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ANTI QUORUM SENSING ACTIVITY OF KAYU MANIS LEAVES EXTRACTS (*Cinnamomun burmannii* Ness. Ex Bl.) AGAINST *Pseudomonas aeruginosa*

DAYA *ANTI QUORUM SENSING* EKSTRAK DAUN KAYU MANIS (*Cinnamomun burmannii* Ness. Ex Bl.) TERHADAP *Pseudomonas aeruginosa*

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ABSTRACT

Quorum sensing is a communication system among bacterial cells which correlates with biofilm formation. Biofilm can protect bacteria from environment including antibiotic of which can cause higher antibiotic concentration of 100 up to 1000 times. Inhibition of quorum sensing is expected to inhibit the biofilm formation. The cinnamon bark (Cinnamomum burmanii Ness. Ex Bl.) has been known to have antibacterial and antibiofilm activities. Leaves are available abundantly which urges a research to find out the activity as anti quorum sensing against Pseudomonas aeruginosa. The succesive maceration of dried pulverized leaves produced hexane, ethyl acetate, and methanol extracts. Antibacterial activity was observed by microdillution method with MTT assay. Afterwards, the active extract was examined for anti quorum sensing activity by diffusion method in cetrimide Agar. Quorum sensing activity was shown by dark zone (opaque) growth around sample application, observed under UV light of 366 nm. TLC bioautography method was done to observe the active spots by using silica gel F254 as the stationary phase, chloroform-methanol (6:1 v/v) as the mobile phase, loading sample used was 1.25 mg and 30 min of plate contact duration. The ethyl acetate extract inhibited growth of P. aeruginosa with shown by MIC at 8 µg/µL Quorum sensing as well as growth inhibition activities were observed at loading samples 12,5 and 25 mg/wells, while at 6,25 mg the extract only exhibited quorum sensing inhibition. Presences of substances having phenolic, flavonoid, alkaloid and aldehyde/keton as functional groups were detected by TLC method of the extract but no active spot identified on bioautography.
Keywords: Quorum sensing, Pseudomonas aeruginosa, Cinnamomum burmanii, and Bioautography

ABSTRAK

Quorum sensing adalah sistem komunikasi antar sel bakteri. Perkembangan biofilm diatur oleh quorum sensing. Biofilm dapat melindungi bakteri dari kerusakan akibat antibiotik sehingga konsentrasi antibiotik harus ditingkatkan 100 sampai 1000 kali lipat. Penghambatan quorum sensing diharapkan dapat mencegah pembentukan biofilm. Kulit kayu manis (Cinnamomum burmanii Ness. Ex Bl.) memiliki aktivitas antibakteri dan antibiofilm. Ketersediaan daun yang melimpah menyebabkan dilakukan penelitian untuk mengetahui apakah daun kayu manis memiliki daya anti quorum sensing terhadap Pseudomona aeruginosa. Serbuk kering daun kayu manis dimaserasi bertingkat berturut-turut menggunakan n-heksana, etil asetat dan metanol. Setelah masing-masing ekstrak dievaporasi, daya antibakteri diamati dengan metode mikrodilusi menggunakan penambahan MTT. Ekstrak yang aktif diamati daya anti quorum sensing nya dengan metode difusi pada cetrimide Agar. Aktivitas antiquorum sensing ditunjukkan dengan daerah buram di bawah sinar UV 366 nm.

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KLT-bioautografi dilakukan untuk mengetahui senyawa yang aktif dalam fraksi tersebut dengan menggunakan fase diam silika gel F254, fase gerak kloroform-metanol (6:1 v/v), loading sampel sebanyak 1,25 mg dan waktu kontak lempeng dengan medium selama 30 menit. Penelitian menunjukkan bahwa fraksi etil asetat memiliki aktivitas antibakteri terhadap P. aeruginosa dengan nilai KHM 8 μg/mL. Aktivitas penghambatan quorum sensing dan pertumbuhan ditunjukkan pada loading samples 12,5 dan 25 mg/sumuran, sedangkan pada 6,25 mg ekstrak hanya menunjukkan aktivitas penghambatan quorum sensing. Keberadaan senyawa yang memiliki gugus fungsi fenolik, flavonoid, alkaloid dan aldehid/keton terdeteksi dengan metode KLT tetapi tidak terdeteksi bercak aktif pada bioautografi.
Kata kunci : quorum sensing, Pseudomonas aeroginosa, Cinnamomum burmanii, bioautografi.

INTRODUCTION

Quorum sensing is a bacterial cell to cell communication system occured either in Gram negative and positives. *Acylated homoserine lactone* (HSL) was reported to be as a signal for this communication. Biofilm formation is one of bacterial fuction regulated by quorum sensing (Whiteley *et al*., 1999).

Biofilm is a bacterial community attached to a surface to form a micro environment which further gives protection against antibiotics, host immune system, etc. This can cause higher antibiotic concentration needed up to 100 times to eradicate the bacteria (Costerton *et al*., 1994, Koch and Hoiby, 1993). *Pseudomonas aeruginosa* is an opportunistic pathogen reported in many nocosomial infections (Dzen *et al.,* 2003). Biofilm formed by this bacteria can cause persistent lung infection in cyctic fibrosis patients and also eye infection related to contact lenses usage (Costerton *et al*., 1999).

Hertiani *et al*. (2011) reported that the essential oils of cinnamon bark exhibited antibiotilm against *Streptococcus mutans* of which a phenolic was detected as antibacterial active. According to Khan *et al*. (2009) the cinnamon oil exhibited bacterial anti quorum sensing activity. Moestafa (1998) reported the major constituents of *C. burmanii* were linalool 24,33%, cinnamoil acetate 10,75%, caryophylene 9,08%, dan trans-cinnamaldehyde 7,29%. According to Wang *et al.* (2008) the chemical constituents of cinnamon oil extracted from the bark had similarity to that from the leaves. The cinnamaldehyde was observed to be significantly active in reducing biofilm formation on *E.coli* dan *P.aeruginosa* (Niu and Gilbert, 2004). It is interesting to find out whether the leaves are active as an anti quorum sensing against *Pseudomonas aeruginosa.*

METHODOLOGY

Materials

Cinnamon leaves were collected from a Cinnamon Farming in Semarang, Indonesia. Cetrimide Agar (E Merck), silica gel F254 precoated TLC plates, Ceftazidim, *Pseudomonas aeruginosa* ATCC 27853, MTT (3-(4,5-dimethylthiazole-2-il) 2-5-diphenyltetrazolium-bromide), cinnamaldehyde standard, UV lamps, 254 and 366 nm.

Methods

Five hundred (500) g of dried-pulverized leaves was macerated in hexane for 24 h. Following solvent evaporation, hexane extract was collected. The residue was macerated in ethyl acetate to yield ethyl acetate extract. Afterwards, the residue resulted from ethyl acetate extract was dried and macerated further in methanol. Each macerates was evaporated to yield solvent-free extract.

Each extract was diluted in DMSO to obtain concentrations of 1, 2, 4, and 8 /μL. Nutrient broth, and bacterial suspension (1.5 x 10^8 CFU/mL) were added to reach a total of 100 μL. Incubation was taken place at 36.6^{0}C for 18-24 h. Controls used were media, media+bacteria, media+DMSO +bacteria, media+antibiotic+bacteria. After 18-24 h incubation, each well was added with MTT and observed for the color changes afterwards visually. Experiments were done in triplicate.

Base Agar was made by pouring sterile cetrimide Agar (30 mL) into a 14 cm diameter petri dish and left to solidify. Top Agar (10 mL) containing 100 μl bacterial suspension (1,5 x 10^8 CFU/mL) was poured, afterwards. Perforator (8 mm diameter) was used to make wells which then filled with 25, 50 or 100 μL of extracts (250 mg/mL stock solution). After being incubated at 30^{0}C for 24 h, anti quorum sensing activity was observed under 366 nm UV lamp. Pigment formation inhibition seen as an opaque zone around wells indicates quorum sensing inhibition while antibacterial activity will be indicated by a transparent zone.

In order to find out the active constituents, active extract was diluted in an appropriate solvent to reach a concentration of 250 μg/μL of which samples was applied onto a silica gel plate as 10 parallel spotting (5 μL each). After elution, the plate was left dried and then was separated as 10 plates for different treatments. One eluted plate

Table I. Rendemen and organoleptics of extracts

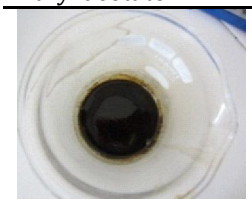
Extract	Colour	Weight	Odor	Rendemen (calculated based on 500 g simplicia)
 n-Hexane	Black	16,078 g	Aromatic, typical odor of cinnamon	3,22 %
 Ethyl acetate	Dark green	15,94 g	Aromatic, typical odor of cinnamon	3,19 %
 Methanol	Dark brown	24,073 g	Aromatic, typical odor of cinnamo.	4,81 %
Total				11,22%

Table II. Result of antibacterial activity of extracts against *P.aeruginosa.*

Extracts	Replication	Results			
		8 µg/µL	4 µg/µL	2 µg/µL	1 µg/µL
n-Hexane	1	Purple	Purple	Purple	Purple
	2	Purple	Purple	Purple	Purple
	3	Purple	Purple	Purple	Purple
Ethyl acetate	1	Clear	Purple	Purple	Purple
	2	Clear	Purple	Purple	Purple
	3	Clear	Purple	Purple	Purple
Methanol	1	Purple	Purple	Purple	Purple
	2	Purple	Purple	Purple	Purple
	3	Purple	Purple	Purple	Purple

Note: purple indicates bacterial growth, clear indicates no bacterial growth observed

was put onto a cetrimide Agar containing bacterial suspension and left for 30 min. Afterwards, the plate was taken out and the Agar was incubated at 37^0C for 24 h. Spot causing pigment inhibition which is observed under 366 nm UV lamp indicates a presence of an anti quorum sensing substance (Nagy, 2010). Other plates were observed for phytochemical assays using 254 and 366 nm UV lamps, as well as spraying reagents: $FeCl_3$, annisaldehyde H_2SO_4, Dragendorff, KOH, $AlCl_3$, 2-4-DNPH. Comparing the active spot and the TLC profile is expected to give information about the type of active compound in the sample.

RESULT AND DISCUSSION

Result of succesive maceration (Table I) showed that total yield of extract was only 11,22% of which the methanol extract had the highest yield. All extracts was tested for the antibacterial activity against *P. aeruginosa*. Table II exhibited that the ethyl acetate extract was the most active extract having a MIC value of 8 µg/µL, while other extracts was not active on the tested sample concentration. Result of assay performed on controls supported that no bias results occurred due to contamination or solvent effect (Table III).

Anti quorum sensing activity was then observed on the active extract, i.e. the ethyl

Table III. Results of controls used in antibacterial assay

Control	Replication	
	1	2
Media	Clear	Clear
Media + bacteria	Purple	Purple
Media + solvent+bacteria	Purple	Purple
Media + antibiotic + bakteria	Clear	Clear
Media + n-hexane extract	Clear	Clear
Media + ethyl acetate extract	Clear	Clear
Media + methanol extract	Clear	Clear

Note: purple indicates bacterial growth, clear indicates no bacterial growth observed

Table IV. Result of quorum sensing inhibition assay of the ethyl acetate extract

Sample	Loading sample (mg)	Growth inhibition zone ±SD (mm, n=3)	Quorum sensing inhibition zone ±SD (mm, n=3)
Ethyl acetate	6,25	ND	13,74 ± 0,49
	12,5	14,4 ±0,67	19,63 ± 0,58
	25	17,68 ± 0,84	24,89 ± 0,68
DMSO	6,25	ND	ND
	12,5	ND	ND
	25	ND	ND
	25	ND	ND

Note: ND = Not detected; Øwells = 8 mm

acetate extract (Table IV). The extract inhibited the pigment formation in all samples. Nevertheless, only at 6.25 μg sample application, the antibacterial activity was absent, while at higher sample application, both activities were observed. This phenomenon can be explained by the fact that in a higher concentration, active substances which interrupt bacterial normal function e.g. through enzyme inactivation can inhibit growth. In a condition where the extract inhibits the bacterial growth, the quorum sensing mechanism is prohibited accordingly.

Inna *et al*. (2010) reported that the antibacterial active substances from cinnamon oil are phenolic and cinnamaldehyde. Eugenol is expected to be the active phenol derivate from the plant. The oil from the leaves was reported by Yuliani and Satuhu (2012) to be dominated by eugenol. According to Yen and Chang (2008), eugenol and cinnamaldehyde showed synergistic effect against the growth of fungi. The antimicrobial activities of those compounds are through cell protein destruction of which disrupt the cell membrane function or inactivate certain enzymes. In correlation with biofilm formation inhibition, phenolics and aldehydes possibly inactivate the related enzyme. It is interesting to find out whether these compounds are existed in the ethyl acetate extract. Considering that both are the components of the essential oils, they are expected to be occurred in the hexane extract. The fact that the hexane extract was not active as antibacterial (Table II) suggests that both compounds were absent in the extract. On the other hand, solubility might be a problem which inhibited non polar substances to exhibit antibacterial activity in aquous media. Beside, phenolic and aldehyde functional groups may cause the compounds becoming more polar and present in the more polar extract, i.e. the ethyl acetate extract. Moreover it is also common that certain chemical components in plant may act as suspending agent, e.g. saponin.

In order to ensure the present of cinnamaldehyde in the extract, a comparative TLC profile was performed with cinnamaldehyde standard. TLC chromatogram showed no similar spot suggests that no cinnamaldehyde present in the extract. Cinnamaldehyde was shown at hRf 75 while 2,4-DNPH positive spot of the ethyl acetate extract was occurred at hRf 37. Probably, the essential oil components were extracted to the hexane extract instead, but due to problem in solubility, the extract did not exhibit antibacterial activity. Furthermore, TLC profile suggests phytochemical contents of the ethyl acetate extract to be phenolic (hRf 25), alkaloid (hRf 41), aldehyde/keton (hRf 37), and flavonoid (hRf 22).

TLC-bioautography was done on the active extract to observe the active spot on TLC and further analyze the chemical group. TLC system used was silica gel F254 as the stationary phase, and a mixture of chloroform : methanol = 6:1 v/v was used as the mobile phase, loading sample was 1,25 mg. Neither quorum sensing inhibition nor antibacterial activity was observed which may be due to too small amount of sample loaded onto the TLC plate (the extract was active at loading sample 6.25 mg). Unfortunately, increasing sample load was inhibited by poor separation on the chromatogram.

CONCLUSION

The ethyl acetate extract inhibited growth of *P. aeruginosa* with shown by MIC at 8 µg/µL. Quorum sensing as well as growth inhibition activities were observed at loading samples 12.5 and 25 mg/wells, while at 6.25 mg the extract only exhibited quorum sensing inhibition. Presences of substances having phenolic, flavonoid, alkaloid and aldehyde/keton as functional groups were detected by TLC method of the extract

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