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Relative assessment of anti-quorum sensing in *Piper betle* leaves extract via pyoverdinin assay

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Abstract. Anti-quorum sensing property has been a popular alternative approach over bactericidal/bacteriostatic property in combating bacterial infection while simultaneously tackling the dilemma of antibiotic resistance. We presented pyoverdinin assay as an initial screening to qualitatively determine the anti-quorum sensing activity in *Piper betle* by measuring the loss of absorbance at wavelength 630 nm. Growth of *Pseudomonas aeruginosa* was proven to be unaffected by the presence of the *P. betle* leaves extract. The regression value (R^2) of the quorum sensing (QS) activity in untreated supernatant of *P. aeruginosa* was 0.9636 and we presented the QS activity in fold-change, normalized to untreated sample for a fair comparison between batch of assays. We further assessed the QS activity in the extracts of *P. betle* leaves and found the QS activity of *P. aeruginosa* grown in the presence of ethyl acetate extract at 200 $\mu\text{g/ml}$ was reduced to 0.6-fold. As the concentrations went lower, higher fold of QS activity was observed, suggesting that *P. betle* leaves extract is demonstrating anti-QS activity at a higher concentration. Further fractionate of ethyl acetate crude extracts resulted in three fractionates with high anti-QS activity with >50% reduction in QS activity and five fractionates with intermediate anti-QS activity. The use of pyoverdinin assay to qualitatively portray the anti-QS activity could shorten the lengthiness of extracting and measuring the signaling molecule yet, produces reliable information to screen for anti-QS activity and guide for further fractionation and purification of bioactive compound.

1. Introduction

Bacterial infection continues to be the major cause of morbidity and mortality worldwide despite the extensive control measure via vaccination and anti-infective agents [1]. The problem is aggravated further when these bacterial pathogens of clinical importance acquire resistance to antibiotics. Some of them are armed with quorum sensing system to coordinate their pathogenesis and *Pseudomonas aeruginosa* is one of them [2].

P. aeruginosa is a Gram-negative bacterium and is one of the Enterobacteriaceae that causes serious healthcare-associated infections. It is an opportunistic pathogen that can cause severe infection in the lung, blood, urinary tract and surgical sites [3]. In cystic fibrosis patients, *P. aeruginosa* causes chronic infection of lower respiratory tract [4]. Back then, antibiotic administration was enough to eradicate *P. aeruginosa* infection but in the early 2000, these antibiotics, namely; aminoglycosides,



cephalosporins, fluoroquinolones and carbapenems; were found to no longer able to resolve the infection and the situation worsen when an outbreak of multidrug-resistant of *P. aeruginosa* was reported in 2004/2005 [3].

It is alarming that antibiotic- and multidrug-resistance bacteria are becoming more typical in the clinic, demanding for better approaches, not only in fighting the bacterial infection but also in containing the bacteria from attaining resistance to the agent [5]. Owing to recent technologies, we now have a better understanding of the bacterial virulence strategies and the induced molecular pathways during the infectious processes, thus providing a platform in search for novel bioactive with ability to interfere with the crucial pathogenicity factors or the virulence-associated traits of bacteria to prevent the emergence of resistance against the novel anti-infective agents [6]. Among the popular alternative approach is the identification of bioactive compound that possesses anti-quorum sensing activity. Quorum sensing is a cell-to-cell communication system that depends on the cell density by means of signaling molecule [7]. Once the threshold concentration of signaling molecule is achieved, a cascade of signaling pathway would be activated leading to the production of virulence proteins that are required for infection process to take place in a host [8].

In this study, we presented pyoverdinin assay as an initial screening to qualitatively determine the anti-quorum sensing activity in *P. betle* leaves extracts against *P. aeruginosa*. Consequently, the information gained would be used in assisting the fractionation towards the isolation of bioactive compounds. *P. betle* is a tropical creeper plant belongs to the family Piperaceae, commonly found in the Southeast Asia region. Its leaf has been widely used for therapeutic purposes and has been reported to possess several biological activities including antidiabetic, antimicrobial, antinociceptive, antioxidant, insecticidal and gastroprotective activities [9,10]. In the ayurvedic practice, *P. betle* leaf is commonly applied on wounds and lesions for its healing effects besides its antiseptic properties [11]. Recent study by Datta S. *et al.* [12] revealed the anti-QS activity in *P. betle* crude extract against *P. aeruginosa*. Nonetheless, the constituents in *P. betle* leaves that blocked the quorum sensing activity in *P. aeruginosa* has not been reported yet, leading to this study. Due to its bioactivity against the QS system in *P. aeruginosa*; rather than direct effect on the bacterium cell; compound isolated from *P. betle* would have the potential to be developed as an alternative medicine to antibiotic while tackling the issue of antibiotic resistance.

2. Materials and method

2.1. *Piper betle* leaves extraction

P. betle leaves were bought from local market and were subjected to solid-liquid extraction in solvent of varying polarities, started with n-hexane, followed by ethyl acetate, ethanol and finally with methanol. For liquid-liquid extraction, method from Cantwell and Lousier was followed [13]. Briefly, 100 g of powdered leaves were macerated in ethyl acetate and concentrated using rotary evaporator. A mixture of water and methanol were then added to the concentrated ethyl acetate extract and vigorously shook to mix well. In a separating funnel, n-hexane was mixed well to previous mixture and let separated. Polar layer was then mixed well with ethyl acetate and the ethyl acetate extract was collected, concentrated using rotary evaporator and stored in -20°C until further use. For column chromatography, the column was packed with silica gel with mobile phase of a mixture of isopropyl alcohol and chloroform. Fractions were collected at every 50 ml and subjected to thin layer (TLC) analysis (data not shown). Fractions of same composition were pooled and concentrated and kept in -20°C. Crude extracts and fractions were reconstituted in DMSO for pyoverdinin assay.

2.2. Strain and media

P. aeruginosa strain 14 (PA14) was used throughout this study. PA14 was maintained on King's B agar or broth for overnight culture. King's B agar was prepared according to the following recipe; 20 g peptone, 1.5 g KH₂PO₄, 15 g agar, 15 ml glycerol, 6 ml 1 M MgSO₄ and ddH₂O to 1 L. Chrome Azurol S (CAS) dye reagent was prepared according to Loudon *et al.* [14]. CAS dye was prepared by

dissolving 0.06 g of CAS in 50 ml of ddH₂O. In another container, 0.0027 g of FeCl₃•6H₂O was dissolved in 10 ml of 10 mM HCl. In a third container, 0.073 g of HDTMA was dissolved in 40 ml of ddH₂O. CAS solution was first mixed well with 9 ml of FeCl₃ in HCl before adding the HDTMA solution. CAS dye reagent was autoclaved and kept in 4°C for up to one month.

2.3. Culture condition

For pyoverdin assay [15], cells were grown overnight in King's B broth at 37°C with agitation. Overnight culture was diluted to OD₆₀₀ 0.05 and inoculated to a fresh King's B broth and grew for 12 to 16 hr at 37°C with aeration. To test for anti-QS activity in *P. betle* leaves, the ethyl acetate extract was added to the King's B broth to a series of final concentration at 25, 50, 100 and 200 µg/ml.

2.4. Growth curve and turbidity test

100 µl of PA14 start culture with OD₆₀₀ 0.05 was added to 250 ml of fresh King's B broth in presence/absence of ethyl acetate extract at 200 µg/ml. The OD₆₀₀ of the culture was taken every hour starting from 0 hour until the growth reached stationary phase. For turbidity test [16], 4 µl of the same PA14 start culture was added to each well of the 24-well plate in presence of series of concentration of the extract ranging from 0.25 to 1024.0 µg/ml. Another four wells were served as controls; in one well, PA14 was grown in absence of the extract while in the second well, PA14 was grown in presence of streptomycin at 10 µg/ml. In the third and fourth well, PA14 was not inoculated in the broth to eliminate any false positive result.

2.5. Pyoverdin assay

Method from Alexander & Zuberer [17] was followed. Briefly, supernatant of overnight culture were filtered through a 0.45 µm nylon filter. An equal amount of filtered supernatant was added to CAS dye reagent and the loss of blue colour was determined through the difference ($t_{\text{initial}} - t_{\text{end}}$) of absorbance at 630 nm using a spectrophotometer.

3. Results

3.1. Effect of Piper betle leaves extract on the growth of PA14

Presence of *P. betle* leaves extract in the growth medium of PA14 showed insignificant influenced of the extract on the proliferation the bacterium, as shown in figure 1. Furthermore, the turbidity test revealed no inhibition of PA14 growth at any of the concentration tested, indicating that the growth of PA14 was not affected by the presence of the *P. betle* leaves extract (Table 1).

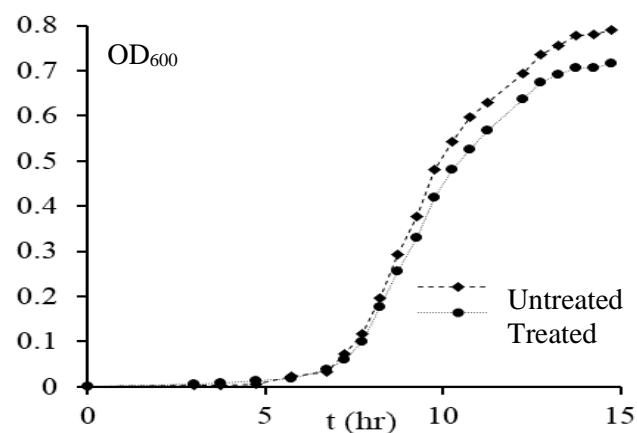


Figure 1. Growth curve of PA14 in absence and presence of extract at 200 µg/ml.

Table 1. PA14 susceptibility to various extract concentration.

	µg/ml	0.25	1.00	4.00	16.00	64.00	256.00	1024.0
PA14 + extract		+	+	+	+	+	+	+
PA14 + broth					+			
PA14 + streptomycin					-			
Broth only					-			
Broth + extract					-			

+ Indicates growth

- Indicates no growth

3.2. Quorum sensing activity in *Pseudomonas aeruginosa*

The QS activity in *P. aeruginosa* was qualitatively assessed based on pyoverdinin activity in scavenging iron from CAS dye [15]. The quorum sensing activity of *P. aeruginosa* grown without treatment for overnight was found to proportionally decrease with an R^2 value of 0.9636; a 19.5% reduction at 0.75-fold dilution, a 29.6% reduction at 2-fold dilution and a 59% reduction at 4-fold dilution. The data was presented in fold-change, normalized to untreated sample to take into account for any slight variation that might have occurred during the process and thus providing fair comparison between batch of assays (Figure 2).

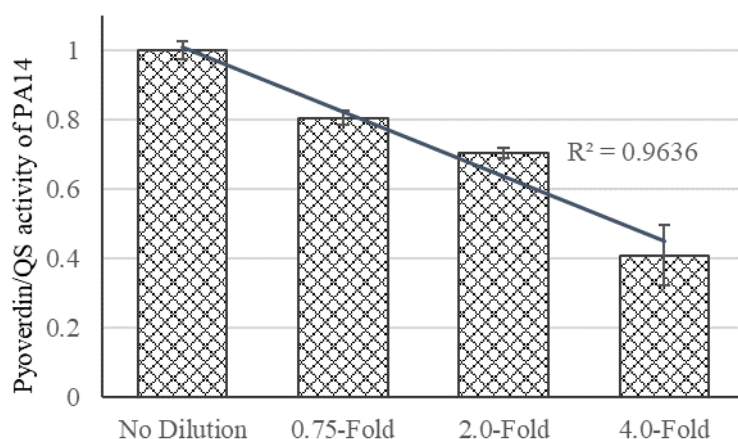


Figure 2. Pyoverdinin activity in fold changes normalized to undiluted sample in depicting the QS activity of PA14

3.3. Anti-quorum sensing activity in *Piper betle* leaves extract

In this study, the anti-QS activity of *P. betle* leaves extracts towards *P. aeruginosa* was determined by the decrease in the QS activity measured in supernatant of *P. aeruginosa* (PA14) grown overnight in presence of *P. betle* leaves extracts [18]. As shown in figure 3A, there was a significant decrease in QS activity compared to that of control; PA14 grown without treatment; when PA14 was grown in the presence of the ethyl acetate crude extract of *P. betle* leaves (40.4% decrease). The presence of methanol crude extract of *P. betle* leaves showed insignificant difference in the activity while presence of ethanol and hexane crude extracts during the growth of PA14 showed significant increase in the QS activity. When PA14 was grown in ethyl acetate extract at varying concentration (Figure 3B), the QS activity was found to significantly reduce to 0.58-fold \pm 0.09 at 200 μ g/ml and to 0.80-fold \pm 0.03 at 100 μ g/ml. As the concentration of ethyl acetate extract went lower, the QS activity was restored. As shown in figure 3C, further fractionates of ethyl acetate extract showed lowered QS activity of PA14 with the first three fractionates (F1, F2 and F3) showed more than 50% decreased in the QS activity, while the activity in F5, F7, F8, F10 and F11 were found to reduce by about 30%. In contrast, F4, F6 and F9 showed insignificant changes in the QS activity in PA14.

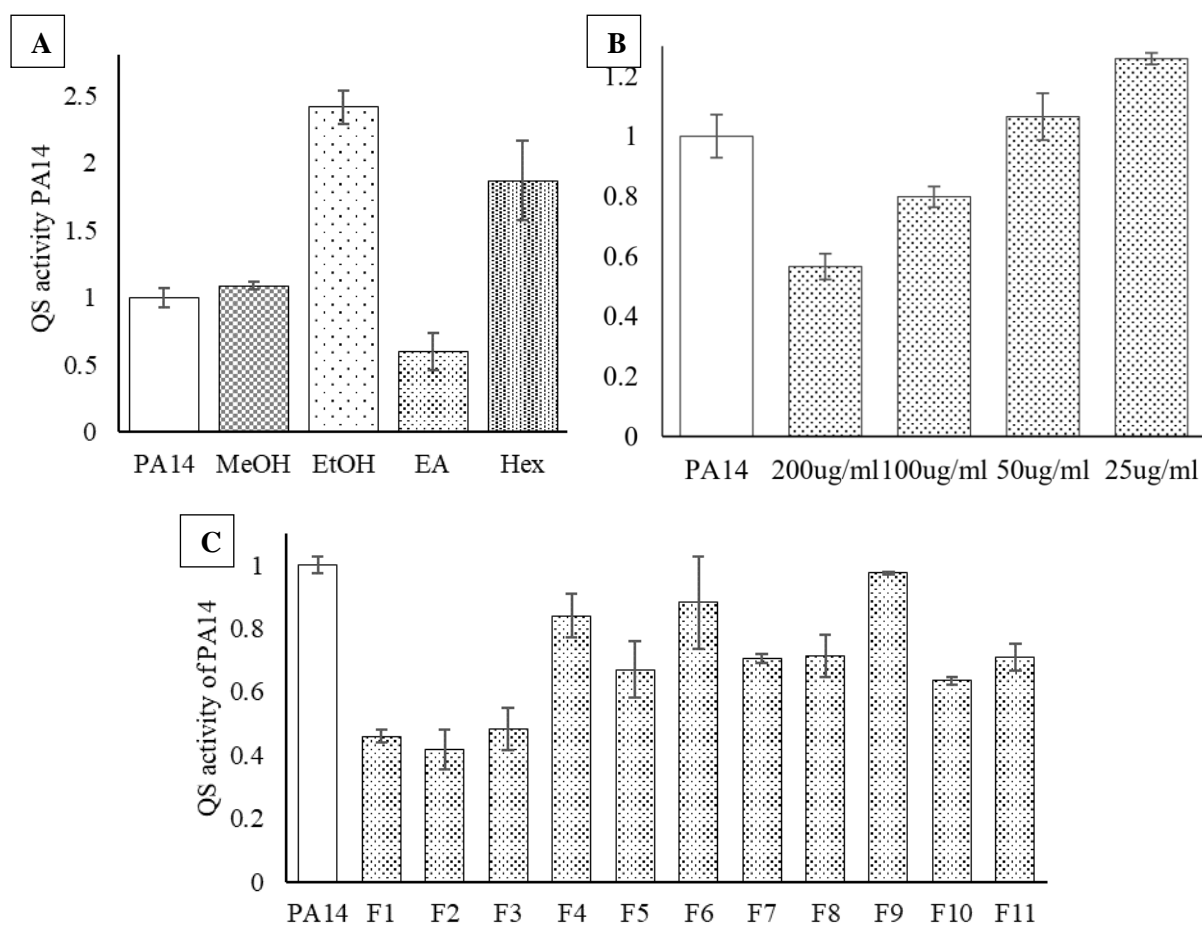


Figure 3. QS activity of PA14 grown overnight with treatment. A: QS activity of PA14 grown in presence of methanol (MeOH), ethanol (EtOH), ethyl acetate (EA) and n-hexane (Hex) extracts at 200 µg/ml. B: QS activity in PA14 grown in ethyl acetate extract at various concentration. C: QS activity in fractionates of ethyl acetate extract tested at 100 µg/ml.

* $p=0.05$, $n=3$ independent experiments.

4. Discussion

Extracts of *Piper betle* leaves were qualitatively examined for its anti-quorum sensing (anti-QS) activity against *Pseudomonas aeruginosa* that was relatively assessed via pyoverdinin assay. Pyoverdinin is a siderophore produced by *P. aeruginosa* to not only serve as an iron-scavenging protein, but also to serve as one of the signaling molecules for the quorum sensing system in *P. aeruginosa*, together among N-(3-oxo-hexanoyl)-L-homoserine lactone, N-butylhomoserine lactone, 2-heptyl-3-hydroxy-4-quinolone and 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde [19, 20, 21, 22, 23]. Signaling by pyoverdinin regulates the production of exotoxin A and endoprotease, both are contributor factors for *P. aeruginosa* to cause disease in a host [19]. We took advantage over this fact to utilize pyoverdinin assay to assess the anti-QS activity in crude extracts of *P. betle* leaves, allowing us to bioassay guide the next fractionation towards isolation of bioactive compounds.

Before we subjected *P. betle* leaves extract to the pyoverdinin assay, we first verified that the extract would not affect the growth of PA14 by its presence in the growth medium. It is important to rule out any influence the extract might have on the growth as we are only interested in bioactive compounds that neither kill nor stop the cell from growing. The time course of OD₆₀₀ showed insignificant difference in the growth curve for both samples, suggesting that the extract has no bactericidal/bacteriostatic towards PA14. In addition, the growth of PA14 in both conditions reached

the stationary phase at approximately the 13th hr and thus, we could safely conclude that the anti-QS we were observing was not due to the killing of the cells.

Pyoverdinin assay was adapted from a method developed to detect siderophore activity in soil bacteria [17]. In the pyoverdinin assay particularly used for this study, pyoverdinin activity was estimated based on the iron removal from the CAS-iron complex by pyoverdinin [15]. When iron form complex with CAS, it gives the solution a deep blue colour. As pyoverdinin removes the iron from the complex, it causes the CAS dye to lose its deep blue colour and the amount of loss is considered as the pyoverdinin activity [15]. Theoretically, the more pyoverdinin present in the PA14 supernatant, the more loss of deep blue colour would be observed, indicating high activity of quorum sensing in any tested sample and vice versa. Therefore, extracts that caused insignificant change of colour at 630 nm would be considered as positive for anti-QS activity.

We first determined the linear regression in dilutions of PA14 supernatant grown without any treatment. The resulted R^2 value was 0.96 (Figure 2), indicating there was a linear relationship between concentration of CAS-iron complex and the light absorption of the CAS dye solution. As expected, a decreasing manner of pyoverdinin activity was observed as the concentration of pyoverdinin reduced; at which a similar pattern of activity should be observed in term of quorum sensing activity as the supernatant became more diluted; justifying the use of pyoverdinin assay to assess the anti-quorum sensing activity in medicinal plants extracts. Furthermore, the obtained QS activity derived from pyoverdinin assay produced a result with low QS activity (>50% reduction), moderate QS activity (30% reduction) and high QS activity (<10% reduction), as shown in Figure 3C. Adonizio *et al.* also demonstrated variation in QS activity in treated *P. aeruginosa* with as much as 91% reduction of activity to as low as 10% reduction by extracts of plants from South Florida [18].

When PA14 was grown in crude extracts of *P. betle* leaves, the QS activity in PA14 grown in presence of ethyl acetate crude extract was found to be significantly reduced to 0.6-fold as compared to the activity in untreated PA14, suggesting the presence of anti-QS activity of the ethyl acetate extract. Meanwhile, methanol, ethanol and n-hexane extracts showed no anti-QS activity against *P. aeruginosa*. The anti-QS ability of ethyl acetate crude extract at 200 and 100 $\mu\text{g/ml}$ reduced the QS activity by almost 40% and 20%, respectively. The anti-QS activity in lower concentration however showed insignificant different to that of untreated PA14. Based on this information we fractionated further the crude ethyl acetate extract of *P. betle* leaves and obtained eleven fractions and subjected them to the pyoverdinin assay. As shown in figure 3C, out of these eleven fractions, three of them showed high anti-QS activity with more than 50% reduction (F1, F2 and F3) and five fractions showed moderate anti-QS activity (F5, F7, F8, F11 and F12). Fractions with high activity were selected for further separation to isolate single bioactive compounds.

In this study, pyoverdinin assay was solely used to guide for further fractionation processes to speed up the isolation and identification of bioactive compounds. We found, among four crude extracts of *P. betle* leaves tested, the ethyl acetate extract showed significant reduction in the QS activity and we went on to fractionate further the ethyl acetate extract. From there, we obtained eleven fractions with three hits (F1, F2 and F3) that would be further separated for single compound isolation. The isolated single compound would be subjected to pyoverdinin assay to qualitatively determine the anti QS properties. The pyoverdinin assay however, would not be able to quantify the actual amount of the QS activity and neither the mechanism of the anti-QS activity. Thorough study must be performed to support initial findings of anti-QS activity via pyoverdinin assay.

5. Conclusion

The use of pyoverdinin assay at initial stage in assessing the anti-QS of potential medicinal plant is feasible due to its simplicity yet, reliable information. This assay reduces the lengthiness of extracting the signaling molecule from the supernatant and purifying it for analysis to just ten minutes. However, the usage of pyoverdinin assay alone in assessing anti-QS activity is insufficient due to the complexity of the quorum sensing system itself which comprises of multiple networks. The application of pyoverdinin assay to screen for anti-QS activity in *P. betle* is appropriate that it served as bioassay to

guide for further fractionation and purification of bioactive compound. Compound with anti-QS activity isolated from *P. betle* leaves would have the potential to be developed as an alternative medicine to antibiotic and put a halt in the antibiotic resistance crisis.

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