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Evaluation of the effect of biosynthetic zinc oxide by VITEX _ agnus castus L on the gene expression of the Quorum sensing genes

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Abstract

The current study was conducted to investigate the effect of zinc oxide nanoparticles (ZnO–np) prepared by physical method, alcoholic extract of Vitex agnus-castus leaf and biosynthetic ZnO–np by using Vitex agnus-castus extracts on the gene expression of Quorum sensing (QS) system of biofilm-producing Pseudomonas aeruginosa. 100 samples were collected from clinical sources and after the identification, 18 clinical isolates were confirmed to be Pseudomonas aeruginosa by PCR using gyrB gene. The effect of MIC for all substances against four biofilm-producing isolates were examined by the microtiter plate method. Furthermore, qRT-PCR was performed to determine three substances inhibitory effect on QS-regulatory genes lasR, , rhlR, and pqsR that control virulence factors secretion. Biosynthetic ZnO–np, ZnO–np and plant extract showed significantly down-regulated the expression level of all biofilm and virulence genes of P. aeruginosa clinical isolates and thus we conclude that Biosynthetic ZnO–np can be used as a quorum sensing inhibitor and an anti-biofilm in P. aeruginosa infections.

Keywords:

real-time, Vitex agnus L, gene expression, citramide, resazurin

Pseudomonas aeruginosa is one of the most bacterial species that has drawn the interest of geneticists, biochemists, and microbiologists due to the variety and intensity of the virulence factors it possesses. P. aeruginosa, which causes 10% of all nosocomial infections, is the fourth most frequent bacteria linked to hospital acquired illnesses, according to the National Nosocomial infections Surveillance System (2,16). P. aeruginosa is a common microbe that can infect people with impaired immune systems and cause nosocomial illness (8). Bacteria and their exopolysaccharide matrix developing on a surface are known as biofilms (22) .

For the formation of biofilms, a bacterial attachment must be made, and this is the first step in their formation when bacteria stick to the surface. This helps them form biofilms and external polysaccharides. These cells have many advantages that distinguish them from other planktonic cells, as these cells In the biofilms can acquire features Hereditary, such as antibiotic resistance. Bacterial cells in aeruginosa communicate with each other through a process called quorum sensing, which plays a role in the pathogenesis of bacterial diseases. There are three major quorum sensing systems in aeruginosa that communicate with each other through signals called auto-inducer. Quorum sensing inhibitors reduce the production of virulence factors and inhibit them without interrupting their growth, as they work by disrupting the Quorum sensing systems in bacterial cells, so no or low resistance can be achieved(17). The use of nanoparticles in medicine, such as the treatment of infections diseases, is the result of advances in

technology that have occurred in recent years. Zinc oxide exerts strong antimicrobial activity as it reduces skin infections(18,19). Rhamnolipids, pyocyanin, pyoverdin, hemolysins, elastase, and proteases were among the virulence components whose production was statistically significantly reduced by ZnO. Additionally, ZnO showed a notable decline in the relative expression of the QS-regulatory genes lasI, lasR, rhll, rhlR, pqsA, and pqsR(20). Despite the tremendous development in the science of chemical medicines and their popularity and the emergence of large numbers of them for treatment, the last period witnessed a return to the use of medicinal herbs in the treatment of diseases as natural alternatives because they have many known therapeutic properties(9). Medicinal plants are used to inhibit biofilm formation, as the results of one experiment indicate that an essential oil extracted from anise (Pimpinella anisum) plant has the ability to reduce biofilm formation by a rate ranging from 26 to 86.41% (26) . In a study, It was proved that the herbal extract, Phyllanthus emblica, has an inhibitory effect on biofilm formation, as this extract provides new information for treatment against antibiotic-resistant bacteria (11). One of the medicinal plants that was dealt with in this study is Vitex agnus plant, and it is considered one of the famous medicinal plants, which is widely used in the Iraqi environment. Vitex agnus contains active ingredients such as flavonoids, glycosides, and triptoides. It has the ability to grow rapidly and resist drought (12). The current study aims to investigate the effect of biosynthetic zinc oxide using Vitex agnus_castus plant, zinc oxide, and a plant extract, each on Quorum sensing genes.

Materials And Methods

Sample collection and isolate identification

From Al-Kindi Hospital, 100 clinical specimens were collected from various wards over a period from October to February. All samples were cultured on Cetrimide agar media then the isolates were identified by standard microbiological techniques (colony morphology, Gram staining and biochemical reactions). The selective medium for P. aeruginosa was improved by Brown and colleagues, who showed that the selective medium better enhances the production of pyocyanin (13).

Molecular Diagnosis

Identification of Pseudomonas aeruginosa using PCR technique by gyrB gene detection

DNA Extraction and PCR conditions

Molecular detection of P.aeruginosa encoding gyrB was used to confirm the presence of P.aeruginosa isolates. Bacterial DNA was extracted using a wizard Genomic DNA Purification Kit from (Promega, Madison, WI.) PCR amplification was carried out using green master mix from Promega. The amplified products were electrophoresed in a 1.5% agarose gel and then stained with ethidium bromide. Table 1 displays the PCR primers and PCR test conditions.

Table 1: PCR conditions and primer used in this study

Gene	Primer sequence	Annealing Tm	Product size (bp)	Reference
gyrB	F-CCTGACCATCCGTCGCCACAAC- R-CGCAGCAGGATGCCGACGCC	62	220	(30)
LasR	F:CAGTCACTGTACCCAGAGCG R: GGTCAGCCCATACACCAGG	55	118	This study
rhlR	F :CTTTTTGCTGTGGTGGGACG R : GGCGTAGTAATCGAAGCCCA	56	118	This study
pqsR	F:CTGATCTGCCGGTAATTGG R:ATCGACGAGGAACTGAAGA	57	142	(20)
rpsL	F: GCAAGCGCATGGTCGACAAGA R: CGCTGTGCTCTTGCAGGTTGT	57	201	(27)

Biofilm formation assay

The 18 isolate that were positive for the gyrB gene were selected to test their biofilm production using Quantitative method MPA (Microtiter plate assay). The test was carried out when it was being prepared (14,38) step by step. Briefly, using brain heart infusion broth, the isolate were inoculated and incubated at 37°C for 18 hours. After that, 300 microliters of broth were placed in a flat bottom and inoculated with 10ul of bacterial broth. After that, the plate was incubated at a temperature of 37Celsius for 18 hours, using phosphate-buffered saline, pH 7, and the plates were washed for one hour. The plates were air-dried at 60 degrees Celsius and stained with crystal violet in a volume of 300ul microliters (0.25) for one minute after washing. The use of acetic acid with a volume of 300ul and a percentage (33%) to destain the samples.

Resazurin_based turbidimetric assay and Minimum Inhibitory concentration (MIC) determination

Using the Microtiter plate method, 100 ul of Double Moller Hinton was placed in each well of the Plate, and then 100 ul of the substance to be determined for its mic was placed in each row of the microtiter and mixed with Broth, and by sterilized pipette we transferred 100 ul of the mixture from the first well to The second well, and from a second well to the third well, and continue in the same way to Well 11. Finally, removed 100 ul from a mixture of well 12. Hence 11 and 12, negative and positive control, respectively. Then, we add 10 ul from the bacterial suspension to every well except for the well 11. After incubated the plate for a full night, add 5ul of Resazurin to all the well and incubate it for 4 hours. Color changes (from blue to pink) were noticed and noted. The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration at which the color changed.

Detection of Quorum sensing system genes in Pseudomonas aeruginosa

Purity, DNA concentration, and agarose electrophoresis were estimated after genomic DNA extraction from bacterial isolate.

Amplification of pqsR, lasR and rhlR genes, Preparation of PCR mixture

The pqsR, lasR, and rhlR genes are found using the PCR method. A total amount of 25 ul was used to build up each PCR mixture.

Table 2: PCR reaction condition of lasR, rhlR, pqsR, and rpsL genes

Primer	Initial denaturation	Denaturation	Annealing	Elongation	Cycles	Final extension
LasR	95/5 min	94/30,sec	55/30sec	72/30sec	35	72/7min
rhlR	95/5 min	94/20sec	58/20sec	72/30sec	40	72/5min
pqsR	95/5 min	94/30sec	57/30sec	72/20sec	35	72/7min
rpsL	95/5 min	94/30sec	57/30sec	72/20sec	35	72/7min

Total RNA extraction

According to the manufacturer's Instructions, RNA was extracted from the P. aeruginosa isolates using a GENEzol TM TriRNA Pure Kit.

Quantitation of RNA

According to Desjardins and Conklin (15), Using a nanodrop to measure the concentration and purity of RNA, 1ul is placed in a nanodrop.

One step quantitative real time PCR assays (qRT_pcr)

Thermal cycling procedure and reaction setup

The studies were carried out for the more suitable cDNA synthesis and annealing temperature utilizing a one-step RT-qPCR system in order to evaluate the gene expression of the lasR, rhlR and pqsR genes. Before use, all reagents were gently mixed, thawed on

ice, and centrifuged at a moderate speed. Table 3 displays RT-PCR mixes with appropriate component volumes in a microcentrifuge tube. The internal control was the housekeeping gene rpsL

Table 3: GoTaq®1step TR_qPCR reaction mix

Master mix	Volume (ul)
GoTaqR qPCR master mix,2x	10
Goscript RT mix (50)	0.4
Mgcl2	0.4
Forward Primer	1
Reverse primer	1
Nuclease free water	4
RNA template	20

Using the GoTaq®1 – Step RT qPCR kit, perform quantitative real-time PCR (qRT PCR) as follows: In a sterile tube , the component volumes were combined .To reduce accretion , the mixtures were carefully and thoroughly mixed using a vortex mixer after each addition. Each smart cycler PCR tube received the proper amount of reaction mixture RNA template was added to the smart cycler PCR tube (or free nuclease water for the no – template control reactions). The tubes were sealed , quickly centrifuged to collect the contents of the bottom wells , and shielded from prolonged exposure to light or high temperatures . Temperature, time, and the number of repetitions for each.

Table 4: Program for the general thermocycler (RT-PCR) utilized in the study

Steps	Temperature	Duration	Cycles	Reference
Reverse transcription	37	15min	1	Company instructions
Reverse transcriptase inactivation and GoTaq® DNA Polymerase activation	95	10min		Company instructions
Denaturation	95	30sec		
Annealing	57* 55** 57*** ****58	30sec 30sec 30sec 30sec	35 35 35 40	(20) This study (27) This study
Extension	72	30sec		

PqsR*, LasR**, rpsL***, rhlR****

Expression level for the isolate was examined using the delta CT method.

Preparation of the plant extract.

After collecting, washing and drying the leaves of the plant, they are ground to form a fine powder, and to form the plant extract, 25 g of the powder is boiled in 250 ml of distilled water for 15 minutes, then filtered, and the filtered extract is kept at a temperature -4.

Nanoparticles Biosynthesis of zinc oxide (ZnO NPs)

According to the method (3) with some modifications. 100 ml of the previously prepared plant extract was heated to 60-70 degrees Celsius on the magnetic stirrer, when the temperature reached 60 degrees Celsius, 10 g of zinc nitrate (Zn (NO3) was added to it, and it was boiled until It turned into a creamy white paste. After Then the paste Is washed with distilled water and placed In a heated horn at a temperature of 400 ° C for two Hours, after which a white powder of ZnONPs loaded with active compounds will form from the plant extract.

Preparation of zinc oxide nanoparticles

The solid zinc metal of high purity (99.9%) was used and formed In the form of a disc by means of a hydraulic press, after which the solid zinc disc was cleaned by washing it with distilled water for 5 minutes to get rid of the impurities on it, and then the zinc disc was placed at the bottom of the glass container with tongs Special and immersed in distilled water, as the volume of distilled water is (5 ml) and the distance of the solid target from the laser lens is about (1.5 cm), the glass container is placed on a rotating base, this disk rotates continuously and works to rotate the glass container that contains the submerged zinc steel disk

in distilled water to ensure that the laser beams do not fall on one site on one surface(4).

Statistical Analysis:

The Statistical Analysis System- SAS (2018) 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 significant compare between means in this study.

Results And Discussion

Isolation and Identification od Pseudomonas aeruginosa

100 clinical samples were collected in this study from burns from different age groups of males and females. After culturing the clinical samples on the selective medium for bacteria, only 28 samples developed on citramide appeared out of 100 clinical samples. In another study higher results were obtained than the results of the current study, where 61 out of 144 isolate were diagnosed as aeruginosa (21). Only 15% of the total number of burn swabs (or 50%) had P. aeruginosa on them, according to the findings.(32). For additional confirmation of the type of bacteria, biochemical tests were performed, and the result was that 25 were positive for oxidase tests.The findings revealed that the cells appeared as Gram – negative bacilli. Cytochrome oxidase, an enzyme crucial to the electron transport chain, is found In bacteria that may be found using the oxidase test. Oxidase negative Enterobacteriaceae are distinguished from oxidase positive Pseudomadaceae by this (7).

Molecular Diagnosis

Identification of Pseudomonas aeruginosa using PCR technique by gyrB gene detection

DNA Extraction and PCR conditions

After extracting the DNA of the samples used in this study, they all contained DNA. For using the PCR technique to check for the gyrB gene's existence. By gel electrophoresis in 0.8% agarose, extraction genomic DNA was qualitatively verified as bands, as illustrated in **Figure 3-1**. Nanodrop calculated the DNA's purity and concentration. Additionally, it was noted that the concentration varied between 232.83 and 428.25 ng/l and that the purity ranged from 1.76 to 1.9. for 18 isolates, the gyrB gene fragment was amplified using the PCR method to validate the Identity. The PCR products' results were verified by comparing their molecular weight to a 100 bp DNA ladder by analyzing the bands on a 1.5% agarose gel electrophoresis, with a PCR product size single amplicon 956 bp band. According to the current findings, 15 isolate from the entire burn sample included gyrB. These results were lower than those by Othman (5), who claimed that P. aeruginosa made up 30% of 985 burn samples from Sulaymaniyah hospitalized patients. According to a study by AL-Sheikhly (6), P. aeruginosa was found in 36.67% of burn samples .

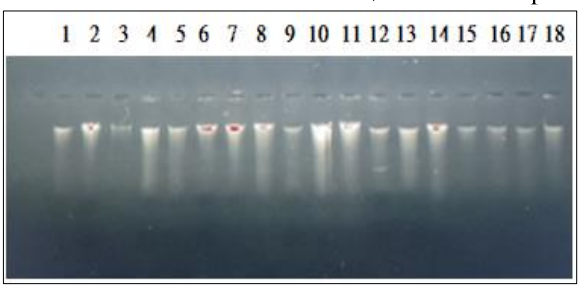

Figure 1. DNA bands from aeruginosa Isolates were electrophoresed on agarose gels containing 0.8% agarose for 30 minutes at 100 volts before being stained with ethidium bromide.

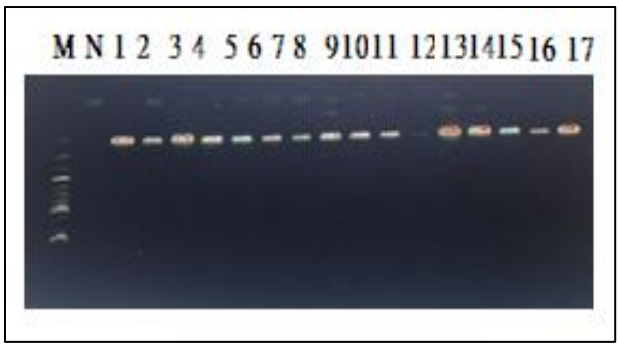

Figure 3_2: lane 1–15 pseudomonas aeruginosa isolate; M: Marker DNA ladder (100bp); and N: Negative control. Agarose gel electrophoresis of amplified PCR product for the Identification of gyrB gene () run on 1.5% agarose (60 min at 70 volt)

Biofilm formation assay

Since absorbance was measured using a microplate reader, absorbance values represent the thickness of the biofilm that the tested isolates produced on the microtiter well's surface. Evidently, research isolate showed considerable variation In their ability to produce biofilms. There is a big difference in the formation of biofilms in bacterial isolates, due to the difference in the genetic composition of these isolate, as well as the gene expression of biofilm genes, and biofilm is one of the virulence factors for the survival of bacteria and is difficult to eliminate(28), and the growth rate of bacteria after incubating them with broth is one of the reasons for the difference in their composition For biofilm, there are bacteria whose growth rate is higher, and therefore the number of bacterial cells is more, so their formation of biofilm is higher than bacteria whose growth rate is low. After a biofilm experiment for 18 isolate to see their ability to form a biofilm, 15 isolates produced biofilm-producing phenotypes were obtained, while two of the isolates were unable to produce a biofilm. On the contrary, there is a study conducted by(21), the results were as follows: 33 isolates are weak biofilm producers, 18 are medium and 7 are strong producers . In contrast to (27) that reported 68 % of the isolates formed biofilm.

Table5: shows the ability of bacterial Isolates to form a biofilm.

Non producer	Weak producer	Moderate	Strong	Total
2(13.3)	3(20%)	3(20%)	7(46.6)	15

Resazurin_based turbidimetric assay and Minimum Inhibitory concentration (MIC) determination

This method was used to determine the inhibitory concentration of each plant extract, Zinc oxide and biosynthetic zinc oxide. The Muller Hinton broth was used in a two-fold dilution procedure to calculate the MIC. The MIC values for each isolate were determined to be the lowest concentrations at which no growth takes place. Resazurin was permanently converted to resorufin by oxidoreductase in the presence of available and metabolically active bacteria cells. The same method was used by the (22) to determine the minimum inhibitory concentration of larva extract against MRSA. The larval extract of L. cupring was Inhibitory against all tested bacteria, according to the resazurin-based TB assay, while the larval extract of 5 peregrina and M. domestica was exclusively inhibited against the MRSA, with a MIC of 100 mg ml

Table 6: concentration of MIC used in this study

Isolate code	MIC (ul/mg) plant	MIC (ul/mg) Nano+	MIC(ul/mg) Nano
1	500	500	500
2	16	1000	500
3	8000	500	8000
4 5	125 125	125 125	500 125

And in another research conducted by Moustafa , the MIC of zinc oxide was determined, as It prevented the growth of ZnO aeruginosa from growing at a concentration of 8mg/ml (20).

Molecular study if Quorum sensing genes in Pseudomonas aeruginosa

Polymerase chain reaction amplification for detection pqsR, lasR, rhlR and rpsL

To discover the genes responsible for antibiotic resistance and biofilm formation, this study was conducted on 5 bacterial isolate using the polymerase chain reaction technique. Specific primers for each gene were used for the detection of the presence of housekeeping gene pqsR, lasR, rhlR, and rpsL. The results were that the four bacterial were positive for the four genes(pqsR, lasR, rhlR, and rpsL)

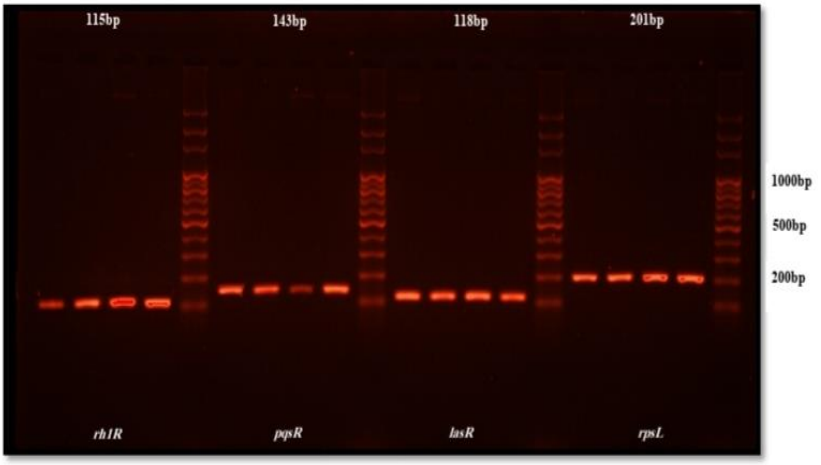

Figure 3. Agarose gel electrophoresis of amplified PCR product for the detection of genes run on 1.5 % agarose (70 volt for 90 min.) stained with ethidium bromide.

Extraction total of RNA

Four P. aeruginosa isolates were total RNA obtained to compare the expression of the lasR , rhlR and pqsR genes before and after each Isolate was treated with nanomaterials, plant and loaded nanoparticles at sub-MIC doses. The RNA was extracted using the GENEZol TM TriRNA Pure Kit, and the purity ranged from 1.44 to 2.16 with a concentration range of 32.32 to 1017.24ng/ul

Effect of biosynthetic zinc oxide on the expression of pqsR, lasR and rhlR In Pseudomonas aeruginosa isolates: P. Aeruginosa has emerged as the primary causative agent of nosocomial infections, necessitating the swift and effective implementation of effective infection control procedures to stop its spread. It demonstrates both innate and acquired multi-drug resistance to antibiotics through various mechanisms (33). Targeting QS has gained popularity as an alternative to anti-microbial therapy because of its role in controlling the production of virulence factors and pathogenesis in Aeruginosa(31) .In this study, zinc oxide was biosynthesized by using the extract of the plant extract.

After treating the four bacterial isolate with biosynthetic zinc oxide to know its effect on the inhibition of genes, excellent results were obtained. Biosynthetic zinc oxide showed its ability to inhibit the gene expression of rhlR, pqsR and lasR. The 2 – AACt technique was used to compare the relative expression of the genes controlling the generation of virulence factors in isolates that had been treated with ZnO and those that had not. After ZnO sub-MIC treatment, the relative expression levels of , lasR, rhlI, and pqsR were drastically decreased.

This substance can be used to manufacture antibiotics to treat patients with aeruginosa. Previous investigations have demonstrated ZnO – np's antibacterial properties .The antibacterial effect of biosynthesize ZnO – np against multidrug resistant P. aeruginosa isolates has been studied by Ali et al. The MIC values for clinical isolates obtained from various sources ranged from 1600 to 3200 ug / mL. [34]

Zinc oxide synthesized using prodigiosin as a reducing agent showed inhibitory results for Pseudomonas and its maximum inhibit diameter was 29mm at a concentration of 320ug/mL(15) .

Effect of zinc oxide on the expression of pqsR, lasR and rhlR In Pseudomonas

aeruginosa isolates: The relative expression of the QS-regulatory genes LasR, rhlR, and pqsR significantly decreased in ZnO. Moreover, ZnO greatly decreased Pseudomonas aeruginosa pathogenicity.

After the real time procedure for the samples treated with zinc oxide, we obtained an inhibition of the Quorum sensing genes, and there was a significant difference in the gene expression of the samples before and after the treatment. ZnO nanoparticles can be employed either as an adjuvant to traditional antimicrobials or as a quorum sensing inhibitor in Pseudomonas aeruginosa infections.

ZnO revealed a statistically significant reduction in the production of QS – controlled virulence factors rhamnolipids , pyocyanin , pyoverdin , hemolysins , elastase and proteases . Furthermore , ZnO exhibited a significant decrease in the relative expression of QS – regulatory genes LasR, rhlR, rhll, pqsR, pqsA and pasR (20).

RT-PCR was used to examine how ZnO nanoparticles affected the expression of several genes involved in the development of biofilm and virulence factors. All biofilm and virulence genes of P. aeruginosa clinical isolates were considerably downregulated by the nanoparticles. After being exposed to ZnO nanoparticles, the fold change decrease of the quorum sensing genes, LasR, rhll, and pqsR was 10.4, 6.3, and 8.7 fold, respectively (p value 0.0001)(37)

ZnO-np can damage bacterial cell membrane integrity, decrease cell surface hydrophobicity, and down-regulate the transcription of oxidative stress-resistance genes in bacteria, according to research by Pati et al. [35]

Effect of extract plant on the expression of pqsR, lasR and rhlR in Pseudomonas aeruginosa isolates: After treating the five bacterial isolate using Vitex agnus extract, Interesting results were obtained, as the plant extract affected the gene expression of Quorum sensing genes, as It reduced the gene expression of (rhlR, pqsR and lasR) genes. Because the plant contains compounds and oils that have inhibitory effectiveness.In another study that supports our current study, it was found that there are 31 compounds in the oil, such as

1,8 – cineole (50.9 %) , sabinene (10.8 %) , a – pinene (9.0 %) , terpinen – 4 – ol (4.8 %) , p – cymene (4.2 %) , limonene (2.5 %) , and a – terpineol (2.3 %)and their inhibitory effectiveness has been proven for many types of bacteria, such as Escherichia coli , Pseudomonas aeruginosa , Bacillus subtilis , and Staphylococcus aureus (36) .

The Vitex agnus of plant can be used to manufacture ointments and creams to treat burns and wounds infected with aeruginosa. In another research conducted by Abdel-Amir using Vitex agnus, the result of inhibition of the growth of two types of bacteria, Staphylococcus aureus and E.coli, was obtained, where he obtained an inhibition diameter of 26 mm for E.coli and 28 mm for Staphylococcus aureus(9). A study by Abdullah Habbab showed that the Vitex agnus contains essential oils that had antifungal activity and can be used as natural preservatives in various products as well as potential antifungal agents, but there must be studies to identify the compound responsible for the antifungal activity and confirm its safety Use this essential oil(25).

Figure 4 : qPCR amplification plots of pqsR expression curve for Pseudomonas aeruginosa isolates before and after ZnO, biosynthetic ZnO and extract plant treatment

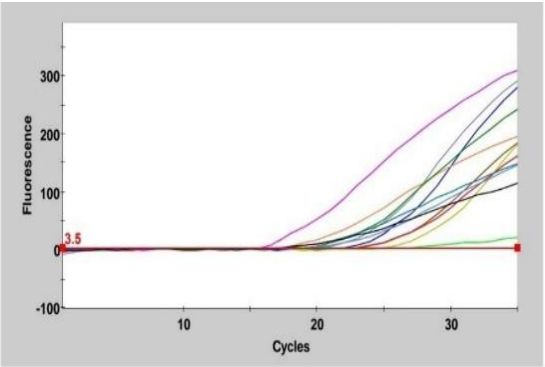

Figure 5 : qPCR amplification plots of rhlR expression curve for Pseudomonas aeruginosa isolates before and after ZnO, biosynthetic ZnO and extract plant treatment

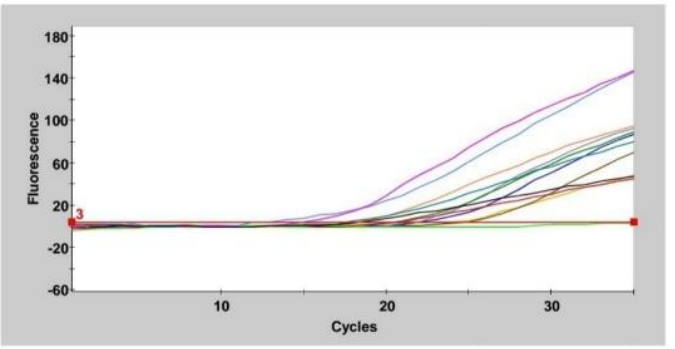

Figure 6: qPCR amplification plots of LasR expression curve for Pseudomonas aeruginosa isolates before and after ZnO, biosynthetic ZnO and extract plant treatment

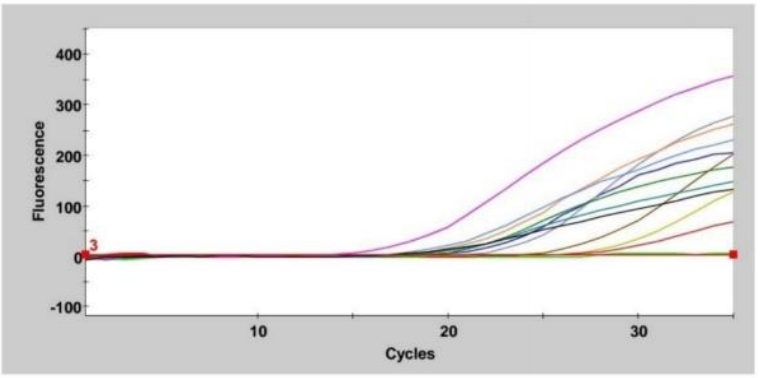

Table 7: fold change of pqs gene after treatment (Mean ± SE of fold change of pqs gene)

Isolate code	Fold change (*)	Fold change (+**)	Fold change (***)
PA1	0.4352±0.17	0.0104±0.002	0.2482 ±0.07
PA2	0.8585±0.24	0.0791±0.21	0.3186 ±0.09
PA3	0.2624±0.04	0.0038±0.0001	0.2624 ±0.07
PA4	0.28±0.9265	0.0181±0.002	0.1528 ±0.05
LSD(P_Value)	0.791(0.001)	0.582(0.0001)	0.339 (0.188)
	P<0.01	P<0.01	NS

NS=Non significant

Table 8: fold change of rhlR gene after treatment (Mean ±SE of fold change of rhlR)

Isolate code	Fold change (*)	Fold(**+) change	Fold change(***)
PA1	0.1062±0.03	0.1065±0.001	0.1767 ±0.05
PA2	0.1111 ±0.04	0.0287 ±0.001	0.2030 ±0.08
PA3	0.0057 ±0.002	0.0001 ±0.0001	0.0228 ±0.003
PA4	0.0583 ±0.02	0.0027 ±0.001	0.0722 ±0.02
LSD(P_Value)	0.692 ** (0.0001)	0.217 ** (0.0001)	0.144 (0.092)
	P<0.01	P<0.01	NS

Table 9: fold change of LasR gene after treatment (Mean ±SE of fold change of LasR)

Isolate code	Fold change (*)	Fold change (+**)	Fold change (***)
PA1	0.4352 ±0.17 b	0.1065±0.03	0.9395±0.24
PA2	0.8585 ±0.24 b	0.1111 ±0.04	0.7526 ±0.21
PA3	0.2624 ±0.09 b	0.0057 ±0.002	0.8122 ±0.30
PA4	0.9265 ±0.28 b	0.0583 ±0.02	0.7737 ±0.22
LSD(P_Value)	0.791 ** (0.0064)	0.692 ** (0.0001)	0.718 ** (0.0001)
	P<0.01	P<0.01	**P<0.01**

*=ZNO,+**=ZNO NP, ***=extract plant, Significant (P⩽0.05), Highly Significant (P⩽0.01).

Fold change represents the gene expression value of the gene after treatment. If the value is less than 1, this indicates that the gene has been inhibited after treatment, and this is the goal.

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