EFFECIENCY OF SILVER NANOPARTICALES AS ANTIBACTERIAL AGAINST *AEROMONAS HYDROPHILA* ISOLATED FROM INFECTED COMMON CARP

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ABSTRACT

The present investigation was carried out to investigate the antibacterial efficiency of silver nanoparticles (AgNPs) in vitro against *Aeromonas hydrophila* using disc diffusion assay and minimum inhibitory concentration. The pathogenic *A. hydrophila* was isolated from infected common carp, usually diagnosed by chemical methods, and Avitek 2 compact device were used to confirm the diagnosis. The effectiveness of the prepared AgNPs was tested by chemical and biological (green synthesis using lemon extract) methods and were diagnosed by Fourier-transform infrared spectroscopy (FTIR), UV-Visible spectroscopy, Transmission electron microscopy (TEM), scanning electron microscope (SEM), which was spherical shape of the nanosilver and the size ranged between 30-50 nm. Results of disc diffusion assay showed that the chemical synthesized of AgNPs in 18hr recorded the highest inhibition zone followed by the bio-synthesized AgNPs and Oxytetracycline respectively. After 24 hr the highest inhibition zone was registered in Oxytetracycline, however after 5 days bio-synthesized AgNPs showed the higher inhibition zone which was significantly different($P \leq 0.05$) in comparison to other products. Based on these results, both bio and chemical synthesized of AgNPs were effectively act as antibacterial against *A.hydrophila*. However, green synthesis using lemon extract is considered better antibacterial with low MIC than chemical AgNPs because lemon extract is regarded eco-friendly and also the low cost product compared to chemical AgNPs synthesis.

Keywords: antimicrobial–avitek2-*cyprinus carpio*- minimum inhibitory concentration-nanosilver

مجلة العلوم الزراعية العراقية -2022 :53(3):589-597 سهيل وآخرون

دراسة فعالية الفضة النانوية كمضاد بكتيري ضد بكتريا *Aeromonas hydrophila* المعزولة من اسماك الكارب الشائع المصابة

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المستخلص

تم إجراء الدراسة الحالية لمعرفة التأثير المضاد للبكتيريا لجسيمات الفضة النانوية في المختبر ضد بكتريا *Aeromonas. hydrophila* باستخدام طريقة الانتشار القرصي والتركيز المثبط الأدنى. تم عزل بكتيريا *A. hydrophila* الممرضة من الأسماك المصابة ، و تشخيصها بالطرائق الكيميائية ، تم استخدام جهاز Avitek 2 لتأكيد التشخيص. واختبار فعالية AgNPs والمحضرة الطرائق الكيميائية والبيولوجية (باستخدام خلاصة الليمون)وتم تشخيصهما بواسطة FTIR و UV و TEM و SEM ،بين أن Ag-NPs كان كروي الشكل و حجمه 30-50 نانومتر. أظهرت نتائج اختبار الانتشار القرصي أن الفضة النانوية المحضرة كيميائيا سجلت أعلى منطقة تثبيط في 18 ساعة تلاها الفضة النانوية المحضرة بيولوجيا ثم الأوكسي تتراسيكلين على التوالي. بعد 24 ساعة، تم تسجيل أعلى منطقة تثبيط في الاوكسي، ولكن بعد 5 أيام أظهرت الفضة النانوية المحضرة بيولوجيا اعلى تثبيط والتي اظهرت اختلاف معنوي($P \leq 0.05$) مقارنة بالمركبات الأخرى. اظهرت الفضة النانوية المحضرة كيميائيا وبيولوجيا التأثير الفعال ضد *A. hydrophila* ولكن، يعتبر التحضير البيولوجي باستخدام مستخلص الليمون أفضل من الكيميائي كمضاد بكتيري مع انخفاض MIC لأن مستخلص الليمون يعد صديقًا للبيئة وكذلك منتجًا منخفض التكلفة مقارنةً بالتحضير الكيميائي للفضة النانوية.

الكلمات المفتاحية: مضادات الميكروبات، الفايتك، الكارب الشائع، الحد الأدنى للتركيز المثبط، الفضة النانوية

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INTRODUCTION

Bacterial diseases are among the most common diseases in intensive fish farming (1).The genus Aeromonas, is among the most common fish diseases, with relatively high resistance to antibiotics (26). *A. hydrophila* is a Gram-negative, free-living, heterotrophic and rod-shaped bacterium with size ranging from .1-3µm in diameter and 1.0-3.5 µm in length. This bacterium was normally found in fresh-water and odd occasionally in seawaters. They are amid the most common species of fish especially those in pond systems containing recirculation (18) cause various diseases in fish named as hemorrhagic septicemia, dropsy, epizootic ulcerative syndrome, hemorrhagic enteritis, and red body disease (15, 1). Artificial feed supplements are added with antibiotics much more in intensive and semi-intensive culture systems for protection the spread of diseases and improvement of food conversion rate. This leads to development an increased resistance of bacteria to antibiotics pathogens in the aquaculture system (13). Many diverse alternative products such as probiotics, prebiotics, plants, essential oils, algae phages, minerals, and nanoparticles have been tested. Nanotechnology is the newest and one of the most promising area of researches in modern medical science (5). Nanoparticles are usually a cluster of atoms ranging between 1-100 nm in size and exhibit new and improved properties based on size, distribution and morphology than larger particles of the bulk materials (7). Silver and Ag NPs occupy a prominent place in the series of such metals which are used as antimicrobial agents from time immemorial. In recent years, studies have been reported that nanoparticles as a promising alternative to antibacterial reagents because of their exhibited antibacterial activity in several biomedical applications, including drug and gene delivery (31). Nanoparticles with one dimension of 100 nm or less in size are now being increasingly utilized for medical applications and are of great interest as an alternative approach to control infectious agents (7). Silver and its compounds have been used for antibacterial and therapeutic applications for thousands of years. Biosynthesized Ag-NPs are considered cost-effective, eco-friendly, safe and alternative tools for biological control. Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of the cell membrane and death of the cell. There is formation of “pits” on the cell surface, and there is accumulation of the nanoparticles on the cell surface (20). This property can enhance biological and chemical activity, hence this provides Ag nanoparticles for their broad-spectrum and highly efficient antimicrobial (8). These carried a great revolution in the biological and medical fields in the modern area as compared to other materials (33). One of the possibilities is to use nanoparticles as antimicrobial drug in aquaculture but their potential use for disease control is not fully explored yet. Therefore, the aim of the present study is to investigate the antibacterial effect of silver nanoparticles in vitro against *A. hydrophila* isolated from infected common carp by using disc diffusion assay and minimum inhibitory concentration.

MATERIALS AND METHODS

Aeromonas hydrophila isolation

Samples were collected from gill, kidney and skin of infected fish. Samples were cultured on tryptic soy agar. Brian heart infusion agar, 5% sheep blood agar and MacConkey agar where the bacterial colonies were densely grown and inoculated in a Rimler-shot medium then incubated at 25 ° C for 24-48 h under aerobic conditions. After incubation, pure yellow haemolytic colonies Appeared. The bacteria were identified as *A. hydrophila* on the basis of colony morphology,Gram-staining, biochemical characteristics and Avitek2 compact**.**

Preparation of silver nanoparticles

Biochemical preparation of silver nanoparticles: The main aim of green synthesis is to minimize the use of toxic chemicals to prevent the environment from pollution (28). It’s done by dissolve 2.5g of silver nitrate ($AgNO_3$) in 800ml of distilled water , heating the solution up to 95-100 °C for boiling after that addition start (0.32g+ 125ml) ml from sodium citrate ($C_6H_5O_7Na_3$) that Moliere (0.01Mu) this added drops by drops slowly and regularly withe moving by magnetic stirrer and maintain high temperature for a period of 90 min, following reaction equation is calculated;

$$4Ag^{+}+C_6H_5O_7Na_3+2H_2O \rightarrow 4Ag\downarrow+C_6H_5O_7H_3+3Na^{+}+H^{+}+O_2\uparrow$$

during preparation begin mirror sliver appearance in wall container . After lose time of adding solution let without heat only move for 15 min. The solution is kept in a dark place to precipitate, after that it is washed with distilled water twice, filtered with filter paper and dried with a temperature of 40°C for 15 minutes The diagnosis was carried out with FTIR and TIM, according to method of Dadosh (11) and modified by Al-abodi et al. (2).

Biosynthesis preparation of silver nanoparticales: Biosynthesis preparation of $AgNO_3$NPs **(**as environmentally friendly way) was carried out using locally fresh lemon (citrus lemon) following the procedure of Swain *et al.* (29) with slight modification. Briefly, lemon juice was mechanically extracted from fresh lemons and then strained through affine nylon mesh followed by cold centrifugation at 5,000 rpm for 25 min at 4°C. After centrifugation, supernatant was collected and processed for synthesis of Ag nanoparticles. The Ag nanoparticles were prepared by adding lemon extract to 10^{-2} molar concentration of $AgNO_3$ at two ratio of 2:3 and 3:2 then, solutions containing two combinations of lemon extract and $AgNO_3$ were heated up to 70 °C on magnetic starrier for 3 hr during which change in color of the solution from pale yellow to reddish brown took place indicating the formation of silver nanoparticles.

Fourier Transform Infrared Spectroscopy (FTIR): Powder of MK-AgNPs was diluted with KBr (1:100), and the transmittance spectrum was recorded. FTIR measurements were carried in the diffuse reflectance mode at 4 cm−1 resolutions using the PerkinElmer FT-IR spectrometer (Spectrum Two, PerkinElmer Life and Analytical Sciences, CT, USA) (26).

Transmission Electron Microscopy (TEM)

The grid was prepared by placing aqueous suspension of MK-AgNPs on a TEM grid. It was allowed to air-dry overnight before imaging. Separate images were taken at 200 KV and magnification 20,000x to 100,000x at the University of Sophisticated Instrumentation Facility (USIF), AMU, Aligarh, using a transmission electron microscope (JOEL-2100, Tokyo, Japan) (24).

Scanning Electron Microscopy (SEM)

For SEM analysis, fine powder of MK-AgNPs was used. The images were recorded using the JSM-6510LV scanning electron microscope, at an accelerating voltage of 20 kV at the University Sophisticated Instrumentation Facility (USIF), AMU, and Aligarh. The analysis of elemental composition of MK-AgNPs was performed using the INCAx-sight EDAX spectrometer (Oxford Instruments) equipped with an SEM (24).

Effect of AgNPs on bacterial growth using Disc Diffusion Assay

Effect of AgNPs on *A. hydrophila* growth was done following mike pit or disc diffusion assays using the protocol of Bauer *et al.* (9). bacterial cultures was incubated for 24-48 hr on TSA (oxiad) plates and sterile filter paper discs containing 0.5 mg 0.75 mg and of nanoparticles were placed above the culture and then the plates were incubated at 28°C for 72-120 h after that, the zone of inhibition were recorded. Based on the findings of inhibition zone after incubation, the results were interpreted either as positive or negative. The nanoparticles showing inhibitory activities were further evaluated to determine minimum inhibitory concentration (MIC).

Determination of Minimum Inhibitory Concentration (MIC): The nanoparticles showing positive inhibitory activities in the preliminary disc diffusion assays were further processed to find out the MIC against *A. hydrophila.* The MIC values were calculated as per glass microbiological tube dilution method. Proportion nutrient broth (DCM) tub after that was added with individual nanoparticles Dual dilution from 0.1g/ml (0.05 0.025, 0.0125, 0.00625and 0,00312g/ml). Then, 100 µg of bacteria (at $1.4x10^{-7}$ CFU/ml) were added into the nanoparticles containing nutrient broth and then incubated at 28°C for 18-72 hr then, bacterial growth was monitored by measuring OD at 600 nm in UV spectrophotometer (22). The MIC values were determined as the lowest concentration of nanoparticles showing complete inhibition of bacterial growth after 18-72hr incubation and also disc diffusion in MHA for different concentration of $AgNO_3$ two way, show inhibition zone recorded by ruler.

Statistical Analysis

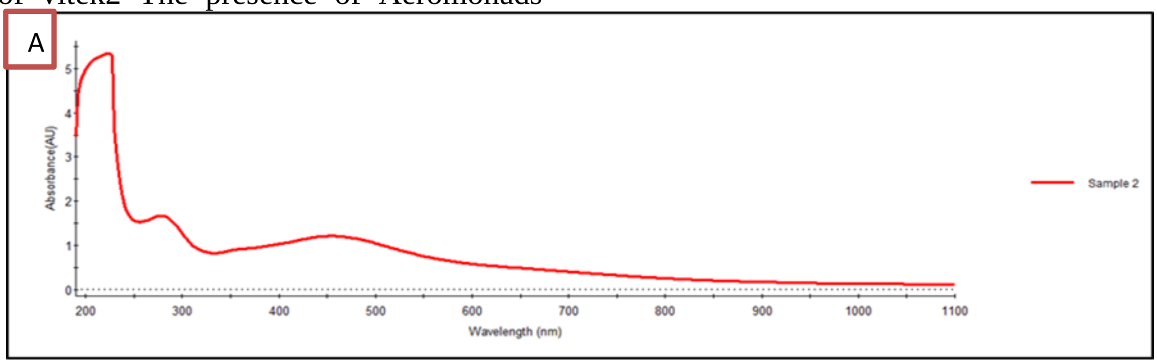
The Statistical Analysis System- SAS (27). program was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to allow significant comparisons among means P values less or equal to 0.05 were considered significantly different.

RESULTS AND DISCUSSION

Aeromonas hydrophila* isolates from *Cyprinus carpio: An inoculum from affected tissues was streaked into agar (TSA agar) plates led to heavy growth of bacterial colonies which showed similar morphological characteristics to that inoculated into the Rimler-Shott media and grew into yellow colonies, than for characteristics growth on BHI agar, blood base agar, Tripal sugar iron urea's agar, MacConky and SIM agar. The isolation of *A. hydrophila* was identified by vitek2 kit rapid identification system. According to the result of vitek2 test, the isolates were identical to the reference of Bergey's Manual of Determinative Bacteriology concerning the Characterization (based on their morphological and biochemical database (BioMerieux inc,frace) results 96-99 exultant (23).In this study A. *hydrophila* isolates from C,carpio were found to be gram negative, motile, fermentative, oxidase positive, catalase positive, lactose negative, glucose positive, maltose positive urea's negative, gelatin positive H2S production negative, scores positive, α hemolysis and β hemolysis on blood agar for 7 days as Aeromonads in the primary characterization tests. These results are in agreement with many researchers (6, 25, 3,12,4, 34). And wall use of vitek2 The presence of Aeromonads particularly *A. hydrophila* in healthy and diseased fish (*C,carpio*) obtained from different regions suggested an epizootic. Torres et al. (30) isolated *Aeromonas spp*. from healthy *Orechromis niloticus* tissues such as kidney, spleen and liver. This result is in line with Muduli et al. (21).

Characterization of silver Nanoparticles

Formation of silver Nanoparticles: Sliver nitrate solution is color less and sedum citrait no color after boiling the silver nitrate suspension and starting to add the sodium citrate, the color gradually changes to maroon and then to dark gray, and a silver mirror is on the walls of the flask with the chemical preparation in the green method after adding the silver nitrate to the lemon juice extract of lemon is pale yellow in color at a ratio of 3: 2 and by heating the temperature to the desired gray color. Dark, this color change in both ways indicates the formation of the silver nanoparticles extract of lemon is pale yellow in color. The presence AgNPs was further characterized by UV and indicated a prominent peak at 450nm the range of AgNPs (Fig.1). While, TEM images showed the morphological properties and surface appearance of AgNPs nanoparticles synthesis by chemical reaction. The nanoparticles have a nearly spherical shape, smooth surface and size ranged between 30-50nm (Fig.2). SEM –energy dispersive x-ray of AgNPs were identified and characterized using scanning electron microscopy equipped with energy dispersive x-ray and showed characterization of Nano syntheses in TEM, FTIR and SEM shape spherical and small size this result agreed with Ibrahim et al. (14) who showed that the nanoparticles were roughly spherical or circular in shape, while the average size of the nanoparticles ranged between 8 and 20 nm by SEM.



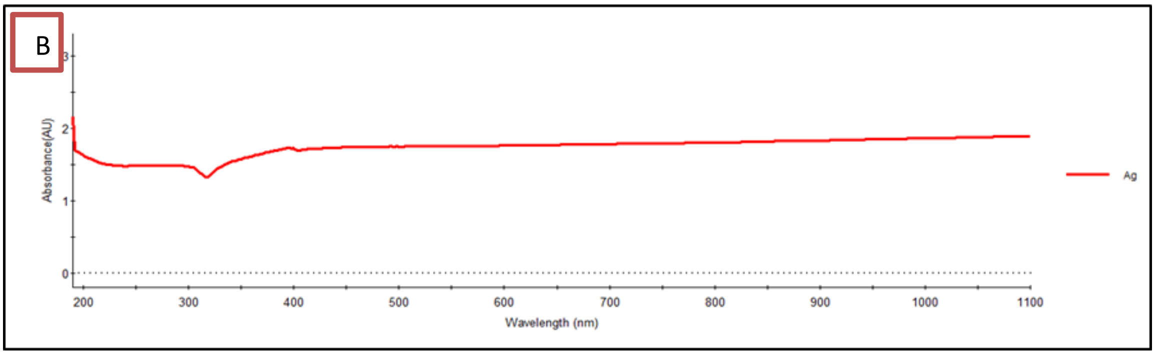


Figure 1. Ultraviolet-visible spectrum A: show the peak at 450 nm. B: the $AgNO_3$.

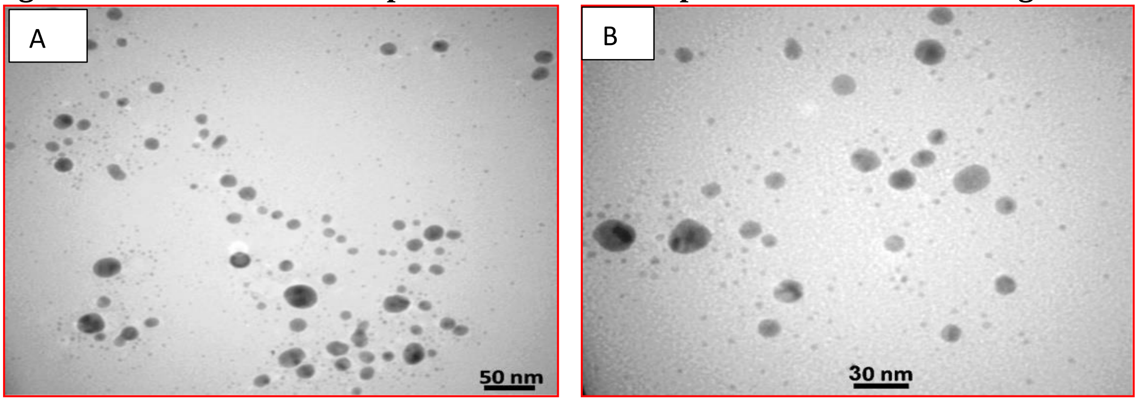


Figure 2. TEM image of AgNPs of A &B show nanoparticles have a spherical shape, smooth surface and the size about 50, 30 nm

Determination of Inhibition Zone by Disc Diffusion Assay: The AgNPs prepared by different combinations exhibited antibacterial activity (Fig. 3), Results showed that the in 18hr the chemical synthesized of AgNPs recorded the highest inhibition zone (1.5±0.04mm) followed by the bio-synthesized AgNPs and OTC (1.2±0.0 and 0.8±0.02mm) respectively which showed no significant difference compared to lemon (1±0.02mm). After 24 hr the OTC showed significantly ($P \leq 0.05$) increased (2±0.06mm) inhibition zone compared with chemically AgNPs and lemon (1.2±0.03mm). After 5 days, the bio-synthesized AgNPs showed the highest inhibition zone (2.5±0.07mm) which was significantly different in comparison to other products (Tab.1). These results are in agreement with Swain *et al.* (29) and Julinta *et al.* (16) whom also found that different commercial as well as laboratory synthesized metal and metal oxide nanoparticles were screened for their antimicrobial activities against a wide range of bacterial and fungal agents including certain freshwater cyanobacteria. The effects of OTC can be explained by their place of action: that is inhibits cell growth by inhibiting translation (19). It prevents amino-acyl tRNA from binding to the A-site from Ribosome by attachment to the 30th ribosome unit. The binding is reversible. OTC is lipophilic and can It easily passes through the cell membrane or is passively spread through the cell membrane or passively diffuse through purine channels in the bacterial membrane(19). Therefore, it is possible to establish bacterial resistance by inhibition of the enzyme, flux, ribosome protection, decreased permeability and ribosome mutation. While AgNPs acts on cell wall degradation, as well as an the production of cellular RNA and DNA (10). In this study, the synthesized AgNPs using lemon extract as a suitable reducing agent demonstrated excellent antibacterial activity against *A. hydrophila* with very low MIC values, which might be due to their agglomerating nature during commercial preparations as compared to green synthesized method. The antimicrobial activity of Ag might be attributed to the facts that the small particle size of Ag could enhance its solubility and the presence of extracellular Ag could interfere with the intracellular Ca metabolism and cause cellular damage (17). Further AgNPs might be also responsible in enhancing the activity via photocatalysis or generation of reactive oxygen species (32).

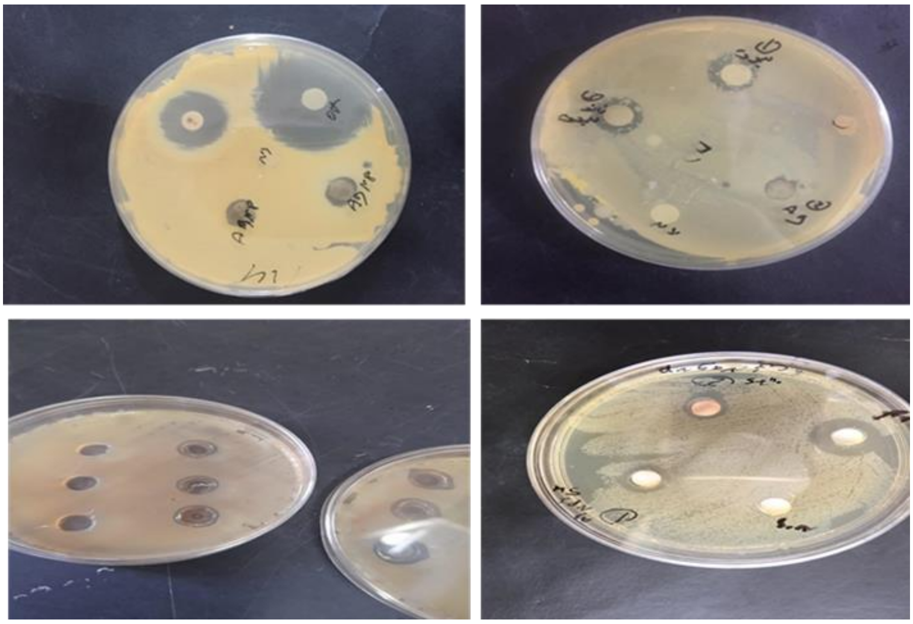


Figure 3. Disc diffusion assay showing zone of inhibition exhibited by various nanoparticles combinations against *A. hydrophila* isolates

Table 1. Results of inhibition zone using disc diffusion test

Name	Inhibition zone 18hr (mm)	Inhibition zone after 1days (mm)	Inhibition zone after 5days (mm)
Lemon Extract	1.0 ±0.02 ab	1.2 ±0.03 b	0.8 ±0.02 c
Normal saline	0 ±0.00 c	0 ±0.00 c	0 ±0.00 d
Oxytetracycline	0.8 ±0.02 b	2.0 ±0.06 a	0.85 ±0.02 c
AgNPs Biosynthesis 5%	1.20 ±0.03 ab	1.5 ±0.04 ab	2.5 ±0.07 a
AgNPs Chemical synthesis 5%	1.5±0.04a	1.2±0.03b	1.4±0.04b
LSD value	0.507 *	0.516 *	0.461 *

Results are Mean ± SE. Means having different letters in same column are differed significantly. * (P≤0.05), NS: Non-Significant.

Determination of minimum inhibitory concentration (MIC): The MIC values for commercial and laboratory synthesized nanoparticles against *A. hydrophila* were found that prepared in 3:2 ratio of lemon extract: AgNPs that is 0.75mg/ml by measuring OD at 600nm in UV spectrophotometer (Tab.2) and AgNPs biochemical is 0.125mg/ml dilution method (Fig.4) and measuring by UV spectrophotometer. The lowest concentration OD method was found 0.075mg/ml, where the readability of the absorbance was 0.1 in contrast to the rest of the other concentrations as shown in Tab. 2.

Table 2. Results of MIC for AgNPs green synthesis by UV- spectrophotometer

Concentrations	Absorption C+	Absorption C-
0.075mg/ml	0.1	0.1
0.0375mg/ml	7.3	9.64
0.018mg/ml	7.87	9.84
0.009mg/ml	7.95	20.9

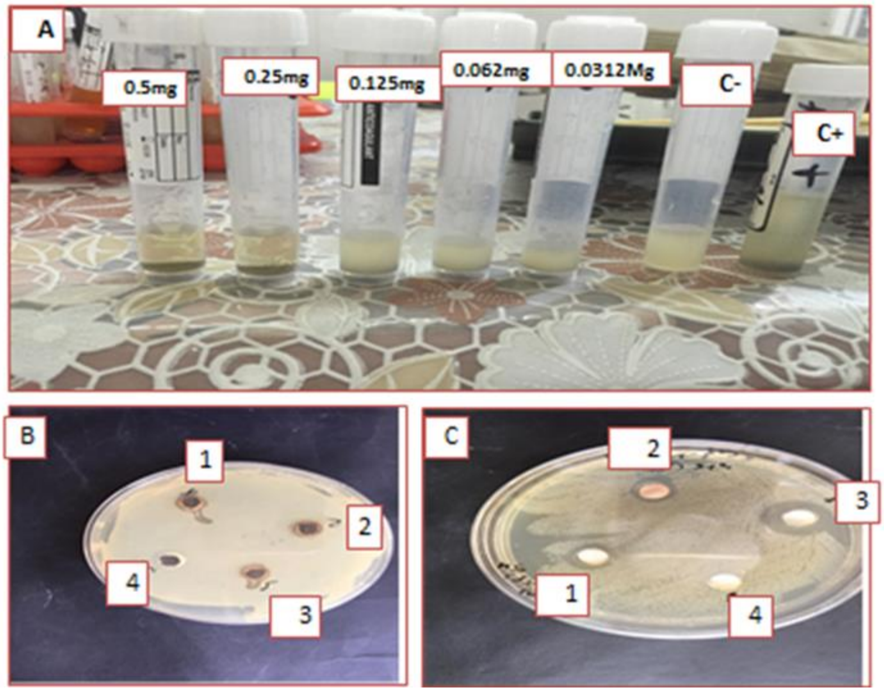


Figure 4. A; MIC for biochemical AgNPs dilution (0.5mg/ml, 0.25mg/l, 0.125mg/ml, 0.062mg/ml, 0.031mg/ml) method B: by well diffusion C: disc diffusion (1,0.5mg/ml,2, 0.25mg/ml, 3,0.125mg/ml and4. NS).

CONCLUSION

silver nanoparticle were successfully synthesized by chemical method and very simple method is presented in this study. The bio-synthesized AgNPs showed that the highest inhibition zone which was significantly different in comparison to other products. The synthesized Ag nanoparticles using lemon extract as a suitable reducing agent demonstrated excellent antibacterial activity against *A. hydrophila* with very low MIC values. Both BIO AgNPs and CHM AgNPs synthesized were effectively acted as antibacterial agent *A.hydrophila*. However, green synthesis using lemon extract is considered better than CHM AgNPs because lemon extract is regarded eco-friendly and also the low cost products compared to biochemical AgNPs synthesis.

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