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Research Article
Quorum Quenching Potential of the Indigenous Kalimantan Fern *Stenochlaena palustris* Against *Pseudomonas aeruginosa*

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Abstract

Background and Objective: Plant-derived compounds that disrupt bacterial quorum sensing (QS) provide alternatives to combat antibiotic resistance. This quorum quenching (QQ) strategy reduces virulence without inhibiting growth, thus minimizing resistance. Kelakai (*Stenochlaena palustris* (Burm.) Bedd.), an endemic Kalimantan plant traditionally used by the Dayak people, was evaluated for QQ activity against *Pseudomonas aeruginosa*. **Materials and Methods:** Ethanolic extract of *S. palustris* leaves was prepared by maceration with 96% ethanol. The extract was tested for inhibition of *P. aeruginosa* motility, pyocyanin production and LasA activity. Antibiofilm effects were assessed using the microtiter plate method, EPS quantified, chemical profiling performed by Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS) and acute toxicity evaluated in *Rasbora lateristriata* embryos. The results from three independent trials were analyzed in SPSS using one-way ANOVA followed by Dunn's test, with significance set at $p<0.05$. **Results:** At 0.25 mg/mL, the extract inhibited swarming and twitching by 58.04±0.46% and 64.98±0.25%, reduced LasA activity to 58.27±0.70% and reduced pyocyanin to 49.96±1.15%. Although antibacterial activity was weak, at a concentration of 0.25 mg/mL the extract caused 52.47±0.58% inhibition of *P. aeruginosa* biofilm formation and *P. aeruginosa* EPS was reduced to 67.40±0.54% of the untreated control. At a concentration of 1 mg/mL, pre-formed biofilm was eradicated as much as 58.52±0.23%. The LC-HRMS identified (-)-Strychnine, D-(+)-Pipecolinic acid and Ethyl oleate as major constituents. Toxicity testing revealed developmental toxicity in embryos at 0.06 mg/mL. **Conclusion:** *Stenochlaena palustris* ethanol extract showed strong QQ activity against *P. aeruginosa*, underscoring its potential as a natural anti-infective. Further isolation of active compounds with reduced toxicity is required for therapeutic development.

Key words: *Stenochlaena palustris*, quorum quenching, *Pseudomonas aeruginosa*, antibiofilm, LC-HRMS, *Rasbora lateristriata*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic bacterium able to infect diverse tissues and cause both acute and chronic diseases. In recent years, multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of this pathogen have risen markedly. The Infectious Diseases Society of America (IDSA) designates these MDR and XDR variants as difficult-to-treat (DTR) organisms because of their extensive resistance, particularly against antibiotics such as carbapenems and fluoroquinolones[1-3].

The ability of *P. aeruginosa* to cause disease is largely governed by its quorum-sensing (QS) network, which coordinates the regulation of multiple virulence determinants. These include biofilm development, surface motility mediated by flagella and pili, the synthesis of pigments such as pyocyanin, secretion of rhamnolipids, release of cytotoxins and the production of extracellular enzymes, including proteases, elastases and phospholipases. The QS additionally contributes to antibiotic resistance and tolerance. This cell-to-cell signaling system relies on diffusible molecules known as autoinducers, which must accumulate to a threshold level before triggering gene expression[4,5]. Inhibiting QS has emerged as a promising anti-virulence approach, aiming to weaken bacterial pathogenicity without directly impairing growth, thereby limiting selective pressure and resistance development[6].

Plant-derived natural products remain important sources in contemporary drug discovery. Numerous medicinal plants are known to produce secondary metabolites with strong antimicrobial properties. Interestingly, certain metabolites act as quorum-sensing inhibitors or quorum quenching (QQ) agents, disrupting bacterial communication and virulence regulation. Therefore, plant extracts exhibiting QQ activity are considered promising candidates for the development of new anti-infective therapies[7].

Stenochlaena palustris (Burm.) Bedd., locally called Kelakai, is a medicinal fern native to Kalimantan, Indonesia and represents part of Borneo's diverse flora. This plant commonly grows in freshwater habitats, swamps and shaded forest areas, especially around Pangkalan Bun, Central Kalimantan. Previous phytochemical analyses demonstrated that Kelakai contains a broad spectrum of biologically active substances, including nutritional components (proteins, calcium, potassium, phosphorus, manganese, iron and vitamins A and C) and secondary metabolites such as beta-carotene, tannins, flavonoids, steroids and alkaloids. For generations, the Dayak communities-renowned for their rich ethnobotanical heritage-have traditionally consumed Kelakai leaves both as a food source and as herbal medicine. Traditionally, it is used to alleviate fever, manage anemia and diarrhea, enhance wound healing, function as an antibacterial and antioxidant and promote lactation in postpartum women[8,9].

The study was designed to determine whether Kelakai leaf ethanolic extract (KLEE) can inhibit quorum-sensing-controlled virulence features of *P. aeruginosa*, particularly those related to biofilm formation, motility, pigment biosynthesis, extracellular enzyme activity and the protective exopolysaccharide (EPS) matrix were quantified. The chemical profile of the extract was characterized via Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS), a sensitive technique that enables comprehensive metabolomic profiling and identification of active secondary metabolites[10].

The acute toxicity of the extract was evaluated using yellow rasbora (*Rasbora lateristriata*), a freshwater species known for its high sensitivity to aquatic pollutants and its reliability as a bioindicator of environmental contamination. Organisms selected for toxicity testing should fulfill several requirements, including chemical sensitivity, consistent availability in different sizes throughout the year, ease of laboratory cultivation, economic relevance and suitability for standardized bioassays[11]. Yellow rasbora meet these criteria and, in addition, are abundant and commonly consumed, which further supports their use in toxicity evaluations[12,13].

MATERIALS AND METHODS

Study area: This research was conducted at the Cell Biology-Microbiology Laboratory of the Faculty of Pharmacy UGM, Animal Developmental Structure Laboratory, Faculty of Biology UGM and the BRIN Gunung Kidul Yogyakarta Laboratory, from March to August, 2025.

Materials: The materials used were *Stenochlaena palustris* leaves, *Rasbora lateristriata* embryos, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, aquadest (WaterOne®), 96% ethanol, crystal violet, DMSO, Chloramphenicol (Sigma-Aldrich), Luria Bertani medium ((Sigma-Aldrich), Bacteriological Agar (Sigma-Aldrich), microplate flat-bottom polystyrene 96 well (Iwaki, Japan), micropipette, 1.5 mL eppendorf tube (Onemed®), micropipettes (Glyson), microtiter plate reader (Accuris), Incubator (LabLine), incubator with orbital shaker S1500 (Stuart, UK), Biosafety Cabinet (LabTech), autoclave (Sakura, Japan), microscope (Leica Type DM750).

Methods: Plant Material Collection and Extraction Procedure Fresh *S. palustris* (Kelakai) leaves were collected from Handil, Tamapole, Muara Jawa, Kutai Kartanegara Regency, East Kalimantan, Indonesia (coordinates: -0.7494473, 117.2670138) between March and April, 2025. Botanical identification and authentication were carried out at the Faculty of Forestry and Tropical Environment, Mulawarman University and a voucher specimen (No. 0015057301) was deposited for future reference.

The leaves were thoroughly washed and oven-dried at 40°C for 48 hrs to prevent enzymatic degradation and microbial growth. This drying temperature was selected within the optimal 20-40°C range recommended for preserving thermolabile phytoconstituents in leaf tissues. Compared with shade air-drying, low-temperature oven drying (30-50°C) shortens processing time while maintaining the stability of bioactive compounds[14]. After drying, the material was ground into a fine powder using a mechanical grinder. The powdered leaves were macerated in 70% ethanol at a 1:10 (w/v) ratio (1 g powder per 10 mL solvent) to produce crude Kelakai leaf ethanol extract (KLEE). The extract was filtered, concentrated under reduced pressure using a rotary evaporator and stored for further analysis. A stock solution was prepared by dissolving the dried extract in Dimethyl Sulfoxide (DMSO) at 100 mg/mL, followed by sterile filtration through a 0.2 µm membrane and storage at 4°C.

Effect on *P. aeruginosa* motility: *Pseudomonas aeruginosa* employs pili and flagella to facilitate surface-associated motility, with the specific type of movement influenced by medium viscosity and partially regulated by quorum sensing. To evaluate the effect of Kelakai leaf ethanol extract (KLEE) on quorum-sensing-related motility phenotypes, swarming and twitching assays were performed following the procedure of Bergeau *et al.*[15] with minor modifications. All assays were conducted in triplicate.

Briefly, LB agar plates containing 0.5% for the swarming motility assay or 1% agar for the twitching motility assay and supplemented with sub-inhibitory concentrations of KLEE were dried at 30°C for 3 hrs before inoculation. For swarming assays, a loopful of *P. aeruginosa* culture (~10 CFU/mL) was gently spotted onto the agar surface using a sterile blunt-ended toothpick. For twitching assays, the inoculum was introduced by stabbing through the agar to the base of the Petri dish with a sterile pointed toothpick. Following incubation at 37°C for 18-24 hrs in an upright position, motility was assessed by measuring the average length of dendritic extensions (swarming) or the diameter of the expansion zone (twitching). To improve visualization of twitching motility, the agar layer was carefully removed and the underlying bacterial film was stained with 1 mL of 1% (w/v) crystal violet.

Inhibition of pyocyanin production: The impact of Kelakai leaf ethanol extract (KLEE) on *Pseudomonas aeruginosa* pyocyanin production was assessed following a modified protocol adapted from Najafi *et al.*[16] and Yin *et al.*[17]. Bacteria were cultured in Luria-Bertani (LB) medium to a density of approximately 1×10 CFU/mL. For each assay, 2.5 mL of LB medium containing different concentrations of KLEE (0.06-1 mg/mL) was prepared in sterile Erlenmeyer flasks and inoculated with 25 µL of bacterial suspension. Dimethyl Sulfoxide (DMSO) served as the negative control. Cultures were incubated at 37°C with shaking at 200 rpm for 24 hrs. After incubation, cultures were centrifuged at 10,000 rpm for 15 min and the supernatant was filtered through a 0.22 µm membrane to obtain a sterile, cell-free fraction. For pyocyanin extraction, 800 µL of supernatant was combined with 600 µL of chloroform and vortexed for 2 min. A blue-green organic phase confirmed successful extraction. Subsequently, 500 µL of the chloroform layer was transferred to a microtube and mixed with 200 µL of 0.2 N hydrochloric acid. Acidification at 37°C for 30 min yielded a pink aqueous phase, characteristic of pyocyanin. The mixture was centrifuged at 10,000 rpm for 2 min and the upper aqueous layer was collected for quantification. Aliquots of this fraction were transferred to a 96-well microplate in triplicate for further analysis, while the organic phase was discarded.

Assessment of LasA protease (staphylolytic) activity: The influence of Kelakai leaf ethanol extract (KLEE) on *P. aeruginosa* LasA protease activity was evaluated using a staphylolytic assay adapted from Qaralleh[18] and Benaissa *et al.*[19] with minor modifications. An overnight culture of *Staphylococcus aureus* (50 mL) was boiled in a water bath for 10 min to inactivate the cells. The culture was then centrifuged at 10,000 rpm for 10 min, after which the supernatant was discarded and the pellet resuspended in 10 mM sodium phosphate buffer (Na_2PO_4, pH 4.5). The suspension was diluted in the same buffer to reach an optical density of ~0.8 at 600 nm (OD600).

To measure LasA activity, 100 µL of *P. aeruginosa* cell-free supernatant (from cultures grown with or without KLEE) was added to 900 µL of the prepared *S. aureus* suspension. Turbidity reduction was monitored by recording OD600 at 0, 10, 20, 30, 40, 50 and 60 min. All assays were conducted in triplicate. The percentage inhibition of LasA protease activity was determined by comparing OD600 reductions in treated samples against the untreated control.

Determination Of planktonic minimum inhibitory concentration (PMIC): The reference strain *P. aeruginosa* ATCC 27853 was employed in this study. Stock cultures were prepared by cultivating the bacterium in Luria-Bertani (LB) broth at 37°C for 18-24 hrs until reaching the mid-logarithmic phase. The planktonic minimum inhibitory concentration (PMIC) of the plant extract was determined using the broth microdilution technique in triplicate. Five serial twofold dilutions of the extract (1-0.06 mg/mL, w/v) were prepared in LB medium with Dimethyl Sulfoxide (DMSO) as the solvent. For each assay, 10 µL of bacterial suspension was added to 96-well microplates, yielding a final inoculum of ~5×10 CFU per well. The total reaction volume was adjusted to 200 µL, including extract, medium and inoculum. Controls included: LB with inoculum (negative control), sterile LB medium only (media control) and chloramphenicol at 1 ppm (positive control). Plates were incubated at 37°C for 18-24 hrs, after which bacterial growth was quantified spectrophotometrically at 600 nm (OD600).

Growth inhibition (%) was calculated by comparing the OD values of treated wells with the negative control. The following formula was applied, incorporating baseline absorbance to account for extract coloration:

$$\text{Inhibition (\%)} = \frac{[(ODgc24-ODgc0)-(ODt24-ODt0)]}{(ODgc24-ODgc0)}\times 100$$

Where:

ODt24 = OD of the test well at 24 hrs
ODt0 = OD at 0 hr
ODgc24 = OD of growth control at 24 hrs
ODgc0 = OD of growth control at 0 hr

The mean inhibition from replicates was used to determine the PMIC, with the concentration causing ≥50% growth suppression defined as PMIC50[20].

Assessment of biofilm formation inhibition and determination of minimum biofilm inhibitory concentration MBIC: After 24 hrs of incubation at 37°C, planktonic cells and non-adherent material were discarded and wells were washed three times with phosphate-buffered saline (PBS) to remove unattached bacteria. Plates were then air-dried at room temperature for 10 min. Biofilms were stained with 125 µL of 1% crystal violet for 15 min, followed by rinsing with tap water to remove excess dye. Bound stain was solubilized with 200 µL of absolute ethanol and absorbance was recorded at 600 nm using a microplate reader[21]. Biofilm inhibition (%) was calculated by comparing the optical density (OD) of treated wells with that of the vehicle control. Results from replicate wells were averaged to determine the minimum biofilm inhibitory concentration (MBIC), defined as the lowest extract concentration achieving a reduction in biofilm biomass (MBIC50):

$$\text{Inhibition (\%)} = \left[1-\left(\frac{\overline{X}ODt-\overline{X}ODmc}{\overline{X}ODvc}\right)\right]\times 100$$

Where:

ODt = OD of test well (595 nm)
ODvc = OD of vehicle control (595 nm)
ODmc = OD of media control (600 nm)

This approach ensures accurate quantification of biofilm biomass while correcting for background absorbance[22].

Evaluation of biofilm disruption and determination of minimum biofilm eradication concentration (MBEC): The ability of the KLEE to disrupt pre-formed biofilms was assessed following a modified protocol based on Haney *et al.*[23]. Mature biofilms of *P. aeruginosa* were established by incubating bacterial cultures in 96-well microtiter plates for 24 hrs under static conditions. After incubation, non-adherent planktonic cells and medium were carefully removed. Fresh LB medium containing the plant extract at various concentrations was then added to each well. The plates were incubated for an additional 24 hrs to allow the extract to act on the established biofilm structure. Following the incubation, biofilm biomass was quantified using the crystal violet staining method, as described previously. The percentage of biofilm eradication was calculated relative to untreated controls to determine the minimum biofilm eradication concentration (MBEC).

Extraction and quantification of exopolysaccharides (EPS): Extracellular polysaccharides (EPS), a major structural element of the *Pseudomonas aeruginosa* biofilm matrix, were quantified using a modified version of the method by Ghanaim *et al.*[24]. Cell-free supernatants from cultures treated with different concentrations of KLEE or untreated controls, were mixed with three volumes of chilled absolute ethanol to precipitate EPS. The mixtures were incubated at 4°C for 24 hrs to ensure complete precipitation. EPS pellets were then collected by centrifugation at 10,000 rpm for 15 min and resuspended in Milli-Q water. Quantification of EPS was carried out using the phenol-sulfuric acid method. In brief, 1 mL of EPS solution was combined with 1 mL of 5% cold phenol, followed by the rapid addition of 5 mL of concentrated Sulfuric Acid (H_2SO_2). After incubation, the intensity of the resulting color, which is directly proportional to carbohydrate content, was measured spectrophotometrically at 490 nm.

LC-HRMS analysis of plant extract: Liquid Chromatography-High-Resolution Mass Spectrometry (LC-HRMS) was applied to characterize the bioactive compounds in KLEE, providing high mass accuracy and detailed structural resolution. This analysis aimed to confirm the molecular identity of active constituents, gather structural information and detect impurities or degradation products, which are essential considerations in the pharmaceutical development of natural extracts. Chromatographic separation was carried out using an ACQUITY UPLC I-Class system fitted with an ACQUITY UPLC HSS C18 column (2.1×150 mm and 1.8 µm). Mass detection was performed on an Xevo G2-S QToF spectrometer, with instrument control and data processing managed through UNIFI software. For sample preparation, the crude extract was dissolved in a polar solvent to a final volume of 1300 µL at a moderate concentration to optimize ionization. The solution was vortexed for 1 min, centrifuged at 13000 rpm for 2 min to separate phases and the supernatant filtered through a 0.22 µm PTFE syringe filter (25 cm). A 10 µL aliquot of the filtrate was then transferred into an LC vial and introduced via the autosampler. Gradient elution was performed with two mobile phases: (A) 5 mM ammonium formate, pH 3.0 and (B) 0.1% formic acid in acetonitrile. The gradient began with 87% A for 0.5 min, decreased to 50% at 10 min, then to 5% at 10.75 min, held for 1.5 min and re-equilibrated to 87% A at 12.5 min, maintained until 15 min. Data acquisition employed positive electrospray ionization (ESI) in full-scan mode over m/z 50-600 Da[24,25].

Acute toxicity assessment in yellow rasbora (*Rasbora lateristriata*) embryos: Acute toxicity of KLEE was assessed in *Rasbora lateristriata* (yellow rasbora) embryos following OECD Guideline No. 236 for the Fish Embryo Acute Toxicity (FET) Test. A total of 200 fertilized eggs were distributed into five groups, each containing 20 embryos with two replicates per group. Embryos were selected at 7 hrs post-fertilization (hpf), corresponding to the early developmental stage (70-75% epiboly). Treatments included serial concentrations of KLEE (1.0, 0.5, 0.25 and 0.125 mg/mL) alongside a control. Exposures were maintained for 48 hrs. Toxicological assessment was performed to estimate the median lethal concentration (LC_{50}). Embryonic development and toxicity endpoints were evaluated at 24, 48, 72 and 96 hpf through microscopic observation at 40× and 100× magnification. The recorded parameters included survival rate, hatching success and heartbeat frequency, which were compared across treatment and control groups[12,13].

Data analysis: All experiments were performed in at least three independent replicates. Data were analyzed using SPSS software. The effects of Kelakai leaves ethanol extract across treatment and control groups were evaluated by One-way Analysis of Variance (ANOVA), followed by Dunn's test to determine pairwise differences between treatments and the control. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Inhibition of bacterial motility: This study examined the effect of KLEE on quorum-sensing–regulated motility in the opportunistic pathogen *P. aeruginosa*. The inhibitory effect of Kelakai leaf ethanolic extract (KLEE) on the quorum-sensing-regulated motility of *P. aeruginosa* is shown in Fig. 1. Figure 1 illustrates the visible reduction in swarming and twitching motility patterns on LB agar plates supplemented with various concentrations of KLEE compared to the control. As summarized in Table 1, treatment with 0.25 mg/mL KLEE significantly impaired bacterial movement. Swarming motility was reduced to 58.04±0.46% of the untreated control ($n = 3$, $p<0.05$), while twitching motility decreased to 64.98±0.58% of the control ($n = 3$, $p<0.05$). These observations indicate that KLEE significantly impaired flagellar- and pili-mediated movements, both of which are regulated by quorum-sensing mechanisms.

LasA staphylolytic assay: Quorum sensing regulates the secretion of extracellular proteases, including LasA staphylolysin, a serine endopeptidase that cleaves the pentaglycine bridges in *S. aureus* peptidoglycan, causing lysis. KLEE inhibited LasA activity in a concentration-dependent manner (Fig. 2). After 60 min, treatment with 0.25 mg/mL reduced activity to 58.27±0.7% of the control ($n = 3$, $p<0.05$). At 1 mg/mL, LasA activity was further reduced to 10.51±0.5% ($n = 3$, $p<0.05$). These results highlight the ability of KLEE to attenuate *P. aeruginosa* virulence by disrupting QS-regulated protease secretion.

Pyocyanin assay: We investigated the influence of KLEE on the production of pyocyanin, a key virulence factor of *P. aeruginosa*. The extract demonstrated an inhibitory effect of *P. aeruginosa* pyocyanin production in a concentration-dependent manner. At a KLEE concentration of 0.25 mg/mL, pyocyanin levels decreased to 49.96±1.15% of control values ($n = 3$, $p<0.05$), whereas at a concentration of 0.5 mg/mL, pyocyanin production was further reduced to 38.25±0.82% of control ($n = 3$, $p<0.05$) (Fig. 3). These results indicate that KLEE interferes with QS-regulated secondary metabolite production, thereby diminishing synthesis of this toxic blue-green pigment. Such inhibition not only indicates the potential role of KLEE as a quorum-sensing modulator but also underscores its promise as a natural therapeutic candidate for attenuating bacterial virulence.

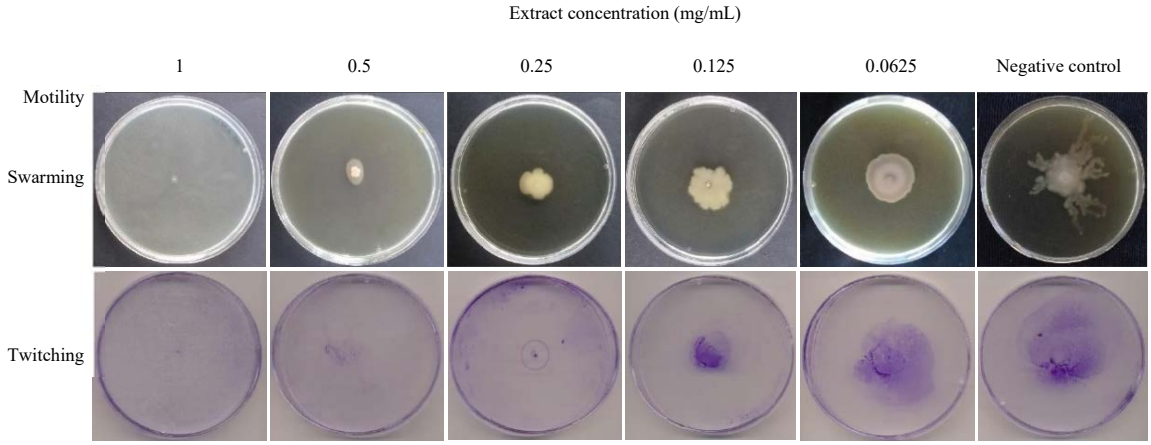

Fig. 1: Effect of various concentration of Kelakai leaves ethanol extracts (KLEE) on swarming and twitching motility of *P. aeruginosa*

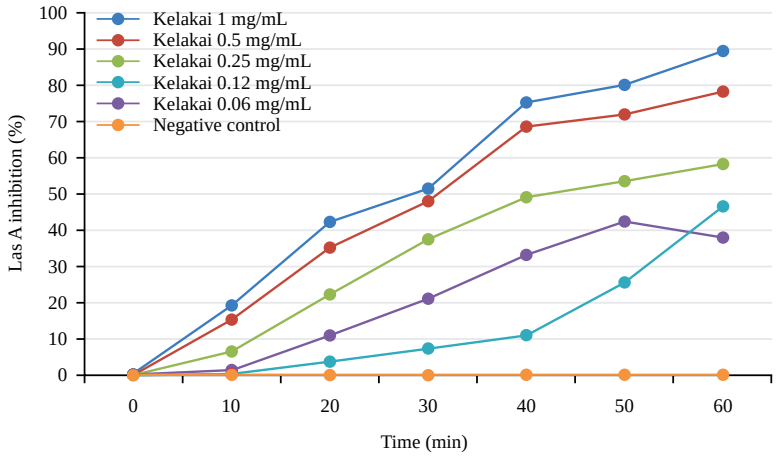

Fig. 2: Inhibition of LasA staphylolytic activity by different concentrations of KLEE
Percentage of LasA inhibition was calculated with respect to control OD at 600 nm

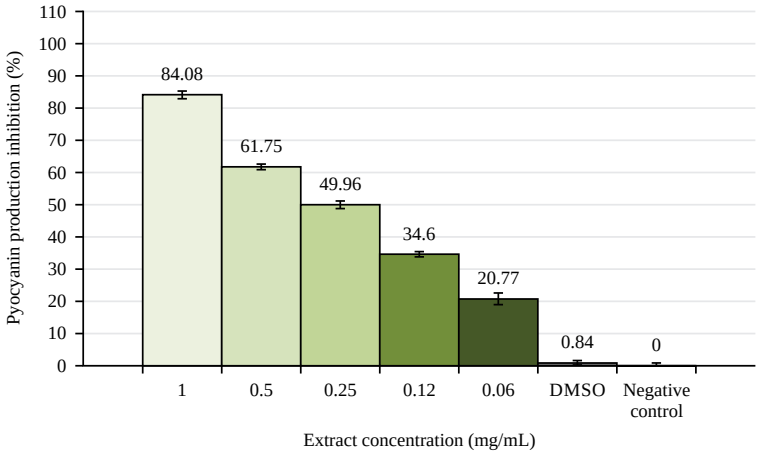

Fig. 3: Inhibition of *P. aeruginosa* pyocyanin production by different concentrations of KLEE
Percentage of inhibition was calculated with respect to control OD at 520 nm

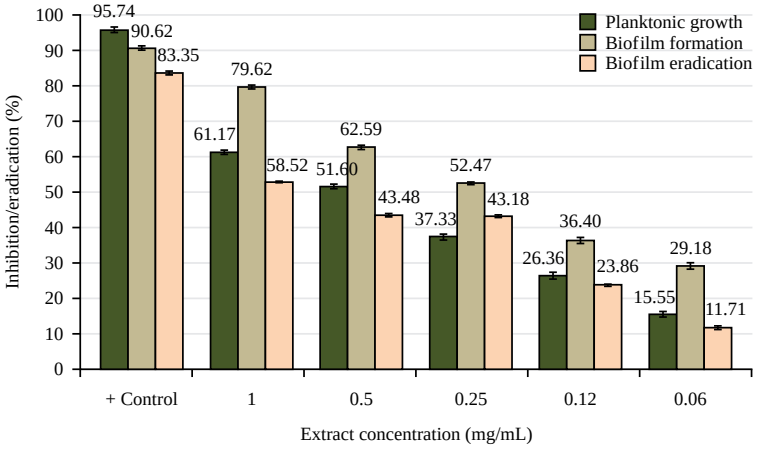

Fig. 4: Percentage of inhibition in planktonic growth, biofilm formation and biofilm eradication of *P. aeruginosa* by KLEE at different concentrations

Chloramphenicol at concentration of 1 ppm was used as positive control. The standard deviation in the percentages is indicated by bar

Table 1: Efficacy of KLEE against *P. aeruginosa* swarming and twitching motility after 24 hrs incubation

	Motility inhibition (%)	
Extract concentration (mg/mL)	Swarming	Twitching
1	98.16±0.58	100±0.12
0.5	71.26±0.82	97.91±0.33
0.25	58.04±0.46	64.98±0.58
0.125	41.95±0.82	43.25±0.12
0.0625	33.90±0.82	11.41±0.46
Untreated (negative control)	0±0	0±0
Chloramphenicol 1 ppm (positive control)	100±0.12	100±0.62
DMSO (vehicle control)	0.1±0.74	0.34±0.82

Efficacy of plant extract on planktonic growth, biofilm inhibition and biofilm eradication of *P. aeruginosa*: The highest concentration selected for testing (1 mg/mL) was based on the recommendation by Ríos and Recio[26], who suggested that crude extracts with minimum inhibitory concentration (MIC) values above 1 mg/mL or isolated compounds with MIC values exceeding 0.1 mg/mL, should generally be avoided. Planktonic growth inhibition was assessed by measuring the reduction in absorbance of treatment wells, which indirectly reflects bacterial biomass, compared to the negative control (untreated wells, defined as 0% activity). The PMIC values were determined using probit analysis.

As presented in Fig. 4, the ethanolic extract of KLEE reduced planktonic growth of *P. aeruginosa* by 51.60±0.82% at 0.5 mg/mL (PMIC50). Beyond planktonic growth, we also examined the extract's effects on biofilm formation and eradication. Biofilm quantification was carried out using the crystal violet assay, a widely adopted, inexpensive and rapid method suitable for high-throughput screening in microtiter plates[27].

The results showed that KLEE exhibited strong, dose-dependent activity against *P. aeruginosa* biofilms. At 0.25 mg/mL, the extract inhibited biofilm formation, decreasing to 52.47±0.58% relative to the control ($n = 3$, $p<0.05$), whereas a higher concentration was required to disrupt established biofilms. Specifically, at a concentration of 1 mg/mL, KLEE eradicated 58.52±0.23% of pre-formed biofilms relative to the untreated group ($n = 3$, $p<0.05$). These findings indicate that KLEE possesses both preventive and therapeutic activity against biofilm-associated infections.

Effect of plant extracts on *P. aeruginosa* EPS production: The EPS production during biofilm growth was significantly reduced by KLEE (Fig. 5). At 0.12 mg/mL, EPS content declined to 49.39±0.54% of control values and at 0.25 mg/mL it decreased further to 32.60±0.54% ($n = 3$, $p<0.05$).

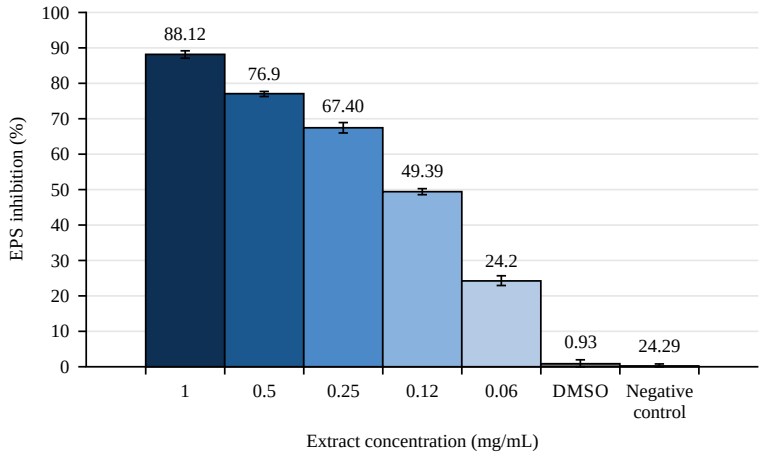

Fig. 5: Effect of KLEE on EPS production of *P. aeruginosa*

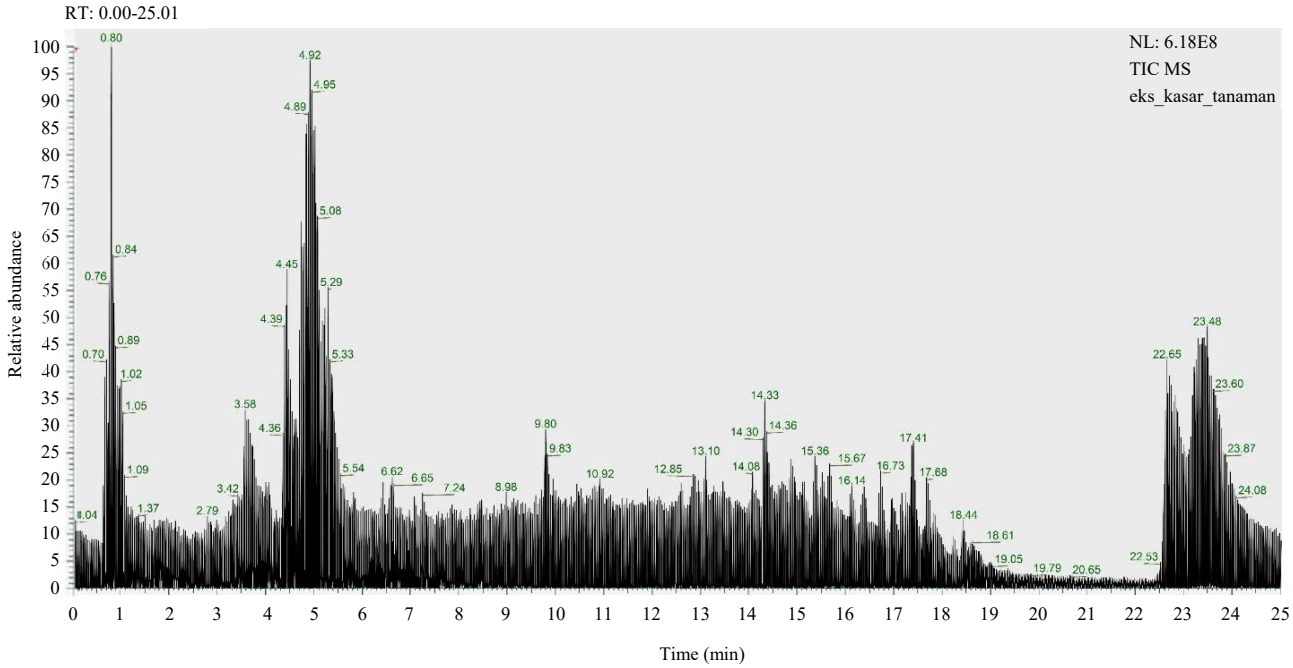

Fig. 6: Chromatogram of metabolite profile analysis of KLEE using LC-HRMS

Chemical composition investigation of Kelakai leaves ethanol extract using LC-HRMS: Chromatogram of the metabolite profile analysis using LC-HRMS is shown in Fig. 6. Based on the results of the metabolite profile analysis using LC-HRMS, 368 compounds were identified in both negative and positive ionization modes. Filtering based on a full match in three annotation sources-predicted compound, mzCloud search and ChemSpider search and a best match score above 70, resulted in the identification of 20 compounds with the highest (max) peak areas (Table 2).

Strychnine was the dominant compound with a relative peak area of 17.31%. Following that, the compounds pipecolinic acid, ethyl oleate, 1-stearoylglycerol and stearic acid were the next most dominant compounds with a relative peak area percentage of >1% (Table 2).

Toxicity testing of extract on *Rasbora lateristriata* embryo: Animal experiments were approved by the Ethical Clearance Committee for Preclinical Research, Universitas Gadjah Mada (Approval No. 00008/III/UN1/LPPT/EC/2025) and conducted in accordance with Institutional and National Animal Care Guidelines.

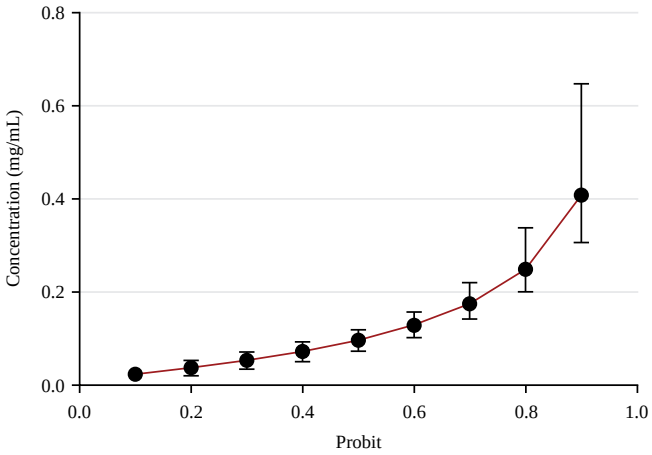

Fig. 7: Probit analysis of KLEE

Table 2: Twenty compounds with the highest area from the metabolite profile analysis results using LC-HRMS

No.	Name	Formula	Annot. delta mass (ppm)	Calc. MW	RT (min)	Area (×10^6)	Relative area (%)	mzCloud best match
1	(-)-Strychnine	$C_{21}H_{22}N_2O_2$	-1.79	334.16753	4.792	5,402.83	17.31	99.9
2	D-(+)-Pipecolinic acid	$C_6H_{11}NO_2$	0.38	129.07903	0.834	1,262.87	4.05	99.6
3	Ethyl oleate	$C_{20}H_{38}O_2$	-1.06	310.28685	17.706	434.53	1.39	99.3
4	1-Stearoylglycerol	$C_{21}H_{42}O_4$	-0.90	358.30799	15.374	415.03	1.33	96.2
5	Stearic acid	$C_{18}H_{36}O_2$	-0.89	284.27128	17.421	395.59	1.27	78.2
6	4-Methoxycinnamic acid	$C_{10}H_{10}O_3$	-0.07	178.06298	14.883	280.26	0.90	98.0
7	Methyl palmitate	$C_{17}H_{34}O_2$	-0.37	270.25578	16.973	222.17	0.71	97.9
8	D-(+)-Proline	$C_5H_9NO_2$	1.90	115.06355	0.813	215.13	0.69	99.7
9	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}H_{30}O_3$	0.15	294.21954	12.939	201.01	0.64	98.5
10	All trans retinal	$C_{20}H_{28}O$	-0.35	284.21391	14.418	130.14	0.42	92.7
11	1-Stearoylglycerol	$C_{21}H_{42}O_4$	-0.94	358.30797	15.588	128.78	0.41	97.2
12	4-Methoxybenzaldehyde	$C_8H_8O_2$	1.25	136.05260	1.499	122.13	0.39	74.2
13	4-quinolyl(5-vinyl-1-azabicyclo[2.2.2]oct-2-yl)methanol	$C_{19}H_{22}N_2O$	-0.76	294.17299	5.399	111.16	0.36	88.4
14	Ethyl myristate	$C_{16}H_{32}O_2$	0.07	256.24025	17.842	103.95	0.33	85.1
15	Ethyl oleate	$C_{20}H_{38}O_2$	-1.06	310.28685	17.923	87.86	0.28	99.2
16	Nicotinic acid	$C_6H_5NO_2$	1.63	123.03223	1.031	80.85	0.26	98.2
17	Bis(2-ethylhexyl)adipate	$C_{22}H_{42}O_4$	-1.54	370.30774	17.179	71.65	0.23	97.9
18	D-(-)-Quinic acid	$C_7H_{12}O_6$	-4.55	192.06251	0.796	55.97	0.18	89.4
19	Phenacetin	$C_{10}H_{13}NO_2$	0.90	179.09479	1.305	55.66	0.18	98.2
20	Oleoyl ethanolamide	$C_{20}H_{39}NO_2$	-0.40	325.29795	14.103	47.99	0.15	99.0

Full match pada predicted compound, mzCloud search and Chem Spider search; best match >70, MS2: DDA for preferred ion

LC50 results: Lethal concentration data are presented in Fig. 7 for the KLEE. The LC50 value is determined at a concentration of 0.097 mg/mL. The concentration gradient steadily increases from the LC10 (probit 0.1) to the LC90 (probit 0.9). The concentration that would probably kill 10% of the population is around 0.023 mg/mL. On the other end of the spectrum, statistically, the concentration to kill 90% of the population is 0.409 mg/mL.

Morphological abnormality: Figure 8 shows that the presence of abnormalities such as impaired swim bladder, pericardium edema, yolk sac edema, craniofacial malformation and spinal abnormality can be observed in the KLEE treatment group. An impaired swim bladder was found at all KLEE treatment concentrations. Spinal abnormality was only observed at concentrations of 0.06, 0.25 and 0.5 mg/mL. Craniofacial malformation was only detected at a concentration of 0.5 mg/mL. Pericardium edema and yolk sac edema in fish embryos are reversible abnormalities (it can return to normal once the toxicant is removed), whereas spinal abnormality and craniofacial malformation are irreversible abnormalities (cannot revert to normal). In the solvent control group, no abnormalities were found in *R. lateristriata* embryos. This indicates that the use of the solvent does not affect the embryos.

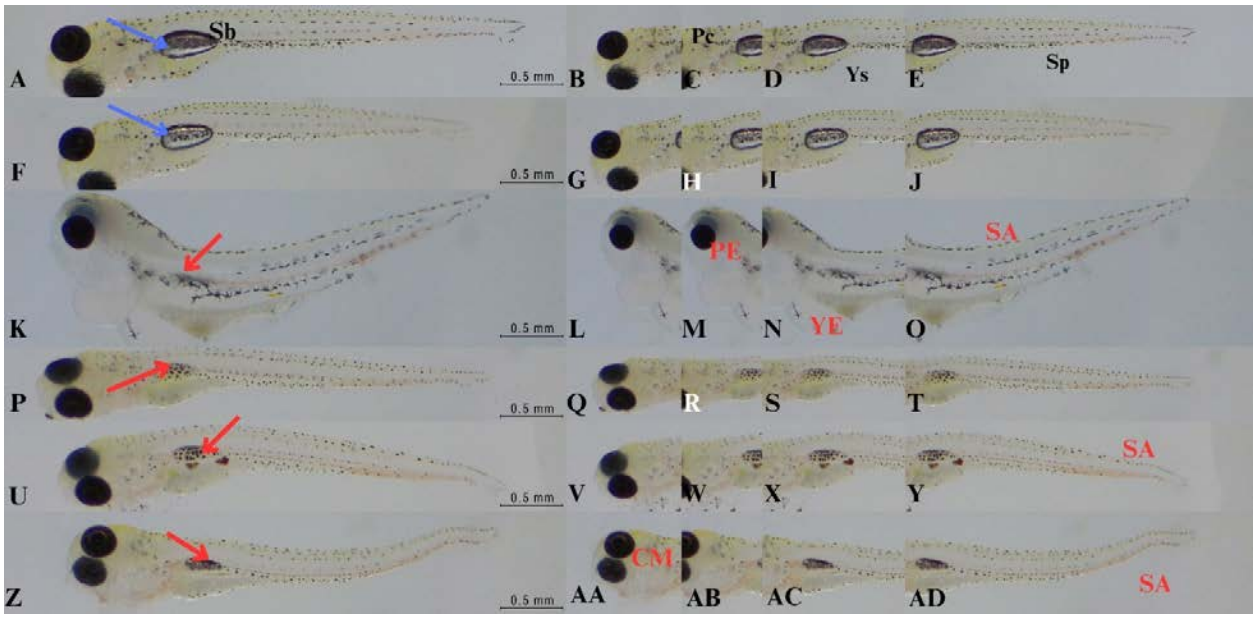

Fig. 8: Morphology of *Rasbora lateristriata* embryos at 96 hpf (hours post fertilization)
A-E: Control, F-J: Solvent control, K-O: Embryo treated KLEE 0.06 mg/mL, P-T: Embryo treated KLEE 0.12 mg/mL, U-Y: Embryo treated 0.25 mg/mL, Z-AD: Embryo treated KLEE 0.5 mg/mL. Sb: Swim bladder, Pc: Pericardial, Ys: Yolk sac, Sp: Spinal, PE: Pericardium edema, YE: Yolk sac edema, CM: Craniofacial malformation, SA: Spinal abnormality, Blue arrow: Normal swim bladder and Red arrow: Impaired swim bladder

Heartbeat rate: Based on the normality test, the data obtained were not normally distributed; therefore, a non-parametric test was used. The non-parametric test applied was Dunn's multiple comparison test. The results of Dunn's test indicated a significant difference between the control group and the KLEE treatment group in terms of the heartbeat rate of *R. lateristriata* embryos.

In accordance with the results shown in Fig. 9, the application of KLEE was found to influence the cardiac rhythm of *R. lateristriata* embryos when administered at concentrations higher than 0.12 mg/mL. No significant alteration in heartbeat rate occurred at 0.06 mg/mL, indicating this dose was non-toxic to embryonic heart function. At 96 hpf, embryos treated with KLEE exhibited a concentration-dependent reduction in heartbeat rate. Meanwhile, no difference was detected between the solvent and control groups, signifying that the solvent used did not interfere with cardiac activity.

Hatching rate: According to Fig. 10, the hatching rate of *R. lateristriata* embryos treated with KLEE showed no difference compared to the control. All groups had a hatching rate above 90% and all embryos had hatched by the 24 hpf observation. This indicates that KLEE treatment did not affect the hatching rate of *R. lateristriata* embryos. *Rasbora lateristriata* embryos normally hatch at 24 hpf. Therefore, KLEE treatment did not cause a delay in hatching.

Survival rate: Based on Fig. 11, the survival rate of *Rasbora lateristriata* embryos was affected by KLEE treatment. The higher the KLEE concentration, the lower the embryo survival rate, as indicated by the percentage survival rate, with the 0.5 mg/mL KLEE treatment showing the lowest survival rate. The solvent control group had a 100% survival rate, the same as the control group. This indicates that the solvent used did not affect the survival rate of *R. lateristriata* embryos.

Kaplan-Meier Graph: The Kaplan-Meier graph is used to illustrate the percentage of survival at specific time intervals. In this study, the time interval used in the graph was 24 hrs. The Kaplan-Meier graph for the bark extract treatment is presented in Fig. 12. The lines for the solvent control and negative control overlap, making the solvent control line (Solvent C, pink) not visible. This indicates that the mortality rate between these two groups is the same. The majority of mortality occurred when the observation reached 72 hrs. The 0.06 mg/mL concentration (light blue line) showed slight mortality within the first 24 hrs before decreasing to 75% survival at 72 hrs, then further dropping to 70% at the 96 hrs observation. The 0.12 mg/mL concentration (green line) did not cause any mortality until the 72 hrs observation. At that point, a mortality rate of 55% was recorded, reducing the survival proportion to 45%. By the final observation (96 hrs), an additional 10% decrease resulted in 35% survival. At a concentration of 0.25 mg/mL (red line), no mortality was

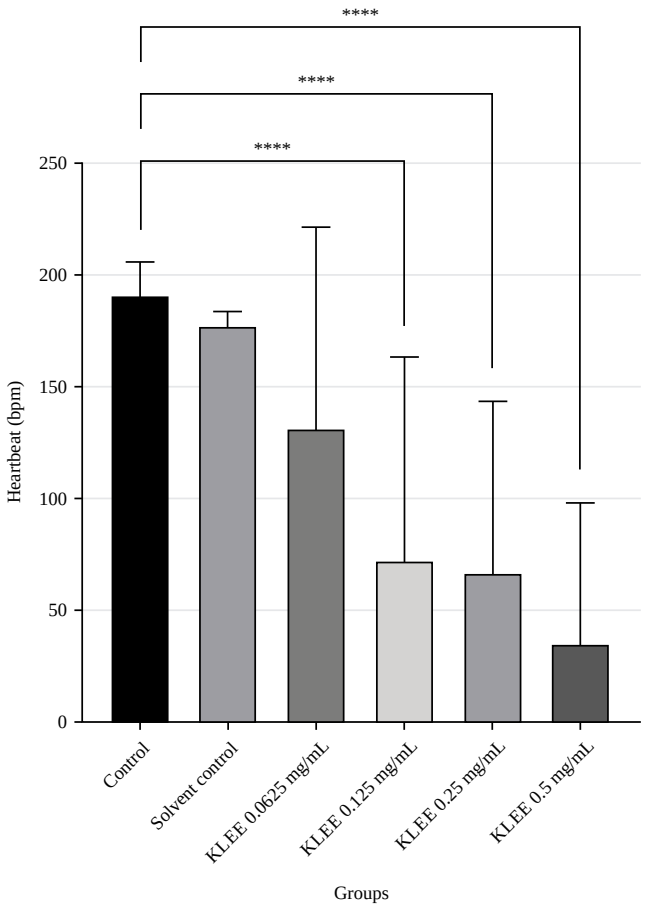

Fig. 9: Heartbeat rate graph of *Rasbora lateristriata* embryos at 96 hpf treated with Kelakai leaves ethanol extract (KLEE)

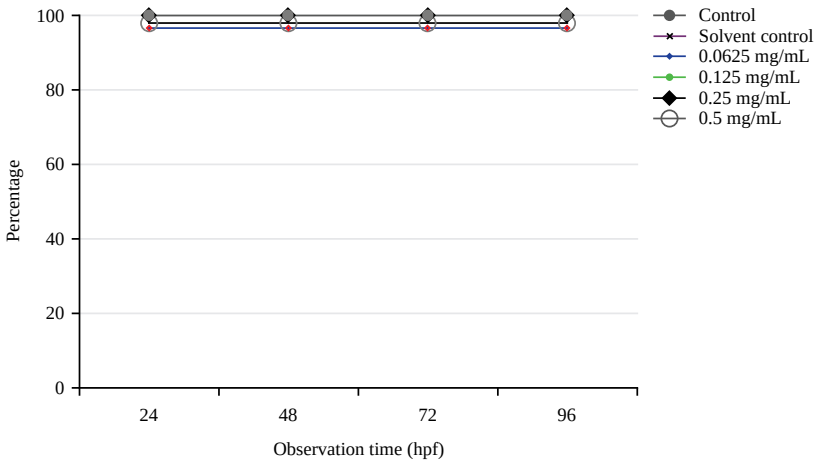

Fig. 10: Hatching rate graph of *Rasbora lateristriata* embryos treated with Kelakai leaves ethanol extract (KLEE) over the observation period

observed in the first 48 hrs. However, a drastic increase in mortality occurred at the 72 hrs observation, dropping survival from 65 to 35% and further decreasing to 10% at the 96 hrs observation. For the 0.5 mg/mL concentration (purple line), the observed mortality at 72 hrs was not as drastic as at 0.25 mg/mL, with only a 40% reduction. However, survival continued to decline, leaving only 10% survivors at the final observation (96 hrs).

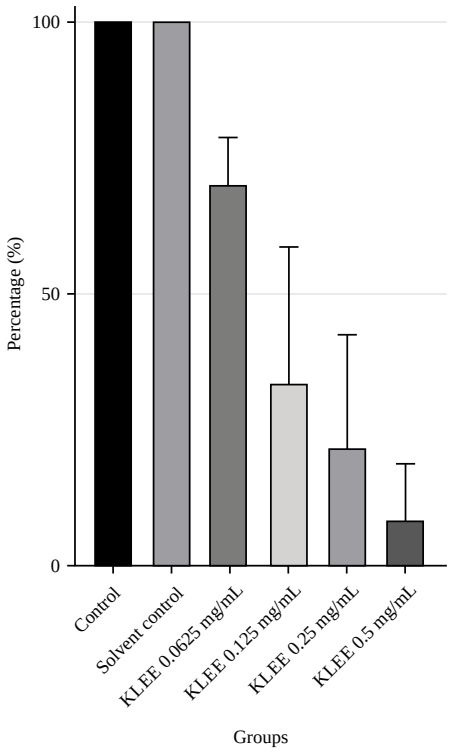

Fig. 11: Survival rate graph of *Rasbora lateristriata* embryo at 96 hpf treated with Kelakai leaves ethanol extract (KLEE)

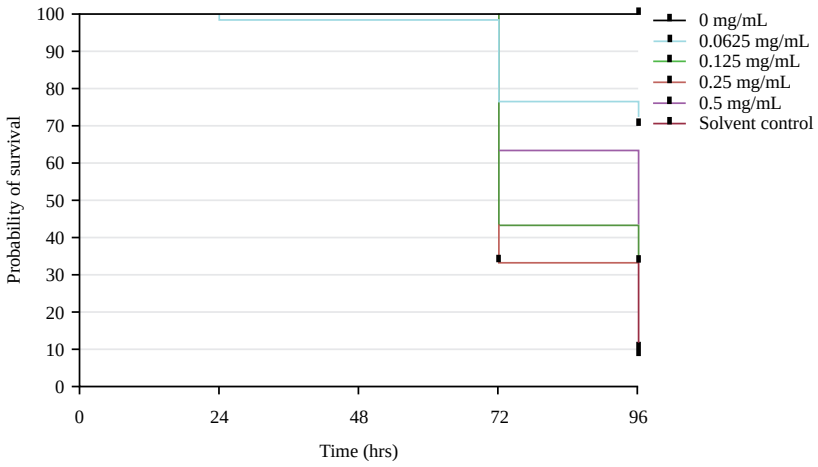

Fig. 12: Proportion of embryo survivability in Kelakai leaves ethanol extract (KLEE) treatment groups

From the results, found that the ethanolic extract of Kelakai leaves ethanol extract (KLEE) demonstrated both concentration-dependent efficacy and toxicity in *R. lateristriata* embryos. Probit analysis revealed an LC50 value of 0.097 mg/mL, indicating that the extract exhibits moderate toxicity within the tested range. Morphological assessments showed reversible abnormalities such as pericardial and yolk sac edema, alongside irreversible defects including spinal abnormalities and craniofacial malformations at higher concentrations. Furthermore, KLEE significantly reduced heartbeat rate at concentrations above 0.125 mg/mL, though no effect was observed on hatching rate, which remained above 90% across all groups. Survival analysis confirmed that higher extract concentrations (particularly 0.25-0.5 mg/mL) markedly decreased embryo viability, with most mortality occurring after 72 hrs of exposure.

This study investigated the antiquorum sensing (QS) or quorum-quenching (QQ) potential of Kelakai leaves ethanol extract (KLEE) as an alternative strategy to control pathogenic infections. These findings align with the emerging paradigm of anti-virulence therapy, where interference with bacterial communication pathways such as quorum sensing (QS) is targeted instead of conventional bactericidal strategies[4,6].

The QS in *P. aeruginosa* involves a hierarchical network comprising the las, rhl and pqs systems, which collectively control the expression of virulence genes responsible for motility, enzyme secretion and biofilm development[28]. Natural products can interfere with QS by blocking autoinducer synthesis, preventing receptor binding or degrading signaling molecules[29]. Several medicinal plants have been documented as QS inhibitors, including *Allium sativum*, *Zingiber officinale*, *Camellia sinensis*, *Azadirachta indica* and *Syzygium aromaticum*[30,31]. Specific metabolites, such as methyl gallate from *Mangifera indica*, have been shown to suppress violacein in *Chromobacterium violaceum* and QS-regulated virulence factors in *P. aeruginosa*[32].

In this work, KLEE markedly reduced pyocyanin production and impaired motility, confirming its QS-disruptive effects. Such activity aligns with quorum quenching (QQ) mechanisms, in which plant-derived molecules act as AHL mimics that competitively occupy LuxR-type receptors. Classic examples include halogenated furanones from *Delisea pulchra*, which destabilize LuxR regulators and malabaricone C from *Myristica cinnamomea*, which inhibits LasR, RhlR and CviR despite not blocking AHL synthesis[31,33].

The KLEE displayed both antibacterial and QQ properties. At higher doses, direct growth inhibition was observed, raising the possibility that antibiofilm and QQ effects may result from either shared or distinct constituents. Motility plays a critical role in colonization and biofilm maturation: Swarming is a density-dependent cooperative migration involving flagella and rhamnolipids, while twitching relies on type IV pili. These movements influence biofilm structure and persistence[34]. Consistent with this, KLEE reduced swarming and twitching, decreased pyocyanin levels and inhibited both the formation and disruption of biofilms in *P. aeruginosa*. The significant inhibition of swarming and twitching motility by KLEE may thus indicate the activity of antibacterial and/or anti-QS compounds that hinder coordinated multicellular migration. These motilities rely on rhamnolipids and type IV pili, whose expression is regulated by the rhl and las circuits. Interference with these pathways suppresses surface colonization and biofilm initiation[34,35]. Such effects suggest that compounds in the extract interfere with QS pathways and weaken biofilm stability, possibly by altering cell wall or membrane properties, hydrophobicity or the extracellular matrix, thereby reducing adhesion and enhancing dispersal[35].

Disruption of QS (Quorum Sensing)-mediated signaling not only interferes with bacterial motility, but also reduces several other virulence factors, including elastase, rhamnolipids, cyanide and pyocyanin, which depend on rhamnolipids (RLs) and 3-(3-hydroxyalkanoyloxyalkanoic acids) (HAAs)[36]. Inhibition of LasA protease and pyocyanin production further substantiates the disruption of the las system. Pyocyanin is a redox-active metabolite that contributes to oxidative stress and host tissue damage, while LasA staphylolytic protease promotes nutrient acquisition and competition with Gram-positive bacteria. The significant decrease in both metabolites under KLEE treatment suggests that extract constituents likely act as antagonists or mimics of acyl-homoserine lactones (AHLs), thereby preventing activation of LuxR-type receptors[30,31].

The substantial decrease in biofilm formation and EPS production indicates that KLEE not only interferes with initial adhesion but also impairs biofilm maturation. The EPS serves as the scaffold of the biofilm, enhancing mechanical stability and antimicrobial tolerance. Its reduction by up to ~68% suggests compromised matrix synthesis or secretion, which may stem from the inhibition of pel and psl operons responsible for exopolysaccharide biosynthesis[21,24]. Interestingly, KLEE also demonstrated moderate bacteriostatic activity, with a PMIC of 0.5 mg/mL. This dual effect, combining anti-QS and mild antibacterial action, can be advantageous in reducing virulence without imposing high selective pressure for resistance[24]. Such combination mechanisms have been reported for plant-derived QS inhibitors like methyl gallate, quercetin and kaempferol, which simultaneously interfere with AHL signaling and biofilm structural integrity[32,36].

Based on these findings, chemical profiling was performed using chromatography and LC-HRMS. LC-HRMS analysis revealed that KLEE contains diverse bioactive constituents, including (-)-strychnine, pipecolic acid, ethyl oleate, 1-stearoylglycerol and stearic acid. Each of these compounds may contribute to QS inhibition through distinct mechanisms. Strychnine is a terpene alkaloid of the Strychnos genus, has been associated with modulation of bacterial membrane potential and enzymatic activity. It is colorless, odorless, intensely bitter and chiefly toxic through antagonism of postsynaptic glycine receptors in the spinal cord, causing involuntary muscle spasms. Although toxic in mammals, its structural features, planar aromatic rings and nitrogen-containing heterocycles, are consistent with AHL mimetics that compete for receptor binding. Its detection in biological samples of opioid users has been reported, suggesting occasional adulteration[37].

Pipecolic acid (Pip; 2-piperidinecarboxylic acid) is a chiral, non-proteinogenic amino acid derived from lysine catabolism that accumulates in plants during pathogen attack, participates in systemic acquired resistance and functions in osmotic regulation, suggesting potential cross-kingdom effects on bacterial signaling and stress regulation. It also contributes to immune regulation in plants and humans[38-41].

Ethyl oleate (EO), formed enzymatically from oleic acid (α/β-hydrolases), serves as a pheromone in *Apis mellifera* and has been reported among non-cytotoxic metabolites of *Trichoderma atroviride* with putative antibacterial and antioxidant effects[42,43]. Additionally, 1-stearoylglycerol isolated from *R. aquatic* shows antibacterial activity against a broad panel of Gram-positive and Gram-negative bacteria, including *P. aeruginosa*, possibly by weakening EPS matrix cohesion. Stearic acid, present in *Opuntia ficus-indica* essential oils and in *Ziziphus spina-christi*, has been associated with antimicrobial effects (e.g., against *Saccharomyces cerevisiae*) and reported cytotoxic/antiviral activities in specific models[44]. Both EO and stearic acid capable of integrating into bacterial membranes, altering surface hydrophobicity and perturbing QS signal diffusion[43]. Together, these compounds likely produce synergistic effects, attenuating signal perception, interfering with membrane-associated receptor dynamics and reducing biofilm matrix integrity[35].

The embryotoxicity of KLEE in *R. lateristriata* underscores the necessity of evaluating the therapeutic index of crude plant extracts. The observed LC50 of 0.097 mg/mL denotes moderate toxicity, potentially attributable to strychnine and long-chain fatty acids. The developmental toxicity observed at higher concentrations is likely due to the combined effects of multiple constituents within the crude extract acting additively or synergistically. Nevertheless, the absence of hatching delay and the reversibility of certain morphological abnormalities (e.g., yolk sac and pericardial edema) suggest that toxic responses are concentration-dependent and partly reversible. Similar embryonic models (e.g., zebrafish) have been used to screen phytochemicals for eco-toxicological safety before pharmacological development. Thus, isolating and characterizing the individual active compounds may reduce overall toxicity and improve safety for host cells, thereby supporting their potential development as antimicrobial or therapeutic agents. Future work should focus on bioassay-guided fractionation to isolate the active constituents with minimal toxicity. Fractionation could separate the QS-inhibitory fractions from the toxic ones, allowing safer formulations or encapsulation strategies to enhance delivery and selectivity.

Stenochlaena palustris offers unique advantages; it is endemic to Kalimantan, abundant and culturally integrated in traditional medicine. Thus, valorization of this species supports both scientific innovation and local biodiversity conservation. Moreover, the integration of QQ strategies using natural products could complement existing antibiotics in combinatorial therapy, where QQ agents suppress virulence while antibiotics target bacterial survival. Such synergistic approaches have shown promise in mitigating chronic infections and reducing antibiotic usage.

CONCLUSION

The KLEE effectively attenuates *P. aeruginosa* virulence by suppressing QS-regulated traits: pyocyanin production, swarming/twitching motility and biofilm formation, while also showing activity against established biofilms. The LC-HRMS indicates a chemically diverse extract, including alkaloids, fatty-acid derivatives and non-proteinogenic amino acids that may act on complementary targets within QS and biofilm pathways. Given the developmental toxicity observed in the embryo model and the likelihood that multiple constituents contribute to both efficacy and toxicity, future work should prioritize bioassay-guided fractionation to isolate and standardize the principal QQ agents, followed by rigorous cytotoxicity and safety profiling and, ultimately, formulation optimization to enable safe therapeutic development.

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SIGNIFICANCE STATEMENT

This study shows that the endemic Kalimantan fern *S. palustris* (Kelakai) exerts strong quorum-quenching and antibiofilm effects against *P. aeruginosa*, suppressing key virulence outputs (pyocyanin, LasA, swarming/twitching).

These findings position Kelakai as a promising natural source for anti-virulence strategies, while motivating isolation of safer active constituents for therapeutic development.

REFERENCES

1. Cosentino, F., P. Viale and M. Giannella, 2023. MDR/XDR/PDR or DTR? Which definition best fits the resistance profile of *Pseudomonas aeruginosa*? Curr. Opin. Infect. Dis., 36: 564-571.
2. Kothari, A., R. Kherdekar, V. Mago, M. Uniyal and G. Mamgain *et al*., 2023. Age of antibiotic resistance in MDR/XDR clinical pathogen of *Pseudomonas aeruginosa*. Pharmaceuticals, Vol. 16. 10.3390/ph16091230.
3. Boni, S., G.H. Marin, L. Campaña, L. Marin, S. Risso-Patrón and G. Marin *et al*., 2022. Association between consumption of fluoroquinolones and carbapenems and their resistance rates in *Pseudomonas aeruginosa* in Argentina. Interdiscip. Perspect. Infect. Dis., Vol. 2022. 10.1155/2022/3924212.
4. Zhao, X., Z. Yu and T. Ding, 2020. Quorum-sensing regulation of antimicrobial resistance in bacteria. Microorganisms, Vol. 8. 10.3390/microorganisms8030425.
5. Liao, C., X. Huang, Q. Wang, D. Yao and W. Lu, 2022. Virulence factors of *Pseudomonas aeruginosa* and antivirulence strategies to combat its drug resistance. Front. Cell. Infect. Microbiol., Vol. 12. 10.3389/fcimb.2022.926758.
6. Naga, N.G., D.E. El-Badan, K.M. Ghanem and M.I. Shaaban, 2023. It is the time for quorum sensing inhibition as alternative strategy of antimicrobial therapy. Cell Commun. Signaling, Vol. 21. 10.1186/s12964-023-01154-9.
7. Moradi, F. and N. Hadi, 2021. Quorum-quenching activity of some Iranian medicinal plants. New Microbes New Infect., Vol. 42. 10.1016/j.nmni.2021.100882.
8. Mashar, H.M., S.A. Damiti, Dali, Y. Ysrafil, I. Ismail and A. Sukrianur, 2022. Education on the potential of kelakai as antibacterial traditional medicine. J. Mandala Pengabdian Masyarakat, 3: 72-79.
9. Dewi, A.A.D. and R.P. Bekti, 2023. Utilizing the potential of the kelakai plant (*Stenochlaena palustris*) as an organic compost material [In Indonesian]. J. Pertanian Peradaban, 3: 28-33.
10. Lima, G.S., N.M. Lima, J.V. Roque, D.V.A. de Aguiar and J.V.A. Oliveira *et al*., 2022. LC-HRMS/MS-based metabolomics approaches applied to the detection of antifungal compounds and a metabolic dynamic assessment of orchidaceae. Molecules, Vol. 27. 10.3390/molecules27227937.
11. APHA, 2005. Standard Methods for the Examination of Water and Wastewater. 21st Edn., American Public Health Association, Washington, D.C., USA, ISBN-13: 978-0875530475, Pages: 1288.
12. Alqurashi, A.S., L.M. Al Masoudi, H. Hamdi and A. Abu Zaid, 2022. Chemical composition and antioxidant, antiviral, antifungal, antibacterial and anticancer potentials of *Opuntia ficus-indica* seed oil. Molecules, Vol. 27. 10.3390/molecules27175453.
13. Gebrehiwot, H., U. Ensermu, A. Dekebo, M. Endale and T.N. Duke, 2024. *In vitro* antibacterial and antioxidant activities, pharmacokinetics, and *in silico* molecular docking study of phytochemicals from the roots of *Ziziphus spina-christi*. Biochem. Res. Int., Vol. 2024. 10.1155/2024/7551813.
14. Roohinejad, S., M. Koubaa and S.M.T. Gharibzahedi, 2025. Drying technologies for *Stevia rebaudiana* bertoni: Advances, challenges, and impacts on bioactivity for food applications-A review. Foods, Vol. 14. 10.3390/foods14162801.
15. Bergeau, D., S. Mazurier, C. Barbey, A. Merieau and A. Chane *et al*., 2019. Unusual extracellular appendages deployed by the model strain *Pseudomonas fluorescens* C7R12. PLoS ONE, Vol. 14. 10.1371/journal.pone.0221025.
16. Najafi, M., M.N. Moghaddam and E. Yousefi, 2021. The effect of silver nanoparticles on pyocyanin production of *Pseudomonas aeruginosa* isolated from clinical specimens. Avicenna J. Med. Biotechnol., 13: 98-103.
17. Yin, L., Y. Zhang, F. Azi, J. Zhou and X. Liu *et al*., 2022. Inhibition of biofilm formation and quorum sensing by soy isoflavones in *Pseudomonas aeruginosa*. Food Control, Vol. 133. 10.1016/j.foodcont.2021.108629.
18. Qaralleh, H., 2019. Thymol rich *Thymbra capitata* essential oil inhibits quorum sensing, virulence and biofilm formation of beta lactamase producing *Pseudomonas aeruginosa*. Nat. Prod. Sci., 25: 172-180.
19. Benaissa, A., A. Khadir, A.N. Tamfu, S. Kucukaydin and N. Latti *et al*., 2024. Biofilm disruption and virulence attenuation effects of essential oil from endemic Algerian *Cistus munbyi* (cistaceae) against clinical strains of *Pseudomonas aeruginosa*. Nat. Prod. Commun., Vol. 19. 10.1177/1934578X241245234.
20. Ayub, R., M. Umer, A.A. Maan, B. Rasool and M.K.I. Khan *et al*., 2020. Antibiotics, acid and heat tolerance of honey adapted *Escherichia coli*, *Salmonella* typhi and *Klebsiella pneumoniae*. Foods, Vol. 9. 10.3390/foods9030311.
21. Cepas, V., Y. López, E. Muñoz, D. Rolo and C. Ardanuy *et al*., 2019. Relationship between biofilm formation and antimicrobial resistance in gram-negative bacteria. Microb. Drug Resist., 25: 72-79.
22. Chen, A.Q., Z.Q. Long, Y. Xiao, Y.M. Feng and Y. Zhou *et al*., 2024. Application of natural product-based quorum sensing inhibitors in plant pathogen control: A review. Arabian J. Chem., Vol. 18. 10.1016/j.arabjc.2024.106050.
23. Haney, E.F., M.J. Trimble and R.E.W. Hancock, 2021. Microtiter plate assays to assess antibiofilm activity against bacteria. Nat. Protoc., 16: 2615-2632.

24. Ghanaim, A.M., H.I. Mohamed and A.E. El-Ansary, 2025. Production and characterization of exopolysaccharides from *Pseudomonas aeruginosa* AG01 with some medical potential applications. Microb. Cell Fact., Vol. 24. 10.1186/s12934-025-02730-z.
25. Kurniawati, F., D. Fitria, A.R.U. Nainggolan and P.A. Wardani, 2022. Identification and characterization of kratom plants through the LC-HRMS QToF marker compound content profile approach and determination of retention index values using GCMS [In Indonesian]. Eruditio: Indonesia J. Food Drug Saf., 3: 79-90.
26. Rios, J.L. and M.C. Recio, 2005. Medicinal plants and antimicrobial activity. J. Ethnopharmacol., 100: 80-84.
27. Wilson, C., R. Lukowicz, S. Merchant, H. Valquier-Flynn and J. Caballero *et al*., 2017. Quantitative and qualitative assessment methods for biofilm growth: A mini-review. Res. Rev.: J. Eng. Technol., 6: 1-25.
28. Qin, S., W. Xiao, C. Zhou, Q. Pu and X. Deng *et al*., 2022. *Pseudomonas aeruginosa*: Pathogenesis, virulence factors, antibiotic resistance, interaction with host, technology advances and emerging therapeutics. Signal Transduction Targeted Ther., Vol. 7. 10.1038/s41392-022-01056-1.
29. Cardiliya, A.P., A. Selvaraj, M.J. Nanjan and M.J.N. Chandrasekar, 2022. Plant based natural products as quorum sensing inhibitors in *E. coli*: Acritical review. Mini-Rev. Med. Chem., 22: 1380-1394.
30. Hetta, H.F., Y.N. Ramadan, Z.I. Rashed, A.A. Alharbi and S. Alsharef *et al*., 2024. Quorum sensing inhibitors: An alternative strategy to win the battle against multidrug-resistant (MDR) bacteria. Molecules, Vol. 29. 10.3390/molecules29153466.
31. Alum, E.U., B.H. Gulumbe, S.C. Izah, D.E. Uti, P.M. Aja, I.O. Igwenyi and C.E. Offor, 2025. Natural product-based inhibitors of quorum sensing: A novel approach to combat antibiotic resistance. Biochem. Biophys. Rep., Vol. 43. 10.1016/j.bbrep.2025.102111.
32. Naga, N.G., A.A. Zaki, D.E. El-Badan, H.S. Rateb, K.M. Ghanem and M.I. Shaaban, 2023. Inhibition of *Pseudomonas aeruginosa* quorum sensing by methyl gallate from *Mangifera indica*. Sci. Rep., Vol. 13. 10.1038/s41598-023-44063-0.
33. Zheng, X., J. Liu and X. Wang, 2025. Quorum signaling molecules: Interactions between plants and associated pathogens. Int. J. Mol. Sci., Vol. 26. 10.3390/ijms26115235.
34. de Sousa, T., M. Hébraud, O. Alves, E. Costa and L. Maltez *et al*., 2023. Study of antimicrobial resistance, biofilm formation, and motility of *Pseudomonas aeruginosa* derived from urine samples. Microorganisms, Vol. 11. 10.3390/microorganisms11051345.
35. Mulat, M., R.J.S. Banicod, N. Tabassum, A. Javaid and A. Karthikeyan *et al*., 2025. Multiple strategies for the application of medicinal plant-derived bioactive compounds in controlling microbial biofilm and virulence properties. Antibiotics, Vol. 14. 10.3390/antibiotics14060555.
36. Escobar-Muciño, E., M.M.P. Arenas-Hernández and M.L. Luna-Guevara, 2022. Mechanisms of inhibition of quorum sensing as an alternative for the control of *E. coli* and *Salmonella*. Microorganisms, Vol. 10. 10.3390/microorganisms10050884.
37. Buchanan, J., C. Lyle, M.R. Castenada, J. Altholz and R. Ralph *et al*., 2025. Strychnine as an emerging adulterant in the illicit fentanyl drug supply. AJPM Focus, 10.1016/j.focus.2025.100410.
38. Al-Rooqi, M.M., Ehsan Ullah Mughal, Q.A. Raja, R.J. Obaid and A. Sadiq *et al*., 2022. Recent advancements on the synthesis and biological significance of pipecolic acid and its derivatives. J. Mol. Struct., Vol. 1268. 10.1016/j.molstruc.2022.133719.
39. Wang, C., R. Liu, G.H. Lim, L. de Lorenzo and K. Yu *et al*., 2018. Pipecolic acid confers systemic immunity by regulating free radicals. Sci. Adv., Vol. 4. 10.1126/sciadv.aar4509.
40. Kucukkalyon, S.M. and B.S. Dinler, 2025. Pipecolic acid priming promotes salt stress tolerance via regulating antioxidant defense system and sugar metabolism in barley plants. Cereal Res. Commun., 53: 1479-1494.
41. Kumari, M., P. Sharma and A. Singh, 2025. Pipecolic acid: A positive regulator of systemic acquired resistance and plant immunity. Biochim. Biophys. Acta, Gen. Subj., Vol. 1869. 10.1016/j.bbagen.2025.130808.
42. Castillo, C., H. Chen, C. Graves, A. Maisonnasse, Y.L. Conte and E. Plettner, 2012. Biosynthesis of ethyl oleate, a primer pheromone, in the honey bee (*Apis mellifera* L.). Insect Biochem. Mol. Biol., 42: 404-416.
43. Saravanakumar, K., R. Chelliah, S.R. Ramakrishnan, K. Kathiresan, D.H. Oh and M.H. Wang, 2018. Antibacterial, and antioxidant potentials of non-cytotoxic extract of *Trichoderma atroviride*. Microb. Pathogen., 115: 338-342.
44. Orbán-Gyapai, O., E. Liktor-Busa, N. Kúsz, D. Stefkó, E. Urbán, J. Hohmann and A. Vasas, 2017. Antibacterial screening of *Rumex* species native to the Carpathian Basin and bioactivity-guided isolation of compounds from *Rumex aquaticus*. Fitoterapia, 118: 101-106.