Inhibition Of *Pseudomonas aeruginosa* Virulence Factors Expression Regulated By Quorum Sensing System Using Ethyl Acetate Extract Of Temu Ireng (*Curcuma aeruginosa*)

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**Abstract.** *Pseudomonas aeruginosa* is an opportunistic human pathogen that can cause serious problem in immunocompromised persons. Difficulty in treating *P. aeruginosa* infection is due to its abilities to develop multi drug resistance towards various antibiotic. *Quorum sensing* system is a potential target for *P. aeruginosa* infection because this system regulate the expression of virulence factors in this bacteria. In previous study, ethyl acetate extract of Temu Ireng rhizomes (*Curcuma aeruginosa*) was proved to inhibit quorum sensing in *Chromobacterium violaceum*. This study was aim to know the effect of *C. aeruginosa* extract towards expression of *P. aeruginosa* virulence factors which were regulated by quorum sensing, such as protease LasA activities, LasB and biofilm formation. Protease LasA activity assay was conducting using *Staphylococcus aureus* cell lysis activity. Protease LasB activity was measured by elastin congo red assay, and the formation of biofilm was monitored using PVC biofilm formation assay method. The result showed that ethyl acetate extract of *C. aeruginosa* rhizomes can decrease *P. aeruginosa* virulence controlled by quorum sensing, namely protease LasA, LasB, and biofilm formation by 66.92%, 37.8%, and 46% respectively.

1. **Introduction**

Quorum sensing is a system of communication between bacterial cells mediated by diffused molecules, which are released into the surrounding environment and can be sensed by the entire population so that they can act in harmony for certain purposes. This system regulates gene expression in response to cell density in the population. Quorum sensing is known to regulate gene expression associated with various physiological functions such as virulence, bioluminescence, sporulation, biofilm formation, pigment production, antibiotic production and interaction with higher organisms [1-3].

Many pathogenic bacteria use the quorum sensing system to regulate the expression of virulence factors. this arrangement ensures that bacteria invade the host only when the amount has reached the quorum so that the host's defense system can be overcome. one of the pathogenic bacteria that uses the quorum sensing system for virulence is *Pseudomonas aeruginosa*. some virulence factors of *P. aeruginosa* which are under the regulation of the QS system are LasA and LasB Protease, pyocyanin
pigment, and biofilm formation [4,5]. P. aeruginosa normally inhabit humans and act as saprophytes in the intestines and skin. However, P. aeruginosa infection is a serious problem in humans whose immune system decreases, especially hospital patients suffering from cancer, cystic fibrosis and burns [6,7]. Pseudomonas aeruginosa can cause infection in urinary tract, respiratory tract, surgical scars, gastrointestinal tract, bones and joints, soft tissues, and various systemic infections.

Infectious diseases to date are still a problem for tropical countries. The re-emergence of infectious diseases that were previously extinct due to the development of pathogenic resistance to anti-microbial compounds. The search for new anti-microbial compounds continues to race with the development of resistance. One approach to overcome resistance is the search for new targets for antimicrobial compounds. QS system is one of the targets expected to be developed for the control of infectious diseases by bacteria that use this system for virulence control. The inhibition of the QS system is expected not to trigger the development of resistance, because the bacteria are not inhibited by growth but only the communication system is blocked, so that virulence factors are not expressed. Compounds which have the ability to interfere with the QS system are called anti-pathogenic drugs [8]. Some secondary metabolites from plants have been known to have the ability to block bacterial QS systems, such as cathcine, vanillin, zingerone, curcumin, etc [9].

Fitri (2010) had screened several plants for anti QS activity. The ethyl acetate extract of curcuma aeruginosa proved to have anti QS activity toward C. violaceum as a reporter strain. In this study we conducted further research to observe the effect of C. aeruginosa rhizome ethyl acetate extract to attenuate the virulence factors of P. aeruginosa which are under regulation of QS system, namely LasA protease, LasB elastase, and biofilm formation.

2. Experimental
2.1. Extraction of Temu Ireng Rhizome
The Extraction of Temu ireng (C. aeruginosa) rhizome was conducted by maceration method using ethyl acetate as a solvent. Dried rhizome was pulverized into powder and then immersed in the solvent for 48 hours. The filtrate was separated by filtering using filter paper and separating funnel and then concentrated using a rotary evaporator at a temperature of 60°C. The extract was dissolved in DMSO (Dimethyl Sulfoxide) with a concentration of 20 mg/ml w/v then sterilized by filtration using a 0.45 µm porous membrane. The solution is stored as stock in the freezer.

2.2. Anti QS assay on Chromobacterium violaceum
Qualitative assay of quorum sensing inhibition was carried out by the well diffusion method  (Adonizio et al., 2006; Hussaini and Mahasneh, 2009). Chromobacterium violaceum is cultured overnight (16-18 hours) in LB broth medium then 100µL of the culture was spread on to LB agar medium. The wells with 6mm diameter were made on the LB agar. Extract was dissolved in DMSO (Dimethyl Sulfoxide) with a concentration of 20 mg/ml w/v then sterilized by filtration using a 0.45 µm porous membrane. The solution is stored as stock in the freezer.

The wells were incubated at 30°C for 24 hours.

As a control, 20µL ethanol were included. As a comparison of antibiotic effect, tetracycline was added with a concentration of 10µg /mL into the well. Quorum sensing inhibition is indicated by the presence of a colorless cell growth zone ring around the well and quantify as the inhibition diameter. The diameter of the quorum sensing inhibition was measured from the outer edge of the disk to the end of the inhibition zone.

2.3. Pseudomonas aeruginosa Culture Maintenance and Preparation
Pseudomonas aeruginosa was grown on LB Agar media (LA) and incubated at 37°C. The culture was stored in a refrigerator as a stock culture. Just before used, bacteria was grown on LA using the streak plate method and incubated for 24 hours. A total of 1 bacterial colony were transferred to 20 mL LB media as inoculum and incubated at 37°C with shaking for 12 hours. Culture was then diluted 100 times in minimal AB media (for LasB protease activity testing) and LB media (for growth measurement, LasA protease activity and total protein) and the growth was measured using
spectrophotometry at 600nm wavelength. When it reaches OD600 ~ 1.7, bacterial culture were grouped into three treatments: 1. culture without any supplementation 2. culture with supplementation of DMSO as a control and 3. culture with supplementation of extract. The final concentration of extract in the culture was 1 mg / ml. The activity of LasA and LasB as well as total protein was assayed using supernatant from bacterial culture at the end of the stationary phase. Meanwhile, The growth was measured every 2 hours for 24 hours.

2.4. Total Protein Assay
Total protein was determined by the Bradford method (1976) using Bovine Serum Albumin (BSA) as a protein standard. The total protein was then used to calculate LasA and LasB protease activity units.

2.5. Protease Activity Assay
LasA protease activity was determined by measuring the lytic activity of P. aeruginosa culture supernatant against S. aureus cells. A total of 60 ml of 12 hours aged S. aureus culture was boiled for 10 minutes then centrifuged for 10 minutes at 10,000xg. The pellets were resuspended in 10 mM phosphate buffer pH 4.5 to reach OD _600_ ~ 0.8. A total of 300 µl each of supernatant P. aeruginosa in LB medium (control and treatment) was added to 2,700 µl of S.aureus suspension. OD _600_ measurements were performed after 0, 10, 20, 30, 45 and 60 minutes of incubation. LasA protease activity is defined as a change in the OD _600_ value S. aureus per hour per mg of protein.

Elastolytic activity (LasB) was determined using the elastin congo red method. As much as 300µl of P. aeruginosa culture supernatant in AB minimal media is mixed with 2,700 µl elastin congo red buffer containing 20 mg of elastin congo red per liter and incubated with shaking for 3 hours at 37 °C. The mixture was centrifuged to separate the insoluble elastin congo red. Absorbance measurements were carried out at a wavelength of 495 nm. LasB protease activity was defined as OD _495_ per hour per µg protein.

2.6. Biofilm Formation Inhibition Assay
The effect of C. aeruginosa extract on biofilm formation was measured by the PVC biofilm formation assay method (O Toole and Kolter, 1998). The overnight culture of P.aeruginosa bacteria was resuspended in AB medium with 0.2% glucose and 0.5% casamino acid added with or without C. aeruginosa rhizome extract and diluted to reach OD _600_ value of 0.1.

The culture was transferred into polystyrene microtiter plate, 100 µL for each well and incubated for 10 hours at 30 °C. Culture medium was discarded, then the microtiter plate well was washed five times using distilled water to remove planktonic bacteria and biofilms that were not firmly bound. After being washed, 150 µL crystal violet included in each well and incubated 45 minutes. Crystal violet discarded and Microtiter plate was washed using sterile distilled water. Biofilm formed resuspended in 200µL 95% ethanol and then absorbance was measured using a spectrophotometry at 600 nm.

3. Result and Discussion
The ethyl acetate extract of C. aeruginosa rhizome had antiQS activity which was showed by the inhibition of violacein production in Chromobacterium violaceum (Figure 1)
Figure 1. Anti QS activity of *C. aeruginosa* rhizome ethyl acetate extract on *Chromobacterium violaceum* violacein production: A. 400 µg/ml B. 600 µg/ml C. 800 µg/ml D. 1000 µg/ml

Ethanol extract of *C. aeruginosa* inhibited violacein production of *Ch. violaceum* as showed by the halo around the well which is indicating the growth of colourless cells. This QS inhibition zone was different from growth inhibition zone of tetracyclin since the bacteria still grew, the zone was not clear. The optimal concentration that inhibited QS was 1000 µg/ml which produced the largest inhibition zone. This concentration was then used to determine the attenuation of *P. aeruginosa* virulence factors.

The OD600 showed that the supplementation of DMSO and *C. aeruginosa* extract 1 mg/ml had no effect on the growth of *P. aeruginosa* bacteria (Figure 2). These results indicated that if the *P. aeruginosa* virulence factor expression decrease in subsequent tests, it was due to the inhibition of the quorum sensing system, not a decrease in the number of bacterial cells. Growth measurements were also carried out to determine the incubation time needed for bacterial culture to achieve optical density values of 1.7 which is the time for *C. aeruginosa* extract addition, to minimize the effects of extracts on bacterial growth at the beginning phase of growth. According to Adonizio (2008), supplementation of extracts at the beginning phase could affect the growth of *P. aeruginosa* so that the starting point for quorum sensing regulation between treatments did not occur at the same time.

Figure 2. Growth curve of *P. aeruginosa* bacteria

The *C. aeruginosa* extract decreased the *P. aeruginosa* LasA activity. The biggest decrease occurred at 60 minutes incubation time (Figure 2). LasA protease activity in the the extract treatment decreased 66.92% comparing to the control. LasB protease activity was also decrease with the extract
treatment (Figure 3). Not as high as in the LasA activity, approximately 37.8% decrease was observed in LasB activity of *P. aeruginosa* with the supplementation of extract, comparing to the control. The DMSO not significantly reduced the activity of LasA or LasB protease, suggesting that the decrease was due to the activity of the extract.

**Figure 3.** Las A activity of *P. aeruginosa*

**Figure 4.** Las B activity of *P. aeruginosa*

Biofilm formation assay results showed differences in biofilm production in the control and extract treatment (Figure 4). Biofilm formation in the extract treatment was lower than the control treatment. *P. aeruginosa* biofilm formation significantly decrease 46% in the presence of the extract.

**Figure 5.** Biofilm formation of *P. aeruginosa*
Two proteolytic enzyme LasA and LasB mediated elastase activity in P. aeruginosa (Galloway 1991). These enzymes work synergistically in degradation of elastin, fibrin, interferon, complement and collagen. LasA is a serine protease which cuts elastin in certain Gly-Gly sequences and makes it more accesible to LasB. Protease LasA also has high staphylolytic activity (Kessler et al. 1993), which can provide selective benefits for P. aeruginosa in competition with S. aureus. Protease LasB (also called elastase), a zinc metalloprotease, is a dominant and most powerful protease released by P. aeruginosa. Protease LasB can degrade elastin, an extracellular matrix proteins, to help the invasion of this bacteria into the host (Galloway 1991). This protease is also able to degrade and inactivate proteins from the immune system, including IgG and IgA (Heck et al. 1990), complement components (Hong & Ghebrehiwet 1992), and cytokines such as interferon gamma and tumor necrosis factor (Park et al. 1996). These activity play an important role in the virulence of these bacteria (Storey, 1998).

Pseudomonas aeruginosa possesses two QS systems: the LasR-LasI and the RhlR-RhlI, using N-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL) and N-butyryl-L-homoserine lactone (BHL) as signal molecules. The LasR-LasI system controls the virulence factors LasA and LasB (Gambello, 1991; Storey et al., 1998), however rhlR also controls activity to a lesser extent (Brint & Ohman, 1995; Pearson et al., 1997). Several reports also showed the regulation of biofilm formation under the QS system. The effect of quorum sensing on biofilm formation can vary in different bacterial species. In P. aeruginosa, the quorum sensing system influences biofilm formation at the stage of maturation and dispersion (Davies, 1998; Heydorn, 2002). Besides, the biofilm effectiveness in the pathogenicity of bacteria is increasing as the cell density increase, cell to cell interaction in the biofilm acquiring the bacteria to be able to communicate each other, and this is conducted using QS signals.

Since many virulence factors expression are regulated under the QS system, a therapeutic compound could target this system to inhibit pathogenicity. The compound could be an analog to signal molecules, or targeting the receptor. One well known anti QS compound, the halogenated furanones, inhibit the virulence of P. aeruginosa by displacing the signaling molecule from its receptor (Manefield et al. 2002). In this study, compounds that inhibit quorum sensing and inhibition mechanism was not identified yet. Anti quorum sensing activity can also occur due to the influence of two or more chemical compounds and is not an activity of a single compound (Adonizio, 2008). Rukayadi and Hwang (2005) stated that curcumin compounds can inhibit quorum sensing in C. violaceum. Curcumin is also one of the active compound of C. aeruginosa. Corresponding with this study, curcumin could be an anti QS compounds in C. aeruginosa. Flavonoids specifically inhibit quorum sensing via antagonism of the autoinducer-binding receptors, LasR and RhlR (Paczkowski et al. 2017). In order to identify the compounds in C. aeruginosa that act as anti QS in P. aeruginosa, bioassay guided fractionation combined with chemical profile tests such as thin layer chromatography should be conducted.

4. Conclusion
The administration of C. aeruginosa rhizome ethyl acetate extract 1 mg / ml can reduce the expression of P. aeruginosa virulence factor in the form of LasA protease activity, LasB protease and biofilm formation, which is regulated under QS system, without affecting the growth.

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