

# The interference of *Moringa oleifera* leaf extracts to modulate quorum sensing-facilitated virulence factors

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**Abstract.** Suhartono S, Ismail YS, Muhayya SR. 2019. The interference of *Moringa oleifera* leaf extracts to modulate quorum sensing-facilitated virulence factors. *Biodiversitas* 20: 3000-3004. The infections attenuated by the presence of virulence factors might be facilitated by quorum sensing (QS) mechanism. The continuous treatments of bacterial infections with current drugs, however, has developed such pathogenic bacteria more antibiotic-resistant, encouraging to searching for better alternative treatment, such as the use of plant-derived bioactive constituents, including kelor (*Moringa oleifera* L.). In the present research, kelor (*M. oleifera*) ethanolic leaf extract was evaluated for their potency as an antibacterial agent to inhibit bacterial virulence factors mediated by quorum sensing mechanism against *Chromobacterium violaceum* and *Pseudomonas aeruginosa* PAO1. A phytochemical examination of *M. oleifera* leaf-ethanolic extracts has been conducted resulting positive detection of alkaloids, steroids, terpenoids, flavonoids, and phenolics or tannins. The extracts had MIC and MBC values of 10 mg/mL and 20 mg/mL, respectively, for both tested bacteria. The ethanolic extracts of *M. oleifera* leaves also showed potent inhibition of quorum sensing by significantly reducing violacein and pyocyanin production as well as interference of swarming motility and biofilm formation.

**Keywords:** *Moringa oleifera*, quorum sensing, virulence factors

## INTRODUCTION

Bacterial infections are still a major concern generating significant implications in various circumstances. The infections are attenuated by the presence of virulence factors facilitated by quorum sensing (QS) mechanism leading to the increasing pathogenicity and persistence of the infection. The QS mechanism allows the bacteria to have a cell to cell signaling and communication to express particular genes, i.e. virulence factors, after reaching a specific threshold of population density (Kalia 2013). The bacterial cells release and detect small molecules known as autoinducers (AIs) that modulate the behavior expression of virulence factors including biofilm formation, swarming motility, and bioluminescence (Ćirić et al. 2018). Biofilm-formation, for instance, has been associated with the occurrence of antibiotic-resistant pathogenic bacteria leading to the skyrocketing annually burden of healthcare to be \$94 billion with more than a half of million death (Wolcott et al. 2010).

The continuous treatment of bacterial infections with current drugs, however, has developed such pathogenic bacteria, in sessile form or in a biofilm structure, to be more antibiotic-resistant (Llor and Bjerrum 2014). This encourages to searching for better alternatives for treatment, such as the use of natural molecules or plant-derived products. These phytochemicals, including kelor (*Moringa oleifera* L.) leaves, have been studied for their potency to inhibit microbial infections (Abdulkadir et al. 2018) and draw more attention as a modulatory agent in

interference of QS signaling (Koh et al. 2013; Noumi et al. 2017). *M. oleifera* is a herb originally from Sub Himalayan mountains of Northern India and is widely spread in the lowlands and highlands of tropical and subtropical countries (Saini et al. 2016). *M. oleifera* leaves are oval-shaped with small sizes arranged compound in a single stalk with flowers which are yellowish-white with the green hood and bloomed all year long (Alegbeleye 2017). Moreover, *M. oleifera* recognized as the miracle plant is globally used for various purposes including food ingredients, medicines, water purification, animal fodder, fertilizer, and biofuel (Daba 2016). In Indonesia, *M. oleifera* leaves have been ethnobotanically consumed as vegetables with distinctive flavors and applied as traditional medicines to treat high blood pressure (Bahriyah et al. 2015). In Aceh, *M. oleifera* is traditionally used as a fever remedy in addition to as foods and medicines in Pulo Breueh tribe (Wardiah et al. 2015). In addition, the later study revealed that *M. oleifera* leaves are known to have phytoactive compounds showing their potentials to be antimicrobial agents (Shahriar et al. 2012). It is speculated that biological function of plant-derived compounds are considered as safe and not trigger any toxicity effects on human cells (Koh et al. 2013).

The objectives of the research were to analyze the phytochemical compounds of *M. oleifera* leaf extracts (MLE) and to evaluate their potency to inhibit bacterial virulence factors mediated by quorum sensing mechanisms, such as biofilm formation.

## MATERIALS AND METHODS

### Plant material and extraction

A total of five kilograms of kelor (*Moringa oleifera* L.) leaves were collected from Aceh Besar, Indonesia and then were cleaned and washed. The leaves were then air-dried for four weeks followed by grinding and sieving into powder. The simplicial powder was then extracted using maceration method in 1: 10 ratio. A total of 600 g of simplicial was mixed and homogenized in a flask containing six liters of ethanol; sealed and incubated for five days. The mixture was then filtered to yield macerates and lees. The resulting macerates were stored in a place protected from light; while the lees were undergone the same maceration process for another five days using 1.5 L ethanol. All the obtained macerates were concentrated using a rotary evaporator at temperatures below 50°C to obtain a 100% concentrate.

### Phytochemical screening

Phytochemical screening assay of ethanolic extract of *M. oleifera* leaves was intended to examine various phytochemical contents found in them. The extract samples were qualitatively analyzed, including the presence of alkaloid, flavonoid, terpenoid, as well as tannin compounds.

### Bacterial strains and culture condition

*Pseudomonas aeruginosa* PAO1 and *Chromobacterium violaceum* were used in this study to determine the effect of ethanolic extracts of *M. oleifera* leaves. All bacterial strains were cultivated and maintained in Luria Bertani (LB) (Difco, USA), Mueller Hinton Broth (Difco, USA), Mueller Hinton Agar (Difco, USA), and Tryptic Soy Agar (Oxoid, UK) and incubated at 30 °C (*C. violaceum*) and 37 °C (*P. aeruginosa*).

### Antibacterial activity

Antibacterial activity of *M. oleifera* leaf extract was conducted using a previously described method (Hossain et al. 2015). The extracts were determined for their minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC). Briefly, various concentrations (0.31, 0.63, 1.25, 2.50, 5.00, 10.00, and 20.00 mg/mL) of *M. oleifera* leaf extracts as well as tetracycline (a concentration range of 0.50 – 256.00 mg/mL) as positive control were inoculated into separate wells of 96-well microplates before adding the target bacteria inoculum with densities of  $5 \times 10^5$  CFU/mL and incubated for 24 h. The MIC was determined by the lowest concentration of the extract inhibiting the increment bacterial number compared to the initial number of bacteria. The MBC was determined by inoculating 20 µL of the  $\leq$  MIC wells onto MHA plates and incubated for 24 h at room temperature. MBCs were considered as the lowest concentrations that inhibit the bacterial target growth completely.

### Quorum sensing inhibition

Quorum sensing inhibition was conducted through violacein quantification, pyocyanin inhibition, swarming motility inhibition, and biofilm viability assay.

Violacein quantification was performed following a previously described method (Hossain et al. 2015). A concentration range (1/2 x MIC, 1 x MIC, and 2 x MIC) of *M. oleifera* extract as well as 10 µg/mL of 3% H<sub>2</sub>O<sub>2</sub> as a positive control was transferred into flasks containing LB broth before they were inoculated with  $5 \times 10^5$  CFU/mL of *C. violaceum* culture. The flasks were then incubated at 30 °C with 150 rpm in a shaking incubator for 24 h before the violacein was extracted and quantified using the previous method (Choo et al. 2006).

Pyocyanin inhibition was determined by overnight culturing of *P. aeruginosa* in 5 mL of LB broth supplemented with various concentration range (1/2x MIC, 1 x MIC, and 2 x MIC) of *M. oleifera* leaf extracts. The culture was then centrifuged at 10,000 x g for 10 min and the supernatant was collected before mixing with 3 mL of chloroform. The aqueous part was removed and mixed with 0.2 M HCl before the mixture was homogenized and centrifuged. The formed pink layer was collected and measured for its optical density at 520 nm.

Inhibition of swarming motility was conducted by inoculating *P. aeruginosa* onto the center of LB agar plates containing 0.50% agar and 0.50% glucose supplemented with various concentration range (1/2x MIC, 1 x MIC, and 2 x MIC) of *M. oleifera* leaf extracts. The plates were then incubated for 48 h at 37°C and the swarm zone was calculated.

The biofilm viability was assayed after overnight culturing *P. aeruginosa* in a 96-well microplate containing 10 µL LB broth and 0.5% glucose. After incubation, the plates were rinsed with 1 x PBS (phosphate buffer saline) solution and transferred into new plates containing various concentration range (1/2x MIC, 1 x MIC, and 2 x MIC) of *M. oleifera* leaf extracts in LB broth. After incubation (24 h at 37 °C), the microplate was washed three times with sterile phosphate buffer saline (PBS). A 200 µL of 95% ethanol was gently poured in the microplate and left for 15 minutes before draining and drying. The staining process was conducted using 125 µL of 0.1% crystal violet for 10 - 15 minutes, rinsed off using sterile distilled water and dried for several hours. The dried microplate was then filled with 125 µL of 30% glacial acetic acid and incubated for 15 minutes. A total 125 µL of the 30% glacial acetic acid was added and the optical density (OD), which described the quantity of biofilm formation, was measured at a wavelength of 570 nm using a microtiter plate reader (Bio-Rad, CA, USA).

### Statistical analysis

Data were presented as mean  $\pm$  standard deviation of two replicate assay. An analysis of variance (ANOVA) was conducted for the determination of statistical significance using XLSTAT2016 (Addinsoft, New York). *P*-values of less than 0.05 were considered to be statistically significant and different letter depicted the statistical significance.

## RESULTS AND DISCUSSION

### Extraction of *Moringa oleifera* leaves

The total extract and the total extraction yield obtained from a 900 g of simplicial of *M. oleifera* leaves by 96% ethanol were 210.5 g and 23.38%, respectively. The phytochemical screening of ethanolic extract of *M. oleifera* leaves was carried out qualitatively. The extracts revealed the presence of flavonoids, phenols (tannins), terpenoids, steroids, and alkaloids; whereas saponins were absent (Table 1).

### Bacterial inhibition

The MIC and MBC of ethanolic extracts of *M. oleifera* leaves against *C. violaceum* and *P. aeruginosa* are shown in Table 2. The MIC of ethanolic extracts of *M. oleifera* leaves was 10.00 mg/mL against both *C. violaceum* and *P. aeruginosa*. The MBCs of the extracts against both the tested bacteria were twice their MICs (20 mg/mL).

### Quorum sensing inhibition

Quorum sensing inhibition consisted of violacein quantification, pyocyanin inhibition, swarming motility inhibition, and biofilm viability assay. The percent violacein inhibitions were 29.91%, 41.88%, and 73.03% in 1/2 x MIC, 1 x MIC, and 2 x MIC of *M. oleifera* extract-treated cultures, respectively, compared to the negative control without addition of the extract. The positive control, H<sub>2</sub>O<sub>2</sub> 3%-treated culture inhibited 99.87 % of violacein pigment. There was a significant effect on the addition of the ethanolic *M. oleifera* leaf extracts towards the violacein production of *C. violaceum* (Figure 1).

Pyocyanin production inhibition against *P. aeruginosa* supplemented with various concentration range (1/2x MIC, 1 x MIC, and 2 x MIC) of *M. oleifera* leaf extracts is shown in Figure 3. The corresponding percent pyocyanin inhibitions were 27.94%, 83.72%, 94.59% in 1/2 x MIC, 1 x MIC, and 2 x MIC of *M. oleifera* extract-treated cultures, whereas H<sub>2</sub>O<sub>2</sub> 3% inhibited 90.85% of violacein pigment. There was a significant effect on the addition of the ethanolic *M. oleifera* leaf extracts towards the pyocyanin production of *P. aeruginosa* (Figure 2).

The swarming motility for *P. aeruginosa* treated with ethanolic extract of *M. oleifera* leaves is presented in Figure 3. The inhibition of swarming motility was 18 mm, 10 mm, and 15 mm in 1/2 x MIC, 1 x MIC, and 2 x MIC of *M. oleifera* extract-treated isolate, respectively, compared negative control which had 24.5 mm.

The result for biofilm availability assay is shown in Figure 4. There was a significant effect on the addition of the ethanolic *M. oleifera* leaf extracts towards the biofilm activity of *P. aeruginosa*. The *M. oleifera* extract-treated cultures with 1/2 x MIC, 1 x MIC, and 2 x MIC had respective percent biofilm inhibitions of 16.89%, 12.40%, 51.10%, compared to the negative control without addition of the extract. The positive control of H<sub>2</sub>O<sub>2</sub> 3%-treated culture inhibited 90.85% of biofilm formation.

**Table 1.** The phytochemical analysis of ethanolic extracts of *M. oleifera* leaves

Phytochemical assays	Results
Alkaloids	Positive
Steroids	Positive
Terpenoids	Positive
Saponins	Negative
Flavonoid	Positive
Phenolics/Tannins	Positive

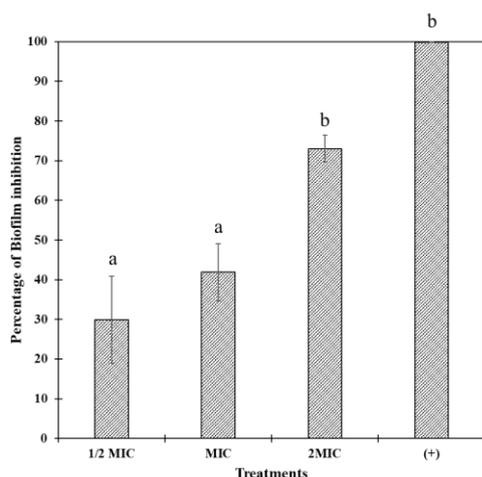
**Table 2.** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ethanolic extracts of *M. oleifera* leaves against *C. violaceum* and *P. aeruginosa* (PAO1)

Bacterial targets	MIC (mg/mL)	MBC (mg/mL)
<i>Chromobacterium violaceum</i>	10	20
<i>Pseudomonas aeruginosa</i> PAO1	10	20

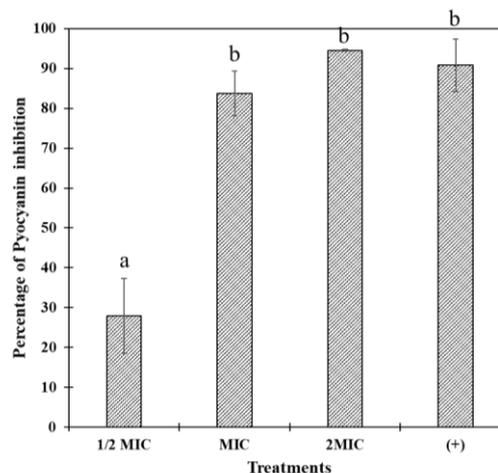
## Discussion

Searching for natural-based product to overcome bacterial pathogens is growing due to the increasing rate of the pathogens in terms of their resistance and virulence. Plant-derived bioactive compounds become potential option since the plants are rich in phytochemicals possessing antimicrobial activities. Additionally, plant-derived constituents are also superior to others since they are produced by the plants that lack immune systems and may have evolved to secrete anti-QS or quorum quenching compounds used to overcome QS pathogens (Koh et al. 2013). One of the plants possessing bioactive compounds is *M. oleifera*. Almost all parts of *M. oleifera* have been widely known for its nutritional and functional features as a food source as well as medicine (Saini et al. 2016; Abdulkadir et al. 2018). The mechanism of how the plant reduced the bacterial infections also gains more attention, particularly those who are mediated by quorum sensing mechanism such as biofilm formation. In the present study, *M. oleifera* leaf extracts were evaluated for their potency to inhibit bacterial quorum sensing-based virulence factors.

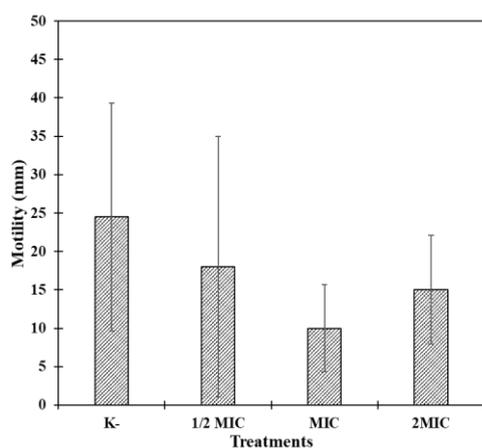
The ethanolic extracts of *M. oleifera* leaves revealed the presence of flavonoids, phenols (tannins), terpenoids, steroids, and alkaloids; whereas saponins were absent (Table 1). This finding is in accordance with another study (Ikalinus et al. 2015) suggesting phytochemical screening of *M. oleifera* leaves showed positive results for all the mentioned compounds, except for saponins. Some of the phytochemicals, such as flavonoids, harbor significant disruption properties for biofilm formation (Raorane et al. 2019).



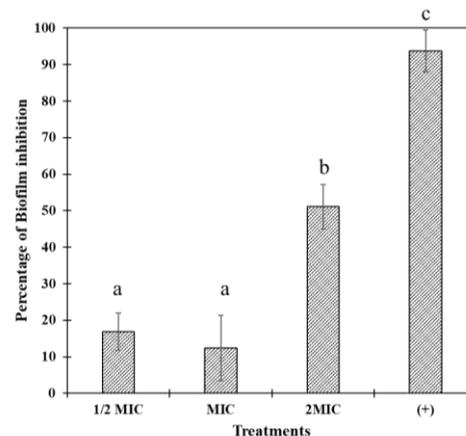
**Figure 1.** The percent violacein inhibitions of *C. violaceum* treated with ethanolic extracts of *M. oleifera* leaves with the concentration of 1/2 MIC (5 mg/mL), 1 x MIC (10 mg/mL), and 2 x MIC (20 mg/mL). (+): positive control of the addition of H<sub>2</sub>O<sub>2</sub> 3%. The treatments followed by the same letters above each column are not significantly different ( $P \leq 0.05$ )



**Figure 2.** The percent pyocyanin inhibitions of *P. aeruginosa* PAO1 treated with ethanolic extracts of *M. oleifera* leaves with the concentration of 1/2 MIC (5 mg/mL), 1 x MIC (10 mg/mL), and 2 x MIC (20 mg/mL). (+): positive control of the addition of H<sub>2</sub>O<sub>2</sub> 3%. The treatments followed by the same letters above each column are not significantly different ( $P \leq 0.05$ )



**Figure 3.** The inhibition of swarming motility of *P. aeruginosa* PAO1 treated with ethanolic extracts of *M. oleifera* leaves with the concentration of 1/2 MIC (5 mg/mL), 1 x MIC (10 mg/mL), and 2 x MIC (20 mg/mL). (K-): negative control *M. oleifera* extract-free *P. aeruginosa*.



**Figure 4.** The percentage of biofilm inhibitions of *P. aeruginosa* PAO1 treated with ethanolic extracts of *M. oleifera* leaves with the concentration of 1/2 MIC (5 mg/mL), 1 x MIC (10 mg/mL), and 2 x MIC (20 mg/mL). (+): positive control of the addition of H<sub>2</sub>O<sub>2</sub> 3%. The treatments followed by the same letters above each column are not significantly different ( $P \leq 0.05$ )

In the present study, the MLE showed Gram-negative bacterial inhibition at the lowest concentration of 10 mg/mL (Table 2). This result was aligned with the previous study (Hossain et al. 2017) highlighting the same MIC value for their *N. tetragona* extracts against *P. aeruginosa* which had slightly higher concentration than the recommended maximum efficiency concentration of 8.0 mg/mL (Ncube et al. 2012). This might indicate the potency of MLEs application as a modulating agent for quorum sensing-based virulence factor.

For the quantitative study of the pigment production of violacein, the present study exhibited significant inhibitions

of *C. violaceum* demonstrating the concentration-dependent MLEs. This result was in line with the previous investigation (Hossain et al. 2017) which reported a similar trend for the crude extract of water lily against *C. violaceum*. This justifies the potentials of MLEs as quorum sensing inhibitors as *C. violaceum* widely used in the quorum-sensing inhibitor assay. Violacein production is QS-regulated by an operon system consisting of set of genes, namely *ioD*, *vioC*, *vioB* and *vioA* genes (August et al. 2000) so it is assumed that the extract might affect the regulation of the operon.

The ability of MLEs to inhibit pyocyanin production and swarming activity was also determined in the current study. Pyocyanin is a blue toxin essential for penetration and it destroys other cells during the *P. aeruginosa* infections, whereas swarming is an orchestrated migration across the surface of solid or semi-solid media facilitated by quorum sensing mechanism. In the present study, the MLEs exhibited a significant dose-dependent inhibition on pyocyanin production (Figure 3) which signifies the fact that MLEs might contain *rhl* system inhibitory properties that disrupt the expression of virulence factors including pyocyanin. In *P. aeruginosa*, virulence factors are synthesized by regulating *rhl* system comprising of transcriptional activator and autoinducers directing the expression of rhamnolipids and production of secondary metabolites including pyocyanin (De Kievit and Iglewski 2000). Rhamnolipids are one of the decreasing-surface tension chemicals allowing *P. aeruginosa* to have swarming activity in addition to the bacterial physical structures, such as pili and fimbriae (Tremblay and Déziel 2008). A slight difference from pyocyanin production, there is no significant concentration-dependence on the swarming activity in this study (Figure 4). However, the increasing MLEs tended to reduce the migration of *P. aeruginosa* highlighting there might be an interference of the extracts in the flagella-facilitated motility of the bacteria leading to the decreased swarming activity.

Another biological process facilitated by QS mechanisms is in biofilm formation. In the current research, there was a significantly reduced biofilm formation in a concentration-dependent fashion (Figure 4). The extract might play role in the disruption of bacterial attachment on the surface as this step is critical for bacteria to initiate a biofilm formation. Moreover, some of the phytochemicals, such as flavonoids, contained in the *M. oleifera* extract have been declared to possess anti-biofilm activities toward *Staphylococcus aureus* due to its ability to inhibit intercellular adhesion genes *icaA* and *icaD* (Lee et al. 2013), a significant disruption factor for biofilm formation.

In conclusion, the activity of MLEs on the interruption of bacterial growth as well as QS-facilitated virulence factors including the production of violacein and pyocyanin, swarming motility, and biofilm formation are indicative for providing insights of the potency of *M. oleifera* leaves as a modulating agent for attenuating the expression of the factors leading to decreased infections. Future co-application of this extract along with their existing conventional antimicrobials is promising as the extracts target specific QS-facilitated virulence factors. Further investigation is necessary to elucidate the molecular mechanism of QS interruption of the extract.

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