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Antimicrobial peptides from the Asian harvester ants of the genus *Monomorium*: *In vitro* screening for antimicrobial activity

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Insect antimicrobial peptides (AMPs) have received considerable attention in the last two decades as potential scaffolds for synthetic antimicrobial compounds with wide ranging applications. Among insects, the hymenopterans comprising of ants, wasps and bees are well known for their extraordinary diversity of chemical defenses. In the present study, three species of ants of the genus *Monomorium* (Hymenoptera: Formicidae) were screened for their antimicrobial peptides. The whole body extracts of worker ants were prepared using polar solvents and screened for antimicrobial activity against standard bacterial strains. Active crude extracts were subjected to fractionation using RP-HPLC to generate the peptide profile of the extracts. Peptides ranging in mass from 500 Da to 3500 Da were identified using MALDI-TOF analysis. The three species of ants used in this study were found to be a promising source for bioprospecting antimicrobial peptides.

Keywords: Antimicrobial activity, antimicrobial peptides, HPLC, MALDI-TOF, *Monomorium* spp.

Introduction

Insects are the most successful of all terrestrial organisms and occupy virtually every conceivable ecological niche despite being in constant conflict with microbes[1,2]. In fact the resistance of insects to microorganisms has been known for a long time. A well studied part of their resistance mechanism is the production of antimicrobial peptides[3]. Antimicrobial peptides are small, mostly linear, synthesized in the insect's fat body as part of innate immune defence mechanism[4]. The antimicrobial peptides are cationic in nature with 10 to 50 amino acid residues with a net positive charge constituting more than 50% hydrophobic residues of total amino acid sequence[5]. These peptides play an important role in host defence mechanism, protecting insects from microbial infections. These peptides are reported to have broad spectrum antimicrobial activity against bacteria, fungi and some viruses[6]. One aspect of antimicrobial peptide research, which is of particular interest, is their possible use as an alternative to synthetic antibiotics, which could be extremely important with increasing antibiotic resistance in human, animals and plants[7].

Hymenopterans with approx 1,20,000 described species comprise of ants, wasps and bees, and are well known for their extraordinary chemical diversity and defensive chemicals[8]. Ants belong to the family 'Formicidae', a taxonomically diverse group comprising more than 13,000 species belonging to 20 subfamilies. Ants produce complex mixtures of proteins and peptides in the venom, metapleural gland secretions and hemolymph[9,10]. These peptides and proteins exhibit a wide range of activities, such as, antimicrobial, haemolytic, cytolytic, paralytic, insecticidal, *etc*[7,11]. Like other hymenopterans ants are eusocial and live in highly populated colonies, increasing the risk of frequent encounters with pathogenic microorganisms. In defense to these invading pathogens, ants have evolved antimicrobial peptides against both Gram-negative and Gram-positive bacteria[12-19]. So far, only 71 antimicrobial peptides have been

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isolated and characterized from ants belonging to few genera. Considering the species diversity and distribution, ants are a potential source for prospecting biologically active compounds, particularly antimicrobial peptides[20].

In the present study, we investigated the antimicrobial properties from genus *Monomorium* (Formicidae: Hymenoptera), commonly known as the Asian harvester ant, widely distributed in India and Sri Lanka. Colonies are polygynous with many queens and several hundred workers per nest, with an average body size of 3-4 mm. These ants live in large colonies and the worker ants provision their subterranean nests with seeds of monocots, mostly Poaceae. Hypothesizing that antimicrobial peptides, in addition to other chemicals, could be playing a major role in protecting the ants against the invading microbial pathogens, we investigated these ants for antimicrobial peptides.

Materials and Methods

Ant Collection

The worker ants of genus *Monomorium* were collected using an aspirator during January, 2012 from crop fields at the campus of the University of Agricultural Sciences, GKVK, Bangalore (13°081´ N; 77°571´ E). The ants were identified to their species by using standard taxonomic tools[21]. The voucher specimens of all the three species of ants have been deposited with the Bioprospecting Referral Collection at the School of Ecology and Conservation, University of Agricultural Sciences, Bengaluru. The collected ants were separated from nest debris and soil particles after inactivating them by holding them in a refrigerator at 4°C for 15 min. Ants were rinsed with Millipore water and transferred to an aqueous solution containing 0.1% trifluroacetic acid (TFA) at – 4°C until processed.

Preparation of Crude Extract

Crude extracts of worker ants of each of the three species were prepared using polar solvents. Ants were disrupted in aqueous solutions containing 0.1% TFA for 30 min at 4°C, followed by disruption by ultrasonic waves using a sonicated water bath for 120 sec. The supernatant of each extract was collected by centrifugation at 10,000× g for 10 min at 4°C. The ant remnants were further subjected to sequential extraction in aqueous solution containing increasing concentrations of acetronitrile (ACN), *i.e.*, 25, 50, 75 and 100%. The supernatant from each extraction step was pooled and concentrated using a vacuum concentrator. The concentrated sample was lyophilized and stored at –20°C for further analysis.

Antimicrobial Assays

The antimicrobial activity of crude extracts was evaluated against Gram-positive bacterial strains (*Staphylococcus aureus* MTCC 3160 & MTCC 9542) and Gram-negative bacterial strains (*Escherichia coli* MTCC 2692, *Pseudomonas aeruginosa* MTCC 8076, *Vibrio cholerae* McV09 & enterotoxigenic *E. coli*) obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Inhibitory activity was determined by the Kirby-Bauer disk diffusion assay. A 10 mL nutrient broth containing 1.5% agar (Bacteriological, Himedia) was inoculated with 50 µL of a pure overnight grown culture of bacterial strain (OD=0.1 at 600 nm, *i.e.*, approx 1×10^6 colony forming unit) and poured into 90 mm Petri plates. Sterile disks of 6 mm diam (Himedia SD067) impregnated with 20 µL of sample solutions at a concentration of 10 mg/mL were placed on the surface of solidified nutrient agar. The inhibition zone diameters were measured by subtracting the disk diameter, after incubating the Petri dishes at 37°C for overnight. Tetracycline (30 µg, Himedia SD037) was used as positive control and the solvent used for reconstitution of the sample was used as the negative control.

RP-HPLC Profiling

The active crude extracts were subjected to fractionation using a Shimadzu HPLC (LC20 AT) equipped with C_{18} reversed-phase column (Phenomenex: 5 µm particle size; 250 × 4.6 mm^2 column). The sample was prepared in Milli Q water containing 0.1% TFA at a concentration of 25 mg/mL. The flow of mobile phase was set at 1 mL/min and the solvent system was 0.1% TFA in Milli Q water (Solvent A) and 80% aqueous ACN with 0.1% TFA (Solvent B), and the flow of the mobile phase was 1 mL/min. The elution was carried out with a linear gradient of 5-95% of solvent B over 60 min and the UV detection of the eluted peptide peaks was monitored at 214 nm using a UV-DAD detector (SPD-M20A).

MALDI-TOF Analysis

The peptide analysis of active crude extracts was carried out by using Matrix Assisted Laser Desorption and Ionization-a linear Time-of-Flight (MALDI-TOF) mass spectrometry (MS). Samples were subjected to MALDI-TOF MS analysis using Ultraflex TOF/TOF mass spectrometer. The spectra were recorded in positive ion mode over a mass range of *m/z* 500 to 4000 using α-cyano-4-hydroxy-cinnamic acid as a matrix.

Results and Discussion

Ant Collection

Three species of ants belonging to the genus *Monomorium*, *M. indicum*, *M. criniceps* and *M. scabriceps*, were collected in the present study (Fig. 1). During the collection of ants the nesting behavior and foraging patterns were also recorded. These ants construct subterranean nests and provision the nests with grass seeds during the month of November to February. The seeds collected by the ants are stored in separate chambers, the 'granaries', which are separated from brood chambers. An interesting feature of this provisioning method is that the grains stored in the sub-terranean nests by ants are completely free from mould[22].

Fig. 1 (a-c) — Three species of *Momomorium* ants.[a. *M. indicum*; b. *M. criniceps*; & c. *M. scabriceps*]

Antimicrobial Activity of Crude Extracts

The crude extracts of *Monomorium* ants showed antibacterial activity against both Gram-positive and Gram-negative bacteria. The disk diffusion assay results showed a clear zone of inhibition around paper disks impregnated with crude extracts. However, the antimicrobial activity of extracts from different ant species showed considerable variation in activity against different microbial strains. Among the three ant species tested for antimicrobial activity, *M. criniceps* showed strong antimicrobial activity against the test organisms (*E. coli* MTCC 2692: 228.01±30.84 mm^2; enterotoxic *E. coli*: 98.96±28.65 mm^2; *P. aurogenosa* MTCC 8076: 59.62±13.58 mm^2; *V. cholerae* MoV 09: 549.45±41.24 mm^2; *S. aureus* MTCC 3160: 269.52±10.53 mm^2; *S. aureus* MTCC 9542: 155.04±10.20 mm^2), followed by *M. scabriceps* and *M. indicum* (Table 1).

The antimicrobial activity of *M. scabriceps* against different bacterial strains was recorded as 88.50±16.95 mm against *E. coli*, 29.80±3.99 mm against enterotoxic *E. coli*, 11.71±2.80 mm against *P. aurogenosa*, 405.18±21.10 mm against *V. cholerae*, 246.65±41.82 mm against *S. aureus* (MTCC 3160) and 140.39±9.74 mm against *S. aureus* (MTCC 9542). Among all the three ant species tested *M. indicum* showed weak antimicrobial activity against Gram-positive bacteria with 138.38±51.54 mm against *S. aureus* (MTCC 3160), 123.78±16.11 mm against *S. aureus* (MTCC 9542). However, the inhibition zone of crude extract was smaller than the zone of inhibition observed around standard antibiotic tetracycline disks (100 mcg, Hi-media). These results clearly suggest that the extracts from *Monomorium* ants possess

Table 1 — Screening of crude extracts of ants of the genus *Monomorium* for antimicrobial activity against selected bacterial strains by Kirby-Bauer disk diffusion assay

Microbial strains	Zone of inhibition (mm)			
	M. indicum	*M.criniceps*	*M. scabriceps*	*Positive Control*
Gram-positive bacteria				
Staphylococcus aureus (MTCC 3160)	138.38±51.54	269.52±10.53	244.65±41.82	741.44±182.68
S. aureus (MTCC 9542)	123.78±16.11	155.04±10.20	140.39±9.74	910.91±58.80
Gram-negative bacteria				
Escherichia coli (MTCC 2692)	64.84±5.92	228.01±30.84	88.50±16.95	401.98±33.09
E. coli Enterotoxic	38.25±10.92	98.96±28.65	29.80±3.99	315.80±3.36
Pseudomonas aurogenosa (MTCC 8076)	21.37±-10.42	59.62±13.58	11.71±2.80	195.92±29.22
Vibrio cholerae (MoV 09) (SI)	451.12±53.46	549.45±41.24	405.18±21.10	1254.19±106.09

*Data represents the mean of three replications
SI: Clinically isolated strain

antimicrobial activity, making them good candidates for prospecting antimicrobial peptides. The antibacterial activity of extracts from the three species of ants suggests that the further purification and characterization of peptides from the crude samples may result in identifying potential antimicrobial peptides.

HPLC Profiling and MALDI-TOF Analysis of Peptides

The HPLC profiling of active crude extracts resulted in elution of peptide peaks at 214 nm. Most of the peptide peaks were eluted within 30 min of the run time indicating that the peptides eluting are mostly polar in nature (Fig. 2). To confirm the presence of peptides in the crude samples, the samples were subjected to MALDI-TOF analysis using Ultraflex TOF/TOF mass spectrometry. The results of MALDI-TOF analysis conform the presence of peptides and the peptides ranging from 500 Da to 3000 Da were recorded (Fig. 3). Based on the peptide mass range (537 Da to 3026 Da), these peptides appear to be small when compared to similar AMPs isolated from other ant species[5,20].

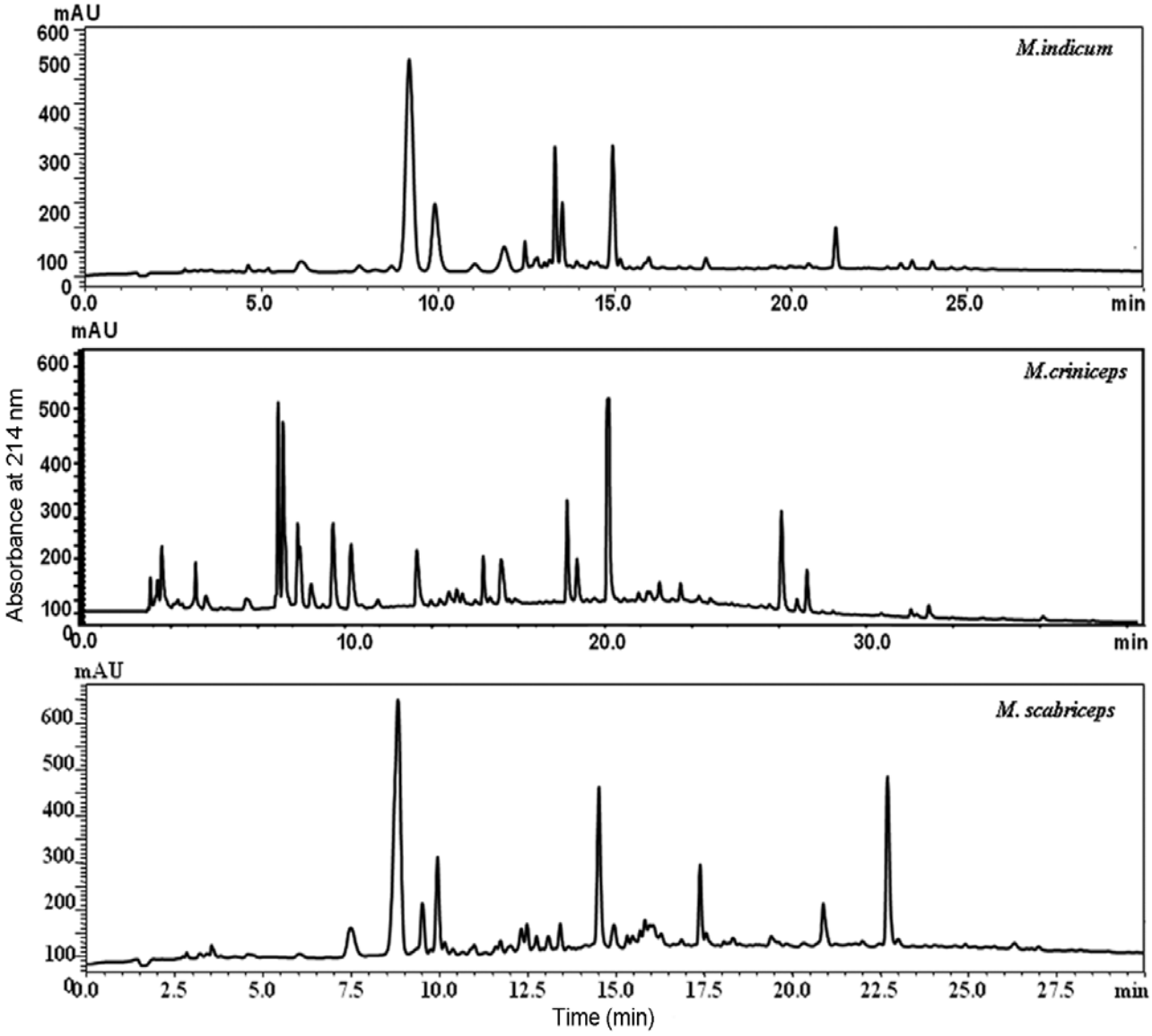

Fig. 2 — HPLC profiling of crude extracts of *Monomorium* ants. [The active crude extracts were subjected to fractionation using a Shimadzu HPLC (LC20 AT) equipped with C_{18} reversed-phase column. The elution was carried out with a linear gradient of 5-95% of ACN for 60 min at a flow rate of 1 mL/min. Eluted peptide peaks were monitored at 214 nm using a UV-DAD detector (SPD-M20A).]

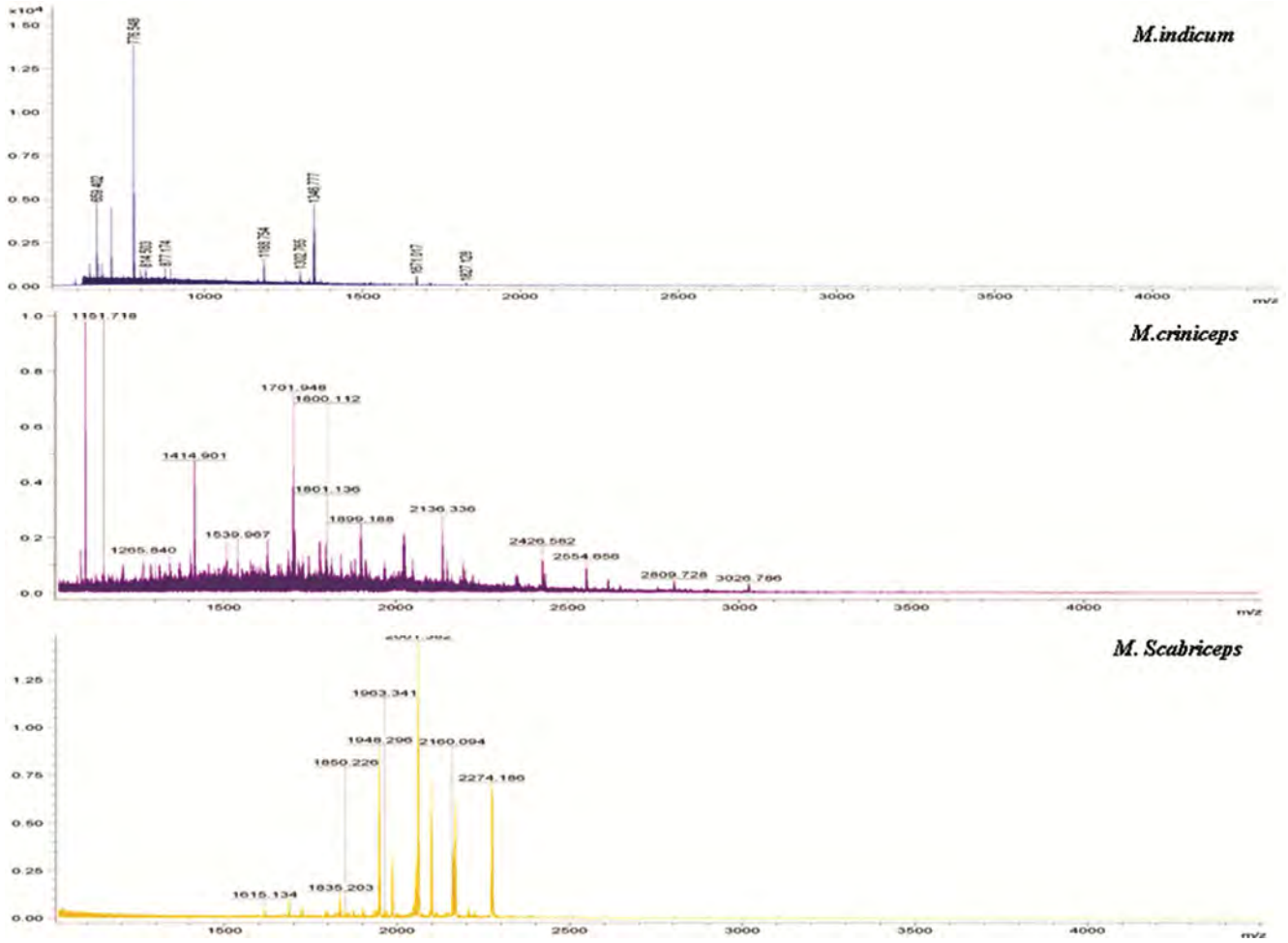

Fig. 3 — Peptide mass analysis of crude extracts of *Monomorium* ants. [Samples were subjected to MALDI-TOF MS analysis using Ultraflex TOF/TOF mass spectrometer. The spectra were recorded in positive ion mode over a mass range of m/z 500 to 4000.]

Conclusion

Ants are a potential source of antimicrobial peptides and so far several antimicrobial peptides have been isolated from ants belonging to different genera. Based on the preliminary screening in the present study the ants of the genus *Monomorium* appear promising for their antimicrobial peptides.

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