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Quenching of virulence factors and expression of LasI and RhlR genes of quorum sensing by natural compounds in Pseudomonas aeruginosa isolates

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***Abstract*---**Salicylic acid (SA) and cinnamaldehyde (CA) have the ability to kill microorganisms, but at certain concentrations, they can reduce virulence factors without affecting bacterial growth. This study aimed to know activity of SA and CA in its sub-inhibitory levels on virulence factors and on expiration of QS genes LasI and RhlR. Minimum inhibitory concentration (MIC) of CA and SA were determined. The sub-MIC of SA and CA were utilized to examine their inhibitory effect on biofilm formation, Pyocyanin, and gene expression levels of LasI and RhlR. the result showed both compounds had an inhibitory effect on biofilm formation, Pyocyanin with significant differences. The mean (±SD) of Biofilm formation was decreased from 0.208 (±0.156) to 0.119 (±0.097) with SA and to 0.098 (±0.079) with CA, while the pyocyanin was reduced from 4.76 (±2.96) to 4.28 (±2.27) with SA and to 1.59 (±1.21) with CA. the expression levels of LasI and RhlR decreased; the mean of RhlR cycle threshold (CT) were increased from 19.8 to 21.5, and 22.28. while (CT) of LasI was increased from 20.7 to 21.16and 21.8 with SA, CA respectively. These impressive results breathe hope in the fight against resistant P. aeruginosa by suppressing its QS-regulated virulence factors.

***Keywords*---**biofilm, cinnamaldehyde, pyocyanin, quorum sensing, salicylic acid.

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative opportunistic bacterium that contributes significantly to health care associated infections. The CDC has reported it as a major cause of pneumonia, the third cause of urinary tract infection, the eighth microbe usually isolated from infections of the blood circulation (Flockton *et al.*,2019) .In addition, it is responsible for deadly infections in patients with cystic fibrosis and immune compromised (Diggle & Whiteley,2021). The pathogenesis *P. aeruginosa* dependent on a number of virulence factors that help to enable for host colonization .Among the virulence factors are: biofilm, pyocyanin, elastase, proteas, and rhamnolipid (Rossolini & Mantengoli,2005). Biofilm formation is a dynamic process that enables free-living bacteria to protect themselves from and become resistant to medications, as well as to host immunological responses (Shah *et al.*,2019). Furthermore, Pyocyanin synthesis is another virulence factor that directly causes oxidative stress. proportionate to the disease's severity (Winsor *et a*l.,2016).

The production of these virulence factors is mostly related to quorum sensing (QS), is partially attributed to the capability. Whole bacterial populations of this bacterium in order to coordinate their activity with cells to cells Communication. Over 10% of the *P. aeruginosa* genome is regulated by QS, including biofilm formation, antimicrobial resistance, and the generation of virulence determinants such elastases, pyocyanin, cyanide, and exotoxins (Talon,2012). *P. aeruginosa* uses two dominant QS systems: las (*LasR*-*LasI*) and *rhl* (*RhlR*-*RhlI*). *LasI* synthase catalyzes the synthesis of N-(3-oxododecanoyl) homoserine lactone, *RhlI* catalyzes the synthesis of N-butyryl-homoserine lactone, which induces their respective transcription regulators. *LasR* and *RhlR* are responsible for the activation of a number of QS-controlled genes (Slonczewski & Foster.,2013). There are several natural compounds that are used to inhibit quorum sensing. Plants contain chemical compounds that act as antibacterial and have recently been shown to inhibit bacterial quorum sensing, such as salicylic acid (SA), cinnamaldehyde (CA). These natural compounds can inhibit the gene expression of regulated genes. QS and involved in bacterial virulence, which leads to reduced biofilm formation, pigment production, and proteases

Materials and Methods

Isolation and Identification of P. aeruginosa

This study included the collection of 102 samples, from Fallujah and Ramadi Teaching Hospital in Anbar Provinces /Iraq. from September to December 2021. The Sample sources were burns, wounds, urinary tract infection and sputum. and transferred to the laboratory of the Department of Biology/ college of the Science / University of Anbar.to diagnosing microscopically and biochemically.

Antimicrobial susceptibility testing

According to Clinical and Laboratory Standards Institute guidelines, antimicrobial susceptibility testing was done for the *P. aeruginosa* clinical isolates using the disk diffusion method (CLSI,2020).The clinical isolates were tested for their

susceptibility to the following antibiotics (Mast Group/England): Amoxicillin (30 µg), cefepime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), Cefotaxime (30 µg), gentamicin (10 µg), Imipenem (10 µg), amikacin (30 µg), and meropenem (10 µg).the diameter of inhibition zones was measured and compared with special tables according to CLSI, 2020.

CA and SA stock solution preparing and Minimum inhibitory concentration determination

The salicylic acid and cinnamaldehyde stock solution from Sigma-Aldrich prepared by dissolving CA in DMSO (1:1) to become 1000 µg/ml. while SA used 10 mg to become 1000 µg/ml. The minimum inhibitory concentration (MIC) in test CA and SA against *P. aeruginosa* was determined using resazurin microtiter plate assay in aseptic conditions. Plates are being prepared, and all the wells of microtiter plates were filled with 100 µl of BHI Broth, then 100 µl of CA and SA stock solution were added each separately to the first row, and 100 µl was transferred from the first to the next well, resulting in serial concentrations. 10 µl from overnight of *P. aeruginosa* cultures after the suspensions were adjusted to McFarland standard turbidity added to the wells. The plates were incubator at 37° C for 24 h. The MIC was determined as the concentration at which there was no change in color following 2 h incubation of the overnight growth with 0.015% resazurin. One dilution below the MIC was regarded as the sub-MIC concentration and was used to evaluate the ability of the CA and SA to inhibit the virulence activity.

The phenotypic inhibitory activity of CA and SA on these virulence factors estimation

Biofilm assays

Overnight of *P. aeruginosa* 0.1 at OD600 (20 µl) was transferred to all wells in a microtiter plate containing 180 µl of BHI broth, three replicates were made for each sample. With the addition of 200 µl of BHI broth only in three wells used as a negative control. plate covered and incubation was at 37C for 24 h. the planktonic cells were removed by washing three time with PBS, then leave to dry at room temperature for 15 min. The adherent cells were fixed by adding 200 µl methanol to each hole and left for 20 min, after which the methanol was removed and left to dry. stained with 0.1% of crystal violet for 15 min. Then the dye was carefully removed by PBS and left to dry. 200 µl 96% ethanol was added and the plates were read at 600nm using ELISA reader. To determine the inhibitory effect of CA and SA on biofilm formation was performed using the previously described method with the addition of sub-MICs of CA and SA. The inhibition of biofilm formation was calculated by the equation below. Inhibition rate: % inhibition = $(OD_{control}-OD_{test} / OD_{control})$ *100% (Rajkumari *et al.*,2018).

Pyocyanin assay

An overnight culture was used to inoculate in BHI medium and incubated for 24 h under continuous shaking at 37 °C. The supernatant was collected by by centrifugation at 10,000 rpm, and filter sterilized. the pyocyanin concentration

estimated in absence and presence the inhibitors by transferring 5 ml of the prepared filtered supernatant to 3 ml of chloroform for separate the pyocyanin pigment from the medium. and vortexed for the color changed to greenish blue. than the samples were centrifuged (10,000×g for 10 min). and 3 ml of the resulting blue colored liquid in the chloroform layer was transferred to a new tube and Added 1ml of 0.2M HCl to the chloroform layer and vortex for30 seconds. The blue chloroform part should become clear and the small aqueous part from the added acid to turn pink or deep red. Then,2ml of the result carefully transferred to cuvette and the absorbance measured at 520 nm, the experiment was repeated three times, and the concentration was calculated in µg/ml by (10): Concentration of pyocyanin (µg/ml) = OD_{520} x factor (17.72) (Debritto *et al.,*2020).

Gene expression analysis

The most virulent *P. aeruginosa* clinical isolates were cultivated in Luria Bertani medium supplemented with sub- MICs of CA and SA until (24 h); the stationary growth phase (OD 600; 0.5 McFarland). The total RNA was isolated by using TRIzol™ Reagent according to the protocol described by the manufacturer's instructions. The WizScript ™ RT FDmix Kit (Wizbio solution/Korea), was used to convert total RNA to complementary DNA (cDNA) According to the manufacturer's instructions, Quantitative Real-Time PCR was used to estimate the effect of CA and SA on expression of QS genes *LasI* and *RhlR* in treated and untreated cultures, The reaction mixture was prepared as in the manufacturer's instructions(Wiz Pure ™ qPCR Master Mix (SYPER)). Then the thermal cycling was programmed as follows; cDNA synthesis at 50 ℃ for 15 min 1 cycle, Thermo-Start activation at 95 ℃ for 60 min 1 cycle, 40 cycles of denaturation at 95 ℃ for 20 sec, annealing at 50℃–60 ℃ for 20 sec, and extension at 72 ℃ for 20 sec. The reaction volume was set to 25 µL, then loaded into the thermal cycler then the reverse transcription run started. Melt curve has been performed to confirm the specificity of the reaction. The quantified gene expressions were normalized to an expression of the housekeeping gene recA because there is no difference in the expression levels of this gene between treated and untreated cultures. The gene expression level of treated *P. aeruginosa* has been calculated relative to that in the untreated *P. aeruginosa* using 2−ΔΔCt method (Livak & Schmittgen,2001).

Table 1
primers sequencing

NO.		Primer Name	Size (bp)	Reference
LasI	F	CTACAGCCTGCAGAACGACA	168	(Luo *et al.*,2016)
	R	ATCTGGGTCTTGGCATTGAC		
RhlR	F	AGTTGCTGACCCAGAAGCTG	181	(Malgaonkar,2019)
	R	TGGATGTTCTTGTGGTGGAA		
recA	F	GCGGTGAAAGAAGGTGATGA	120	(Bai *et al.*,2020)

Statistical analysis

the data analyzed according to the simple experiment system applied using the Complete Randomized Design (CRD) with three replications, and using the statistical program GenStat-Tenth Edition,version-10.3.0.0 as the significant

differences of the mean were tested using the LSD (least significant difference test), P-values < 0.05 were statistically significant.

Results and Discussions

The results included 50 isolates were *P. aeruginosa*, after being diagnosed microscopically and biochemically. More *P. aeruginosa* isolates was found in wound (68%) ratio, while in burn, sputum, otitis media and UTI were (53%), (52%), (49%), and (36%). respectively. The susceptibility test showed an isolate highly resistance against Amoxicillin (100%), Amikacin (98%), and moderate resistance to Cefotaxime, gentamycin (86%), meropenem (30%)., Cefotaxime (26%). and low resistance towards Imipenem (10%) and cefepime (10%), Ceftazidime (12%), Ciprofloxacin (8%). The Sub-MIC of CA and SA were 0.25 µg/ml and 250 µg/ml respectively.

The biofilm was tested using ELISA reader, and the results indicated that most of the isolates were biofilm-producing, as 98% (48/50) were biofilm-producing in different range between weak, Moderate and strong after comparing the readings with negative control. The Sub-MIC of CA and SA reduced the biofilm formation, and the mean (±SD) of Biofilm formation was decreased from 0.208 (±0.156) to 0.119 (±0.097) with SA and to 0.098 (±0.079) with CA.the mean of Biofilm inhibition rate was 53% and 44% of the biofilm were prevented by CA and SA, respectively, as figure 1. The results of showed that many of the isolates were able to produce pyocyanin with different concentrations. The highest concentration of pyocyanin was 8.22 µg/ml, while the lowest concentration was 0.99 µg/ml. during The Sub-MIC of SA and CA there was a decrease in the pigment formation. the mean (±SD) of pyocyanin was reduced from 4.76 (±2.96) to 4.28 (±2.27) with SA and to 1.59 (±1.21) with CA. the mean of inhibition of pyocyanin was 10% with SA, and 66.4% with CA, Compared to control, figure2. This compounds also inhibited virulence activity on the genotypic level. At the sub-MIC concentration of SA and CA, the mean of *RhlR* cycle threshold (CT) were increased from 19.8 to 21.5, and 22.28 respectively. and the mean CT of LasI was increased from 20.7 to 21.16and 21.8 respectively. The mean of fold decrease of LasI, expression was from 1.0 to 0.6 and 0.3, with SA and CA respectively. And *RhlR* expression was 1.5 and 0.6 respectively.

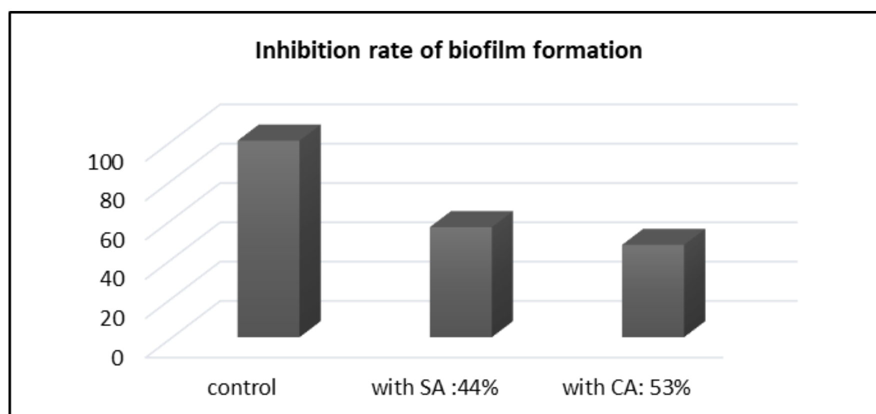

Figure 1. effect the natural compounds on biofilm formation in *P. aeruginosa*

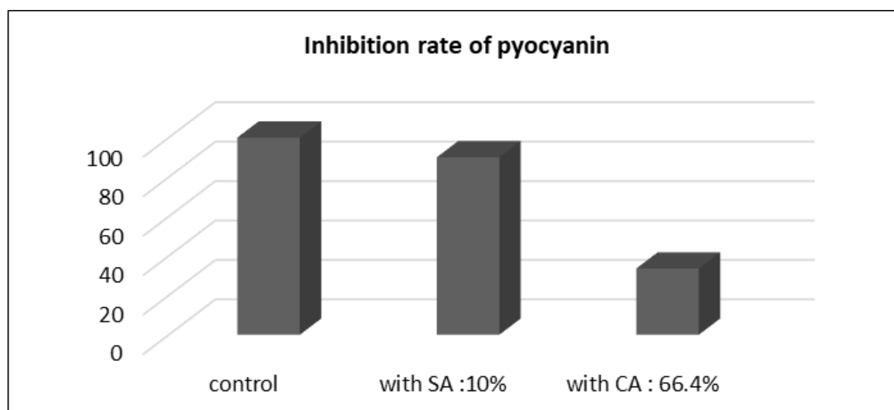

Figure 2. effect the natural compounds on Pyocyanin production in *P. aeruginosa*

P. aeruginosa is an opportunistic bacteria that can cause a variety of infections**.**This is especially true in immunocompromised patients (Hwang &Yoon,2019).The QS network regulates bacterial virulence**.** Capability to form biofilms It includes transcriptional regulators as Las and Rhl, which are activated by their Natural autoinducers (Grabski & Tiratsuyan,2018). QS-deficient isolates ceased to amplify *lasR* and *rhlR* genes so it is still capable of causing infections by other methods. Isolates having *lasR* and *rhlR* are a crucial regulator of virulence factors and biofilms in *P. aeruginosa* while the *lasR* gene positively controls biofilm formation, proteolytic activity, pyocyanin production and rhamnolipid biosurfactant synthesis. The QS regulatory *RhlR* gene did affect protease and rhamnolipid creation positively (Gomes *et al.*,2011).

Las system plays an important role in early stages of biofilm formation, the mutation in the las synthase gene resulted in deficient, uniform, thin and undifferentiated biofilms. The rhl system share capital biofilm maturation through deposition of rhamnolipid(Zhong *et al.*,2020).The *rhl* system regulates protease and elastase when pyocyanin and rhamnolipid are present. C12-HSL/*lasR* controls the production of virulence factors such as elastase (*lasB*), toxA, and the *lasA* protease(Hegazy *et al.*,2020). The treatment of *Pseudomonas* isolates with natural inhibitors of CA and SA led to the suppression of the gene expression of the genes of the quorum system *lasI* and *rhlR*, with significant differences at a probability of 0.05, compared to the housekeeping gene recA, which acts as an internal control and is not affected by the treated compounds. Those results were compatible with (Yang *et al.*,2009),as the SA suppression the gene expression of QS genes. the compound CA was more inhibiting of those genes than the SA. which effectively inhibited both the regulatory proteins (*RhlR*) and the AHL synthases (*LasI*). Both CA and SA reduced the production of virulence factors at the phenotypic and genetic levels in different proportions. The results showed that compound CA had the most effect on inhibiting the formation of biofilm and pyocyanin, without affecting bacterial growth.

Conclusion

The CA and SA have activity to reduce the virulence factors significantly at both genotypic and phenotypic levels and CA was more efficiency than SA. These impressive results could breathe hope in the fight against resistant *P. aeruginosa* by suppressing its QS-regulated virulence factors.

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