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Small Peptides from solitary wasps exhibit antibacterial activity against Xanthomonas Campestris cv oryzae: Relevance for plant protection

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Small Peptides from solitary wasps exhibit antibacterial activity against *Xanthomonas Campestris cv oryzae:* Relevance for plant protection

Abstract

Insects, the largest and most diverse class within the phylum Arthropoda, represent an extensive yet underexplored source of antimicrobial peptides (AMPs), especially from non-model groups like solitary wasps. Despite their ecological diversity and complex venom systems, their potential as sources of bioactive compounds for managing plant pathogens remains largely unexamined. In this study, AMPs were examined from four solitary wasp species: *C. bengalense, S. madraspatanam, D. conoideum*, and *P. flavopictus*. Crude body and venom gland extracts were tested for antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Among the species tested, only the body extract of *S. madraspatanam* (12.84 µg/spot) showed broad-spectrum activity against *S. aureus* (57.83 ± 2.2 mm²) and *E. coli* (41.31 ± 1.8 mm²), while the venom extract of *P. flavopictus* (10.75 µg/spot) demonstrated selective activity against *S. aureus* (100.08 ± 1.8 mm²). Bioassay-guided RP-HPLC purification of *S. madraspatanam* yielded five fractions, with only Fraction-3 exhibiting antibacterial activity, including against the plant pathogen *Xanthomonas oryzae pv. oryzae* (10.27 ± 0.26 mm²). MALDI-TOF analysis identified a dominant peptide at m/z 861.049. In contrast, individual fractions of *P. flavopictus* were inactive; however, combinations of fractions showed synergistic antibacterial effects, with the mixture (1+2+3) demonstrating significant inhibition against *S. aureus* (24.54 ± 1.4 mm²). No antifungal activity was observed against *Sclerotium rolfsii*. These results demonstrate that solitary wasps are a promising source of antimicrobial peptides, with both individual bioactive components and synergistic interactions playing a role in antibacterial activity, underscoring their potential for developing sustainable strategies against plant pathogens.

Keywords: Antibacterial activity, Insect-derived AMPs, Biocontrol, Peptide fractions, Bioactivity, Peptide synergy

Introduction

Antimicrobial peptides (AMPs) are small, gene-encoded proteins, usually containing 10–100 amino acid residues, that play a key role in the innate immune response of various organisms, including the order Hymenoptera (such as those from bees and wasps) [1–3]. These peptides are recognized as vital, evolutionarily conserved defense mechanisms that provide immediate protection against bacteria, viruses, fungi, and parasites [4, 5]. Hymenopteran AMPs are often cationic (positively charged) and amphipathic, allowing them to interact with and disrupt the negatively charged lipid membranes of pathogens [6, 7]. By exploring the potent chemical arsenal of Hymenoptera, we uncover not just alternative biocontrol agents but a revolutionary blueprint for next-generation biopesticides that can balance intensive agricultural output with ecological health.

Plant diseases caused by diverse pathogens, including bacteria, fungi, viruses, and nematodes, represent a major constraint to global agricultural productivity and food security. Recent estimates indicate that more than 30% of global crop yield is lost annually due to plant diseases, resulting in economic losses worth hundreds of billions of dollars [8] . Annually, plant pathogens impose a staggering US$220 billion tax on the global economy, representing a primary bottleneck to sustainable intensification and planetary food security [9, 10]. Within this landscape, Fungal pathogens drive the most pervasive agricultural losses, accounting for 10–23% of annual yields and 10–20% of post-harvest attrition [11, 12]. While bacterial pathogens cause lower volumetric losses (2–15%), they pose a critical "double threat" through rapid horizontal gene transfer and a lack of non-toxic field treatments [13].

The selection of test organisms in the present study was based on their biological relevance and widespread use as model and economically important pathogens. *Staphylococcus aureus* and *Escherichia coli* were chosen as representative Gram-positive and Gram-negative bacteria, respectively, due to their well-characterized cell envelope structures and their routine use in antimicrobial screening assays to evaluate broad-spectrum activity [14, 15]. These organisms serve as standard indicators for assessing the efficacy and membrane-targeting potential of antimicrobial peptides. In addition, *Xanthomonas oryzae pv. oryzae,* the causative agent of bacterial leaf blight in rice, was selected as a model plant pathogen due to its significant economic impact and its role as a major constraint in rice production systems worldwide [16]. Extensive research has been conducted to identify novel antimicrobial agents targeting *Xanthomonas spp*., including plant-derived compounds [17–19], microbial metabolites [20, 21], and synthetic peptides [22, 23]; however, the exploration of insect-derived antimicrobial peptides, particularly from Hymenopteran species, remains limited. Previous studies have demonstrated that insect antimicrobial peptides exhibit strong activity against human pathogens [24–26], but their application against plant-pathogenic bacteria is comparatively underexplored. Therefore, evaluating the efficacy of wasp-derived peptide fractions against both standard bacterial models and agriculturally relevant pathogens provides a comprehensive framework for assessing their potential as broad-spectrum and plant-specific antimicrobial agents.

Building upon our previous findings regarding the antimicrobial potential of insect-derived peptides in mitigating pomegranate post-harvest losses [27, 27], the present study further expands this investigation to four solitary wasp species a) *Chalybion bengalense* b) *Sceliphron madraspatanam and* c) *Delta conoideum* d) *Phimenes flavopictus.* We report the isolation, purification, and characterization of bioactive peptide fractions from both crude body and venom gland extracts. The antibacterial efficacy of these fractions was evaluated against *E.*

coli, *S. aureus*, and the rice blight pathogen *X. oryzae pv. oryzae*. Following initial screening, bioactive peptides were purified via Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) and characterized using MALDI-TOF MS. Our results demonstrate that solitary wasp-derived antimicrobial peptides (AMPs) represent a promising, sustainable alternative for the management of recalcitrant plant pathogenic bacteria.

Materials and Methods

Specimen collection and ethical considerations

Four solitary wasp species, *Chalybion bengalense*, *Sceliphron madraspatanam*, *Delta conoideum*, and *Phimenes flavopictus* were collected from various locations around the University of Agricultural Sciences (UAS), GKVK, Bengaluru, India, based on their abundance in the area (Table 1). Specimens were collected from habitats including crop fields, gardens, old buildings, flowering plants, and nearby irrigated lands. Individual wasps were manually collected using an insect net and immediately placed into separate sterile bottles. Taxonomic identification was performed based on morphological characteristics using standard taxonomic keys and confirmed with the help of an entomologist from the Department of Entomology, UAS, GKVK. To minimize ecological disturbance, only a limited number of individuals were collected, and care was taken to avoid disturbing nesting sites or surrounding habitats. The collection was conducted strictly for research purposes, ensuring that natural populations were not adversely affected. The specimens were kept alive in separate bottles immediately after collection. They were inactivated by placing them at −5 °C for about 30 minutes and then stored at −80 °C until further use.

Collection locations and number of samples of solitary wasp species used in the study

Sl. No.	Wasp Species	Family	Collection Locations	Number of Samples
1	*Chalybion bengalense*	Sphecidae	GKVK Campus Bangalore, India	246
2	*Sceliphron madraspatanam*	Sphecidae	National Seed Project, GKVK; Zonal Agricultural Research Station, GKVK; University of Horticultural Sciences, Bagalkot Bangalore, India	33
3	*Delta conoideum*	Vespidae	National Seed Project, GKVK; Zonal Agricultural Research Station, GKVK Bangalore, India	39
4	*Phimenes flavopictus*	Vespidae	Department of Horticulture, GKVK; North Block, Bangalore, India	20

Venom Gland Isolation and Crude Extract Preparation

Venom glands were isolated from inactivated female wasp specimens (venom apparatus derived from the ovipositor) by holding the sting at the terminal end of the abdomen with forceps and gently pulling to extract the gland. The isolated venom glands were immediately transferred into glass vials containing an organic solvent mixture (1:1 acetonitrile: distilled water with 0.1% Trifluoroacetic acid [TFA]) and stored at −80 °C until further use. For crude extract preparation, the wings and legs of each wasp were carefully dissected and discarded, and the remaining body parts, excluding the venom glands, were collected. These tissues were then placed in the same solvent mixture (1:1 acetonitrile: distilled water with 0.1% TFA) and stored at −80 °C until further processing for crude extract preparation.

Preparation of Venom Gland and Body Extracts

Both the venom glands and the wasp body tissues preserved in solvent were subjected to ultrasonic disruption using a probe sonicator (Qsonica Q700, USA) to facilitate the extraction of bioactive compounds. Sonication was performed at 80% amplitude in pulse mode (10 s ON / 10 s OFF) for a total duration of 60 seconds for venom glands and 3 minutes for body tissues, while the samples were kept on ice to prevent overheating. The resulting yellowish aqueous

extracts were transferred to fresh Eppendorf centrifuge tubes and centrifuged at 10,000 rpm for 15 minutes at 4 °C to remove insoluble debris. The supernatants were carefully collected into new tubes, and the centrifugation step was repeated once more for the body tissue extracts to obtain a clear, foam-free aqueous phase. The extracts were then freeze-dried in a lyophilizer at −40 °C to remove excess solvent and obtain a powdered extract. The dried extracts were weighed and reconstituted in fresh acetonitrile: water (1:1, containing 0.1% TFA) and stored at −80 °C until further analysis.

Protein estimation and Antibacterial assay for the venom gland and crude body extract

Protein concentration of venom gland and crude body extracts was determined using the Bradford assay. A 2 mg/mL BSA stock solution was prepared with distilled water, stored at −20°C, and diluted (1:10) to obtain a 0.2 mg/mL working standard. Serial volumes (2–20 µL) of the working standard were dispensed into a 96-well microplate and adjusted to 20 µL with distilled water, while 5 µL of crude samples were added in separate wells. Subsequently, 180 µL of Bradford reagent was added to each well, incubated in the dark for 15–20 min, and absorbance was recorded at 595 nm using a microplate reader. Protein concentrations were calculated from a standard calibration curve generated using BSA. The antibacterial activity of crude extracts was evaluated against *S. aureus* (MTCC 3160) and *E. coli* (MTCC 2692), CSIR-Institute of Microbial Technology (CSIR-IMTech), Chandigarh, India, using a spot-on-lawn assay. Overnight cultures (OD_{600} = 0.1) were mixed with molten nutrient agar and poured into sterile 90 mm Petri plates, allowed to solidify, and 5 µL of test samples were spotted onto the agar surface. Tetracycline served as a positive control, while acetonitrile: water (1:1, v/v) containing 0.1% TFA was used as a negative control. Plates were incubated at 37°C overnight, inhibition zones were observed as clear annular regions surrounding the application spot. To achieve more accurate quantification, particularly in cases of non-uniform or diffuse inhibition

patterns, the inhibition annulus was expressed as area (mm^2) rather than diameter. The area of inhibition was calculated using the relationship:

$$A = \pi (R^2 - r^2)$$

Where R represents the radius of the total inhibition zone (including the clear zone), and r represents the radius of the central application spot. Measurements were obtained using a calibrated Vernier calliper, and all experiments were performed in triplicate.

Purification and characterization of antibacterial peptide fractions

Lyophilized crude extracts were purified using Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) on a C18 column (Phenomenex; 250 × 4.6 mm, 5 μm) with a binary solvent system comprising solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). Separation was achieved using a gradient program of 0–5 min (5% B), 5–25 min (5–60% B), 25–30 min (60–95% B), 30–35 min (95% B), followed by re-equilibration to 5% B (35–40 min) at a flow rate of 1.0 mL/min, column temperature of 30°C, and injection volume of 100 μL, with detection at 214 nm. Fractions were collected based on peak profiles within defined retention windows and pooled from three runs, lyophilized, and stored at −20°C; protein concentration was determined using the Bradford assay at 595 nm with a bovine serum albumin (BSA) standard curve. Bioactive fractions were characterized by MALDI–TOF mass spectrometry (Bruker Daltonics Ultraflex TOF/TOF) using a 337 nm nitrogen laser, where samples reconstituted in 0.1% TFA were mixed (1:1) with α-cyano-4-hydroxycinnamic acid (CHCA) matrix (in 50% acetonitrile/0.1% TFA), spotted by the dried-droplet method, and air-dried prior to analysis; spectra were acquired in positive and negative reflector modes over an m/z range of 400–2700 Da with MS/MS performed using LIFT technology and 2000 laser shots per spectrum, externally calibrated using a peptide standard mixture (angiotensin II,

bradykinin 1–7, ACTH fragments 1–17 and 18–39, and bovine insulin), and processed using FlexAnalysis software (version 2.0).

In vitro Antibacterial assay on plant pathogenic bacteria

Purified peptide fractions obtained from *Sceliphron madraspatanam* were evaluated for antimicrobial activity against *Xanthomonas oryzae pv. oryzae* using a spot-on-lawn assay. The bacterial culture was grown to mid-log phase and uniformly spread onto sterile nutrient agar plates to obtain a confluent lawn. Peptide fractions were quantified and reconstituted in ACN: H_2O (1:1), and a fixed volume (10 µL) of each fraction was applied onto the agar surface, corresponding to the following peptide loads: Fraction-1 (1.63 µg/spot), Fraction-2 (1.54 µg/spot), Fraction-3 (2.22 µg/spot), Fraction-4 (2.55 µg/spot), and Fraction-5 (2.28 µg/spot). Tetracycline (5 µg/10 µL) served as the positive control, while ACN: H_2O (1:1; 10 µL) was used as the negative control. Plates were incubated at 28 ± 2 °C for 12 h to allow peptide diffusion and interaction with the bacterial lawn. Antimicrobial activity was quantified by measuring the zone of inhibition (mm^2). All treatments were performed in triplicate, and mean values were used for comparative analysis.

Data analysis

All experiments were performed in triplicate, and results are expressed as mean ± SD. Differences among treatments were analyzed using one-way or two-way ANOVA, depending on the experimental design, followed by Tukey's HSD post hoc test for multiple comparisons. Statistical significance was set at $p < 0.05$. Analyses were performed using R version 4.5.1

Results

Protein content of venom gland and whole body extract

The total protein concentration of crude body and venom gland extracts of solitary wasps was determined. Overall, protein levels were higher in body extracts than in venom gland extracts, likely due to the presence of diverse structural and enzymatic proteins in whole-body tissues, whereas venom contains a more limited set of specialized bioactive peptides (Table 2). Among the venom gland extracts, *Phimenes flavopictus* exhibited the highest protein concentration (2.150 mg/mL), while *Chalybion bengalense* showed the lowest (1.005 mg/mL). In contrast, the highest protein concentration in body extracts was observed in *C. bengalense* (3.351 mg/mL), whereas *Sceliphron madraspatanam* recorded the lowest (2.056 mg/mL) (Fig. 1). These variations indicate species-specific differences in protein abundance and composition, which may influence their antimicrobial potential.

Table 2 Total protein concentration of crude extracts from solitary wasps

Sl. No.	Species	Extract Type	Protein concentration (mg/mL)
1	*Chalybion bengalense*	Body	3.351
2	*Chalybion bengalense*	Venom gland	1.005
3	*Sceliphron madraspatanam*	Body	2.569
4	*Sceliphron madraspatanam*	Venom gland	2.056
5	*Delta conoideum*	Body	2.864
6	*Delta conoideum*	Venom gland	1.513
7	*Phimenes flavopictus*	Body	2.651
8	*Phimenes flavopictus*	Venom gland	2.150

Antibacterial activity of venom gland and whole body extract

The antibacterial activity of crude body and venom gland extracts from *C. bengalense, S. madraspatanam*, *D. conoideum*, and *P. flavopictus* against *S. aureus* and *E. coli* using a spot-on-lawn assay revealed that only the body extract of *S. madraspatanam* (12.84 µg/spot) exhibited significant inhibition of both *S. aureus* (57.83 ± 2.2 mm^2) and *E. coli* (41.31 ± 1.8 mm^2), indicating broad-spectrum activity, whereas its venom extract showed no activity. In contrast, the venom gland extract of *P. flavopictus* (10.75 µg/spot) displayed strong antibacterial activity against *S. aureus* (100.08 ± 1.8 mm^2) but not against *E. coli*, suggesting specificity toward Gram-positive bacteria, while its body extract remained inactive (Fig. 1 and 2). No antibacterial activity was observed in either the body or venom extracts of *C. bengalense* and *D. conoideum* (Supplementary Fig. 1 and Fig. 2), with responses comparable to the negative control (ACN: H_2O). The positive control, tetracycline, showed the highest inhibition against both pathogens, confirming assay reliability, and statistical analysis ($p < 0.05$) indicated significant differences among treatments.

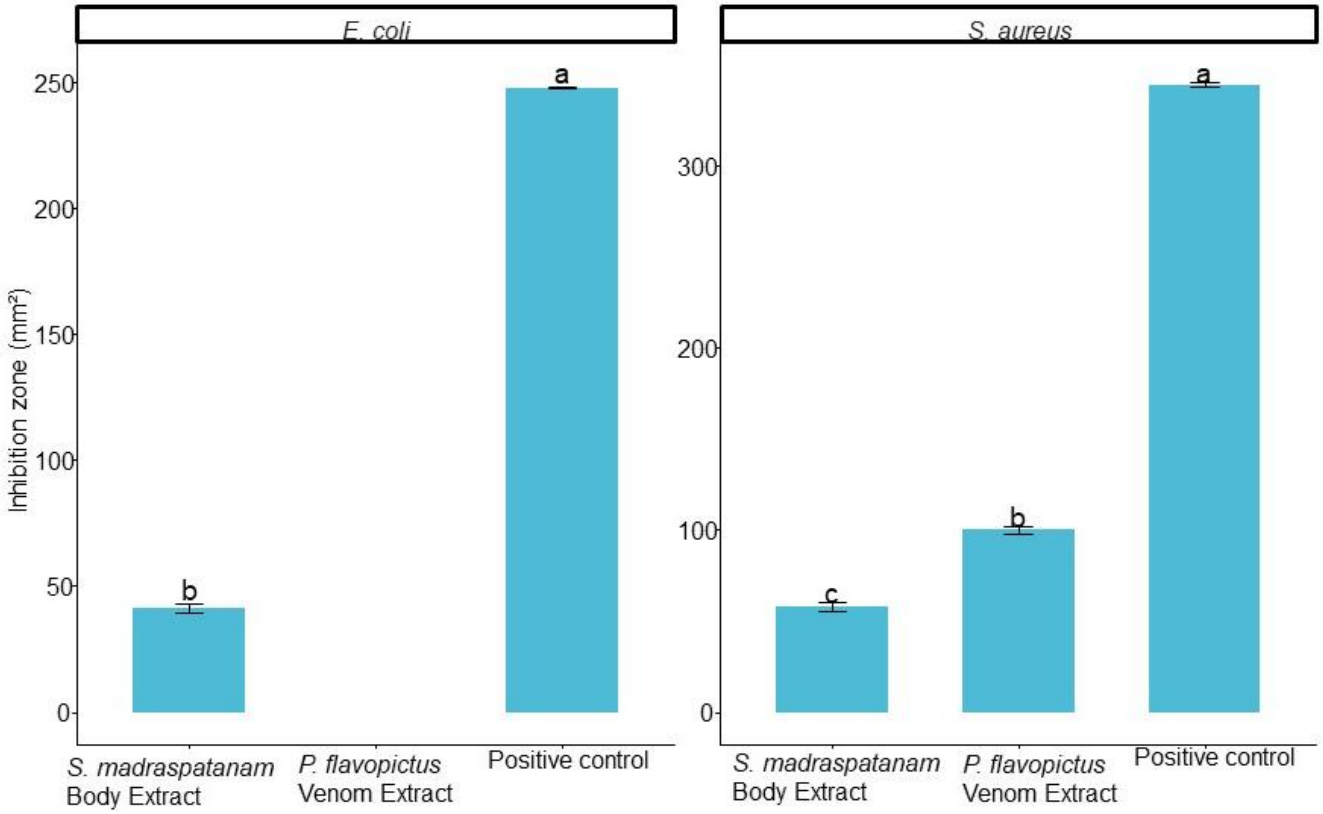


Fig. 1 Antibacterial activity of selected crude extracts against *E. coli* and *S. aureus***.** The body extract of *S. madraspatanam* exhibited moderate antibacterial activity against both pathogens, whereas the venom extract of *P. flavopictus* showed activity only against *S. aureus*. The positive control displayed the highest inhibition in both cases. Data are presented as mean ±

SD. Different letters above bars indicate statistically significant differences among treatments within each pathogen, based on Tukey's HSD test ($p < 0.05$).

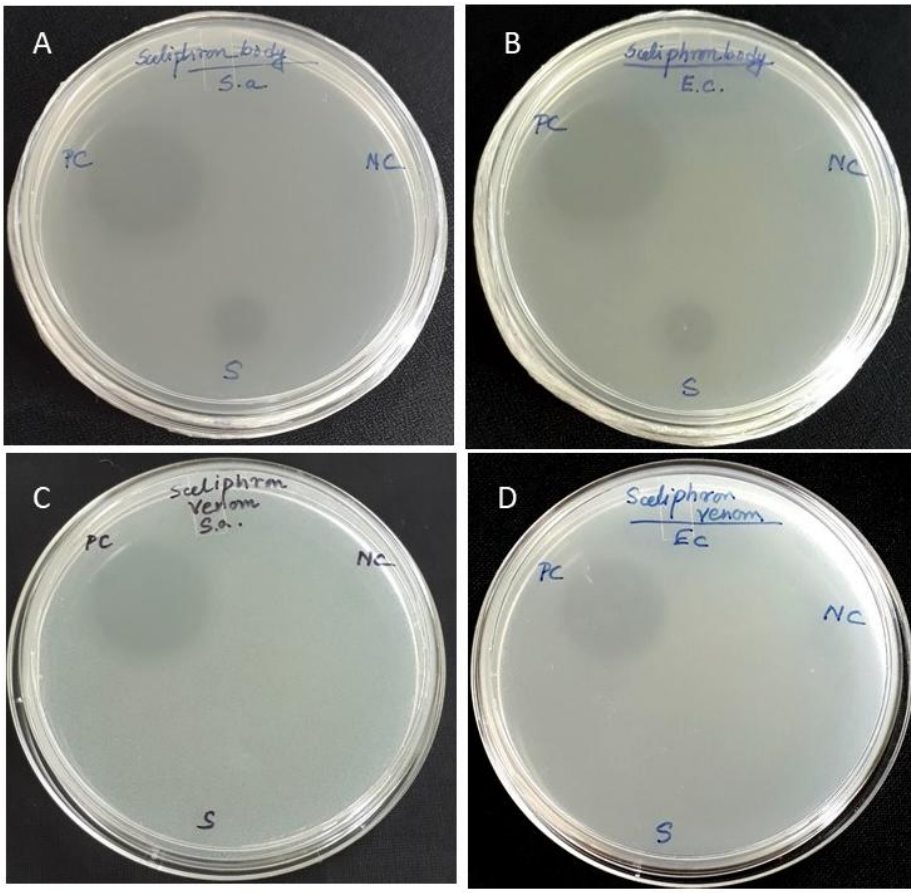
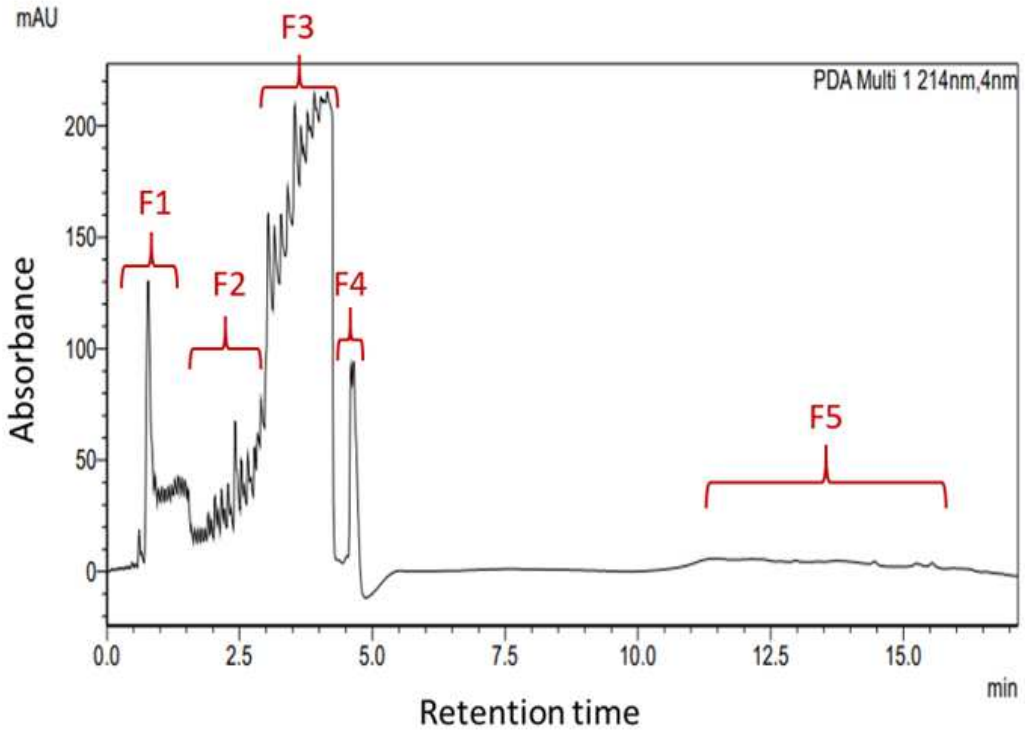


Fig. 2 Antibacterial efficacy of *S. madraspatanam* extracts against Gram-positive and Gram-negative pathogens. Representative images of spot-on-lawn assays following 12 h incubation at 37°C. (A–B) Inhibitory activity of crude body extract (12.84 µg/spot) against (A) *S. aureus* and (B) *E. coli.* (C–D) Inhibitory activity of venom gland extract (10.28 µg/spot) against (C) *S. aureus* and (D) *E. coli*. Labels indicate: PC, positive control (Tetracycline; at 2.5 µg/spot for *S. aureus,* and 5 µg/spot for *E. coli*); NC, negative control ACN: H_2O, 1:1 v/v); and S, sample extract. Clear zones around PC and S indicate bacterial growth inhibition.

Purification of antibacterial peptide fractions

Based on the results obtained from the initial antibacterial screening, the body extract of *S. madraspatanam* and the venom gland extract of *P. flavopictus* were identified as promising sources of AMPs and were therefore selected for further purification and characterization. The RP-HPLC chromatographic profile of *S. madraspatanam* was monitored at 214 nm, revealing multiple distinct peaks (Fig. 3). Based on retention time and peak distribution, five major fractions were manually collected: Fraction-1 (0.5–2 min), Fraction-2 (2–3 min), Fraction-3 (3–4.5 min), Fraction-4 (4.7–5 min), and Fraction-5 (11–16 min). Protein quantification of these fractions indicated variation in peptide content (Table 3). Similarly, the RP-HPLC elution profile of *P. flavopictus* venom extract, monitored at 214 nm, showed distinct peaks corresponding to three major fractions: Fraction-1 (0.5–5 min), Fraction-2 (10–15 min), and Fraction-3 (19–25 min) (Fig. 4). The protein concentration of the collected fractions also varied, as summarized in Table 4. The presence of multiple peaks in both species indicates the

253 separation of peptides with differing physicochemical properties, which may contribute to
254 variations in antimicrobial activity.



255

256 **Fig. 3** Purification of whole-body crude extract of *S. madraspatanam*. Multiple peaks were
257 observed between retention times of 0–16 min, with varying peak heights and areas. Fractions
258 were collected from peaks indicated as (F1–F5).

259 **Table 3** Protein concentration, volume used, and protein content per treatment of RP-HPLC
260 fractions from *S. madraspatanam* extract

Fraction	Protein concentration (µg µL^{-1})	Volume used (µL)	Protein content per treatment (µg)
Fraction 1	0.327	5.0	1.63
Fraction 2	0.309	5.0	1.54
Fraction 3	0.445	5.0	2.22
Fraction 4	0.510	5.0	2.55
Fraction 5	0.457	5.0	2.28

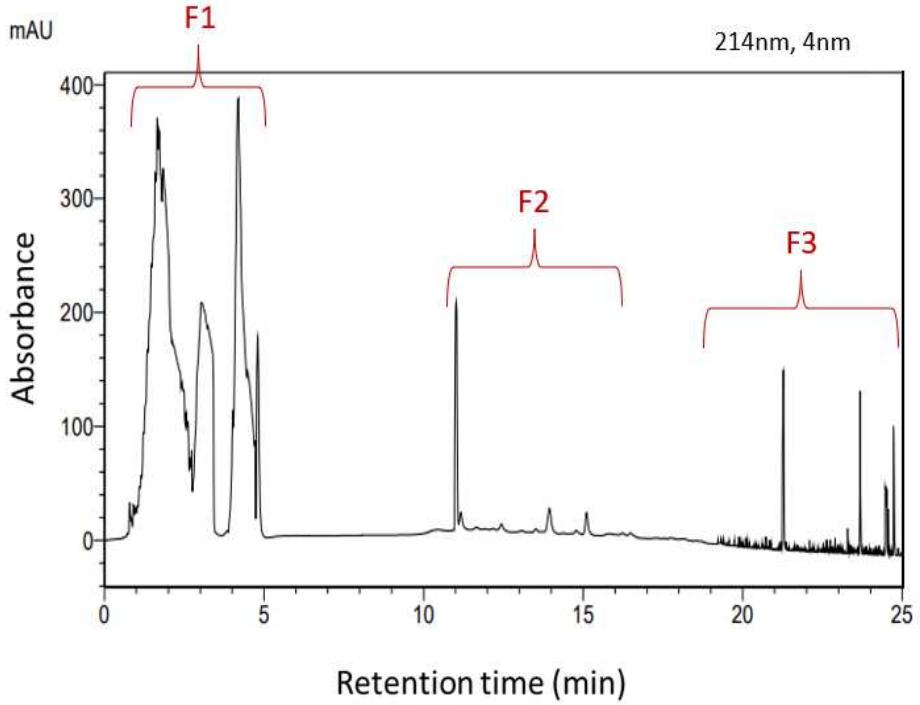


Fig. 4 Purification of *P. flavopictus* venom extract. Multiple peaks were observed between retention times of 0–25 min, with varying peak heights and areas. Fractions were collected from peaks indicated as (F1–F3).

Table 4 Protein concentration, volume used, and protein content per treatment of RP-HPLC fractions from *P. flavopictus* venom extract.

Fraction	Protein concentration (µg µL^{-1})	Volume used (µL)	Protein content per treatment (µg)
Fraction 1	0.320	5.0	1.60
Fraction 2	0.410	5.0	2.05
Fraction 3	0.260	5.0	1.30

Antibacterial activity of peptide fractions obtained from *S. madraspatanam* body extract

Among the five RP-HPLC fractions obtained from *S. madraspatanam*, only Fraction-3 exhibited antibacterial activity against *S. aureus* and *E. coli* (Fig. 5), while Fractions 1, 2, 4, and 5 showed no inhibition, comparable to the negative control. Fractions were tested at protein contents ranging from 1.54 to 2.55 µg/spot (Table 3), whereas tetracycline was applied at 2.5 µg/spot for *S. aureus* and 5 µg/spot for *E. coli.* Fraction-3 produced a higher zone of inhibition against *S. aureus* (17.81 ± 1.6 mm²) than *E. coli* (5.29 ± 0.6 mm²). The positive control (tetracycline) exhibited substantially greater inhibition against both organisms (*S. aureus*: 347.26 ± 0.6 mm²; *E. coli*: 248.83 ± 0.6 mm²) (Fig. 6). Statistical analysis indicated that Fraction-3 showed significantly higher antibacterial activity compared to other fractions ($p < 0.05$), as indicated by distinct letter groupings. Overall, antibacterial activity was restricted to Fraction-3, with a stronger effect observed against *S. aureus* than *E. coli*.

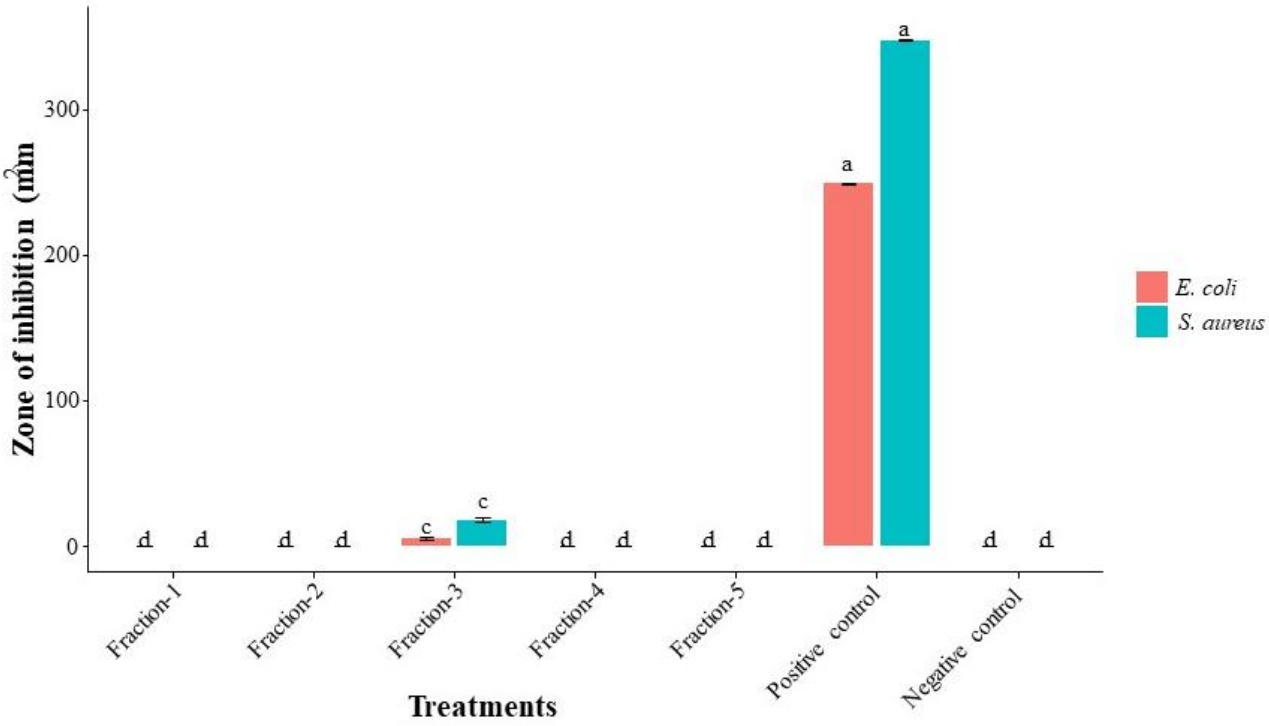
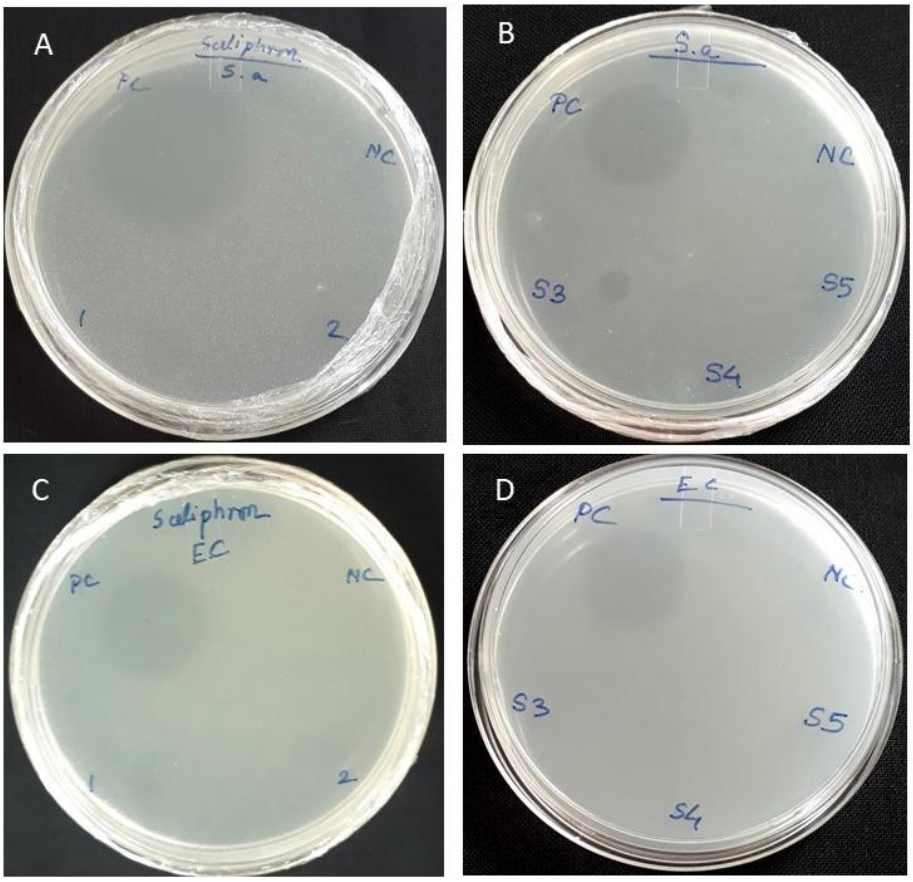


Fig. 5 Antibacterial activity of RP-HPLC peptide fractions from *S. madraspatanam* extract against *S. aureus* and *E. coli*. Zone of inhibition (mm²) is expressed as mean ± SD. Tetracycline

and ACN:H_2O served as positive and negative controls, respectively. Data were analyzed using two-way ANOVA followed by Tukey's HSD post hoc test. Different lowercase letters above the bars indicate statistically significant differences, where bars sharing the same letter are not



significantly different and those with different letters differ significantly at $p < 0.05$.

Fig. 6 Representative in vitro antibacterial activity of RP-HPLC–purified fractions from *S. madraspatanam* assessed by the spot-on-lawn assay against *S. aureus* (A, B) and *E. coli* (C, D). Plates A and C correspond to Fractions 1 and 2, while plates B and D correspond to Fractions 3, 4, and 5. Among the tested fractions, only Fraction 3 exhibited a distinct zone of inhibition against both test organisms, indicating antibacterial activity. Labels indicate: PC, positive control (tetracycline; 2.5 µg/spot for *S. aureus* and 5 µg/spot for *E. coli*); NC, negative control (50% acetonitrile : water, 1:1 v/v); and S (or S1–S5), sample fractions. Clear zones surrounding PC and sample spots indicate inhibition of bacterial growth, whereas the absence of such zones in NC confirms lack of antimicrobial effect of the solvent control.

Antibacterial activity of peptide fractions obtained from *Phimenes flavopictus* venom gland extract

No detectable antimicrobial activity was observed against *E. coli* and *S. aureus* when individual fractions of *P. flavopictus* venom gland extract were tested. In view of this lack of activity, selected fractions were combined in different permutations and subsequently re-evaluated to assess potential synergistic effects. Upon combinatorial testing, distinct antimicrobial activity was observed against *S. aureus* (Fig. 7). Among the tested combinations, the fraction mixture (2+3) exhibited moderate activity with a zone of inhibition of 4.31 ± 0.4 mm^2, while the combination (1+2+3) demonstrated a substantially higher activity with a zone of inhibition of 24.54 ± 1.4 mm^2. In contrast, all other combinations, including (1+2) and (1+3), as well as individual fractions, showed no detectable inhibition. The crude extract displayed measurable antibacterial activity (100.08 ± 1.8 mm^2), whereas the positive control, tetracycline, showed

the highest inhibition (34.22 ± 1.3 mm^2). The negative control (ACN:H_2O, 1:1) exhibited no activity. Statistical analysis using one-way ANOVA revealed significant differences among treatments ($p < 0.05$), with distinct grouping based on significance letters. The enhanced activity observed in combined fractions, particularly (1+2+3), suggests a potential synergistic interaction among bioactive components present in the *P. flavopictus* venom fractions (Fig. 8).

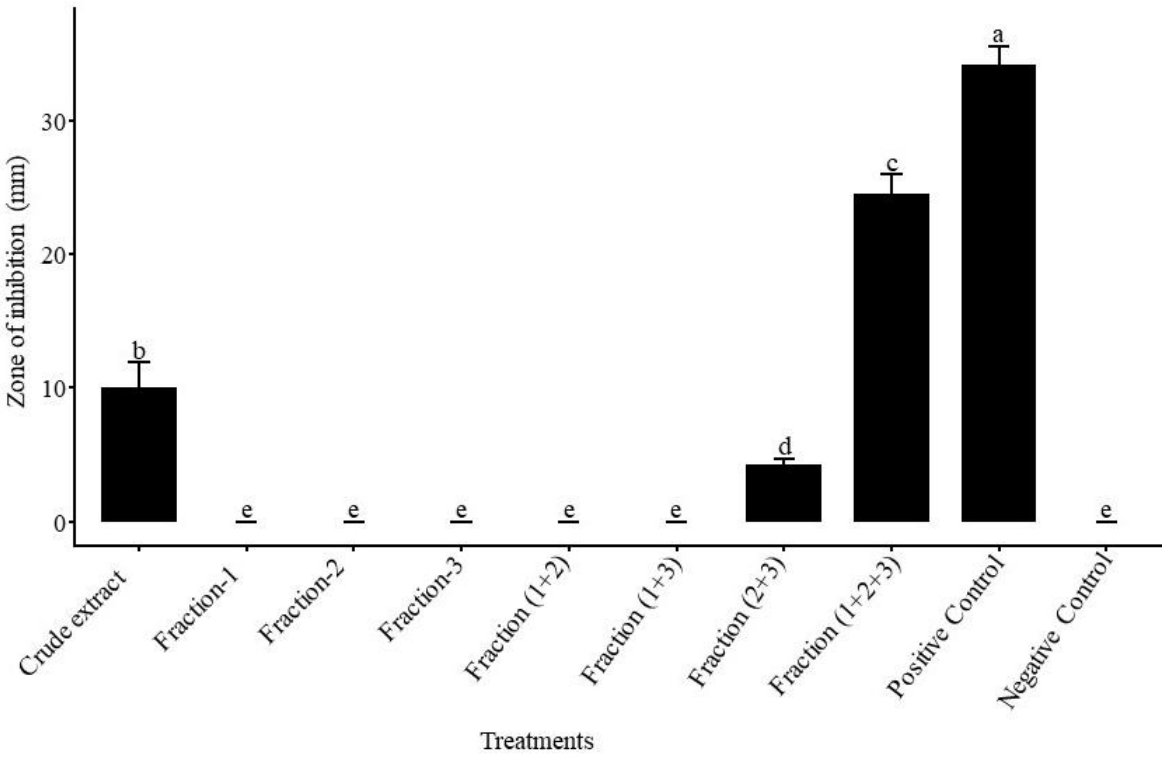


Fig. 7 Antibacterial activity fractions of *P. flavopictus* against *S. aureus*. Protein amounts applied per spot were: Fraction-1 (1.6 µg/spot), Fraction-2 (2.05 µg/spot), Fraction-3 (1.3 µg/spot), Fraction-(1+2) (1.82 µg/spot), Fraction-(1+3) (1.45 µg/spot), Fraction-(2+3) (1.67 µg/spot), and Fraction-(1+2+3) (1.64 µg/spot). Tetracycline was used as the positive control and ACN: H_2O (1:1) as the negative control. Values represent mean ± SD; different letters indicate significant differences ($p < 0.05$, one-way ANOVA followed by Tukey HSD analysis)

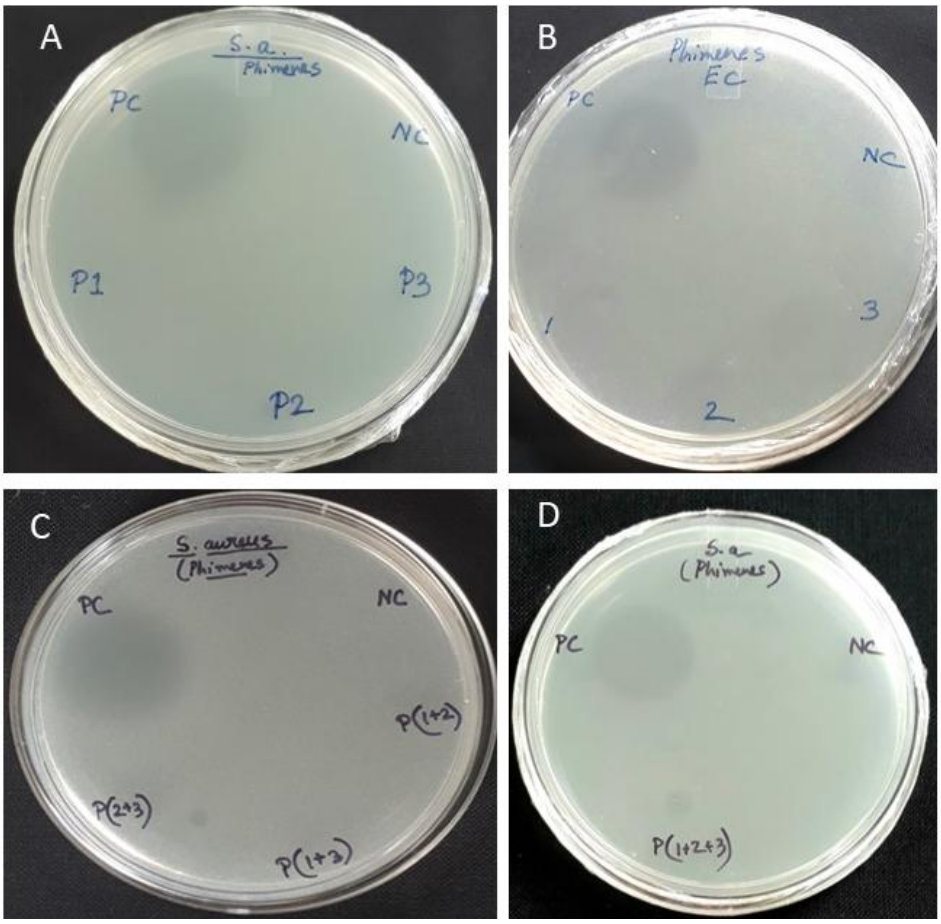


Fig. 8 Representative in vitro antibacterial activity of RP-HPLC–purified fractions from *P. flavopictus* evaluated by the spot-on-lawn assay against *S. aureus* (A, C, D) and *E. coli* (B). Panel A shows individual fractions (P1, P2, P3) tested against *S. aureus*, while panel B shows individual fractions (1, 2, 3) against *E. coli*, with no detectable zones of inhibition in either case. Panel C represents combinatorial fractions [P(2+3), P(1+2) and P(1+3)] against *S. aureus*, which exhibited minimal or no antibacterial activity. In contrast, panel D shows the combined fraction P(1+2+3), which produced a distinct zone of inhibition against *S. aureus*, indicating enhanced antibacterial activity due to possible synergistic effects. Labels indicate: PC, positive control (tetracycline; 2.5 µg/spot for *S. aureus* and 5 µg/spot for *E. coli*); NC, negative control (50% acetonitrile:water, 1:1 v/v); and P (P1–P3 or combinations), peptide fractions. Clear

zones surrounding PC and sample spots indicate inhibition of bacterial growth, whereas the absence of such zones in NC confirms the lack of antimicrobial activity of the solvent control.

Bioassay against plant pathogenic bacteria

Among the tested fractions, Fraction-3 (F3) of *S. madraspatanam* exhibited clear antibacterial activity against *Xanthomonas oryzae pv. oryzae,* with a mean zone of inhibition of 10.27 ± 0.26 mm^2 (Fig. 9). This suggests the presence of bioactive antibacterial peptides capable of inhibiting pathogen growth. In contrast, fractions F1, F2, F4, and F5 did not exhibit any detectable zone of inhibition under the tested conditions, indicating a lack of antimicrobial activity (Fig. 10). Furthermore, peptide fractions derived from *P. flavopictus* did not show any antibacterial activity against *X. oryzae pv. oryzae* under the tested conditions (Supplementary Fig. 3). The positive control (tetracycline, 5 µg/spot) showed a significantly higher zone of inhibition (335.68 mm^2), confirming the susceptibility of the test organism, whereas the negative control (ACN: H_2O, 1:1) showed no inhibition, thereby validating the experimental conditions.

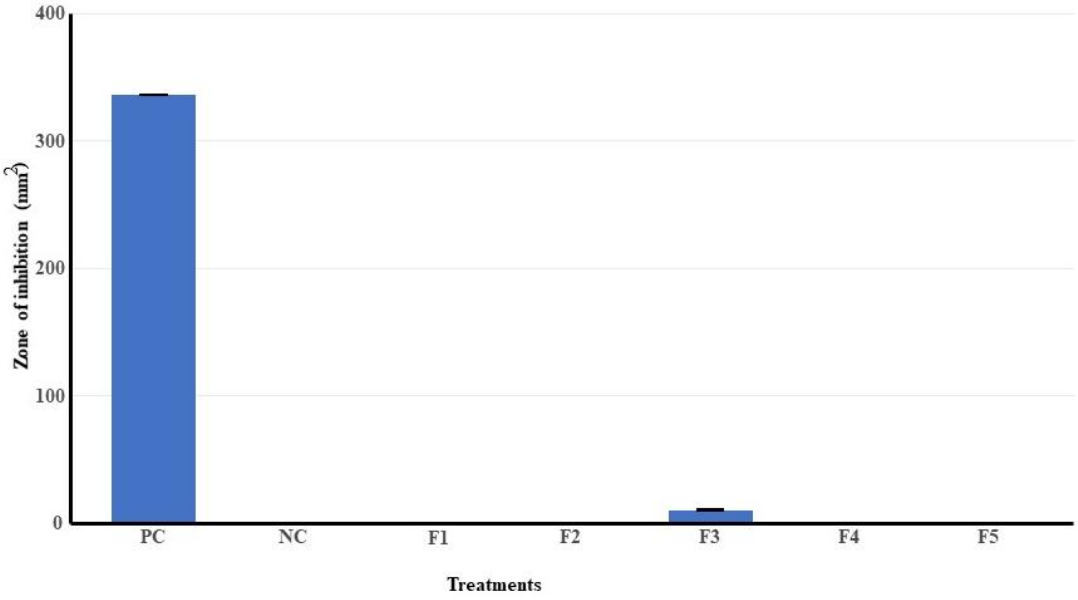


Fig. 9 Targeted in vitro screening of purified peptide fractions from *S. madraspatanam* for antimicrobial activity against *X. oryzae pv. oryzae.* Each fraction was applied at the following concentrations: F1 (1.63 µg/spot), F2 (1.54 µg/spot), F3 (2.22 µg/spot), F4 (2.55 µg/spot), and F5 (2.28 µg/spot). Among the tested fractions, only Fraction-3 (F3) exhibited measurable antimicrobial activity (10.27 ± 0.26 mm²), whereas other fractions showed no detectable inhibition. The positive control (PC; tetracycline, 5 µg/spot) showed a pronounced zone of inhibition, while the negative control (NC; ACN: H_2O, 1:1) showed no activity. Data are presented as mean ± standard deviation (n = 3).

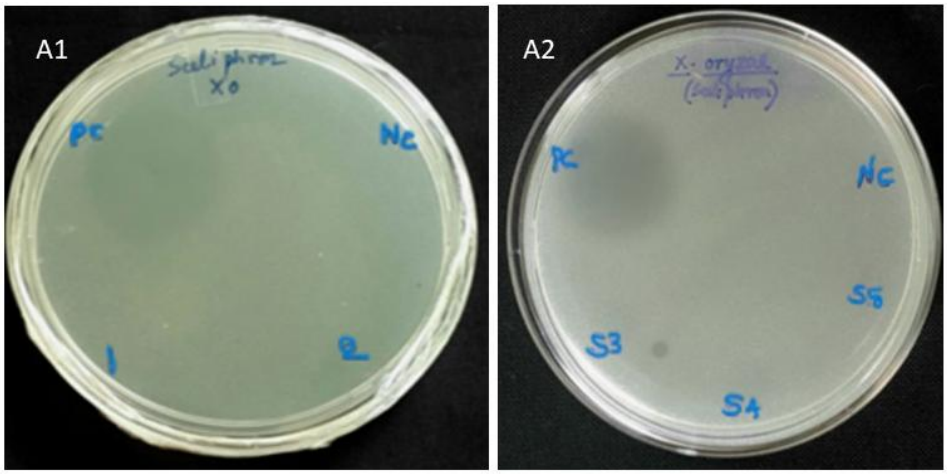


Fig. 10 Representative in vitro antibacterial activity of RP-HPLC–purified fractions from *S. madraspatanam* against *X. oryzae* evaluated by the spot-on-lawn assay. Panel A1 shows Fractions 1 and 2, while panel A2 shows Fractions 3 (S3), 4 (S4), and 5 (S5). Among the tested fractions, Fraction 3 exhibited a discernible zone of inhibition, indicating antibacterial activity against *X. oryzae,* whereas other fractions showed no detectable effect. Labels indicate: PC, positive control (tetracycline); NC, negative control (50% acetonitrile:water, 1:1 v/v); and S (S1–S5), sample fractions. Clear zones surrounding PC and active sample spots indicate inhibition of bacterial growth, whereas absence of such zones in NC confirms the lack of antimicrobial activity of the solvent control.

MALDI-TOF Characterization of Bioactive Peptide Fraction

Based on the results obtained in the bioassay of peptide fractions, only fraction 3 of *S. madraspatanam* exhibited antibacterial activity against the major plant pathogen *X. oryzae*. Detailed characterization was performed for this fraction. The MALDI-TOF MS spectrum of Fraction 3 (Fig. 11) displayed several peptide peaks in the m/z range of 568–1066. Among them, the most prominent peak was recorded at m/z 861.049, suggesting that this peptide represents the major component of the active fraction. Other notable peaks were observed at m/z 650.056, 656.121, and 672.056 (Table 5), with comparatively high intensities, whereas additional peaks at m/z 568.280, 634.408, 877.036, and 1066.399 were detected with lower abundance. The predominance of the m/z 861.049 peptide correlates with the antibacterial activity exhibited by Fraction 3 against Xanthomonas oryzae. In contrast, the remaining fractions did not exhibit significant antibacterial activity in the bioassay.

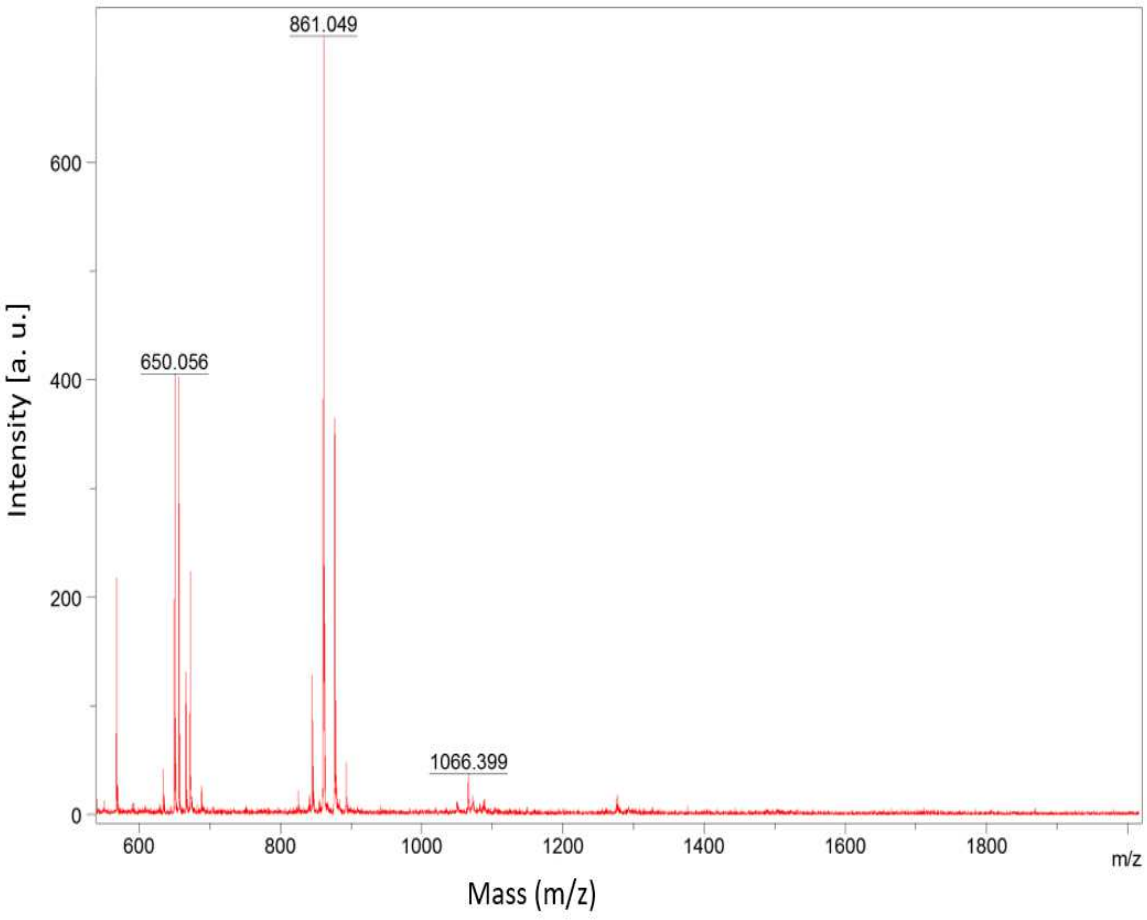


Fig. 11 MALDI-TOF MS spectrum of bioactive Fraction 3 purified from *S. madraspatanam*, showing prominent peptide peaks in the range of m/z 568–1066. The dominant peak at m/z 861.049 is likely associated with the observed antibacterial activity against *X. oryzae*.

Table 5 MALDI-TOF-generated peptide masses identified from Fraction 3 of *S. madraspatanam*

Sl. No.	m/z (Da)	S/N Ratio	Resolution (Res.)	Intensity	Peak Area
1	568.280	27	1762	218	94.3
2	634.408	5	1806	42	25.6
3	650.056	50	1858	403	199
4	656.121	50	1831	403	196
5	672.056	27	2172	216	122
6	861.049	89	2856	714	529
7	877.036	46	4508	365	277
8	1066.399	5	2077	35	34.1

m/z: mass-to-charge ratio; S/N: signal-to-noise ratio; Res.: resolution. Higher S/N values indicate greater confidence in peak detection

Discussion

The present study investigates solitary wasps as a promising yet underexplored source of antimicrobial peptides (AMPs), with *S. madraspatanam* and *P. flavopictus* exhibiting notable antibacterial activity. This aligns with recent comprehensive reviews emphasizing that Hymenopteran venoms contain diverse bioactive peptides, particularly mastoparans and related compounds, which possess strong antimicrobial properties and therapeutic potential [28, 29].

The observed higher protein concentration in whole-body extracts compared to venom gland extracts is consistent with earlier reports indicating that venom comprises a relatively small proportion of total protein but is enriched in functionally specialized peptides [30]. Importantly, our results demonstrate that antibacterial activity is not directly correlated with total protein

content, reinforcing previous findings that biological activity is driven by peptide specificity and structure rather than overall abundance [31].

Studies on insect-derived AMPs, which are usually cationic and amphipathic and can damage bacterial membranes, are consistent with *S. aureus* and *E. coli* [26, 32, 33]. For instance, mastoparan-derived peptides have been shown to exert potent bactericidal effects against *E. coli* both in vitro and in vivo [34]. Similarly, antimicrobial peptides isolated from Hermetia illucens demonstrated broad-spectrum activity through membrane permeabilization, supporting the mechanism likely involved in our study [35]. The stronger antibacterial activity observed against *S. aureus* compared to *E. coli* in our study, particularly for Fraction-3 of *S. madraspatanam* (17.81 ± 1.6 mm² vs. 5.29 ± 0.6 mm²) can be attributed to structural differences in bacterial cell envelopes. Gram-negative bacteria such as *E. coli* possess an additional outer membrane rich in lipopolysaccharides (LPS), which acts as a permeability barrier that limits the penetration of antimicrobial peptides, thereby reducing their susceptibility [36, 37].

In contrast, the selective antibacterial activity of *P. flavopictus* venom against *S. aureus* aligns with earlier findings that mastoparans and related peptides often exhibit preferential activity toward Gram-positive bacteria [38, 39]. Structural studies have shown that these peptides possess amphipathic α-helical conformations that facilitate interaction with lipid membranes, leading to pore formation and cell lysis [40]. Moreover, the predominance of mastoparan-like peptides in wasp venoms, which account for a significant fraction of venom composition, further supports their role in antimicrobial activity [41].

The absence of antibacterial activity in *C. bengalense* and *D. conoideum* underscores the species-specific variability in venom composition. Previous studies have reported that venom composition is influenced by ecological factors such as prey type and defensive strategies,

leading to significant differences even among closely related species [25]. This variability likely explains the differential antibacterial activity observed in the present study.

Fractionation of *S. madraspatanam* extracts revealed that antibacterial activity was confined to a single RP-HPLC fraction (Fraction-3), suggesting the presence of a specific bioactive peptide. Similar observations have been reported in solitary wasp venoms, where a single dominant peptide can be isolated after minimal fractionation due to relatively simple chromatographic profiles [42]. The MALDI-TOF analysis in our study identified a peptide at m/z 861.049 as the dominant one; we cannot confirm it as solely responsible for antifungal activity. Validation with purified or synthetic peptides and assessment of possible synergistic effects remain essential next steps.

The synergistic antibacterial activity observed in *P. flavopictus* venom, where the combined Fractions 1, 2, and 3 showed activity at a lower protein concentration (1.64 µg/spot) than the inactive individual Fraction-2 (2.05 µg/spot), indicates a cooperative, multi-component mechanism typical of Hymenopteran venoms. The loss of activity in individual fractions and its restoration upon recombination suggest that peptide interactions are essential for effective membrane disruption. Similar synergy has been reported in wasps such as Vespa tropica and Polybia paulista, where mastoparan-like peptides act as membrane permeabilizers, facilitating the action of other antimicrobial components [43, 44]. Recent studies also confirm that AMP combinations enhance membrane disruption and reduce resistance compared to single peptides [45, 46], reflecting an energy-efficient evolutionary strategy in venom systems [15]. Mechanistic studies have shown that such peptides can collectively destabilize lipid bilayers more effectively than individual components [47] .

The observed antibacterial activity of Fraction-3 against *X. oryzae pv. oryzae* highlights the potential application of wasp-derived peptides in agriculture. Insect AMPs have been

increasingly recognized as eco-friendly alternatives to conventional pesticides due to their biodegradability and targeted mode of action [25]. However, the comparatively lower activity relative to tetracycline indicates that optimization strategies, such as peptide engineering or formulation improvements, may be necessary to enhance efficacy.

No antifungal activity was observed for any of the fractions of *S. madraspatanam* and *P. flavopictus* against *Sclerotium rolfsii* under the tested conditions (Supplementary Fig. 4). This observation is consistent with previous reports indicating that many insect-derived antimicrobial peptides exhibit predominantly antibacterial activity, while antifungal activity is comparatively less common and often restricted to specific peptide classes [48]. However, as antifungal efficacy is highly dependent on peptide structure and target specificity, the possibility of activity against other fungal pathogens cannot be excluded.

Despite these promising findings, several limitations must be acknowledged. First, the study is restricted to in vitro assays, and the efficacy of these peptides under in vivo or field conditions remains to be validated. Second, peptide characterization was limited to mass spectrometry profiling, and the absence of sequence identification prevents detailed structure–function analysis. Approaches such as LC-MS/MS, de novo sequencing, and peptide synthesis are required for precise characterization. Additionally, the mechanism of action was not experimentally validated in this study, although membrane disruption is the most likely mode based on existing literature. Finally, the limited number of bacterial strains tested restricts the broader applicability of the findings, particularly against clinically relevant multidrug-resistant pathogens.

Overall, the present study strengthens the growing body of evidence that solitary wasp venoms are a rich source of antimicrobial peptides with significant potential in both biomedical and agricultural applications. Future research should focus on peptide sequencing, structural and

functional characterization, toxicity profiling, and in vivo validation to fully exploit their potential as next-generation antimicrobial agents.

Conclusion

This study establishes solitary wasps as a potent, underexplored reservoir of antimicrobial peptides (AMPs) with high specificity for plant-pathogenic bacteria. Our findings identify *S. madraspatanam* and *P. flavopictus* as primary sources of bioactive compounds, with the latter demonstrating significant synergistic interactions among peptide fractions. The characterization of a dominant peptide (m/z 861.049) effective against *X. oryzae pv. oryzae* underscores the potential of these molecules as sustainable alternatives to synthetic bactericides. While the current results are limited to in vitro antibacterial efficacy, they provide a critical foundation for future peptide sequencing and in vivo validation. Ultimately, these wasp-derived AMPs offer a promising biocontrol strategy for the management of devastating crop diseases.

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