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Original Article

Attenuation of *Pseudomonas aeruginosa* Virulence by Some Indonesian Medicinal Plants Ethanolic Extract

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ABSTRACT

Context: One strategy to address the emergence of infectious diseases supported by the increasing cases of microbial antibiotic resistance is the development of anti-pathogenic drugs, a quorum quenching compound(s) capable of inhibiting microbial communication (quorum sensing inhibitor). *Pseudomonas aeruginosa* is one of the most common source of chronic opportunistic infections, which demonstrates the most consistent resistance to antimicrobial agents. Quorum sensing (QS) controls secretion of virulence factors in *P. aeruginosa*. **Aim:** This study aims to discover quorum sensing inhibitors (QSI) from some Indonesian medicinal plants ethanol extract to analyze their inhibitory activities against QS-mediated virulence factors in *P. aeruginosa*. **Settings and Design:** *In-vitro* experimental study-laboratory setting **Materials and Methods:** Indonesian medicinal plant ethanolic extracts were tested for their capability to inhibit *P. aeruginosa* motility, biofilm formation using microtiter plate method, pyocyanin and LasA production using LasA staphylolytic assay. **Statistical analysis used:** Statistical significance of the data were determined using one way ANOVA, followed by Dunnett's test. Differences were considered significant with P values of 0.05 or less. **Results:** Ethanolic extract of *T. catappa* leaves and *A. alitilis* flower capable to inhibit *P. aeruginosa* motility as well as pyocyanin production and biofilm formation. Both extracts also showed capability in reducing LasA protease production. **Conclusion:** *T. catappa* and A. *alitilis* are an interesting sources of innovative plant derived quorum quenching compound(s), thus can be used in the development of new antipathogenic drug.

Key words: Ethanol extract, Anti-pathogenic drugs, Quorum quenching, Quorum sensing inhibitor, *Pseudomonas aeruginosa*.

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INTRODUCTION

Quorum sensing is a process of cell to cell communication in bacteria mediated by a small diffusible molecule called auto inducers (oligopeptides in Gram positive and *N*-acyl homoserine lactone (AHL) in Gram negative bacteria). These auto inducers diffuse freely from the bacterial cell and accumulate in the surrounding environment. When a threshold concentration (quorum) has been reached they diffuse back into the cell and regulate transcription of specific genes as in a response to their changing environmental conditions.[1] Fungi, like bacteria, also use quorum sensing to affect population-level behaviours such as biofilm formation and pathogenesis.[2]

Examples of cellular processes modulated by quorum sensing are biofilm formation,[3] LasA protease production, pigment production, and motility.[4] These traits have also shown to be involved in the pathogenicity of bacteria.[5] It has been suggested that targeting the quorum sensing system by interruption of bacterial communication, instead of killing bacteria, is an example of an antipathogenic effect and may give a solution to antibiotics resistance.[6] Therefore, anti-quorum sensing (anti-QS) compounds can be of great interest in the treatment of bacterial infections.[7]

Plants have long been a source of medicines and continues to contribute significantly to the development of today's pharmaceuticals for therapeutics and source of new bioactive compounds.[8] Indonesia harbours a very high flora of diverse species used in traditional ways as medicine.[9] Previous anti-infective studies on Indonesian medicinal plants have focused mainly on antimicrobial drug discovery perspectives. However, no systemic effort has been made to explore its anti-QS activity. Furthermore, shifting the focus from antibacterial activity to anti-QS properties may disclose new quorum quenching compounds.[10] For this reason, research in determining anti-QS activity of a compound is generating potential for development of a new therapeutic.

Motility of *P. aeruginosa* in aqueous and dry environments has been shown to be associated with its virulence. In the presence of a quorum sensing inhibitory compound, the motility and therefore the virulence will be limited.[11] Six different forms of bacterial movement have been described including

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swimming, swarming, twitching, gliding, sliding and darting.[12] These various forms of surface motility enable bacteria to increase their efficiency of nutrient uptake, avoid toxic substances, move to preferred hosts and provide access to optimal colonization sites within, and spread themselves into the environment.[13] Bacterial motility plays a different role in biofilms. They can promote adhesion of the cell to the surface for biofilm maturation process and/or in be involved in the dispersal process.[14] However, motility is not critical to biofilm formation, which was shown for biofilms produced by *P. aeruginosa* PAO1 mutant strain which is lack flagella and type IV pili.[15]

In this study, we provide the screening result of some commonly used Indonesian medicinal plants for anti-QS activity using the *P. aeruginosa* PAO1 in reducing quorum sensing related motility of *P. aeruginosa* PAO1 such as swimming, swarming and twitching motility, biofilm formation inhibition, pyocyanin pigment production, and LasA protease production inhibition. We have found potential anti-QS activity in *Terminalia catappa* leaves and *Artocarpus alitilis* flower ethanol extracts.

SUBJECTS AND METHODS

Bacterial isolate and culture conditions

The bacterial strains used in this study were *P. aeruginosa* PAO1 and *Staphylococcus aureus* Cowan I strain. Bacterial strains were grown on Luria-Bertani (LB) Agar and incubated for 24 h at 30^{0}C. Following the incubation on agar plate, the colonies were transferred to LB broth and incubated for another 24 h at 30^{0}C. Cell density was adjusted to 10^8 CFU/mL by altering the optical density of the suspension to 0.1 at 600 nm.

Medicinal plants and plant extraction

Indonesian medicinal plants were collected from Yogyakarta, Indonesia and its surroundings based on ethnopharmacological information. Plants samples were washed, cut into small pieces and oven dried at 40^{0}C for 48-72 h.[16] The process of drying prevents degradation through metabolic process, and prevents microbial development. The drying temperature may vary from 35^{0}C to 70^{0}C depending on the part of the plant and sensitivity of the active principles. For the leaves, a temperature range of 20^{0}C – 40^{0}C is recommended. Drying plant material in oven with low drying temperatures between 30^{0}C and 50°C is faster than exposure plant materials to fresh air (shaded from direct sunlight), and still capable to protect sensitive active ingredients.[17] The dried plant materials were ground into a fine powder. The pulverized materials were extracted by maceration using 70% ethanol in a ratio of 1g (plant material): 10 mL to obtain crude ethanol extract. Furthermore, extracts were dried and concentrated under reduced pressure using a rotary evaporator. Stock solutions (100 mg/mL) of crude ethanol extract in dimethyl sulfoxide (DMSO) were prepared, filter-sterilized (0.2 µm) and stored at 4°C.

Determination of planktonic minimum inhibitory concentration (PMIC) of plant oils

MIC values for different plant extracts against *P. aeruginosa* were determined by Micro broth dilution method as followed by Khan *et al.*[18] Different concentrations within the range of 0.06 mg/mL – 1 mg/mL were tested. The concentration at which the extract depleted the growth of bacterial by at least 50% was labelled as the $PMIC_{50}$ and was taken to assess the anti QS activity.

Effect of Plant Extracts on Biofilm Formation

Biofilms were formed on polystyrene flat bottom 96-well microtiter plates (IWAKI). To determine biofilm formation inhibition, extracts at sub inhibitory concentration (concentration of $PMIC_{50}$ and under) ranging from 0.06-0.5 mg/mL were used to ensure a concentration that is not affecting the microbial growth. Negative controls (cells + media: LB medium for *P. aeruginosa* PAO1), positive control (cells + media + chloramphenicol), vehicle controls (cells + media +DMSO), and media controls were included. For the positive controls concentrations of 1 mg/mL chloramphenicol was used, prepared by serial dilution techniques. Blanks undergo the same treatment as samples, but without incubation. All tests were performed in triplicate.

Plates were incubated for 24 h at 28^{0}C. After 24 h incubation, the content of the well was aspired, rinsed 3 times with distilled water, and dried at room temperature for 10 min. Then, 125 µL of 1% crystal violet stain was added to the wells for staining for 15 min. The excess stain was rinsed off with tap water and 200µL methanol was added to the wells and transferred to a flat-bottom 96-well plates. Optical density readings were obtained by a plate reader at 600 nm. Biofilm formation inhibition was calculated as % of inhibition by using the formula mentioned below. The % of inhibition of replicate tests was used to determine the final minimum biofilm inhibitory concentration (MBIC) values. The concentration at which the extract depleted the bacterial biofilm by at least 50% was labelled as the $MBIC_{50}$.

$$\% \text{ inhibition} = \left(1 - \left(\frac{\bar{x}\,ODt - \bar{x}\,ODmc}{(\bar{x}\,ODvc}\right)\right) \times 100$$

ODt= optical density (595 nm) of the test well; ODmc: optical density (595 nm) of the media control well; ODvc: optical density (595 nm) of the vehicle control well.[19]

Pseudomonas aeruginosa PAO1 motility test

The plant ethanol extract and essential oils which inhibited violacein production were further tested to explore their effects on quorum sensing related swarming, swimming and twitching motility of *P. aeruginosa* PAO1. Swimming, swarming and twitching motility assays were performed by the method of Rashid and Kornberg.[13] Briefly, LB agar (0.3%) plates (for swimming motility), and LB agar (0.5%) plates (for swarming motility) containing sub-inhibitory concentrations of ethanol extract or essential oils were prepared and allowed to dry for 3-4 h at 30^{0}C. Plates were point inoculated with freshly grown culture cells using a blunt ended sterile toothpick. For twitching motility, LB agar (1%) plates were used bacterial cells were inoculated by using a sharp end toothpick and stabbing through the agar to the bottom of the petri dish. After 24 h of incubation in upright position at 30^{0}C, the extent of motility was determined by measuring the diameter of the bacterial colony.

Las A staphylolytic assay

Las-A protease activity was determined by measuring the ability of culture supernatants to lyse boiled *S. aureus* cells. A 100µL aliquot of *P. aeruginosa* LB culture supernatant with or without plant extracts added to 900µl of boiled *S. aureus* suspension. OD600 was determined after 0, 5, 10, 15, 20, 25, and 30 min. Activity was expressed by measuring OD600 value.

Pyocyanin inhibition assay.

Pyocyanin was extracted from *P. aeruginosa* culture supernatant and measured by the method as described by Ra'oof and Latif.[20] Briefly, a 5-ml sample of culture grown for 72 h, in LB medium containing plant extract tested, was centrifuged, and the supernatant extracted with 3 ml of chloroform and then re-extracted into 1 mL of 0.2 mol/L HCl to give a pink to deep red solution. The absorbance of this solution was measured at 520 nm. Concentrations expressed as pyocyanin produced in l g/mL of culture supernatant were determined by multiplying the optical density at 520 nm (OD520) by 17.072.

Statistical methods

The data were initially analysed by a normal distribution using the one-sample Kolmogorov-Smirnov test. Following the confirmation of normal distribution, statistical significance of the data was determined using one-way ANOVA, followed by Dunnett's test. Differences were considered significant with *P* values of 0.05 or less.

RESULT

Effects of plant ethanol extracts on planktonic growth and biofilm formation of *P. aeruginosa* PAO1.

The maximum plant extract concentration of 1 mg/mL of plant ethanol extracts for testing was chosen based on the previous study by Rios and Recio[21] who reported that extracts should be avoided exhibiting minimum inhibitory concentration (MIC) values higher than 1 mg/mL or isolated compounds exhibiting MIC values higher than 0.1 mg/mL. The inoculums concentration used in this study is 10^5 CFU/mL. According to Clinical and Laboratory Standards Institute (CLSI) guidelines,[22] a $5x10^5$ CFU/mL inoculum provides an acceptable challenge dose for assessing the biological activity of anti-microbial agents and is large enough to provide statistically satisfactory data. If the inoculum is too small, significant bacteria resistance may not be detected. As demonstrated by Barry *et al.*[23] the growth phase may not have significant effect on MIC determination assay, however, it does critical in the MBC assay because if a stationary phase of inoculums is used, the number of surviving cells tends to increase after 24 h incubation and makes the MBC result artificially high.

The percentage of planktonic growth inhibition was measured from the reduction in absorbance level of the treatments wells which is indirectly measure the bacterial cells biomass, compare to negative control well (without the presence of the test compounds). The negative control was assumed to have 0% activity compare to the compounds tested. Extract concentration around $PMIC_{50}$ and below (sub-PMIC) were used for further tests, to ensure a concentration that is not affecting the microbial growth.

As shown in Table 1, most of the crude extracts used in this study have anti-bacterial activity against planktonic growth of *P. aeruginosa* PAO1 except ethanol extract of languas rhizome, *A. alitilis* fruit and *A. muricata* leaves.

In addition to testing of the plant extracts for inhibition of planktonic growth we also have investigated their effect on biofilm formation. Crystal violet staining has been widely adopted by microbiologist to investigate biofilm formation and attachment of microorganisms to diverse surfaces. This staining method is inexpensive, relatively quick, and adaptable for use in high-throughput screening with microliter plates.[24]

Using the crystal violet method, we have found that the inhibition of biofilm formation was dose dependent in *P. aeruginosa* PAO1. Plant ethanol extract concentration of 0.5 mg/mL is the lowest concentration which shows 50% inhibition on *P. aeruginosa* biofilm formation (Table 1). Three extracts i.e. *A. alitilis* flower, *M. indica* leaves and *T. catappa* leaves, tested inhibit 50% of *P. aeruginosa* PAO1 biofilm formation at concentration of 0.5 mg/mL.

Inhibition of bacterial motility

We investigated if the extracts which inhibited quorum sensing had any effect on quorum sensing related motility in the human opportunistic pathogen *P. aeruginosa* PAO1. Our result, shown in Table 2, indicated that ethanol extracts of *A. alitilis* flower and *T. catappa* leaves at a concentration of 0.5 mg/mL significantly reduced the swimming motility of *P. aeruginosa* PAO1 by 47.38±0.76 % (P<0.005) and 74.68±0.86 % (p<0.005), respectively. The swarming motility of *P. aeruginosa* PAO1 was also reduced as much as 56.81±0.28 % (p<0.005) and 67.8±0.28 % (p<0.005) when extracts at concentrations of 0.5 mg/mL were applied. The same concentrations of the extract also showed a decrease in twitching motility namely, 62.09±0.28 % (p<0.005) and 75.8±0.00 % (p<0.005) respectively.

Table 1: Antibacterial and anti-biofilm activity of plant extracts against *P. aeruginosa*.

Plant extract	Local name	Plant part	Planktonic antibacterial activitivy ($PMIC_{50}$) in mg/mL*	Antibiofilm formation activity ($MBIC_{50}$) in mg/mL*
Languas galanga	Laos	Rhizome	0.5	-
Centella asiatica	Pegagan	Leaves	0.5	1
Artocarpus alitilis	Sukun	Flower	1	0.5
		Fruit	-	-
Andrographis paniculata	Sambiloto	Leaves	1	1
Annona muricata	Sirsak	Leaves	-	-
Muntingia calabura	Talok (Kersen)	Leaves	-	1
Mangifera indica	Mangga	Leaves	1	0.5
Terminalia catappa	Ketapang	Leaves	-	0.5

Table 2: Antimotility efficacy of some medicinal plants ethanol extract against *P. aeruginosa*.

Plant Extract	Part	Concentration (mg/mL)	Motility (mm)		
			Swimming	Swarming	Twitching
C. asiatica	Leaves	1	54.37 ± 0.57	52.42 ± 0.28	46.72 ± 0.00
A. alitilis	Flower	0.5	47.38 ± 0.76	56.81 ± 0.28	62.09 ± 0.28
	Fruit	1	29.57 ± 0.57	47.00 ± 0.76	44.75 ± 0.00
A. paniculata	Leaves	1	44.37 ± 0.76	51.40 ± 0.00	52.87 ± 0.57
A. muricata	Leaves	1	27.39 ± 0.57	40.25 ± 0.76	38.76 ± 0.57
M. calabura	Leaves	1	33.52 ± 0.42	60.47 ± 0.28	62.35 ± 0.42
M. indica	Leaves	0.5	42.65 ± 0.00	33.56 ± 0.57	48.05 ± 0.57
T. catappa	Leaves	0.5	74.68 ± 0.86	67.80 ± 0.28	75.80 ± 0.00

Pyocyanin inhibition assay

We tested the effect of plant extracts on pyocyanin pigment production in *P. aeruginosa*. Our result suggest that *T. catappa* leaves ethanol extract showed inhibition of pyocyanin pigment formation by 63.41 ± 0.6 % (p<0.005) (Figure 1), whereas at the same concentration *A. alitilis* flower ethanol extract showed inhibition of 63.41 ± 0.6 % (p<0.005) (Figure 2), respectively.

LasA Staphylolytic

Inhibition of LasA protease in *P. aeruginosa* by plant extracts tested was assessed via LasA staphylolytic assay. *T. catappa* leaves ethanol extract exhibited inhibition of LasA protease production by 73.2 ± 0.6% (p< 0.005), whereas at the same concentration *A. alitilis* flower ethanol extract showed inhibition of 70.6 ± 0.8 % (p< 0.005) (Figure 3) , respectively.

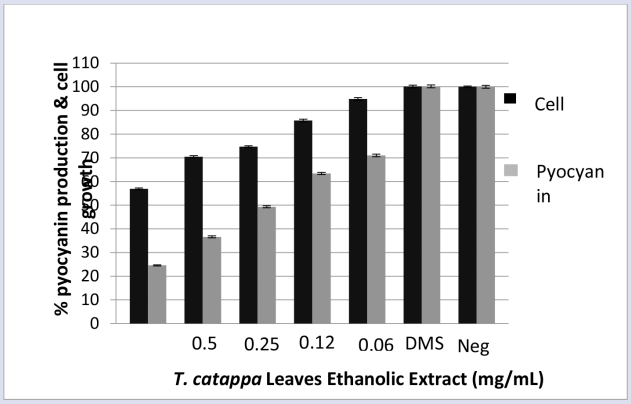

Figure 1: Efficacy of *T. catappa* flower ethanol extracts in various concentration against pyocanin production in *P. aeruginosa*. 0.3% v/v DMSO was used as vehicle control.

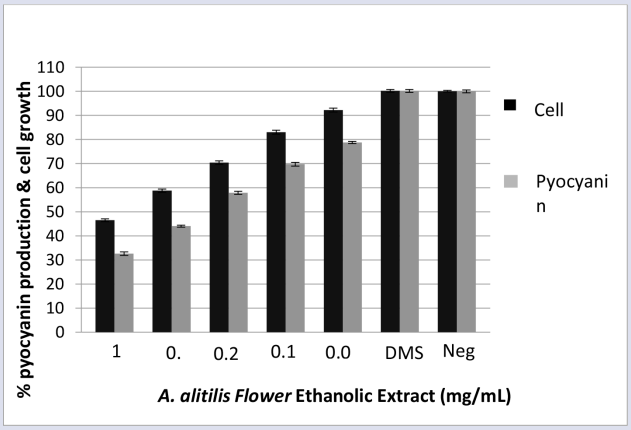

Figure 2: Efficacy of *A. alitilis* flower ethanol extracts in various concentration against pyocanin production in *P. aeruginosa*. 0.3% v/v DMSO was used as vehicle control.

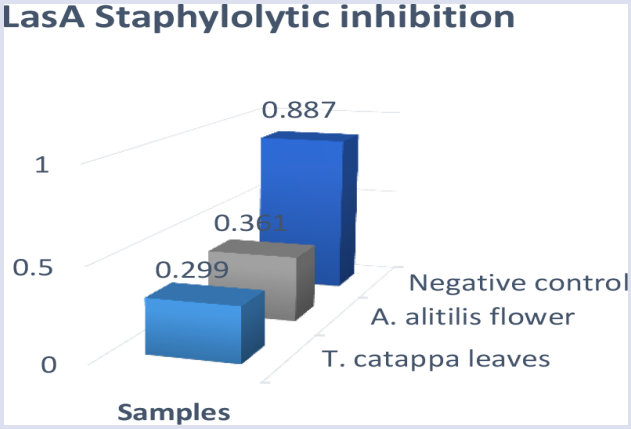

Figure 3: LasA protease activity.

DISCUSSION

The aim of this study was to determine the anti-QS potential of ethanol extracts and essential oils from Indonesian medicinal plants with a potential to a possible use in controlling detrimental infections. Quorum sensing in *P. aeruginosa* has been well studied. This organism has two QS systems, LasR/I and RhlR/I systems. The lasI produces diffusible extracellular signal, N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) which binds with LasR to activate a number of virulence genes and biofilm maturation, and to regulate the expression of LasI. In the Rhl system, the RhlI synthase produces N-butyryl-L-homoserine lactone (C4-HSL), which interacts with RhlR protein and activated C4-HSL-RhlR complex, further stimulates the expression of RhlI, virulence genes and biofilm associated genes and biofilm associated genes.[25]

P. aeruginosa produces a third signalling molecule, 2-heptyl-3-hydroxy-4(1H)-quinolone, called Pseudomonas quinolone signal (PQS). It is produced by the *pqs* (*pqsABCDE*) operon and pqsH gene, wherein the *pqsABCDE* operon products synthesize 2-heptyl-4-hydroxyquinoline (HHQ), and the PqsH converts HHQ into PQS. PQS diffuses in and out of the cell, accumulates in the environment, and when a threshold concentration reached, it binds to the regulator protein, PqsR, which modulates genes encoding virulence factors and the synthesis of PQS itself, resulting in autoinduction.[25]

There are several reports in literature that propose different mechanisms of quorum sensing inhibition by natural products, either by inhibition of the signal molecule biosynthesis,[26] preventing the binding of the AHL molecules to its receptors[6] or enzymatic inactivation and biodegradation of the quorum sensing signalling molecules.[27]

Plants are the sources of varied bioactive metabolites which useful for the development of successful and effective drugs. Various plants have demonstrated the ability to interfere with microbial QS systems that further and control its virulence. Vanilla (*Vanilla planifolia*), garlic (*Allium sativum*), weeping bottlebrush (*Callistemon viminalis*), Zaragoza mangrove (*Conocarpus erectus*), graceful sandmat (*Chamaesyce hypericifolia*), black olive (*Bucida buceras*), Florida clover ash (*Tetrazygia bicolour*), and southern live oak (*Quercus virginiana*), showed quorum sensing inhibitory properties against *C. violaceum* and *Agrobacterium tumefaciens*.[5] Study from Zahin *et al.*[13] Chong *et al.*[28] and Priya *et al.*[29] also revealed quorum sensing property of *Mangifera indica*, *Punica granatum*, *Myristica cinnamomea* and *Phyllanthus amarus*, which showed efficacy in regulating violacein production of *C. violaceum* and inhibiting motility of *P. aeruginosa*. The result in our study have revealed that the ethanol extract of *T. catappa leaves* and *A. alitilis* flower significantly inhibit the quorum sensing mechanism of *P. aeruginosa* PAO1 as indicated by a reduction in pyocyanin production, inhibition of *P. aeruginosa* motility, reduction in LasA protease production and inhibition of *P. aeruginosa* biofilm formation which is an important trait for its pathogenicity. It is concluded that anti-QS is as important as antibacterial activity as it will unlikely cause resistance problems as it does not pose selection pressure.

CONCLUSION

In conclusion, the finding from this study conclusively demonstrated the *in vitro* antiquorum sensing activity of *T. catappa* and *A. alitilis* ethanol extract, makes them as an interesting sources to be used in the development of new antipathogenic drug. Further purification of the active compounds may be suggested on the basis of the present study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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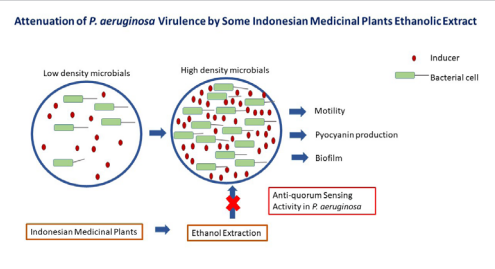
GRAPHICAL ABSTRACT



SUMMARY

- Quorum sensing is a process of cell to cell communication in bacteria mediated by a small diffusible molecule called auto inducers. *Quorum sensing* regulate bacterial cell activities in order to be able to survive in the environment. Among the many activities controlled by *quorum sensing* is the expression of virulence factors by *pathogenic* bacteria. Targeting the quorum sensing system by interruption of bacterial communication, instead of killing bacteria, is an example of an antipathogenic effect and it may also give a solution to the emergence of bacterial resistance to current antibiotics. This research aimed to evaluate anti-QS activity of some Indonesian medicinal plants ethanol extract against QS-mediated virulence factors in *P. aeruginosa*. Our result showed that ethanolic extract of *T. catappa* leaves and *A. alitilis* flower capable to inhibit *P. aeruginosa* motility as well as pyocyanin production and biofilm formation. Both extracts also showed capability in reducing LasA protease production. We conclude that the ethanol extract from *T. catappa leaves and A.alitilis flower* are an interesting sources of innovative plant derived anti-quorum sensing agents, thus can be used in the development of new antipathogenic drug.

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