Effect of Garlic Extract Treated with Silver Nanoparticles on *Escherichia coli* Isolated from UTI and Diarrheal in Human

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Abstract:

The current study included testing the effect of silver Nanoparticles with garlic extract on *E. coli* isolated from cases of urinary tract infection and diarrhea in humans. Where 51 samples were collected from hospitals in Babil Governorate, 11 isolates of *E. coli* were revealed. Silver nanoparticles AgNPs were synthesized in a biological way using garlic extract, then the formation of silver nanoparticles was detected by changing the color from yellow to brown, and the formed silver nanoparticles were characterized using ultraviolet visible spectrophotometry (UVI-vis) which appeared absorption atom (silver nanoparticles at 410 nm and green fabrication of nanosilver with garlic extract at 325 nm). Fourier-transform infrared (FTIR) analysis indicated the presence of carboxyl groups, amines, amides, and polyphenols in the composition of AgNPs, while scanning electron microscopy showed the size of green AgNPs ranging between (78-145nm). Then, the silver particles classified as antibacterial were studied, and their effect on bacterial growth was measured by two methods, well diffusion methods by digging and macro- dilution method . The results showed that the silver particles had an effective effect on isolates of *E. coli*. After activation of efflux pump genes of *E.coli* isolates, efflux gene expression (AcrA) was measured by quantitative polymerase chain reaction (R.T.q PCR) after treatment with green AgNPs of *E. coli* isolates and it gave a high genetic change. The aim of this study to evaluate the antimicrobial activities of biosynthesized AgNPs against of *E.coli* .

Keywords: Silver nanoparticles; Biosynthesis; *Allium sativam*; *E.coli,* FTIR .

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Introduction:

Escherichia coli are commensal bacteria found in the human colon, and they seldom cause illness. *E. coli* pathotypes, on the other hand, are specific clones of E. coli that are pathogenic as a result of gaining virulence factors. Enterohaemorrhagic E. coli (EHEC) is a family of intestinal pathotypes that produces the shiga toxin (STEC). Bloody diarrhea (haemorrhagic colitis), non-bloody diarrhea, and the potentially fatal heamolytic uremic syndrome (HUS) are all symptoms of STEC infections (Kaper *et al*., 2004). STEC is mostly found in ruminants' intestines, and illness is most commonly brought on by eating tainted meat and produce. Shiga toxin (Stx) is the main toxin generated by STEC, and it is divided into several serotypes based on lipopolysaccharide (O) and flagellar (H) antigens. O157:H7 strain generates Stx and has a locus of enterocyte effacement (LEE) pathogenicity island that codes for additional virulence factors that cause human illness (Clements *et al*., 2012). Although the exact mechanism(s) of action of silver nanoparticles, which have been widely used in medicine, are still unclear, it is known that they have antibacterial characteristics (Lee *et al*., 2014). Numerous physical, biological, and pharmacological applications can make use of nanoparticles that have been engineered to be more effective. Due to its beneficial properties,

nano-Ag has gained widespread recognition as a powerful antibacterial agent. According to several studies, this antimicrobial action is mediated by the prevention of bacterial cell division, which ultimately leads to cell death by damaging bacterial membrane structure (Li *et al*., 2010). Allium sativum Linn, also known as garlic, is a member of the Liliaceae family and is classified as an onion. It has received a lot of attention from modern medicine because it contains more than 200 chemicals and is frequently used to protect the human body from a variety of diseases due to widespread beliefs about its effectiveness in enhancing overall wellbeing (Bayan *et al*., 2014) . Allicin is the primary bioactive substance present in raw garlic that has been crushed or extracted in water (allyl 2 propenethiosulfinate or diallyl thiosulfinate). which function as anti-infective drugs against fungus, parasites, and bacteria (Abramovitz *et al*., 1999; Karunanidhi *et al*., 2019).

Materials and Methods:

1. Bacterial Isolates Collection

A total of 11 *E.coli* isolated were collected from differ clinical specimen in Babylon province during the period from October to December 2021. These specimens were collected from 51 sample obtained from human suffered from different infection by taking swabs from diarrheal and UTI infection, were collected from AL-hayshemia Hospital and AL-Qasim Hospital.

2. Preparation of the Garlic Extract

The Extraction was performed in a 500 mL Soxhlet extractor applying reflux technique. For extraction, 40 gram was extracted with the ethanol solvent (70%). The system was immersed in a Precisterm thermostatic bath. When the boiling point of 70% ethanol was reached, the reflux was repeated for eight cycles. The extraction process was continued for 7 h. After filtration of total extracts with Whitman No.3 filter paper, the extracts were evaporated to dryness in rotary evaporator apparatus at a temperature (80°C) until all the solvent had been removed to obtain an alcohol-free extract sample then weighed the yield. The final product was stored in freezer at -20 C until use (Cañizares *et al*., 2004).

3. Synthesis of green AgNPs

After preparing 25 mL of prepared garlic extract (10% concentration), about 750 mL of AgNO3 (10 mM) was added drop wise. The mixture was then gently stirred using a magnetic stirrer at a speed of 800 rpm for 120 minutes at 30-50°C. By using high speed centrifuge, the green AgNPs were concentrated and purified, then in order to remove any interactive biological molecules, it was rinsed with D.I. Three times were needed to complete the separation of the green AgNPs, after which it was dried and prepared for characterization. Formation of green AgNPs will be marked by the color change from colorless solution to a signature brown solution according to Sun *et al*., (2014). And the green synthesized AgNPs kept in dark condition during synthesis and use to avoid any oxidation of new product AgNPs.

4. Characterization of green-synthesized AgNPs:-

The identification and verification of the creation of silver nanoparticles. Using a Perkin Elmer-Lambda 950-UV-visible spectrometer, UV-Vis spectra were taken in the 200 to 800 nm region. By employing a BRUKER D8 advance with a Cu target (λ 1.54 Å), X-ray powder diffraction (XRD) was used to analyze the crystal structure and phase produced for as-synthesized AgNPs. The diffraction patterns were collected at 40 kV and 40 mA, with a 0.05º step size and a count time of 3 s. FESEM (QUANTA FEG 250) with energy dispersive X-ray spectroscopy was used to analyze the morphology of the as-produced AgNPs (EDX). The Perkin Elmer spectrophotometer was used to gather Fourier transforms infrared (FT-IR) spectra with a spatial resolution of 4 cm-1 in the transmission mode, in the range of 400 to 4000 cm-1. In order to determine the surface chemistry of the reduced AgNPs and the presence of biofunctional moieties in garlic extract, the samples were produced using the KBr pellet method.

5. Antibacterial activity of AgNPs and garlic extract.

For testing antibacterial activity, *E.coli* (Gram-negative) were attained from the laboratory of Microbiology, AL-Qasim Green University , veterinary medicine collage . The isolates were identified and the results were confirmed (Koneman *et al*., 1992). Bacterial isolates were

rehydrated on nutrient agar from the stocks prior to bacterial investigations. In the current study, Mueller Hinton Broth (MHB), Mueller Hinton agar (MHA), and locally sourced garlic extract were used.

6. Estimation of MICs and MBCs for AgNPs and garlic extract.

The tube broth dilution technique, as advised by the Clinical and Laboratory Standards Institute, was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of five distinct E. coli isolates. The lowest dose of the medicinal substance that prevented the development of the microorganisms used in the experiment was identified as the minimum inhibitory concentration (MIC). The minimum bacterial concentration (MBC) is the amount of antimicrobial agents needed to completely get rid of a certain bacterium. By incubating the isolated bacteria in tubes containing broth and serial dilutions of the medication ingredients for 24 hours at 37°C, researchers were able to obtain the MIC value. Fresh cultures were used to create the bacterial inoculum, which was produced in 0.9 percent sodium chloride. MIC value was estimated by visual method. While MBC determined via sub culturing last clear MIC tubes onto growth medium and enumerated to determine viable CFU/ml. The experiment was performed in triplicate for each isolate and negative and positive control was also included.

Statistical analysis

Using the available statistical package SPSS-26, data analysis was conducted (Statistical Packages for Social Sciences- version 26). Descriptive statistics: mean± standard deviation SD. Statistical analysis of data was performed on the basis of ANOVA (one way analysis) with least significant difference LSD was detected to compare between groups.

Results :-

1. *E. coli* isolation by culture and biochemical tests

11 out of 51samples (21.56%) were isolated, where. 4 (16%) from 25 represented UTI , while 7 (26.92%) from 26 represented diarrhea infections in human .show in table 1.

Table (1) Percentage of *E. coli* isolated from Human

Specimen Human	Number	Positive result	Negative result	Percentage
UTI	25	4	21	16%
Diarrheal	26	7	19	26.92%
Total	51	11	40	21.56%

2. Green synthesis of green AgNPs using Allium sativum Extract:-

After 24 hours, the Allium sativum extract and AgNO3 combination showed a color shift from colorless to brown figure (1). During the incubation of Allium sativum extract without AgNO3, no color change was noticed. Since, the appearance of a brown color in AgNO3-treated Allium sativum suggested the formation of AgNPs and that confirmed the reduction of Ag+ to Ag^0.

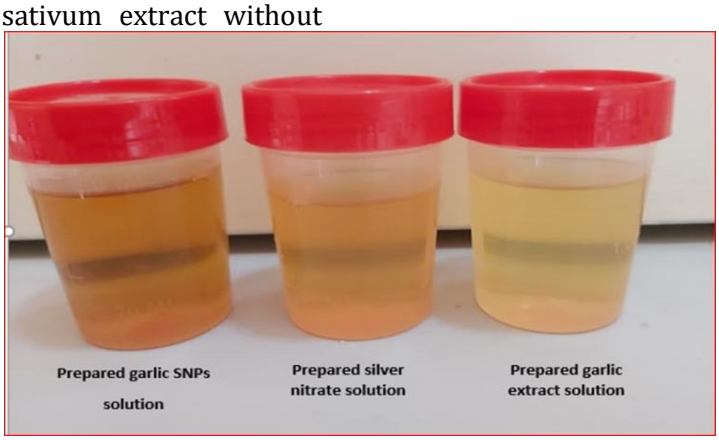
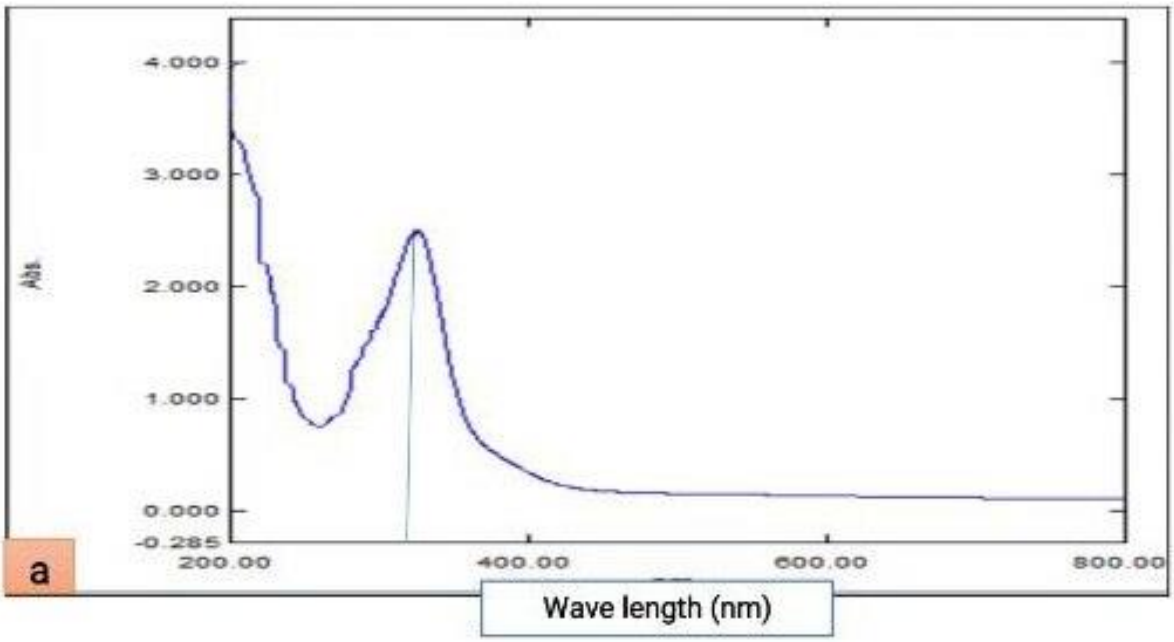


Figure (1) A photograph demonstrating how the color of AgNO3 changed when alcoholic Allium sativum Extract was added to it.

3. Characterization of green AgNPs

a. UV–Visible Spectroscopy

UV–visible spectroscopy is a primary step in confirmation of the synthesis of Ag-NPs as well as the color change to dark brown. The UV–visible spectra of samples Ag-NPs were recorded with 24 hours with scanning from 200 to 800 nm. The UV-VIS absorption was analyzed after centrifuging and re-dispensing the particles in deionized water. Garlic extract was used to biosynthesize the Ag-NPs, in which there was just one clearly visible surface plasmon resonance band at 325 nm (Figure 2a). A narrow plasmon absorption band seen in the 200–800 nm range, as shown in Figures (2b), is the feature of silver solution that is most distinctive.



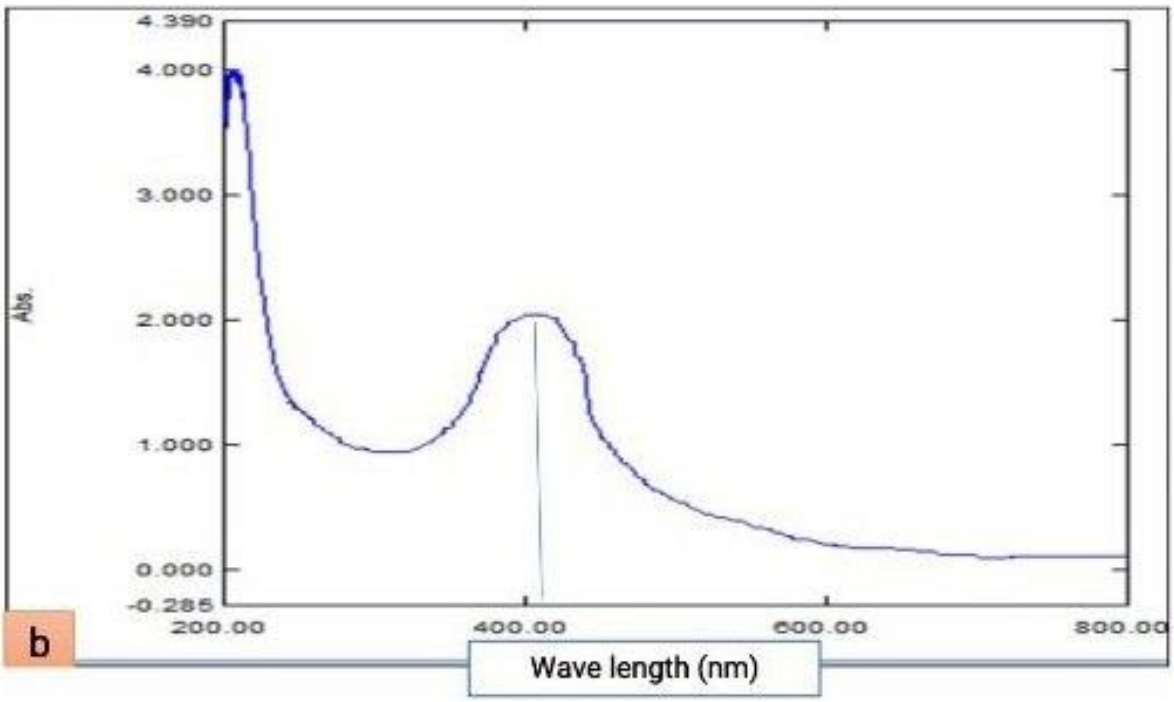


Figure (2): UV-VIS Absorbance Spectroscopy for (a) Green AgNPs from Garlic extract.(b) AgNPs.

b. X-ray diffraction (XRD) Analysis:

Is a rapid analytical technique that can tell you how big each unit cell is and is used to characterize and identify crystalline materials made of manufactured nanoparticles. These pronounced Bragg peaks might be the product of an Ag-NP capping agent. The bio-synthesized (Garlic extract) and chemical synthesized of Ag-NPs were characterized by the XRD. Figures (3).

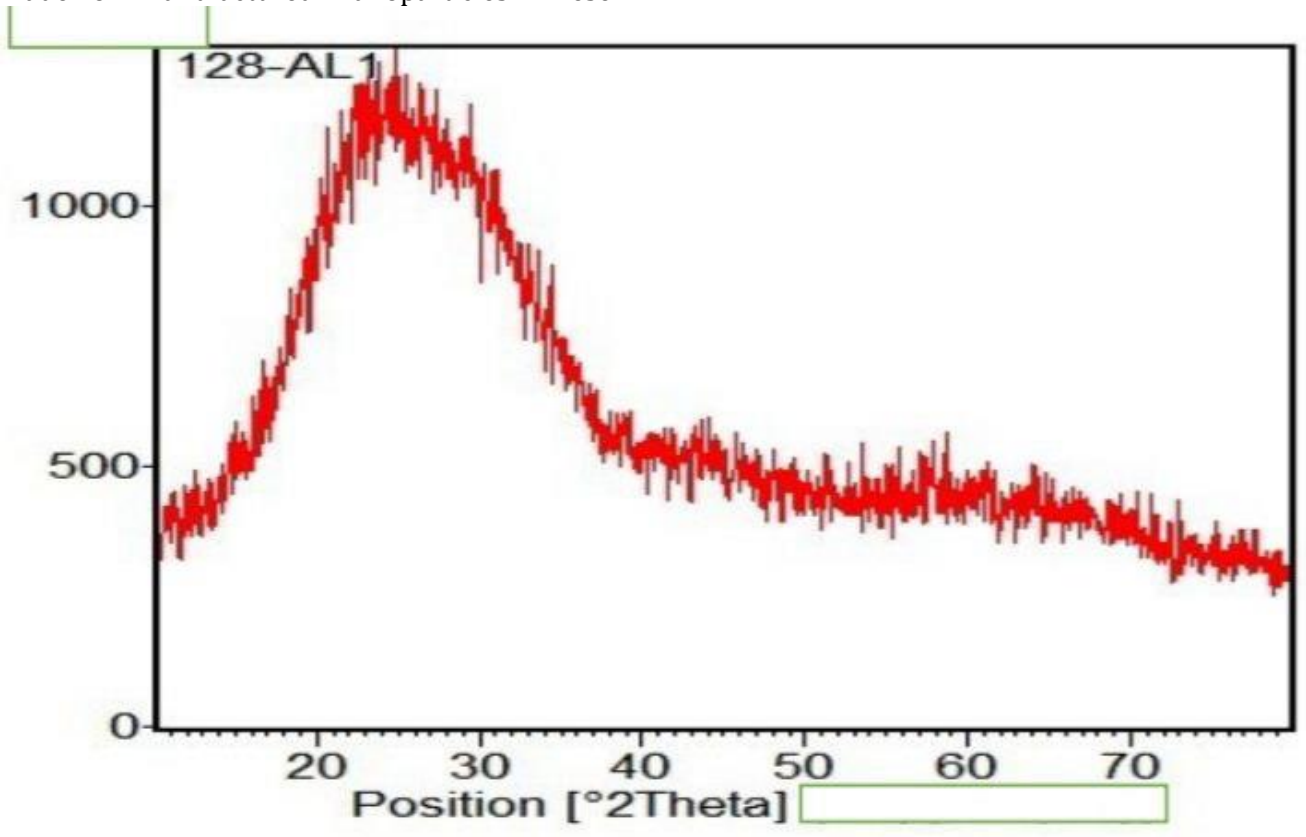


Figure (3): X-ray diffraction pattern of green synthesized AgNPs from Garlic extract

c. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR analysis of samples was performed in the range of 600–4000 cm-1. FTIR spectroscopy showed that samples analysis, had prominent bands of absorbance at peaks (3286.70, 2102.41, 1631.78, 956.69, 771.53, 644.22, 613.36 and 563.21) cm-1 for garlic-AgNPs (Figure 4a). For AgNPs, the absorption bands occurred at (2916.37, 2854.65, 1747.51, 1651.07, 1570.06, 1516.05, 1462.04, 1373.32, 1188.15, 1083.99, 1002.98, 794.67, 748.38, 678.94, 636.51, 582.50) cm−1 (Figure 4b).The absorption bands 3286 cm−1 belongs to the stretching vibration of intermolecular hydrogen bonded O–H group in alcohols and phenols, and the bands at 2916.37 cm−1 assigned to aldehydic C–H stretching, while the bands at 2102.41 cm−1 was attributed to carbon dioxide. While the bands at 1747.51-1651.07 cm−1is related to the stretching vibration of carbonyl (C=O). while the bands at 1570.06, 1516.05 band is related to the stretching vibration of C=C. The absorbance band at 1462.04 cm-1.

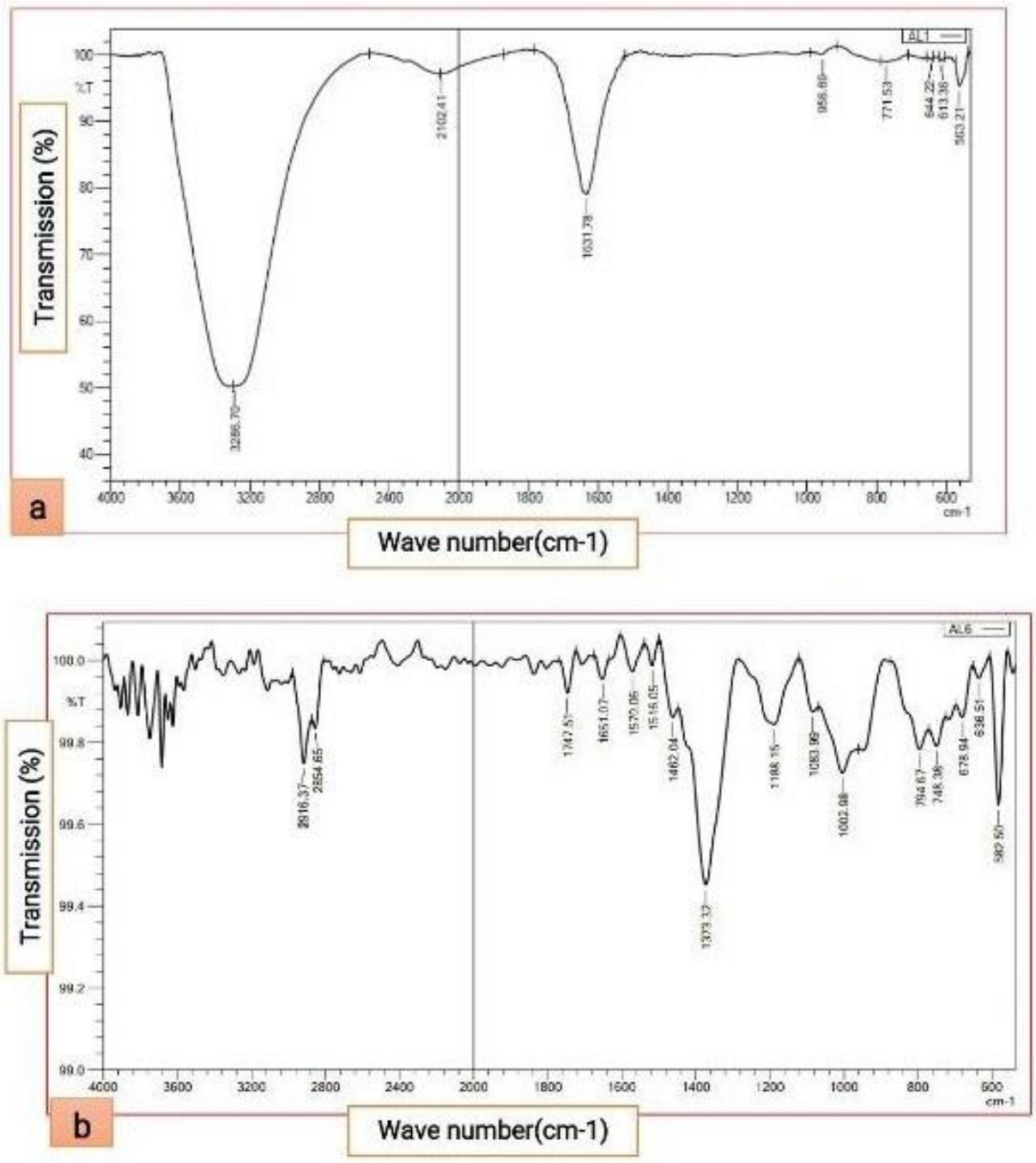


Figure (4) FTIR Spectra Pattern of (a) AgNPs Synthesized from garlic extract.(b) AgNPs.

d. Scanning Electron Microscope (SEM) analysis:

The particle size and morphology of the garlic silver-NPs were studied by SEM. The morphological features (shape and size) of Prepared garlic silver nanoparticles were estimated by SEM analysis. The surface deposited of garlic AgNPs are clearly seen at (40,000x). With optimizing synthesis process, the size of selected synthesized garlic extract AgNPs average exhibited the mean size of 118.2±1.12 with range of 78-145 nm shape was spherical and irregular as in figure (5).

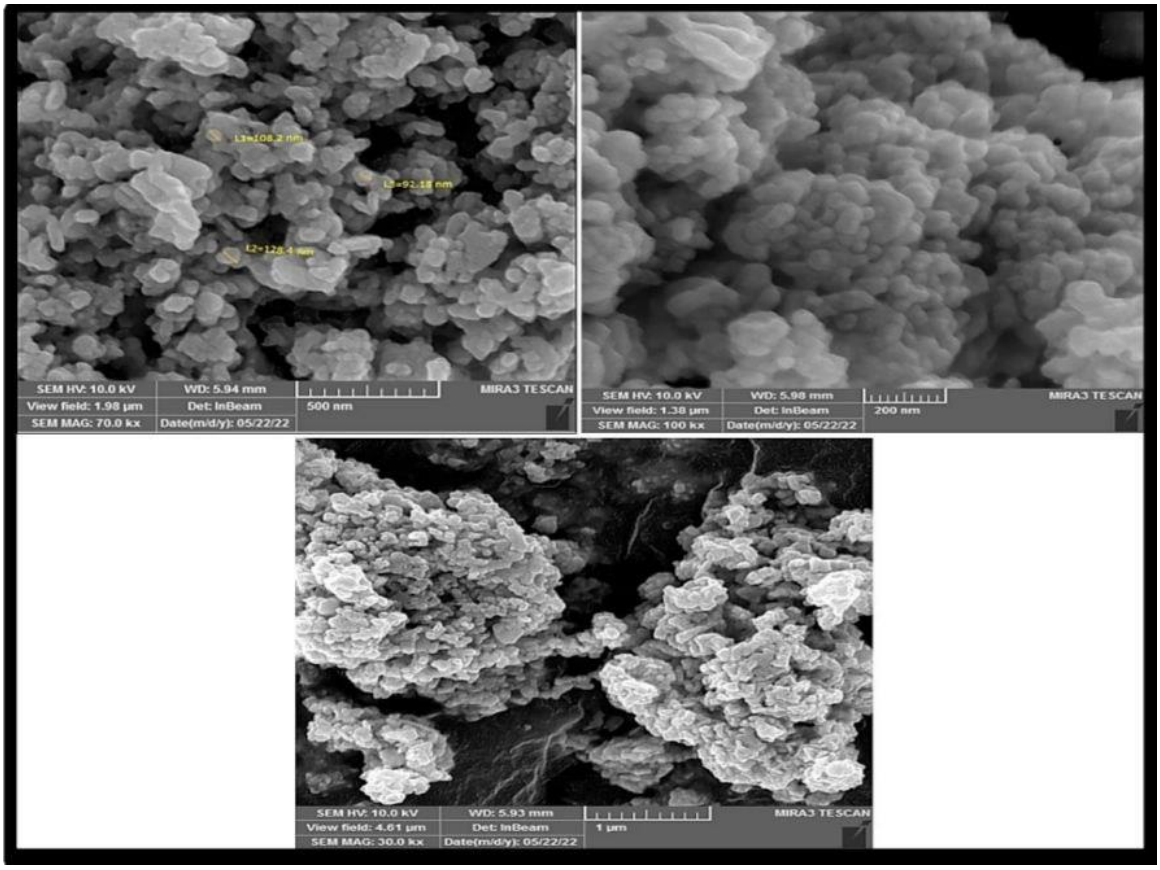


Figure (5) SEM image of AgNPs synthesized by garlic extract (a) size was 108.2nm in spherical shape (b) the clarity of the layers stacked on top of each other (c) Cauliflower - like in shape.

4. Antibacterial activity of green AgNPs Against E. coli Isolates by Agar Well Diffusion Test.

The activity of AgNPs was examined against E. coli isolate by using agar well diffusion method, and tested by using different concentration (5,10,20 µg/mL). Also, AgNPs tested alone and in combination with garlic extract. The activity of AgNPs alone was significantly effective P<0.05 in concentration dependent manner, and recorded the highest zone of inhibition in concentration 20 µg/mL that about 19±0.47.

Table (4.3) Antibacterial activity of tested substances by agar will diffusion method

Groups	Concentration (µg/mL)		
	5	10	20
Garlic extract	8.9±0.23Cb	10.6±0.26Bb	14.6±0.16Ac
Silver nanoparticles	13.8±0.24Ba	18.4±0.16Aa	19±0.47Ab
AgNPs and Garlic extract	14±0.21Ca	19±0.21Ba	20.6±0.16Aa
LSD (P<0.05)	0.713		

Capital letters denote to the vertical statistical analysis

Small letters denote to the horizontal statistical analysis

Different letters denote to the significant difference at P<0.05.

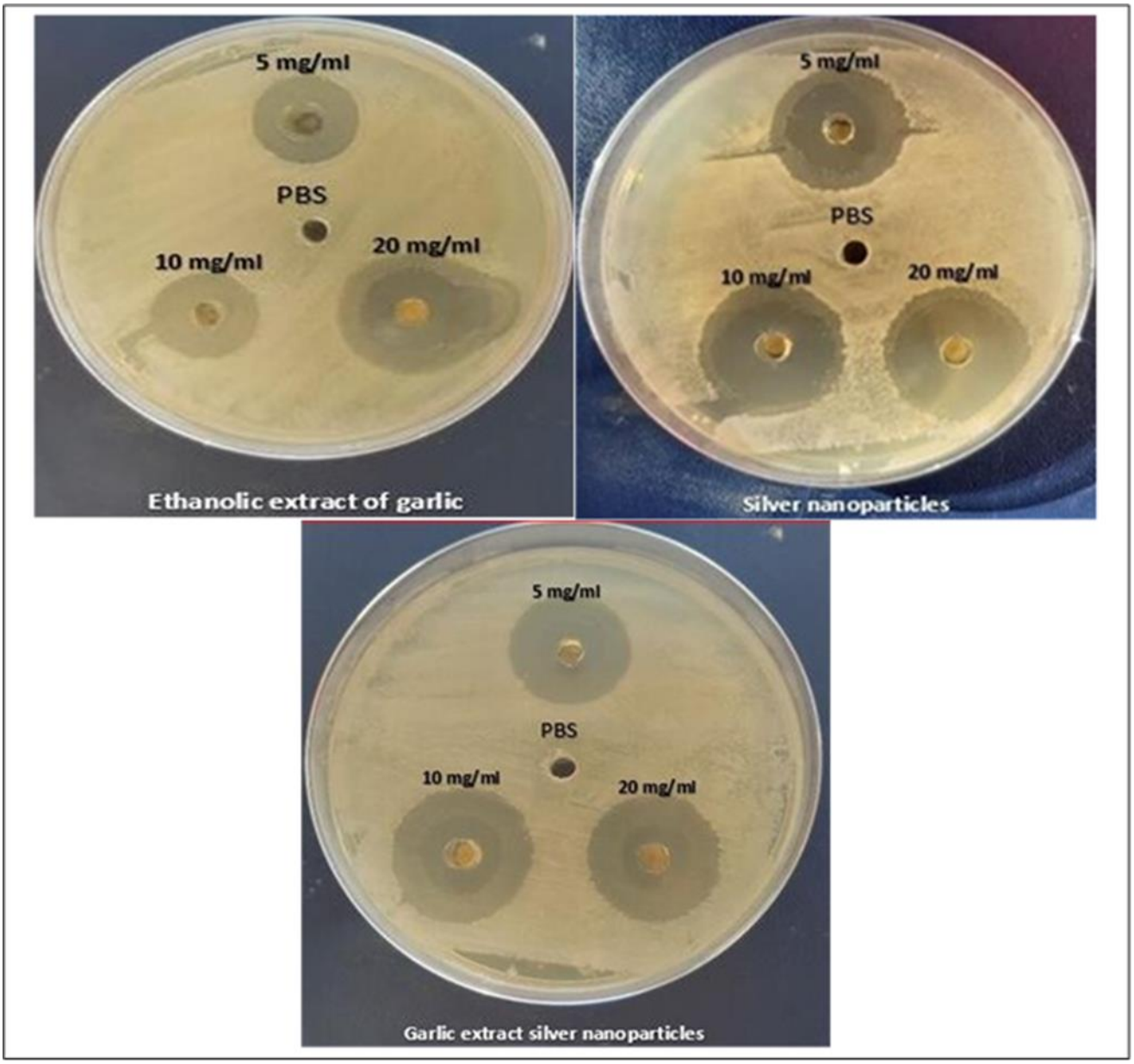


Figure (7) :- antibacterial activity of biosynthesized AgNPs and garlic extract against *E.coli* isolates by agar well diffusion.

5. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentrations (MIC) determined by tube dilution method, against ten tested bacterial isolates. The observed MIC values for test substances of ten E. coli isolates analyzed statistically and are showed in table (4). From the data recorded in the table below, there were significant $P<0.05$ differences in MIC value between three groups that recorded 1.43±0.167, 35.2±5.22 and 76±14.25 µg/mL for Garlic extract, AgNPs and Garlic extract and for AgNPs Respectively. Additionally, MBC value revealed significantly differences between groups. Since they recorded 115.2±8.53, 205.41±83.44 and 332.8±51.2 µg/mL for AgNPs and Garlic extract, Garlic extract and for AgNPs respectively.

Table (4) Antibacterial activity of tested substances by macro-dilution method.

Substance	MIC value (µg/mL)	MBC value(µg/mL)
Garlic extract	819.2±83.6A	1433.6±167.2A
Silver nanoparticles	83.2±14.25B	332.8±51.2B
ANGps and Garlic extract	35.2±5.22B	115.2±8.53B
LSD (P<0.05)	142.4	293.3

Different letters denote to the significant difference at P<0.05

6. Estimation the Expression of Efflux Pump Genes *(AcrA)* of E.coli by Real-time PCR

The term "relative quantification" refers to the change in target gene expression in comparison to the control group (Livak and Schmittgen, 2001). All of the RT-PCR analysis was thought to be a standard way to look at gene expression of genes that might have an effect. The figures (8) show the fold change in RT-qPCR amplification of *acrA* genes in tested bacteria and controls compared with HKG (*isrA gene*).

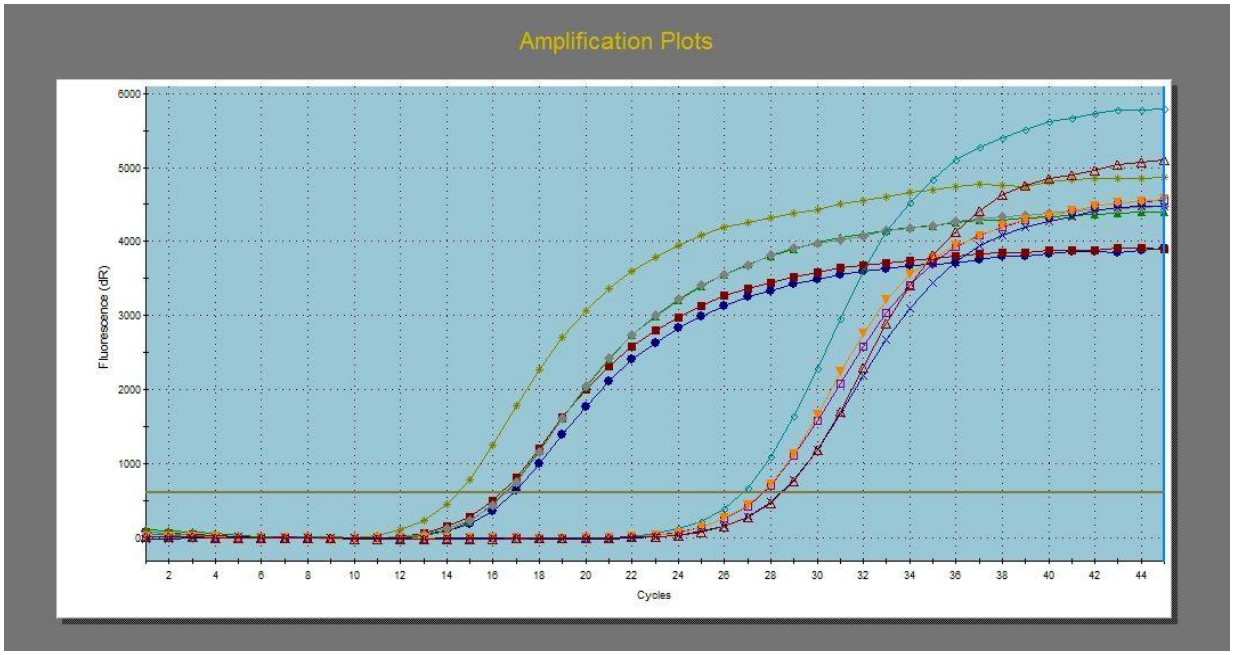


Figure 8: RT- qPCR amplification Efflux pump gene (acrA gene) and HKG (*isrA gene*) in treated and untreated (control) *E. coli* isolates **.**

To estimate fold change gene expression, the differences Ct between *acrA* gene and housekeeping gene are shown in fig. (9) which represent the expression level of (*acrA*) after treated with AgNPs. The expression level of *acrA* significantly different (P<0.05) for *E. coli* were treated with AgNPs in compared to level of gene expression of the same isolates without treating. The results showing the expression of the *acrA gene* using RT-qPCR after Treatment with combination of AgNPs and garlic extract resulted in increase The cycle number and these explain that this extract will inhibit efflux pump system when this bacterium exposed to it.

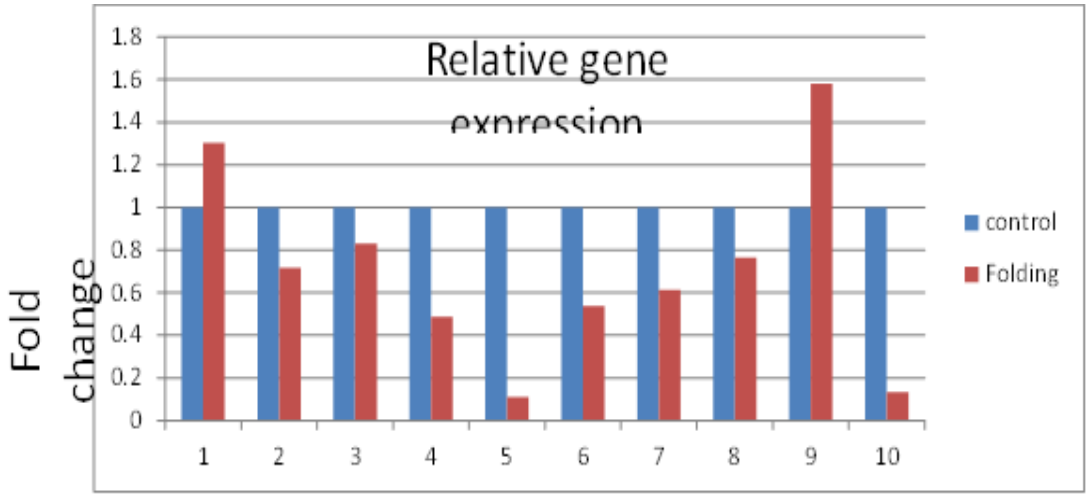


Fig 9: Effect of (AgNPs) on relative genes expression: the fold change of *acrA* gene decrease in *E. coli* isolates after treated with combination of AgNPs and garlic extract.

Discussion:

According to the results of this study, it was revealed that 26.92% of human specimens with diarrhea were infected with E. coli causing diarrhea. And these findings were in agreement with Mahdavi *et al*., (2018). But Khmnh, (2007) reported relatively higher (60%) prevalence. The findings from UTI specimens in human were 16% caused by E. coli bacterial strain, and this result were in agreement with Tajbakhsh *et al*., (2015) who reported E. coli was the most common bacteria in men (17%) and women (34.7%). Additionally, the green synthesized AgNPs are stored in dark conditions throughout the synthesis process to prevent oxidation of the new product AgNPs. Our results are consistent with previous study done by Bouqellah *et al*., (2019) who found that the plant extract from Allium. Also, our results in agreement with Ayad *et al*., (2019) who reported reduction of Ag+ to Ag^0 by plant extract and change in color from colorless to brownish color AgNPs. *A. Sativum* can reduce Ag+ ions into silver particles, which results in color changes from light yellow to brownish once the reaction with Ag+ ions is complete. This procedure demonstrated that the reducing agent produced in the extract completely reduced the Ag+ ions. The UV-visible spectrum of Ag NPS and garlic Ag nanoparticles scanned in between the wavelength 200-800 nm and exhibited a maximum absorption were found to be 410 nm for AgNPs while garlic silver nanoparticles exhibited 325 nm (Figure 2)The type, size, and morphologies of the produced NPs, the medium's and temperature's dielectric constant, as well as their interparticle distances, all have a remarkable impact on the SPR absorbance (Mahmoud and Al-Ghamdi, 2012). The XRD results suggested that the crystallization of the bio-organic phase occurs on the surface of the Ag-NPs. In most cases, particle size effects are to blame for the widening of peaks in solids' XRD patterns. Broader peaks reflect the influence of the experimental circumstances on the nucleation and development of crystal nuclei and indicate smaller particle size. The crystalline nature of AgNPs was clearly by the peak broadening. Ag-NPs showed distinctive peaks at the 2θ value of 27.56°, 29.80°,32.25°, 43.94°, 57.81° and 64.30° in bio-synthesized (Garlic extract). This result indicated that the AgNPs have good crystallinity and purity structure. These patterns correlate to the numbers 122, 111, 200, 220, and 311 in that order. Lattice planes were examined, and reference values from the Joint Committee on Powder Diffraction Standards were compared (JCPDS: 89-3722).The XRD patterns displayed are consistent with earlier reports on microstructures (Ayad *et al*., 2019). The functional groups between the reducing agent (garlic extract) and AgNO3 were identified using FTIR analysis in order to anticipate their role in the production of silver nanoparticles. All of these functional groups were depicted as peaks in the FTIR Spectra in the figure (4). Related to the stretching vibration of C-N. 1373.32 cm-1 which related to stretching vibration of C-O. The absorbance band at 1188.15, 1083.99, 1002.98 cm-1 related to stretching vibration of C-O. In

addition to the bands 956.69 cm−1 assigned to C=CH2. And the bands near 771.53, 644.22, 613.36 cm−1 means C–H bending vibrations and amine groups (NH). Changes in the functional groups in active biomolecules might suggest their involvement in synthesized of Ag-NPs (Das *et al*., 2019). The particle size and morphology of the garlic silver-NPs were studied by SEM. The fact that the particles varied in size suggests that the method used to create AgNPs using a green synthesis method is versatile in manufacturing nanoparticles with diverse size distribution profiles and particle size spectra. In particular, both sonication and churning break apart the particles' agglomeration to make them more dispersed (Sarafraz *et al*., 2014). The interactions between the bio-organic capping molecules attached to AgNPs through hydrogen bonds and electrostatic interactions produced the SEM image of AgNPs (Alsarhan, 2017). From the other hand, the combination of and garlic extract revealed also efficacious effect in concentration dependent manner and recorded the largest zone of inhibition in concentration 20 µg/mL, Also, when comparison between groups; the significant P<0.05 inhibition effect showed in combination group (AgNPs and Garlic extract) in concentration 20 µg/mL which recorded synergistic activity in about 20.6±0.16, while there are no significances between combination groups and AgNPs groups in concentrations 5,10 µg/mL. Furthermore, garlic extract group recorded the lowest antibacterial activity when compared with AgNPs and Garlic extract. Table (3) and figure (7). These results of this study were agreed with the results of Chauhan *et al*., (2015) who found that AgNPs have antibacterial activity against *E. coli*, *P. aeruginosa*, and Salmonella sp. From the other hand our results agreed with Anandalakshmi *et al*., (2016) and El-Refai *et al*., (2018) who reported that the AgNPs synthesized by using the mixture of plant extracts showed more potent antimicrobial activity than the AgNPs. AgNPs and naturally occurring chemicals in ginger, garlic, and capsicum may have combined antibacterial actions to produce increased bactericidal activity. It has been shown that medicinally significant biomolecules contained in plant extracts, such as amino acids, proteins, vitamins, alkaloids, phenolics, saponins, tannins, and terpenoids, are involved in the reduction and stability of silver ions (Kulkarni and Muddapur, 2014). This might involve the release of Ag+ ions from AgNPs that may attach to the positive charged cell wall, thus leading to the deformation of the proteins and resulting in cell death (Kim *et al*., 2009; Reda *et al*., 2019). Minimum inhibitory concentrations (MIC) determined by tube dilution method, against ten tested bacterial isolates. The results of this study were in accordance with (Charti *et al*., 2021) who recorded MIC value of green synthesized AgNPs against *E. coli* about 39.06 ± 0,02 µg/mL. From other hand disagreement with (Charti *et al*., 2021) in the MBC value results. From the other hand, (Halawani, 2016) recorded MIC value of green synthesized AgNPs against E. coli about 59 µg/mL and it is less efficacious than the MIC in the present study. So we suggest that the plant extract (garlic extract) used in the present study have great reducing effect to produce more effective AgNPs. The previous research showed that Gram-negative bacteria were more susceptible to AgNPs than Gram-positive bacteria. This could be because their cell walls' composition varies (Chatterjee *et al*., 2015). It was reported that the activity of AgNPs against Gram-negative bacteria was stronger than Gram-positive bacteria (Cheng *et al*., 2018; Devagi *et al*., 2020) which was confirmed by results of the present study. All of the RT-PCR analysis was thought to be a standard way to look at gene expression of genes that might have an effect. The figures (9) show the fold change in RT- qPCR amplification of *acrA* genes in tested bacteria and controls compared with HKG (*isrA gene*). Change in efflux activity in Enterobacter spp. by AgNPs has been reported in context of EmmDR efflux pump belonging to multiple antibiotic and toxic extrusion (MATE) superfamily] (He *et al*., 2013).The results of this study were in agreement with Behdad *et al*., (2020) who found efflux pump genes were down-regulated significantly after the treatment with green AgNPs. Furthermore, the study of Hasson and Abady, (2019) revealed that gene expression of biofilm encoding genes (SmaI and acrA) for biofilm forming and MDR bacteria (Serratia fonticola and Pantoea sp.) were down regulated when treated with biological AgNPs. There are two potential pathways for metal NPs' inhibition of efflux pumps. The first potential method prevents the ejection of antibiotics outside the cells by directly attaching metal nanoparticles to the efflux pumps' active region. Another potential mechanism is the interruption of efflux kinetics. AgNPs may operate here as a

competitive inhibitor of antibiotic for the binding site of efflux pumps (Gupta *et al*., 2017).

Conclusion:

This study has consistently demonstrated the effective ability to biosynthesize silver nanoparticles by Allium sativum extract against *Escherichia coli*. nanoparticles can be functionalized with different molecules in order to improve their antibacterial effect. AgNPs showed strong antibacterial effectiveness. As a result, they might be employed as an antibacterial agent and a viable alternative to traditional antibiotics. These data support the use of AgNPs as antibacterial; however, appropriate strategies for their use must be studied and developed without aggravating the situation of the emergence of resistant strains.

conflict of interest

The authors declared that there was no difference in interests.

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