



Piper betle Leaf Extract Exhibits Anti-virulence Properties by Downregulating Rhamnolipid Gene Expression (rhlC) of *Pseudomonas aeruginosa*

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Abstract

BACKGROUND: *Piper betle* (*P. betle*) is widely used as a traditional medicine in Indonesia, with anti-quorum sensing and anti-biofilm activity. We investigated the impact of methanolic leaf extract of *P. betle* against *Pseudomonas aeruginosa*'s (*P. aeruginosa*) virulence factor, which associated with rhamnolipid (rhl) genes,

METHODS: Minimum biofilm inhibitory concentration of the extract was determined at a concentration of 0.4% by agar dilution assay. The expression of rhlA and rhlC gene was assessed by using real-time polymerase chain reaction.

RESULTS: All *P. aeruginosa* isolates contained rhlA, rhlB, and rhlC genes, which associated with rhl production. The expression of the rhlC gene decreased after administration of *P. betle* leaf extract at concentration of 0.4%, with beta coefficient was 0.662 ($p = 0.019$).

CONCLUSION: The methanolic leaf extract of *P. betle* shows inhibition of rhlC gene expression, indicating the anti-rhl properties of *P. betle* against *P. aeruginosa* infection.

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is Gram-negative bacteria which associated with a high fatality rate and antimicrobial resistance [1]. Biofilm formation is one of the main mechanisms of *P. aeruginosa* to protect itself from host's immune response and killing by antibiotics [2]. Quorum sensing (QS) pathway is an important determinant for biofilm formation of *P. aeruginosa*, including Las, rhamnolipid (rhl), pseudomonas quinolone signal, and integrated quorum-sensing signal systems [3], [4]. The rhl system is regulated by three genes: rhlA, rhlB, and rhlC, which encode corresponding proteins necessary in synthesis of rhl [5]. rhl plays important role in biofilm formation, especially for *P. aeruginosa* [4].

Piper betle (*P. betle*) (Indonesian name Sirih hijau) is widely used as a traditional medicine in Indonesia. Its leaf extract has been investigated as potent anti-biofilm and also exhibited anti-QS activity, although

the precise mechanism of action remains unclear [6], [7]. One of the most abundant compounds inside *P. betle* is eugenol, which exhibits powerful antioxidant activity [8]. Eugenol could suppress the expression of *P. aeruginosa* virulence factor such rhl and also inhibit biofilm formation [9], [10]. However, the influence of eugenol against rhl gene regulation has not been elucidated yet. We aimed to investigate the impact of methanolic leaf extract of *P. betle* against *P. aeruginosa*'s virulence factor, which associated with rhl gene.

Methods

Bacterial isolate

P. aeruginosa clinical isolate was obtained from the bacterial culture collection in the Department

of Clinical Microbiology, Faculty of Medicine, Brawijaya University and identification was confirmed by the Microbact™ Gram-negative system.

Plant material and extract preparation

The *P. betle* leaves were collected from Malang, East Java, Indonesia. The species of *Piper* was identified and confirmed by plant taxonomist of units of pulmonary toxicity dose Materia Medica, Batu, as herbarium unit in East Java, Indonesia. The extraction process was executed in Technical Chemistry Laboratory of State Polytechnic of Malang, Indonesia. The leaves were shade dried and ground into fine powder (30 g), then macerated in 100 ml absolute methanol for 72 h by the extraction apparatus. The extract was evaporated to dry at 40°C by using a rotary evaporator and then stored at 4°C for further use.

Determination of the minimum biofilm inhibitory concentration (MBIC)

Agar dilution method was used to determine the MBIC of methanolic leaf extract of *P. betle*. Bacteria isolates were incubated for 24 h inside brain heart infusion liquid medium, then diluted to achieve 0.5 Mc Farland. Then, the suspension was challenged with *P. betle* extract and incubated so that the biofilm formation and MBIC could be determined [11]. Two-fold serial dilutions of the extract (0%, 0.05%, 0.1, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, and 1.00%) were prepared in Mueller–Hinton agar. Ten microliters of bacterial inoculum were delivered onto agar with a final inoculum of 10⁴ CFU/spot. Bacterial plates were incubated at 37°C and evaluated after 24 h.

Detection of rhamnosyltransferase gene

Gene expression of rhlA and rhlC were assessed using two-step quantitative real-time polymerase chain reaction (PCR) in the Institute of Tropical Disease of Airlangga University, Surabaya, Indonesia [12]. Before PCR, RNA was first transcribed into cDNA, and in a second step, the quantitative real-time PCR was performed using a MyGo Mini Real-time PCR System in combination with GoTaq® qPCR Master Mix. Primers used for real-time PCR are listed in Supplementary Table 1. Before gene expression analyses, primers were checked for appropriate product amplification, and primer efficiencies were determined and target

specific efficiencies were then used for calculation of relative expression ratios. All primers used met the requirements as well as the tolerable efficiency range of 90–110% or were redesigned if otherwise. The bacteria were challenged by *P. betle* extract within 24 h before real-time PCR evaluation.

Statistical analysis

Gene expression data were obtained using the MyGo Mini Real-time PCR System software which is installed in Windows and subsequently analyzed using the same software, which calculates relative gene expression based on the delta-delta Ct method. Linear regression method was used to assess the correlation between *P. betle* extract and rhl gene expression using SPSS 23.0. Significant differences were accepted when $p < 0.05$.

Results and Discussion

All *P. aeruginosa* isolates in this study contained rhlA, rhlB, and rhlC genes (Figure 1), which associated with rhl production. A previous report in our laboratory demonstrated that *P. betle* leaf extract was able to inhibit biofilm growth, with minimum inhibitory concentration reached by 0.4% concentration. The expression of rhlA gene did not significantly change after exposure to *P. betle* leaf extract, with a beta coefficient 0.242 ($p = 0.384$). *P. betle* leaf extracts have eugenol as an active compound; however, the concentration is not high enough to suppress rhlA gene expression. rhlA protein is responsible for 3-hydroxyalkanoxy-alkalonic acid (HAA) synthesis [13].

Optimization test for rhlA and rhlC gene expression produced a normal curve at an annealing temperature of 60°C, while quantification of the rhlB gene did not succeed in obtaining a normal curve, although the annealing temperature had been manipulated. It could be associated with the primary storage factor, but this possibility could be ruled out, as all three primers have the same storage requirements and also stored in the same manner and temperature. The rhlB gene is an intermediate gene, which activated by HAA and produced a catalyst from the rhamnosyltransferase subunit (RhIB) to form a mono-rhl. The expression of the catalyzed gene may be so short that it cannot be measured [14].

As rhamnosyltransferase enzyme, RhIB is responsible for the conjugation of HAA with dTDP-L-rhamnose to mono-rhl and RhIC. Then, RhIC will convert mono-rhl to di-rhl. The rhlA and rhlB genes are located on a single operon along the rhlRI system, while rhlC is located 2.5 Mb under the rhlRI operon [14]. It might explain why the rhlA gene was not directly affected by *P.*

Supplementary Table 1: Primers for rhamnosyltransferase gene detection

Notation	Sequence 5'→3'	Expected length of the amplified fragment (bp)
rhlA F	GATCGAGCTGGACGACAAGTC	95
rhlA R	GCTGATGGTTGCTGGCTTTC	
rhlB F	GCCCACGACCACTTCGAC	226
rhlB R	CATCCCCCTCCCTATGAC	
rhlC F	ATCCATCTCGACGGACTGAC	159
rhlC R	GTCCACGTGGTTCGATGAAC	

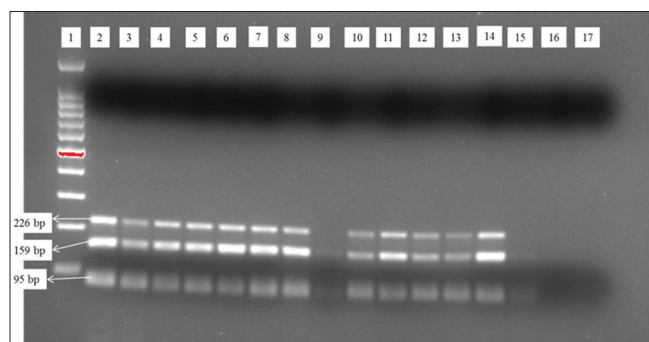


Figure 1: Identification of genes *rhlA*, *rhlB*, *rhlC* 1: DNA marker 100 bp; 2: *Pseudomonas aeruginosa* (14018); 3: *Pseudomonas aeruginosa* (14018); 4: *Pseudomonas aeruginosa* (13842); 5: *Pseudomonas aeruginosa* (13842); 6: *Pseudomonas aeruginosa* (14040); 7: *Pseudomonas aeruginosa* (14040); 8: *Pseudomonas aeruginosa* (14076); 9: *Pseudomonas aeruginosa* (14076); 10: *Pseudomonas aeruginosa* (14230); 11: *Pseudomonas aeruginosa* (14230); 12: *Pseudomonas aeruginosa* (14150); 13: *Pseudomonas aeruginosa* (14150); 14: positive control or wild-type strain (27853); 15: positive control or wild-type strain (27853); 16: negative control; 17: negative control. Gene identification used simple PCR method on a number of isolates, indicated that each clinical isolate had *rhlA* (95bp), *rhlB* (226bp), and *rhlC* (159bp) genes

betle extract administration. The *rhlB* gene expression could not be assessed because of an unidentifiable normal curve. However, *rhlB* gene does not play a single role in the QS system but works together with *rhlA* gene to form di-*rhl*. The *rhlC* gene is responsible for the formation of active *rhl* [4].

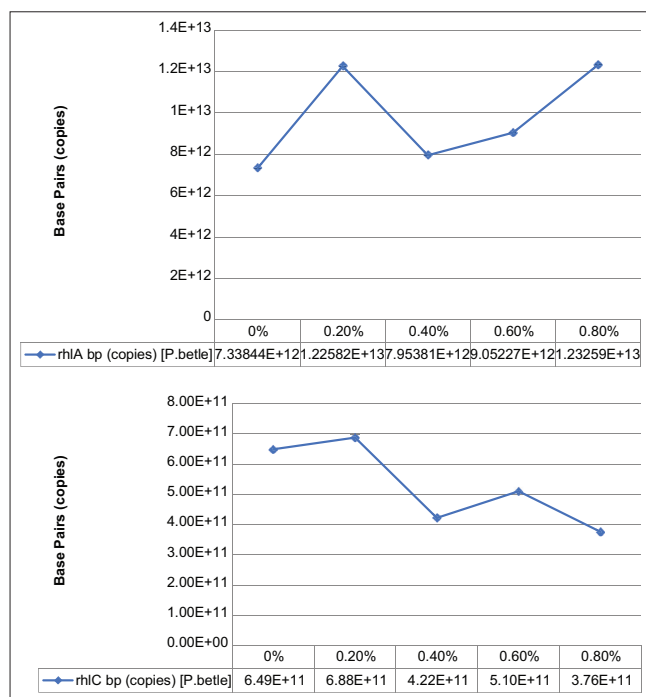


Figure 2: (a) Graph of *rhlA* gene quantification after exposure to *Piper betle* leaf extract. (b) Graph of *rhlC* gene quantification after exposure to *Piper betle* leaf extract

The expression of *rhlC* gene decreased after administration of *P. betle* leaf extract at concentration of 0.4%, with a beta coefficient was 0.662 ($p = 0.019$) (Figure 2). The expression of this gene might be influenced by *P. betle* leaf extract. Eugenol that is

present abundantly in *P. betle* leaf extract has the strongest potential effect on *rhl* itself and also the *rhlC* gene expression. It was consistent with a previous study, which showed that *P. betle* leaf extract will inhibit the expression of *rhlC* gene at MBIC concentration [15]. Bacteria which used in this study might be highly virulent so that *rhlA* gene was not affected by *P. betle* leaf extract administration.

Conclusion

P. betle methanolic leaf extract showed inhibition of *rhlC* gene expression, indicating anti-*rhl* properties against *P. aeruginosa* infection. Further studies are required to define appropriate dosing regimens for *P. aeruginosa* infections and to assess novel treatment strategies, including combination therapies with appropriate antibiotics.

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