PROCEEDING

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International Conference on Biological Science
Faculty of Biology Universitas Gadjah Mada 2011
(ICBS BIO-UGM 2011)

ADVANCES IN BIOLOGICAL SCIENCE

Education for Sustainable Development-based Tropical Biodiversity Management and Conservation for Supporting Human Prosperity

September 23rd-24th 2011
Yogyakarta, INDONESIA

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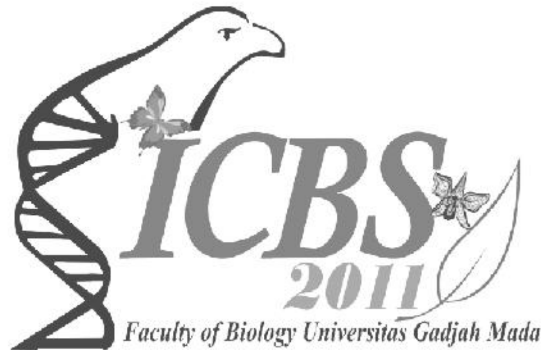

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FACULTY OF BIOLOGY
UNIVERSITAS GADJAH MADA

I-MHERE
PROJECT

PROCEEDING ICBS BIO-UGM 2011

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PREFACE

Proceeding of the **International Conference on Biological Science Faculty of Biology Universitas Gadjah Mada 2011 (ICBS BIO-UGM 2011), Advances in Biological Science: Education for Sustainable Development-based Tropical Biodiversity Management and Conservation for Supporting Human Prosperity,** organized by and held at the Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia on September 23-24, 2011. The conference addressed a range of important research from various fields in biological science likely to play role tropical biodiversity management and conservation for supporting human prosperity. Three kinds of session were held at the conference: plenary session featuring keynote and invited papers, oral presentation session, and poster presentation session. This proceeding features a number of papers presented in these sessions, which represent 5 themes covered in the conference, i.e. genetics and molecular biology, ecology and conservation, systematics and evolution, physiology and developmental biology, and biomedics.

Many people have been involved in the production of these Proceedings, which is started in June 2011 with the launching of a call for abstracts. The abstracts were reviewed by both internal and external reviewers . Those selected abstracts were called for either oral or poster presentations and invited to submit full papers.

Lastly, on behalf of the organizing commite we would like to all participants for their kindness to be part of this conference. We would like to acknowledge each partnerships and sponsorship that involve during this event. I believe that this proceeding still has some weaknesses, therefore any constructive comments are welcome. We hope that the papers contain in this proceeding will prove helpful toward improving the scientific atmosphere. See you in the next two year ICBS 2013.

Yekti Asih Purwestri

Chair of the Organizing Commitee

WELCOMING SPEECH FROM CHAIR PERSON OF THE ORGANIZING COMMITTEE

Distinguish guests
- Executive Director of Indonesia-Managing Higher Education for Relevane and Efficiency (I-MHERE) Project
- Keynote speaker, invited speakers, participants, sponsorships, ladies and gentlemen

Good morning and May God shower us with His blessing.

On behalf of the Conference Organizing Committee, I extend a warm welcome to all participants to the second **International Conference on Biological Science Faculty of Biology Universitas Gadjah Mada 2011 (ICBS BIO-UGM 2011), Advances on Biological Science : Education for Sustainable Development-based tropical biodiversity management and conservation for supporting human prosperity.** Bio-conservation becomes a critical issue not only in Indonesia but also in global community. A good understanding on Education for Sustainable Development- based tropical biodiversity management is necessary to have the right policy regarding bio-conservation action.

For this year, the organizing committee has put together an interesting Scientific Program to accommodate the areas of Biology. The Program comprises of 6 plenary sessions of keynote and invited speakers. The parallel session of 82 oral presentations and more than 50 poster presentations. I realize that you are fully dedicated to the sessions but I do hope that you all will also take time to enjoy Yogyakarta, the multicultural city and may enjoy the special Merapi scenery, the most active volcano in the world.

I would like to take the opportunity to thank Prof Hubert Gijzen (Director of UNESCO-Jakarta) as a keynote speakers and also to these following invited speakers, Hao Yu, Ph.D (National University of Singapore), Prof. Christ Austin (Charles Darwin University, Australia), Prof. Yasumasa Bessho, Ph.D (Nara Institute of Science and Technology, Japan), Dr. Yam Tim Wing (Senior Researcher Orchid Breeding and Conservation Singapore Botanic Gardens), Drs. Langkah sembiring, M.Sc. Ph.D (Faculty of Biology, Universitas Gadjah Mada) for delivering their valuable scientific information.

To make this program happen, I would like to gratefully acknowledge to Indonesia-Managing Higher Education for Relevane and Efficiency (I-MHERE) which support this conference. We also thank to the valuable contributions from personal and institutional sponsorship and funding including Ms. Sachiko Iida, PT Diastika Biotekindo, PT Roche, Prima Grafika Yogyakarta., and Drs. Agus Suryanto - Indogama Yogyakarta.

I also gratefully thank to the Dean and Vices Dean of Biology Faculty, Universitas Gadjah Mada for giving us opportunity and support to organize this conference. My deep appreciation to the Steering Committee, the Academic Reviewers (internal and external: Dr. Sentot Santoso from Institut fuer Klinische Immunologie und Transfusionsmedizin, Justus Liebig Universitaet Giessen, Germany and Prof. Yasumasa Bessho, Ph.D from Gene Expression Research, Biological Sciences, Nara Institute of Science and Technology, Japan), members of the Organizing Committee for their strong support, active participation, cooperation and hard works in preparing and organizing this event a success.

It is inevitable that there is a lack in organizing this conference and I profoundly apologize to all invited speakers, oral and poster presenters, attendants, donators and committee members.

I wish you a pleasant and rewarding two days of scientific discussion.

Thank you,

Yekti Asih Purwestri
Chair person of the Organizing Committee

OPENING REMARKS FROM THE DEAN of THE FACULTY OF BIOLOGY

Bismillahirrahmaanirrahiim.

Director of UNESCO Office Jakarta, Prof. Dr. Hubert Gijzen,
Executive Direktor of Indonesian-Managing Higher education for relevance and Efficiency (I-MHERE) Project
Honorable speakers and distinguished guest, dear participants,

Assalamu'alaikum wr.wb., may God give us healthy and happier life

Welcome to Yogyakarta, the city of youth, education, and culture. It's been an honour for me to be here in front of you to open the prestigious **International Seminar with the special theme of "Advances in Biological Science: Education for Sustainable Development-based Tropical Biodiversity Management and Conservation for Supporting Human Prosperity",** that invited our honorable speaker from the UNESCO as the keynote, Prof. Hubert Gijzen, Ph.D honorable invited speakers Dr. Yam Tim Wing From Singapore Botanic Garden, Singapore; Prof. Yasumasa Bessho, MD, Ph.D from NAIST, Japan; Prof. Christopher M. Austin, Ph.D from Charles Darwin University, Australia; Dr. Yu Hao from National University of Singapore, and Dr. Langkah Sembiring MSc, from the Faculty of Biology, Universitas Gadjah Mada, Indonesia.

My special gratitute to the speakers who have spent your time travelling to Indonesia in your such busy activity. This international seminar atrracts more than 400 scholars and students mostly come from Indonesia, and some participants come from abroad. This occassion is such a good opportunity for us to share our experiences in research and good practices of ESD based research and community service done, that could inspire students and other researchers, furthermore our keynote speaker today is the Director of UNESCO Jakarta Office, who will talk about Science, Technology and Innovation-an Engine for Sustainable Development.

Honorable and distinguished participants,

The seminar theme taken today is in line with vission of the Faculty of Biology UGM as the center of excellence for higher education that generates biologists who respect to our tropical biodiversity. Since 2010, Faculty of Biology UGM had obtained an ESD based research grant from the World Bank, through I-MHERE (Indonesian Management of Higher Education for Efficiency and Relevance) project. In this project has been conducted 3 activities, these are: improvement of publication and research quality, improvement of integrated collaboration research in tropical diversity with other Institutions, and community based activities that respect to biodiversity conservation. As stated in UNESCO HE information brief, the challenge for higher education in the context of ESD is to innovate the traditional learning environment and learning processes in such a way that they do not only support learning process in the formal education, but also in informal learning.

Our environment is now facing many dilemmas starting from global financial and economic crises highlights the risks of unsustainable economic development models and practices based on short-term goals. These aspects triger economic disparity between the poor and the rich countries, many complex societal contexts, and finally environmental degradation.

Education for Sustainable Development (EfSD) promotes quality education and its inclusive for all people. It is based on values, principles, and practices necessary to respond effectively to current and future challenges. UGM has shown commitment in Education for

Sustainable Development and will continue to conduct ESD in the future. I hope that this Conference will continue to serve as a sustainable forum to provide opportunities for teachers, lecturers, researchers and professionals to share experience and present research activities and action programs. To everyone present here, I wish you have a productive and significant Conference that will benefit humankind, civilization as well as knowledge.

Lastly, I would like to extend my sincere appreciation and profound gratitude to the Director of UNESCO Jakarta and NAIST Japan for their supports. My special thanks should also go to the steering and organizing committee for their hard work in making this event a success. Thank you very much.

Yogyakarta, September 23rd, 2011

Sincerely yours,

Dr. Retno Peni Sancayaningsih, MSc.

WELCOMING SPEECH FROM EXECUTIVE DIRECTOR I-MHERE UGM

Honorable Dean of Faculty of Biology UGM, Dr. Retno Peni Sncayaningsih, M.Sc.
Distinguish Keynote speaker Prof Hubert Gijzen (Director of Unesco in Indonesia)
Distinguish Dr. Yam Tim Wing (Singapore), Prof. Yasumasa Bessho (Japan), Prof Christ Austin (Australia), Dr. Langkah Sembiring (UGM),
Dr. Yu Hao (Singapore)
Distinguish all of participants

Assalamu'alaikum wr.wb.

Welcome to Yogyakarta and participating in International Conference on Biological Science, by Faculty of Biology UGM.

This seminar was supported by IMHERE UGM (Indonesia Managing Higher Education for Relevancy and Efficiency). As we know, UGM get a competitive grant from World Bank trough Directorate General of Higher Education, from 2009 – 2012, and proposed program entitled "Education for Sustainable Development toward World Class Research University" by establishment of Center of Excellence (CoE) on 3 selected academic units, namely (i) "Tropical Biodiversity", in Faculty of Biology (ii) "Medical Herbal and Supplements" in Faculty of Pharmacy and (iii) "Reduction Emission from Deforestation and Degradation (REDD)" in Faculty of Forestry.

Faculty of Biology has attempted for enhancement of the research quality on tropical biodiversity, development of the integrated research on utilizing biodiversity resources to enhance the EfSD and development of network capacity for national and international collaboration on research and community services through Regional Centre of Expertise (RCE) Yogyakarta.

This prestigious international seminar is one of our strategic activities to achieve better key performance indicator, especially in international publication and international research collaboration. As a new paradigm of competitive grant that developed by World Bank, called "Performance Based Contracts", achievement of our key performance indicator in this year was 190% compare to targeted indicator for three years activities. We would like to continuing our "Research based Learning and Services for sustainable reputation as World Class Research University.

Please be enjoy to discuss and active participating in this seminar.

Wassalamu'alaikum wr.wb.

Sincerely yours,

Executive Director I-MHERE UGM

Dr. Cahyono Agus Dwikoranto, M.Agr.Sc.

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PLENARY SESSIONS

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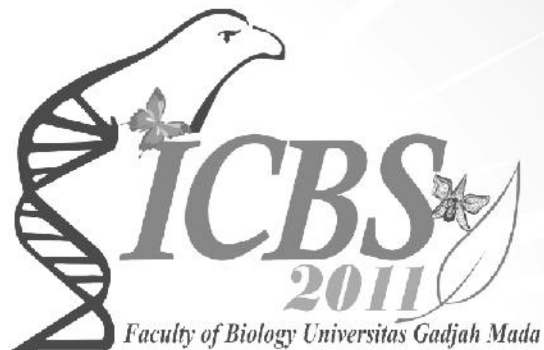

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FACULTY OF BIOLOGY
UNIVERSITAS GADJAH MADA

I-MHERE
PROJECT

O-BM09

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**

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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 ° 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 ° 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°C, 9000 rpm for 20 minutes. Pellet resulted was dissolved in 750 mL ethyl acetate and centrifuged at a temperature of 20°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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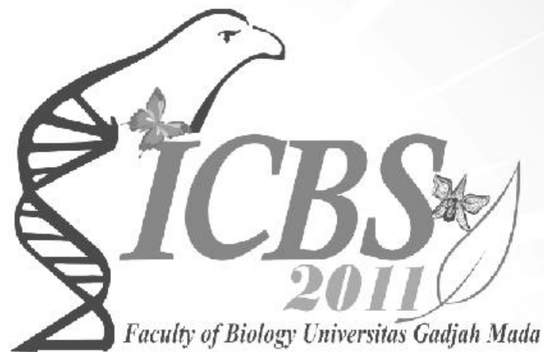

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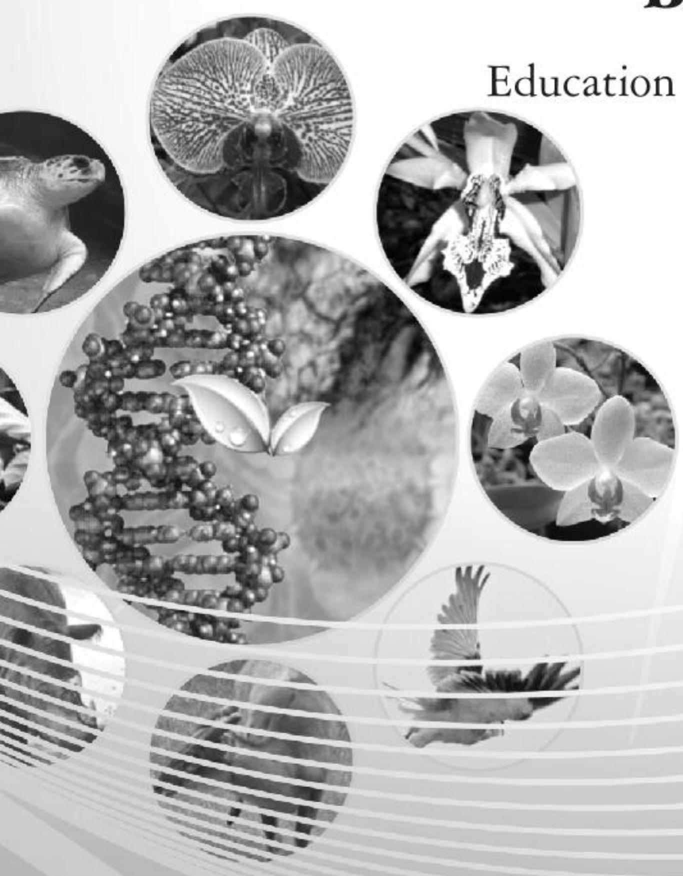

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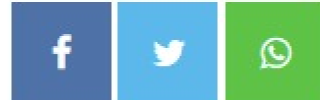
Berdasarkan data dari Sekertaris Panitia, ibu Ardaning Nuriliani, S.Si., M.Kes., konferensi ini dihadiri lebih dari 400 orang, berasal dari berbagai negara antara lain Indonesia, Jepang, Australia, Belanda dan Afrika. Sebanyak 82 makalah akan disampaikan dalam bentuk presentasi oral dan 59 makalah disampaikan dalam bentuk poster. Semua makalah tersebut meliputi 5 kelompok topik yaitu 1) *Molecular Biology, Genetic and Bio-informatics*; 2) *Ecology and Conservation*; 3) *Systematic and Evolution*; 4) *Physiology and Developmental Biology*; dan 5) *Bio-medics*, imbuh beliau.

Pada konferensi yang dibuka secara resmi oleh Dekan Fak. Biologi UGM (Dr. Retno Peni Sancayaningsih, M.Sc.), Prof. Hubert Gijzen (*Director of UNESCO in Indonesia*) didaulat untuk menjadi pembicara kunci pada acara ini. Beliau menyampaikan bahwa kemajuan teknologi hendaknya sejalan dengan tindakan konservasi alam. Acara ini dihadiri lima pakar sebagai pembicara tamu, yaitu: Dr. Yam Tim Wing (Singapore Botanic Garden, Singapore) yang akan membawakan makalah tentang *Physiology and Developmental Biology*, Prof. Yasumasa Bessho (Graduate School of Biological Sciences, Nara Institute of Science and Technology, Japan) dengan makalah bertema *Biomedics*, Prof. Chris Austin (School of Environmental and Life Sciences, Charles Darwin University, Australia) untuk bidang *Ecology and Conservation*, Drs. Langkah Sembiring, M.Sc., Ph.D (Faculty of Biology, Universitas Gadjah Mada, Indonesia) dengan makalah yang bertema *Systematic and Evolution*, dan Dr. Yu Hao (Department of Biological Science, National University of Singapore, Singapore) akan membawakan makalah untuk bidang *Molecular Biology, Genetic, and Bioinformatics*.

Sebagai rangkaian acara ICBS 2011, sehari sebelumnya (22/09) telah diadakan *Stadium General* tentang *Animal Cell Culture* oleh Prof. Yasumasa Bessho. Acara yang dilaksanakan di Ruang Sidang Pascasarjana Fakultas Biologi ini kemudian dilanjutkan dengan Praktek laboratorium di Fasilitas penelitian Bersama (FALITMA) Fak. Biologi UGM. (**ZR**)



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O-BM09

Screening of Indonesia Medicinal Plants Producing Quorum Sensing Inhibitor

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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°C, 9000 rpm for 20 minutes. Pellet resulted was dissolved in 750 mL ethyl acetate and centrifuged at a temperature of 20°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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Screening of Indonesia Medicinal Plants Producing Quorum Sensing Inhibitor

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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°C, 9000 rpm for 20 minutes. Pellet resulted was dissolved in 750 mL ethyl acetate and centrifuged at a temperature of 20°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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