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Screening of Indonesia Medicinal Plants Producing Quorum Sensing Inhibitor

Sulistyo Emantoko*, Kestrilia Rega, Ida Bagus Made Artadana, Tjie Kok, Ernest Suryadjaja, Hendry Rusady, Anny Liana, Savitri

Fakultas Teknobiologi, Universitas Surabaya
E-mail : emantoko@ubaya.ac.id
* Author for correspondence

Abstract

Antibiotic resistance of bacteria lead to create different way in the pathogen bacteria handling such us inhibit their quorum sensing mechanism. The goal of this study is to search quorum sensing inhibitor of seven Indonesia medicinal plants. The experiment was conducted by extracting the plants using ethyl acetate subsequently tested on reporter carrying luxR homologous and luxCDABE genes. Reporter luminescence used as indicator of quorum sensing inhibition. The results show that ethyl acetate extracts of buah adas (*Foeniculum vulgare)*, bunga lawang (*Illicium verum*), selasih (*Ocimum basilicum*), temu ireng (*Curcuma aeruginosa)*, temu giring (*Curcuma heyneana)*, dan temu lawak *(Curcuma xanthorriza)* mampu menginhibisi quorum sensing pada *Pseudomonas aeruginosa*. Further analysis was done by observing several metabolites which directly influenced by quorum sensing. The experiment was design by growth *Pseudomonas aeruginosa* at LB medium occurring fennel seeds ethyl acetate extract in the various concentration. Number of biofilms, rhamnolipid and activity of LasA produced by *Pseudomonas aeruginosa* were then measured. The experiment shown LasA activity inhibition reaching 100% was obtained at growth media containing 1.52 mg / ml extract. There was a decrease at inhibition activity when the extract concentration was added above this value. Meanwhile, 19% inhibition of rhamnolipid production occurred at concentrations of ethyl acetate extract of 2.03 mg / ml in growth media. Different results obtained in the production of biofilm which is induced by fennel seeds ethyl acetate extract at the level 123%.

Keywords: Medicinal Plants, Quorum Sensing Inhibitor, Las A, Rhamnolipid, Biofilm

Introduction

Infectious diseases handling, is currently facing many challenges with the emergence of strains of pathogenic bacteria which are resistant to antibiotics. *Pseudomonas aeruginosa* (PA) is one of the pathogen bacteria which have resistance to many antibiotics (multi-drug resistance). Antibiotic resistance arise because the given treatment mechanism try to kill the bacteria. To this treatment, pathogenic bacteria will make a survival mechanism by eliminating antibiotic toxicity and lead the emergence of antibiotic resistance.

Quorum sensing (QS) is bacterial mechanisms which regulate specific proteins expressions by calculating bacterial density in the environment. Genes which are regulated by quorum sensing mechanism will only express when bacteria have reached a high density. Several genes whose expression was regulated by this mechanism i.e. the formation of antibiotics, the formation of flagella, formation bioiflm and genes associated with virulence properties. The emergence of the fact that the virulence factor is influenced by QS raises new hope to overcome bacterial pathogen by utilizing this mechanism.

Several previous studies show some approach in the QS inhibition. Two approaches which are widely used are to destroy the AHL (Acyl Homoserine Lactone) on QS using amidase, asilase, or laktonase[1,2,3] and using AHL analog

which is compete with the AHL in interacting with regulatory proteins[4,5]. *Delisia pulchra* known as the most effective substances to inhibit QS[6]. However, at high concentrations this compound is toxic. This underlies QS inhibitors further exploration. Indonesia medicinal plants, which are traditionally serves in the treatment of infectious diseases, thought to have potential in inhibiting QS of PA.

MATERIALS AND METHODS

Preparation of Medicinal Plant Ethyl Acetate Extracts

Medicinal plants are dried and ground up into powder. One gram of finely powdered herbs were weighed and added to 5 ml of ethyl acetate and shaken on a shaker at room temperature for 24 hours. After 24 hours, ethyl acetate is evaporated with a rotary evaporator. Dried extract was weighed and dissolved in 5 ml of ethanol pa[7].

Preparation of AHL PA

24-hour culture of 10 ml of PA O1 centrifuged at 11 000 rpm for 10 minutes. Supernatant was sterilized using 0.2 µm Whatman membrane filters and is called AHL PA (Adonizio, 2007)[8].Penyiapan Kultur Reporter *Escherichia coli* XL1 pSB1075

Preparation of Reporter

A single colony of Escherichia coli pSB1075, inoculated into 10 ml Luria Bertani Broth-amp medium. After incubated in a shaker incubator at 120 rpm at 37 º C for 18 hours, then the culture used as a reporter on a test using a microplate (Lucyana, 2008)[7].

Effectiveness Test of Ethyl Acetate Extracts of Medicinal Plants For QS Inhibitors

Concentration variation of ethyl acetate extract was obtained by performing variations of the volume of the extracts were added to the microplate well, prior adjusted with absolute ethanol up to 210 µL. Solvent is then evaporated by placing microplate in a preheated oven at ± 40-50 0C for 24 hours. Microplate which has been dried, added with 41.7 µL and 100 µL culture AHL reporter PA O1. Each well adjusted with LB media to a final volume of 210 µL per well. Reporter culture as much as 41.7 µL with 100 µL (AHL) O1 PA culture supernatant used as a negative control. The microplate then incubated at 37 º C for 1 hour.

Analysis of QS Inhibitor Effectiveness

QS inhibitor acitivity observed by following reporter luminescence at each different concentration of the extract. The luminescence observed by capturing existing light using X-ray film negatives. The negative films are processed at one of the clinical laboratory located in Surabaya. Interpretation of the results was done with the help of the program MILDA (Digital Automated Microplate Analyzer luminescence). In general, the program will provide a high value on the black color negative film. The black color also shows that there is greater luminescence on micrioplate well in that section. One of the medicinal plants that showed the bestQS inhibitor activity , then tested to see its effect on the formation of protease LasA, rhamnolipid and biofilm.

Las A protease Test

O1 PA that has been incubated on a wide variety of concentrations of ethyl acetate extract of fennel for 12 hours was centrifuged at 11 000 rpm for 20 minutes (4oC). Supernatant obtained subsequently sterilized using 0.02 µm Whatman membrane filter. A total of 750 µL sterile supernatant was added to the microtube which contained 6.75 µL of culture SA (Optical Density / OD 0.6). This mixture was incubated at room temperature and measured value of A600 at 30, 60 and 90 minutes after incubation.

Biofilm Formation Test

PA O1 12-hour culture was transferred into an erlenmeyer in which there has been the ethyl acetate extract of fennel that has evaporated. Cultures were incubated further for 3 days. Biofilms then taken using filter paper. Biofilm number was determined by measuring a constant weight of biofilm on filter paper.

Rhamnolipid Formation Test

Supernatant of 12 hours O1 PA culture was acidified at pH 2 and centrifuged at 4^{o}C, 9000 rpm for 20 minutes. Pellet resulted was dissolved in 750 mL ethyl acetate and centrifuged at a temperature of 20^{o}C with a speed of 9000 rpm for 10 minutes. A total of 500 mL of organic phase then adding into a new microtube, and then heated at 70 °C. After the ethyl acetate evaporated, into the microtube was added 100 µL and 900 µL aquades and orcinol reagent prior heated at a temperature of -80 °C for 30 minutes. The solution obtained is cooled for 15 minutes and measured absorbance at 421 nm.

RESULTS AND DISCUSSION

Six medicinal plants selected in this study are: fennel fruit (Foeniculum vulgare), bunga lawang (*Illicium verum*), selasih (*Ocimum basilicum*), temu ireng (*Curcuma aeruginosa)*, temu giring (*Curcuma heyneana)*, temu putih (*Curcuma zeodaria)* dan temu lawak *(Curcuma xanthorriza.* Based on preliminary test was conducted to all the plants, they do not provide antibiotic activities against the reporter used in the research. This result shown that luminescence differences in this study come from different expression at the luminescence gen rather than come from the differences of reporter numbers.

The reporter has lasR gene[9], which is the QS regulator in PA. LasR protein produced by the reporter is able to bind to the auto inducer of C-12-HSL (N-Dodecanoyl-L-Homoserine lactone) thus forming a complex that is able to activate expression of the reporter luminescence. In this study, C-12-HSL, obtained from cultured PA O1 24 hours ethyl acetate extract. AHL extracted from the supernatant culture media using ethyl acetate[8]. Ethyl acetate extracts of medicinal plants expected to have AHL analogues which is compete with C-12-HSL in interacting with LasR. Las -AHL analouge complex will minimize the complex of C-12-HSL-LasR formed, so it will reduce the reporter luminecence[10].

The result of reporter luminescence inhibiton was summarized at Table 1. Almost all medicinal plants provide luminescence inhibiton to the reporter on the selected concentration. Luminescence decreased up to 65.4% compare to the control is the highest luminescence inhibition. It was shown by the ethyl acetate extract of fennel plant at concentration 19 mg / ml. Meanwhile temu giring show a relatively low luminescence inhibition at each concentration tested. Inhibition of reporter luminescence did not appear linear at all concentrations of tested plant

extracts. In plants such as fennel inhibition of luminescence increases as the concentration of ethyl acetate extracts were added decreased from 23.8 mg / ml to 19.0 mg / ml. However, the luminescence inhibition decreased when the concentration of ethyl acetate lowered back to 14.3 mg / ml. Something similar happened to temulawak. At the temu putih there was an increase luminiscence inhibition on each decreased concentrations of ethyl acetate extract of the plant. Instead there is a reduction in luminescence inhibition on any reduction in the concentration of ethyl acetate extracts of four other plants.

The phenomenon of luminescence changes in the different of concentration of ethyl acetate extract in accordance with the results obtained by some previous researchers. In general the greater the concentration of QS inhibitors, then the intensity of light produced will smaller[11,12] . This happens because more and more analog AHL on ethyl acetate extracts, causing a growing number of proteins that will be occupied by the LasR AHL analog and minimize the chance LasR to bind to the C-12-HSL. AHL analog complex - not able to induce expression of LasR luminescence, so it will minimize luminisensi happens.

On the other side of the maximum QS inhibition at a certain concentration which further decreased in the higher concentration also experienced by several other researchers. In general it has been observed the same molecule capable of inducing QS it also can be QS inhiibitor. AHL analog molecules are not purely antagonist, but also has partial agonist properties. Geske et.al[13] using synthetic AHL analogues found that 60% of compounds that are antagonists also have agonist properties at specific concentrations. Two opposite properties in the same molecule is due to disturbance of balance in the hydrogen bonds that occur and that there is steric hindrance between the AHL and the receptor analog (regulatory proteins).

Table 1. Luminescence Inhibition Result

Medical Plants	Concentration (mg/ml)	Luminescence Inhibition (%)	Medical Plants	Concentration (mg/ml)	Luminescence Inhibition (%)
Fennel	23.8	59.2	Temu Giring	38.1	16.3
	19.0	65.4		19.0	3.9
	14.3	53.9		9.5	7.9
Lawang	23.8	34.1	Temu Putih	38.1	-0.3
	19.0	19.2		19.0	4.9
	14.3	-3.2*		9.5	28.8
Selasih	23.8	59.8	Temu lawak	38.1	16.5
	19.2	47.9		19.0	23.5
	14.3	44.0		9.5	6.3
Temu Ireng	38.1	44.1			
	19.0	39.7			
	9.5	7.7			

* = minus means it was increase at reporter luminescence

Reporter luminescence inhibition is indirect evidence that the ethyl acetate extracts of medicinal plants have attempted inhibit the activity of PA QS O1. To obtain direct evidence of the existence of barriers QS, we explored further the influence of ethyl acetate extracts of plants to the production of proteases LasA, rhamnolipid and biofilm, which are also influenced by QS. Medicinal plants selected for this testing is the fruit of fennel, since the ethyl acetate extract of the fruit of these

plants provide the largest reduction in reporter luminescence test. The test results obtained are summarized in table 2 and table 3.

Tabel 2. LasA Activity Test Result

Concentratin (mg/ml)	Bacterial Concentration at Certain Time (Minutes)			% Decrease of SA Optical Density	
	30	60	90	60	90
Kontrol	0.12	0.11	0.11	8.33	8.33
0.15	0.12	0.11	0.11	8.33	8.33
0.25	0.11	0.10	0.09	9.09	18.18
0.51	0.11	0.10	0.09	9.09	18.18
1.02	0.11	0.10	0.09	9.09	18.18
1.52	0.11	0.11	0.11	0	0
2.03	0.13	0.13	0.13	0	0

Las A used by PA, when the bacterium infects its host cell. In SA, these enzymes will break the amide bond between D-alanine-D-alanine thus destroying the SA peptidoglycan[14]. SA-LasA mixed, thus going through lysis and SA culture absorbance will decline after some incubation time. In this study the variation between SA and LasA incubation performed in the range of 30, 60 and 90 minutes. LasA activity was calculated by considering the absorbance of the SA at any time of the experiment. Table 2 shown no LasA activity, until the concentration of 0.15 mg / ml of ethyl acetate extract. The decrease of absorbance increase in the concentration of ethyl acetate extract of 0:25 mg / ml to 1:02 mg / ml and no visible decrease in absorbance at the concentration of ethyl acetate extract of 1:52 mg / ml and 2.03/ml.

The greater% decrease in absorbance indicates more active LasA. At concentrations that increased the range of 0:15 mg / ml to 1:02 mg / ml seems the addition of Las activity A. This shows the nature of agonist ethyl acetate extract of fennel. But on the contrary at higher concentrations ie at 1:52 mg / ml and 2:03 mg / ml seen any antagonist properties ethyll acetate extract of fennel. This phenomenon reinforces the effect of ethyl acetate extract of fennel as shown in Table 1.

Judging by the results obtained in this study which showed that the concentration of 1:52 mg / ml of ethyl acetate extract of fennel has been able to stop the decline in OD SA, meaning that production of LasA by PA has been stopped close to 100% at this concentration. It is far more effective than Andonizio[15] research who conduct tests on a variety of spices and get highest QS inhibition to extract B. Buceras (black olive) which is able to reduce LasA production by 96% in concentration 1g/ml crude extract. Further studies on the ethyl acetate extract of fennel plant needs to be done to further confirm these results related to types of compounds contained in the ethyl acetate extract of fennel.

Tabel 3 Result of Rhamnolipid and Biofilm Production Inhibition

Concentration (mg/ml)	Rhamnolipid		Biofilm	
	Absorbance ± Sd	Production Decrease (%)	Weight (gram) ± Sd	Production Decrease (%)
Kontrol	0,42 ± 0,005	0	0,017 ± 0,000529	0
0.15	0,40 ±0,008	4.8	0,015 ± 0,001504	11.8
0.25	0,36 ±0,004	14.3	0,021 ± 0,002762	-17.7*
0.51	0,39 ± 0,007	7.1	0,026 ± 0,000351	-52.9
1.02	0,36±0,004	14.3	0,028 ± 0,000351	-64.7
1.52	0,35 ± 0,006	16.7	0,031 ± 0,001513	-94.1
2.03	0,34 ± 0,003	19.1	0,038 ± 0,001670	-123.5

* = minus means addition in the production of certain metabolite

Metabolite which influenced the next QS studied is rhamnolipid and biofilm. As the surfactant, rhamnolipid provide role when bacteria will stick to the surface of the host tissue. It is expected that ethyl acetate extract has an analog AHL molecules will decrease the production of rhamnolipid PA O1. The same is expected in the formation of biofilms produced by the PA as an agent colonizes their place. The results of experiments on both types of metabolites are summarized in Table 3.

Table 3 generally shown opposites thing of plant extract influence to the PA metabolite production. The extract will effect to the reduction of rhamnolipid production (except for the concentration 0:51 mg / ml of the fennel extract). Another thing is that plant extract induce biofilm production with increasing concentrations of ethyl acetate extract of fennel. This shows that the same molecule has the possibility to give different effects on the mechanism of QS PA.

In general this phenomenon is almost the same as the QS regulation scheme which is proposed by the Christian et.al (1998)[16] who explained that C-12-HSL is able to repress RHL on the PA system, which in turn suppresses the production of rhamnolipid. At the same time the C-12-HSL also trigger the activation of genes involved in biofilm formation. Given the initial screening in this study was conducted using a reporter who has a welding system, then the reporter will be more sensitive to the analog C-12-HSL on the welding system and not the C-4-HSL on RHL systems. This shows also that the AHL analogues compounds contained in the ethyl acetate extract of fennel can interact with the regulator of biofilm production and rhamnolipid.

On the other hand the results of a study similar to the phenomenon in this study are found in the compound indole. Indole class of compounds able to repress virulence factors such as rhamnolipid, pyocyanin and pyoverdin[17]. Yet it is precisely these compounds trigger antibiotic resistance and biofilm formation on the PA. The mechanism of how the biofilm production-induced has not been found to be clear, it's just been proven that these compounds do not trigger the stress on the bacterial culture. The author estimates that there are compounds in the ethyl acetate extract of fennel seeds that have the ability as the indole group.

CONCLUSION

1. Ethyl acetate extracts of fennel fruit (Foeniculum vulgare), anise (Illicium verum), basil (Ocimum basilicum), Intersection ireng (Curcuma aeruginosa), meeting dribbles (Curcuma heyneana), and temu lawak (Curcuma xanthorriza) have capability to inhibit Pseudomonas aeruginosa quorum sensing.

2. Ethyl acetate extract of fennel 2:03 mg / ml inhibit of Pseudomonas aeruginosa rhamnolipid production up to 19:05% and induces the production of biofilms up to 123.53%. Ethyl acetate extract of fennel 1:52 mg / ml is able inhibit of Pseudomonas aeruginosa Las A production up to 100%.

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