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Antibacterial Effects of Green Synthesized Silver Nanoparticles Using Anise (*Pimpinella anisum L.*) Against *Pseudomonas aeruginosa*

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Abstract

Background: *Pseudomonas aeruginosa* is a ubiquitous and opportunistic pathogen that has garnered significant attention in the medical community due to its remarkable ability to develop resistance to a wide range of antibiotics.

Aim: This study was conducted to identify the antibacterial activity of both the synthesized AgNPs-Anise (*Pimpinella anisum* L.) and the traditional antibiotics against *P. aeruginosa*.

Materials and methods: After Anise (*P. anisum* L.) extraction, AgNPs was prepared, validated through the UV spectrophotometer, transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR); and then, used to prepare the AgNPs-Anise solution. Finally, *P. aeruginosa* isolates were re-cultured on Muller-Hinton agar (MHA) plates to examine the antibacterial activity of the four different concentrations of the AgNPs-Anise (50, 100, 200 and 400 μg/ml) solution as well as five traditional antibiotics including Gentamicin (GEN), Ceftazidime (CAZ), Amikacin (AK), Levofloxacin (LE), and Ciprofloxacin (CIP).

Results: Based on values of inhibition zone (mm), significant elevation ($p \leq 0.0001$) in antibacterial activity of different concentrations of AgNPs-Anis solution was seen at 50 μg/ml (20.83 ± 1.08 mm) while reduction was observed at 200 μg/ml (17.17 ± 0.79) when compared to other concentrations; 100 μg/ml (18.17 ± 0.79) and 400 μg/ml (19.83 ± 1.11). In comparison with traditional antibiotics, values of antibacterial activity of all AgNPs-Anis concentrations were significantly lowered than detected by AK (22.21 ± 1.1 mm), CAZ (18.14 ± 2.14 mm), CIP (29.3 ± 1.73 mm), GEN (22.5 ± 0.78 mm), and LEV (24.78 ± 2.08 mm). Among the selected antibiotics, antibacterial activity were increased significantly in CIP and decreased in CAZ comparing to others AK, GEN, and LEV.

Conclusion: The findings of the current study revealed that AgNPs-Anise was significantly having less antibacterial effects than conventional antibiotics. However, it's important to double-check specific applications and formulations, as the effectiveness can vary based on the type of nanoparticles and antibiotics used. Also, the use of NPs with antibiotics enhances the antibacterial effects while reducing the required dosage of antibiotics. This combination improves treatment efficacy and can help combat antibiotic resistance, as nanoparticles can deliver antibiotics more effectively to target pathogens.

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Keywords: AgNPs, Medicinal plants, Antibiotics, Nanotechnology, Iraq

Introduction

Nanotechnology has gained importance in many fields of science and technology in recent years [1]. Nanoparticles are particles with a size of 1 to 100 nanometers [2]. Some metal nanoparticles such as silver, gold and platinum are broadly applied in medicine and pharmaceutical industries as well as common consumables such as detergents and cosmetics [3, 4]. Nanoparticles are also used in the production of new generation of vaccines as both antigen nanocarriers and adjuvants [5-7]. Nowadays, nanoparticle-based vaccines have attracted a lot of attention due to their high-efficiency in stimulating the humoral and cellular immune responses as well as their low risks for human consumption [8-10].

Silver nanoparticles (AgNPs) are widely used in recent years due to their low toxicity compared to alternative chemical compounds [11]. Moreover, using biological methods for nanoparticle synthesis such as by microorganisms and plants with their high potential of reducing metal are considered as eco-friendly, and cost-effective compared to conventional means of synthesis [12, 13]. Although chemical methods are easier to perform than green synthesis and have higher efficiencies, their applications for nanoparticles synthesis are deemed more toxic and detrimental to the environment. Alternatively, plant extracts can be suitably scaled up for large scale biosynthesis of AgNPs in a controlled manner, according to their size, shape, and sensitivity [14-16]. So far, green synthesis of nanoparticles using plant extract of *Andrachne cordifolia* [17], *Azadirachta indica* [18], *Medicago sativa* [19], *Gliricidia sepium* [20], *Aloe vera* [21], *Chenopodium album* [22], *Capsicum annuum* [23], *Citrus sinensis* [4], *Cinnamon zeylanicum* [24] have been reported, to name a few.

Pimpinella anisum L. (Apiaceae), also known as aniseed or anise is an annual aromatic herb and a grassy plant with white flowers and small green to yellow seeds [25]. It is native to the eastern Mediterranean region while it has been used in traditional Iranian medicine as a remedy for carminative, neurologic, anticonvulsant, respiratory disorders, disinfection epilepsy, galactagogue, anti-asthma, and dyspnea [26]. Anise extracts have flavonoids, phenols and proteins while they also have antioxidant activities [26, 27]. The antioxidant activity of anise and the presence of agents such as flavonoids, proteins and phenols in it lead to the reduction of Ag+ ions to nanoparticles [16, 28]. The biosynthesis of silver nanoparticles is a complex process during which their quantity and quality are affected by many factors, such as pH, temperature and time [29]. Here, this study was conducted to identify the antibacterial activity of both the synthesized AgNPs-Anise (*P. anisum* L.) and the traditional antibiotics against *P. aeruginosa*.

Materials and methods

Ethical approval

This study approved by the Scientific Committee of the Department of Microbiology in the College of Veterinary Medicine, University of Basrah (Basra, Iraq).

Identification of P. aeruginosa

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The specimens were directly inoculated onto brain heart infusion broth and incubated at 37ºC for 24h, then inoculated in the MacConkey agar plates and incubated at 37°C for 24h to distinguish between the ferment and non-ferment lactose bacteria also the isolates were cultured on chrome agar media. The colonies from essential cultures were purified by subculture onto nutrient agar and incubated at 37ºC for 24h. Then, all isolates were stained with Gram's staining and examined by a light microscope. A small amount of pure growth was transferred with a wooden stick into a clean slide, and then a drop of 3% catalase reagent (H_2O_2) was added. The evolution of gas bubbles indicates a positive result. A disc of filter paper was saturated with a little freshly made (1%) solution of oxidase reagent (tetramethyl p-phenylene-diamine dihydrochloride), then a colony was picked up with a sterile wooden stick and smeared over the saturated filter paper. A positive result was indicated when an intense deep purple color appeared within 5-10s. Heavy inoculums were lined above the superficial slope of Triple sugar iron agar and stabbed into the button, incubated aerobically at 37°C for 24h. Interpretation of the consequences was improved by the change of color at surface and button, with or without H_2S production.

Anise extract preparation

Anise plants were purchased from local market in Basrah city. Seeds and stems of anise were weighed carefully and rinsed with tap water to remove excess dirt, dust, and mud, and then dried at room temperature. After adding 100mL of deionized double-distilled water, to 5 g anise seeds and stems and boiling them at 100°C for 3 min, the mixture was cooled down at room temperature and then filtered with Whatman filter paper. The filtered extract was stored at 4°C [20].

AgNPs Biosynthesis

Pure $AgNO_3$ was purchased from Basrah city and the solution of 1 mM $AgNO_3$ was prepared for the biosynthesis of AgNPs. To complete the biosynthesis process, 5mL of the anise extract were added to 250 ml $AgNO_3$ (1 mM) in an Erlenmeyer flask, incubated in dark at room temperature on a shaker (140 rpm) for 96h [20]. Then, four different concentrations were prepared from the $AgNO_3$-Anis including 50, 100, 200 and 400 μg/ml.

FTIR

To study the structure and formulation of the synthesized AgNPs and the extract, FTIR (PerkinElmer65) was used in the range of 4000-400 cm-1 with resolution of 0.01 cm-1 [30]. The powder of AgNPs and the extracts were prepared as described above for TEM. The obtained powders were then mixed with KBr with a ratio of 2/50 to achieve a relatively homogenized solution. The solutions were then examined with FTIR spectrometer (Figure 1).

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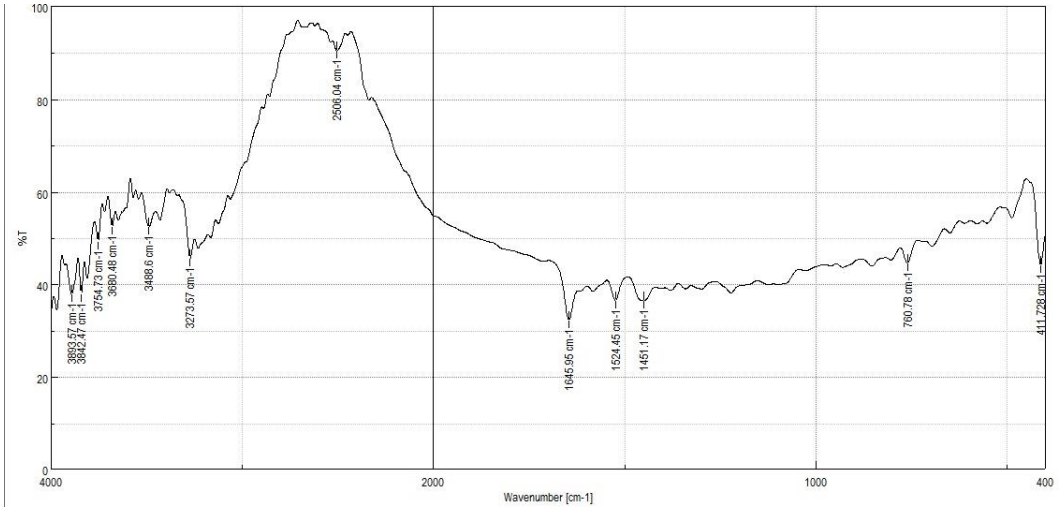

Figure (1): FTIR spectrometer for the prepared AgNPs

Measurement of antibacterial activity

Antibacterial assays were carried out on *P. aeruginosa* by standard agar well diffusion and minimum inhibitory concentration (MIC) [31]. The unfrozen bacteria were incubated on brain heart broth (BHB) for 24h at room temperature and then were cultured on brain heart agar (BHA) for 24h at 37°C. Densities of 0.5 Macfarlane were produced from each bacterial culture. The bacteria were swabbed uniformly onto separate Muller-Hinton agar (MHA) plates by sterile cotton swabs. For agar well diffusion method, aqueous AgNPs was produced from the powdered AgNPs in 50 and 100 µg/ml densities. A sterile micropipette was used and 50 µl of each density was poured onto each well in all plates, and also 20 µl and 50 µl of the direct non-centrifuged aqueous AgNPs were poured onto each well. For the disk diffusion method, 50 µl aqueous from the prepared powdered AgNPs and non-centrifuged AgNPs were added to blank disks. Five traditional antibiotics including GEN, CAZ, AK, LE, and CIP in addition to the four different concentrations of AgNPs-Anis (10 µl) were incubated onto the plates of *P. aeruginosa* isolates for 24h at 37°C. After ending of incubation period, the inhibition zones were measured in mm [31].

Statistical analysis

One way Analysis of Variance (ANOVA) in the GraphPad Prism Software was applied to detect significant differences between study values at $p<0.05$ (32).

Results

Based on values of inhibition zone (mm), significant elevation ($p\leq0.0001$) in antibacterial activity of different concentrations of AgNPs-Anis solution was seen at 50 µg/ml (20.83 ± 1.08 mm) while reduction was observed at 200 µg/ml (17.17 ± 0.79) when compared to other concentrations; 100 µg/ml (18.17 ± 0.79) and 400 µg/ml (19.83 ± 1.11), (Figures 2, 3).

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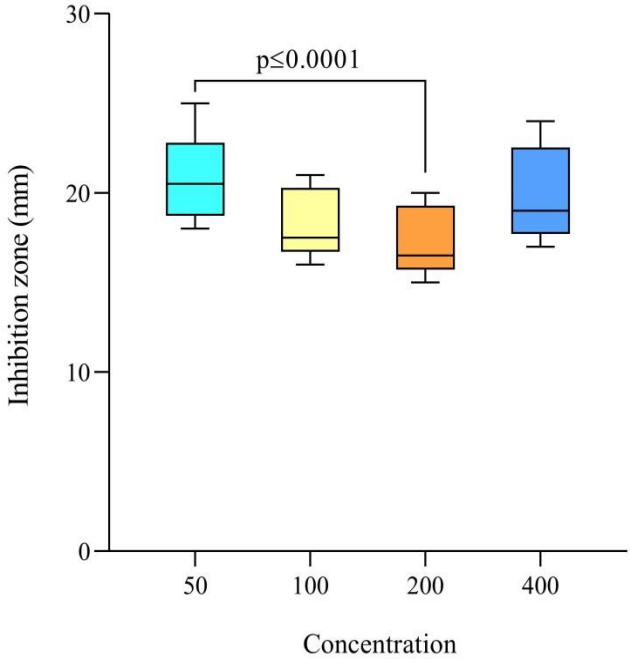

Figure (2): Antibacterial activity of different concentrations of AgNPs-Anis solution

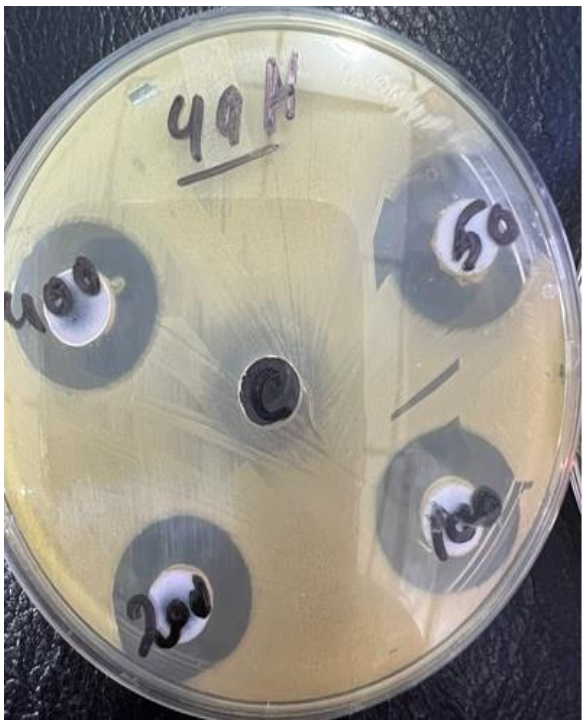

Figure (3): Antibacterial activity of different concentrations of AgNPs-Anis solution on *P. aeruginosa* isolates

In comparison with traditional antibiotics, values of antibacterial activity of all AgNPs-Anis concentration; 50µg/ml (20.83 ± 1.08 mm), 100µg/ml (18.17 ± 0.79 mm), 200µg/ml (17.17 ± 0.79 mm) and 400µg/ml (19.83 ± 1.11 mm) were significantly (p≤0.0001) lowered than detected by AK (22.21 ± 1.1 mm), (Figure 4).

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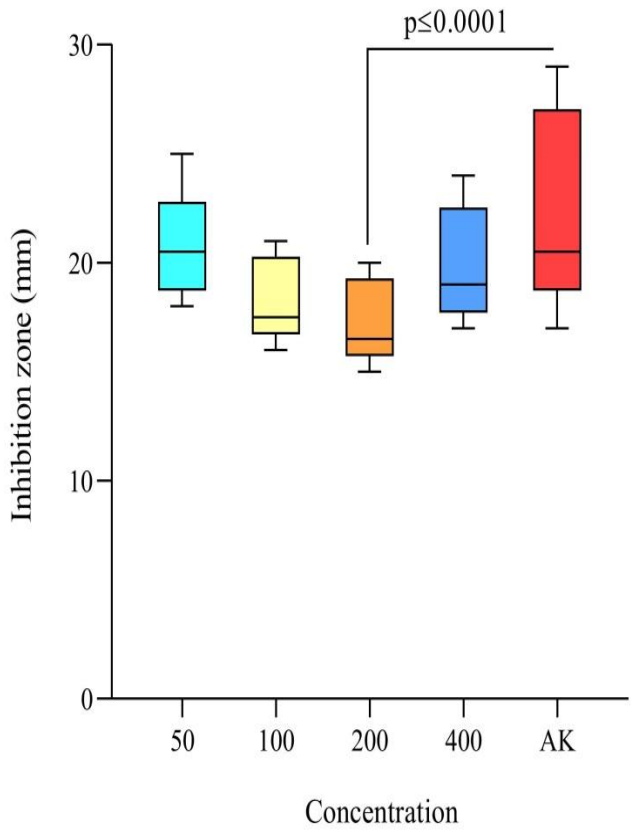

Figure (4): Antibacterial activity of different concentrations of AgNPs-Anis solution in comparison with the activity of AK

Significantly (p≤0.0001), the antibacterial activity of CAZ (18.14 ± 2.14 mm) was higher than detected for all AgNPs-Anis concentrations; 50µg/ml (20.83 ± 1.08 mm), 100µg/ml (18.17 ± 0.79 mm), 200µg/ml (17.17 ± 0.79 mm) and 400µg/ml (19.83 ± 1.11 mm), (Figure 5).

Regarding the antibacterial activity of CIP (29.3 ± 1.73 mm), there were significant reduction (p≤0.0001) in values of all AgNPs-Anis concentrations; 50µg/ml (20.83 ± 1.08 mm), 100µg/ml (18.17 ± 0.79 mm), 200µg/ml (17.17 ± 0.79 mm) and 400µg/ml (19.83 ± 1.11 mm), (Figure 6).

Efficacy of GEN (22.5 ± 0.78 mm) on *P. aeruginosa* was significantly elevated (p≤0.0001) when compared to antibacterial activity values of all AgNPs-Anis concentrations; 50µg/ml (20.83 ± 1.08 mm), 100µg/ml (18.17 ± 0.79 mm), 200µg/ml (17.17 ± 0.79 mm) and 400µg/ml (19.83 ± 1.11 mm), (Figure 7).

The antibacterial activity of LEV (24.78 ± 2.08 mm) was increased significantly (p≤0.0001) when compared to those of all AgNPs-Anis concentrations; 50µg/ml (20.83 ± 1.08 mm), 100µg/ml (18.17 ± 0.79 mm), 200µg/ml (17.17 ± 0.79 mm) and 400µg/ml (19.83 ± 1.11 mm), (Figure 8).

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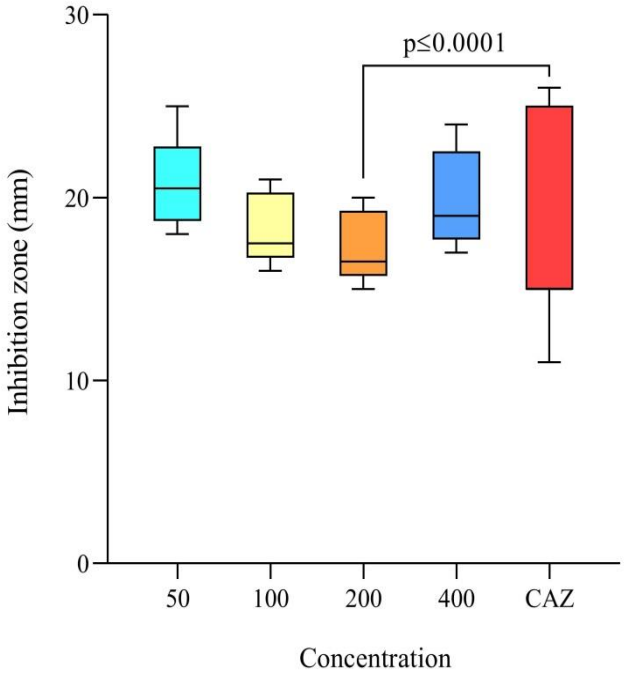

Figure (5): Antibacterial activity of different concentrations of AgNPs-Anis solution in comparison with the activity of CAZ

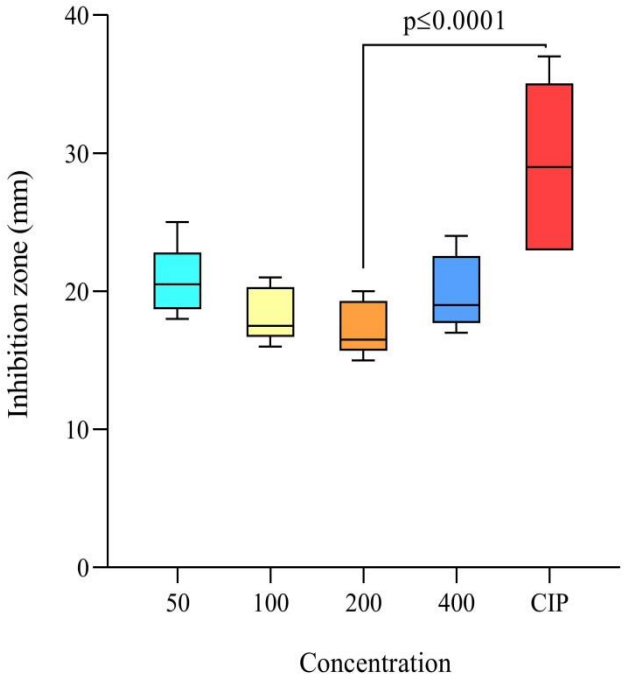

Figure (6): Antibacterial activity of different concentrations of AgNPs-Anis solution in comparison with the activity of CIP

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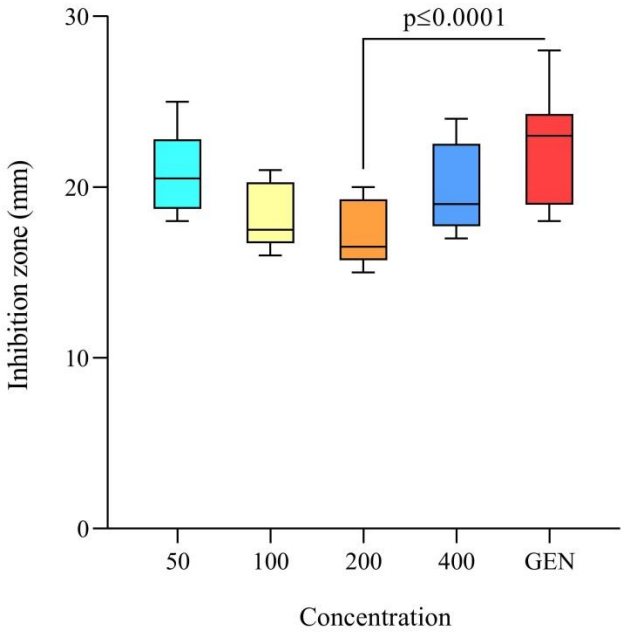

Figure (7): Antibacterial activity of different concentrations of AgNPs-Anis solution in comparison with the activity of GEN

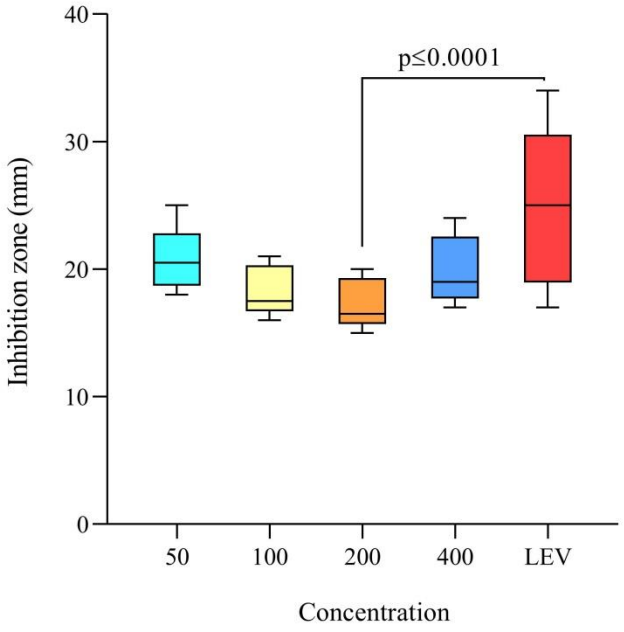

Figure (8): Antibacterial activity of different concentrations of AgNPs-Anis solution in comparison with the activity of LEV

Among the values of traditionally applied antibiotics, values of antibacterial activity were significantly ($p \leq 0.0001$) increased in CIP (29.3 ± 1.73 mm) and decreased in CAZ (18.14 ± 2.14 mm) in comparison to those of AK (22.21 ± 1.1 mm), GEN (22.5 ± 0.78 mm), and LEV (24.78 ± 2.08 mm), (Figures 9, 10).

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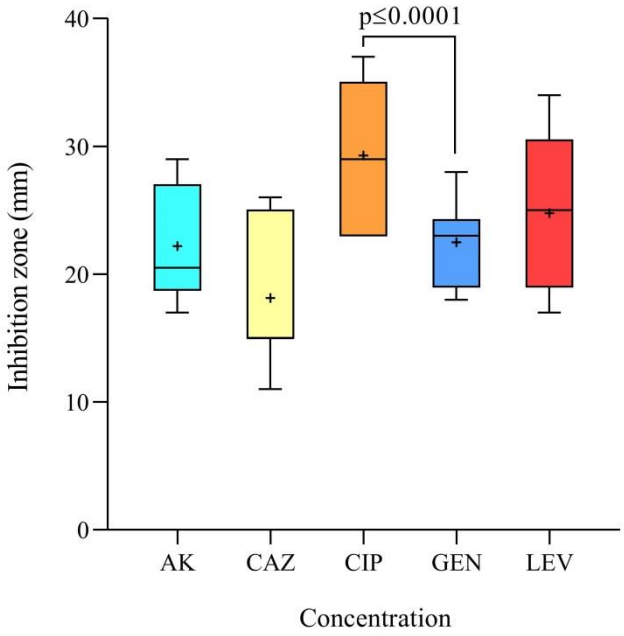

Figure (9): Results of antibacterial activity of traditionally used antibiotics in current study on *P. aeruginosa* isolates

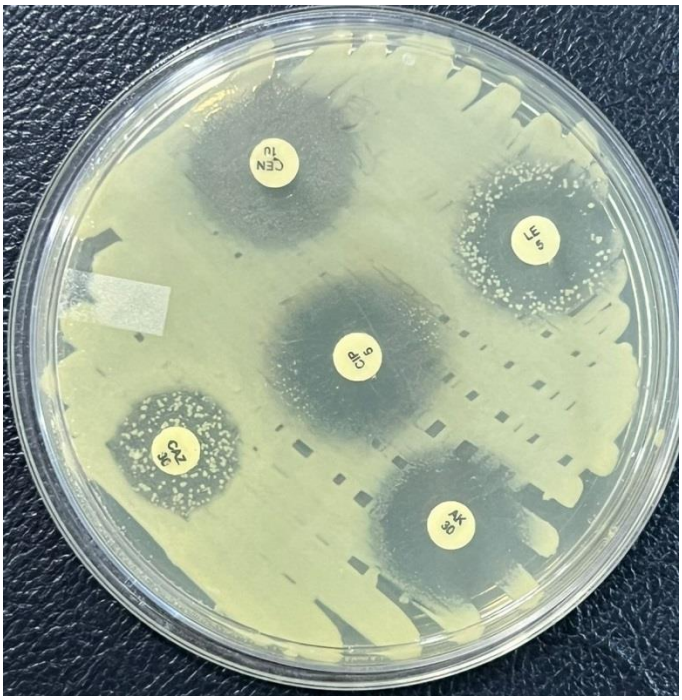

Figure (10): Antibacterial activity of traditionally antibiotic on *P. aeruginosa* isolates grown on MHA

Discussion

The high antibiotic resistance of *Pseudomonas aeruginosa* makes it critical to develop alternative antimicrobial agents that are effective and affordable [33]. One of the many applications of AgNPs is their use as an antimicrobial agent against bacteria resistant to common antibiotics [34]. It is strongly recommended that ecologically friendly techniques are

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used to synthesize AgNPs such as green synthesis approaches that involve both plants and microorganisms [35]. In the current study, green AgNPs biosynthesized using Anis plant demonstrated that AgNPs-Anis had significantly variable potent antibacterial effects on *P. aeruginosa* at four different concentrations. However, the higher antibacterial activity for AgNPs-Anis was seen at lowered concentration (50µg/ml) which might attributed to several factors, including their high surface area-to-volume ratio, which enhances interaction with biological systems, and their ability to penetrate biological membranes effectively. Furthermore, lower concentrations can lead to a more effective delivery of the nanoparticle's active components, minimizing toxic effects and maximizing therapeutic benefits due to reduced competition for binding sites. It should be noted that the relationship between concentration and potency can vary greatly depending on the specific nanoparticle type, its properties, and its applications. Other studies indicated that no significant difference in the antimicrobial activity of AgNPs on the different groups evaluated (Gram-positive versus Gram-negative and resistant to antibiotics versus susceptible), suggesting that AgNPs have a broad-spectrum bactericidal effect [36]. One of the mechanisms suggested to explain the action of AgNPs on Gram-negative bacteria is in binding to their cell membranes and increased permeability due to structural changes that would result in cell lysis [37].

This study showed that the efficacy of different concentrations of AgNPs-Anis was lowered than recorded by the traditional applied antibiotics. Several studies suggested that NPs can actually augment the potency of antibiotics as they serve as carriers that enhance the delivery and effectiveness of antibiotics, allowing them to fight pathogens more effectively through various mechanisms [38-40]. The retaining power of NPs in the body exceeds that of antibiotics, which may lead to more lasting therapeutic effects. However, the low potency of NPs compared to antibiotics can be attributed to several factors, including their variability in targeting specific bacteria, the effectiveness of the delivery mechanism, and the potential for rapid bacterial adaptation or resistance. While NPs can enhance the action of antibiotics and combat bacterial pathogens through various pathways, they may not always achieve the desired level of antibacterial activity on their own. Additionally, the complexity of the interactions between NPs, bacterial cells, and the host environment can affect their efficacy [41, 42]. Antibiotics generally have a higher potency compared to nanoparticles when it comes to direct antibacterial action; however, NPs are increasingly recognized for their potential to enhance antibiotic efficacy, especially in combating antibiotic resistance [43]. Other studies showed that NPs can exert significant antibacterial effects on both Gram-positive and Gram-negative bacteria and are being explored as alternatives or adjuncts to traditional antibiotics [44-46].

Conclusion

The findings of the current study revealed that AgNPs-Anise was significantly having less antibacterial effects than conventional antibiotics. However, it's important to double-check specific applications and formulations, as the effectiveness can vary based on the type of nanoparticles and antibiotics used. Also, the use of NPs with antibiotics enhances the antibacterial effects while reducing the required dosage of antibiotics. This combination improves treatment efficacy and can help combat antibiotic resistance, as nanoparticles can deliver antibiotics more effectively to target pathogens.

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