



## Effect of salicylic acid on biofilm formation and on some virulence factors in *Pseudomonas aeruginosa*

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### Abstract

*Pseudomonas aeruginosa* is an opportunistic pathogen resisting to several antibiotics. The resistance has been associated with bacterial biofilm formation favored by the presence of exopolysaccharides (EPS) and the ability to express many virulence factors. In our study, we investigated the use of salicylic acid (SA) to affect biofilm formation by use of crystal violet staining method (CVSM), swarming motility, pyocyanin and rhamnolipids production by *P. aeruginosa* ATCC 27853 and five clinical isolates of the same species. The results showed that MIC and MBC of SA against the studied bacteria were recorded around 10mM and 20mM respectively and the biofilm formation was highly decreased when incubated with the sub-inhibitory SA concentrations. Where we recorded an inhibition of 62,97% at the concentration of 4mM of SA for the reference strain *P. aeruginosa* ATCC 27853. Pyocyanin production and motility assay showed that sub-inhibitory concentrations of salicylic acid significantly decreased pyocyanin production and swarming motility, with relative swarming motility of 33% for *P. aeruginosa* ATCC 27853 at concentration of 4mM compared to non treated strain. Then the rhamnolipids production detected for only one isolate (P<sub>2</sub>) in the utilized medium PPGAS among the tested isolates was completely inhibited by 2mM or more of SA.

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## Introduction

*Pseudomonas aeruginosa* is an opportunistic bacterium causing nosocomial infections, especially in immune compromised patients. It colonizes lungs, urinary tract, kidneys, and also adheres to catheters, causing inflammation and septic shock in hospital patients (Donalan, 2011). The capacity of *P. aeruginosa* to cause a wide variety of diseases depends partially on its ability to express many virulence factors. The principal factor rendering the pathogen resistant to antibiotics and environmental fluctuations is the formation of biofilms. They are a complex structure composed of microbial cells adhered to a surface and enclosed in a matrix of hydrated extracellular polymeric substances (EPS). Biofilms have significant implications in medical settings, primarily due to their resistance to antimicrobial agents and to host immune system, resulting in chronic infections and medical equipment contaminations (Harmsen *et al.*, 2010).

Bacterial behavior within biofilms is regulated by the phenomenon of quorum sensing (QS) (Heurlier *et al.*, 2006). It's a regulatory mechanism that enables bacteria to make collective decisions with respect to the expression of a specific set of genes (Kalia, 2013). The main components of QS system are the QS signal synthase, the signal receptor and the signal molecule (Williams, 2007). In *P. aeruginosa*, there are two known complete conventional QS system, *las* and *rhl*. The synthase LasI and RhlI produce the homoserine lactones 3OC12-HSL and C4-HSL respectively, which complex with their correspondent transcriptional regulators, LasR and RhlR to modulate the transcription of 5-10% of the entire *P. aeruginosa* genome (Dekimpe and Deziel, 2009). Pesci *et al.* (1999) discovered a third *P. aeruginosa* quinolone signaling system (PQS). PQS belongs to the family of the 4-hydroxy-2-alkylquinolines (HAQ). Two operons are implied in the synthesis of the HAQ: *pqsABCDE* and *phnAB*, (Deziel *et al.*, 2004) the expression from these two operons is controlled positively by the factor transcriptionnel associated virulence PqsR (also called MvfR) (Deziel *et al.*, 2005).

The 4-hydroxy-2-heptylquinoline is transformed into PQS by PqsH of which the expression is controlled by LasR (Deziel *et al.*, 2004; Gallagher *et al.*, 2002). The PQS-PqsR system is controlled positively by LasR and negatively by Rhl. Pyocyanin is one of the main virulence factors in *P. aeruginosa* regulated by quorum sensing and synthesized by several strains of the genes at high cell densities, associated with biofilm formation (Costerton *et al.*, 1999). The Swarming motility is a complex phenomena also regulated by quorum sensing playing an important role in the pathogenicity and biofilm formation of *P. aeruginosa*. It requires flagella, type IV pili, and biosurfactants production (Pamp *et al.*, 2007). Among the best known biosurfactants produced by *P. aeruginosa* are Rhamnolipids. They are surface-active molecules considered as a key in swarming motility and by decreasing the surface tension between the cells and its environments.

Salicylic acid (SA) is the major phenolic acid plant product, a secondary metabolite which occurs in some fruits and vegetables. Besides its well-known function in regulating plant defense, a recent study demonstrated that the non-steroidal anti-inflammatory drug (SA) reduced biofilm formation of *Staphylococcus epidermidis* (Polonio *et al.*, 2001), *Bacillus subtilis* (Rudrappa *et al.*, 2007), *P. aeruginosa* and *Staphylococcus aureus*. It affects motility and reduces other virulence factors in *P. aeruginosa* (Bandara *et al.*, 2006; Da *et al.*, 2010). The SA is also known by interfering with global regulators and the expression of *S. aureus* virulence factors (Riordan *et al.*, 2007).

The main goal in the present work was to study biofilm formation with and without SA and to explore the effects of SA on the growth, swarming motility, pyocyanin and rhamnolipides production in six isolates of *P. aeruginosa*.

## Materials and methods

### Bacterial strains

The tests were carried out using a reference strain *P. aeruginosa* ATCC 27853 and five clinical isolates of *P. aeruginosa* isolated from a health center in Sidi Lakhdar Mostaganem (Algeria).

The isolates were identified by some phenotypic tests and completed by the API 20NE system (bioMérieux Marcy-l'Etoile, France).

#### *Effect of salicylic acid on the growth of P. aeruginosa*

To determine whether the presence of SA had some effects on the bacterial growth in liquid medium, the minimum inhibitory concentration (MIC) was determined using the broth macrodilution method (Moroh *et al.*, 2008). 200  $\mu$ l at  $10^6$  cells/ml of five isolates and one strain of *P. aeruginosa* ATCC 27853 were inoculated with 2 ml trypticase soy broth (TSB) supplemented with 0, 0.5, 1, 2.5, 5, 10, 20 and 40mM SA. After 24 h incubation at 37°C, the Minimal inhibitory concentration (MIC) was calculated as the lowest concentration of SA that inhibited visible growth of the microorganism. The MBC was determined by spreading out 0.1 ml of the tubes contents of concentration  $\geq$  MIC on Mueller-Hinton agar. The MBC is the smallest concentration which permits the survival of 0.01% or more of the bacterial suspension after 24 h incubation.

#### *Effect of salicylic acid on biofilm formation*

The studied strains of *P. aeruginosa* were screened for their ability to form biofilms. The quantitative assays on biofilm formation were performed in glass tubes. 200  $\mu$ l of bacterial suspension (OD  $\approx$  0.08 at 620nm) was inoculated in tubes containing 2 ml of TSB (as a control) and TSB with the (0.5, 1, 2 and 4mM) of SA. Biofilms were allowed to develop under static conditions at 37°C quantified after 24h, by spectroscopic determination at 580 nm after a crystal violet staining (1% w/v) (Djordjevic *et al.*, 2002).

#### *Swarming assay*

Swarming motility was evaluated on modified BM2 glucose plates (62 mM potassium phosphate buffer pH 7, 0.5% casamino acids, 2 mM MgSO<sub>4</sub>, 10  $\mu$ M FeSO<sub>4</sub>, 0.4% (w/v) glucose) containing 0.5% (w/v) agar (Overhage *et al.*, 2008). The SA at 0, 0.5, 1, 2 and 4mM was incorporated into the growth medium (tempered at 45 °C). A sub-inhibitory concentration was used to ensure that the effects on motility inhibition were not due to antimicrobial activity. 3  $\mu$ l aliquots of the log phase cultures at  $10^6$  cells/ml were

inoculated onto the centre of each plate that was subsequently incubated overnight at room temperature. The motility halos were measured after 24hours incubation. Three plates were used to evaluate the motility of each bacterium and three independent experiments were performed.

#### *Effect of salicylic acid on pyocyanin production by P. aeruginosa*

The pyocyanin assay was performed using Luria Bertani broth (LB; tryptone 1%, yeast extract 0.5% and NaCl 1.0%). To determine the effect of SA on pyocyanin production, 200  $\mu$ l of the bacterial suspension (OD  $\approx$  0.08 at 620nm) of *P. aeruginosa* ATCC 27853 and five isolate were inoculated in tubes with 3 ml Luria Bertani broth (LB) supplemented with 0, 0.5, 1, 2 and 4mM of SA. The tubes were incubated for 48 h at 37°C under shaking conditions (180 rpm). Pyocyanin was extracted as described in the literature (Essar *et al.*, 1990). 2.5ml culture was extracted in 1.5 ml of chloroform and transferred to a clean tube. 0.8 ml of 1 N HCl was added and gently shaken to bring the pyocyanin to the pink aqueous phase. The OD at 520nm of the aqueous solution was measured and the pyocyanin concentration (mg/ml) was determined by multiplying this measurement by 17.07 (Drenkard, 2003).

#### *Effect of SA on Rhamnolipids production*

Rhamnolipids production was tested on Proteose Peptone-Glucose-Ammonium Salts (PPGAS) medium: NH<sub>4</sub>Cl : 1 g/L, KCl : 1.5 g/L, TRIS-HCl : 19 g/L, MgSO<sub>4</sub>.7H<sub>2</sub>O : 0.4 g/L, Glucose : 5 g/L, Peptone : 10 g/L. The experiment was conducted in 250ml flasks containing 50ml of PPGAS medium. The flasks were inoculated with 1 ml of the bacterial suspension ( $10^6$ cells/ml) of six isolate of *P. aeruginosa* and incubated under shaking conditions (180 rpm) at 30 °C for 3 and 8 days. To determine the influence of SA on rhamnolipids production, the bacterial suspensions were inoculated with PPGAS medium supplemented with 0, 0.5, 1, 2 and 4mM of SA. The cells suspensions were centrifuged at 8000g for 20 min to prepare the cell-free supernatant (CFS). 300  $\mu$ l of the CFS was extracted 2 times with 600  $\mu$ l of diethyl ether, the 2 extracts are gathered and

evaporated dry to yield rhamnolipids. The rhamnolipids were dissolved in 100  $\mu$ L of distilled water. The concentration of rhamnolipids was determined using the orcinol assay (Wilhelm *et al.*, 2007). By mixing 100  $\mu$ L of diluted solution of rhamnolipids (purified with liquid–liquid extraction) with 100  $\mu$ L of orcinol at 1.6% and 800  $\mu$ L of sulphuric acid at 60%. The mixture was heated at 80°C for 30 minutes, absorbance was measured spectrophotometrically at 421 nm. The concentration of rhamnolipids was calculated according to L-rhamnose standard curve (0 to 50 mg/l) and multiplying the result with a coefficient of 3.4 obtained from the correlation of pure rhamnolipids/rhamnose (Benincasa *et al.*, 2004).

#### Crystal violet analysis

The quantitative analysis of biofilm formation was performed using crystal violet staining of the attached cells, the supernatant in the tubes were aspirated and rinsed 3 times with distilled water and fixed by drying at room temperature until they were fully dried out. The total of 2 ml of 1% crystal violet was added into each tube for 15 min. The excess stain was washed off 3 times with distilled water. The crystal violet that

stained the attached cells was distilled with 2 ml of 95% ethanol at room temperature for 2 minutes. The optical density (OD at 580nm) of 2 ml distilled solution was examined using a spectrophotometer (Djordjevic *et al.*, 2002). The absorbance value is positively correlated to the amount of the bacterial adhesion or biofilm. All tests were performed in three triplicate and the absorbance readings were averaged.

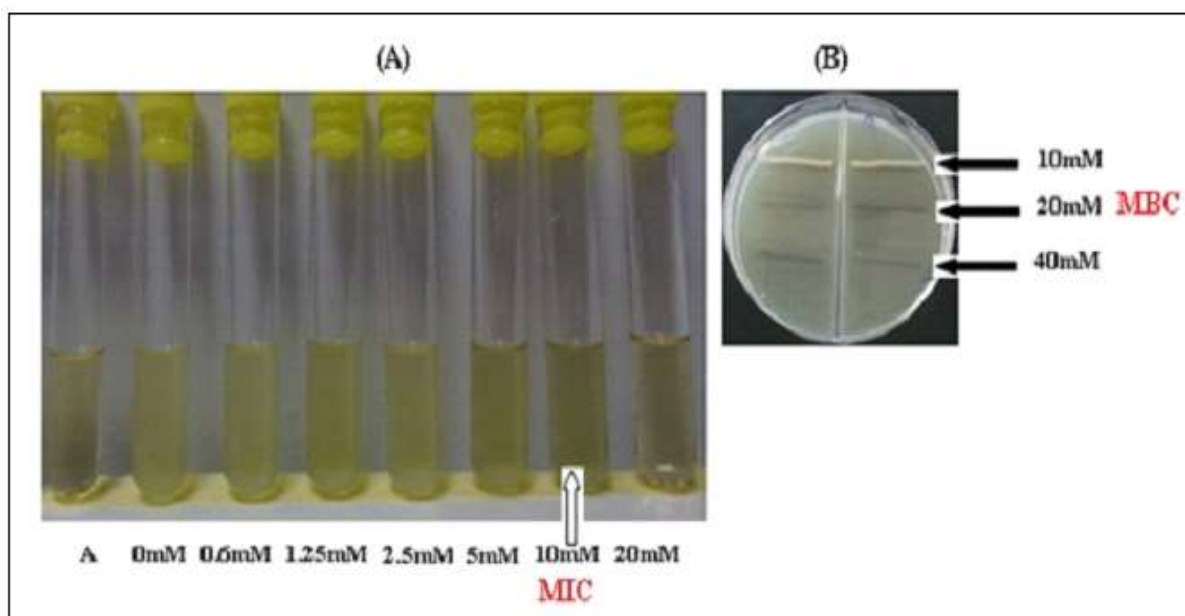
#### Statistical analysis

The experimental results were expressed as mean  $\pm$  standard deviation of three replicates. The data were evaluated by analysis of variance using Statbox 6. The comparison of means was performed using the least significant difference were considered statistically significant if  $P < 0.05$ .

## Results and discussion

#### Minimum inhibitory concentration

The antibacterial activity of SA was examined against five isolates of *P. aeruginosa* and *P. aeruginosa* ATCC27853. MIC assay results indicated that SA concentrations  $\geq 10$  mM inhibited strains growth in all tubes when viewed with naked eye. The MBC of the phenolic acid (SA) was around 20mM (Fig. 1).

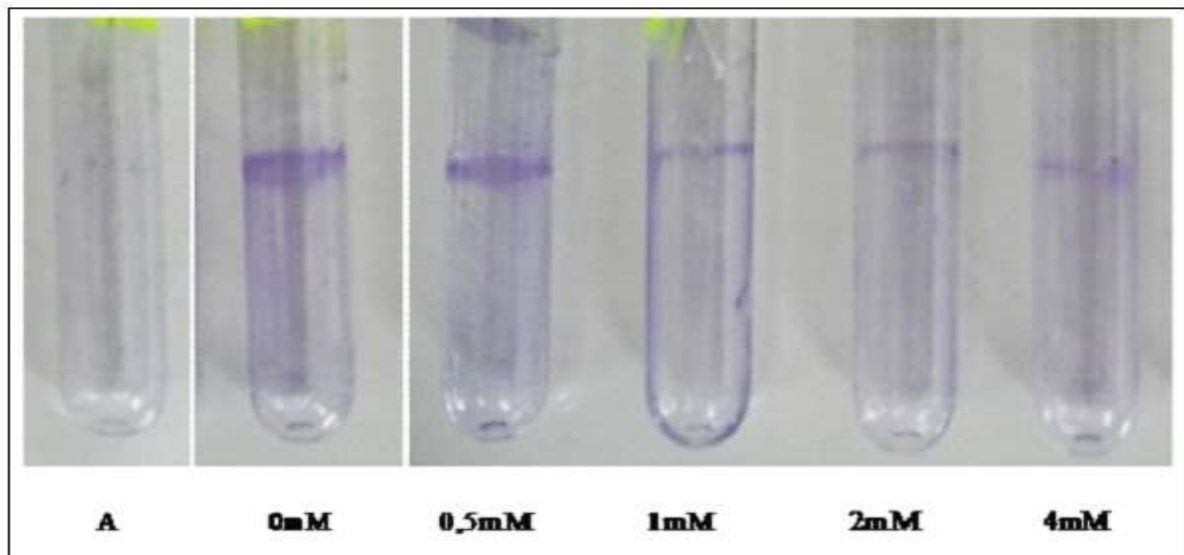


**Fig. 1.** Variation of turbidity due to the growth of *P. aeruginosa* ATCC 27853 according to the concentration of Salicylic Acid (A) and minimal bactericidal concentration (B).

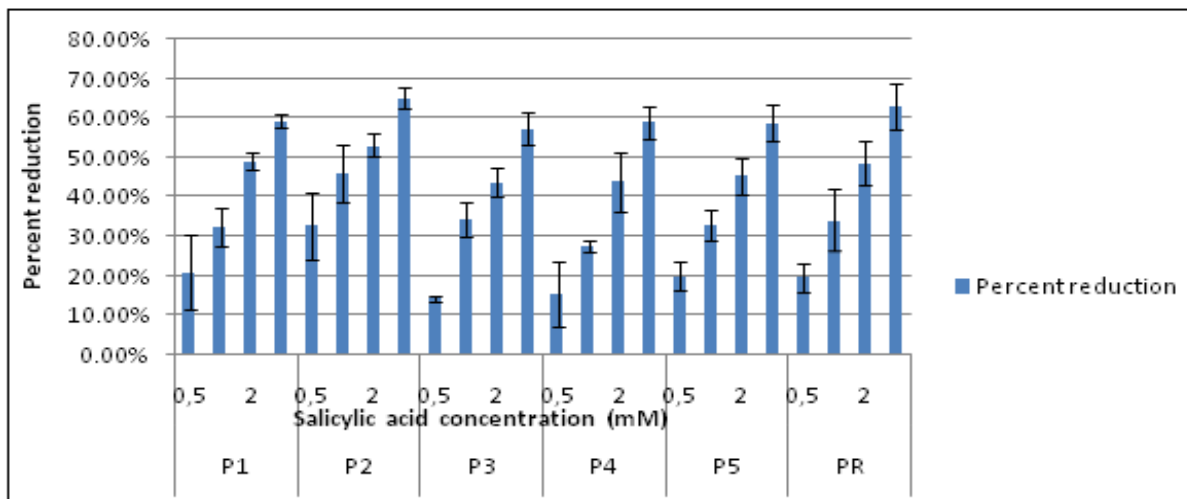
The medicinal properties of salicylic acid have been known since ancient times. There are studies on the inhibition of planktonic cell growth with SA demonstrated significant antimicrobial properties (Delaney *et al.*, 1994; Kus *et al.*, 2002; Prithiviraj *et al.*, 2005; Samuel *et al.*, 2011).

#### Biofilm formation assay

Biofilm development is associated with antibacterial resistance and can contribute to severe infections (Høiby *et al.*, 2010). To test whether the presence of SA affects the biofilm formation on *P. aeruginosa*, we carried out an in vitro experiment in which SA was supplemented into the culture medium with different concentrations (0.5, 1, 2, and 4mM).



**Fig. 2.** Effect of the Salicylic Acid on the biofilm formation by *P. aeruginosa* ATCC 27853, A: negative control, 0mM: positive control.



**Fig. 3.** Reduction of biofilm formation by *P. aeruginosa* ATCC 27853 and five isolates of *P. aeruginosa* in the presence of 0, 0.5, 1, 2 and 4mM of SA.

The obtained result reported that the addition of SA can reduce biofilm formation in *P. aeruginosa* compared to the untreated control (Fig. 2 and 3). A number of studies have found that SA can reduce

biofilm formation in *P. aeruginosa* (Prithiviraj *et al.*, 2005; Mei *et al.*, 2010; Samuel *et al.*, 2011). Films of the salicylic acid-releasing polymers were found to inhibit biofilm formation, as shown by

bioluminescent and GFP in *P. aeruginosa* and *E. coli* strains (Nowatzki *et al.*, 2012). Rosenberg *et al.* (2008) and Nowatzki *et al.* (2012) found that salicylic acid-based and realising polymers significantly reduced biofilm formation by *Salmonella enteric* serovar *typhimurium* and *E. coli*.

One hypothesis that could explain the observed biofilm inhibition is that the SA is somehow affecting quorum sensing (QS) known as cell to cell

communication which is required for the differentiation of the individual cell to the mature biofilm (Davies *et al.*, 1998). Data provided by some studies showed that mutants with lack of QS form biofilms that are more unstructured, and susceptible to chemical agents compared to wild types (Bjarnsholt *et al.*, 2005). *P. aeruginosa* QS is composed of *las*, *rhl* and *pqs* circuits under control of *lasI/R* system (Rasmussen and Givskov, 2006; Jimenez *et al.*, 2012).

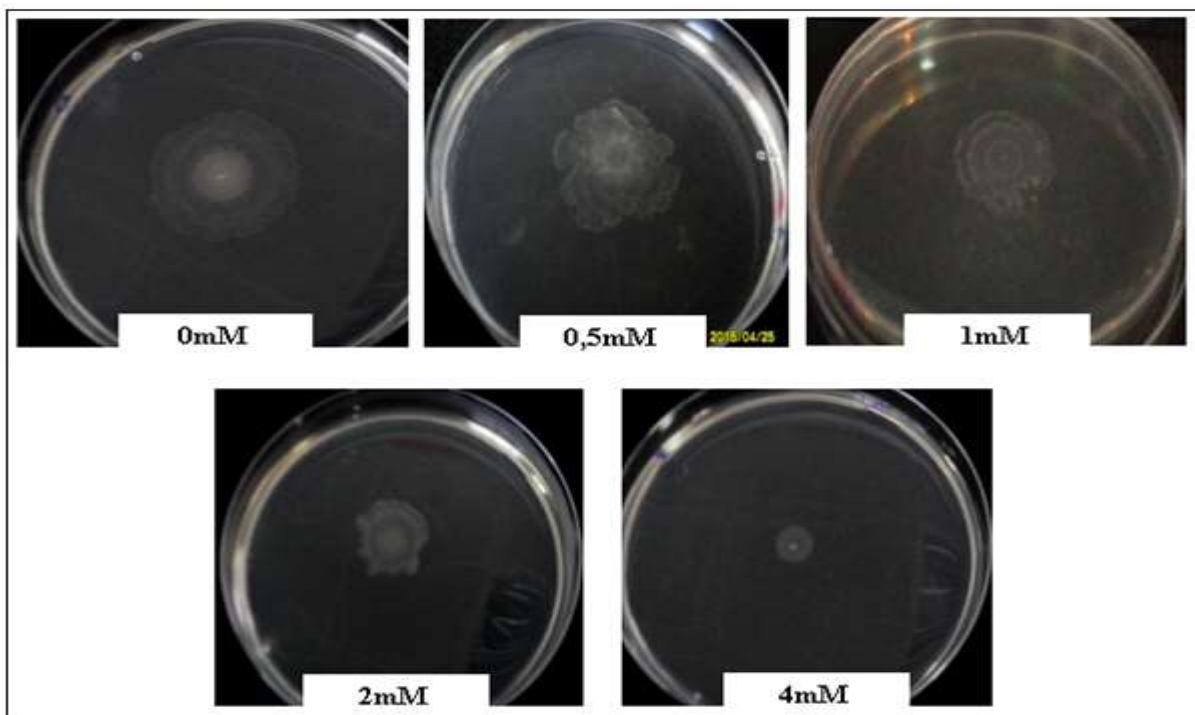


Fig. 4. Effect of SA on swarming motility in *P. aeruginosa* ATCC 27853.

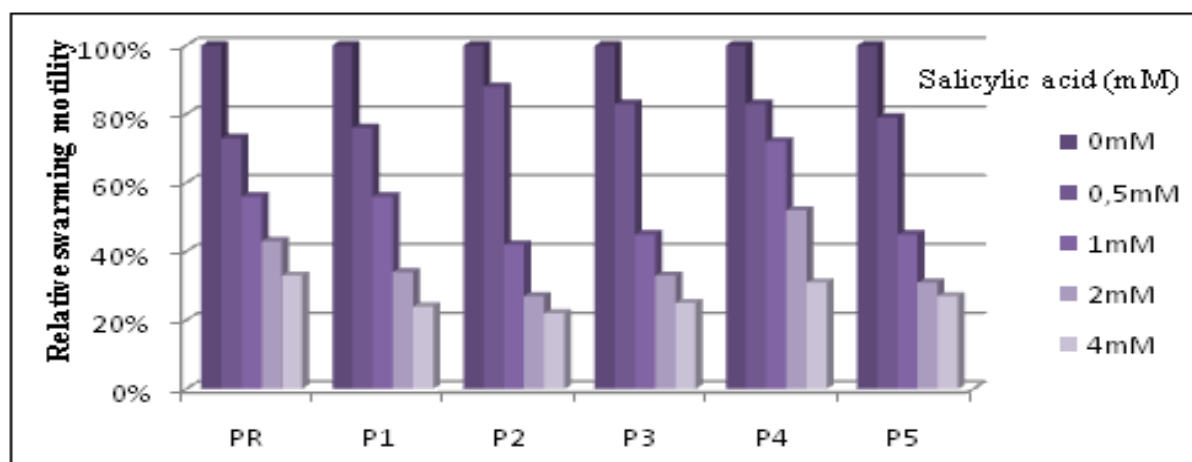


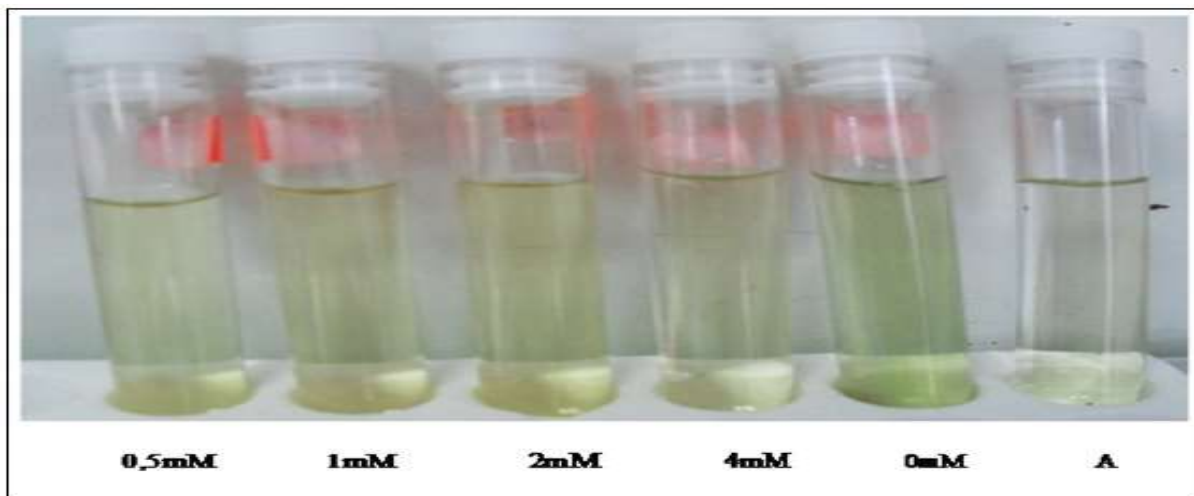
Fig. 5. Quantification of swarming motility by *P. aeruginosa* ATCC 27853 and five isolates of *P. aeruginosa* when exposed to increasing concentrations of salicylic acid.

Interference with QS can affect the biofilm development and make the bacteria more susceptible to anti-microbials (Landini *et al.*, 2010). Yang *et al.* (2009) reported that SA suppressed the expression of genes associated with the las/rhl quorum sensing autoinducer 1 (AI1) homoserine lactone-based signalling system in *P. aeruginosa*.

#### *Effect of SA on swarming motility*

*P. aeruginosa* motility plays a vital role in creation of biofilms in its initial attachment (Arora *et al.*, 2005).

The sub-inhibitor concentrations (0.5, 1, 2 and 4 mM) of SA decreased swarming motility in all studied strains of *P. aeruginosa* (Fig. 4 and 5). Samuel *et al.* (2011) conducted the motility assays on wild-type strains, flagella and type IV pili mutants and found that salicylic acid had significant effects in decreasing swarm, swim and twitch motility for all strains tested. The swarming motility has been shown to be important for the early stages of biofilm formation (Shrout *et al.*, 2006).



**Fig. 6.** Effect of the salicylic acid on the pyocyanin production by *P. aeruginosa*. A: negative control.

It occurs by overcoming the long-range repulsive forces that facilitate close approach to the surfaces, enabling accelerated surface adhesion (Karatan and Watnick, 2009). Houry *et al.* (2010) also reported the importance of motility in *B. cereus* biofilm formation in microtiter plates.

Swarming is a complex motility regulated by quorum sensing that occurs on semi-solid surfaces, and requires flagella, type IV pili and biosurfactant production (Pamp and Tolker-Nielsen, 2007).

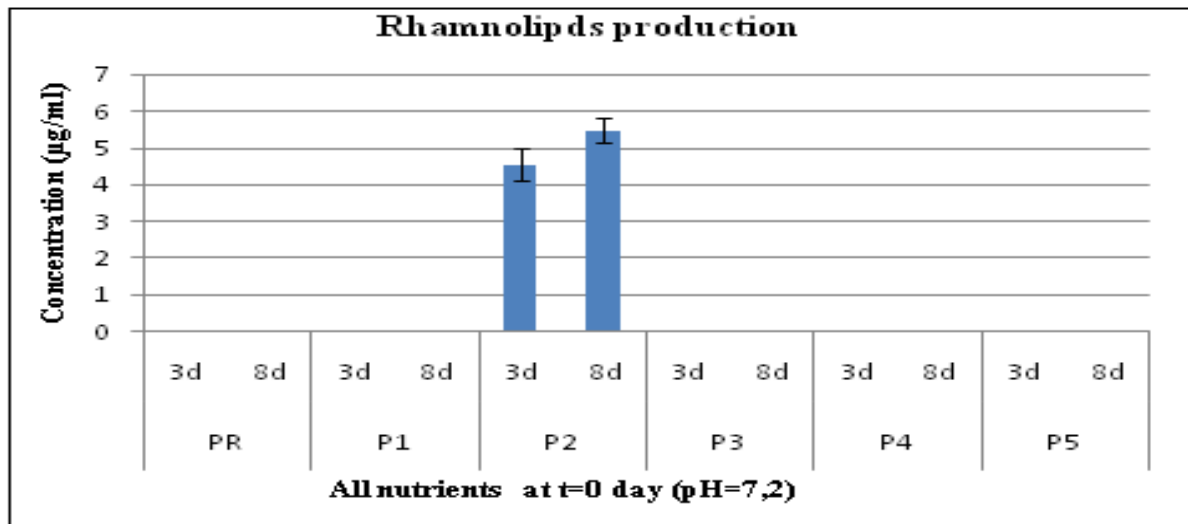
#### *Effect of salicylic acid on pyocyanin production*

Pyocyanin is a blue-green phenazine produced by several strains of *P. aeruginosa* (Dietrich *et al.*, 2006). It is one of the many controlled molecules by the QS, synthesized at high cell densities, and associated with biofilm formation (Costerton *et al.*, 1999). We tested the effect of SA on pyocyanin production by supplementing the growth medium with different concentrations.

Addition of SA to the bacterial growth medium reduced the production of pyocyanin (Fig. 6).

Prithiviraj *et al.* (2005) showed while the addition of 0.1 mM SA reduced pyocyanin production by  $\approx 50\%$ , and 1.0 mM SA resulted in more than an 80% reduction of pyocyanin production with no apparent effect on the growth of the bacteria.

They also evaluated the effect of SA derivatives, including acetyl salicylic acid, salicylamide, and methyl salicylate, and benzoic acid, on pyocyanin production by PA14. All the SA derivatives led to reduce pyocyanin production, and the levels of reduction caused by methyl salicylate, salicylamide, and benzoic acid were close to those caused by SA. Prithiviraj *et al.* (2005) indicated that Salicylic acid down regulates rhlR and lasA in *P. aeruginosa* PA14 and cause subsequent inhibition of pyocyanin, protease, and elastase activities (Yang *et al.*, 2009; Guo *et al.*, 2013).

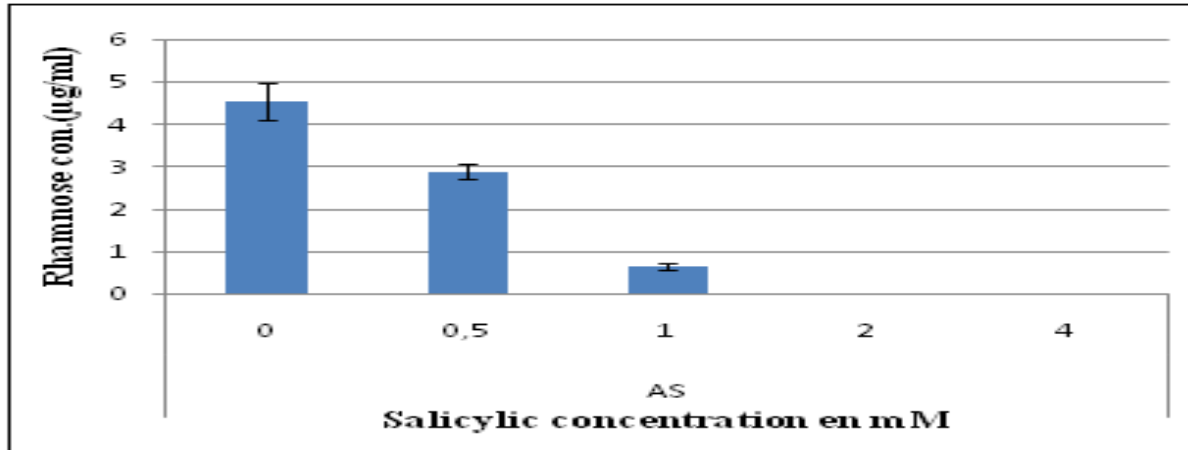


**Fig. 7.** Rhamnolipids production by *P. aeruginosa* ATCC 27853 (PR) and five clinical isolates of *P. aeruginosa*.

#### Rhamnolipids production

Rhamnolipids are biosurfactants produced by *P. aeruginosa* (Soberon-Chavez *et al.*, 2005). They are extracellular amphiphilic glycolipides used by this bacterium like factors of virulence. They support the swarming because they act as wetting agents which reduce the surface stress (Caiazza *et al.*, 2005).

As show in Fig.7, Only one isolates (P2) showed a production of rhamnolipids. They were extracted from the cell free supernatant of the 3-day bacterial culture with yield of 4,5 mg/l. The other isolates did not show rhamnolipids production in the utilized medium PPGAS (Fig. 8).



**Fig. 8.** Effect of salicylic acid on the production of rhamnolipids by *P. aeruginosa* (P<sub>2</sub>).

Nagant *et al.* (2013) indicated that rhamnolipids production was highly related to bacteria species and to culture conditions. As also cited by Juan *et al.* (2001) *P. aeruginosa* UG2 was examined under different culture conditions show that the Rhamnolipid yield was affected by the nature of the carbon sources, the nutrient concentrations, pH, and age of the culture.

Our results indicated when using SA that rhamnolipids production was highly infected and the MIC was at 2mM (Fig. 8). Rhamnolipids are produced during the formation of the biofilm under the control of quorum sensing (QS) system (Jensen *et al.*, 2007). The expression of the genes *rhlB* and *rhlA* coding for a rhamnosyltransférase implicated in the synthesis of the rhamnolipids was decreased in the presence of an antimicrobial peptide (Ochsner *et al.*, 1995).



## Conclusion

Following the obtained results we can conclude that SA inhibits bacterial growth depending on concentrations. The MIC and MBC of SA were recorded around 10mM and 20 mM respectively. We can also retain that SA decreased biofilm formation by the used *Pseudomonas*. The swarming motility and pyocyanin production by the studied *Pseudomonas* were also affected by the SA. The production of rhamnolipids was completely inhibited in (P2) the only one isolate producing them in PPGAS medium.

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