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Biosynthesis of silver nanoparticles using Thyme vulgaris leaves extract and its antibacterial activity

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Abstract

In the current research, an eco-biosynthesis method for synthesizing silver nanoparticles (AgNPs) is reported using thymus vulgaris leaves (T. vulgaris) extracts. The optical and structural properties of the nanoparticles were determined using uv-visible, x-ray diffraction (XRD) and field emission scanning electron microscope (FESEM). In addition, the synthesis factors such as temperature, molar ratio of silver nitride and thymus vulgaris leaves extract was investigated. The XRD pattern presented higher intensity for the five characteristic peaks of silver. FESEM images for same samples indicated that the particle size was distributed between 24-56 nm. The mixtures were used to inhibit two kinds of bacteria which are Gram negative (E. coli (and Gram positive (S-aureus) as tested for antibacterial activity by agar well diffusion method. The results showed the effectiveness of the synthesized AgNPs on inhabitation the growing up of the bacteria and their isolates.

Key words

Silver nanoparticle, Thyme, FESEM, antibacterial activity, escherichia coli.

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التخليق الحيوي للجسيمات النانوية الفضية باستخدام خلاصة أوراق الزعتر والفعالية المضادة للبكتيريا

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الخلاصة

تم في هذا البحث تحضير جسيمات الفضة النانوية بطريقة صديقة للبيئة هي الطريقة البيولوجية باستخدام مستخلص نبات الزعتر، وتمت دراسة تأثير عدة عوامل منها عامل تأثير درجة حرارة التحضير، وعامل تأثير نسبة الخلط بين محلول نترات الفضة ومحلول مستخلص أوراق نبات الزعتر على الخصائص التركيبية والبصرية لجسيمات الفضة النانوية المحضرة عن طريق مطياف الاشعة فوق البنفسجية والمرئية UV-Visible، تحليل نمط الاشعة السينية (XRD)، والمجهر الالكتروني الماسح (FESEM). اظهرت فحوصات الاشعة السينية من خلال الحصول على أعلى شدة وكذلك على عدد 5 من القمم التي تعود لجسيمات الفضة النانوية. اوضحت صور FESEM لنفس العينات إلى أن حجم الجسيمات كان ضمن الحجم النانوي ويتراوح بين 24-56 نانومتر. تم استخدام المحلول لدراسة اثر جسيمات الفضة في عملية تثبيط نوعين من البكتريا سالبة الكرام *Escherichia coli* و بكتريا موجبة الكرام *Staphylococcus aureus*. أظهرت جسيمات الفضة النانوية منطقة تثبيط واسعة للبكتريا من AgNPs في جميع تحليلها للبكتريا (*E.coli* و *Staph*) باستخدام طريقة اختبار نشاط مضاد للبكتريا لجسيمات الفضة النانوية بطريقة نشر اجار ضد الكائنات الحية الدقيقة.

Introduction

Nanotechnology, as a general rule, is adapted to obtain a high degree of precision in the functions, sizes and forms of materials. Controlling the reaction or directing the involved molecules and atoms in the reaction will refine the traditional materials in

addition to a decrease of the consumption of energy [1]. American Society for Testing and Materials ASTM defined nanoparticles as the particles of two or three dimensions ranged from 1 to 100 nM [2]. The chemical and biological properties of the nanoparticles depend on the individual atoms or molecules that consteuct the nanoparticles. The nanoparticles chemical and biological properties vary corresponding to the atoms/individual molecules and the materials. Generally, nanoparticle exhibits new or modified properties in the term of size, distribution and morphology [3]. Nanoparticles are potentially applicable to be used for electronical, optoelectrical magnetic, and storage devices [4]. Among nanoparticles, silver nanoparticles (AgNPs) have unique properties that can be adopted in many applications such as antimicrobial material, biological sensors, fiber-composite materials and electronic devices. Silver nanoparticles are reported to be synthesised by many physical and chemical methods [5]. The nanoparticles can be synthesized in different types via adopting the proper protocol to meet the requirements of various nanoparticle applications. Those protocols can be divided into top-down path and bottom-up path categories [6]. The bottom-up path technique forms the nanoparticles via accumulation from the bottom, while top-down path produces the nanoparticles by breaking down the bulk material [7]. Consequently, the biosynthesis method is a bottom-up approach which includes oxidation-reduction of the reactants. Microorganisms and plant extracts are utilized in nanoparticles synthesizing (green synthesis) [8]. Green synthesis or phytosynthesis is an emerging synthesizing approach in nanoscience and nanotechnology [9]. green synthesis of nanomaterials includes three mechanisms that use different plants and solvents to extract the effective compound from the plant [10]. The main mechanism is the phytochemical reduction (in) the plant. The phytochemicals include many compounds such as terpenoids, ketones, aldehydes, amides, flavones, and carboxylic acids. The function of flavones, organic acids, and quinone's are the immediate reduction of silver ions. Phytochemicals directly reduce the ions and release hydrogen to form silver nanoparticles [11]. Green synthesis is an eco-friendly process (bottom-up approach) that includes the use of microorganisms (bacteria, yeast, fungi, algae and viruses) and plants or their extracts [12, 13]. Adopting plants in the synthesis of AgNPs has drawn the attention of researchers due to the rapid, ecofriendly, economical protocol and non-pathogenic. In addition, it is a single step biosynthetic process [14]. A large number of plants are reported to facilitate silver nanoparticles.

Many types of bacteria are causes infections and different types of diseases for human. Among them, Staphylococcus aureus, and Escherichia coli are popular types [15]. Their increased resistance into the bactericides and antibiotics has been reported recently because of the development of resistant strains. In addition, some antimicrobial agents are reported to be irritant and toxic. This increases the need to create ways to formulate new, safe and cost-effective types of biocidal materials. In literature, it has been reported the antimicrobial efficiency of nanoparticles as bactericidal materials [16, 17]. More recent, Klabunde and co-workers stated that highly reactive metal oxide nanoparticles excellently present a biocidal action toward Gram-positive and Gram-negative bacteria [18]. In this work, silver nanoparticles

biosynthesis were used as bacteria inhibition method without any side effects.

Experimental work

1. Synthesis of the plant extract

Thymus vulgaris leaves were vigorously washed with distilled water then dried using oven. After drying, the leaves were grinded using electrical miller and stored in nylon bags at room temperature for future use. In order to produce the plant extract, 10 g of the dried leaves powder was mixed with 100 ml of distilled-water in Erlenmeyer glass flask using magnetic stirrer for 1 hour at room temperature. 0.4 micron bacterial filters were used for sterilization of the extract. Then the pure filtered solution was dried at 35˚C. After which, in order to prepare 10000 ppm of stock solution of the extract, 1 g of the dried extract was mixed with 100 ml of distlled-water under magnetic stirring for 15 min for full dissolve of the extract which can be used later to prepare different concentrations.

2. Synthesis of silver nanoparticles

2.1 Changing of temperature factor

Silver nanoparticles were synthesized by changing the synthesis temperature. Typically, 10 ml of $AgNO_3$ of 1mM was mixed with 1 ml of T. vulgaris at different temperatures of 20˚C, 60˚C and 80˚C, respectively.

2.2 Changing $AgNO_3$/ extract mixing ratio

Silver nanoparticles were synthesized by changing the mixture ratio of $AgNO_3$ to extract. Where 10 ml of $AgNO_3$ of 1mM was mixed with T. vulgaris extract to produce mixing ratio of $AgNO_3$: extract 2:10, 3:10 and 4:10.

The colloidal nanoparticles were characterized with a UV-visible spectrophotometer (Shimadzu UV-Vis 1800 spectrophotometer using a double beam UV-visible spectrophotometer (PD-303 UV)) in order to detect the surface Plasmon resonance property (SPR) of AgNPs, at room temperature. The structural evolution of colloidal nanoparticles was executed using X-ray diffractometer [Shimadzu XRD-6000, AS (3k.NOPC)] using Cu-Kα-radiation of wavelength (λ=0.15418 nm) operating at 40 kV and 30 mA. Debye-Scherrer formula ($D=k\lambda/\beta\cos\theta$), where D is the diameter of the particle, k is a constant= 0.94, λ is wavelength of X-ray source, β is the full width at half maximum (FWHM) and θ is the Bragg angle) was used to measure the average crystallite size. The field effect scanning electron microscope (FESEMtype Jeol JSM-6460 LV microscope) was used to study the morphology and microstructure of the colloidal nanoparticles.

3. Effect of AgNPs on the bacteria

The activity of bacterial inhibition on the two types of bacteria (*E.coli*) and (*S-aureus*) (provided from the Biology department, University of Anbar) was carried out using well Agar diffusion method. The bacterial suspension was prepared and diffused on the surface of Neutrian ager using a swab and then it was left for 30 min at room temperature. Each Petri dish of the ager contains 5 holes of 6 mm diameter. One of these holes contains the bacterial suspension while the others contain diluted suspension. In addition, each hole was incorporated with 75 µl of the diluted suspension of AgNPs. The Petri dishes were left at

room temperature for a specific period of time before incubation at 37 ˚C for 18-24 hours. Then the diameters of the inhibition areasaround the holes were measured in millimetres [19].

Results and discussion

1. Characterization

1.1 Changing of temperature

Formation of silver nanoparticles were firstly identified through the change of the color. Where the presence of the dark red is a preliminary evidence of AgNPs formation (see Fig.1). Synthesis temperature is one of the factors which affect the formation of AgNPs which was confirmed through study the UV-Vis, XRD and FESEM.

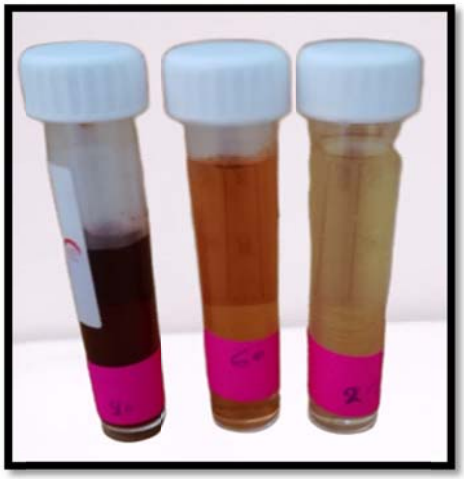

Fig.1: Change the colour of AgNPs during synthesising.

Fig.2 illustrates the UV-Vis spectra of AgNPs which were synthesized at different temperatures. The absorbance was measured at wavelength of 200-700nm. With increasing the temperature, the intensity of the surface plasmon resonance (SPR) increased. Where the broadness of the absorption spectra for SPR indicates the size and shape of the nanoparticles [20]. The optimized synthesis temperature was 60 ˚C which was in agreement with the results reported by Kredy and Adnan [21].

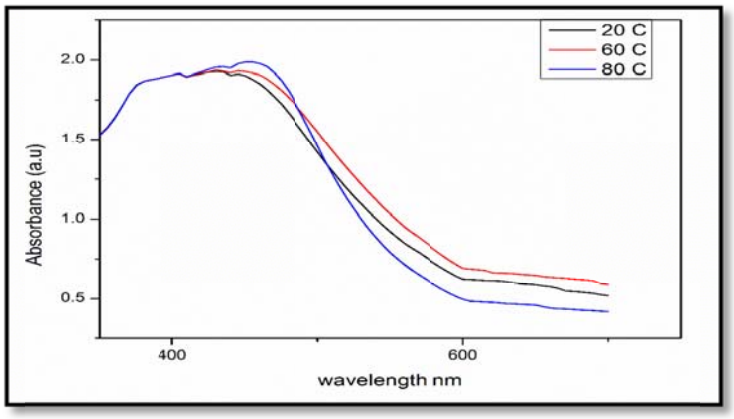

Fig.2: Absorbance as a function of wavelength of 1mM of silver nanoparticles synthesized at different temperatures.

Fig.3 illustrates XRD pattern for the synthesized nanoparticles. The patterns show five characteristic peaks (111), (200), (220), (311) and (222) for the angles 2θ of 77.68˚, 38.48˚, 88.7˚, 44.30˚ and 64.75˚, respectively. The presence of those peaks indicates the fcc structure of the nanoparticles which is identical to the reported peaks of JCPDS pattern (file 0784-04). The XRD pattern of the samples prepared at 60˚C showed the highest intensity. It has no further peaks which indicates the pure synthesis of the nanoparticles [22]. The obtained results were in agreement with that reported by Akinsiku A. [23]. The particle size was calculated using Scherrer equation which was found to be between 18-22 nm.

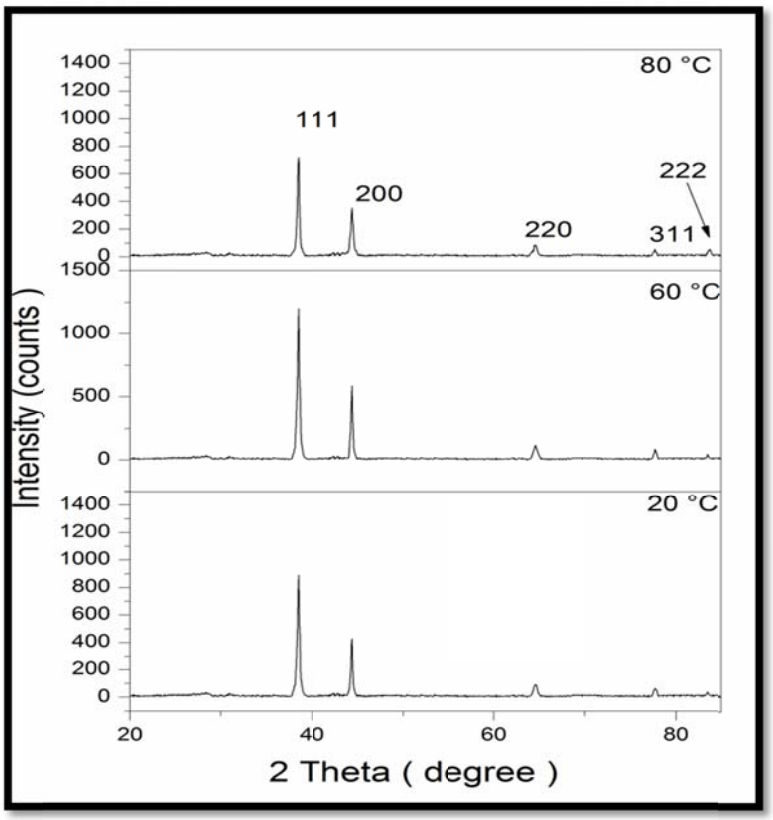

Fig.3: XRD pattern of AgNPs synthesised at different temperatures.

FESEM imaging was performed to investigate the shape and size of AgNPs. Fig.4 illustrates FESEM images of the biosynthesized nanoparticle deposited on silicon substrates. The majority of the particles were distributed in nanoscale range (1-100 nm). In addition, it can be seen from the images that the particles are spherical with diameter in the range of 24-56 nm, which are suitable for the medical application usage. However, some particles of different shapes existed as expected due to the aggregation of Ag nanoparticles during precipitation [24]. In addition, it has been observed that the temperature of synthesizing affected the shape of the nanoparticles.

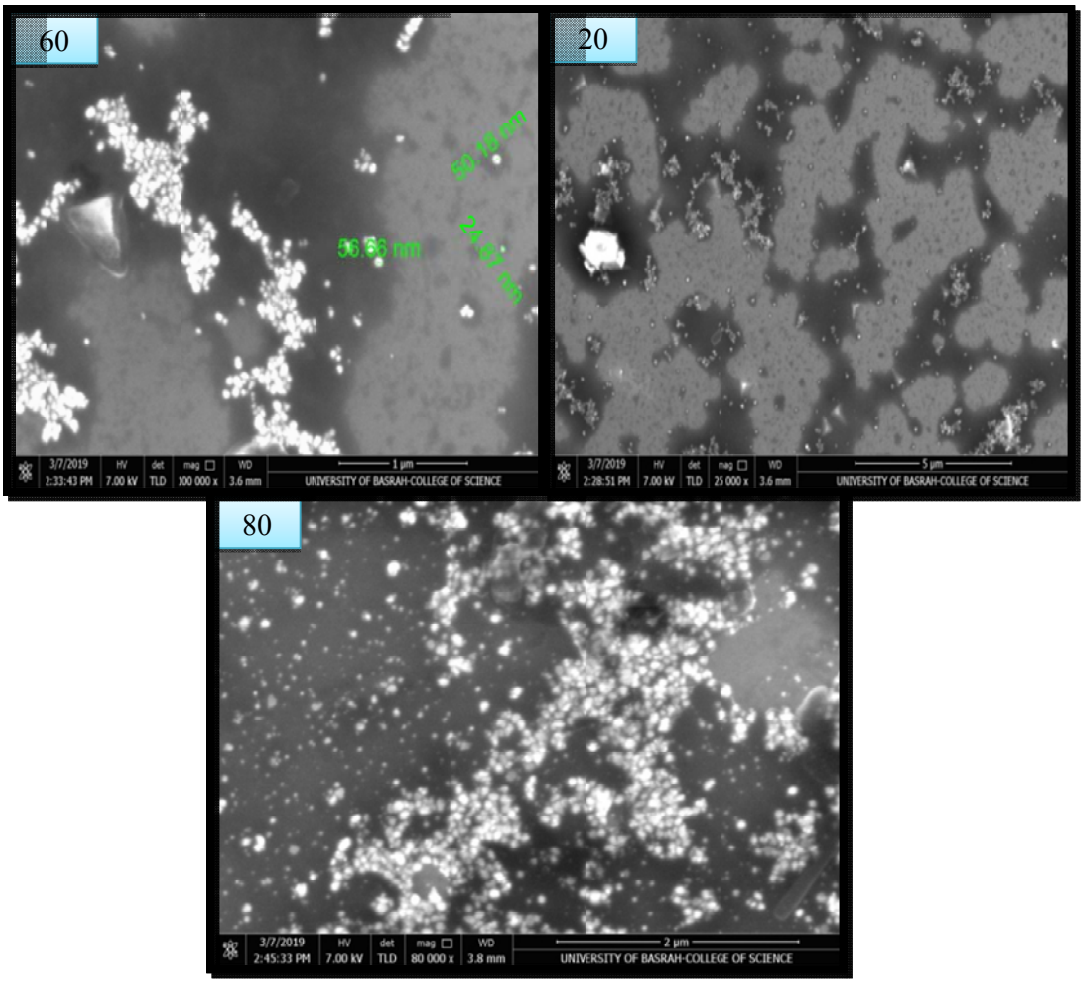

Fig.4: FESEM images of AgNPs synthesised at different temperatures.

1.2 Effect of mixing ratio

The effect of mixing ratio was investigated by Uv-Vis absorption spectra, XRD and FESEM microscopy. The SPR of UV spectra was between 435-445 nm (see Fig.5).

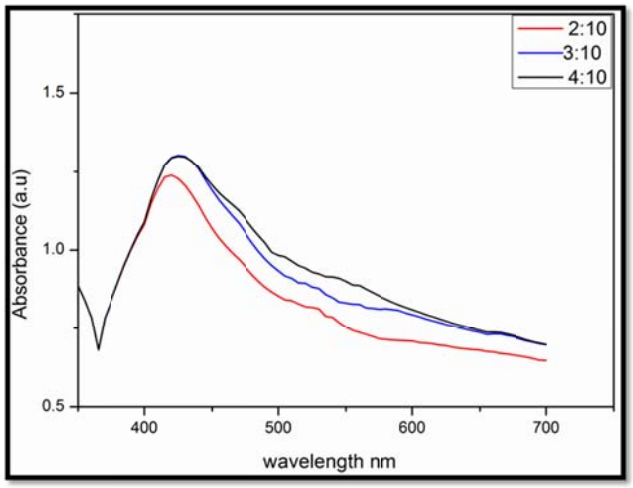

Fig.5: UV-Vis absorption spectra of AgNPs synthesized by changing the mixing ratio.

Fig.6 illustrates XRD pattern of the synthesised samples at different mixing ratios (2:10, 3:10 and 4:10) of AgNO3 and T. vulgaris extracts. It is observed the presence of peaks (111), (200) and (220) for the angles 2θ of 38.48˚, 44.30˚ and 64.75˚, respectively for the mixing ratio (2:10). While two peak existed for the mixing ratio of (3:10) and only one peak at (111) for the mixing ratio of (4:10). However, all the obtained patterns indicates the fcc structure of the nanoparticles which agrees with JCPDS (see file 04-0784).

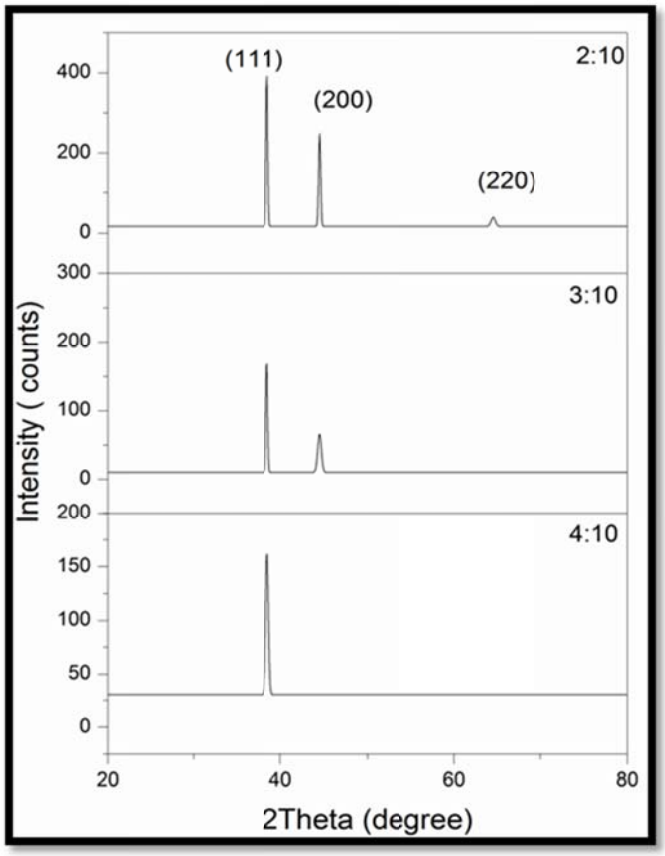

Fig.6: XRD patterns of AgNPs synthesized by changing the mixing ratio.

Fig.7 shows the FESEM images of the synthesized AGNPs with respect to the change in the mixing ratio. The nanoparticles were in the nanoscale with particle sizes distributed in the range 13-53 nm. Again, the majority of particles were of spherical shape with the presence of some other shapes. The particle size changed with the mixing ratio.

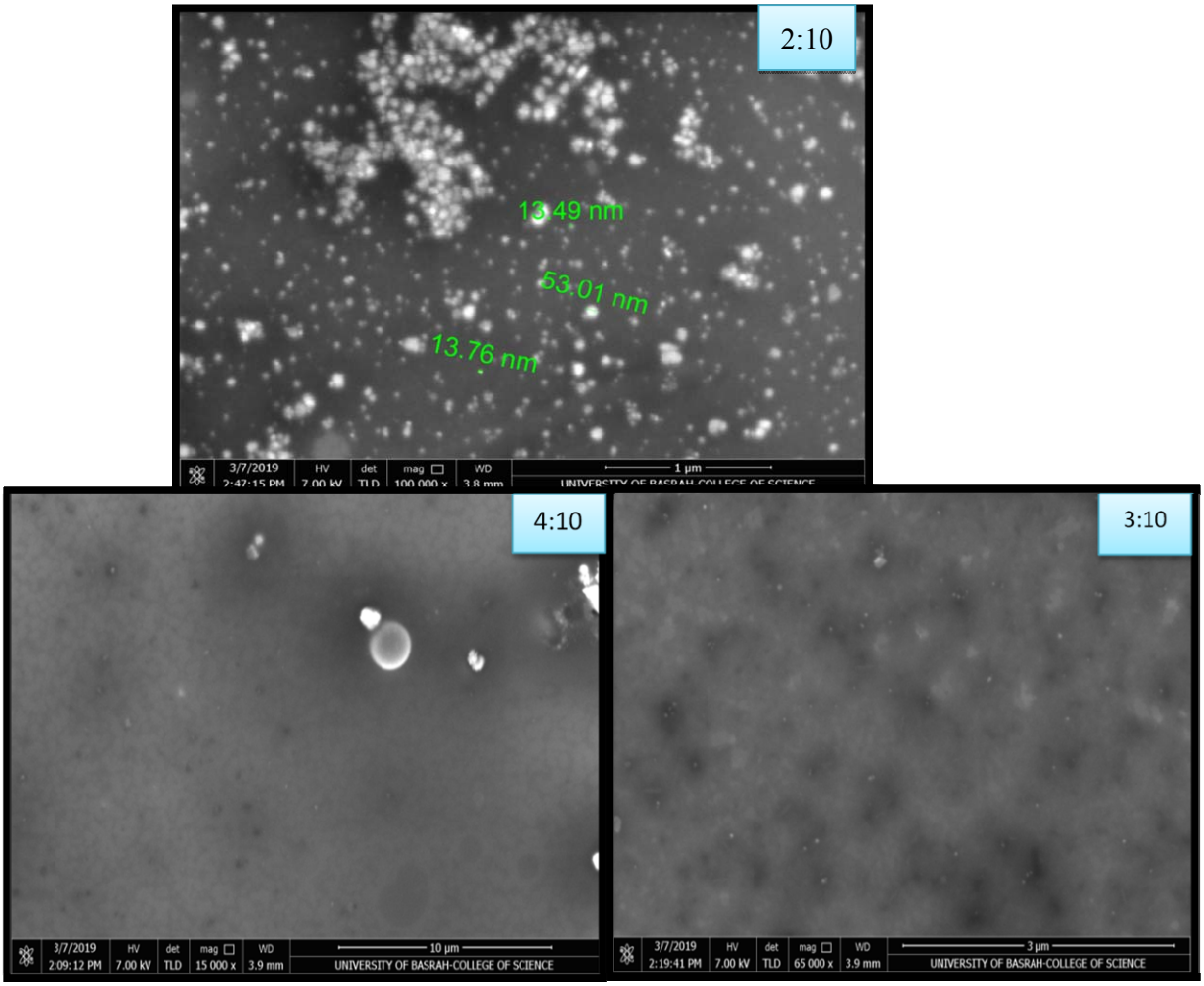

Fig.7: FESEM images of AgNPs which synthesized by changing the mixing ratio.

2. Inhibiting activity of the bacteria

The Agar well diffusion method was used to evaluate the antimicrobial activity of Ag nanoparticles. The results showed high inhibition against Gram-positive bacteria of *S-aureus* and against Gram-negative bacteria of *E.coli*. Table 1 and Fig.8 show the mean of inhibition of *S-aureus* bacteria and the diameter of inhibition area for the synthesized AgNPs at 60 ˚C and 1 mM for the mixing ratio of 1:10, 2:10, 3:10 and 4:10.

Table 1: Inhibition rate of Staphylococcus aureus bacteria with respect to mixing ratio.

Diluting % / Mix. ratio	100	80	65	50	30	Average
1:10	18	18	17.5	16.5	16	17.2
2:10	17	17	16.5	16	16	16.5
3:10	17	17	16.5	16	15	16.2
4:10	17	16.5	15	15	14.5	15.7

It has been observed that the inhibition rate of the bacteria was highly related to the particle size of the Ag nanoparticles. The smaller the nanoparticle size (high surface area), the larger the number of particles

clustered on the surface of the cells. That will increase the toxicity of the micro-organisms by increase their effect on the permeability of the cell membrane and leads to cell death [25]. It was also noticed that the mixing ratio of the nanoparticles (the number of Ag nanoparticles inside the solution) affected the inhibition rate.

It has been noticed the difference in inhibition activity of the Ag nanoparticles in the case of *E.coli* (see Fig. 9). The difference in the effect of the antibacterial is due to the difference in the structure of the cell wall, where the cell wall of the gram positive bacteria contains a single layer, while the cell wall of Gram-positive bacteria composed of a rigid thicker multiple layer of peptidoglycan, as it prevented the nanoparticles from entering into cell wall. Table 2 illustrates the effect of the concentration of the Ag nanoparticles which were synthesized at 1mM at 60˚C and of mixing ratio of 1:10, 2:10, 3:10 and 4:10, respectively (inhibition rate of E coli is measured in mm). The mechanism of the interaction between the nanoparticles and the living cells includes electrical attraction between the micro-organisms which carry negative charge and the nanoparticle ions which have positive charge.

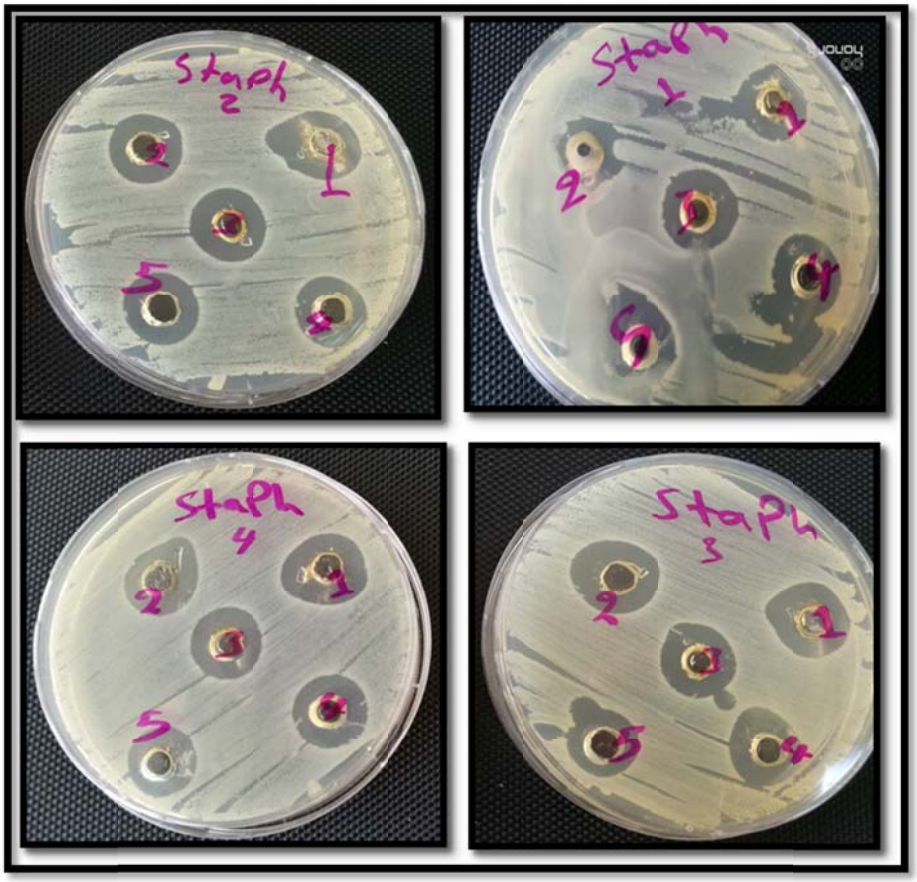

Fig.8: Inhibition activity of AgNPs against S.aureus bacteria.

Table 2: Inhibition rate of E.coli bacteria with respect to mixing ratio.

Diluting % / Mix. ratio	100	80	65	50	30	Average
1:10	16.5	16	15.5	15.5	14.5	15.6
2:10	15.5	15	14.5	14.5	14	14.8
3:10	15.5	15.5	15	14.5	14	15.6
4:10	15	15	14.5	14.5	13.5	14.5

This attraction makes the Ag nanoparticles can attach to the bacterial cell membrane, causing structural changes or functional damages. The membrane includes many sulfur containing proteins, which could be the preferential sites for Ag particle attachment due to sulfur-Ag affinity [23].

The inhibition mechanism of AgNPs to the bacterial growth occurred through inhibiting the ability of DNA gene expression of the proteins and the necessary enzymes for producing Adenosine triphosphate (ATP). Therefore, the cell becomes inactive [26-28]. The results of the current study confirmed the outcome of the study carried out by Husam M. and his group [21] in which the inhibition activity is higher in the case of *S.aureus* than that of *E.coli*.

Fig.9: Inhibition activity of AgNPs against E.coli bacteria.

3. Conclusion

Thyme leaf extracts was adopted as a bio-reduction agent of silver nitrate solution for green synthesis of silver nanoparticles (AgNPs). The particle size of AgNPs was found to be

correlated with the metal ion concentration, where low concentrations produce smaller AgNPs size. The results of UV-Vis spectroscopy stated that the surface Plasmon resonance of AgNPs is located at the wavelength of 550 nm, while FESEM analysis reveals the well dispersion of AgNPs with a spherical shape and of average particle size distributed between 18-22 nm. The synthesized AgNPs showed to exhibit inhibition activity toward Gram-positive S.aureus and Gram-negative E.coli bacteria. The highest inhibition was recorded for the solvent of silver nitride of 1:10 ratio.

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