization were; UV-vis peak at 350 nm; X ray diffraction amorphous crystalline at size 7-10 nm; and AFM grain nanoparticles at size 27nm. The four bacterial spp. gained (50 isolates) were examined to the antibacterial profile of gram-positive bacterial isolates showed sensitivity(mm) to: Ciprofloxacin (21.52± 0.2), Gentamicin (20.54 ± 0.2), Levoxacine (20.57 ± 0.15), Norfloxacin (22.59 ± 0.2), Oxacillin (16.1± 0.2) and resistance to Tetracycline (0.00). Whereas, the antibacterial profile of gram-negative bacterial isolates displayed sensitivity (mm) to: Ciprofloxacin (20.2±0.2), Gentamicin (18.11± 0.1), Levofloxacin (18.22± 0.12), Norfloxacin (20.2±0.2), but resistance to Oxacillin and Tetracycline (00.0). The sensitivity profile of AgNP showed; (20.54 ± 0.2), (16.11± 0.1) against gram positive and gram-negative bacterial isolates respectively. Accordingly, AgNP shown sensitivity to gram positive and gram-negative bacteria included the resistance isolates. The AgNPs as antimicrobial agents holds promise for the use to sanitation process in poultry industry.

Keywords **—** Antibacterial activity; Antimicrobial resistant in Iraq; and Silver nano particles.

INTRODUCTION

Over the time, the indiscriminate uses to antimicrobial agents causes decline in their effectiveness due to resistance of bacteria towards these agents. Antimicrobial resistance appears through inactivating antimicrobial agents or reduction in their therapeutic efficiency. Accordingly, the antimicrobial resistance represents the principals' reasons for the prolonged period of infection, increased rates of morbidity, and to extra-economic load on health systems (1,2,3). In the recent years, coinciding with the emergence of nanotechnology, scientific researchers focused on working to alternate the commercial antimicrobial products with metal nanoparticles. Since many metal nanoparticles showed advantages as antimicrobial agents and have superiority over other materials. The nanoparticles could be improved upon their application subject like; the purpose of uses, the period of uses, and the firmness of nanoparticles (4,5). In the medicine projects, numerous nanoparticles of organic origin have been implemented as carriers of medicines and nutrients besides, to reduce doses of administered medications and maintain nutrients (6,7). Some of nanomaterials have been approved as antibacterial agents on gram negative and gram-positive bacteria like; silver (Ag), Zinc (Zn), and Titanium (Ti). Thus, these nanoparticles have been applied in marketable products; cosmetics, disinfectants and types of household apparatuses (8,9).

Ancient nations were used silver in food preservation and to therapy. They remarked the therapeutic effect of silver metal to profuse healing of wound and injuries (10,11). The marked efficacy of silver nanoparticles against a wide range of pathogenic bacterial spp., candidate them a promising unconventional antibiotic (12,13,). Besides, to decline the abuse of antibiotic in veterinary medicine (14). The antimicrobial activity of silver nanoparticles AgNPs is principally owing to their oxidation state, (15).

The antibacterial activity of AgNPs is a complicated process, it represents multi-layered procedure relating thru physical and biochemical responses. In addition, factors related to nanoparticles such as; the metal nano particles size less than 10nm are able to penetrate bacterial cell wall more easily, the crystal particles shape interact more destructively with bacterial surface, the metal nano particles surface charge particularly the positive charge of particles are most active to interact with the negative charge inside the bacterial cell. Finally, the concentration of nanoparticles when the dose of nanoparticles is sufficient to inhibit growth of bacteria but not toxic to animal body (16).

Reduction of silver nitrate salt to generate silver nanoparticles using aqueous solution of sodium citrate, involves reducing of silver ion (Ag+) to silver atom (Ag0) to form metallic AgNPs, has been proposed as safe antimicrobial agents by many researchers (17,18,19). Currently, silver nanoparticles have been introduced in veterinary medicine when the AgNPs recognized in their antibacterial activity against both gram negative and gram positive bacteria (20, 21). Therefore, compounds of silver nanoparticles have been applied for animal management, infection cure and wound healing. Hence, synthesis or creation of new ingredients with antimicrobial effect still a challenge in the industry of medicines and necessary to avoid antibiotic resistance. So, this study designed to inspect antibacterial influence of AgNPs synthesized by chemically reducing of silver nitrate with sodium citrate salt on circular bacteria discharged through broiler residues to the environment and participate to overcome the AMR issue.

MATERIALS AND MTHODS

Chemicals and reagents: silver nitrate-powder and sodium citrate were purchased from Sigma Aldrich (Saint Louis, MO, USA). Antibiotic disks, filter papers and Media for bacterial culture; Nutrient agar (NA); *Salmonella- Shigella* agar (SSA), Eosin Methylene Blue Agar (EMB); MacConckey agar (Mc. A); Mueller Hinton Agar (MHA); and Nutrient broth (NB), were purchased from Hi Media Laboratories Pvt. Ltd., Mumbai, India.

Samples collection: Fifty cloacal swabs were collected from live birds (local breed broiler) kept in cages and prepared to slaughter and sale in five local marketplaces (Bayaa, washash, Hay al Amel, Al doura, and Saydia) | Baghdad, according to ethic committee of the "Industrial application and materials technology research center" garneted ethic approval (Ref.:115\0001). The swabs were kept in prepared nutrient broths (swab\broth) and transferred to bacteriology laboratory in the "Industrial application and materials technology research center", al Janiya , Baghda, for processing within one hour (14).

Preparation of silver nano particles by chemical reduction with sod. Citrate: chemical reduction of $AgNO_3$ was conducted beneath persistent heating to Tri-sodium citrate C6H5O7Na3. To prepare 1mM of AgNO3 solution; 17.0mg of AgNO3 was added to 250 ml deionized water in a volumetric flask and was placed in the microwave oven for continuous heating and absolute dissolving of the solution. Then, 10 ml (1%) solution of Tri-sodium citrate that previously prepared and sterile was added drop by drop to AgNO3 solution (17.0mg dissolved by 250ml dd water) with vigorously mixing. The obtained mixture was heated to 70^{O}C until the colorless solution altered to yellowish brown. Then, the solution was removed from heater stirrer and left at room temperature with stirring to cool at 25^{O}C (17).

Preparation of AgNPs soaked filter paper pieces: to investigate the antibacterial effect of AgNPs, filter papers were cut into pieces of 0.5 cm in diameter and kept in sterile petri-dish. Then, the pieces of filter paper were autoclaved and cooled at room temperature. On need, the sterile filter papers were deepened with 5ml of AgNPs homogenous solution (pH adjusted to 6.4) and incubated at 37^{O}C for 60 minutes before use in agar diffusion assay (17).

Characterization of silver nanoparticles AgNPs: the procedures for characterization of the synthesized AgNPs was relying on the previously described researches, (22,23). The reading of UV– vis spectra was observed thru (Model Lambda 365 Perkin Elmer CHINA), The Spectrum range 190-1100 nm double beam., Spectrum resolution 0,5 nm, test mode (A, T, R and Energy). The analysis of X-ray diffraction (XRD) was carried out using an X-ray diffractometer (DX-2700BH-Detector Drift Silicon, Co Instrument Haoyuan. Ltd, CHINA). The analysis of atomic force microscopy AFM was done by (Model TT-2, CHINA). Silver nanoparticles (AgNPs) has been examined and the data were analyzed by use of Software to process the images allowing for measurement of particles size, shape and distribution. Surface morphology of biologically produced Ag-NPs was studied using atomic force microscopy (AFM), (24).

Isolation and identification of bacterial isolates: the required media to isolate and identify the bacterial strains that collected by cotton swabs from cloacal of birds were prepared and sterile to be ready to use. Intended for bacterial culture and growth; Nutrient broth (NB) was prepared in universal bottles (20ml). For bacterial isolation and identification; Nutrient agar (NA), MacConkey agar (Mac), Eosin Methylene Blue (EMB), Salmonella- Shigella agar (SSA), Mannitol Salt agar (MSA), and blood agar were prepared. Then, Muller Hilton agar (MHA) was prepared for antibacterial activity.

The collected swabs were dipped into nutrient broth and incubated (24-48) hrs at 37°C for the proliferation of bacteria.

The loopful of fresh bacterial growth transferred from nutrient broth and streaked on nutrient agar then, incubated at 37°C for 24 h. A single colony was gained via sub culturing of bacteria on the nutrient agar plates after that, transferred to selective media such as MS agar, Mac agar, EMB agar, SSA agar and blood agar. Subsequently, all plates were incubated at 37°C for 24-48 hrs. For observing the bacteria, gram stain technique was conducted; characteristics colonies from specific medium were smeared on sterilized glass slides and stained with gram staining materials. followed standard procedure (25).

Antibacterial assays: antimicrobial activity of antibiotics and AgNPs against gram negative; *Escherichia coli*, *Salmonella* spp., *Pseudomonas* spp., and gram-positive bacteria *Staphylococcus* spp. was assessed for antibiogram thru agar diffusion assay.

The agar diffusion assay was performed using commercial antibiotic disks against the collected bacterial isolates on Mueller Hinton Agar MHA plates. In this study, the antibacterial activity of antibiotics in compression with AgNPs was evaluated. The conventional broad-spectrum antibiotics were selected to analyze the effect of antibiotics agents and AgNPs. Based on the Clinical and Laboratory Standards Institute CLSI standard, antibiotics were used at

particular concentrations as follows: Ciprofloxacin (10µg), Gentamicin (10µg), Levoxacine (5µg), Norfloxacin (30 µg), Oxacillin (10 µg), and Tetracycline (10 µg). To estimate the efficacy of AgNPs, the paper disk that prepared and soaked with the AgNPs previously was used. For agar diffusion assay; a single colony of each collected isolate was grown overnight on nutrient broth using a rotary shaker (200 rpm) at 37 °C. The inocula were equipped by diluting the overnight cultures with 0.9% NaCl to a 0.5 McFarland standard and were applied to the plates along with the standard and replaced commercial disks of antibiotics. After incubation at 37 °C for 24hrs, a zone of inhibition (ZOI) was measured by subtracting the disk diameter from the total inhibition zone, and compared with guidelines of clinical and laboratory standard institutes CLSI (17).

Statistical Analysis

The statistical analysis was estimated as the mean ± standard deviation (Mean ± SD) of the experimental data. The data were analyzed using the SPSS for analysis of variance ($p < 0.05$) using T- test on a two-way basis (26). The number of repetitions for the analyses was twice.

Result and discussion

The result in (Table1) show that; 20(40%) of isolates were *E. coli* since they fermented lactose sugar on MacConkey agar, the colonies found a rounded with metallic sheen when grow on EMB agar while, 14(28%) isolates were *Salmonella* spp., and produced gas on SS agar. Six (12%) isolates were *Pseudomonas* spp, able to grow on blood agar and the colonies showed *β* hemolysis besides, the growing colonies showed pigments and grape like odor on nutrient agar. In addition to 10(20%) isolates were *Staphylococcus* spp., yellow colonies arranged as bunches of grapes and able to grow on MS agar.

Table 1. Isolates obtained from bacterial isolation

Bacterial spp.	Medium growth	Gram stain	Frequencyy	Percent
E coli	MacCkonkey agar	neg	20	40
Salmonella,	*Salmonella Shigella* agar	neg	14	28
Psedoumonas	Blood agar	neg	6	12
Staphylococucus	Manitol Salt agar	pos	10	20

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Through light microscopy, all stained smears that acquired red stain were gram negative and appeared; Short rods, no spore-forming bacteria while, smears that acquired a blue stain were gram positive bacteria appeared in grape-like clusters.

Result in (Figure 1) show: synthesis and characterization of silver nano particles by the color change that represents the main sign of silver nanoparticles creation. Thru reduction of silver nitrate salt (AgNO3) that conducted by sodium citrate $C_6H_5O_7Na_3$, and the appearance of the "AgNO3 and sod. citrate "mixture in a yellowish-brown color indicated the formation of silver nanoparticles in the solution.

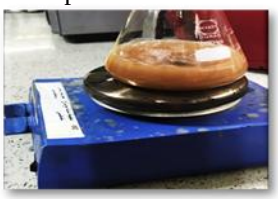

Figure1. Reduction of silver nitrate by sodium citrate and the appearance of a yellowish-brown color in the $AgNO_3$ indicated the formation of silver nanoparticles.

The results revealed in (Figure 2) show: the characterization to the synthesized silver nanoparticles by UV-Vis spectrum. The UV-Vis absorption spectra optical densities of the formed AgNPs were examined using UV-Vis spectrum at wave length ranged 20 – 1100 nm recorded a resonance at 300-450 nm.

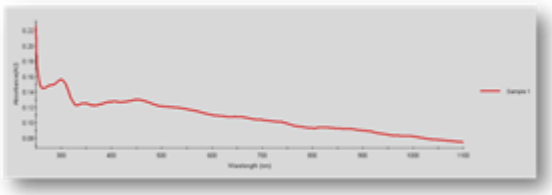

Figure 2. Reading of OD through UV.vis to the synthesized silver nanoparticles on 350 wave length.

Result show in (Figure 3): the characterization to the synthesized silver nanoparticles by X-ray diffraction (XRD). The patterns of AgNPs when chemically reduced silver nitrate with sod.citrate presented; the diffraction peaks of 20.895,23.373, 27.273, 28.993, 30.594, 32.008, 35.168, 36.208, 38.648, 40.99, 43.809, 45.396, 47.799, 52.921, 58.607, 62.218.

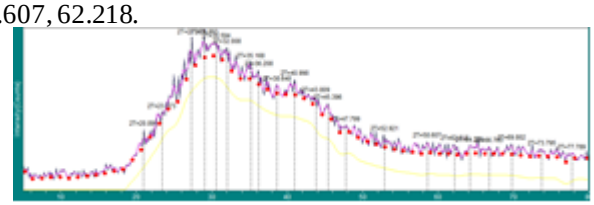

Figure 3. characterization of XRD to the synthesized silver nanoparticles synthesized by reduction of silver nitrate with sod.citrate, amorphous crystalline at size 7-10 nm .

Outcomes show in (Table 2): the atomic force microscopy analysis AFM was utilized to check the surface roughness and morphology of the silver nanoparticles.

Result in (Figure 4) show: the magnification is proportional to the intricacy of the particle size with that of the AFM tip and on the sample preparation. The white patches represented the spherical nanoparticles which appeared in the micrograph and the 3D image showed the surface roughness due to the biomolecules or the organic compounds on the silver nanoparticles. A careful inspection of the micrograph revealed that the particles were spherical, with a smooth surface and the grain size of silver nanoparticles was 27nm.

Table 2. Silver nanoparticles created by chemical reduction of silver nitrate with sod. citrate.

Statistical summary					
Parameters	Unit	Mean	Std dev	Min	Max
Projected area	µm²	0.0009659	0.01151	4.325e-06	0.2099
Z-maximum	nm	27.62	4.757	23.36	52.54

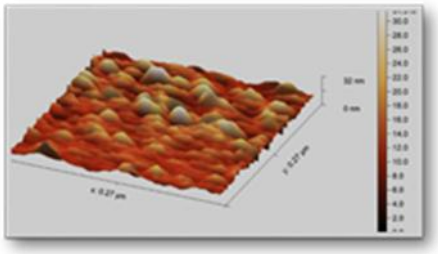

Figure 4. finding of AFM to the grain size of silver nanoparticles (chemical reduction of silver nitrate with sod.citrate) was 27nm.

Result in (Figure 6) of the antibacterial profile show: the gram-positive bacterial isolates sensitivity to antibacterial desks showed high sensitivity (mm) to: Ciprofloxacin (21.52± 0.2) Gentamicin (20.54 ± 0.2), Levoxacine (20.57 ± 0.15), Norfloxacin (22.59 ± 0.2), moderate sensitivity to Oxacillin (16.1± 0.2) and resistance to Tetracycline (0.00). Whereas, the antibacterial profile of gram-negative bacterial isolates presented in (Figure 7) show, high sensitivity (mm) to: Norfloxacin (20.2±0.2), Ciprofloxacin (20.2±0.2), and moderate sensitivity to Gentamicin (18.11± 0.1), Levofloxacin (18.22± 0.12) but resistance to Oxacillin and Tetracycline (00.0) respectively.

The result in (Table 3) of sensitivity profile of AgNP show; (20.54 ± 0.2), (16.11± 0.1) against gram positive and gram-negative bacterial isolates.

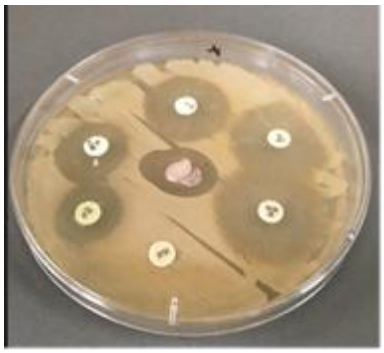

Figure 6. the Antibacterial activity of silver nanoparticles product (silver nitrate reduced by sod.citrate) on gram positive isolated bacteria.

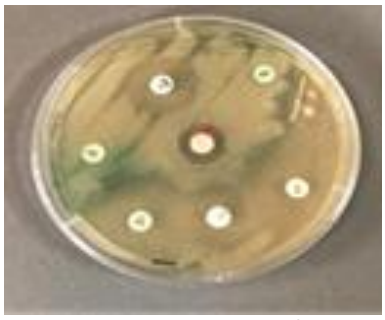

Figure 7. the Antibacterial activity of silver nanoparticles product (silver nitreate reduced by sod.citrate) on gram negative isolated bacteria.

Table 3. the antibacterial profile of gram-positive and gram negative bacterial isolates.

Antibacterial agent/	Inhibition zone/mm/ gram pos bacteria	Inhibition zone/mm/ gram neg bacteria
Ciprofloxacin	21.52± 0.2	20.2±0.2
Gentamicin	20.54 ± 0.2	18.11± 0.1
Levoxacine	20.57 ± 0.15	18.22± 0.12
Norfloxacin	22.59 ± 0.2	20.2±0.2
Oxacillin	16.1± 0.2	00.0
Tetracycline	0.00	00.0
silver nanoparticles	20.54 ± 0.2	16.11± 0.1

Outcome in (Fiure 8) revealed that; gram positive bacterial isolates shown a significant ($p<0.05$) level of sensitivity with an inhibitory zone against AgNP (20.54mm), Ciprofloxacin (21.52 mm), Gentamycin (20.54 mm), Levoxacine (20,57), and Oxacillin (16.1) while, the zone of inhibition was (00.0) for all the isolates against Tetracycline. As well gram negative bacteria revealed a significant ($p<0.05$) level of sensitivity with inhibitory zone 16.11mm, 20.2mm, 18.11mm, 18.22, and 20.2 for AgNP, Ciprofloxacin, Gentamycin, Levoxacine and Norfloxacin, respectively. Whereas such zone of inhibition was (00.0) for all the isolates against amoxicillin and Tetracycline.

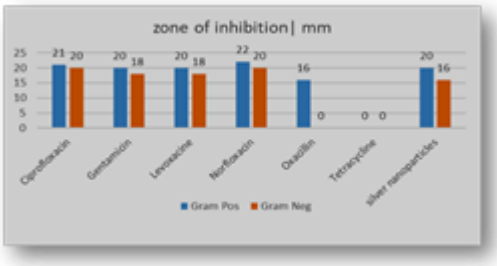

Figure 8. the antibiogram of gram positive and gram negative bacteria a significant ($p<0.05$) level of sensitivity with an inhibitory zone against AgNP and commercial antibiotic discs**.**

In the present study, the solution of silver nanoparticles (silver nitrate reduced with sodium citrate salt) showed superiority as an antibacterial agent against gram-positive bacteria than as an antibacterial agent against gram-negative bacteria. Even though the created silver nanoparticle displayed

efficacy as antibacterial agent against gram negative and gram positive bacterial isolates that dismissed in floors and cages of broiler. Therefore, antibacterial resistance towards conventional cleaners can be overcome when applied of silver nanoparticle as commercial compound for sanitation of floors and cages of domestic birds.

The bacteria were isolated from five live bird markets within Baghdad city at the aim of collecting numerous species of bacteria that resistance to different antibiotics. The obtained isolates from sampling markets were: *Staphylococcus* spp., *E. coli*, *Salmonella* spp. and *Pseudomonas* spp, as representatives of commonly available poultry bacteria. The isolation and identification of the bacteria were performed thru bacterial culture, gram staining and differential isolation. The Mannitol Salt agar MS, Eosin Methylene Blue agar EMB, *Salmonella Shigella* agar were used as selective medium for *Staphylococcus* spp, *E. coli* and *Salmonella* spp. respectively whereas, blood agar was used for cultivation and identification of *Pseudomonas* spp. In MS agar medium colonies were arranged as bunches of grapes with a yellowish tinge in the medium which reduced mannitol salt of the medium and thus suggestive for the growth of *Staphylococcus aureus*. In case of MacCkonkey agar medium colonies were found round shaped with green metallic sheen which is a characteristic feature for the growth of *E. coli*. While in SS agar round shaped blackish bacterial colonies were found that indicated the presence of *Salmonella* spp. On gram staining bacteria isolated from MS agar, round purple color bacteria in bunched grape-like cluster paired in chain were observed that confirm the gram-positive *Staphylococcus aureus*. While staining from EMB agar revealed single short rod-shaped pink color bacteria confirmed gram negative *E. coli*. Besides, staining from SS agar, a distinct short pink color rod was found indicating gram negative *Salmonella* spp., (27). In this study, the result of isolation agreed with result reported by (23,24, 26 and 27).

The yellowish-brown color appeared by reduction of silver nitrate with sod. citrate pointed to the formation of silver nanoparticles in the solution. It is well known that AgNPs exhibit a dark yellow to brown color in water, arising from excitation of surface plasmon vibrations in the metal nanoparticles, (17, 28, 29). Reading of OD through UV.vis to the synthesized silver nanoparticles was recorded a resonance at 300-450 nm. Since the surface Plasmon resonance (SPR) absorbance relies heavily on the size, shape, and environment by which the nanoparticles are formed. The plasmon bands are broad with an absorption tail in the longer wavelengths by increasing the concentration of AgNO3, indicating an enhancement in particle size distribution of the synthesized nanoparticles. The characterization patterns of AgNPs synthesized by reduction with sod. citrate, by XRD was related to crystallographic planes of face centered cubic (fcc) structure for the silver powder sample. The peak of the silver structure indicated the purity of synthesized silver nanoparticles without any additional diffraction peaks.

The product of silver nanoparticles (AgNPs), when silver nitrate reduced by sod. Citrate has been examined and the data were analyzed by use of Software to process the images allowing to measure the particles size, shape and distribution. Surface morphology of chemically produced Ag-NPs was proceeded using atomic force microscopy AFM. A careful inspection of the micrograph revealed that the particles were spherical, with a smooth surface, and the clusters of AgNP with dimension around 27 nm was observed. Accordingly, the results of characterization to synthesized silver nanoparticles by reduction with sod.citrate were agreed with results reported by (23, 24, 28, 29, 30, 31 and 32).

In this study, the synthesized AgNPs product, (reduced silver nitrate by sod.citrate) was used for the investigation of its antibacterial activity against multi-resistant clinical strains of *Enterobateriaceace* strains, *Staphylococcus* spp., *Pseudomonas aeruginosa*, isolated from birds in local markets in Baghdad city. Besides, comparison of the created AgNPs with the commercial antibiotic disks. The synthesized AgNPs (1mM of AgNO3 solution reduced with (1%) solution of tri-sodium citrate), was effective against all pathogens (gram-negative and gram -positive) bacteria with a significant ($p<0.05$) level of sensitivity. Whereas, some strains of gram-negative and gram -positive) bacteria showed resistance against the compared commercial antibiotics; the gram positive bacterial isolates showed moderate sensitivity to Oxacillin (16.1± 0.2) and resistance to Tetracycline (0.00). While, gram negative bacterial isolates showed resistance to Oxacillin and Tetracycline (00.0), respectively. The higher sensitivity of gram-positive bacteria refers to bacterial cell wall structure in comparison with gram negative bacteria. While, gram neg bacteria have a complex double barrier composes of: a lipid bilayer comprising lipopolysaccharides (LPS) which acts as a bodily shield and selective porins, that represent channels and the antibiotic should pass through so large molecules such as erythromycin and penicillin cannot diffuse. As well, the presence of periplasmic space which's a gap amid the outer and inner membranes, and consider the store of beta-lactamases that responsible on mechanism of resistance. Then, eliminate antibiotics before they reach the actual cell body. The gram-positive bacteria have simpler cell wall: composes of; porous peptidoglycan, which's a very thick layer of peptidoglycan, like a mesh or a sponge rather than a solid wall. It cannot prevent molecules from disseminating through. Besides, lack to outer membrane since the absence of that extra lipid shield, antibiotic agent has a direct path to the bacteria cell membrane and the interior machinery (27). Results of this study agreed with results of (32, 33,34 and 35). Therefore, the product of this study (a prepared solution of AgNPs thru reduction of silver nitrate by sod.citrate), considers as a promise antibacterial agent in can add to a formula of detergent against both gram negative and gram positive bacteria (resistant to antibiotics, AMR,) that discharged by the birds in the floors of the poultry farms and transported avian cages to, eliminate bacterial contamination of the environment.

CONCLUSION

This study concluded that sodium citrate is a simple, low cost, and eco-friend method for synthesized silver nanoparticles. silver nanoparticles showed have been effective antibacterial activity against both gram positive and gram negative bacteria isolates from poultry cages. The bacteria

isolates show variable resistance to commercial antibiotic due to improper antibiotic use in poultry farms. These result finding that silver Nanoparticles have highlight potential to alternative antibiotic agents. Although further need to confirm their safety and application in the poultry industry in vivo studies.

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Conflict of Interest

The authors declare no conflicts of interest.

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