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Exploring the venom of *Ectatomma brunneum* Smith (Hymenoptera: Formicidae)

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Abstract

Ant venom has several functions, including predation, communication, defense against predators, and action against pathogens. There is a scarcity of data about ant venom components that could provide support for understanding the mechanisms of action. The objective here was to identify the amino acids and proteins in the venom of the predatory ant *Ectatomma brunneum* and to evaluate its antimicrobial activity. The amino acids were analyzed by liquid chromatography, with diode array detection, and were identified using amino acid standards. The two-dimensional (2D) gel electrophoresis fractionation approach was used to identify the proteins, together with MALDI-TOF/TOF mass spectrometry and protein databases. The antimicrobial activity of the venom was evaluated using the minimum inhibitory and minimum microbiocidal concentrations. The venom of *E. brunneum* contained free amino acids, with a high amount of alanine. The 2D gel analysis showed 104 spots, of which 21 were identified and classified according to biological function, as follows: venom proteins, nontoxic reservoir protection, cellular maintenance proteins, and proteins with unknown function. The venom showed antimicrobial activity, inhibiting the growth of all the bacteria and fungi tested. The results provide new insights into ant venom components and antimicrobial activity.

Keywords Allergen · Innate immunity · Proteomics

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Introduction

Ants are invertebrate predators with evolutionary adaptations for venom production (Touchard et al. 2016). In ants, the venom is used for colony defense, social communication, and protection against pathogens (Orivel et al. 2001; Aili et al. 2014; Touchard et al. 2016; Baracchi and Tragust 2017; Tragust et al. 2020). Although solitary foraging behavior is strongly linked to phylogeny, ants have powerful venoms for incapacitating victims (Pie 2004; Kolay et al. 2020; Correia et al. 2022).

Ant venom is a complex mixture of hydrocarbons, salts, sugars, amines, alkaloids, acids, free amino acids, peptides, and proteins (Santos et al. 2011, 2017; Fox et al. 2012; Aili et al. 2014, 2016; Touchard et al. 2016). Its components can vary intraspecifically according to environmental changes (Touchard et al. 2015, Bernardi et al. 2017a), age (Haight and Tschinkel 2003), and social caste in the Hymenoptera (Touchard et al. 2015).

Like other venoms, those from ants are a potential source of novel compounds for pharmacological use, mainly due to their antimicrobial and immunological activities, as reported for the venoms of *Solenopsis invicta* (Buren) (Jouvenaz et al. 1972; Li et al. 2012), *Pachycondyla goeldii* (Forel), currently called *Neoponera goeldii* (Orivel et al. 2001), *Myrmecia pilosula* (Smith) (Zelezetsky et al. 2005; Dekan et al. 2017), *Odontomachus bauri* (Emery) (Silva et al. 2015), *Tetramorium bicarinatum* (Nylander) (Téné et al. 2016), and *Dinoponera quadriceps* (Kempf) (Lima et al. 2016).

Although the studies mentioned above have contributed to knowledge of ant venoms, they were limited to the identification of only a few components, limiting understanding of the modes of action, allergenicity, and pharmacological properties (Touchard et al. 2016). This gap in the data is mainly due to the limited amount of venom that can be extracted from a single individual (Lima and Brochetto-Braga 2003; Pluzhnikov et al. 2014; Aili et al. 2014, 2017), together with the laborious nature of dissection of the venom gland (Fox et al. 2015). The development of sophisticated approaches for isolation of specific components, together with advances in mass spectrometry tools, have improved research concerning the venom of these insects (Aili et al. 2014, 2017).

The purpose of this study was to quantify free amino acids, identify proteins, and evaluate the antimicrobial activity of *E. brunneum* venom.

Materials and methods

Collection area and study material preparation

The ants were collected in a transition zone between the Atlantic Forest and Cerrado biomes, in the municipality of Dourados, in the south of Mato Grosso do Sul State, Brazil. A total of 900 workers of *E. brunneum* were randomly collected from different colonies during foraging activity, in January 2015. All the samples were obtained by active collection of foragers of similar body size (Bernardi et al. 2017a), avoiding age-related variation in the venom chemical profile, since the foraging function is performed by older workers (Hölldobler and Wilson 1990). The ants were obtained in different fields to minimize collection of workers from the same colony.

In the laboratory, the ants were cryo-anesthetized at – 4 ℃ and then dissected under a stereomicroscope (S6D, Leica, Germany), using fine-tipped tweezers. The dissection began with extraction of the stinger, which after being carefully pulled in the opposite direction to the abdomen, exposed the venom reservoir. The reservoir was then isolated and washed with ultrapure water.

The amount of venom extracted from the individuals was quite variable, as could be visually observed by the volume of the venom reservoir, making it difficult to quantify it during the experimental process.

During the dissection, the venom reservoirs were placed in vials, together with 50 µL of cold ultrapure water, causing rupture of the reservoir membrane by osmotic shock. After the extraction step, the samples were centrifuged at 13,700×*g* for 10 min at 4 ℃ (NT 805, Novatecnica, Brazil), to separate the reservoir membrane debris from the venom. The supernatant was then separated, filtered through a 0.45 µm filter (Millipore, USA), and frozen at − 20 °C for subsequent analysis. Specifically for the antimicrobial tests, crude venom was lyophilized (Alpha 1.2/LD-plus, Christ, Germany) for dry weight quantification and preparation of the tested concentrations.

The identification of amino acids and proteomic analysis of the *E. brunneum* venom were performed with 100 ants per replicate in each analysis. The experiments were performed in triplicate. Antimicrobial activity was tested using the venom obtained from 300 individuals. Three aliquots of this sample were employed to perform the test replicates. The chemicals used were of analytical grade and were purchased from Sigma-Aldrich (USA), Bio-Rad (USA), Dynamics (Brazil), Labsynth (Brazil), Merck (Germany), and Oxoid (Brazil). All buffers were prepared using ultrapure water (Master All 2000 ultra-purifier, Gehaka, Brazil).

Analysis of the free amino acids of the venom

Amino acid analysis was performed according to Torres et al. (2018), with some adaptations. Briefly, for derivatization, 200 µL of the lyophilized venom sample, 3 mL of sodium borate buffer (pH 9.0), and 2 µL of diethyl ethoxymethylenemalonate (DEEM) were used. The solution was stirred and incubated at 50 ℃ for 50 min, followed by filtering through a 0.20 µm nylon membrane (Chromafil Xtra, Macherey–Nagel, Germany).

Analyses were performed with a liquid chromatograph (CL-6AD, Shimadzu, Kyoto, Japan) equipped with a diode array detector (DAD) and a C18 ODS Hypersil column (4.6/150, Thermo Electron Corporation, USA). Mobile phase A was a solution of 25 mM acetic acid and 0.02% (v/v) sodium azide in water (pH 6.0), while mobile phase B was 100% (v/v) acetonitrile (ACN). The phases were prefiltered through 0.2 µm nylon membranes and the column was maintained at a constant temperature of 18 ℃. The injection volume was 20 µL. The eluent flow rate was 0.9 mL/min and the gradient applied was as follows: 4% to 12% B in 3 min, 12% B for 10 min, 12% to 31% B in 17 min, and 31% B for 5 min.

Identification was performed by comparison of the sample peaks with amino acid standards (alanine, arginine,

cysteine, isoleucine, methionine, proline, serine, threonine, tryptophan, and valine), considering the retention time and the ultraviolet–visible (UV–Vis) spectrum in the wavelength range 200–800 nm. For quantitative analysis, the external standards method was used for the 10 amino acids evaluated.

Venom proteomics

The total protein content in the venom was determined by the Bradford method (1976), using bovine serum albumin as standard (25.48 µg/µL). The venom samples (100 µg protein) were applied by rehydration for 10 h on 7 cm immobilized pH gradient ribbons (IPG, GE Healthcare, USA), with linear pH 3–10 change, in rehydration solution containing 2% (v/v) IPG buffer (pH 3–10), 40 mM dithiothreitol (DTT), and DeStreak solution (GE Healthcare, USA). The first dimension of the gel was performed using an IPGphor Ettan III system (GE Healthcare, USA), with a voltage program of 300 V for 12 h, 1000 V for 30 min, 5000 V for 2 h, 5000 V for 1 h, and 200 V for 1 h. After the isoelectric concentration, the IPG ribbons were incubated in equilibration solution containing 6 M urea, 75 mM hydroxymethyl aminomethane hydrochloride (Tris–HCl) (pH 8.8), 29% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), and 0.002% (v/v) bromophenol blue. The ribbons were then maintained for a further 15 min under stirring in 1% (w/v) DTT and 2.5% (w/v) iodoacetamide.

The second dimension (2D) was performed with 14% polyacrylamide gel, using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), according to the methodology described by Laemmli (1970) and the instructions for the Mini-Protean Tetra Cell (Bio-Rad, USA), using a Mini Protean II vertical vat (Bio-Rad, USA), under 80 W electrical current for approximately 100 min. Subsequently, the gels were stained for 24 h with Coomassie brilliant blue G-250, as described by Santos et al. (2017), and were stored at 21 °C in preservation solution (5% (v/v) acetic acid). Scanning of the 2D gel was performed with an Image Scanner III (GE Healthcare, USA), in 16-bit transparency mode, with red-blue colors and 600 dpi resolution for documentation. The images were analyzed using Image Master 2D Platinum v.7 software (GE Healthcare, USA).

The spots obtained from the two-dimensional gel were excised and dehydrated in a solution containing 50% (v/v) acetonitrile (ACN) and 20 mM ammonium bicarbonate (AMBIC) (pH 8.0). After dehydration, the gel fragments were sequentially incubated in 65 mM DTT solution, for 30 min at 56 °C, and in 200 mM iodoacetamide solution for 30 min (in the dark) at room temperature, followed by washing in 100 mM AMBIC (pH 8.0) and 100% (v/v) ACN. The gel fragments were treated with 10% (v/v) ACN solution and 40 mM AMBIC containing 25 ng/µL trypsin enzyme (Sequencing Grade Modified Assayed Trypsin, Promega, USA), followed by incubation at 37 °C for 16 h. Extraction of the purified compounds in the gel fragments was achieved by the addition of 5% (v/v) formic acid and 50% (v/v) ACN. The extract obtained was concentrated under vacuum and solubilized in 10 µL of 0.1% (v/v) trifluoroacetic acid (TFA). The samples were desalted using ZipTip tips with C18 resin (Pipette Tips for Sample Preparation, Millipore, USA), according to the manufacturer's instructions, dried under vacuum, and prepared for MALDI-TOF/TOF MS (matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry).

The material obtained from the gel digestion was solubilized in 10 µL of 0.1% (v/v) TFA solution and then mixed into the matrix (2.5 mg/mL α-cyano-4-hydroxycinnamic acid) prepared in 50% (v/v) ACN and 0.1% (v/v) TFA, at a 1:1 ratio (sample: matrix). Aliquots were applied on an MTP AnchorChip 600/384 TF steel plate (Bruker Daltonics, USA) and analyzed by MALDI-TOF/TOF MS, using an Ultraflex III system (Bruker Daltonics, USA). For the first peptide fragmentation (MS), the spectra were acquired in reflection mode (LPPepMix), with a detection range of 500–5000 Da. The method calibration standard used was Peptide Calibration Standard II (Bruker Daltonics, Germany). For the second fragmentation (MS/MS), the spectra were acquired in LIFT mode, in the mass range 40–1878 Da. The spectra were acquired in reflection mode, the ion polarity was positive, the ionization source voltage was 25 kV, the number of laser shots per spectrum was 400, and the standard laser intensity was used.

The MALDI-TOF/TOF mass spectra were processed using FlexAnalysis 3.3 software (Bruker Daltonics, USA) and submitted to analysis using two research software packages: MASCOT (Peptide Mass Fingerprint and MS/MS Ion Search, Matrix Science Ltd., UK) and Peaks DB 7.0 (Bioinformatic Solutions Inc., Canada). The MASCOT software IDs were validated using Scaffold 4.0 software (Proteome Software Inc., USA). The identifications from Peaks 7.0 were also validated, assuming the false discovery rate (FDR) to be equal to 0.0%. The protein and peptide sequences deposited in NCBInr and Swissprot were used, assuming an error of 0.5 Da. The cysteine carbamidomethylation and methionine oxidation reactions were used as fixed and variable modifications, respectively. Metazoa (animals), Insecta, Formicidae, and "proteins from animal venom" (mollusks, snakes, insects, arachnids, and amphibians) were selected as taxa for entry into the databases.

Sequences suggested by the PEAKS Studio 7.0 software (Bioinformatics Solutions Inc., Canada) and those that presented mean local confidence (ALC) of 70% or higher were submitted to the MS Blast research database (http://genetics.bwh.harvard.edu/msblast/). Sequences showing significant alignments with proteins that have been already

described in ant and animal venoms were considered as positive identification.

Venom antimicrobial activity

The antimicrobial activity was tested against bacteria strains acquired from the American Type Culture Collection (ATCC), as follows: *Escherichia coli* (Escherich) (ATCC 38731), *Enterococcus faecalis* (Schleifer & Kilpper-Bälz) (ATCC 29212), *Klebsiella pneumonia* (Trevisan) (ATCC 15305), *Listeria innocua* (Pirie) (ATCC 33090), *Listeria monocytogenes* (Pirie) (ATCC 1011), *Staphylococcus aureus* (Rosenbach) (ATCC 25232), *Staphylococcus epidermidis* (Evans) (ATCC 12228), and *Staphylococcus saprophyticus* (Shaw) (ATCC 15305). The activity was also tested against the fungi strains *Candida albicans* (Berkhout) (ATCC 10231) and *Saccharomyces cerevisiae* (Meyen & Hansen) (isolated by the University Center of Grande Dourados). The tests followed the methodology described by Bernardi et al. (2017b).

Determination of the minimum inhibitory concentration (MIC) was performed using sterile 96-well microplates, to which were added 100 µL of Mueller Hinton broth (for bacteria) or Sabouraud broth (for fungi). For each microorganism tested, 100 µL of the venom was added, with serial dilution in the broth (1000, 500, 250, 125, 62.5, 31.25, 15.62, and 7.81 µg/mL). Finally, a 10 µL aliquot of standardized microorganisms in 0.9% saline was added. The positive controls employed 4 mg/mL of the antibiotic tetracycline (All Chemistry, Brazil) for the bacteria and 4 mg/mL of ketoconazole (All Chemistry, Brazil) for the fungi. As a negative control, ultrapure water was used in the initial dilution of the venom. After preparation and homogenization of the wells, the microplates were incubated for 24 h at 35 °C. The MIC was determined by reading in a microplate spectrophotometer (TP Reader NM, Thermo Plate, USA) at an absorbance wavelength of 580 nm.

Results and discussion

Amino acids

Five free amino acids were identified in the *E. brunneum* venom (Fig. 1, Table 1). Alanine (6.55 ng) was found at the highest concentration, followed by valine (1.26 ng), tryptophan (1.07 ng), isoleucine (0.95 ng), and serine (0.77 ng). The amino acids showed little quantitative variation and no qualitative variation among the samples evaluated, indicating that the occurrence of free amino acids was a common feature of the *E. brunneum* venom.

Similar results were found for the venom of the wasp *Vespa orientalis* (Linnaeus), with four amino acids (alanine, valine, isoleucine, and serine) in common with *E. brunneum* venom, and alanine at the highest concentration (Ikan and Ishay 1973). Alanine is also the main free amino acid in the venom of the honey bee *Apis florae* (Fabricius) (Kumar and Devi 2014). The venom of the ant *Pseudomyrmex triplarinus* (Weddell) contained the amino acids serine, alanine, and tryptophan (Hink et al. 1994), which also occurred in the *E. brunneum* venom. Besides being found in Hymenoptera, free amino acids have also been reported in the venoms of spiders (Margaret and Phanuel 1988) and scorpions (Russel 1968, Ismail et al. 1974). However, the functions of these chemicals found in the venom remain under discussion.

According to Abe et al. (1989), amino acids may act as neurotransmitters that at high concentrations can paralyze the prey. In social wasps, there are neuroactive amino acids with inhibitory neurotransmitter action, including alanine (Curtis and Watkins 1965; Abe et al. 1989), which may explain the presence of these components in the venom studied here. Moreover, amino acids are sources for many catabolites, such as ammonia, carbon dioxide, fatty acids, glucose, hydrogen sulfide, ketone bodies, nitric oxide, urea,

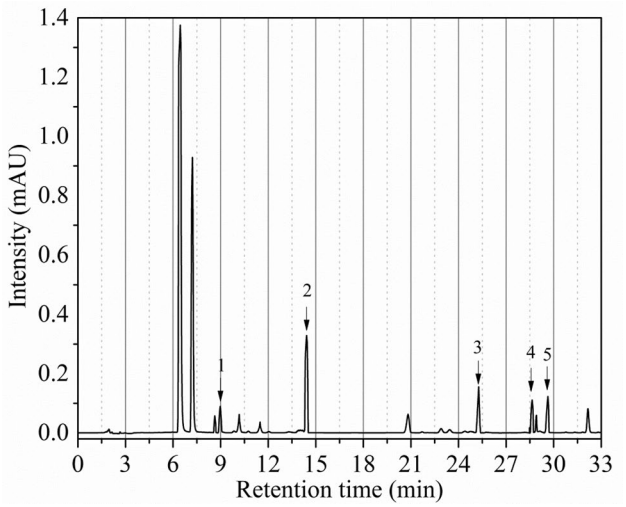

Fig. 1 Representative chromatogram of free amino acids in the *Ectatomma brunneum* venom

Table 1 Average concentration, per reservoir, of free amino acids present in the *E. brunneum* venom

	Time (min)	Amino acid	Average content/ reservoir (ng)
1	8.83 ± 0.33	Serina	0.77 ± 0.06
2	14.69 ± 0.77	Alanine	6.55 ± 0.77
3	25.31 ± 0.38	Valine	1.26 ± 0.12
4	28.71 ± 0.25	Isoleucine	0.95 ± 0.03
5	29.10 ± 0.26	Tryptophan	1.07 ± 0.04

uric acid, polyamines, and other nitrogenous substances of biological importance (Wu 2009). Hence, amino acids may be a source of biogenic amines used for both defense and predation, commonly found in the venom (Weisel-Eichler and Libersat 2004).

Proteins

The 2D electrophoresis fractionation of three biological replicates showed 92% similarity among them, with 104 spots per sample in the three replicates of all samples, with isoelectric points varying from 4.70 to 9.43 and molecular weights ranging from 3 to 299 kDa (Fig. 2). For identification, 91 of the most highly expressed spots were excised and analyzed by MALDI-TOF/TOF MS.

Twenty-one spots were identified, representing ca. 20% of the detected spots (23% of the excised spots) (Table 2, Fig. 2). The identified proteins represented 69.63% of the volume in the 2D gel. It is noteworthy that the remaining spots did not have any similarity to known database sequences, which highlights the specificity of the ant venom, as well as the low number of available sequences. This is the main bottleneck also reported in other studies of hymenopteran venom (Bouzid et al. 2013; Sookrung et al. 2014; Torres et al. 2014; Aili et al. 2016; Santos et al. 2017).

Many of the spots identified here were analogous to proteins of ants (Table 2), but none from *E. brunneus*, for which only 23 proteins and peptides are described (UniProt Database 2017), three of them being venom components (Pluzhnikov et al. 2014). Tsai et al. (2004) suggested that variation in venom composition occurs due to the differential expression of genes in response to environmental stimuli. The *E. brunneum* venom data obtained by Pluzhnikov et al. (2014) were from samples collected in the Peruvian Amazon, a biome with different environmental conditions and 2500 km distant from the present collection sites in the Pantanal biome, preventing gene flow between these populations. Further evidence was provided by Firmino et al. (2020), in work with *E. brunneum*, where differences in compounds composition were correlated with the geographic distance between populations.

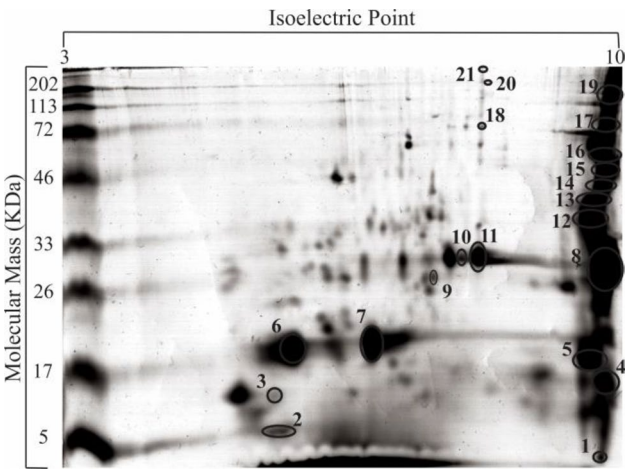

Fig. 2 Representative two-dimensional gel (14%) analysis of the *Ectatomma brunneum* venom, highlighting the proteins identified by MALDI-TOF/TOF MS

The intraspecific chemical profile of *E. brunneum* venom varies among individuals collected in the same region, attributed to slight variations among the collection sites (Bernardi et al. 2017a), where exogenous factors, such as diet, could also contribute to the differences (Mendonça et al. 2019). Investigations of the venoms of the ants *Dinoponera quadriceps* (Kempf) (Cologna et al. 2013), *Odontomachus haematodus* (Linnaeus) (Touchard et al. 2015), and *Paraponera clavata* (Fabricius) (Aili et al. 2017) found quantitative variations in venom components among colonies from the same region. These findings have been hypothesized to be due to genetic polymorphisms or small environmental differences between collection areas (Bernardi et al. 2017a).

Among the 21 identified proteins, 76.19% were significantly homologous with ant, 14.29% with snake, and 9.52% with non-venomous species proteins. However, for the last group, these proteins were detected in different molecular forms in ant species and other venomous species (UniProt Database 2017). Tensin was identified in the ant *Harpegnathos saltator* (Jerdon) (accession code EAI_12409, UniProt Database 2017), while the dual specificity protein phosphatase CDC14A was identified in *Cerapachys biroi* (Forel) (accession code X777_04475, UniProt Database 2017).

The proteins identified in *E. brunneum* could be divided a priori into five groups, according to biological functions: allergenic (spots 5, 8, 10, 12, 13, 14, 15, 17, and 20); enzymes (spots 1, 4, 9, 11, 18, and 21); structural protein (spot 6); DNA and/or RNA acting protein (spots 3 and 7); proteoglycan (spot 2); and unknown function (spots 16 and 19). Considering this classification, the proteins with higher detection rates (those that occurred most frequently in the samples) were allergenic and enzymatic (Fig. 3a). Regarding the relative volumes of the 104 spots detected in the gel, the allergenic proteins had the highest relative volume among those identified, followed by those with unknown function, DNA and/or RNA acting, enzymes, and proteoglycan (Fig. 3b).

The *E. brunneum* venom showed the presence of the venom allergen 3 protein in different molecular forms, as well as the venom allergen 5 protein (Table 2, spots 5, 8, 10, 12, 13, 14, 15, 17, and 20), which together represented 43% of the identified proteins, or 45.90% of the relative concentrations of the proteins detected in the gel. The venom

Table 2 Proteins identified in the *E. brunneum* venom after 2D gel fractionation and MALDI-TOF/TOF MS analysis

Spot	pI	MM (kDa)	Proteína	Access number	Peptide sequence	Confidence of results	Organism
1 + ¢	9.2	3	Ubiquitin-fold modifier- conjugating enzyme 1	J3SDM0_CROAD		62 mascot score	*Crotalus adamanteus* (snake)
2 + ¢	5.2	5	Glypcan-6	V8P0M2_OPHHA		70 mascot score	*Ophiophagus Hannah* (snake)
3 + ¢	5.1	10	U1 Small nuclear ribonucleoprotein A	E1ZWA3_CAMFO		92 mascot score	*Camponotus floridanus* (formiga)
4*#£	9.3	12	Dipeptidyl peptidase 4	E2AF09_CAMFO	VYYLATAPGEPTQR	94,58 (− 10lgP) 2% coverage	*C. floridanus* (ant)
5*§ £	9.0	17	Venom allergen 3-like	XP_011871197	MVQSWYDEVAHFNR	ALC 70%	*Vollenhovia emeryi*(ant)
6* ~	5.4	18	Centromere protein J	V8PI61_OPHHA	VLKNGC (+ 57.02) HLIIFPNG TR	27,15 (− 10lgP) 1% coverage	*O. Hannah* (snake)
7*£	6.4	19	Homeobox protein HB1	A0A026W958_CERBI	QLYNSVDNK	22,70 (− 10lgP) 2% coverage	*Cerapachys biroi* (ant)
8*§£	9.3	24	Venom allergen 3	XP_012222954	SWYDEVAHFNR	ALC 83%	*Linepithema humile*
*§£			Venom allergen 3-like	XP_012222954	RDSSWYDEVAHFNR	ALC 74%	*L. humile* (ant)
9 + ¢	7.1	31	D-glucuronyl C5-epimerase	A0A026WTR0_CERBI		84 mascot score	*C. biroi* (ant)
10*§£	7.3	33	Venom allergen 3-like	XP_011704838	KWSDQGM (+ 15.99) NDAER	ALC 75%	*Wasmannia auropunctata* (ant)
11*#	7.7	34	Dual specificity protein phosphatase CDC14A	A0A091GAC7_9AVES	ASKVMAVNSSSAER	21,63 (− 10lgP) 3% coverage	*Cuculus canorus* (bird)
12*§£	9.1	36	Venom allergen 3-like, partial	XP_011705280	SWYDEVAHFNR	ALC 76%	*W. auropunctata*
*§£			Venom allergen 3-like, partial	XP_011704844	HLLVC (+ 57.02) NYGPAGNF LGER	ALC 73%	*W. auropunctata* (ant)
13*§£	9.1	39	Venom allergen 3-like	XP_014469499	SYLVC (+ 57.02)NYGPAGN M(+ 15.99)LGER	ALC 77%	*Dinoponera quadriceps* (ant)
14*§£	9.2	42	Venom allergen 3-like	XP_011645540	HLLVC (+ 57.02) NYGPAGNF LGER	ALC 70%	*Pogonomyrmex barbatus* (ant)
15*§£	9,2	45	Venom allergen 3-like	XP_011871198	SWYDEVAHM (+ 15.99)NR	ALC 74%	*Vollenhovia emeryi* (ant)
16*£	9.2	54	UPF0550 protein C7orf28-like protein	E2A9V8_CAMFO	FNKLNLAYK	27,73 (− 10lgP) 3% coverage	*C. floridanus* (ant)
17*§# £	9.3	72	Venom allergen 3	VA3_SOLIN	SYLVC (+ 57.02) NYGPAGNF LGER	ALC 71%	*Solenopsis invicta* (ant)
18*£	7.7	82	Dual specificity tyrosine-phosphorylation-regulated kinase	A0A026VWH5_CERBI	WGTAGGLNSGHQSLNSVS GGGGGGGGR	37.70 (− 10lgP) 5% coverage	*C. biroi* (ant)
19*#	9.3	117	Tensin	G7Y462_CLOSI	NGYPKNFLWR	47.93 (− 10lgP) 9% coverage	*Clonorchis sinensis* (Platyhelminthes)
20*§£	7.8	162	Venom allergen 5	VA5_PACCH	NYGPAGNM (+ 15.99)LGER	ALC 78%	*Pachycondyla chinensis* (ant)
21*£	7.7	299	Galactose-1-phosphate uridylyltransferase	E9J1S0_SOLIN	MQDLSDSQQESLAVIM	21.90 (− 10lgP) 3% coverage	*S. invicta* (ant)

+ Identified by Mascot Software

¢ Validated by Scaffold 4.0

*Identified by Peak DB 7.0 software

§Identified by sequence de novo and submitted to MS BLAST research bank

Taxon for database entry: #Metazoa, $ Insecta, £ Formicidae, ~ Animal venom

allergen 3 protein, also known as Sol i 3, described for the fire ant *S. invicta*, belongs to the family of cysteine-rich secretory proteins and is the principal allergen family in terms of number of components, as well as the most frequent cause of post-stinging hypersensitivity reactions for this species (Padavattan et al. 2008), triggering cytokines with

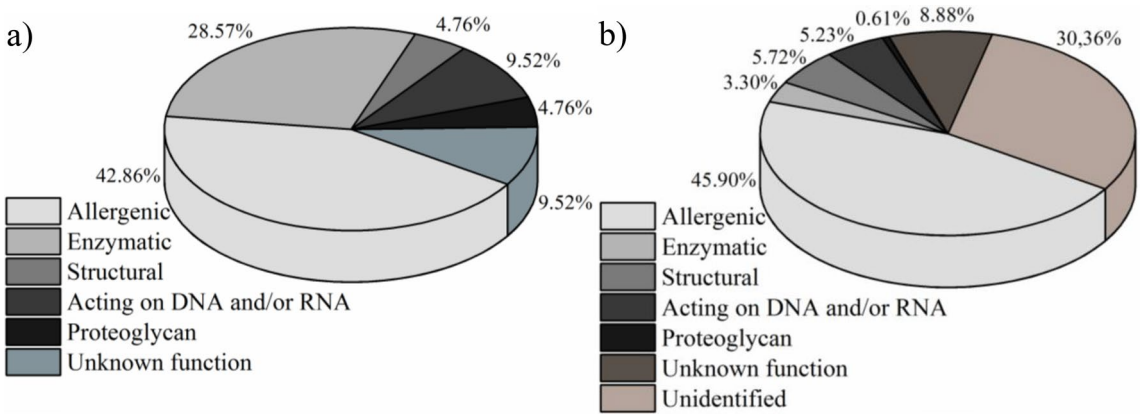

Fig. 3 **a** Classification of the *Ectatomma brunneum* venom proteins related to the functions assigned by the UniProt database (absolute numbers). **b** Relative volumes of identified protein classes and unidentified general proteins in the venom

potential effects on the host immune response (Anderson et al. 2006).

The enzymes were the second most representative class in terms of the number of identified proteins, with some representatives associated with cytolytic activity (Nicholson 2006). In ants, this function in venom may be important for pre-digestion, since adult ants have a predilection for liquid foods (Hölldobler and Wilson 1990; Davidson et al. 2004). The cytolytic action of enzymes present in the venom helps in degradation of the cell membranes of prey, liquefying them, as occurs in the case of spider venom (Nicholson 2006). Hence, the presence of galactose-1-phosphate uridylyl transferase (GALT) (Table 2, spot 21), reported for the first time in animal venom, may reflect a pre-digestive activity. This enzyme acts in the metabolic processing of galactose (UniProt Database 2017), a sugar that is widely distributed in plants, animals, and microorganisms, as a constituent of oligo- and polysaccharides (Ramachandran and Elumalai 2012), which might explain its presence in the venom.

Among the enzymes identified in this study, the multifunctional dipeptidyl peptidase 4 (DPP-4) (Table 2, spot 4) is a serine protease commonly found in the venoms of snakes, scorpions, spiders, wasps, and bees (Danneels et al. 2010). It is responsible for moderate tissue necrosis, as well as for improving the diffusion of venom through the prey tissues. Serine proteases are proteins responsible for inflammatory processes caused by venom, with other functions including digestion activity, stimulation of immunity, and antimicrobial activity (Miyoshi et al. 2004; Zychar et al. 2010; Danneels et al. 2010; Matkawala et al. 2021). However, little information is available concerning insect venom proteases, especially those from ants (Lima and Brochetto-Braga 2003).

The ubiquitin-fold modifier-conjugating enzyme 1 (UFC1) (Table 2, spot 1), also known as E1, is responsible for the immune response (Hershko and Ciechanover 1998; Yoo et al. 2015) and protein degradation in eukaryotes (Hershko and Ciechanover 1998).

The dual specificity tyrosine phosphorylation-regulated kinase and the dual specificity phosphatase CDC14A (Table 2, spots 18 and 11) are proteins belonging to the two largest protein families encoded in the eukaryotic genome (Zhang 2001; Ceulemans et al. 2002), namely the kinase family and phosphatase proteins, respectively. These proteins are responsible for the insertion and removal, respectively, of phosphate groups in proteins, receptors, transporters, and ion channels. They are also associated with programmed cell death, allergy, and innate immunity (Zhang 2001; Ceulemans et al. 2002). Protein kinases have also been identified in the venoms of the wasp *Polybia paulista* (von Ihering) (Santos et al. 2010) and the honey bee *Apis mellifera carnica* (Pollman) (Peiren et al. 2008), playing a putative role in protein phosphorylation, but it was not determined whether the targets are venom toxins, prey proteins, or both. Additionally, some protein kinases may act in inflammatory processes (Myers et al. 1997). Wanandy et al. (2018) reported that forms of arginine kinase in *Myrmecia pilosula* ant venom have the potential to act as allergens, so it is possible that this may be one of the actions of the kinase in *E. brunneum* venom.

The function of tensin protein, another component of the *E. brunneum* venom (Table 2, spot 19), is poorly understood, but there is evidence of its indirect action in protein degradation, since it has been reported to be a substrate for proteases acting in the interaction between the extracellular matrix and cytoskeleton, and in signal translation (Lo 2004). In addition, it has effective antifungal activity (Nielsen et al. 2002).

Centromere Protein J (Table 2, spot 6) is structural (UniProt Database 2017), but it is also co-activator of the nuclear factor-κB protein complex, important for

inflammation, immune response, cell proliferation, and apoptosis (Koyanagi et al. 2005).

Among the proteins identified in this study, two are members of the cellular membrane proteins, with possible functions in tissue protection. Glypican-6 proteoglycan (Table 2, spot 2) and the D-glucuronyl C5-epimerase enzyme (Table 2, spot 9) act in the selective interaction and biosynthesis, respectively, of glycosaminoglycans (UniProt Database 2017), such as those belonging to the heparan sulfate family, which are potent blockers of the cytolytic action of venom (Lomonte et al. 1994). Therefore, they may function as proteins associated with the protection of the structures in contact with the venom.

It should be noted that despite the care taken during the dissection process, part of the reservoir, the convolute gland, muscles, and other structures associated with the sting might have released their contents into the venom. In the wasp *P. paulista*, calponin protein was identified in the venom and was assigned a function in the muscular structure of the stinger apparatus (Santos et al. 2010). More recently, Aili et al. (2017) discussed the venom extraction technique and the presence of proteins from other associated structures, in a study of the venom of the species *P. clavata*. Transcriptomics has revealed that most of the identified transcripts from the venom reservoir are cellular organization proteins (Bouzid et al. 2013; Torres et al. 2014). This might explain the presence in the *E. brunneum* venom of the U1 small nuclear ribonucleoprotein (Table 2, spot 3) and homeobox protein HB1 (Table 2, spot 7), which act in the regulation of DNA transcription (UniProt Database 2017). Therefore, together with the glypican-6 and D-glucuronyl C5-epimerase proteins, they may not have a direct function in the *E. brunneum* venom.

Finally, it was possible to classify the identified proteins of *E. brunneum* venom according to biological functions indicated as venom proteins (spots 1, 4, 5, 6, 8, 10, 11, 12, 13, 14, 15, 17, 18, 19, 20, and 21), nontoxic reservoir protection and cell maintenance proteins (spots 2, 3, 7, and 9), and unknown function protein (spot 16). Following the classification model suggested by Touchard et al. (2016), with modifications, the venom proteins found here could be divided into allergenic proteins, pre-digestion proteins, proteins for venom diffusion, proteins that cause inflammation, and antimicrobial proteins (Fig. 4). There was an impressive number of allergenic proteins in the *E. brunneum* venom (spots 5, 8, 10, 11, 12, 13, 14, 15, 17, 18, and 20), totaling 52% of the identified proteins, which has been claimed to be a conserved proportion in the venom (Bouzid et al. 2013).

Venom antimicrobial activity

The presence in ant venom of proteins and peptides with innate immune activity is a common and essential characteristic in the evolutionary adaptation of these animals (Hancock and Scott 2000; Andersson et al. 2016; Mylonakis et al. 2016). The antimicrobial function of the venom of predatory ants may be due to the need to minimize the potential for infection by bacteria, fungi, and viruses. This is because the prey is transported to the nest soon after immobilization, increasing the infection risk for the colony (Orivel et al. 2001; Baracchi and Tragust 2017; Pereira and Detrain 2020).

The *E. brunneum* venom was tested against gram-positive and gram-negative bacteria, as well as fungi (Table 3), revealing broad-spectrum antimicrobial action, with MIC values ranging from 62.5 to 250 µg/mL and the highest activity against gram-positive bacteria (Table 3). Similar findings were reported for the venom of the ant *M. pilosula*, affecting *E. coli*, *K. pneumoniae*, *S. aureus*, *S. epidermidis*,

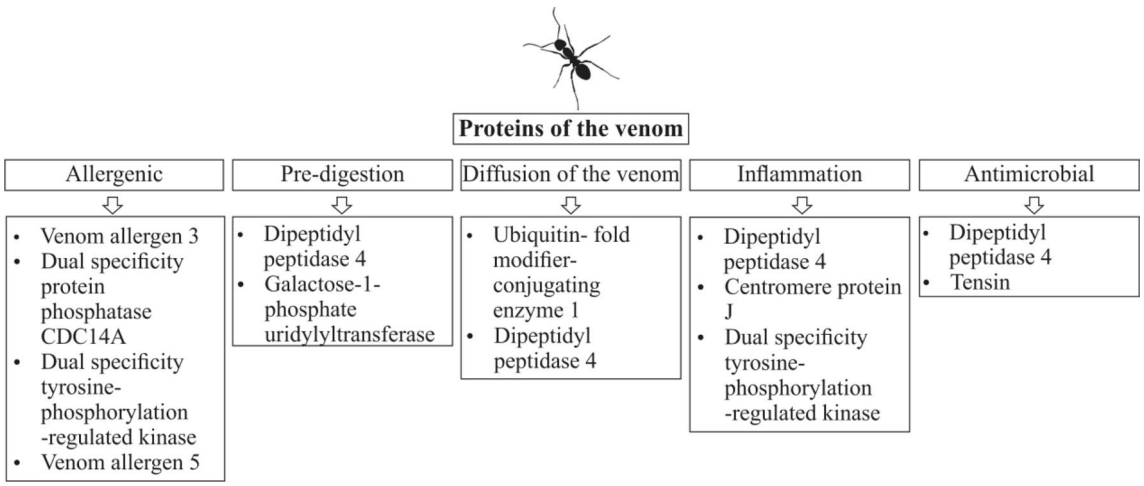

Fig. 4 Functions attributed to the *Ectatomma brunneum* venom proteins

Table 3 Minimum inhibitory concentrations (MICs) for the *E. brunneum* venom

Microorganism	MIC (μg/mL)
Enterococcus faecalis^{+}	250.0
Listeria innocua^{+}	62.5
Listeria monocytogenmes^{+}	62.5
Staphylococcus aureus^{+}	62.5
Staphylococcus epidermidis^{+}	125.0
Staphylococcus saprophyticus^{+}	125.0
Escherichia coli^{-}	62.5
Klebsiella pneumonia $^{-}$	250.0
Candida albicans^{f}	250.0
Saccharomyces cerevisiae^{f}	250.0

+Gram-positive bacteria, – Gram-negative bacteria, *f* Fungus

and *C. albicans* (Zelezetsky et al. 2005), which were also inhibited by the venom tested here.

Ponericin toxins isolated from the venom of the ant *P. goeldii* have shown activity against gram-positive and gram-negative bacteria, as well as fungi (Orivel et al. 2001). Bicarinin, a peptide isolated from the venom of the ant *T. bicarinatum*, was found to be active against 15 microorganisms (Téné et al. 2016), four of which (*E. coli*, *S. aureus*, *C. albicans*, and *S. cerevisiae*) were used in the present study.

Pluzhnikov et al. (2014) reported that the crude venom of *E. brunneum* inhibited the bacteria *Arthrobacter globiformis* (Conn & Dimmick) VKM Ac-1112 and *E. coli* MH1, at concentrations of 7.5 and 30 μg/mL, respectively. It is important to highlight that MIC values can vary according to the different bacteria or strains studied. Nonetheless, the difference between these values and the MIC values obtained in the present study could also be related to genetic characteristics or different collection periods.

In eusocial insects, the genetic homogeneity of the individuals creates ideal circumstances for the dissemination of infectious diseases in the nests. The venom constitutes part of the physiological adaptations acquired to prevent the establishment and dissemination of parasites and pathogens, which, together with organizational and behavioral adaptations, provides social immunity (Cremer et al. 2007; Konrad et al. 2018). Baracchi and Tragust (2017) proposed that natural selection could favor any immunological factor that improves fitness in a specific context. This perspective considers environmental factors as selective forces that can result in intraspecific and interspecific differences in venom composition. In the Hymenoptera, it was essential that the evolution of sociality should be accompanied by the development of antimicrobial compounds (Stow et al. 2007; Hoggard et al. 2011). Therefore, the broad spectrum of antimicrobial activity of the venom is an important evolutionary aspect in these eusocial animals, contributing to the immunity and survival of the colony (Turillazzi et al. 2006).

The present findings contribute to understanding the components of *E. brunneum* venom, which is the fundamental step for elucidation of the activity mechanisms, allergenicity, and antimicrobial activity of ant venoms.

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Declarations

Conflict of interest All authors report no conflicts of interest.

Ethical approval All experimental procedures performed with animals followed the ARRIVE guidelines and were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986, and associated guidelines, EU Directive 2010/63 / EU for animal experiments, and the ethical guidelines of the State University of Mato Grosso do Sul (Brazil).

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