Acta Scientific Microbiology (ISSN: 2581-3226)

Volume 8 Issue 5 May 2025

Research Article

Social Wasp Venom: A Treasure of Antimicrobial Peptides

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Received: April 01 2025

Published: April 21, 2025

Abstract

Antimicrobial peptides (AMPs) are versatile molecules that are vital for combating pathogenic microorganisms, including bacteria, fungi, and viruses. Insects thrive well in microbe-rich environments and rely on their cellular and humoral immune systems for defense. The humoral system, among other constituents, chiefly comprises antimicrobial peptides. Interestingly, the venom of hymenopteran insects has been identified as a rich repository of these peptides. This study aimed to explore the potential of social wasp venom as a source of antimicrobial peptides, focusing on two species viz. *Ropalidia marginata* and Vespa tropica. Venom gland extracts from both species were tested against *Staphylococcus aureus* (MTCC 3160) and Escherichia coli (MTCC 2692), which are gram-positive and gram-negative bacteria respectively. Vespa tropica venom showed antimicrobial activity against both bacteria, while Ropalidia marginata venom was effective only against *Staphylococcus aureus*. Reverse phase- High performance liquid chromatography (RP-HPLC) was carried out with a peptide-specific protocol to separate fractions of peptides from the venom. Seven fractions were obtained from each species. Two fractions from Vespa tropica exhibited antimicrobial activity against both *Staphylococcus aureus* and *Escherichia coli*, while two fractions from Ropalidia marginata were effective only against *Staphylococcus aureus*. Additionally, two fractions from each species showed antibacterial activity against the plant pathogen Xanthomonas oryzae pv. oryzae, while no antifungal activity was observed against *Sclerotium rolfsii*. Further analysis of the active fractions using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) revealed the presence of six peptides in each species with m/z 932.280 to m/z 1729.650 for *Ropalidia marginata* and m/z 1386.182 to m/z 1695.805 for Vespa tropica. This study elucidates the antimicrobial potential of social wasp venom against a range of bacterial pathogens, suggesting its potential in both therapeutic and agricultural contexts.

Keywords: *Ropalidia marginata*; *Vespa tropica*; Antimicrobial peptides; RP-HPLC; *Escherichia coli; Staphylococcus aureus*; *Sclerotium rolfsii*; MALDI-TOF

Introduction

Throughout history, living organisms have encountered significant health obstacles, from ancient times to the recent COVID-19 pandemic. In the earliest eras, the lack of advanced medical knowledge and technology left populations vulnerable to pathogens and diseases. However, a pivotal turning point came with the emergence of antibiotics, marking a "golden age" in medicine where these treatments offered newfound hope in combating illnesses. Regrettably, this era of triumph was short-lived as the rise of multidrug-resistant strains posed formidable challenges, highlighting the ongoing evolutionary arms race between medicine and microbial adversaries [1]. According to the World Health Organization (WHO, 2022), approximately 4.95 million deaths annually are attributed to resistant bacteria, underscoring the gravity of these es-

calating public health challenges. The situation may worsen, with a projected death toll of approximately 10 million per year by 2050 if the alarming increase in multidrug-resistant strains remains unchecked [2,3]. In this regard, antimicrobial peptides (AMPs) have emerged as promising alternative therapeutic options, opening new avenues for drug development. Predating the widespread use of antibiotics, AMPs represent the oldest frontline defenses and are integral components of the innate immune system [1].

Antimicrobial peptides (AMPs), also referred to as host defense peptides, are short, generally positively charged, amphipathic molecules present across a wide range of life forms, from microorganisms to humans. Typically, composed of 7 to 100 amino acid residues, they exhibit broad-spectrum antimicrobial activity against bacteria and fungi, while also demonstrating efficacy against viruses, protozoa, and cancer cells. Unlike traditional antibiotics, AMPs operate through unique mechanisms of action, making it challenging for microbes to develop resistance against them [4]. Antimicrobial peptides (AMPs) exhibit a plethora of functions within the host, many of which are often subtle or overlooked. They assist insects in combating viral infections and play a role in regulating brain-controlled processes such as sleep and non associative learning. By influencing neuronal health, communication, and activity, they can impact the functioning of the insect nervous system. Furthermore, changes in the repertoire of AMPs and their decreased specificity are associated with the aging process and lifespan of insects. AMPs contribute to maintaining gut homeostasis by regulating the population of endosymbionts and reducing the presence of foreign microbiota [5].

Insects flourish in diverse environments abundant in microbes, relying on their cellular and humoral immune systems for defense. The humoral system, which includes antimicrobial peptides among other components, plays a significant role in this defense mechanism. Notably, within the insect kingdom, venom production is predominantly attributed to members of the order Hymenoptera. It is intriguing that the venom of hymenopteran insects serves as a significant source of these peptides. The venom composition depends on its function. Social wasps typically utilize their venom for defense and self-protection. Over numerous years of evolution, the venom of social wasps has been refined to inflict more painful stings and elicit stronger immune and allergic reactions than that of solitary wasps [6]. Solitary wasps primarily employ their venom for predation rather than defense. By utilizing neurotoxic and antibacterial components within their venom, most solitary wasps swiftly immobilize their prey, preserving it for consumption by their larvae [7].

Wasp venom is a sophisticated blend of biologically active substances, encompassing high molecular weight proteins, small peptides, bioactive amines, and amino acids. Peptides, constituting up to 70% of dried venom, play a crucial role. In the venoms of social wasps, three prominent peptide categories stand out: mastoparans, which trigger mast cell degranulation; chemotactic peptides, which promote the migration of polymorphonucleated leukocytes; and kinin-related peptides, which are renowned for inducing pain and enhancing vascular permeability. Notably, mastoparan, a bioactive tridecapeptide, predominates and might possess antimicrobial properties [8].

Mastoparans usually consist of 14 amino acids with a significant presence of hydrophobic and basic residues, adopting amphipathic α-helical configurations. Several studies on mastoparan-related peptides have demonstrated their robust antibacterial efficacy and ability to inflict substantial damage to the cell membrane. For instance, mastoparan-X exhibited minimal inhibitory concentration (MIC) values of 2.5 μM for *Lactococcus lactis* and 8 μM for *E. coli* [9]. Analogous peptides have been discovered in the venoms of social wasps belonging to the Polistes genus: mastoparan in *Polistes jadwiga* and dominulin A and B in *P. dominulus*. These peptides demonstrate efficacy against both gram-positive and gram-negative bacteria by creating pores in the cell membrane [10].

Another instance of a venom peptide displaying antimicrobial properties is crabrolin, which consists of 13 amino acids. Initially identified in the venom of the European hornet *Vespa crabro* and categorized as a chemotactic factor, crabrolin exhibits efficacy against specific bacteria. Its antimicrobial activity is influenced by the presence of hydrophobic groups and positive charges, while maintaining an α-helical conformation is essential for sustaining hemolytic activity [11,12]. In contrast to those of mastoparan peptides, the antibacterial potential of chemotactic peptides has been relatively underreported. Three chemotactic peptides were isolated and characterized from *V. magnifica* venom, demonstrating

antibacterial activity against both bacteria and fungi. For peptide 5e, the MICs against *E. coli*, *Staphylococcus aureus*, and *Candida albicans* were 30, 5, and 25 µg/mL, respectively. Peptide 5f and peptide 5 g exhibited similar MICs against *E. coli, S. aureus*, and *C. albicans* as peptide 5e. All three chemotactic peptides from wasp venom exhibit the typical characteristics of antibacterial peptides, including an abundance of positively charged residues and amphiphilic structures [13].

Despite its toxic nature, wasp venom has gained significantly less attention than bee venom. Nevertheless, numerous studies have highlighted that wasp venom holds great potential as a source of novel bioactive compounds for various pharmacological, therapeutic, and agricultural applications [14,15]. Extensive research conducted in recent decades has unveiled the pharmacological properties of wasp venom. Currently, various potential venom-based therapies for treating infectious diseases, immune-related disorders, and tumors are undergoing rigorous investigation [15,16]. Insect antimicrobial peptides (AMPs) exhibit remarkable potency, with their IC50 falling within the submicromolar or low micromolar range. Despite this, there are currently no insect-derived AMPs available on the market. Nonetheless, these AMPs hold promise as an alternative to antibiotics and are ripe for exploitation in pharmaceutical applications [4].

This study was taken up to prospect novel antimicrobial peptides from two social wasp species, *Ropalidia marginata* and *Vespa tropica,* to explore the potential of social hymenopteran insects as a source of antimicrobial peptides and to assess their antimicrobial activity against gram-positive bacteria, gram-negative bacteria and plant pathogen.

Materials and Methods

Study site and collection of wasps

This research focused on investigating the antimicrobial properties of venom gland extracts from social wasps through a bioassay-guided process of isolation and purification. The study was carried out at the University of Agricultural Sciences, Gandhi Krishi Vignana Kendra in Bengaluru, Karnataka, India (12° 57' N 72° 35' E, 930 m MSL). Two species of social wasps were chosen for the study: *Ropalidia marginata* and *Vespa tropica*.

Ropalidia marginata, a polistine wasp species, was sourced by collecting adult wasps from live, active nests, as well as from crop fields and old buildings within the GKVK campus, using a hand net. *Vespa tropica*, a hornet species, was collected from crop fields and areas near old buildings in the GKVK campus, particularly during seasons of abundance, using a similar method. All collected specimens were identified using standard taxonomic tools in the Niche Area for Excellence in Taxonomy of Insects and Mites, GKVK. Voucher specimens were archived at the University of Agriculture in Bengaluru, India.

Venom collection and preparation of crude extract

To ensure minimal degradation of the peptides, venom glands were collected from fresh live wasps for preparation of the crude peptide extract. The extraction process involved dissecting the venom glands from individual wasps by gently holding the sting with forceps and pulling out the gland with a slight jerk. Prior to dissection, the wasps were briefly frozen and thawed by placing them in a freezer for a few seconds. The dissected venom glands were then stored in a solution consisting of 1:1 acetonitrile: water (v/v) with 0.1% trifluoroacetic acid (TFA) and maintained at -20°C until further use. Once a sufficient number of venom glands were collected, the venom extraction process commenced by homogenizing the glands using a probe sonicator or ultrasonic processor. Ultrasonic frequencies (>20 kHz, Ultrasonic water bath Sidilu, India) were applied in pulse mode for 30 seconds. Subsequently, the aqueous solution was carefully decanted into fresh Eppendorf centrifuge microtubes. The extract underwent centrifugation at 10,000 rpm for 15 minutes at 4°C, facilitating the separation of any remaining wasp tissue. This centrifugation process utilized radial acceleration, causing denser particles to settle at the bottom of the tube, while low-density substances floated to the top [17]. The supernatant, containing the venom extract, was then collected in fresh tubes. To remove excess solvent from the crude extract, the concentration was achieved using a vacuum concentrator at 30°C. The concentrated sample was subsequently lyophilized at -40°C and stored at -80°C for further analysis.

Protein estimation

The total protein content of venom samples extracted from wasps was determined utilizing the Bradford method [18]. Bovine serum albumin (BSA) served as the standard to establish a regression curve. This standard curve derived from the BSA preparation was utilized as a reference to quantify the total protein content within the venom extract. To conduct the assay, 5 µL of the sample and 195 µL of Bradford reagent were added to each well of a flat-bottom microplate or a 96-well ELISA plate, followed by an incubation period of 15-20 minutes. During this time, the sample underwent a color change to blue, indicating the binding of the dye to the proteins present, thereby indicating the protein abundance. Subsequently, the absorbance was measured at 595 nm using an ELISA microplate absorbance reader. By plotting a standard graph and determining the regression equation from the absorbance values of various concentrations of BSA standards, the protein content was calculated using the regression equation.

Antimicrobial assay

Evaluation of the antimicrobial activity of crude venom extracts

The antimicrobial assay was conducted using the spot-on-lawn method to assess the efficacy of venom gland extracts against standard bacterial strains, namely the gram-positive bacteria *Staphylococcus aureus* (MTCC 3160) and gram-negative bacteria *Escherichia coli* (MTCC 2692). These bacterial strains were obtained from the Institute of Microbial Technology (IMTECH) in Chandigarh, India.

Protocol for the antimicrobial assay

The antimicrobial activity of the crude extract was assessed using the spot-on-lawn assay method. Initially, a loopful of pure culture of bacterial strains was inoculated into sterile nutrient broth in 5 mL test tubes and incubated overnight at 37°C. After 6-8 hours of incubation, the optical density (OD) of the inoculated culture was measured using a visible spectrophotometer to ensure a turbidity of 0.1 OD at 600 nm, indicating a bacterial cell density of approximately 1×10^6 colony forming units. The resulting bacterial cultures were then used for bioassays.

For the spot-on-lawn assay, microbial culture plates were prepared by pouring 10 mL of nutrient agar (NA) into each pre-sterilized petri plate and allowing them to solidify. Subsequently, 20 µL of the pure bacterial culture (OD = 0.1 at 600 nm) from the culture broth was poured into pre-sterilized conical flask containing pre-sterilized, liquidified, and cooled semisolid media. After thorough mixing, the media was poured onto labelled nutrient agar plates and allowed to solidify further. Positive controls (PC) were established by pipetting 5 µL of tetracycline (50 µg/50 µL for *Staphylococcus aureus* MTCC 3160 and 100 µg/50 µL for *Escherichia coli* MTCC 2692) onto the test plates, while the negative control (NC) consisted of 50% acetonitrile water (50% acetonitrile: 50% water) used for the preparation of the crude extract. For the experimental groups, 5 µL of the crude extract was pipetted onto each semisolid media plate for both test organisms. Similarly, 5 µL each of the positive and negative controls were pipetted onto the respective test plates. The plates were then placed in a BOD incubator at 37°C for 12 hours to allow for growth. After incubation, inhibition zones were observed and recorded by measuring the diameter of the clear zone in the petri plates against the microorganisms using a Vernier caliper.

The inhibition area was calculated using a standard formula and is expressed in mm^2 (Andrews, 2002).

Inhibition annule (mm^2) = πr^2 {where, r = radius of the inhibition zone in mm and $\pi = 22/7$}

Isolation and purification of the active fractions

The crude venom extracts from both species were subjected to preparative reverse-phase high-performance liquid chromatography (RP-HPLC) for the fractionation and purification of venom components. A C18 column was used, along with HPLC-grade acetonitrile (CH3CN), Milli-Q water, trifluoroacetic acid (TFA), methanol, and a solvent delivery system with binary gradient capability, coupled with a UV detector. The injection volume was 20 µL, the sample temperature was maintained at 4°C and the column temperature was 40°C. A diode array detector (DAD) at 214 nm, 4 nm (1.00) was used. The mobile phase solvents consisted of Buffer A: 0.1% (v/v) TFA in water and Buffer B: acetonitrile/ACN (CH3CN), 80%:20% (water: 0.1% TFA). The binary gradient program for

peptide fractionation was as follows: 0-5% ACN for 5 min, 5-95% ACN for 50 min, 95% ACN for 5 min, and 95-5% for 5 min. Using a C-18 reverse-phase analytical column (Phenomenex: 5 mm particle size; 250×4.6 mm column) installed on a Shimadzu HPLC, 20 µL of crude extract was injected into the column. Fractionation was carried out using a linear binary gradient method at a flow rate of 1 ml/min. A UV-DAD detector (SPD-M20A) monitored the UV detection of the eluted peptide peaks at 214 nm during the 65 min run period for the linear gradient elution of solvent B. Fractions were collected repeatedly to obtain sufficient quantities for subsequent bioassays, based on their retention time. All collected fractions were lyophilized and stored at -80°C until further use.

Antimicrobial activity of the collected fractions

A total of seven fractions were collected from each crude extract of *Ropalidia marginata* and *Vespa tropica* through preparative RP-HPLC. Protein estimation of these lyophilized fractions were done and were subsequently subjected to antimicrobial bioassays against *S. aureus* (MTCC 3160) and *E. coli* (MTCC 2692) using the spot-on-lawn assay method. This approach aimed to identify the active fractions that exhibit antimicrobial activity.

Antimicrobial activity of the fractions against plant pathogens

Antibacterial assay

To evaluate the antibacterial activity of the purified fractions against the gram-negative bacterium *Xanthomonas oryzae* pv. oryzae, were obtained from the Department of Pathology, GKVK, UAS-Bengaluru, Karnataka, and spot-on-lawn assays were conducted as previously described. As a positive control, 5 µL of tetracycline at a concentration of 100 µg/100 µL was used for *Xanthomonas oryzae* pv. oryzae.

Antifungal assay

To assess the antifungal activity of the purified HPLC fractions, they were tested against the plant pathogenic fungus Sclerotium rolfsii, obtained from the School of Ecology and Conservation Lab, GKVK, Karnataka. The Kirby-Bauer disc method was employed to evaluate the antifungal activity of the HPLC fractions.

Kirby-Bauer disk diffusion assay

The fungal strain *Sclerotium rolfsii* was grown in potato dextrose agar (PDA) media in 90 mm pre-sterilized petri plates for 3-4 days using spores obtained from the SEC Lab, GKVK. After obtaining a pure subculture from the spores, the mycelia were utilized as a source of inoculum for subculture, which was then subjected to testing using the Kirby-Bauer method. The fungus was allowed to grow for 2-3 days in PDA media until the mycelia covered approximately half of the petri plates. Sterile disks of 6 mm diameter (Himedia SD067) were impregnated with 20 µL of the samples to be tested for antifungal activity and were placed on the surface of the agar. A control plate was maintained, and the solvent used for sample preparation served as the negative control. All petri plates were then incubated for an additional 1-2 days in a BOD (Biochemical Oxygen Demand) incubator to allow further mycelial growth. The inhibition zone around the paper discs impregnated with the samples was observed, and the diameters of the inhibition zones were measured using a Vernier caliper. The data were recorded, and the zone of inhibition was calculated by subtracting the diameter of the disk from the diameter of the inhibition zone using the following formula.

Inhibition annule (mm^2) = II (R+r) (R-r)

Where, R = radius of the inhibition zone in mm, r = radius of the discs in mm and $\pi = 22/7$

MALDI-TOF analysis

Peptide mass analysis of the active fractions of venom extracts was conducted using Matrix assisted laser desorption and ionisation (MALDI TOF) mass spectrometry, a widely utilized technique for determining the mass of peptides and proteins. This ionization technique involves directing laser energy onto a matrix containing the sample in dry droplet form to generate ions from large molecules with minimal fragmentation. The mass of the ionized fragments was then measured using a computer program. MALDI TOF spectra were obtained using a Bruker Daltonics Ultraflex TOF/TOF system equipped with a nitrogen laser at a wavelength of 337 nm. The bioactive fraction, specifically fraction 4 of both *Ropalidia marginata* and *Vespa tropica*, was lyophilized, and the peptide samples were resuspended in 20 µL of an aqueous solvent containing 0.1%

trifluoroacetic acid (TFA). Approximately 0.5 µL of the bioactive fraction was mixed with an equal amount of matrix solution (α-cyano-4-hydroxycinnamic acid). The mixture was then placed on a glass plate and dried at room temperature. The MS/MS spectra were recorded using LIFT technology, employing a calibration reference list of peptides. The data analysis was performed using Flex Analysis software, Bruker Daltonics, Germany, version 2.0. Spectra were recorded in positive ion mode over a mass range of m/z 800 to 1800, and the data were collected and analyzed.

Results

Extraction of antimicrobial peptides from social wasps

In the present study, 440 *Ropalidia marginata* and 250 *Vespa tropica* wasps were dissected to isolate their venom glands and extract crude venom. The protein concentration of these extracts was determined using the Bradford assay. Additionally, the antimicrobial properties of these extracts were assessed (Figure 1).

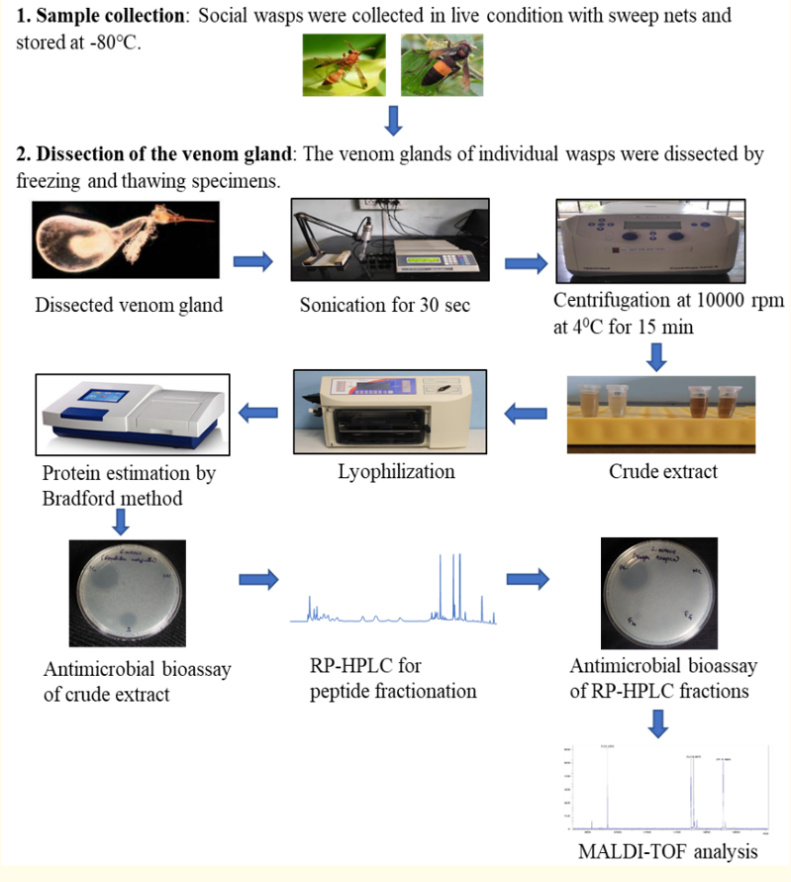


Figure 1: Flow chart illustrating the protocol adopted for preparing crude venom extract and bioassay guided RP-HPLC fractionation from social wasps used for screening of antimicrobial peptides.

Total protein content of crude venom extracts

The Bradford assay was utilized to determine the total protein concentration of crude venom extracts from two species of wasps. *Vespa tropica* had a relatively higher total protein concentration at 4.71 mg/ml, whereas *Ropalidia marginata* had a slightly lower concentration at 3.07 mg/ml.

Evaluation of the antimicrobial activity of crude venom extracts

The antimicrobial efficacy of the crude venom extract against *S. aureus* and *E. coli* was evaluated. The crude venom extracts from *Ropalidia marginata* and *Vespa tropica*, at concentrations of 15.35 µg and 23.55 µg respectively, exhibited clear inhibition zones

of 51.56 ± 1.28 mm^2 and 39.62 ± 1.12 mm^2 respectively against *Staphylococcus aureus*. However, only *Vespa tropica* crude venom extract at concentration of 23.55 µg displayed a clear zone of inhibition against *Escherichia coli*, measuring 31.29 ± 1.71 mm^2. On the other hand, 15.35 µg of *Ropalidia marginata* extract showed no antimicrobial activity against *Escherichia coli*, as seen by the complete absence of any zone of inhibition on the bacterial lawn (Figure 2,3).

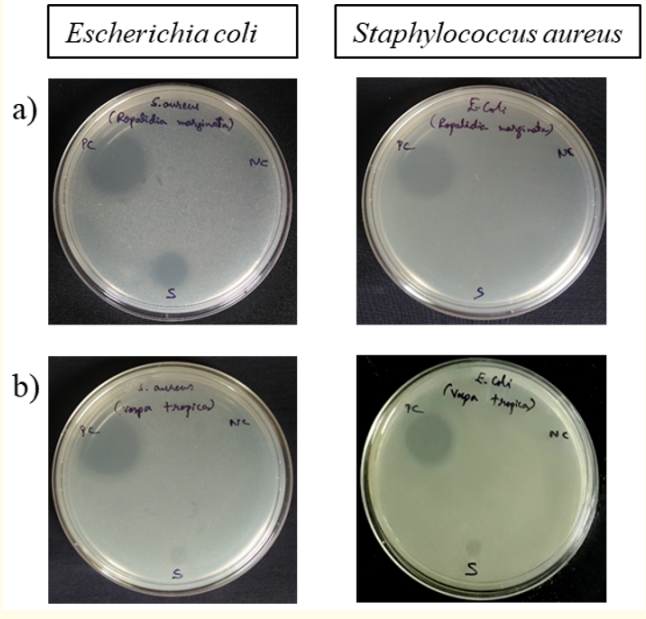


Figure 2: Antimicrobial activity (in vitro) of crude venom gland extracts from a) *Ropalidia marginata* and b) *Vespa tropica* against the *Escherichia coli* Strain MTCC: 2692 and *Staphylococcus aureus* Strain MTCC: 3160 by the "Spot on lawn bioassay" method after 12 hr of treatment (PC-Positive Control-Tetracycline) (NC- Negative Control-acetonitrile 50%: water 50%).

Peptide profiling of crude venom gland extracts from social wasps by RP-HPLC

To identify the active fractions exhibiting antimicrobial effects against *Staphylococcus aureus* and *Escherichia coli*, lyophilized venom samples were subjected to Preparative Reverse phase high performance liquid chromatography (RP-HPLC), with absorbance measured at 214 nm, a wavelength specific for detecting peptides or protein fragments [19]. RP-HPLC analysis revealed multiple potential peptide peaks in both the *Ropalidia marginata* and *Vespa tropica* extracts, which eluted at various retention times. Seven fractions were obtained from each crude venom extract (Figure 4). Subsequent RP-HPLC fractionation was carried out iteratively to ensure that sufficient quantities of each fraction were collected for assessing antimicrobial activity in pure fractions.

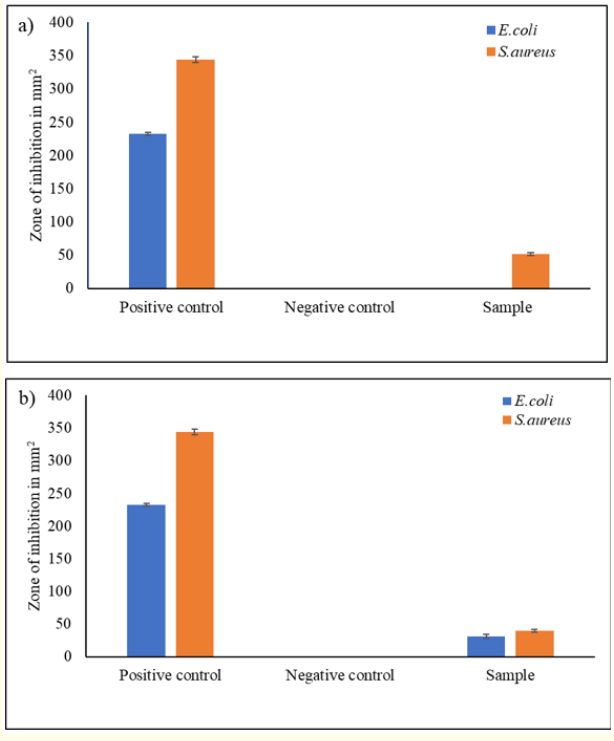


Figure 3: *In vitro* antimicrobial activity of crude venom gland extracts of a) *Ropalidia marginata*, and b) *Vespa tropica* as determined by spot on lawn assay against *Escherichia coli* and *Staphylococcus aureus* at 12 h post treatment (PC-Tetracycline and NC- acetonitrile 50%: water 50%).

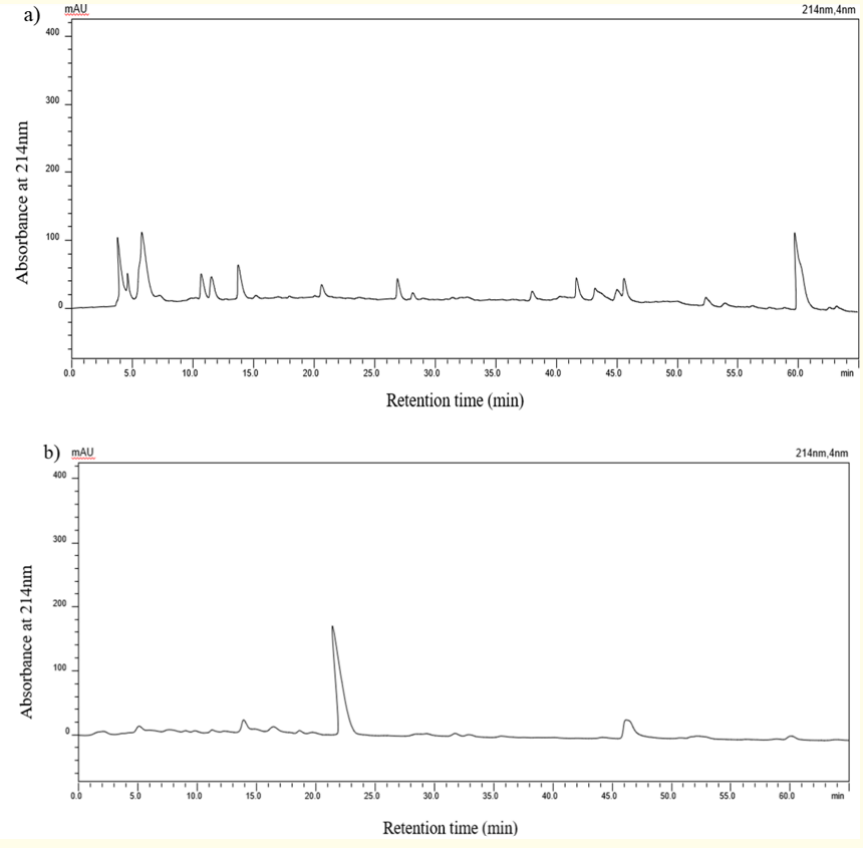


Figure 4: RP-HPLC profile of crude venom extracts from a) *Ropalidia marginata* and b) *Vespa tropica* subjected to Preparative Reverse-Phase HPLC with a gradient (0-5% ACN for 5 min, 5-95% for 50 min, 95% ACN for 5 min and 95-5% for 5 min) of acetonitrile and water containing 0.1% TFA over 65 min at a flow rate of 1 ml/min, eluted peaks were monitored at 214 nm, where several peaks were pooled into seven fractions.

Protein estimation of pure peptide fractions

Repeated collection of fractions from RP-HPLC yielded a greater quantity of the fractions along with the solvent, which were then lyophilized. Thus, the obtained dry samples were reconstituted in 50% acetonitrile (ACN) and water (50:50) and subjected to protein estimation using the Bradford method. Bovine serum albumin (BSA) served as the protein standard to generate a calibration curve, enabling the calculation of protein concentration for each individual fraction. However, all the fractions exhibited relatively lower protein concentrations compared to the crude venom extract. For *Ropalidia marginata*, the protein concentrations in each fraction(F) were as follows: F1 (0.28 mg/ml), F2 (0.27 mg/ml), F3 (1.18 mg/ml), F4 (0.78 mg/ml), F5 (0.46 mg/ml), F6 (0.26 mg/ml), and F7 (1.69 mg/ml). For *Vespa tropica*, the protein concentrations in each fraction(F) were as follows: F1 (0.42 mg/ml), F2 (0.27 mg/ml), F3 (2.14 mg/ml), F4 (2.13 mg/ml), F5 (0.67 mg/ml), F6 (0.49 mg/ml), and F7 (0.61 mg/ml).

Antimicrobial assays of pure individual RP-HPLC fractions against *Escherichia coli* and *Staphylococcus aureus*

Antimicrobial assays were conducted using the spot-on-lawn method, where 5 µl of the fractions were used to assess antimicrobial activity against the gram-positive bacterium *Staphylococcus aureus* (MTCC3160) and the gram-negative bacterial strain *Escherichia coli* (MTCC 2629).

Antimicrobial activity of RP-HPLC fractions from Ropalidia marginata

Among all seven fractions obtained by RP-HPLC of *Ropalidia marginata* crude venom extract, only fraction-4 at a concentration of 3.9 µg and fraction-7 at a concentration of 8.45µg displayed antimicrobial activity against *S. aureus*, with zones of inhibition of 27.06 ± 1.21 mm^2 and 15.22 ± 0.7 mm^2, respectively. However, none of the fractions exhibited antimicrobial activity against *Escherichia coli*. The mean area for the positive control (Tetracycline) was 243.03 ± 1.57 mm^2 for *Escherichia coli* and 344.47 ± 2.02 mm^2 for *Staphylococcus aureus* (Figure 5,7a).

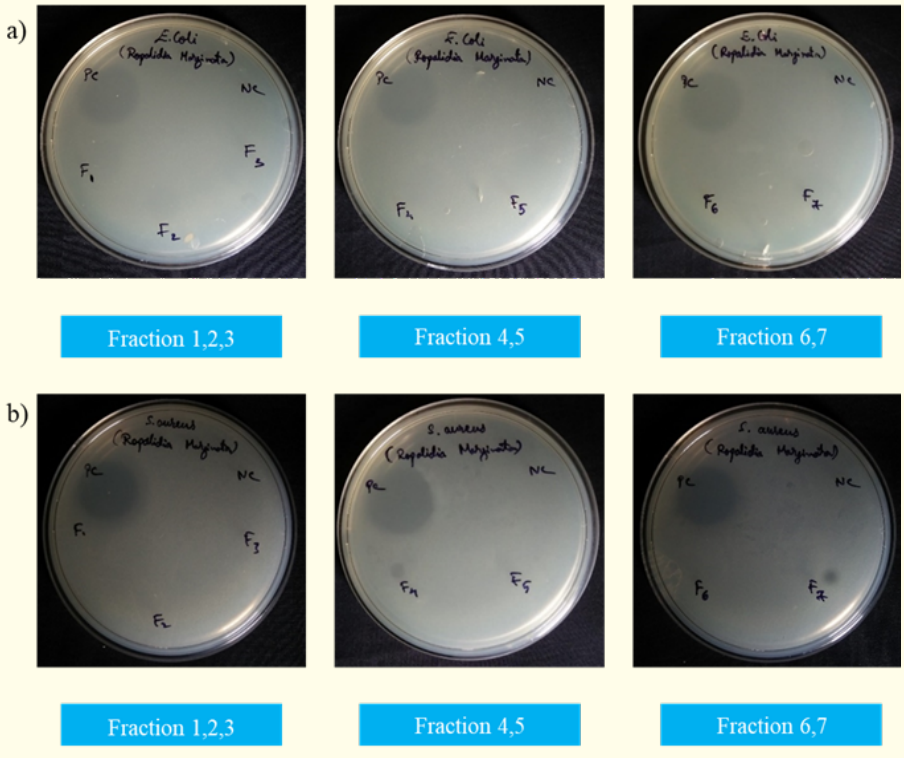
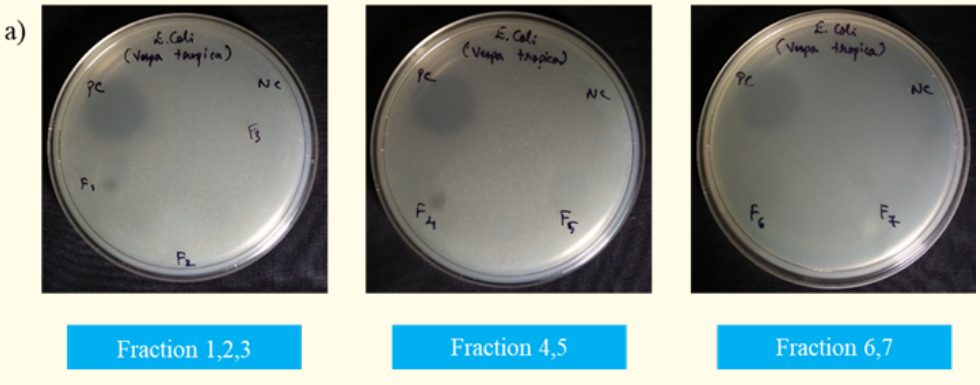


Figure 5: Antimicrobial activity (*in vitro*) of individual fractions isolated by RP-HPLC from the *Ropalidia marginata* venom gland crude extract against a) *Escherichia coli* Strain MTCC: 2692, and b) *Staphylococcus aureus* Strain MTCC: 3160 by the "Spot on lawn bioassay" method after 12 hr of treatment (PC-Positive Control-Tetracycline) (NC- Negative Control-Acetonitrile 50%: water 50%).



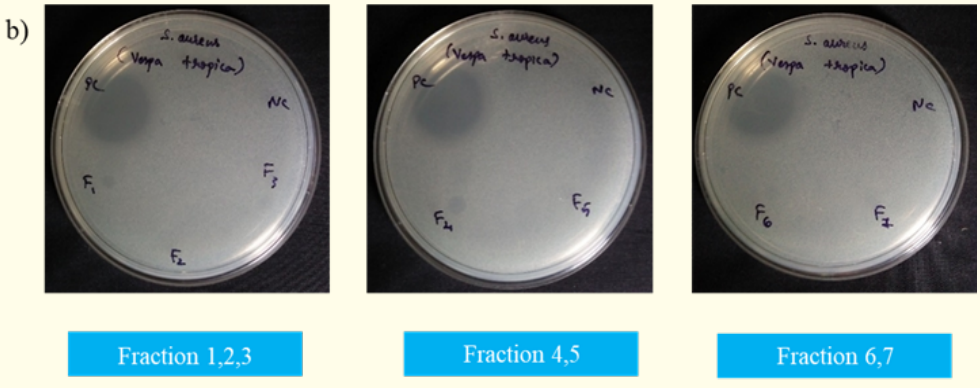


Figure 6: Antimicrobial activity (*in vitro*) of individual fractions isolated by RP-HPLC from the *Vespa tropica* venom gland crude extract against a) *Escherichia coli* Strain MTCC: 2692 and b) *Staphylococcus aureus* Strain MTCC: 3160 by the "Spot on lawn bioassay" method after 12 hr of treatment (PC-Positive Control-Tetracycline) (NC- Negative Control-Acetonitrile 50%: water 50%).

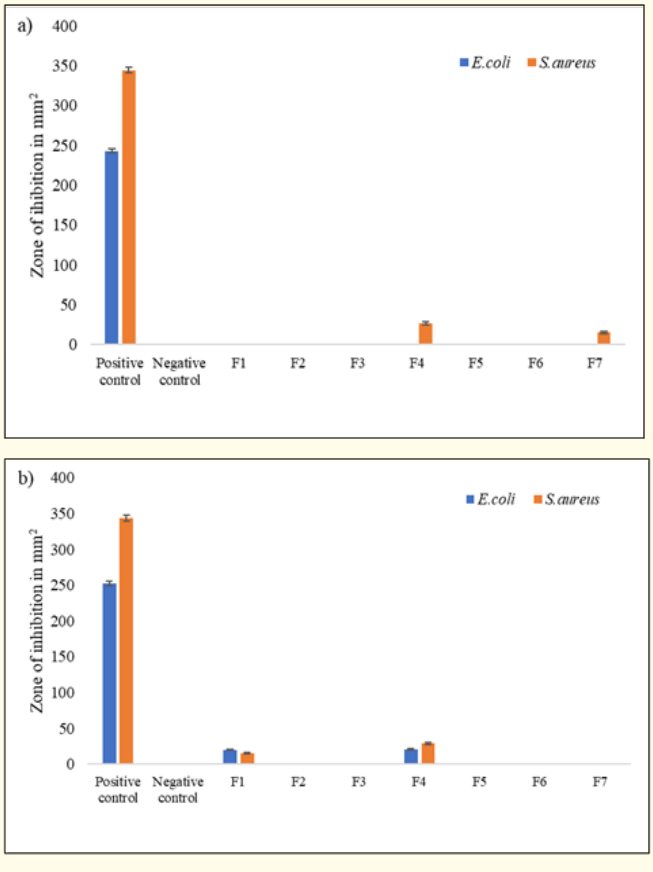


Figure 7: *In vitro* antimicrobial activity of a) *Ropalidia marginata* b) *Vespa tropica* RP-HPLC fractions (F1-F7) as determined by the spot on lawn assay against *Escherichia coli* and *Staphylococcus aureus* at 12 h post treatment. (PC-Tetracycline, NC- Acetonitrile 50%: water 50%).

Antimicrobial activity of Vespa tropica RP-HPLC fractions

Among all seven fractions obtained by RP-HPLC from the *Vespa tropica* crude venom extract, only fraction-1 at a concentration of 2.1 µg and fraction-4 at a concentration of 10.65 µg displayed antimicrobial activity against *Staphylococcus aureus*, with zones of inhibition of 15.22 ± 0.7 mm^2 and 29.24 ± 0.96 mm^2, respectively. Similarly, fraction-1 at a concentration of 2.1 µg and fraction-4 at a concentration of 10.65 µg also exhibited antimicrobial activity against *Escherichia coli*, with zones of inhibition measurements of 20.17 ± 0.53 mm^2 and 20.70 ± 0.53 mm^2, respectively. The positive control (Tetracycline) had a mean area of 252.43 ± 2.14 mm^2 for *Escherichia coli* and 343.82 ± 2.67 mm^2 for *Staphylococcus aureus* (Figure 6,7b).

Antimicrobial assays of individual RP-HPLC fractions against plant pathogens

The HPLC fractions were further tested against the plant pathogenic bacterium *Xanthomonas oryzae* pv. oryzae and the plant pathogenic fungus *Sclerotium rolfsii*, to explore the possibility of application of the obtained bioactive agents against plant disease causing microorganisms.

Antimicrobial activity of RP-HPLC fractions against plant pathogenic bacteria

Antimicrobial assays were conducted using the spot-on-lawn method with 5 µl of the fractions to assess antimicrobial activity against *Xanthomonas oryzae* pv. oryzae. For *Ropalidia marginata*, among the seven fractions tested, only fraction-4 at a concentration of 3.9 µg and fraction-7 at a concentration of 8.45 µg exhibited antimicrobial activity against *Xanthomonas oryzae* pv. oryzae, with zones of inhibition of 30.21 ± 0.96 mm^2 and 31.2267 ± 1.71 mm^2, respectively. The mean area for the positive control (Tetracycline) was 248.28 ± 2.32 mm^2. For *Vespa tropica*, among the seven fractions tested, only fraction-1 at a concentration of 2.1 µg and fraction-4 at a concentration of 10.65 µg displayed antimicrobial activity against *Xanthomonas oryzae* pv. oryzae, with zones of inhibition of 17.62 ± 1.00 mm^2 and 22.37 ± 1.13 mm^2, respectively. The mean area for the positive control (Tetracycline) was 246.26 ± 2.65 mm^2 (Figure 8,9).

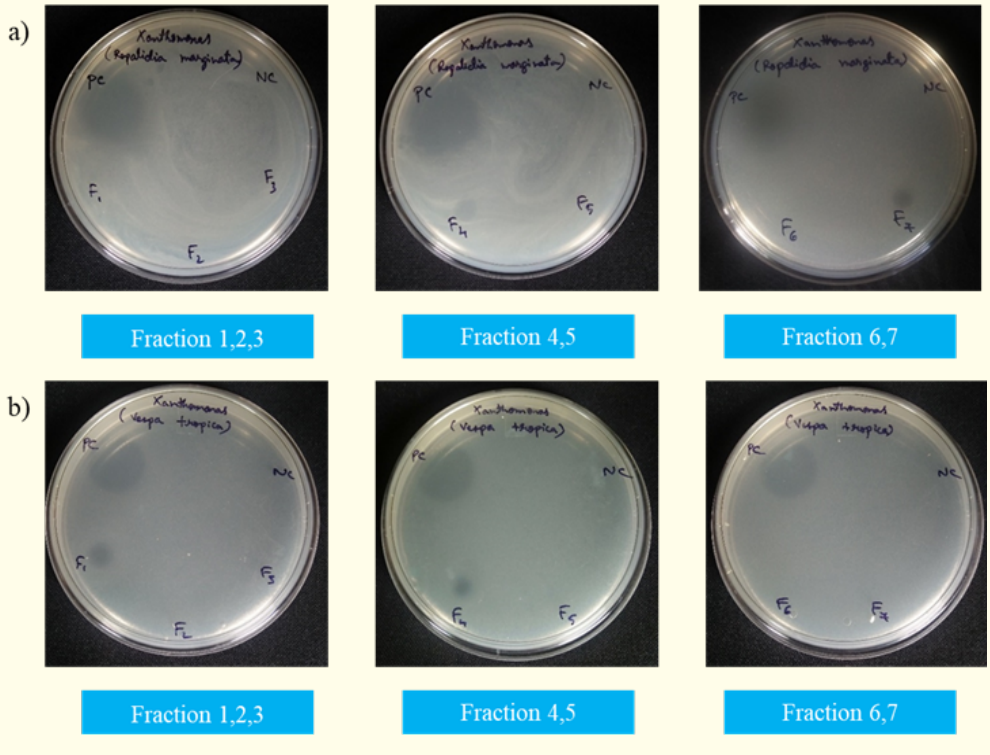


Figure 8: Antimicrobial activity (*in vitro*) of individual fractions isolated by RP-HPLC from a) *Ropalidia marginata* and b) *Vespa tropica* venom gland crude extract against *Xanthomonas oryzae* pv. oryzae by the "Spot on lawn bioassay" method after 12 hr of treatment (PC-Positive Control-Tetracycline) (NC- Negative Control-Acetonitrile 50%: water 50%).

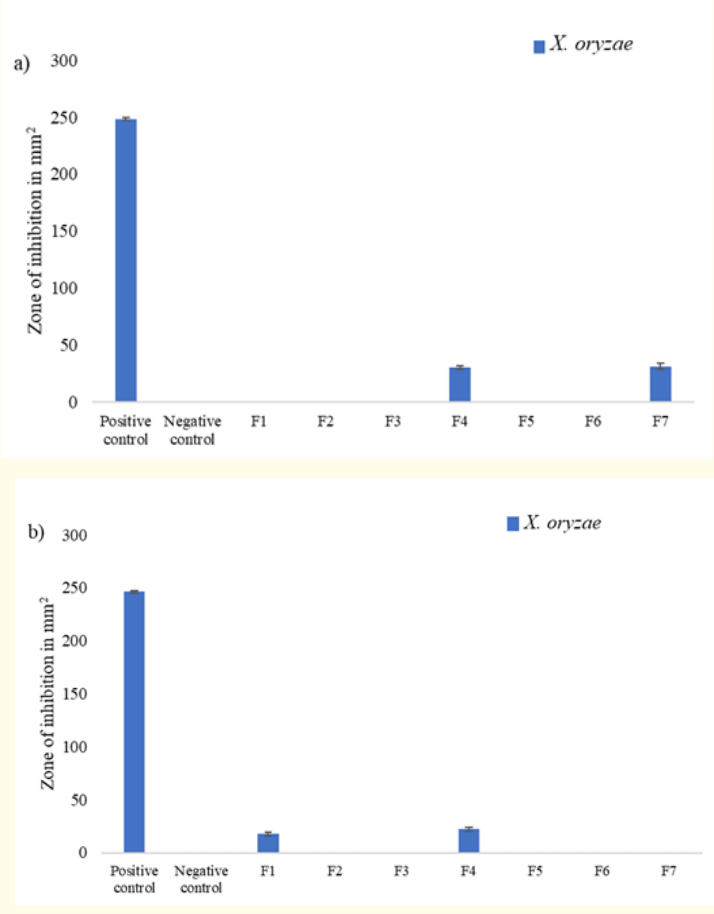


Figure 9: *In vitro* antimicrobial activity of a) *Ropalidia marginata* b) *Vespa tropica* RP-HPLC fractions (F1-F7) as determined by the spot on lawn assay against *Xanthomonas oryzae* pv. oryzae at 12 h post treatment. (PC-Tetracycline, NC- Acetonitrile 50%: water 50%).

Antimicrobial activity of RP-HPLC fractions against plant pathogenic fungi

The fractions obtained from *Ropalidia marginata* and *Vespa tropica* were assessed for their effects on the plant pathogenic fungus *Sclerotium rolfsii* using the Kirby-Bauer disc method. Each sample, along with a negative control consisting of a 1:1 solution of acetonitrile and distilled water with 0.1% trifluoroacetic acid (TFA), was applied at a volume of 20 µl. However, none of the fractions from either species exhibited antifungal activity (Figure 10).

MALDI-TOF analysis

The active RP-HPLC fraction 4 from *Ropalidia marginata* and *Vespa tropica* were subjected to MALDI-TOF analysis using Ultraflex TOF/TOF mass spectrometry, confirming the presence of peptides. In the *Ropalidia marginata* sample, six peptides were identified with m/z 932.280, m/z 1497.772, m/z 1513.821, m/z 1535.646, m/z 1713.880 and m/z 1729.650 in the positive ionization method. Notably, the peptide with m/z 932.280 appeared to be present at a higher concentration compared to the other peptides observed in the MALDI-MS experiment (Figure 11 a). Similarly, in the *Vespa tropica* sample, six peptides were detected with m/z 1386.182, m/z 1513.794, m/z 1535.751, m/z 1551.719, m/z 1686.479 and m/z 1695.805 in the positive ionization method. The peptide with m/z 1513.794 was found to be more abundant compared to the other peptides observed in the MALDI-MS experiment (Figure 11 b).

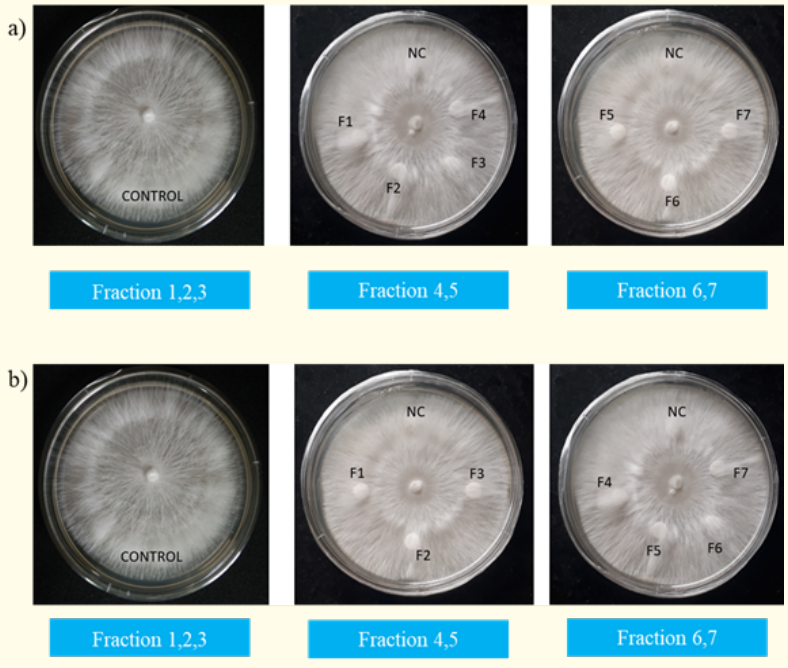
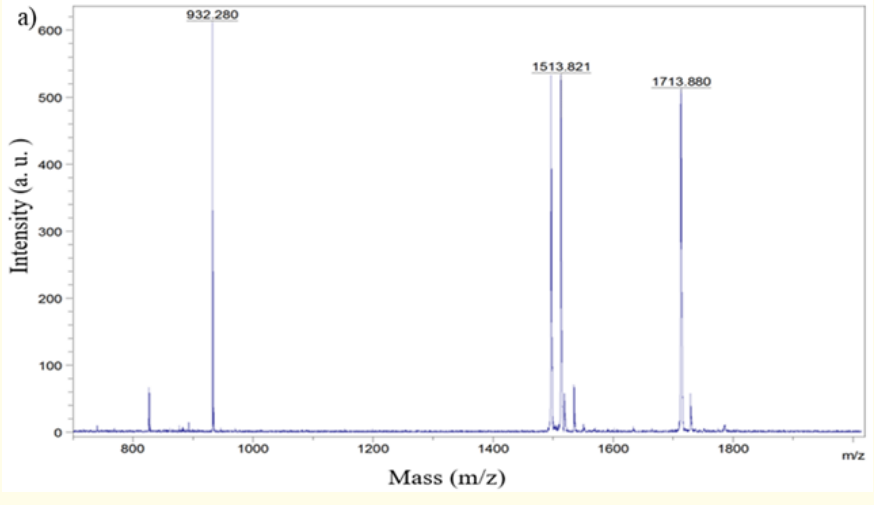


Figure 10: Antimicrobial activity (*in vitro*) of individual fractions isolated by RP-HPLC from a) *Ropalidia marginata*, and b) *Vespa tropica venom* gland crude extract against *Sclerotium rolfsii* by the "Kirby-Bauer disk diffusion assay" method after 12 hr of treatment (Control) (NC- Negative Control-Acetonitrile 50%: water 50%).



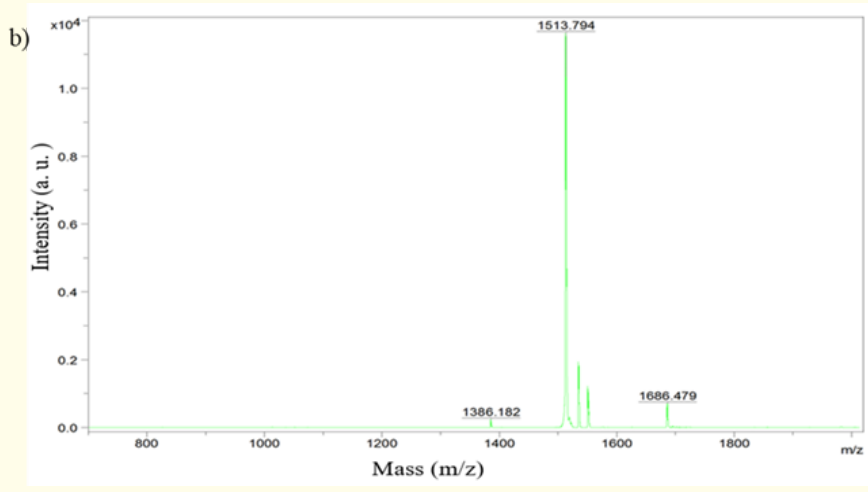


Figure 11: Peptide mass analysis of the bioactive fraction of a) *Ropalidia marginata* and b) *Vespa tropica.* Fraction 4 was subjected to MALDI-TOF MS analysis using an Ultraflex TOF/TOF mass spectrometer. The spectra were recorded in positive ion mode over a mass range of m/z 800 to 1800.

Discussion

As the number of multi-resistant bacterial strains rises, researchers are working hard to find novel classes of antimicrobial compounds with new mechanism of action and optimize them for therapeutic use. Because of their strong antimicrobial activity, cationic peptides, which are known to act as cell penetrating carriers, are interesting compounds. All organisms, from plants to insects to humans, produce these major classes of antimicrobial compounds as a major part of their immediate effective, nonspecific defenses against infections.

The findings presented in this study shed light on the potential of social wasps, particularly *Ropalidia marginata* and *Vespa tropica*, as sources of antimicrobial peptides (AMPs) with broad-spectrum activity. The exploration commenced with the isolation and extraction of venom glands from these wasps, followed by the determination of the total protein concentration in the crude venom extracts and evaluation of their antimicrobial properties against clinically relevant pathogens.

The total protein content of the crude venom extracts revealed notable differences between the two species studied. *Vespa tropica* venom had a higher total protein concentration of 4.71 mg/ml than *Ropalidia marginata* with 3.07 mg/ml. This difference in protein content could be attributed to variations in venom gland size, and venom composition between these species, reflecting their distinct ecological niches and evolutionary adaptations.

Assessment of antimicrobial activity demonstrated that both *Ropalidia marginata* and *Vespa tropica* crude venom extracts at concentrations of 15.35 µg and 23.5 µg respectively, had significant inhibitory effects against *Staphylococcus aureus*, a gram-positive bacterium commonly associated with skin and soft tissue infections. However, only the *Vespa tropica* crude venom extract at a concentration of 23.55 µg displayed activity against *Escherichia coli*, a gram-negative bacterium notorious for causing gastrointestinal and urinary tract infections. The absence of antimicrobial activity against *Escherichia coli* in *Ropalidia marginata* extract suggests species-specific variations in venom composition, highlighting the need for further investigation into the molecular mechanisms underlying these differences. The lack of activity of the extract against *Escherichia coli* can be attributed to many factors, such as the strain of the microbe used, the diversity of antimicrobial peptides in the extracts and their titre, and the range of microbes against which they are active in the wild etc. Moreover, gram-negative bacterial infections are established to be difficult to treat using antibiotics

owing to the presence of lipopolysaccharides (LPS) on their cytoplasmic membrane [20].

Staphylococcus aureus and *Escherichia coli* were not inhibited by *Pimpla turionellae* (Parassitoid wasp) crude venom [21], whereas they were inhibited by *Vespa orientalis* crude venom [22]. Moreover, *Pimpla hypochondriaca* (red legged wasp) crude venom was most active at low concentrations against *Escherichia coli* and *Xanthomonas campestris* [23]. This suggests that the constituents of venom, the AMP's may have different modes of action, and bacterial cell wall which differ between gram-positive and gram-negative bacteria in terms of composition which may affect pore formation by cationic AMPs [24].

To identify the bioactive components responsible for the observed antimicrobial effects, RP-HPLC fractionation was performed on the crude venom extracts. Subsequent antimicrobial assays of individual fractions revealed distinct peptide profiles and differential activity against both gram-positive and gram-negative bacteria. Notably, fractionation enabled the isolation of specific fractions with potent antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli.* Fraction-4 at a concentration of 3.9 µg and fraction-7 at a concentration of 8.4 µg of *Ropalidia marginata* venom extract displayed antimicrobial activity against *Staphylococcus aureus*, with zones of inhibition of 27.06 ± 1.21 mm^2 and 15.22 ± 0.7 mm^2, respectively. However, none of the fractions exhibited antimicrobial activity against *Escherichia coli*. Fraction-1 at a concentration of 2.1 µg and fraction-4 at a concentration of 10.65 µg of *Vespa tropica* venom extract displayed antimicrobial activity against *Staphylococcus aureus*, with zones of inhibition of 15.22 ± 0.7 mm^2 and 29.24 ± 0.96 mm^2, respectively. Similarly, fraction-1 at a concentration of 2.1 µg and fraction-4 at a concentration of 10.65 µg also exhibited antimicrobial activity against *Escherichia coli*, with zones of inhibition of 20.17 ± 0.53 mm^2 and 20.70 ± 0.53 mm^2, respectively. These findings underscore the potential of social wasp venom as a source of novel antimicrobial agents for combating antibiotic-resistant pathogens.

The difference in the activity of the peptide fractions against bacteria observed may be because of their differential mode of action of antimicrobial peptides [25]. The absence of antimicrobial activity of these fractions against *Escherichia coli* can possibly be explained by many factors, such as the strain of the microbe used and the titre of peptides in the fractions. As mentioned earlier, gram-negative bacterial cell walls are more resistant to antibiotics and AMPs compared to gram-positive bacteria [20]. The purified venom toxins of yellow wasp *Polistes flavus* were found to have the minimum inhibitory concentration (MIC) of 12.3 µg/ml against *E. coli* and 49.24 µg/ml against *S. aureus*. Using the agar disc diffusion method, inhibition zones with diameters of 18.36 ± 0.14 mm for *E. coli* and 17.36 ± 0.43 mm for *S. aureus* was observed [26]. Nine antimicrobial peptides (AMPs) were identified from the *Vespa tropica* wasp, including mastoparan-VT1 to VT7 and the vespid chemotactic peptides (VCP)-VT1 and VT2. The minimum inhibitory concentrations (MICs) against *S. aureus* were found to be 5 µg/ml, 5 µg/ml, 10 µg/ml, 80 µg/ml, 20 µg/ml, 10 µg/ml, 1.2 µg/ml, and 2.5 µg/ml for mastoparan-VT1 to VT4, VT6, VT7, VCP-VT1, and VT2, respectively. For *E. coli*, the MICs were 20 µg/ml, 20 µg/ml, 2.5 µg/ml, and 5 µg/ml for mastoparan-VT1, VT2, VCP-VT1, and VT2, respectively [27]. The Wasp venom peptide, synoeca-MP, which is extracted from *Synoeca surinama*, was found to exhibit robust antimicrobial efficacy. The MIC50 and MIC90 values against methicillin-resistant *Staphylococcus aureus* (MRSA) were 1.9 and 2.2 µM, respectively. Similarly, for *Escherichia coli* ESBL, the MIC50 and MIC90 were 2.0 and 2.1 µM, respectively [28]. The results obtained in our study were consistent with those reported in the previous studies mentioned above.

Moreover, the assessment of RP-HPLC fractions against plant pathogens, including *Xanthomonas oryzae* pv. oryzae and *Sclerotium rolfsii*, highlights the broader applicability of social wasp venom-derived peptides in agricultural sectors. *Xanthomonas oryzae* pv. oryzae is a gram-negative bacterium that causes destructive bacterial disease in rice, and the development of an environmentally safe bactericide is urgently needed. Antimicrobial peptides penetrate bacterial membranes, accumulate inside bacteria and then block bacterial functions and induce cell death by interacting with intracellular DNAs and RNAs [29]. The identification of two fractions exhibiting antimicrobial activity against plant pathogens suggests potential applications in crop protection and disease management.

MALDI-TOF analysis further elucidated the peptide composition of the active RP-HPLC fractions, revealing distinct mass-to-charge ratios corresponding to putative antimicrobial peptides. The iden-

tification of specific peptides, such as those with m/z 932.280 in *Ropalidia marginata* and m/z 1513.794 in *Vespa tropica*, provides valuable insights into the molecular basis of their antimicrobial activity and lays the foundation for future studies on peptide structure-function relationships.Eumenitin, a novel antimicrobial peptide, was identified from the venom of the solitary eumenine wasp *Eumenes rubronotatus*. MALDI-TOF MS analysis revealed components with a mass range up to m/z 5000. The major fraction exhibited high purity, featuring a protonated molecular ion peak at m/z 1644.9 [30]. In the wasp venom peptide, synoeca-MP, derived from *Synoeca surinama*, a major compound with m/z 1617.861 was detected using MALDI-TOF [28]. 49 primary MALDI-TOF peaks ranging from 1078 to 3375 m/z from venom samples of 10 species belonging to the Stenogastrinae wasp group was observed [31]. The m/z values identified in our study falls within the range of m/z values previously reported for characterized antimicrobial peptides in the mentioned studies.

In conclusion, this study demonstrated the potential of social wasp venom as a rich source of bioactive peptides with promising antimicrobial properties. The identification and characterization of novel antimicrobial peptides from *Ropalidia marginata* and *Vespa tropica* not only contributes to our understanding of natural host defense mechanisms but also hold significant implications for the development of new therapeutic agents against infectious diseases and agricultural pests. Further research focusing on peptide purification, structural elucidation, and mechanistic studies is warranted to harness the full therapeutic potential of social wasp venom-derived peptides.

Conclusion

Based on the results obtained, it can be concluded that both *Ropalidia marginata* and *Vespa tropica* social wasps produce crude venom extracts with antimicrobial properties. The crude venom extracts from both species exhibited antimicrobial activity against *Staphylococcus aureus*, while only the *Vespa tropica* extract showed antimicrobial activity against *Escherichia coli*. Further fractionation of the crude venom extracts using RP-HPLC revealed individual fractions with varying levels of antimicrobial activity against both bacterial species. Additionally, the active fractions from both species showed promising antimicrobial activity against the plant pathogenic bacterium *Xanthomonas oryzae* pv. oryzae, indicating potential applications in agriculture for controlling plant diseases caused by bacterial pathogens. However, none of the fractions exhibited antifungal activity against *Sclerotium rolfsii*. MALDI-TOF analysis of the active RP-HPLC fractions confirmed the presence of peptides in both *Ropalidia marginata* and *Vespa tropica* samples, suggesting that the observed antimicrobial activity may be attributed to these peptides.

In conclusion, the findings of this study highlight the potential of social wasp venom as a source of novel antimicrobial peptides with applications in medicine and agriculture. Further research is warranted to characterize and elucidate the mechanisms of action of the identified peptides, as well as to explore their potential for development into antimicrobial agents for therapeutic and agricultural purposes.

Conflict of Interest

No conflict of interest exists.

Acknowledgment

RBN acknowledges ICAR-CIPHET, Ludhiana for the support.

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