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Gene Expression Analysis Mexz and Aac6 Genes in Pseudomonas Aeruginosa Isolates Before and After the Treatment with the Agnps Aloe Vera Plant Extract

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Abstract:

RNA was extracted from the pseudomonas aeruginosa isolates (P1, P5, P9, P17, P18, and P29) before treating as a control and after treating with the AgNPs Aloe vera plant Extract. Total RNA was extracted using TRIzol™ Reagent and its concentration was from 80 to 210 ng/µL, the purity of total RNA samples ranged from (1.6 to1.9). mexZ and aac6 genes which are carried by the six P. aeruginosa isolates (P1, P5, P9, P17, and P18) that were used in the investigation of gene expression. These isolates were chosen because they exhibited the highest level of resistance. The influence of the AgNPs Aloe vera leaves extract on the Gene Expression of the mexZ and aac6 Genes in P. aeruginosa Isolates was the primary focus of this research. Primers designed to target the mexZ and aac6 genes in P. aeruginosa have a high degree of specificity for gene expression. P. aeruginosa isolates had their gene expression of the aac6 and mexZ genes measured using real-time PCR before and after being treated with the AgNPs Aloe vera plant extract. Reverse transcription-quantitative PCR (RT-qPCR) was carried out using SYBR green; a fluorescent dye that recognizes any double-stranded DNA including cDNA. Through a two-step RT-qPCR method in order to determine the effect of sub MICs of AgNPs Aloe vera leaves on the expression of mexZ and aac6 gene in P. aeruginosa. The first step is Synthesis cDNA from RNA by used specific primer (hexamer) for the target gene and housekeeping gene in the study, the immediately transformed of genomic RNA to cDNA after extraction accomplished to get a complete transformation without any loss or degradation of RNA in one steps of cDNA synthesis. The second step is using the quantitative PCR (RT-qPCR) device to measure the range of Ct value of this gene under study. The result of real-time quantitative PCR experiments for gene aac6 was inhibited in the gene expression after treatment with AgNps Aloe vera leaves extract. The result of real-time quantitative PCR experiments for gene mexZ was inhibited in the gene expression after treatment with AgNps Aloe vera leaves extract. To reveal the inhibition efficacy of AgNps Aloe vera leaves extract on the P.aeruginosa gene, mexZ and aac6 expression were measured. The results when the isolates were treated with AgNps Aloe vera plant extract with sub-MIC (10 mg/mL) for each sample. It was found that the presence of AgNps Aloe vera leaves extract negatively affects in mexZ and aac6 gene expression. Decreased expression in mexZ and aac6 genes causes to reduce of the efflux pump expression and production of enzyme modify which is one of the causes of antibiotic resistance. Decreased mexZ and aac6 gene expression is an important indicator that it influences an important mechanism of resistance in P. aeruginosa. The main purpose of studying gene expression in the presence and absence of AgNps Aloe vera extract is to investigate the role of these genes in efflux pump formation and enzyme modification production. It is considered one of the most important virulence genes that cause resistance to the aminoglycoside group in P. aeruginosa. Generally, numerous studies have shown that Aloe vera helps wound healing because of its anti-inflammatory, antioxidant, and immuneomodulatory activities.

Key words:

Gene expression,

mexz,

aac6,

pseudomonas aeruginosa,

AgNPs,

Aloe vera

Introduction:

The *P. aeruginosa* accessory genome is made up of extra chromosomal components like plasmids, islands, and blocks of DNA that are incorporated into the chromosome at a variety of different locations (Klockgether *et al.,* 2011). Variations in the core genome as well as variations in the accessory genome have an effect on the virulence factors as well as the antibiotic resistance of any given *P. aeruginosa* strain. (Subedi *et al.*, 2018). The cytoplasmic and periplasmic components of *P. aeruginosa* resistance-nodulation-division (RND) pumps are named multidrug efflux *(Mex)* along with a letter, and the outer membrane porin is named *Opr* along with a letter. *Pseudomonas aeruginosa* expresses twelve RND family efflux pumps, four of which *(MexAB-OprM, MexCD-OprJ, MexEF-OprN*, and *MexXY-OprM)* contribute to antibiotic resistance (Dreier and Ruggerone, 2015). *MexZ* is a transcriptional regulator of the *mexXY* multidrug transporter operon, which confers aminoglycoside resistance on *Pseudomonas aeruginosa*. *MexXY–OprM* is the major factor that contributes to aminoglycoside antibiotic export and resistance. *(Mex AB - oprM)* is responsible for efflux of β-lactams and quinolones (Chevalier *et al.,* 2017). *MexCD-OprJ* is able to pump out β-lactams (Okamoto *et al.*, 2002). The development of aminoglycoside-modifying enzymes is the primary defense mechanism against aminoglycosides (Shaw *et al.*, 1993). These genes have been given the designations aac(6=)-Ia to aacA43 (Vakulenko and Mobashery, 2003). The Aloe Vera extract has been created, and it has been found to be very sensitive to a wide range of bacteria, Gram-negative pathogens, Gram-positive pathogens, and some fungal diseases. (Cock, 2019)

Material And Methods :

Gene expression

Total RNA Extraction

RNA was isolated from sample using TRIzol™ Reagent according to the protocol described by the manufacturer as in the following:

1- Sample lysis

A- The supernatant was discarded, and 0.75 ml of TRIzolTM Reagent was added to the pellet after approximately 1.5 ml of cell culture was precipitated using a microspin centrifuge for 2 minutes at 13000 revolutions per minute.

B- The lysate was homogenized by pipetting up and down several times.

2- Three phase's separation

A- For each tube, 0.2 ml of chloroform was added to the lysate, and then the tube was securely capped.

B- After incubating each mixture for 2 - 3 minutes and centrifuging it for 5 minutes at a speed of 1000 revolutions per minute, the mixture was separated into a lower organic phase, an interphase, and a colorless upper aqueous phase.

C- The aqueous phase containing the RNA was transferred into a new tube.

3- RNA precipitation

A- A volume of 0.5 ml of isopropanol was added to the aqueous phase and incubated for 10 min then centrifuged for 10 min at 10000 rpm.

B- Total RNA was precipitated forming a white gel-like pellet at the bottom of the tube.

C- Supernatant was then discarded.

4- RNA washing

A- For each tube, 0.5 ml of 70% ethanol was added and vortexed briefly then centrifuged for 5 min at 10000 rpm.

B- Ethanol was then aspirated and the pellet was air-dried.

E- RNA solubility Pellet was rehydrated in 50 µl of Nuclease Free Water then incubated in a water bath set at 55–60˚C for 10–15 min.

Estimation of RNA, Concentration and Purity

In order to determine whether or not the samples were suitable for use in subsequent processes, a Quantus Fluorometer was utilized to measure the amount of RNA or cDNA that had been extracted. Mixing 199 µl of diluted QuantyFlour Dye was done for every 1 µl of RNA or cDNA that was used. Incubation for 5 minutes at room temperature in a dark environment resulted in the detection of RNA concentration values.

Quantitative Real-time PCR Assay (RT-qPCR) protocol

This is the main step in the project that is divided into two phases; the first is done through the synthesis of cDNA from RNA through a specific primer for mexZ, aac6, and housekeeping gene transcripts and protoscript cDNA synthesis kit. Newly designed primer through geneious prime software and the primer specifications have been checked by an oligo analyzer tool. The procedure has performed through steps:

Aliquot of 08 µl from each extracted total RNA sample added into the new PCR tube.

ProtoScript reaction mix that contains dNTPs, buffer and other essential components added as 10µl for each sample.

MuLV Enzyme then added into reaction as 2µl per sample.

Add aliquot of 2 µl hexamer.

This mixture was incubated for 1 hour by using the thermocycler, as in Table (1).

Condition	Step 1	Step 2	Step 3	Step4
Temperature	15 ºC	50˚C	60 ºC	4 ºC
Time	20 sec	4 min	20 sec	------

Table1: *Thermal cycler steps of conditions cDNA Reverse Transcription*

It is repeated 12 times, and followed by 95 ºC for inactivation the enzyme (Relative quantitative PCR). The second section of

this protocol has been done by choosing the cDNA samples with the sub MIC concentration of AgNps Aloe Vera plant excrete and control at the same run. There are four PCR tubes for each sample, the first tube before treatment as control, the second tube after treatment with AgNps Aloe Vera plant excrete, and two housekeeping to each one. The detection of quantity based on fluorescent power of SyberGreen. The reaction mix was composed of the component with their quantity as mentioned in Table (2) below: -

Component	Volume per 20µl Reaction
qPCR Master Mix, (SYBR)	10 µl
Forward Primer	1 µl
Reverse Primer	1 µl
cDNA Template	5 µl
Nuclease-Free Water	to 20 µl

Table2: *Components of quantitative real-time PCR used in aac6 and mexZ genes expression experiment*.

Quickly spin for PCR tubes to remove the bubbles and collect the liquid (1 minute at 2000 rpm, then the program for Real-Time PCR was set up with indicated thermocycling protocol as shown in Table (3).

Loop's steps	Temperature	Time	Number of cycles
Initial denaturation	95 ºC	3min	1
denaturation	95 ºC	20 sec	40
annealing	60 ºC	20 sec	
extension	72 ºC	20 sec	
Melt Curve	55-95ºC	.....	1

Table 3: RT-PCR Cycling Program

Delta delta Ct method (Schmittgen and Livak, 2008):

The ΔCt values of the target gene and the reference gene are directly compared using the delta delta Ct (ΔΔCt) approach, which is the method considered to be the most straightforward. The selection of a calibrator sample is required in order to do relative quantification. The untreated sample, the optimal temperature of 37 degrees Celsius, or any other sample that the user wants to use to compare the unknown samples can serve as the calibrator sample. First, the difference in Ct between the target gene and the reference gene in each sample (both the unknown samples and the calibrator sample) is computed using the equation indicated below. This includes both the unknown samples and the calibrator sample.:
ΔCt = Ct target gene - Ct reference gene
Then, the difference between the ΔCt of the unknown and the ΔCt of the calibrator is calculated, giving the ΔΔCt value,
ΔΔCt = (Ct target - Ct reference) sample - (Ct target- Ct reference) Calibrator
The normalized target amount in the sample is then equal to 2−ΔΔCt and this value can be used to compare expression levels in samples (Schmittgen and Livak, 2008). The relative changes in mRNA expression levels were determined by using a comparative threshold cycle (CT) method (2−ΔΔCt).

Result and discussion

Quantitative Real Time-PCR

RNA extraction and gene expression analysis *mexz* and *aac6* genes

RNA was extracted from the *pseudomonas aeruginosa* isolates (P1, P5, P9, P17, P18, and P29) before treating as a control and after treating with the AgNPs *Aloe vera* plant Extract. Total RNA was extracted using TRIzol™ Reagent and its concentration was from 80 to 210 ng/µL, the purity of total RNA samples ranged from (1.6 to1.9).
Reverse transcription-quantitative PCR (RT-qPCR) differs from previous approaches for evaluating gene expression due to its accuracy, sensitivity, and rapid results. This method is now widely used in gene expression analysis. It is critical to understand that in a relative quantification study, experiments are usually required to compare the level of gene expression between different samples and to check for the presence of specific virulence genes in pathogenic isolates (Priyanka 2013). *mexZ* and *aac6* genes which are carried by the six *P. aeruginosa* isolates (P1, P5, P9, P17, and P18) that were used in the investigation of gene expression. These isolates were chosen because they exhibited the highest level of resistance. The influence of the AgNPs

Aloe vera leaves extract on the Gene Expression of the *mexZ* and *aac6* Genes in *P. aeruginosa* Isolates was the primary focus of this research. Primers designed to target the mexZ and aac6 genes in *P. aeruginosa* have a high degree of specificity for gene expression. *P. aeruginosa* isolates had their gene expression of the aac6 and mexZ genes measured using real-time PCR before and after being treated with the AgNPs *Aloe vera* plant extract. Reverse transcription-quantitative PCR (RT-qPCR) was carried out using SYBR green; a fluorescent dye that recognizes any double-stranded DNA including cDNA. Through a two-step RT-qPCR method in order to determine the effect of sub MICs of AgNPs *Aloe vera* leaves on the expression of *mexZ* and *aac6* gene in *P. aeruginosa*. The first step is Synthesis cDNA from RNA by used specific primer (hexamer) for the target gene and housekeeping gene in the study, the immediately transformed of genomic RNA to cDNA after extraction accomplished to get a complete transformation without any loss or degradation of RNA in one steps of cDNA synthesis. The second step is using the quantitative PCR (RT-qPCR) device to measure the range of Ct value of this gene under study.
In order to determine how much the mexZ and aac6 genes are expressed, their levels are compared to those of the housekeeping gene. The experiment was carried out on each sample a total of four times, and the quantity of given gene expression for each sample was determined by taking the average of those four measurements. The Ct was calculated by deducting the Ct of the sample from the Ct of the reference gene. This was done in order to determine the level of gene expression. Comparative Ct value is also known as the fold change = 2-Ct method of (Schmittgen and Livak 2008), which is a methodology dubbed a golden equation approach to compare the gene expression in various samples. This method is based on data of relative gene expression. In both the treated and the untreated cases, each sample was correlated to a control gene. This was done to guarantee that the observed differences could be attributed to shifts in the expression of the target gene. The results are presented in Table 4 and Table 5. Using the livak equation 2-ΔΔct which is a suitable method to investigate the comparative variations in gene expression from real-time quantitative PCR experiments (Schmittgen and Livak 2008). Ct value was determined as the point (cycle) at which the amplification plot crossed the threshold line.

Sample	CT Treatment	CT Untreated	ΔCTE	ΔCTC	ΔΔCt	Expression Fold Change2^-ΔΔCt
C	0	0	0	0	0	1.00 ±0.00
1	22.51	22.62	2.84	2.45	0.39	0.763129604 ±0.20
5	24.48	22.32	3.81	2.31	1.5	0.353553391 ±0.12
9	25.45	24.24	3.38	1.68	1.7	0.307786103 ±0.08
P-value	--	--	--	--	--	0.1063 NS
NS: Non-significant						

Table 0: *Fold change of aac6 gene in p. aeruginosa isolates before and after the treatment with the AgNPs aloe vera plant extract*

Sample	CT Treatment	CT Untreated	ΔCTE	ΔCTC	ΔΔCt	Expression Fold Change2^-ΔΔCt
C	0	0	0	0	0	1.00 ±0.00
17	25.35	23.65	3.42	0.38	3.04	0.121581868 ±0.08
18	23.75	19.01	0.86	-2.11	2.97	0.127626516 ±0.08
29	24.67	22.6	3.83	3.09	0.74	0.598739352 ±0.17
P-value	--	--	--	--	--	0.0729 NS
NS:Non-Significant						

Table 1:*Fold change of mexz gene in p. aeruginosa isolates before and after the treatment with the AgNPs aloe vera plant extract*

The result of real-time quantitative PCR experiments for gene aac6 was inhibited in the gene expression after treatment with AgNps *Aloe vera* leaves extract as shown in Figure 1

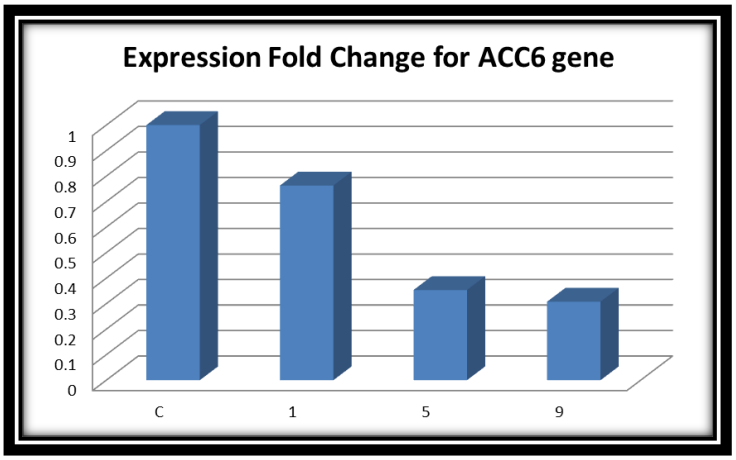

Figure 1: *Expression fold change for aac6 gene*

The result of real-time quantitative PCR experiments for gene *mexZ* was inhibited in the gene expression after treatment with AgNps *Aloe vera* leaves extract as shown in Figure 2.

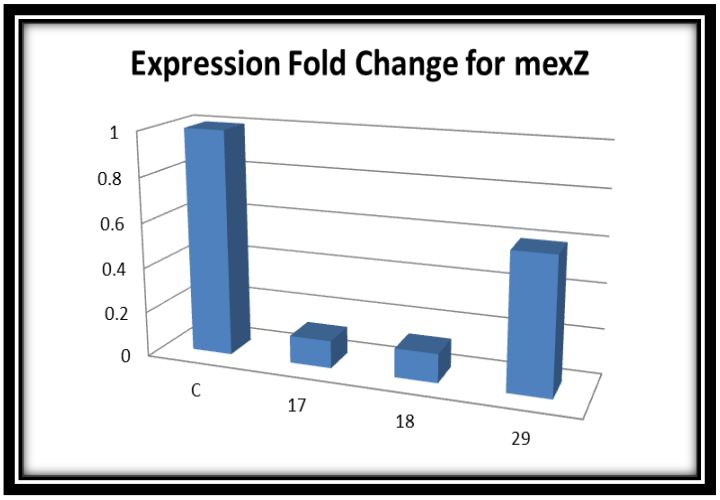

Figure 2: *Expression fold change for mexz gene*

To reveal the inhibition efficacy of AgNps *Aloe vera* leaves extract on the *P.aeruginosa* gene, *mexZ* and *aac6* expression were measured. The results when the isolates were treated with *AgNps Aloe vera* plant extract with sub-MIC (10 mg/mL) for each sample were presented in Table 4 and Table 5 respectively. It was found that the presence of AgNps *Aloe vera* leaves extract negatively affects in *mexZ* and aac6 gene expression. Decreased expression in mexZ and aac6 genes causes to reduce of the efflux pump expression and production of enzyme modify which is one of the causes of antibiotic resistance. Decreased *mexZ* and *aac6* gene expression is an important indicator that it influences an important mechanism of resistance in *P. aeruginosa.* The main purpose of studying gene expression in the presence and absence of AgNps *Aloe vera* extract is to investigate the role of these genes in efflux pump formation and enzyme modification production. It is considered one of the most important virulence genes that cause resistance to the aminoglycoside group in *P. aeruginosa.* Generally, numerous studies have shown that *Aloe vera* helps wound healing because of its anti-inflammatory, antioxidant, and immuneomodulatory activities.
The study of (Liang *et al.* 2021) demonstrated the effect that an extract of the *Aloe vera* plant had on the expression of genes in the host cells by showing an increase in the rate of gene expression for vascular endothelial growth factor (VEGF) and its receptor at the wound site. During the process of wound healing, VEGF is one of the growth factors that regulate cell signaling and the activity of the extracellular matrix. Compared with this study, which showed the effect of *Aloe vera* plant extract on host cells, specifically on increasing VEGF gene expression, this indicates that the effect of AgNps *Aloe vera* plant extract on bacteria cells, especially on the level of gene expression, is very relevant when applied in the treatment of wounds and burns. It is one of the dozens of healing properties of the *Aloe vera* plant like anti-cancer, anti-oxidation, anti-bacteria, lipid-lowering, anti-hypertensive, anti-diabetic, anti-inflammation, liver protection, immune regulation, anti-ulcer, anti-viral, and so on. In the treatment of burns, wounds, tumors, ulcers, constipation, dental issues, diabetes, metabolic syndrome, acquired immune deficiency syndrome, herpes, and psoriasis, it is also one of the medicinal herbs that is employed the most frequently. Additionally, it finds application in the cosmetics & skin care products industries.

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