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ANTIMICROBIAL RESISTANCE PROFILES OF BACTERIA ISOLATED FROM POULTRY DROPPINGS AND TREATED BY AGNPS GREEN SYNTHESIS FROM *THYMUS KOTSCHYANUS*

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Abstract

Poultry is considered one of the most kinds of meat that is consumed all over the world, and it uses large quantities of antimicrobialsamong flocks of poultry to prevent and to treat disease, as well as for promote growth. Poultry pathogens that are resistant to antimicrobials may lead to treatment failure, leading to losses of economic, as well as a risk to human health and a high risk to the environment because the nutrients and microorganisms presentin high concentrations in the waste materials. Here, in this report, try toisolate resistance profiles of pathogenic bacteria isolated from poultry droppings and test their antimicrobial sensitivity patterns and trying to treatments by using AgNPs extracted from *Thymus kotschyanus* extract as the alternative treatments. *Salmonella typhi* isolated from ten samples of poultry dropping as a pathogenic bacteria among Enterobacteriaceae. Various analytical technique including UV-Vis Spectroscopy, Fourier Transform Infrared (FTIR) Measurements, X ray diffraction (XRD) pattern, scanning electronmicroscopy (SEM) tocharacterized AgNPs. AgNPs showed Antibacterial effects by using agar disk diffusion as a test to determined the antibacterial activities of synthesized AgNPs against the *Salmonella typhi* MDR.

Key words: Multi Drug Resistance, Poultry, Green synthesis, silver nanoparticles, *Thymus kotschyanus*.

Introduction

The industry of poultry is type of important, greater and growing with fastest agricultural industries in all world. So, this can be contributed to an increasing needed for poultry products including egg and meat. While slaughtering chicken, carcasses may be contaminated by dropping materials from the chicken's intestines and this drooping may containing pathogenic bacteria. Transferred of bacterial infections happened through such process or consumption of cooked chicken meat (Havenstein *et al.*, 2003; Panisello *et al.*, 2000; Ukut *et al.*, 2010; Omulo *et al.*, 2015). Dropping poultry is a source and reason of human microbes and pathogens, like *Listeria monocytogenes, Salmonella*, *E.coli and Campylobacter jejuni* that can infect and contaminate all fresh products and their environments that lead to food borne outbreaks (Wilkinson *et al.*, 2011; Kim *et al.*, 2012; Moore *et al.*, 1995; Enticknap *et al.*, 2006; Wilkinson, 1997; Chinivasagam *et al.*, 2010).

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The main problem which faces the industry of poultry is the wide spreads tack up of poultry wastes such as, manures and dropping which may led to problems of disposal, pollution and contamination problems except evolved in economically and environmentally sustainable development techniques (Bolan *et al.*, 2010). Fresh products have been contaminated by the deterioration of pathogenic bacteria in the agricultural environment as the main cause of many food poisoning cases (Doyle and Erickson, 2008).

Dropping poultry contains residues of antimicrobial and different of resistant bacteria; when used as fertilizer, the effects and level of these anti-microbial drug and bacteria in the environment cause for concern (Ali *et al.*, 2009). Foods and environmental sources containing of resistant of bacteria to one or more antibiotics used in medicine of human and veterinary and in production of food and animal (Arathy *et al.*, 2011). About 8,164,662 kg of antibiotic drugs are used in each year in farming of most animal (70% of which is used for non-curativeaims

like promoting growth and preventing disease) than those to only 1,363,636 kg used in medicine of human (Sridevi Dhanarani *et al.*, 2009). Resistance of bacteria to antibiotics are developed, for this reason should take into consideration their uses of different agents of antimicrobial, in particular, in medicine of veterinary, agriculture and nutrition (Caprioli *et al.*, 2000). Now, the major risk reasons for increased bacterial drug resistance is an major and highly use of drugs and antibiotics that similarly in each animals than humans. In animals, agents of antimicrobial are used not for treatment and production against bacterial infections only, but also as stimuli for growth. For the reasons mentioned, the antibiotics resistance should monitor in bacterial infected human also in animal origin source bacteria (Kolár *et al.*, 2002).

Various methods have been used to creating of different metal nanoparticles. A chemical material toxic has been used in the traditional and conventional chemical methods, while to eliminate dangerous material in chemical methods, the methods of nanoparticles green synthesis are used, also green synthesis chemistry presented one branch of nano-science techniques to make it important to uses (Khataee and Mansoori, 2011; Albrecht *et al.*, 2006; Song and Kim, 2009). Green synthesis of metallic nanoparticles, specially by different parts of plants that include seed, root, fruit and leave extract where the focus is on reducing agent and capping agent (Awwad and Salem, 2012; Sham *et al.*, 2014; Choi *et al.*, 2008; Curtis *et al.*, 2006). Characters of Agnan oparticlesas antibacterial activities made it important one of the metals (Shaham *et al.*, 2017 and Lewinski *et al.*, 2008). Green bio-synthesis of Ag nanoparticles were studied from different plant parts like olive leaves, (Khalil *et al.*, 2014) mulberry leaves, (Awwad and Salem, 2012) plant leaves extract of Indian ones (Banerjee *et al.*, 2014) *Thymbra spicatas*, (*Veisi et al., 2018) Stachy slavandulifolia*, (Shahriary *et al.*, 2018) *greentea*, (Veisi and Ghorbani, 2017) *Sesamum indicum* seeds, (Mohasseli and Pourseyedi, 2013) *banana peel*, (Ibrahim and Rad, 2015) *oak fruit* barks, (Veisi *et al.*, 2016) and *Eucalyptus leaves* (Rasooli and Mirmostafa, 2003) Lamiaceae family isorigin of the *Thymus Kotschyanus* species the and used widely in traditional Indian medicine (Sulaiman *et al.*, 2013). Reason of our constant interest in the metal nanoparticals bio-synthesis (Veisi *et al.*, 2016). In this study, we focused on the isolation of pathogenic bacteria from poultry drooping from different location of chicken farm and their drug sensitivity patterns, green bio-synthesis of Ag nanoparticles by *T. kotschyan*us extract as reducing agent then characterized it using different techniques to analytic. Also, the antibacterial activity of synthesized AgNPs were study against pathogenic bacteria isolated from poultry dropping as atraditional treatments.

Materials And Methods

Sample collection, Isolation and Identification of microorganisms

Samples of fresh poultry droppings were collected from chicken keepers residing in 5 localities situated within Al-Hilla city, Babylon. The samples were collected by using sterile spatula and deposited into closed sterile containers before transporting them to our laboratory for further processing, on the same day. Two grams of each sample was suspended in about 2 ml of sterile normal saline and left for 5 minutes to sediment. Fifty micro liters of supernatant was drawn and spread-plated onto freshly prepared and culturing on Nutrient, blood and Mac Conkey's agar plates (Himedia, India) for 24-48 h at 37°C for isolation and purification. Allisolates were confirmed by Vitek-2 compact system (Biomérieux) (CLSI, 2018).

Antibiotic susceptibility and multiple antibiotic resistance (MAR) determination

Each isolate of identified bacterial under study was study their antibiotic resistance profiling against 4 antibiotic disk frequently used viz *Salmonella typhi*. Trimethoprim (5µg), Ampicillin (10µg), Meropenem (10µg) and Ceftriaone (30µg). All tests were applied in plates containing Mueller-Hinton agar (Carl Roth, Germany) by using method of disk-diffusion of Kirby-Bauer. Each identified bacterial isolate was re-suspended to compared with 0.5 McFarland as standard turbidity [equivalent to 1.5×10 colony forming unit per milliliter (cfu/ml)]; before to subjecting them to antibiotic sensitivity analysis according to the guidelines of Clinical Laboratory and Standards Institute (CLSI), zones of inhibition around each disc were measured and recorded as Sensitive, Intermediate and Resistant (Egorova and Revina, 2000).

Processing of Plant Extract:

Parts from *T.kotschyanus* plant collected and washed for many time by using deionized distal water, then dried at (25°C) by incubator, by using mortar powdered dried plant to made powdered. Soaked of 5 gram of powder in (50 ml) water in boiling degree for (30 min) until changing their color of solution to pale yellow color. Filtered the product extract by using filter of Whatman (no. 1) and maintained at (4°C). *Thymus Kotschyanus* extract was used for the synthesis silver nano particles by reduction the (Ag+) silver ions to (Ag0) silver nanoparticles.

Processing $AgNO_3$ Precursor

To preparation of AgNPs precursor, suspended (3.34mg) of $AgNO_3$ salt in (30ml) of distilled water, to result (1 mM) of $AgNO_3$. Allowed stir the solution in a magnetic stirrer for (15 min). Aqueous solution of (1 Mm) of silver nitrate was prepared; and this solution was used for silver nanoparticles syn thesis.

Silver Nanoparticles Synthesis

For synthesis of Ag nanoparticles, prepared *Thymus Kotschyan* extract added to aqueous solution of $AgNO_3$ in 1:1 ratio. Mixture solution putted in a magnetic stirrer to stirred then boiled for an hour at (80°C). After that, the color of mixture reaction changes from pale yellow color to color of dark brown indicating the reduction of (Ag+) silver ions to (Ag0) AgNPs (Fig. 1). At final, centrifuged the solution mixture at (5,000 rpm) for (20 min) and the pellets materials collected and dried in oven by hot air at (65°C) for (20 min).

Characterization of Silver Nanoparticles

The formation, stability, size and shape of silver nano particle are synthesized in aqueous solution are monitored by UV-Vis Spectroscopy (Sharma *et al.*, 2009; Nabikhan *et al.*, 2010). Immediately when it I and sprepared the reaction mixture the spectrum was taken. Characterized the dried silver nanoparticles synthesized from *Thymus kotschyan* extract by X-Ray diffractometer; to proposes the crystalline nature of AgNPs synthesized (Karamian and Kamalnejad, 2019). Chemical composition of the synthesized Ag nanoparticles was determine by using FTIR spectrometer. FTIR is used to identify the functional groups present with in *Thymus kotschyan* extract, that have responsible role in reduction Ag nanoparticles (Forough and Farhadi, 2010). Scanning Electron Microscopy of the obtained samples of plant extract was performed to know the shape and size of AgNPs, by prepared a thin films of the prepared samples by dropping on a foil and then removing of excess solution by butting in an oven at (80°C) (Karamian and Kamalnejad, 2019).

Table 1: Antimicrobial susceptibility pattern of *Salmonella typhi* isolated from poultry dropping (N=10) at Hilla, City.

Bacterial Isolats	Antimicrobial Agents Tested			
	CRO-30	TMP-5	AMP-10	MEM-10
Sal-1	*R	R	R	R
Sal-2	R	R	R	R
Sal-3	R	R	R	R
Sal-4	R	R	R	R
Sal-5	R	R	R	R
Sal-6	R	R	R	R
Sal-7	R	R	R	R
Sal-8	R	R	R	R
Sal-9	R	R	R	R
Sal-10	R	R	R	R
*R= Resistance				

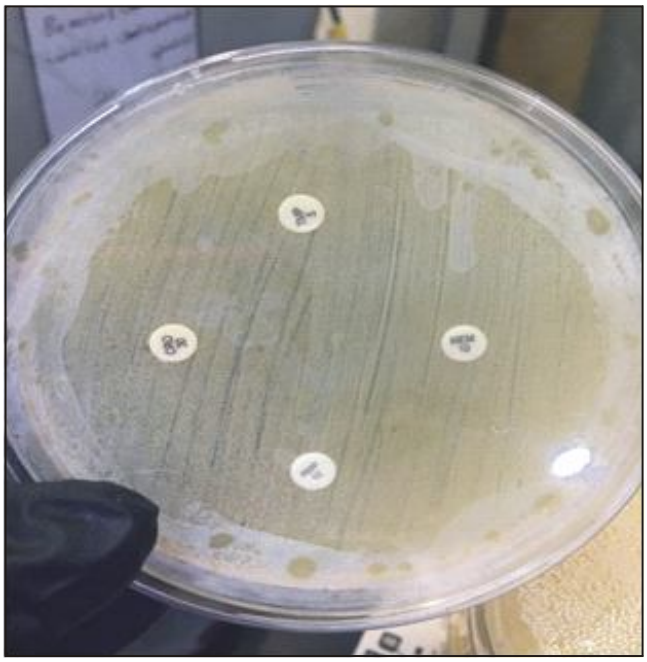

Fig. 1: Sensitivity test of 4- antibiotics against *S. typhi* isolates.

Results and Discussion

Pathogenic bacteria isolated from poultry dropping

Ten isolates of *Salmonella typhi* pathogens was isolated and identified from poultry dropping taken from 5 different area. Allisolates were confirmed by biochemical tests and by Vitek-2 compact system (Biomérieux).

Susceptibility profiles of the isolated bacteria

The sensitivity patterns of the *Salmonella typhi* isolated from poultry dropping to antibiotics frequently used for treatment this bacteria. Different antibiotic disks Trimethoprim (5µg), Ampicillin (10µg), Meropenem (10µg) and Ceftriaone (30µg) were purchased from (Bioanalyse, Turkey). All 10-bacterial isolates are

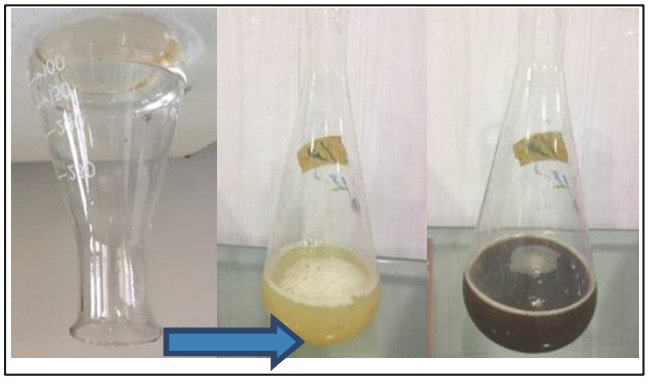

Fig. 2: a-$AgNO_3$ solution, b-*Thymus Kotschyanus* extract and reaction mixture c-($AgNO_3$ + plant extract).

resistance to all antibiotic disc are used in the test and that the selected antibiotics disc were not effective against all bacterial isolated under study according to the (CLSI, 2018), as in fig. 1 and table 1.

Characterization of the synthesis AgNPs

Changing the color of mixture solution in period of reaction from pale yellow color to the dark brown color indicate to bio-synthes is of AgNPs from *Thymus Kotschyanus* extract and confirmed by uv-vis spectrophotometer (Perkin Elmer, Lambda 25) with (200-700 nm) wave length. Fig. 1 shows spectrum of UV-Vis in the solution containing synthesized AgNPs. According to the result, single and strong peak obtained at (340–360 nm) are shown and indicated to bio-synthes is and formation of AgNPs in the solution mixture (Sharma *et al.*, 2009; Nabikhan *et al.*, 2010).

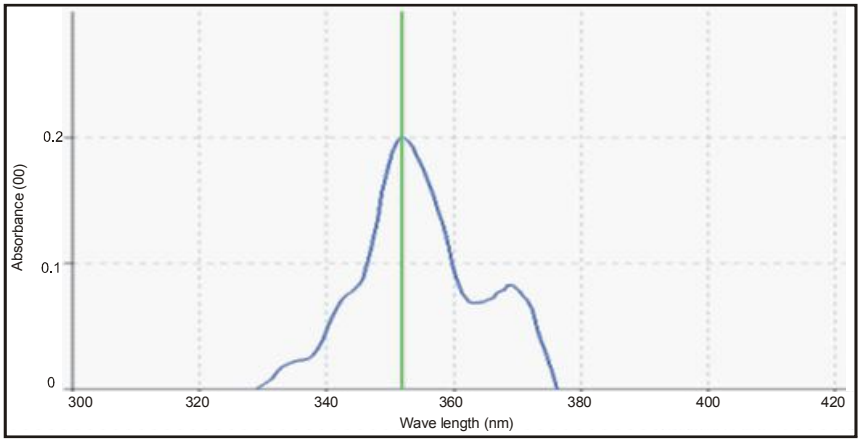

Fig. 3: UV-Vis Spectrum for AgNPs synthesis by *Thymus Kotschyanus* extract.

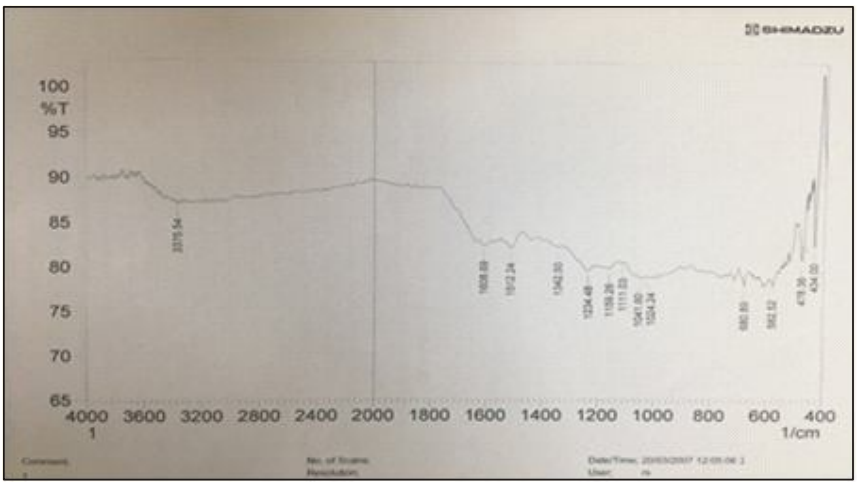

Fig. 4: FT-IR of AgNPs synthesis by *Thymus Kotschyanus* extract.

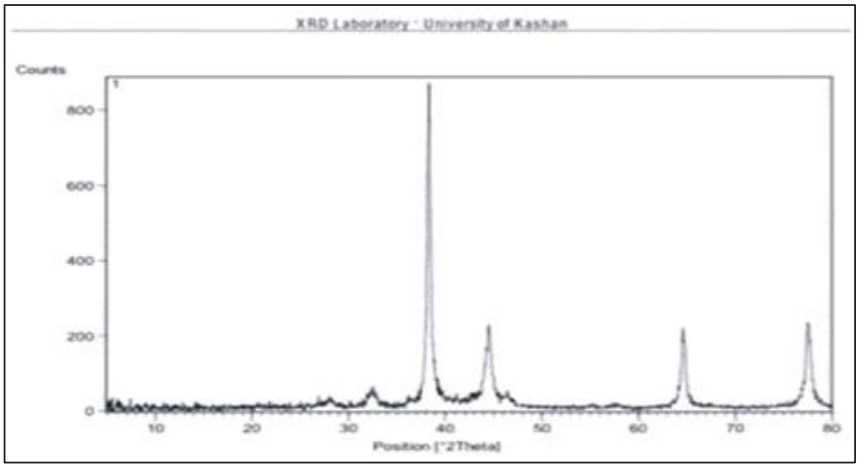

Fig. 5: XRD of AgNPs synthesis by *Thymus Kotschyanus* extract.

Samples FT-IR spectrums were made with range of (400-4000cm^{-1}) (SHIMADZU) instrument. To determined the functional group in the samples, as in fig. 3, spectrum of *T. kotschyanus* extract show strongly peak at 3375 cm^{-1} this result indicate accrue stretching of the amino group N H bond and due to present hydroxyl group (O H stretch), 1608 cm^{-1} (C=O stretch), 1512 and 1234 cm^{-1} (C C stretch) and 1041 cm^{-1} (C Nstretch). So, from previous observation the presence of proteins in *Thymus Kotschyanus* extract is confirms and phenols act as a stabilizing and reducing agent for Ag nanoparticles (Forough and Farhadi, 2010).

Synthesized AgNPs crystal structure measured by using XRD technique by using (6000 XRD), at a step, with a voltage (40 KV) and electric current (30 mA), scanning rate of 2^{o} with (2θ/min) and range of 2θ (30^{o} to 80^{o}). Fig. 5 shows pattern of XRD of bio-synthesized Ag nanoparticles. Observed the peaks at (38.5, 44.5, 65.5 and 77.5) in the 2θ range (0-80°), that is parallel to (111, 200, 220 and 311), indicating that AgNPs is metallic and have crystal structure (Karamian and Kamalnejad, 2019).

Scanning electron microscope SEM (type- S-1640 HITACHI company/ Japan) uses to recognized morphological features and size of nanoparticles by SEM image of biosynthesized AgNPs fig. 6 show formation of homogenous AgNPs with the average diameter was (21.64-123.14 nm) with spherical in shapes. The larger size of AgNPs indicate to the aggregation of smallest size (Karamian and Kamalnejad, 2019).

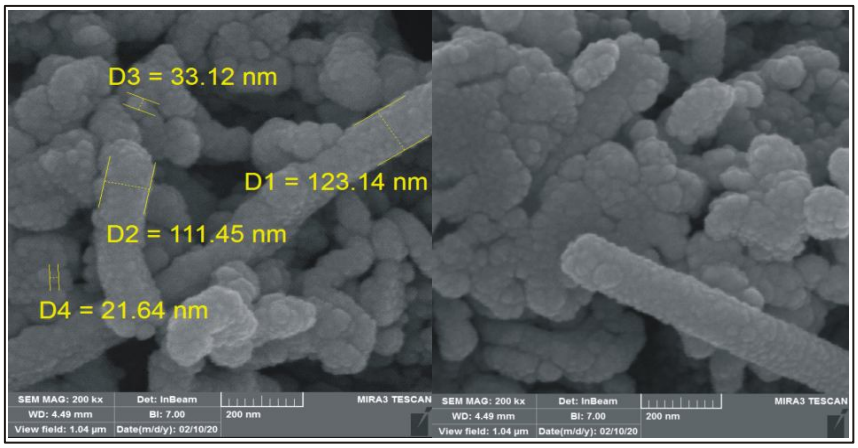

Fig. 6: FE-SEM of AgNPs synthesis by *Thymus Kotschyanus* extract with 21.64 - 123.14 nm in size and spherical in shapes.

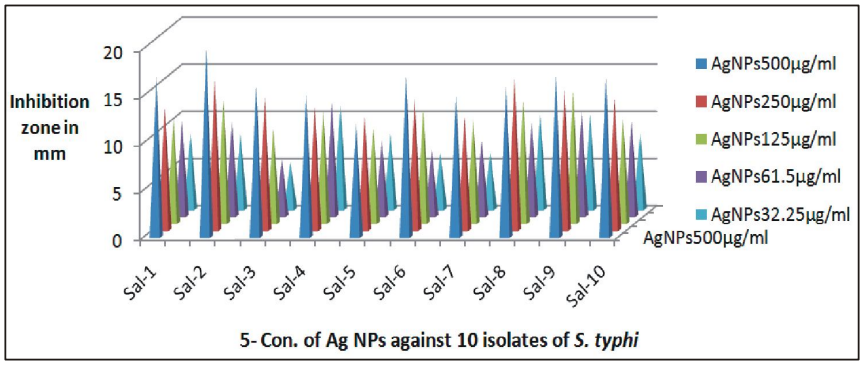

Fig. 7: Antibacterial activity of AgNPs synthesis by *Thymus kotschyanus* extract against 10 isolates of pathogenic *Sal.typhi* isolates (inhibition zone diameter in mm).

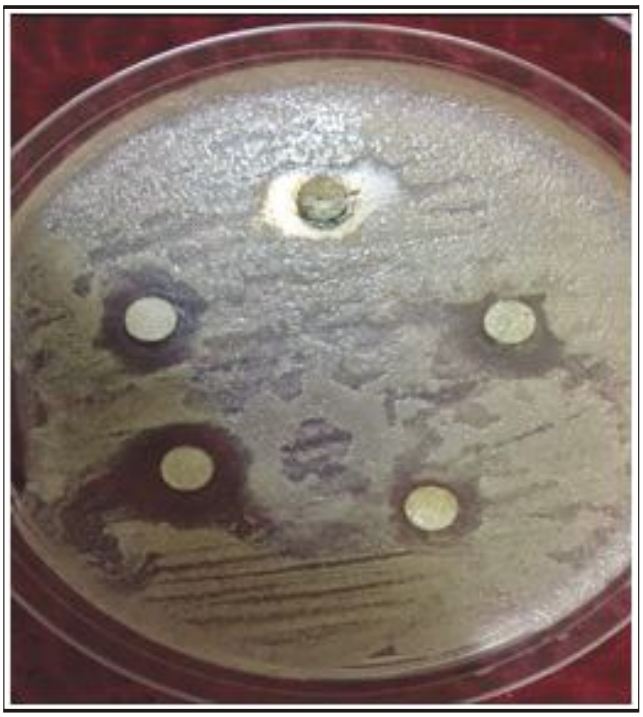

Fig. 8: Inhibition zone of bacterial growth as a result of antibacterial activity of AgNPs synthesis by *Thymus kotschyanus* extract.

Antibacterial Activity test for AgNPs against MDR bacteria

• Agar disk diffusion test : AgNPs shows that powerful broad-spectrumanti bacterial activity against 10-isolates of multidrug resistance *Salmonella typhi* Gram negative bacteria is tested, this results are compared with the effects of different antibiotics on bacterial isolates. The result in table 1 showed that the selected antibiotics were not effective against all 10-bacterial isolates. AgNPs showed clearly inhibition zone diameter with the increase in NPs concentration that even exceeded the activity of selected antibiotics. 500 µg/ml concentration showed highest zone of inhibition against all 10-bacterial isolates, maximum zone of inhibition of 20 mm appeared against Sal-2 that showed high sensitivity even at 31.25µg/ml. Sal-5 the least sensitive isolate to the AgNPs compared with others bacterial isolates. Result in the (Fig. 7,8) appear synthesized AgNPs by *T.kotschyanus* extract have inhibition growth of all bacteria under study, in a dose-dependent manner. The activity of AgNPs antibacterial depends on many mechanisms. First mechanism depends on generated ROS then oxidative stress that lead to protein chemical damage and genetic material (DNA) found in bacteria, Secondly, interactions of electrostatic happened between AgNPs and cell membrane proteins and cell membranes in bacterial may lead to physical damage and then leads to cell death of bacterial, another search suggested that the nanoparticles small size can helped AgNPs antibacterial activity (Kim and Lee, 2005).

According to obtain results, it is possible used AgNPs synthesis from *Thymus kotschyanus* extract to treated the poultry infection (*in vivo*) as alternative treatment instead of antibiotics, due to their activity against pathogenic bacteria and their safety applications in pharmaceutical and medical fields (Hamelian *et al.*, 2018).

Conclusion

This study explained isolation and identification of pathogenic bacteria from poultry dropping and test their antimicrobial sensitivity patterns to indicate MDR isolate, then synthesis of AgNPs by *Thymus Kotschyanus* extract by method ecofriendly, easy and clean with out using

chemical material method. Different techniques were used to characterized AgNPs synthesized. AgNPs synthesized by using herb, with range of diameter (21.64-123.14 nm), have great antibacterial effect against MDR and pathogenic bacteria isolate under study. From previous study AgNPs without any recorded toxicity this advantage contribute to the possibility using AgNPs as alternative treatment than antibiotic to treated MDR pathogenic bacteria to poultry when mixing with poultry feed.

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