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Effect of Chemically synthesis compared to biosynthesized zinc oxide nanoparticles using extract of *Vitex agnus* on the expression of MexAB-OprM efflux pump genes of Multi-Drug Resistance *Pseudomonas aeruginosa*

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Abstract

The current study's goal was to find out how the expression of the efflux pump genes (MexAB-OprM) in MDR *P. aeruginosa* isolates was influenced by chemically manufactured Zinc Oxide Nanoparticles using sol-gel method as compared to biosynthesized Zinc Oxide Nanoparticles using *Vitex agnus-castus* leaves extract. PCR analysis was used to confirmthe the presence of 16S rRNA gene in all 16 *P. aeruginosa* isolates The findings indicated that *P. aeruginosa* tested positive for 16S rRNA (100%), and 7 (43.75%) of the isolates were MDR. All MDR isolates carried MexA, MexB and OprM genes.The *Vitex* extract's GC-MS analysis exposed the existence of active chemicals. The size of the synthesized Zinc Oxide Nanoparticles ranged from 22 to 74 nm. The Minimum inhibitory concentration values for chemically synthesized NPs, biosynthesized NPs, and *Vitex* extract against concentrations were 1024, 256, and 512 µg/mL, respectively, when tested against MDR isolates. Biosynthesized Zinc Oxide Nanoparticles have a greater inhibitory effect on efflux pump genes than chemically synthesized Zinc Oxide Nanoparticles and *Vitex* extract in the expression of MexA, MexB, and OprM genes in samples treated with sub MIC of Zinc Oxide Nanoparticles and plant extract compared to non-treated samples. One way that nanoparticles work against bacteria is by suppressing the expression of the efflux pump genes, which lowers the number of active efflux pumps on the cell surface. As a result, Zinc Oxide Nanoparticles are regarded by the pharmaceutical sector as a potential medicinal option.

Keywords: *Pseudomonas aeruginosa*, ZnO NPs, *Vitex*, *MexA*, *MexB*, *OprM*.

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a broadly spread opportunistic bacteria that is linked to healthcare-associated diseases and can cause serious contagions in plants, animals, and mammals [1,2]. Multidrug-resistant strains' (MDR) appearance is perceived as a serious public health concern [3].There are several virulence mechanisms through which the bacteria have grown [4]. Among them, the overexpression of efflux pump systems is the main mechanism leading to multidrug resistance (MDR) [5]. Antibiotics only experience sub-inhibitory levels in bacteria as a result of efflux pumps, which can

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lower intracellular drug concentrations and prevent antibiotics from reaching their targets in microbial cells. This could lead to other resistance mechanisms, such as changes in the structure of antibiotic targets as a result of mutation or enzymatic deactivation of antibiotics [6]. Efflux pumps have three resistance settings. First, they induce intrinsic resistance in the bacteria by expressing at the basic level. Second, mutant bacteria acquire resistance as a result of pump overexpression. Third, transient overexpression of pumps would result in resistance in strains that proliferate under stress[7] There are six distinct multidrug efflux mechanisms in *P. aeruginosa*: Finding alternatives to antibiotics is instantly required because *Mex*AB-*OprM*, the majority-studied pump of this bacterium, has the widest spectrum of substrates among all bacterial effluxes, including quinolones, tetracycline, chloramphenicol, trimethoprim, β-lactam, β-lactamase inhibitors, macrolides, azithromycin, colistin, detergents, and dyes [8]. Drug-resistant patients may benefit significantly from the use of nanoparticles as a therapeutic alternative in the future [9]. One such material is zinc oxide nanoparticles or ZnO-NPs .Zinc oxide nanoparticles with great biocompatibility, low toxicity, and affordability are utilized in biological applications. In biomedicine, ZnO NPs have shown great potential, especially in the areas of antibacterial and cancer treatment [10]. ZnO NPs were produced via chemical, biological, and physical processes [11]. The biological method is an effective process that substitutes expensive and harmful chemicals with natural compounds (such as plant extracts and microorganisms) that are used as capping, stabilizing, and reducing agents . Physical and chemical methods, on the other hand, are more costly and require a lot of labor and time in addition to creating a lot of secondary waste from the chemical agents used for the reduction and precipitation [12]. Compared to the others, the plant-based green synthesis process is used more frequently. Compared to the fungal and bacteria-mediated syntheses, the plants are safer for biological systems and easier to locate. Additionally, because plant extracts contain active substances such terpenoids, alkaloids, phenols, tannins, and vitamins, they are well-known for their usage in medicine and the environment [13]. The Verbenaceae plant *Vitex agnus-castus*, often known as the chasteberry or chaste tree, is native to the Mediterranean region as well as Asia, Europe, and North America. This herb has been utilized for traditional medicinal reasons to treat gynecological disorders for over 2500 years [14]. Furthermore, a multitude of bioactive metabolites (such as flavonoids, ketosteroids, iridoids, essential oils, etc.) found in *Vitex agnus-castus* exhibit outstanding activity in the environmentally friendly synthesis of ZnO nanoparticles with potent reducing qualities [15]. The current work aimed to explore the influence on the expression level of the efflux pump genes (*Mex*AB-*OprM*) in MDR-*P. aeruginosa* by comparing the biosynthesized ZnO NPS using leaves extract of *V. agnus-castus* to chemically synthesized ZnO NPs as well as alcoholic extract of *V. agnus* leaves.

Materials and Methods

P. aeruginosa isolates and molecular identification

This investigation was conducted on sixteen *P. aeruginosa* isolates from various illnesses that were collected over five months (October 2022–February 2023) from Al Kindy Teaching Hospital in Baghdad. After being incubated at 37°C for 24 hours, cetrimide agar, a selective medium for *P. aeruginosa* isolation, was used to screen all 16 isolates. The results were then authenticated by PCR using particular primer pairs for the 16S rRNA gene of *P. aeruginosa* (Table 1). Genomic DNA was isolated following the Wizard genomic DNA purification kit/Promega manufacturer's instructions. PCR was used to find the 16S rRNA gene using a particular primer, as indicated in table 1. The following protocol was carried out using an Eppendorf, Germany-based Mastercycler/nexus-PCR Thermal Cycler: First, denaturation was done for two minutes at 95 °C. Thereafter, denaturation was done for 35 cycles at 94 °C for 20 seconds, annealing for 20 seconds at 58 °C, and elongation for 40 seconds at 72 °C. Finally, one cycle at 72 °C

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Baghdad Science Journal

for five minutes was conducted. Following an hour of 70-volt electrophoresis on 1.5% agarose gel, the amplified products were stained with ethidium bromide.

Antibiotics susceptibility test

The following six antibiotic classes' susceptibility tests were conducted using the Kirby-Bauer method (1968) on Muller-Hinton agar (Bio lab, Hungary) and described by clinical laboratories standard specified CLSI (2023) which are used for treatment of *P. aeruginosa*. 1. Aminoglycoside (30 µg Gentamicin and 10 µg Amikacin) 2. Carbapenem (10 µg each of Meropenem and Imipenem) 3. Levofloxacin (5 µg) and Ciprofloxacin (5 µg) fluoroquinolones 4. Ceftazidime (30 µg) via Cephalosporin 5. Monobactam (30 µg Aztreonam) and 6. Beta-lactamase inhibitor (100 ug Piperacillin, 100/10 µg Piperacillin-Tazobactam, and 75/10 µg Ticarcillin-Clavulanate).

Detection *P. aeruginosa* efflux pump genes

Using uniplex PCR, four multidrug-resistant bacterial isolates were chosen for the current search to detect the existence of efflux pump genes (*MexA*, *MexB*, and *OprM*) connected to a housekeeping gene (*rpsL*). A 25 µl final volume was used for PCR. 12.5 µl of Amplicon, Denmark's Master mix, 1.5 µl (10pmol/µl) of each primer (provided by Bioneer Co., Korea), 4 µl of template DNA, and 5.5 µl of sterile deionized distilled water were used in this reaction. Tables 1 and 2 include the primer sequence and conditions needed to duplicate these genes. Using a 1 kb DNA ladder (Roche, Germany), the PCR products were electrophoresed on 2% agarose containing 0.5 mg/mL of ethidium bromide for 60 minutes at 70 volts.

Table 1. Primer's sequence used in the present study.

Primer	Primer sequence (5'→3')	Product size (bp)	Tm	Reference
MexA				
Forward	TGACCCTGAATACCGAGCTG	168	60	**Designed**
Reverse	GGTAGTCGGCCTCGTAGGT		59	
MexB				
Forward	AGGTCCAGGTGCAGAACAAG	158	60	**Designed**
Reverse	GTTCGACAGGTCTTCCTTGG		59	
OprM				
Forward	CCATGAGCCGCCAACTGTC	180	58	[16]
Reverse	CCTGGAACGCCGTCTGGAT		59	
rpsL				
Forward	GCAAGCGCATGGTCGACAAGA	201	59	[17]
Reverse	CGCTGTGCTCTTGCAGGTTGTGA		60	
16SrRNA				
Forward	GGGGGATCTTCGGACCTCA	956	58	[18]
Reverse	TCCTTAGAGTGCCCACCCG		59	

Table 2. Conditions of PCR program.

Stages	Temperature (ºC)	Period	Cycles number
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	
Annealing	55* , 57**	30 seconds	35
Elongation	72	20 seconds	
Final elongation	72	7 minutes	1
Note.**MexA, MexB, OprM* ***rpsL*			

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Preparation and Characterization of *V. agnus-castus* extract and synthesized ZnO-NPs

The botanical herbarium at the University of Baghdad/ College of Science for Women verified the species identity of the *V. agnus* leaves, which were purchased from AL-Jadriya gardens in Baghdad. To get rid of the dust particles on the surface, fresh *V. agnus* leaves have been repeatedly cleaned with distilled water. With minor adjustments, the method for extracting *Vitex* leaves was created as described by [19]. The soxhlet system was used, and hexane and methanol were used as solvents, respectively. A firmly closed funnel-shaped filter paper was filled with 100 gm. of leaf powder, and the filter paper was then located in the soxhlet system. 500 ml of solvent was added and allowed to sit for 24 hours at room temperature. To obtain a dark brown crude extract, the extract was focused using a rotary evaporator with a water bath set at 40˚C. Up until it was used, the crude extract was stored in a sterile container at 4˚C. Additionally, an alcoholic *Vitex* leaf extract was made to use a gas chromatography-integrated mass spectrometer (GC-MS) apparatus to identify the active components.

Synthesis of ZnONPs

The main phases of the experiment were preparing ZnO-NPs using two different methods: biosynthesis, as described by [20] with certain modifications, and chemical synthesis using the sol-gel approach, as described by [21]. After heating 100 ml of *Vitex* extract to 60°C using a magnetic stirrer, 10 gm. of zinc nitrate Zn(NO3)2 was added. It is cooked until a creamy white paste forms. Subsequently, the dough is cleaned with distilled water and baked for two hours at 400 °C. Next, ZnONPs filled with active ingredients from the *Vitex* extract will produce a white powder. Using an X-ray diffractometer, the produced ZnO-NPs were subjected to an X-ray diffraction (XRD) test to evaluate the crystal structure of the particles and estimate their size [22]. Additionally, to employing scanning electron microscopy (SEM) to ascertain the phenotypic and structural properties of particles [23].

Antibacterial activity of ZnO-NPs and *Vitex* extract, Minimum Inhibitory Minimum Inhibitory Concentration (MIC) determination

The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration at which the color remained unaltered. Following [24], the MIC was completed. To achieve a concentration, range from 8 to 8192 µg/ml, a stock solution (10000 µg/m) of each chemically manufactured ZnO NPs, biosynthesized ZnO NPs, and *Vitex* extract were serially diluted in 96-well microtiter plates using Mueller Hinton broth. Each bacterial culture (1.5 $\times 10^8$ CFU/ml) was prepared overnight, and 10 µl of the culture was added to each well. For 24 hours, a microtiter plate was incubated at 37°C. Subsequently, each well received 5 µl (6.75 mg/ml) of resazurin dye (Thermo Fisher, USA), and they were incubated for an additional 4 hours at 37°C. The color transition from purple to pink was inspected.

Gene Expression of the Efflux Pump genes using qRT-PCR

For four MDR *P. aeruginosa* isolates, the gene expression of efflux pumps was assessed using the quantitative Real-Time PCR technique both before and after each isolate was treated with chemically produced ZnONPs, biosynthesized ZnO NPs, and *Vitex* extract at sub-MIC.

RNA extraction

Four sterile microcentrifuge tubes with three milliliters of nutritive broth were inoculated with one milliliter of the bacterial inoculum suspension made from fresh cultures of each *P. aeruginosa* isolate. Three of these tubes were treated with one milliliter of sub MIC of each chem.ZnO NPS, bioZnO NPS, and *Vitex* extract; the fourth tube served as a control (not treated). All tubes were incubated for 18–24 hours at 37ºC .The TransGen, Biotech, China TransZol up plus RNA Kit was used to extract RNA following the manufacturer's instructions. In the event of material shortage, RNA was additionally extracted for every isolate of *P. aeruginosa* (gene expression control). RNA concentration and purity were assessed for the extracted RNA quantity using Nanodrop (Quawell, China).

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RT-qPCR reaction and program:

Reverse transcription was carried out using the GoTaq®1-Step RT-qPCR kit, and then the efflux pump genes (*MexA*, *MexB*, and *OprM*) were amplified using quantitative real-time PCR. Following the manufacturer's recommendations, a 20µl volume reaction was used for the operation. The materials needed were 10µl of qPCR Master Mix 2X, 1µl each of the forward and reverse primers, 0.5µl of each GoScript RT Mix and MgCl2, 3µl water, and 4µl of the extracted RNA for reverse transcription. Using the primers listed in Table 1, an RT-PCR thermal cycler was used, and the relative expression values of the efflux pump genes were compared to rpsL, a housekeeping gene. The RT-qPCR procedure is displayed in Table 3. Using the $2^{-\Delta Ct}$ technique, the relative gene expression levels in treated and untreated isolates were compared.

Table 3. Conditions of RT-qPCR program.

Stage	Temperature (ºC)	Period	Cycle
Reverse transcription	37	15 min.	1
Reverse transcriptase inactivation and GoTaq® DNA Polymerase activation	95	10 min.	1
Denaturation	95	30 sec.	40
Annealing	55* 57**	30 sec.	
Elongation	72	30 sec.	

Note. * *MexA*, *MexB* and *OprM*; ***rpsL*

Statistical Analysis

The Statistical Analysis System- SAS (2018) program was utilized to ascertain the impact of different variables on the study parameters. To compare means significantly, the Least Significant Difference (LSD) test Analysis of Variation (ANOVA) was employed. A significant comparison between percentages (0.05 and 0.01 probability) was made using the Chi-square test. An estimate of the correlation coefficient between variables was conducted in this study.

Results and Discussion

Molecular identification of isolates

Sixteen isolates were examined using PCR to determine whether they contained 16S rRNA or not. All of the isolates were positive (100%) for 16S rRNA, as shown in Fig. 1. The current findings coincide with those of [24,25,26]; however, a different study by [27] showed 96% susceptibility. Medical microbiology currently frequently uses 16S rRNA gene sequencing as a rapid and low-cost substitute for phenotypic methods for bacterial identification [28].

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Baghdad Science Journal

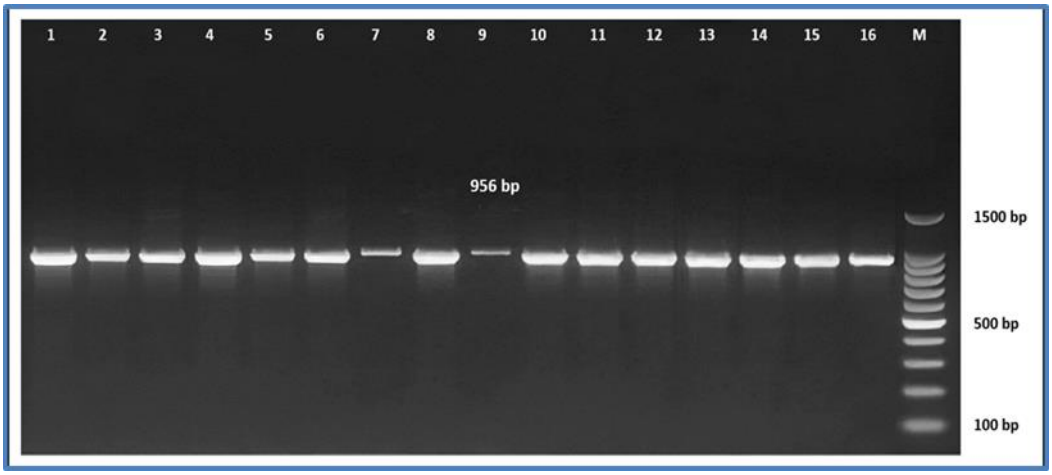

Figure 1. Results of *16srRNA* gene replication in clinical isolates of *P. aeruginosa*. Electrophoresed on 1.5 % agarose for 1 hour at 70 volts.

Antibiotics susceptibility test

Based on the Kirby-Bauer method test for antibiotic susceptibility, the isolates with the highest levels of resistance were Imipenem 12 (75%), Piperacillin 11 (68.75%), Gentamicin 10 (62.5%), Levofloxacin and Aztreonam 9 (56.25%), Meropenem and ceftazidime 8 (50%), Ciprofloxacin, Amikacin and Piperacillin/ Tazobactam 7 (43.7%) and Ticarcillin/Clavulanate 6 (37.5%) (Fig. 2). Seven isolates (43.75%) were predicted to be multidrug-resistant (MDR) based on the CDC's classification of MDR, which is defined as an isolate that is resistant to at least one antibiotic in three or more medication classes.

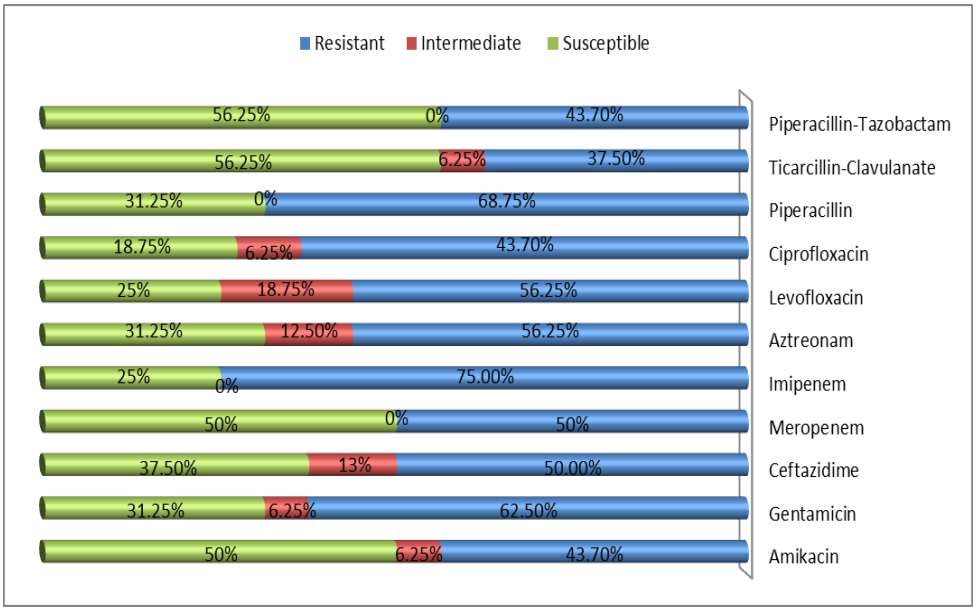

Figure 2. Percentage of Susceptibility of *P. aeruginosa* isolates to 11 antibiotics.

MDR-*P. aeruginosa* isolates were evaluated as 43.75% (n=7) at the time of the current study. According to research by [29,30], 30.2% of the isolates were screened as MDR isolates. Other scientists discovered that 45.3%, 30.1%, and 5.46% of the isolates were also MDR**.** In a burn unit, resistant infections are largely the cause of death. *P. aeruginosa* is one of the bacteria that primarily cause burn infections, although it also shows notable antibiotic resistance. Because of its poor permeability outer membrane, *P. aeruginosa* exhibits intrinsic resistance to a variety of

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Baghdad Science Journal

antimicrobial drugs [31]. However, other mechanisms also contribute to their intrinsic resistance, such as the generation of antibiotic-inactivating enzymes and efflux pumps, which actively eject antibiotics from bacterial cells to lower drug concentrations and cause drug resistance [32,33]. The frequent use of antibiotics is another factor contributing to the rise in multidrug resistance (MDR). This is caused by the transfer of mobile genetic elements carrying resistance genes, such as plasmids encoding β-lactamases, and can happen during antibiotic therapy. Mutations in these elements can result in the overexpression of endogenous beta-lactamases or the expression of particular porins[32].

PCR test results for efflux pumps genes detection

Four of the seven MDR bacterial isolates that tested positive for antibiotic resistance were chosen based on the findings of the antibiotic susceptibility test. Using a traditional uniplex PCR approach, the presence of efflux pump genes (*MexA*, *MexB*, *OprM*) and a housekeeping gene (rpsL) in four MDR *P. aeruginosa* isolates was examined. The findings revealed that every gene (100%) was present in the isolates that were tested (Fig. 3).

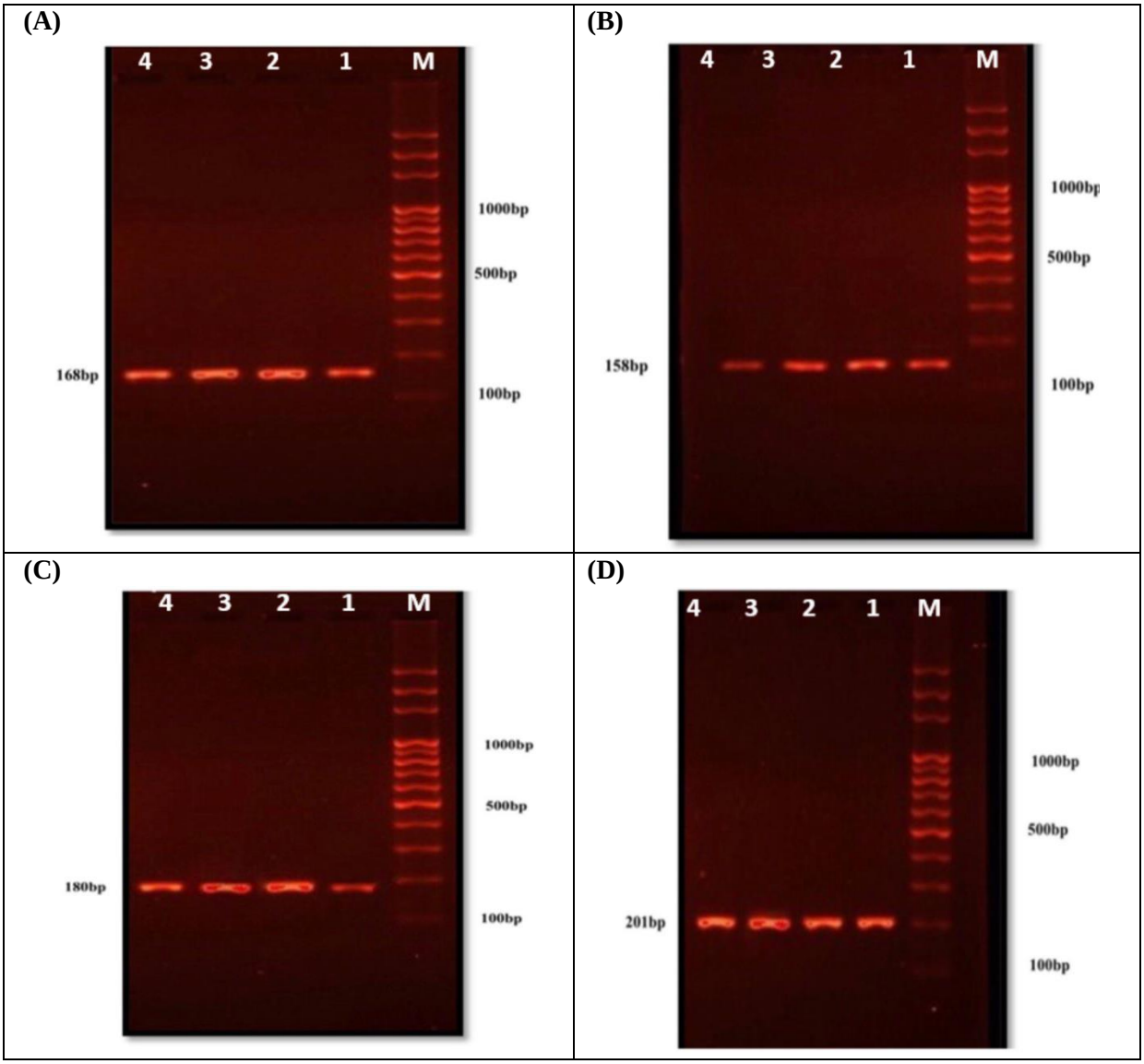

Figure 3. Agarose Gel electrophoresis of amplified PCR product for detection: A) *MexA* (168bp), B) *MexB* (158bp), C) *OprM* (180bp), and rpsL (201bp) gene run on 2%agarose for 60 min at 70 volts, stained with ethidium bromide, lane 1-4 *P. aeruginosa* isolates; M: DNA ladder.

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Baghdad Science Journal

Before examining gene expression, the present study assessed the existence of *MexA*B-*OprM* genes in *P. aeruginosa*. The findings showed that the efflux pump genes were present in all MDR isolates, which was consistent with the findings of [26,34–36] that concluded that *MexA*B-*OprM* genes existed in 100% of *P. aeruginosa* isolates. Furthermore, *MexA*B-*OprM* of *P. aeruginosa* was found in 100% of samples for both the *MexA* and *MexB* operon genes, according to [37], demonstrating the chromosomal location of the *MexA*B-*OprM* efflux pump genes**.**

However, compared to earlier studies by [38,39], which found the presence of the *MexA* and *MexB* genes in 58.8% and 70.58% of the isolates examined, respectively, the current study's percentage of both genes was higher than in the above-mentioned studies. While the results published by [40] agreed with the current study that authorized isolates carried the *MexB* gene 100% of the time, another study conducted in Iran found that the *MexB* gene existed in 53.3% of isolates [41]. These days, it is acknowledged that the efflux pump is one of the key complexes implicated in resistance to the majority of antibiotic classes [42]**.**

Characterization of *Vitex* extract and ZnO NPs

In this research, the development of *Vitex* -ZnO NPs was characterized by a color shift from yellow to brown. After the ZnO salt was fully bio-reduced to ZnO NPs, the solution's color changed for a few hours. Zinc ions are transformed into ZnO NPs by flavonoids and phenolic substances. Phytochemicals can function as both reducing and stabilizing agents because they are non-toxic compounds and antioxidants [43]. These findings were in line with the research by [44,45] that reported color variations in plant-mediated green production of ZnO NPs**.** After GC-MS analysis of the leaf extract components, it was found that the methanolic extract contained a high concentration of phytochemical components (> 50 chemical compounds) with anti-inflammatory and antibacterial properties, including Phytol (C20H40O), Phenol (C6H5OH), Vitamin E (C29H50O2), 4,5-Dichloro-1,3-dioxolan-2-one (C3H2Cl2O3), Squalene (C30H50), and 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy (C6H8O4). These conclusions are consistent with earlier research by [46,47], which confirmed that the alcoholic extract included 36 and 93 chemical components, respectively, indicating that the *V. agnus* extract possesses a broad spectrum of chemical compounds. These substances fall under several chemical classes, such as flavonoids, glycosides, diterpenoids, essential oils, and phenolic compounds. Variations in the genotypes of *V. agnus* may be the cause of the variance in the number of phytochemical substances [48]**.**

The ZnO NPs' XRD patterns are displayed in Fig. 4. The peak locations of the X-ray diffraction are 31.8°, 34.4°, 36.3°, 47.5°, 56.6°, 62.8°, 66.4°, 67.9°, 69.1°, 72.5°, and 76.9°. As reflections, the XRD peaks were identified as (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202). The biosynthesized ZnO NPs' XRD patterns closely matched the earlier finding made by [49].

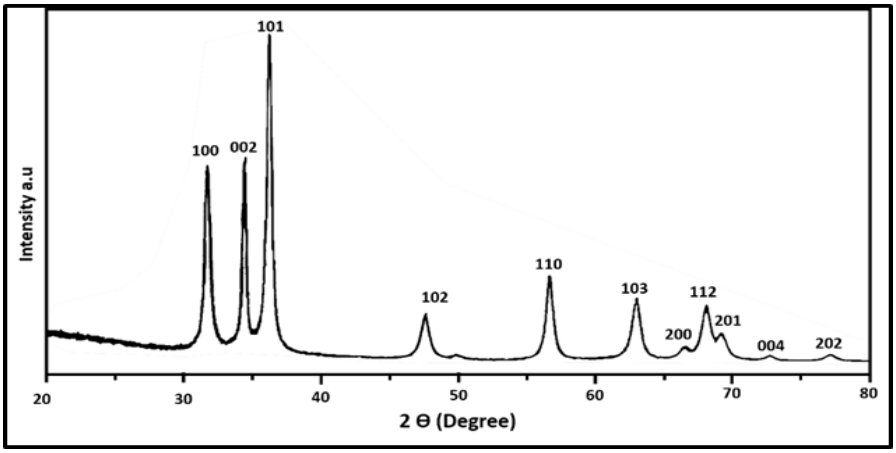

Figure 4. The XRD patterns of ZnO NPs.

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Baghdad Science Journal

In Fig. 5, the scanning electron microscopy (SEM) pictures depict the size and shape of ZnO NP. The particle size was between 22 and 74 nm, with a spherical and compact form. These findings are consistent with those of [50], who also observed a spherical morphology. In contrast, a different study's synthesis of ZnO nanoparticles using plant extract produced particles that were 40–50 nm in size and had a spherical morphology [51].

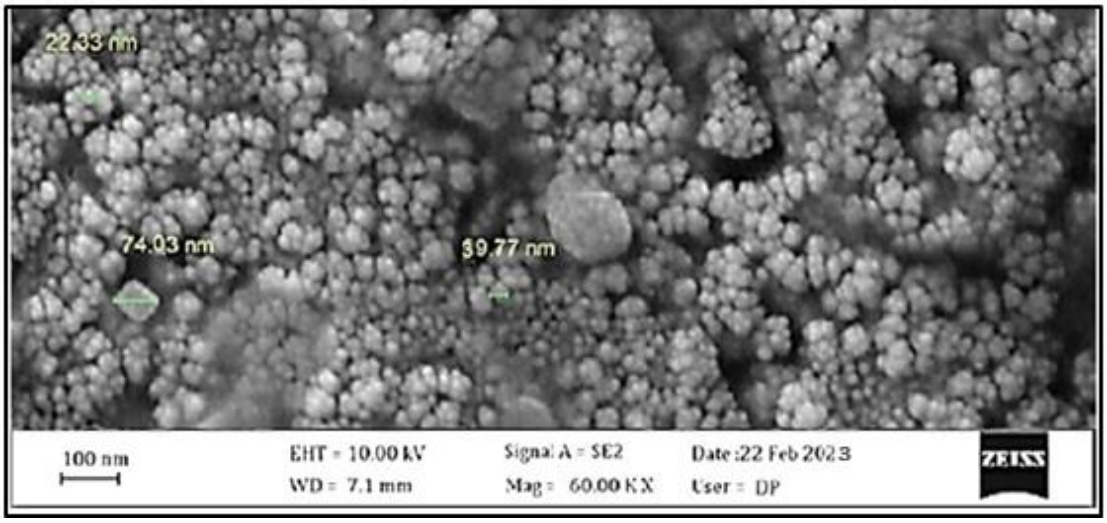

Figure 5. SEM image for ZnO-NPs synthesized

Antibacterial activity study and MIC determination ZnO NPs generated chemically had an antibacterial activity and MIC value of 1024µg/ml against MDR *P. aeruginosa*, while ZnO NPs biosynthesized using *V. agnus* leaf extract had a MIC value of 256 µg/ml and a MIC value of 512 µg/ml. ZnO-NP has been proven to exhibit antibacterial action in a few earlier experiments. Regarding biosynthesized ZnO-NP, [12,52] found that the MIC values varied between 1600–3200 µg/ml and 150µg/ml against isolates of *P. aeruginosa* that were resistant to multiple drugs, respectively. Disparities in ZnO nanoparticle manufacturing techniques and particle sizes could be the cause of variable findings regarding the toxicity of these particles. After researching ZnO-NP with a size of 30-90 nm against *P. aeruginosa* [53], revealed that the MIC was 300µg/ml. According to [54], ZnO nanoparticles smaller than 20 nm have a stronger antigrowth effect than larger ones; this may be because they can more easily pass through the bacterial cell wall. Zinc oxide nanoparticles are generally antimicrobial; they can prevent bacteria from growing by penetrating the cell membrane; additionally, they can lead to oxidative stress, which can harm proteins, carbohydrates, lipids, and DNA [55]. The high negative charge on the cell surface of gram-negative bacteria compared to gram-positive bacteria is another mechanism that could explain the increased antibacterial activity of ZnO-NP. As a result, gram-negative bacteria support the oxidative stress mechanism involved in zinc oxide nanoparticle antibacterial activity [56] When compared to chemically produced ZnO NPs, the results of antibacterial activity indicate that the biosynthesized ZnO NPs have higher inhibitory action. According to [57], chemical synthesis may potentially even now result in the presence of some harmful chemical species adsorbed on the surface that could have negative consequences in healthcare applications. As a result, the Bio-ZnO NPs enter the bacteria and attach themselves to the cell membrane with great ease.

The minimum inhibitory concentration (MIC) of *V. agnus* extract against multidrug-resistant *P. aeruginosa* was 512 µg/ml. The *Vitex agnus-castus* plant is regarded as one of the significant medicinal plants that has drawn significant interest and investigation from scholars [58]. According to [59], the capacity of the essential oil derived from *V. gardneriana* to influence microbial cells shown antibacterial efficacy against *P. aeruginosa*. The antibacterial properties of alcoholic *V. agnus-castus*

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extract can be attributed to flavonoids, tannins, and phenols—active phytochemical compounds found in medicinal plants—or to the hydroxyl groups (-OH) found in phenols, which have the capacity to form hydrogen bonds with the water molecules in bacterial cells, disrupting the bacterial cell's essential functions. Furthermore, the ability of tannins to inactivate enzymes, microbial adhesions, cell membranes that prevent *P. aeruginosa* transport proteins from growing, and other mechanisms may be linked to their action [58]. The specific mechanism by which ZnO-NPs kill bacteria can be understood in a variety of ways. For example, their unique inhibitory mechanisms may stem from their capacity to produce toxic ions (Zn2+), which compromise the integrity of the bacterial cell wall. Once inside the cells, the NPs and free metal ions disrupt biochemical processes by inducing denaturation of proteins and DNA damage [60]. When the bacteria inside the microbial cells are unable to multiply regularly, this will ultimately result in cell death and high amounts of reactive oxygen species (ROS) [61].

Effects of ZnO -NPs and *V. agnus* extract on efflux pump Gene expression

Every bacterial isolate's total RNA was successfully extracted both before and after treatment with. The folding ($2^{-\Delta ct}$) method was employed to quantify the gene expression levels, and the ΔCt value was adjusted to correspond with the housekeeping gene (*rpsL*). Tables 4, 5, 6 demonstrate that the expression of the *MexA*, *MexB*, and *OprM* genes was lower (fold expression <1) in the treated group isolates than in the untreated control group (fold expression =1). Cycle threshold, or Ct value, was used to measure the amplification; low Ct values indicate high expression, and high Ct values indicate reduced expression of the gene (Fig. 6). The quantitatively investigated using melting curve analysis and amplification plot (Fig. 7).

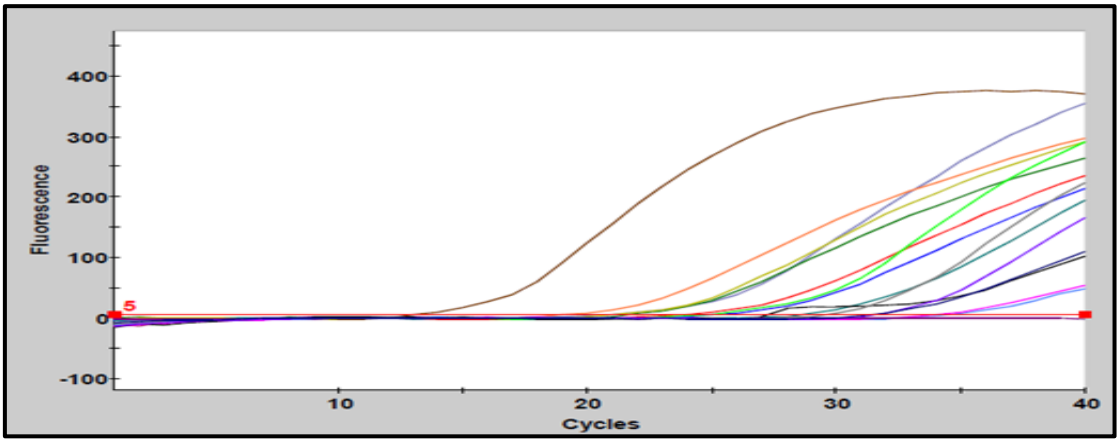

Figure 6. Results of *MexA* gene amplification curve by cycle in RT- PCR.

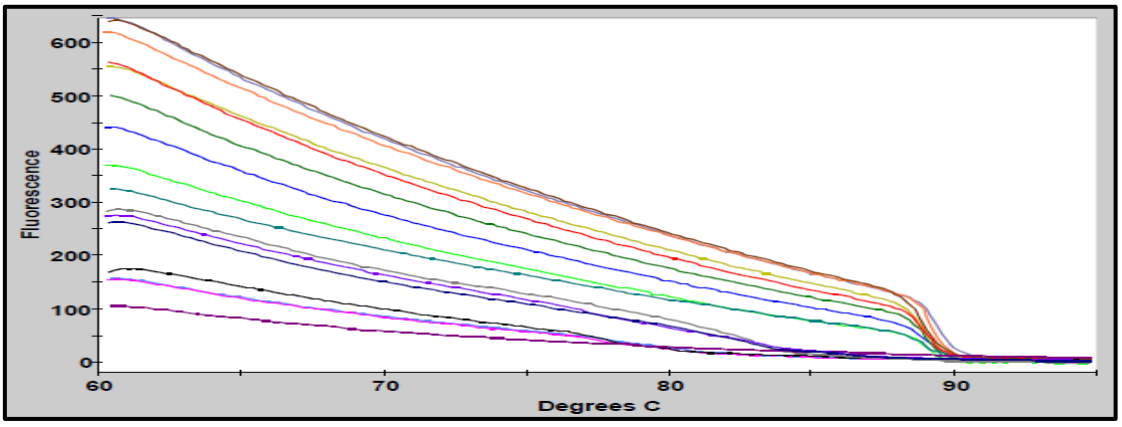

Figure 7. Melting curve analysis of the *MexA* gene. The measured curves of the gene in all samples are consistent and in the form of single-dot

2024, 21(12 Suppl.): 4050-4066
https://doi.org/10.21123/bsj.2024.10976
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Baghdad Science Journal

The overexpression of the efflux pump genes (*MexA*, *MexB*, *OprM*) in the untreated *P. aeruginosa* isolates (= 1) was found to be associated with resistance mechanisms in MDR *P. aeruginosa*, as confirmed by the results of biosynthesized ZnO-NPs, chemically synthesized ZnO-NPs, and *V. agnus* extract. Additionally, *MexAB-OprM* appears to be very helpful during antibiotic pressure in the medical environment and may be vital for the efflux of many drugs of a direct association between antibiotic resistance and the expression of the *MexA* and *MexB* genes was in line with our results [62]. The results displayed in (Fig. 8) indicate that biosynthesized ZnO-NPs has a stronger inhibitory effect on efflux pump genes than chemically synthesized ZnO-NPs and *V. agnus* extract, respectively. The expression of all the efflux pups' genes was dramatically downregulated, except for *OprM*, where the effect of chemically synthesized ZnO-NPs was greater than that of *V. agnus* extract, and *MexB*, where the effects of both biosynthesized ZnO-NPs and *V. agnus* extract were similar.

Since they can change the DNA strand and gene expression, ZnO -NPs and *Vitex* extract appear to affect the efflux pump genes of bacteria by increasing the formation of reactive oxygen species (ROS). Furthermore, RNA is extremely vulnerable to oxidative damage brought on by ROS, in contrast to DNA. By blocking MDR efflux pumps, nanoparticles could improve the effectiveness of conventional antibiotics as a treatment. Conversely, the existence of significant bioactive compounds may be responsible for the inhibitory activity of VA-C extracts [63]; some of these compounds may inhibit or lessen the formation of germ tubes (Pseudohyphae), adhesions, enzyme production, biofilm formation, and quorum sensing, among other virulence factors [64].

Table 4. Comparison of *MexA* gene fold change between groups.

Material name	Group	Means CT of *MexA* gene	Means CT of HK gene	ΔCt	$2^{-\Delta Ct}$	Experimental group /control group	Fold change
Biosynthesized ZnO-NPs	Group1 (control)	21.06	18.84	2.22	0.2146	0.2146/0.2146	1.00 ±0.00
	Group2 (After treatment)	31.74	18.69	13.05	0.0001	0.0001/0.2146	0.0004 ±0.0001
T-test (P-value)		--	--	--	--	--	0.439 * (0.0467)
Chem.synthesized ZnO-NPs	Group1 (control)	21.06	18.84	2.22	0.2146	0.2146/0.2146	1.00 ±0.00
	Group2 (After treatment)	23.82	18.81	5.01	0.0310	0.0310/0.2146	0.1444 ±0.05
T-test (P-value)		--	--	--	--	--	0.502 * (0.0492)
Vitex extract	Group1 (control)	21.06	18.84	2.22	0.2146	0.2146/0.2146	1.00 ±0.00
	Group2 (After treatment)	24.77	18.66	6.11	0.0144	0.0144/0.2146	0.0671 ±0.019
T-test (P-value)		--	--	--	--	--	0.488 * (0.0431)

* ($P \leq 0.05$).

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Baghdad Science Journal

Table 5. Comparison of *MexB* gene fold change between groups.

Material name	Group	Means CT of *MexB* gene	Means CT of HK gene	ΔCt	$2^{-\Delta Ct}$	Experimental group /control group	Fold change
Biosynthesized ZnO-NPs	Group1 (control)	18.27	18.84	-0.57	1.484	1.484/1.484	1.00 ±0.00
	Group2(After treatment)	27.4	18.69	8.71	0.0023	0.0023/1.484	0.0015 ±0.0002
T-test (P-value)		--	--	--	--	--	0.511 * (0.0419)
Chem. synthesized ZnO-NPs	Group1 (control)	18.27	18.84	-0.57	1.484	1.484/1.484	1.00 ±0.00
	Group2 (After treatment)	21.25	18.81	2.44	0.1842	0.18421/1.484	0.1241 ±0.04
T-test (P-value)		--	--	--	--	--	0.485 * (0.0422)
Vitex extract	Group1 (control)	18.27	18.84	-0.57	1.484	1.484/1.484	1.00 ±0.00
	Group2 (After treatment)	27.2	18.66	8.54	0.0026	0.0026/1.484	0.0018 ±0.0003
T-test (P-value)		--	--	--	--	--	0.547 * (0.039)

* (P≤0.05).

Table 6. Comparison of *OprM* gene fold change between groups gene.

Material name	Group	Means CT of *OprM* gene	Means CT of HK gene	ΔCt	$2^{-\Delta Ct}$	Experimental group /control group	Fold change
Biosynthesized ZnO-NPs	Group1 (control)	17.86	18.84	-0.98	1.972	1.972/1.972	1.00 ±0.00
	Group2 (After treatment)	31.89	18.69	13.2	0.0001	0.0001/1.972	0.0005 ±0.0002
T-test (P-value)		--	--	--	--	--	0.532 * (0.045)
Chem. synthesized ZnO-NPs	Group1 (control)	17.86	18.84	-0.98	1.972	1.972/1.972	1.00 ±0.00
	Group2 (After treatment)	22.48	18.97	3.65	0.079	0.1593/1.972	0.0400 ±0.01
T-test (P-value)		--	--	--	--	--	0.439 * (0.0382)
Vitex extract	Group1 (control)	17.86	18.84	-0.98	1.972	1.972/1.972	1.00 ±0.00
	Group2 (After treatment)	20.65	18.66	1.99	0.2517	0.2517/1.972	0.1270 ±0.03
T-test (P-value)		--	--	--	--	--	0.537 * (0.0455)

* (P≤0.05).

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Baghdad Science Journal

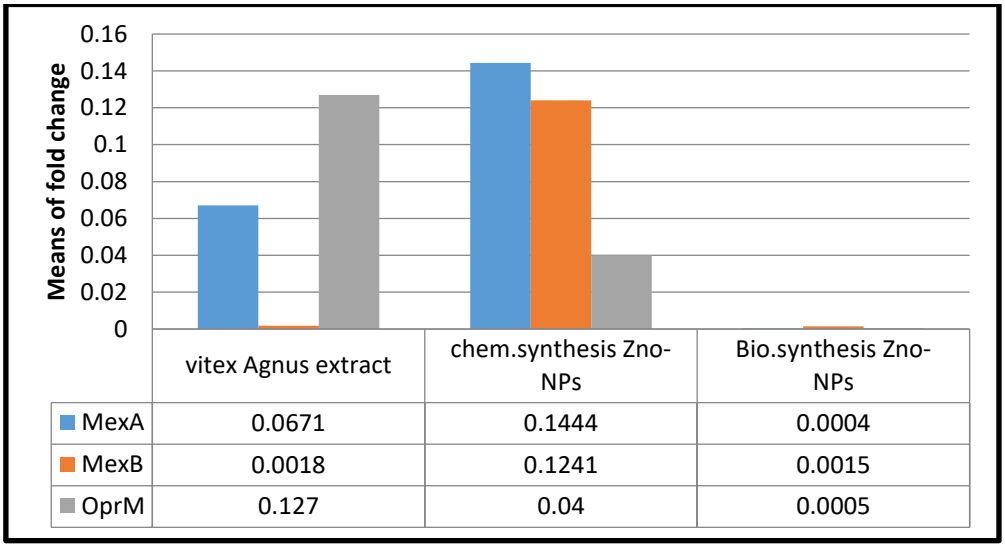

Figure 8. The expression rates of efflux pump genes in the biosynthesized ZnO –NPs, chemically synthesized ZnO –NPs *and V. agnus* extract -treated isolates and a significant decrease in gene expression was observed.

Conclusion

One of the primary causes of *P. aeruginosa* resistance is the *Mex*AB-*OprM* system. A decrease in the level of these efflux pumps reduces the excretion of antibiotics from the cell, which in turn causes lower concentrations of antibiotics to be able to kill cells. The present investigation's results showed that biosynthesized ZnO-nanoparticles using *V. agnus* extract reduced the number of active efflux pumps by reducing the expression of *MexA*, *MexB*, and *OprM* genes more than *V. agnus* extract alone and chemically synthesized ZnO-NP respectively.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Authors sign on ethical consideration's approval (the Code number of health ministry approved 2779).
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

Authors' Contribution Statement

This study was carried out with contribution of all authors.S.T.A. was the one who developed the theoretical idea of the project and fully supervised on work and writing with publishing while the practical part and writing the draft was the responsibility of S.A.K.K.

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تأثير جسيمات أوكسيد الزنك (ZnO) المصنعة كيميائيا مقارنةً بجسيمات أوكسيد الزنك المصنعة حيويا باستخدام مستخلص نبات *Vitex agnus* على تعبير جينات مضخة التدفق (MexAB-OprM) في بكتيريا الزائفة الزنجارية المتعددة المقاومة للمضادات

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قسم علوم الحياة، كلية العلوم للبنات، جامعة بغداد، بغداد، العراق.

الخلاصة

كان هدف الدراسة الحالية هو معرفة كيف تؤثر جسيمات اوكسيد الزنك النانوية المصنعة كيميائيا باستخدام طريقة sol-gel مقارنة بالجسيمات المخلقة حيويا باستخدام مستخلص أوراق نبات *Vitex agnus-castus* على التعبير الجيني لجينات مضخة التدفق (MexAB-OprM) في عزلات بكتريا الزائفة الزنجارية المتعددة المقاومة للادوية MDR. تم استخدام تفاعل البوليميراز المتسلسل PCR للتحري عن جين 16S rRNA في 16 عزلة من *P. aeruginosa* كان اختبار 16S rRNA ايجابيا لجميع عزلات *P. aeruginosa* (100)٪ ، وان 7 (43.75٪) من العزلات كانت من نوع MDR. كما أشارت النتائج إلى أن عزلات MDR تحمل جينات OprM و MexB. MexA و تم فحص المواد الكيميائية النباتية في مستخلص نبات *V. agnus* باستخدام فحص تحليل GC-MS و اظهر الفحص لمستخلص *Vitex* عن وجود مركبات فعالة. و وكانت احجام جسيمات اوكسيد الزنك النانوية المصنعة تتراوح مابين 22 و 74. بلغت قيم MIC لكل من جسيمات اوكسيد الزنك النانوية المصنعة كيميائيا والمخلقة حيويا وكذلك مستخلص Vitex 1024 و 256 و 512 ميكروغرام / مل على التوالي عند اختبارها ضد عزلات MDR تم دراسة التعبير لجينات MexA و MexB و OprM المعاملة مع ZnO NPs ومستخلص نبات *Vitex* بواسطة تقنية RT- PCR.اظهرت جسيمات اوكسيد الزنك النانوية المخلقة حيويا تأثير مثبط أكبر على التعبير الجيني لجينات MexA و MexB وOprM من الجسيمات النانوية المصنعة كيميائيا ومستخلص نبات *V. agnus* في العينات المعاملة بالتركيز المثبط الادنى لكل من جسيمات اوكسيد الزنك النانوية والمستخلص النباتي مقارنة بالعينات غير المعاملة. إحدى الطرق التي تعمل بها الجسيمات النانوية ضد البكتيريا هي قمع التعبير عن جينات مضخة التدفق ، مما يقلل من عدد مضخات التدفق النشطة على سطح الخلية ونتيجة لذلك ، يعتبر ZnO-NPs من قطاع الأدوية خيارا طبيا محتملا..

الكلمات المفتاحية: الزائفة الزنجارية، جزيئات أوكسيد الزنك النانوية، *Vitex*، *MexA*، *MexB*، *OprM* .