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Inhibition of protease activity and anti-quorum sensing of the potential fraction of ethanolic extract from *Sansevieria trifasciata* Prain leaves against *Pseudomonas aeruginosa*

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ABSTRACT *Sansevieria trifasciata* is a plant that is commonly utilized in traditional medicine. The leaves of *S. trifasciat*a show antibacterial properties against *Pseudomonas aeruginosa*. This bacterium is an opportunistic pathogen that can cause serious illness in humans and produce a variety of virulence factors responsible for bacterial pathogenesis with quorum sensing (QS) systems that mediate intracellular communication. Bacteria produce protease through a QS mechanism in which they express signaling molecules to become pathogens. Proteases are extracellular enzymes required for successful infection that mediate biofilm spread through QS and regulate a variety of cellular and physiological functions. This research aimed to evaluate the protease, and anti-QS activities of the ethanolic extract from *S. trifasciata* leaves against *P. aeruginosa* and the expression of QS genes. An azocasein test was used to determine the protease activity in qualitative and quantitative methods. Using real-time quantitative polymerase chain reaction, a study was conducted to investigate the effect of ethanolic extract from *S. trifasciata* leaves on selected QS-regulatory genes at the transcriptional level. The results showed that the potential ethanolic extract from *S. trifasciata* leaves inhibited the protease enzyme activity by as much as 77.1%. The potential ethanolic extract from *S. trifasciata* leaves decreased the expressions of *lasA*, *lasB*, *lasI*, *lasR*, *rhlI*, and *rhlR* with $2^{-\Delta\Delta Ct}$ values of 0.81, 0.93, 0.76, 0.97, 0.90, and 0.55 respectively.

KEYWORDS *Sansevieria trifasciata*; *Pseudomonas aeruginosa*; Protease activity; Anti-quorum sensing

1. Introduction

Sansevieria trifasciata is an herbaceous plant with sword-shaped leaves and distributed in tropical and subtropical areas (Lu et al. 2014; Umoh et al. 2020). The leaves are often used as traditional medicine for various types of diseases, such as colds, diarrhea, coughs, respiratory tract inflammation, venomous snake bites, and hair growth. *Sansevieria trifasciata* has been studied for its antioxidant, antitumor, antidiabetic anaphylactic, and antibacterial activities (Karamova et al. 2022; Berame et al. 2017). *S. trifasciata* leaves have antibacterial activity against various bacteria, including *P. aeruginosa* (Dewatisari et al. 2021; Erhabor et al. 2019; Kingsley et al. 2013).

For numerous years, *P. aeruginosa* has been linked to significant infectious diseases, such as eye and ear infections, it is more resistant to physical and chemical conditions than other pathogenic bacteria (Kose et al. 2014; Retnaningrum et al. 2020). These bacterium exploits damage to the host's defense mechanisms to initiate an infection (Moseley 2022).

P. aeruginosa produces multiple virulence factors. The production of extracellular enzymes is the main mechanism by which bacteria succeed active biofilm dispersion. A protease is one of these enzymes. The production of proteases is linked to the quorum sensing system (QS). QS system regulates intracellular communication, which is responsible for bacterial pathogenicity. *P. aeruginosa* secretes numerous proteases involved in pathogenicity, and most of them are regulated by QS. The QS system regulates the bacterial behavior by changing the gene expression of signaling molecules. The lasI/R system, which controls the activity of the rhlI/R sequence, is a part of *P. aeruginosa*'s QS system. As a result, the entire QS system is structured as a hierarchical network. N-(3-Oxododecanoyl)-L-homoserine lactone (odDHL) and N-butanoyl-L-homoserine lactone (BHL) are distinct acyl-homoserine lactones (AHL) signaling molecules produced

by *P. aeruginosa*. These signaling molecules work together to stimulate virulence responses, such as the synthesis of protease, by triggering *las* and *rhl* (Crabbé et al. 2019; Karthick Raja Namasivayam et al. 2020).

The full elastolytic activity of protease is dependent on four genes: *lasA*, *lasB*, *lasI* and *lasR*. The host cell's epithelial layer is disrupted when the lasA gene creates a protease with virulence features. The elastase enzyme encoded by the *lasB* gene can degrade elastin, collagen, and other matrix proteins in host cells. The expression of the *lasA* and *lasB* genes is regulated by the signaling molecular responsive regulatory systems *lasR* and *rhlR* in *P. aeruginosa* (Lee and Zhang 2015).

It is difficult to eradicate *Pseudomonas aeruginosa* infections due to its association with a high risk of antibiotic resistance. This resistance mechanism requires new sources of antibiotics from plants (Purwestri et al. 2016; Calina et al. 2017; Sathiya Deepika et al. 2018). Although studies have reported the protease and anti-quorum sensing activity mediated by *S. trifasciata* on *P. aeruginosa*, they have not been properly investigated. Scholars suspect that *S. trifasciata* can affect intercellular communication disorders and decrease the production of virulence factors. The potential fraction of ethanolic extract from *S. trifasciata* leaves has been shown to inhibit the formation of biofilms and damage the structure of bacterial cells (Dewatisari 2022). Therefore, it is necessary to measure the protease of *P. aeruginosa* to determine its proteolytic activity.

Several bioactive compounds from *S. trifasciata* leaves have been reported. These compounds such as quinolones, 3,4-dimethoxybenzoic acid, palmitaldehyde, 1,2-benzene-dicarboxylic acid, linoleic acid, quinolone, and delta-undecalactone have antibacterial activities (Yumna et al. 2018). Researchers developed interest in conducting antibacterial testing of probable re-fractionated ethanolic extract of *S. trifasciata* because of these distinct chemical components.

This study aimed to investigate the inhibition of *P. aeruginosa* protease activity after exposure to a potential fraction of ethanolic extract from *S. trifasciata* leaves and evaluate the relative expressions of the QS *P. aeruginosa*-controlled genes.

2. Materials and Methods

2.1. Materials

The material used in this study was a potential fraction of ethanolic extract from *S. trifasciata* leaves obtained via preparative thin-layer chromatography analysis of potential fractions. The leaves were collected from Bandar Lampung, Lampung, Indonesia (5° 21' 30.16" S | 105° 13' 58.62" E) (Dewatisari et al. 2021; Dewatisari 2022). *P. aeruginosa* FBGMU 01 was utilized in the experiment for tested bacteria. This research was conducted at the FAL-ITMA and Biochemistry Laboratory, the Faculty of Biology, and the Integrated Research and Testing Laboratory (LPPT) University Gadjah Mada Yogyakarta, Indonesia.

2.2. Methods

2.2.1 Protease assay

Qualitative test The medium used was nutrient Agar plus 2% skim milk. The medium was poured into a Petri dish and added with the potential fraction of *S. trifasciata* leaf ethanolic extract (4 mg/mL). *P. aeruginosa* isolates were inoculated on a Petri dish containing the medium using the spot method and incubated at 37 °C for 24 h. The medium inoculated with solvent without the potential fraction was used as a negative control. For positive control, the antibiotic ciprofloxacin was used. The protease activity can be observed by a clear zone in the medium (Vijayaraghavan et al. 2013). The clear-zone area in each treatment was calculated, and then compared with that in the control. The calculation of the clear-zone area was as follows:

$$Clear\ Zone\ Area\ (cm^2) = \pi R^2 - \pi r^2 \qquad (1)$$

R : total circle radius (bacteria colony + clear zone) (cm)
r : radius of bacterial colony circle (cm)
π: 3.14

Protease activity (Azocasein assay) Bacterial isolates with a cell number of 1.5×10^8 CFU/mL, equivalent to the turbidity of 0.5 McFarland standard, were grown in 20 mL nutrient broth added with the potential fraction of *S. trifasciata* leaf ethanolic extract. For the negative control, the bacteria were grown without the treatment with the potential fraction of *S. trifasciata* leaf ethanolic extract, and then incubated at 37 °C for 24 h. A total of 10 mL bacterial suspension was obtained and centrifuged at 10,000 g for 10 min at room temperature. As much as 1 mL supernatant was acquired and then placed in test tube already containing 3 mL phosphate buffer solution (pH 8 0.2 M). This mixture was then placed in a water bath until the temperature reached 37 °C. Then 2 mL azocasein was added, which had previously been heated in waterbath at 37 °C. Precisely 4 mL 10% trichloroacetic acid (TCA) was added, and the mixture was centrifuged to form a yellow precipitate. Next 5 mL supernatant was obtained and then added to 5 mL 0.5 M NaOH solution. Finally, the absorbance of the mixture was read at a wavelength of 440 nm.

The enzyme activity was measured every 2 h for 24 h with three replications. The level of enzyme activity was defined as the protease activity that can increase the absorbance measurement by 0.01 every hour under measurement conditions. The unit of activity of the protease used in this study was expressed in U/mL with the formula of Vijayaraghavan et al. (2013):

$$Unit\ of\ activity/mL\ of\ sample\ (U/mL) = (absorbance : 0.01) \times 2 \qquad (2)$$

TABLE 1 List of primers used for qPCR.

Primer		Primers sequence	Ref
lasA	Forward	5' TTCTCGTTGACCTTCCGTGG -3'	This study
	Reverse	5' GCGGACAGTGGTTAGGTTGA -3'	
lasB	Forward	5′-ATCGGCAAGTACACCTACGG -3'	This study
	Reverse	5′-ACCAGTCCCGGTACAGTTTG -3'	
lasI	Forward	5′- GTTTTCGGTTGCTGGCGAAT -3′	This study
	Reverse	5′-TGTCGTTCTGCAGGCTGTAG-3′	
lasR	Forward	5′GGCCTTGGTTGACGGTTTTC-3′	This study
	Reverse	5′-GGGTAGTTGCCGACGATGAA-3′	
rhlI	Forward	5′-TGCTCTCTGAATCGCTGGAA-3′	This study
	Reverse	5′-GTTTGCGGATGGTCGAACTG-3′	
rhlR	Forward	5′-GAAATGGTGGTCTGGAGCGA-3′	(Chanda et al. 2017)
	Reverse	5′-GGAAAGCACGCTGAGCAAAT-3′	
16s rRNA	Forward	5′-GAGGAAGGTGGGGATGACGT -3′	(Husain et al. 2017)
	Reverse	5′-AGGCCCGGGAACGTATTCAC-3′	

2.2.2 Extraction of RNA and real-time quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was carried out to confirm the expression levels of QS-regulatory genes (*lasA*, *lasB*, *lasI*, *lasR*, *rhlI*, and *rhlR*) at the transcriptional level. *P. aeruginosa* RNA was extracted using Favorgen – RNA mini kit reagent. Previously, *P. aeruginosa* was exposed to the potential fraction of *S. trifasciata* leaf ethanolic extract (4 mg/mL) and incubated for 24 h. The control treatment was *P. aeruginosa* without the provision of the potential fraction of *S. trifasciata* leaf ethanolic extract. The concentration and purity of the total extracted RNA were calculated using a spectrophotometer. cDNA was synthesized using a reverse transcriptase kit (Smobio). The primers for the RT-qPCR utilized in this investigation were generated using the Primer Blast online tool, whereas identification primers for *P. aeruginosa* were obtained from https://www.ncbi.nlm.nih.gov/tools/primer blast. The qRT PCR kit used was the Exceltaq Fast qPCR Master Mix (Smobio). Table 1 lists the primers used in this research.

The reaction was initiated by 95 °C pre-denaturation for 2 min followed by 40 amplification cycles (denaturation at 95 °C for 15 s, followed by annealing at 60 °C for 1 min). Gene expressions were calculated relative to the control samples and housekeeping genes (16s rRNA) using the cycle threshold (Ct) ($2^{-\Delta\Delta Ct}$) comparison method (Livak and Schmittgen 2001).

$2^{-\Delta\Delta Ct}$ = [(Ct target gene – Ct gene 16s rRNA) with exposure to extract – (Ct gene target – Ct gene 16s rRNA) *P. aeruginosa* without exposure to a potential fraction of *S. trifasciata* leaves]

2.3. Data analysis

Protease activity test and Anti-QS test were performed in triplicate. The obtained results were qualitatively and quantitatively analyzed. SPSS 26 was used to process the data, and one-way analysis of variance was used to analyze data at a 95% confidence level and followed by a Tukey test at a $p < 0.05$ significance level. The result of Ct value were analyzed using a t-test paired sample.

3. Results and Discussion

3.1. Protease activity test

The positive control showed the protease enzyme activity based on the protease test results. This activity could have resulted from the diameter of the formed clear zone. Given the potential fraction of *S. trifasciata* leaf ethanolic extract, the treatment showed less protease activity (2.29 cm^2) than the positive control (4.23 cm^2). The treatment with ciprofloxacin did not show any clear zones nor bacterial growth. The results of the clear zone area under the three treatments were significantly different (Table 2).

The quantitative measurement of *P. aeruginosa* protease production was carried out only in treatments that showed a decrease in proteolytic activity in the qualitative test and did not inhibit the growth of *P. aeruginosa*. This result is based on the QS inhibitors principle, which denotes the inhibition of the expression of genes encoding

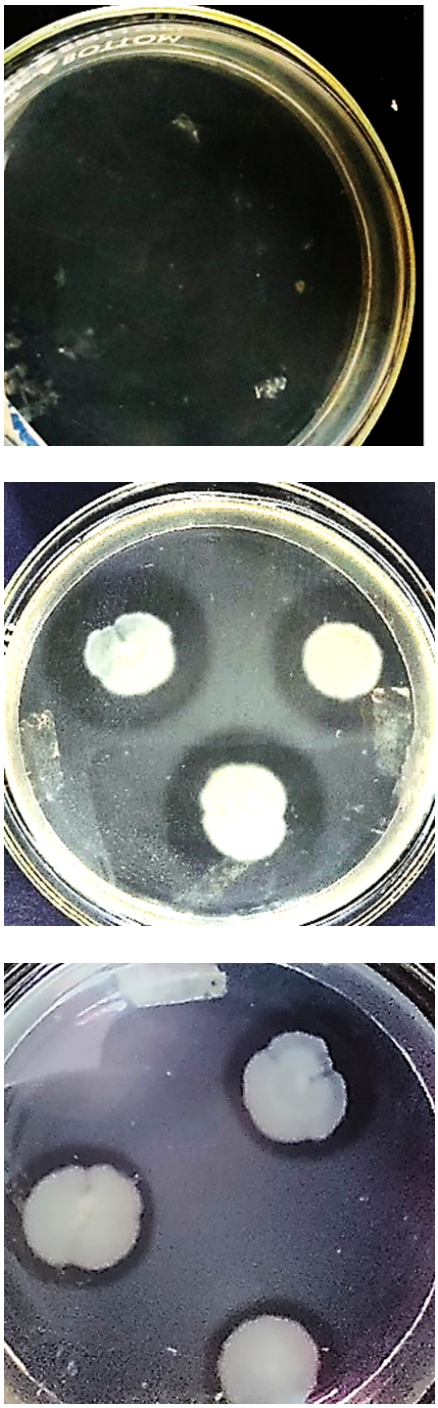
TABLE 2 Results of the *P. aeruginosa* protease clear zone test.

Materials	Diameter colony (cm) ±Standard deviation(SD	Clear zones area(cm^2) ±SD
Ciprofloxacin(-)	0±0.00a	0±0.00a
P. aeruginosa (+)	2.43±0.06c	4.23±1.33c
Potential fraction of *S. trifasciata* leaf ethanolic extract	1.27±0.25b	2.29±0.44b

Note: * The mean standard error of three replications is used to represent antibacterial activity results. Different superscript alphabetic letters differed considerably, according to Tukey's test. ($p < 0.05$).

virulence factors without affecting bacterial growth.

A quantitative test of the protease activity of *P. aeruginosa* was carried out with azocasein as the substrate. Protease hydrolyzes azocasein into peptides and amino acids that exhibit certain colors when NaOH adds. Under measurement conditions, one unit of protease activity was defined as the amount of enzyme that can increase the absorbance measurement by 0.01 per hour. Proteases are hydrolases found in bacteria, and they degrade a wide range of host proteins, thereby aiding bacterial invasion of infected tissues.



(a)

(b)

(c)

FIGURE 1 Qualitative test of protease: a. negative control (*P. aeruginosa* with ciprofloxacin antibiotic treatment), b. positive control (*P. aeruginosa* bacteria without the potential fraction of *S. trifasciata* leaf ethanolic extract), and c. *P. aeruginosa* treated with the potential fraction of *S. trifasciata* leaf ethanolic extract.

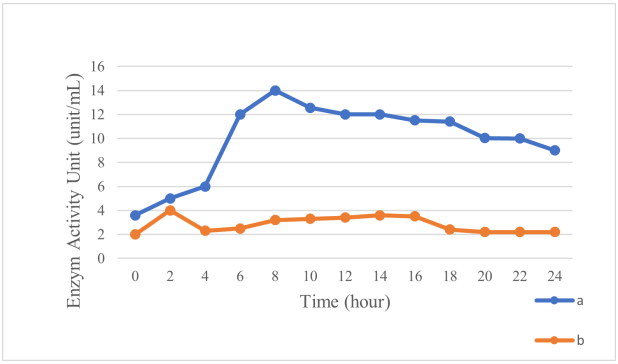


FIGURE 2 Protease activity curve: a. *P. aeruginosa* (control); b. *P. aeruginosa* treated with the potential fraction of *S. trifasciata* leaf ethanolic extract.

The potential fraction from *S. trifasciata* leaf ethanolic extract decreased the protease activity of *P. aeruginosa* (Figure 1). The protease production in the treatment with the potential fraction of *S. trifasciata* leaf ethanolic extract was then compared with the control to obtain the percentage decrease in the enzyme production. In the control, the most significant protease production, which was considered 100%, occurred at the eighth hour. The reduction in protease production was calculated by comparing the protease production after the administration of the potential fraction of *S. trifasciata* leaf ethanolic extract with the control (Figure 2). At the 8th hour, the decrease in *P. aeruginosa* enzyme production with the administration of the potential fraction of *S. trifasciata* leaf ethanolic extract was 77.1%.

This result is consistent with that of previous research (Yarmolinsky et al. 2015; Crabbé et al. 2019; Baburam et al. 2022; Elekhnawy et al. 2022), which showed that plant extracts can inhibit the bacterial QS system. The indicator is the production of inhibited protease, but several plant extracts do not necessarily inhibit cell growth at such concentration. The results of this study were compared with those of previous studies. Mangifera indica extract inhibited *P. aeruginosa* protease activity by 20% (Husain et al. 2017). *P. aeruginosa* protease activity can be reduced by 60% when exposed to linolenic acid compounds (Chanda et al. 2017). The protease activity of *P. aeruginosa* decreased by 87% after exposure to *Andrographis paniculata* extract (Banerjee et al. 2017). As a result, the possible fraction of *S. trifasciata* leaf ethanolic extract that inhibits *P. aeruginosa* protease activity can be used as a plant agent.

3.2. Anti-QS activity mechanism

The relative expression level of six QS-regulated genes (*lasA*, *lasB*, *lasI*, *lasR*, *rhlI*, and *rhlR*) was evaluated in bacteria exposed and not exposed to the potential frac-

tion of *S. trifasciata* leaf ethanolic extract. RT-qPCR data showed that the potential fraction from *S. trifasciata* leaf ethanolic extract significantly ($p < 0.05$) suppressed the levels of transcription of *lasA*, *lasI*, and *rhlR* $2^{-\Delta\Delta CT}$ values of 0.81, 0.76, and 0.55 respectively, compared with the control treatment (Table 3 and Figure 3). Meanwhile, the expression levels of *lasB*, *lasR*, and *rhlI* reduced the expression levels unsignificantly by 0.93, 0.97, and 0.90, respectively (Table 3). These results indicated that the potential fraction of *S. trifasciata* leaf ethanolic extract may inhibit *P. aeruginosa*'s QS system (*las* and *rhl*).

The main regulatory system of *P. aeruginosa* comprises of the LasI protein synthase and the transcriptional regulator LasR. From the results of gene expression, lasI decreased significantly, while *lasR* not showed. The production of the AHL signal molecule N-(3-oxododecanoyl)-l-homoserine lactone (3O-C_{12}-HSL) may decrease with a decrease in *lasI* expression, whereas *lasR*, a signal receptor that is an active transcription factor, experienced an insignificant decrease in activity. Therefore, the possibility of incomplete binding of LasI-LasR resulting in the synthesis of LasA (protease) is also reduced. This can be seen from the significant decrease in *lasA* gene expression. Furthermore, the LasR system activates *lasB* (elastase). The decrease in *lasR* gene expression also caused a decrease in the expression of *lasB*, although both decreased not significantly.

The second QS system of *P. aeruginosa* is *rhl*, which comprises the RhlI and RhlR proteins. The *rhlI* gene expression was not significantly reduced, allowing RhlI synthase to continue producing the AHL N-butyryl-L-homoserine lactone (C_4-HSL). However, the transcriptional regulator of *RhlR* was less active because the expression of the *rhlR* gene was significantly downregulated compared to the expression of other RhlR genes. The inadequate biofilm structure is also made possible by the decreased expression of *rhlR*.

Several studies have also shown that plant extracts can reduce gene expressions. Sarabhai et al. (2013) discovered that ellagic acid derived from *Terminalia chebula* Retz decreased the expression of genes that regulate the formation of extracellular virulence factors in *P. aeruginosa*. Exposure to the extract at 0.5 mg/mL reduced the expression of the autoinducer synthase genes *lasI* and *rhlI* by 89% and 90%, respectively. The extract also reduced the expression of the *lasR* and *rhlR* genes by 90% and 93%, respectively. The potential fraction from *S. trifasciata* leaf ethanolic extract significantly affected the downregulation of genes associated with six interrelated QS system genes (*lasA*, *lasB*, *lasI*, *rhlI*, and *rhlR*) (Figure 3).

QS is essential for *P. aeruginosa* pathogenicity and biofilm formation, rendering it a promising target for antipseudomonal drug development. The inhibition of the *P. aeruginosa* QS system prevents the formation of biofilms and the production of virulence factors (Chanda et al. 2017). *P. aeruginosa* produces virulence factors, such as proteases, which oppose host defenses and directly damage host tissues. Proteases exhibit a high staphylolytic activity in breaking *P. aeruginosa* cell peptide bonds and increase the activity of LasB elastase in elastin degradation of Gly – Gly peptide bonds, and components of connective tissues, blood vessels, and lung tissues (Andrejko et al. 2013; Gellatly and Hancock 2013)

The *las* system regulates protease and elastase expressions, whereas the *rhl* system generates rhamnolipids. The *las* system regulates the secretion of elastase and protease, which is essential for invading host tissues. The rhamnolipids regulated by the *rhl* system contain surface motility, which necessitates the use of a surfactant and the initiation of *P. aeruginosa* biofilm formation (Yin et al. 2022). Two types of AHL signaling molecules are produced by *P. aeruginosa*: odDHL and BHL. By activating *lasI/R* and *rhlI/R*, these signaling molecules use the action of their natural ligands to activate the virulence response (Pattnaik et al. 2018). The transcriptional activator LasR interacts with LasI to induce the synthesis of various virulence enzymes, including LasB elastase and LasA protease. The *rhl* system, which consists of *rhlI* and *rhlIR*, is responsible for the production of pycocyanin. The reduced expression levels of these QS systems in this study were consistent with the decreases the overall virulence determinants mediated by *lasI/R* (elastase and protease) and *rhlI/R* (elastase and protease) (Ha et al. 2014).

The production and activity of *P. aeruginosa* virulence factors are controlled by the *las* and *rhl* regulatory systems in *P. aeruginosa*. Compounds in the potential fraction of

TABLE 3 Relative expression values of target genes obtained by the $2^{-\Delta\Delta CT}$ method.

Parameter	Target Genes±SD					
	lasA	***lasB***	***lasI***	***lasR***	***rhlI***	***rhlR***
ΔCt control	6.08±0.17	12.22±0.15	9.65±0.34	14.59±0.03	18.30±0.00	14.50±0.49
ΔCt potential fraction of *S. trifasciata*	6.38±0.07	12.32±0.43	10.04±0.41	14.63±0.04	18.45±0.09	15.35±0.74
ΔΔCt	0.30	0.11	0.39	0.04	0.15	0.85
$2^{-\Delta\Delta Ct}$	0.81	0.93	0.76	0.97	0.90	0.55
Downregulated gene expression (- $1/2^{-\Delta\Delta Ct}$)	-1.23	-1.08	-1.31	-1.02	-1.11	-1.80

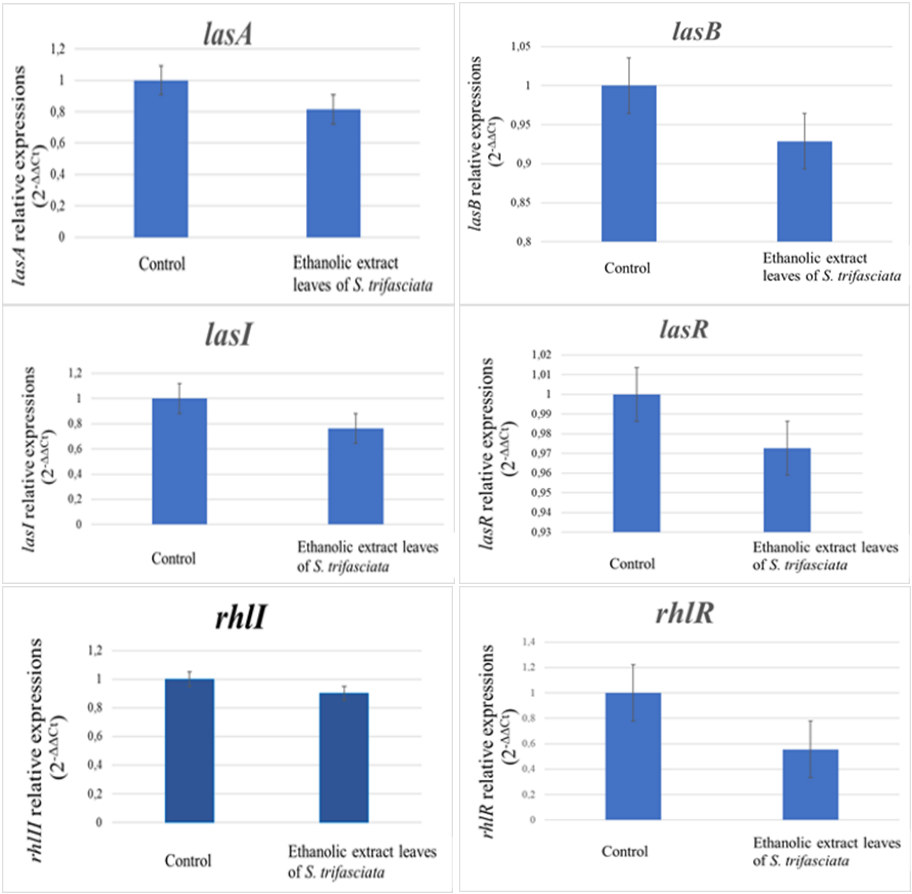


FIGURE 3 Relative expression levels of significant QS regulatory genes in *P. aeruginosa* (*lasA*, *lasB*, *lasI*, *lasR*, *rhlI*, and *rhlR*) under the treatment of the potential fraction of *S. trifasciata* leaf ethanolic extract vs. untreated control ($p < 0.05$).

ethanolic extract interfere with the AHL signal that binds LasI and LasR. This binding downregulates protease and elastase activity expressed by *lasA* and *lasB*, together with *rhl* expression. Decreased *rhl* expression in the QS system results in lower rhamnolipid-producing activity along with proteases. Rhamnolipids facilitate the movement of *P. aeruginosa* on the host surface to form a biofilm and are also involved in the spread and maturation of the biofilm structure. These proteases interfere with cellular function, chelate iron uptake, and increase the virulence of expression in host cells. Therefore, rhamnolipids and proteases, are considered as important indicators in the QS mechanism (Lee and Zhang 2015).

Several studies have been conducted to examine the expressions of QS-controlled genes from plant extracts. Cranberry proanthocyanidins reduce the gene expressions of *P. aeruginosa lasI/R* and *rhlI/R* (Maisuria et al. 2016). According to the RT-PCR analysis, *P. aeruginosa* QS gene expression was downregulated after exposure to *A. paniculata* extract (Banerjee et al. 2017).

The cell-to-cell communication system mediated by AHL can interfere with *P. aeruginosa*'s QS system, paving the way for the prevention of biofilm development, protease activity, and infection. The biofilm's characteristic properties, which are dependent on AHL-dependent extracellular polymeric substance (EPS) production, are another critical factor in the biofilm development process. As a result, the inhibition of AHL may prevent the expression of EPS synthesis and result in weak *P. aeruginosa* biofilm characteristics, which may be related to the inhibition of the QS system (Rashiya et al. 2021).

P. aeruginosa pathogenicity and biofilm development are dependent on QS, making it a promising target for antipseudomonal drug development (Chanda et al. 2017). Plants are excellent sources of novel antimicrobial agents. The investigation of the underlying mechanism is also critical for drug development. According to the results of this research, reductions in the expressions of *lasA*, *lasB*, *lasI*, *lasR*, *rhlI*, and *rhlR* suggest that the potential fraction of *S. trifasciata* leaf ethanolic extract inhibits *P. aeruginosa*'s QS system, and interferes with biofilm development, and virulence factor production. This study provides data and a groundwork for future studies on the cability of S. trifasciata leaf ethanolic extract to reduce *P. aeruginosa* pathogenicity.

4. Conclusions

In this study, a potential fraction of *S. trifasciata* leaf ethanolic extract showed its ability to reduce protease activity by 77.1%. This result was supported by the relative expression analysis with RT-qPCR, in which the genes

that regulate QS and control protease activity (*lasA*, *lasB*, *lasI*, *lasR*, *rhlI*, and *rhlR*) showed decreased expressions in the provision of the potential fraction of S. trifasciata leaf ethanolic extract.

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Authors' contributions

WFD, LHN, ER, and YAP designed the study. WFD carried out the laboratory work, analyzed the data, and wrote the original draft. LHN supervised the study and revised the final version of the document. ER provided critical revision of the article. YAP created the manuscript's illustration and edited the final version. All authors read and approved the final version of the manuscript.

Competing interests

There are no competing interests declared by the authors.

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