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Research Article

Assessing the Proteomic Activity of the Venom of the Ant *Ectatomma tuberculatum* (Hymenoptera: Formicidae: Ectatomminae)

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Ectatomma tuberculatum has one of the most toxic venoms known among ants but there is no detailed study on its characteristics. In light of this, knowing the venom's chemical composition is of paramount importance in order to obtain information about the mechanisms of its components. Several bioactive molecules have already been identified in Hymenoptera venoms, i.e., proteins such as phospholipases, hyaluronidases, and proteinases, as well as peptides. Protein databases show that information on protein components of ant venoms has been recently growing exponentially. In this study, we have identified proteins from the venom of *Ectatomma tuberculatum* by means of 2D PAGE, followed by tandem nanochromatography with mass spectrometry. A total of 48 proteins were identified, of which 42 are involved in metabolic processes, transport, and structural support. Moreover, six of them show similarity with not yet characterized proteins. Nine proteins are related to the attack/defense or maintenance process of the colony (colony asepsis, conservation of venom constituents, venom diffusion on prey, paralysis of prey, alteration of homeostasis, and cellular toxicity). Our findings may contribute to the identification of new natural prototypes of molecules to be synthesized and used in several areas of pharmacology.

1. Introduction

The biotechnological potential of venoms to produce drugs with different applications such as antimicrobials, analgesics, antihypertensives, and insecticides has motivated studies of this kind of products [1]. The use of substances obtained from natural products such as animal venoms, including those from insects, is a practice adopted in folk medicine by numerous native populations since ancient times. They would find the raw materials for these therapeutic resources in nature [2, 3] for the treatment of the most varied diseases [4]. This is due to the immunological, analgesic, antimicrobial, diuretic, anesthetic, and antirheumatic properties of these products [3], which also include several substances with potential in the treatment of cancer, microbial, and viral infections, as well as of possible biocide properties such as those of insecticides [5, 6].

Therefore, the search for substances with biological activity obtained from natural products has been an increasingly valuable alternative to find and produce bioactive compounds with practical application [1, 9, 10]. This has motivated numerous recent scientific studies supported by the pharmaceutical industry to search for new bioactive molecules [6, 11].

A series of bioactive molecules that are important for several fields such as allergy, immunology, microbiology, biotechnology have already been identified in the venoms of

Hymenoptera. Proteins like phospholipases, hyaluronidases, and proteinases have been observed among the identified components, and peptides with diverse activities have already been found in ants, wasps, and bees [1, 12].

Thanks to the richness and diversity of bioactive compounds in their venom, Hymenoptera have been gaining notoriety over the last decades, through research studies that use diverse techniques of characterization and biochemical analysis [1]. Many substances of pharmacological interest have been discovered, along with information regarding their structures, biological activities, and action mechanisms. These substances, which vary in diversity and complexity, include pheromones, hormones, defense substances, enzymes, and peptides [1, 12, 13].

Nevertheless, these insects can cause physiological damage and serious pathologies such as allergies, intense pain, intense sweating, nausea, tachycardia, among other manifestations [14, 15], which in some cases may lead to death of the affected individual [16]. Although the amount of ant venoms produced is small, when compared to that produced by bees and wasps, there is a much larger structural diversity of its components [14, 17]. In light of this, an in depth study of the venoms of Hymenoptera is fundamental as their complex composition and diversity of functions suggest an enormous biotechnological potential for the synthesis of new macromolecules that can be used for therapeutic purposes or even pest control [18].

Among the studies on ants, [19] pointed out the presence of protein allergens with posttranslational modifications in the venom of the Australian ant *Myrmecia pilosula*. Touchard and collaborators [20, 21] have revealed the profiles of venom proteins and peptides of different species of ants through mass spectrometry and high performance liquid chromatography (HPLC), thus reinforcing the thesis that this group of Hymenoptera is promising in the search for molecules to produce new drugs and bioinsecticides. According to Schmidt, Blum, and Overal [22], *E. tuberculatum* has one of the most toxic venoms among ants. Pluzhnikov and collaborators and Arseniev [23, 24] have confirmed the high toxicity of its raw venom and have studied the action of the isolated peptide ectatomin. In order to identify the chemical composition of the venom of *E. tuberculatum* in greater detail, it is moreover extremely important to obtain information on the characteristics of its components and their mechanism of action.

In the present study, proteins of the *Ectatomma tuberculatum* venom have been identified by means of 2D PAGE, followed by tandem nanochromatography with mass spectrometry. A total of 48 proteins have been identified and are discussed for their mechanisms of action. This may help to point out some of these components as a natural prototype of bioactive molecule worthy of synthesizing for pharmaceutical application.

2. Material and Methods

2.1. Protein Extraction from the Ant Glands. Workers of the Ectatomminae ant *E. tuberculatum* have been collected in the experimental fields of the Cacao Research Center (CEPLAC) at Ilhéus, Bahia, Brazil. Additional information on the biology of this species in cacao plantations can be found in Delabie and Hora and collaborators [26, 27]. In the laboratory, 100 workers were anesthetized with CO_2 and dissected for extraction of venom glands.

The reservoirs of these venom containing glands were removed from the sting apparatus by pulling them out of bodies with forceps and microscissors. The venom reservoirs were then carefully washed and suspended in small volumes of a phosphate buffered saline solution with pH 7.8, punctured, and centrifuged at 10.000× g for 10 min at 4°C. The supernatants were collected and maintained at -20°C until use. Venom proteins were extracted from the supernatants according to Rocha and collaborators [28] and the 2D Quant Kit (GE Healthcare) was used to quantify the proteins. Bovine serum albumin (BSA) was used as standard.

2.2. Two-Dimensional Electrophoresis (2D PAGE). For each sample, 350 μg of venom proteins homogenized in rehydration buffer (7 mol·L^{-1} urea, 2 mol·L^{-1} thiourea, 1% CHAPS, and traces of bromophenol blue) have been used with DTT 40 mmol.L^{-1} and 0.5% ampholytes at pH 3-10NL with a final volume of 250 μL.

The samples were subjected to IEF in 13 cm gel strips, pH gradient pH 3-10NL (Amersham Biosciences, Immobiline™ Dry-Strip). The IEF in the Ettan IPGphor 3 (GE Healthcare) system was carried out according to the manufacturer's manual. The 2D Electrophoresis was performed on 12.5% gel in a SE600 Ruby Vertical Electrophoresis System (Hoefer). The running conditions were similar to those used by Mares and collaborators [29], staining in 0.08% colloidal Coomassie [30] for 5 days, followed by destaining in distilled water for 5 days with daily water replacements to remove excess of colloidal Coomassie.

The gels were scanned on ImageScanner II (GE Healthcare) for imaging. Afterwards, it was analyzed using Image Master 2D Platinum 7.0 (GE Healthcare) software to evaluate the number of spots, isoelectric point (pI), molecular mass (MM), and relative abundance of spots on the gels.

2.3. Spot Preparation and MS/MS Analysis. All spots on the gels were excised with the aid of a scalpel, inserted separately into microtubes, and processed according to Silva and collaborators [31].

After peptide digestion and extraction, 4 μL of the sample corresponding to each spot was applied to the nanoAcquity UPLC (WATERS®) and subjected to a C18 "trapping" column of 5 μm, 180 μm x 20 mm, followed by the analytical column of 1.7 μm, 100 μm x 100 mm, under a flow of 0.6 μL/min in a 50 minute run. The peptides were separated according to a gradient of 1% acetonitrile for 1 minute, from 1% to 50% for 40 minutes, from 50% to 85% for 5 minutes, remaining at that concentration for another 2 minutes, then returning to a concentration of 1% in one minute, and remaining in this condition for 2 minutes, totaling 5 minutes of run.

Separated peptides were transferred to the mass spectrometer (Micromass Q-TOF micro), and ionized in a capillary under 3000 V voltage. Next, they were fragmented in

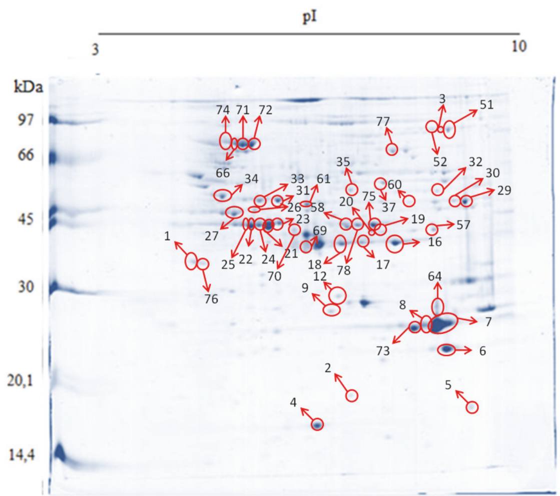

Figure 1: Spots corresponding to the proteins identified in the bidimensional gel of *Ectatomma tuberculatum* venom. Isoelectric point (pI) of 3 to 10, molecular weight (MW) of 14.4 to 97.0 kDa. The numbered spots correspond to proteins identified by mass spectrometry.

positive mode with selection of the minimum intensity of 10 counts, and the 3 of the most intense ions were analyzed by each scan of 1 second, with collision energy ranging from 20 to 95 eV according to the mass/charge (m/z) ratio of the peptides.

The obtained spectra were analyzed by ProteinLynx Global Server 4.2 (WATERS), as well as by MASCOT (Matrix Science), and compared with the SWISSPROT and NCBI databases, respectively. In trypsin hydrolysis, the possible loss of a cleavage site was considered. The tolerance of the peptide masses was ± 0.3 Da, and the mass tolerance of the fragments was ± 0.1 Da.

The peptides identified through the NCBI database had their complete sequence located on the same bank and analyzed with NCBI Blast2GO using the BlastP algorithm for protein identification.

Since the *E. tuberculatum* ant genome has not yet been sequenced, the identification of the proteins was based on the complete genome sequences of organisms phylogenetically close to the species.

3. Results

The 350 μg mass of *E. tuberculatum* venom protein resolved on 2D PAGE resulted in a well-resolved, low-drag spot profile in the range of 14 to 97 kDa and pH of 4 to 9. A higher density of spots appears for the pH range of 5 to 8.5 and molecular weight of 25 to 93 kDa (Figure 1).

Gels analysis in the ImageMaster 2D Platinum 7.0 software showed the number of spots, the pI and molecular mass values, and the spots' expression levels in each replicate. A total of 244 spots were detected in the gels.

From a total of 129 spots treated with trypsin and analyzed by mass spectrometry, 48 proteins have been identified in the venom of *E. tuberculatum*. Among these, six showed similarity with not yet characterized proteins.

Table 1 shows the proteins identified with their respective access codes in the NCBI, molecular mass (MM), isoelectric point (pI), score, and peptide sequence for each spot. *E. tuberculatum* proteins showed sequences similar to the proteins of other Hymenoptera, including wasps, bees, and ants.

4. Discussion

According to the Blast2Go software, 42 proteins identified in *E. tuberculatum* venom can be grouped basically into proteins involved in insect metabolism (annexin B9, nucleoside diphosphate kinase; aconitate hydratase; superoxide dismutase; triose phosphate isomerase; glyceraldehyde-3-phosphate dehydrogenase; succinyl-CoA ligase; fructose-bisphosphate aldolase; arginine kinase; citrate synthase; enolase; ATP synthase; 4-hydroxybutyrate coenzyme A transferase; medium-chain specific acyl-CoA dehydrogenase, and isocitrate dehydrogenase); transport protein (transferrin); and structural proteins (cyclophilin, actin, and chitinase).

Three proteins found in the venom of *E. tuberculatum* are part of a group of compounds with known allergenic effects: triose phosphate isomerase, arginine kinase, and cyclophilin-like protein. Through various techniques of molecular biology, including proteomics studies, allergenic proteins present in Hymenoptera venoms have been identified, as well as their respective action mechanisms.

The triose phosphate isomerase protein, identified in spot 9, relates to the metabolism of carbohydrates, acting as an important enzyme of the glycolytic pathway. It has been studied and classified by [32] as allergenic because it appeared in the serum of patients presenting allergy to bites of the midge *Forcipomyia taiwana* (Ceratopogonidae). Also Hoppe, Steinhart, and Paschke [33] found allergenic enzymes in the protein extract of lychee fruit (*Litchi chinensis*, Sapindaceae) with high homology with triose phosphate isomerase.

Arginine kinase, expressed in spots 21, 22, 23, 24, and 25 as isoforms of the same protein, is an enzyme that catalyzes the reversible transfer of the phosphate group from ATP to supply the need for energy-requiring cells. It was found in the venom of solitary wasps Eumenidae *Eumenes pomiformis* and *Orancistrocerus drewseni* [34], in the venom of social wasps *Polybia paulista* (Vespidae), with evidence of its allergenic action in humans [35] as well as in various other invertebrates such as *Dermatophagoides pteronyssinus* (Acari: Pyroglyphidae), *Blatella germanica* (Insecta: Blattodea: Blattellidae), *Penaeus monodon* (Crustacea: Penaeidae), *Homarus gammarus* (Crustacea: Nephropidae), *Mytilus edulis* (Mollusca: Bivalvia: Mytilidae), and *Plodia interpunctella* (Insecta: Lepidoptera: Pyralidae), a new class of allergens [36]. Yamamoto and collaborators [37] have isolated arginine kinase from the venom of solitary wasp Pompilidae *Cyphononyx dorsalis* and confirmed its paralytic activity in spiders, their natural prey. According to Höfling and Rocha [18], Hymenoptera use their venoms to paralyze or kill their prey, as well as to defend themselves when they feel threatened by vertebrates, including humans. According to [38], *E. tuberculatum* is an active predator of cacao pests, feeding on Chrysomelidae

Table 1: Proteins identified in the *Ectatomma tuberculatum* venom with their respective access codes in NCBI, molecular mass (MM), isoelectric point (pI), score, and peptide sequence of each spot were analyzed in the Mascot program (*Matrix Science*).

Spot	Protein/Source	Accession code NCBI	MM/pI	Score	Peptide sequences
01	Annexin B9/ *Harpegnathos saltator* (Formicidae)	gi|307194598	35836/4.93	68	GIGTTDSTLIR
76	Annexin B9/ *Harpegnathos saltator* (Formicidae)	gi|307194598	35836/4.93	106	GIGTTDSTLIR
02	Nucleoside diphosphate kinase/ *Camponotus floridanus* (Formicidae)	gi|307173082	19547/8.41	68	TFIMVKPDGVQR, VMLGETNPKDSAPGTIR
03	Aconitate hydratase, mitochondrial/ *Acromyrmex echinatior* (Formicidae)	gi|332018721	92836/8.65	101	SEIAGAADQHK, SAFNVTPGSEQIR, NQLTNEWGAVPDVAR
51	Aconitate hydratase, mitochondrial/ *Acromyrmex echinatior* (Formicidae)	gi|332018721	92836/8.65	371	LDFNPVKDR, NQLTNEWGAVPDVAR, VAVPSTIHCDHLIEAQIGGNEDLQR
52	Aconitate hydratase, mitochondrial/ *Acromyrmex echinatior* (Formicidae)	gi|332018721	92836/8.65	406	LDFNPVKDR, SEIAGAADQH, NQLTNEWGAVPDVAR
04	Superoxide dismutase [Cu-Zn]/ *Harpegnathos saltator* (Formicidae)	gi|307204104	14008/6.18	218	LACGVIGITK, TLVVHADPDDLGQGGHELSK
05	Cyclophilin-like protein/ *Nylanderia* nr. *pubens* LZ-2010 (Formicidae)	gi|292397870	18008/8.89	162	VFFDMTADDKPVGR, VIPNFMCQGGDFTNHNGTGGK
09	Triosephosphate isomerase/ *Acromyrmex echinatior* (Formicidae)	gi|332024520	26935/7.71	129	IIYGGSVTAANAK, VIACIGEKLEER
12	Phosphoglycerate mutase 2-like/*Bombus terrestris* (Apidae)	gi|340726229	35071/7.17	90	FLGDEETVK, IIIAAHGNSLR, YGEEQVQIWR, YADGPKPEEFPK
16	Glyceraldehyde-3-phosphate dehydrogenase/ *Camponotus floridanus* (Formicidae)	gi|307181618	35815/7.71	133	VKEASEGPLK, VPVHNVSVVDLTVR, VVSNASCTTNCLAPLAK
17	Glyceraldehyde-3-phosphate dehydrogenase/ *Camponotus floridanus* (Formicidae)	gi|307181618	35815/7.71	194	VKEASEGPLK, IAVFSEREPK, LAKPASYDAIK, VPVHNVSVVDLTVR
18	Glyceraldehyde-3-phosphate dehydrogenase/ *Camponotus floridanus* (Formicidae)	gi|307181618	35815/7.71	174	VKEASEGPLK, IAVFSEREPK, LAKPASYDAIK, VPVHNVSVVDLTVR
69	Glyceraldehyde-3-phosphate dehydrogenase 2/ *Acromyrmex echinatior* (Formicidae)	gi|332026368	37576/8.15	81	IAVFSEREPK
19	Succinyl-CoA ligase [GDP-forming] subunit alpha, mitochondrial/ *Camponotus floridanus* (Formicidae)	gi|307170861	35682/8.93	108	LLEQNKSR, SPAQMGNELLK, GGAQDKINALEK, KAGTEHLGKPVFK, QGTFHCQQAIDYGTK, LIGPNCPGIIAPEQCK
26	Succinyl-CoA ligase [ADP-forming] subunit beta, mitochondrial / *Camponotus floridanus* (Formicidae)	gi|307178345	48972/6.63	125	FDDNAEFR, MCETPEEAK, ICNAVMVTQR, ICNAVMVTQR, IVPIDDLDEAAR, LHGGEPANFLDVGGGASASAVK

Table 1: Continued.

Spot	Protein/Source	Accession code NCBI	MM/pI	Score	Peptide sequences
57	Succinyl-CoA ligase [GDP-forming] subunit alpha, mitochondrial/ *Camponotus floridanus* (Formicidae)	gi|307170861	35682/8.93	158	LIGPNCPGIIAPEQCK
20	Fructose-bisphosphate aldolase-like/ *Apis mellifera* (Apidae)	gi|110748949	39975/7.57	85	LAILENANVLAR
58	Fructose-bisphosphate aldolase-like / *Apis mellifera* (Apidae)	gi|110748949	39975/7.57	85	LAILENANVLAR
75	Fructose-bisphosphate aldolase/ *Harpegnathos saltator* (Formicidae)	gi|307206615	40185/8.31	264	GILAADESTATIGK, GILAADESTATIGKR
78	Fructose-bisphosphate aldolase/ *Harpegnathos saltator* (Formicidae)	gi|307206615	40185/8.31	338	GILAADESTATIGK, GILAADESTATIGKR
21	Arginine kinase / *Acromyrmex echinatior* (Formicidae)	gi|332018357	40032/5.86	370	FLQAANACR, LVTAVNEIEK, LGLTEYQAVK, GTFYPLTGMSK, EMNDGIAELIK, VSSTLSGLTGELK, EGDRFLQAANACR, LIDDHFLFKEGDR, IISMQMGGDLGQVYR, GTRGEHTEAEGGIYDISNK, GTRGEHTEAEGGIYDISNK
22	Arginine kinase / *Acromyrmex echinatior* (Formicidae)	gi|332018357	40032/5.86	238	LIDDHFLFK, EMNDGIAELIK, LVTAVNEIEKR, VSSTLSGLTGELK, IISMQMGGDLGQVYR, GTRGEHTEAEGGIYDISNK, LGLTEYQAVKEMNDGIAELIK, GIFHNDDKTFLVWCNEEDHLR
23	Arginine kinase / *Harpegnathos saltator* (Formicidae)	gi|307197996	39996/5.75	447	LGLTEYQAVK, LIDDHFLFK, IISMQMGGDLGQVYR, SLDGYPFNPCLTEAQYK, GIFHNDDKTFLVWCNEEDHLR
24	Arginine kinase / *Acromyrmex echinatior* (Formicidae)	gi|332018357	40032/5.86	333	LVTAVNEIEK, LGLTEYQAVK, LIDDHFLFK, EMNDGIAELIK, LVTAVNEIEKR, IISMQMGGDLGQVYR, IISMQMGGDLGQVYR
25	Arginine kinase / *Acromyrmex echinatior* (Formicidae)	gi|332018357	40032/5.86	333	LVTAVNEIEK, LGLTEYQAVK, LVTAVNEIEKR, LIDDHFLFKEGDR, IISMQMGGDLGQVYR, GTRGEHTEAEGGIYDISNK, GTRGEHTEAEGGIYDISNK
27	Actin-5, muscle-specific/ *Harpegnathos saltator* (Formicidae)	gi|307197034	42098/5.30	114	GYSFTTTAER, HQGVMVGMGQK, DSYVGDEAQSKR, QEYDESGPGIVHR, VAPEEHPVLLTEAPLNPK, DLYANNVLSGGTTMYPGIADR
29	Probable citrate synthase 1, mitochondrial/ *Harpegnathos saltator* (Formicidae)	gi|307202019	49316/8.94	85	AISQEWAS, SGQVVPGYGHAVLR, VGEVTVDMMYGGMR
30	Probable citrate synthase 2, mitochondrial/ *Bombus impatiens* (Apidae)	gi|350425352	51596/9.03	72	VVPPILLETGK, VGEVTVDMMYGGMR

Table 1: Continued.

Spot	Protein/Source	Accession code NCBI	MM/pI	Score	Peptide sequences
34	ATP synthase subunit beta mitochondrial/ *Harpegnathos saltator* (Formicidae)	gi|307195440	55223/5.32	397	VVDLLAPYAK, TIAMDGTEGLVR, AHGGYSVFAGVGER, FTQAGSEVSALLGR, VALVYGQMNEPPGAR, LVLEVAQHLGENTVR, IMDPNIIGMEHYNIAR, IPSAVGYQPTLATDMGTMQER
32	ATP synthase subunit alpha, mitochondrial precursor/ *Nasonia vitripennis* (Pteromalidae)	gi|269784695	59247/9.18	131	HALIIYDDLSK, TAiAIDTIINQKR, EAYPGDVFYLHSR, VVDALGNPIDGKGPLNNK
31	Enolase/ *Harpegnathos saltator* (Formicidae)	gi|307211488	47379/5.79	276	MGSEVYHYLK, SNGWGTMVSHR, IGMDVAASEFY
33	Enolase/ *Harpegnathos saltator* (Formicidae)	gi|307211488	47379/5.79	230	MGSEVYHYLK, IGMDVAASEFYK, VNQIGSVTESINAHK
61	Enolase/ *Harpegnathos saltator* (Formicidae)	gi|307211488	47379/5.79	400	IGMDVAASEFYK, VNQIGSVTESINAHK, AAVPSGASTGVHEALELR
35	4-hydroxybutyrate coenzyme A transferase/ *Camponotus floridanus* (Formicidae)	gi|307174077	53245/7.99	104	IVGSFCVGSEK, IQPVLTSGAGVVTNR
37	Chitinase-like protein Idgf4/ Harpegnathos saltator (Formicidae)	gi|307207611	55766/8.45	56	EADYPAPIYGSYGR
60	Probable medium-chain specific acyl-CoA dehydrogenase, mitochondrial-like/ *Nasonia vitripennis* (Pteromalidae)	gi|156553409	45544/8.37	67	TNPDPKAPASK
66	Transferrin/ *Harpegnathos saltator* (Formicidae)	gi|307215135	82014/5.66	87	DLDINNVQGLR
71	Transferrin/ *Acromyrmex echinatior* (Formicidae)	gi|332029256	79357/5.47	145	YEAVAVIHK, DNGADITIIDGGSVK, FDCILEKDEAACLK, LTAMGVLTDINNPEYSAR
72	Transferrin/ *Acromyrmex echinatior* (Formicidae)	gi|332029256	79357/5.47	431	DNGADITIIDGGSVK, LTAMGVLTDINNPEYSAR
74	Transferrin/ *Acromyrmex echinatior* (Formicidae)	gi|332029256	79357/5.47	84	DNGADITIIDGGSVK
70	Probable isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial/ *Camponotus floridanus* (Formicidae)	gi|307166568	29723/6.01	74	VAEFAFKYATDNNR
06	Uncharacterized protein SINV_05483/ *Solenopsis invicta* (Formicidae)	gi|322778685	17131/6.70	123	YPLPLADGSGYK, ELETDECFNKYPLPLADGSGYK
07	Uncharacterized protein EAI_10007/ *Harpegnathos saltator* (Formicidae)	gi|307206139	27511/6.70	74	GAMGCGPQETFR
73	Uncharacterized protein EAI_10007/ *Harpegnathos saltator* (Formicidae)	gi|307206139	27511/6.70	85	GAMGCGPQETFR
08	Uncharacterized protein LOC100743840/ *Bombus impatiens* (Apidae)	gi|350427541	24614/6.41	72	YGTGVIVQR

Table 1: Continued.

Spot	Protein/Source	Accession code NCBI	MM/pI	Score	Peptide sequences
64	Uncharacterized protein LOC100743840/ *Bombus impatiens* (Apidae)	gi|350427541	24614/6.41	78	YGTGVIVQR
77	Uncharacterized protein SINV_07147/ *Solenopsis invicta* (Formicidae)	gi|322796407	68188/6.51	97	VYKDLPVGK

(Coleoptera), leaf-cutting ants, and caterpillars. The presence of this protein in the venom of *E. tuberculatum* may have the function of paralyzing the prey, facilitating its transport to the nest, and the feeding of the larvae or other individuals of the colony.

Cyclophilin-like protein was identified in spot 5. Cyclophilins are peptidyl-prolyl cis-trans isomerases, enzymes found in prokaryotic and eukaryotic cells, which catalyze the isomerization of peptide bonds from *trans* to *cis* form in proline residues, facilitating protein folding [39–41]. They are part of a family of homologous proteins with different isoforms that have significant allergenic activity [42]. The role of cyclophilins in the venoms of Hymenoptera is not clear but it is likely that this protein has structural function, acting on the isomerization of proteins present in the venom of *E. tuberculatum*. It may also relate to hypersensitivity reactions caused by venom inoculation.

Superoxide dismutase protein (spot 4) is an enzyme that is part of the ant defense system and has the function of reducing reactive forms of oxygen [43]. Peiren and collaborators [44] and Santos and collaborators [35] have revealed the presence of this enzyme in the venom of *Apis mellifera* bees and in social wasp *Polybia paulista*, respectively. Both studies attribute their presence in Hymenoptera venoms to the fact that this protein acts against natural oxidative stress, avoiding damage to cellular structures, which is also likely to occur in *E. tuberculatum*.

Actin (spot 27) is a structural protein related to cell and cytoskeletal motility [45]. Enolase (spots 33 and 61) is a metalloenzyme that participates in glucose metabolism in the glycolysis stage, and annexin B9 (spots 1 and 76) is part of a family of proteins located below the plasma membrane, bound to the phosphate group of phospholipids in the presence of Ca2+ in membrane fusion processes. These are all described as proteins related to exosomes, extracellular vesicular structures formed by the fusion of an intermediate endocytic compartment with the plasma membrane, responsible for releasing intracellular vesicles [46]. The role of these proteins in venoms is still under evaluation but several components of Hymenoptera venoms are secreted via exosomes, without the need for a signal peptide, such as in solitary wasps *E. pomiformis* and *O. drewseni*, in which these proteins have been found. Actin, also known as a component of snake venom, affects blood pressure and glucose homeostasis of the prey [34] and was also found in the venom of *Bombus terrestris* [47]. Chitinase protein, found in spot 37, is an enzyme that degrades the chitin of the exoskeleton of the prey, playing an essential role for the insects in the period of exuviation, as it acts on the degradation of the anterior exoskeleton, allowing the remodeling of a new chitinous structure [48]. This enzyme is part of a class of compounds that is especially interesting for the plant breeding industry because it has applications in pest control, destroying the chitin present in their exoskeleton [49, 50]. Krishnan and collaborators [51] have verified the presence of this enzyme in the venom reservoir of the parasitoid *Chelonus* sp. (Braconidae). They observed that the chitinase present in the venom facilitates the degradation of the prey's cuticle, allowing the permeation of toxic components such as hyaluronidases, melittin, and phospholipases. According to Jeong and collaborators [52], this protein shows homology with allergenic substances. The presence of chitinase in the venom of *E. tuberculatum* suggests that this protein may be involved in the capture of prey, since this ant feeds on a range of arthropods, which obviously have their exoskeleton composed of chitin.

Transferrin, identified in spots 66, 71, 72, and 74 as isoforms, is a transport protein with high affinity for iron, allowing its transport to several tissues. In *Solenopsis invicta* fire ants, transferrin synthesis occurs in response to external aggressions, such as infections caused by microorganisms. This protein is important for the physiological defense of the insect in the sequestration of iron, a nutrient of extreme importance for microbial metabolism [53]. It acts as an antioxidant agent to control the extracellular ferric levels, suggesting that its presence in Hymenoptera venoms relates to the preservation of the prey, in order to keep it fresh until the moment of feeding the larvae [34]. This protein has also been found in centipede venom and is considered a potent antimicrobial agent [54]. De Gregorio and collaborators [55] have found this enzyme in *Drosophila* sp. (Diptera: Drosophilidae) and associated its expression with the occurrence of septic lesions and infections caused by the Ascomycete fungus *Beauveria bassiana*. *E. tuberculatum* ants feed on small arthropods, which are possible hosts for microorganisms that cause infections to the ants and/or their nests. The presence of transferrin in the *E. tuberculatum* venom may relate to a preventive mechanism of infection by this ant, which, when stinging and handling the prey, inoculates this protein with an antimicrobial effect, thus minimizing the risk of carrying a potentially infected prey into their nest.

Proteins expressed in spots 6, 7, 8, 64, 73, and 77 have been recorded in the databases as "non-characterized" but

Table 2: Proteins found in the venom of *E. tuberculatum* with unknown function in the venoms of Hymenoptera.

Protein	Spot	Action Mechanism
Medium-chain specific acyl-CoA dehydrogenase, mitochondrial-like (E. C. 1.3.99.3)	60	Enzyme that acts on the β-oxidation of fatty acids in mitochondria.
Aconitate hydratase mitochondrial (E.C. 4.2.1.3)	3, 51 and 52 (isoforms)	Catalyzes the isomerization of citrate in isocitrate in the citric acid cycle. Some studies have revealed the presence of this protein in the venoms of several arthropods, such as Vespidae *Vespula maculifrons*, Sicariidae *Loxosceles reclusa* spider, *A. mellifera,* among others, as well as in the cuticle of Hemiptera Coccidae *Ericeruspela* [7, 8].
ATP Synthase (E.C. 3.6.3.14)	32	ATP-forming enzyme complex from ADP and inorganic phosphate during oxidative phosphorylation in respiration and photosynthesis processes.
Citrate Synthase (E.C. 2.3.3.1)	29, 30 and 34	Participates in the first step of the citric acid cycle by catalyzing the condensation reaction of an acetate residue containing two carbons of an acetyl coenzyme A with an oxaloacetate molecule containing four carbons to form a six-carbon citrate.
Fructose-bisphosphate aldolase (E.C. 4.1.2.13)	20, 58, 75 and 78	Involved in carbohydrate metabolism, and responsible for the cleavage of fructose 1,6-bisphosphate in two trioses: glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in glycolysis.
Glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.12),	16, 17, 18 and 69	Participates in glucose metabolism by the Embden-Meyerhof route, catalyzing the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate.
Isocitrate dehydrogenase (E.C. 1.1.1.42)	70	Enzyme that catalyzes oxidative decarboxylation of isocitrate to form α-ketoglutarate in the citric acid cycle.
Nucleoside diphosphate kinase (E.C. 2.7.4.6)	2	Participates in nucleotide biosynthesis, catalyzing the conversion of the nucleoside diphosphate into nucleoside trisphosphate.
Phosphoglycerate mutase 2-like (E.C. 5.4.2.11)	12	Glycolytic enzyme which catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate
Succinyl-CoA ligase (E.C. 6.2.1.5)	19, 26 and 57	Enzyme that participates in the citric acid cycle, catalyzing the conversion of succinyl-CoA to succinate.
4-Hydroxybutyrate coenzyme A transferase (E.C. 2.8.3.9)	35	Enzyme that participates in the metabolic process involving acetyl Co-A, exhibiting transferase activity. It is well studied in bacteria and participates in the fermentation process.

the conserved domains to which these proteins belong have been identified, classifying them into specific superfamilies. Spots 6 (SINV_05483), 7 (EAI_1007), 8 (LOC_100743840), 64 (LOC_100743840), and 73 (EAI_10007) belong to the superfamily LPMO10 (Lytic polysaccharide monooxygenase, cellulose degrading). They form a recently discovered group of oxidative enzymes that carries the important role of degrading polysaccharides such as chitin and cellulose, cleaving the glycoside bonds with the oxidation of the C-1, C-4 carbons, or both [56, 57]. Spot 77 (SINV_07147) belongs to the superfamily GMC-OXRED-C (glucose-methanol-choline oxidoreductase). They aggregate homologous proteins with oxidation-reduction enzymatic activity, which uses FAD as a cofactor [58, 59].

Some proteins found in the venom of *E. tuberculatum* are not part of the protein composition generally found in the venoms of Hymenoptera. It is likely that these proteins are components of the membrane cells of the venom reservoir. Table 2 summarizes the situation for these proteins and their corresponding functions.

The results of the proteomic analysis of the venom of *E. tuberculatum* allowed the identification of nine proteins related to the process of attack/defense and maintenance of the colony. The active components of the venom have been classified into five subgroups:

(1) Colony asepsis—transferrin and annexin B9: they have antioxidant and antimicrobial activity and are used by the insects to prevent infections of stored food and colony members themselves [34, 53, 54].

(2) Protein of the venom constituents—superoxide dismutase and cyclophilin-like protein: they relate to the preservation of the functional integrity of cellular/glandular constituents, preventing oxidation and damage to the cellular structure of the venom bag and its reservoir [35, 40, 41, 44].

(3) Diffusion of the venom in the prey—chitinase, enolase, annexin B9, and actin: the process of diffusion of the constituents of the venom [46].

(4) Prey paralysis—arginine kinase: it causes neurotoxicity, leading to paralysis and subsequent death of prey [38].

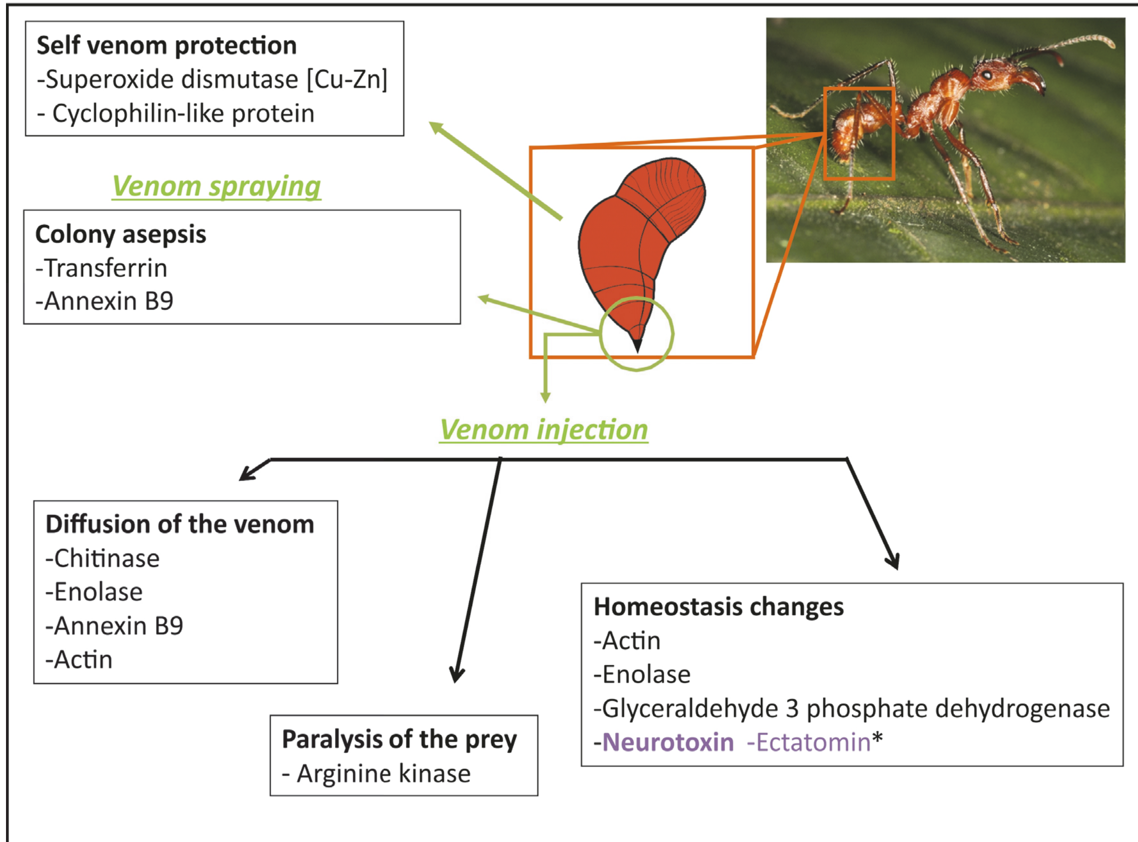

Figure 2: Representation of the action mechanisms proposed for the proteins identified in the venom of *E. tuberculatum*; *peptide ectatomin [23, 25] not identified in the present study.

(5) Alteration of homeostasis and cellular toxicity—actin, enolase, glyceraldehyde-3-phosphate dehydrogenase: they cause damage to different tissue types and alteration of cellular homeostasis and may be neurotoxic [34].

Figure 2 resumes the scheme for the aforementioned functions.

The identification and characterization of the proteins present in the venom of *E. tuberculatum* can aid in the better understanding of how the ants use the protein constituents for different behaviors such as defense against natural and pathogenic enemies, attack on prey, and maintenance of the colony, in addition to correlating the presence of certain allergic components in the venom with effects occurring in humans after inoculation. Furthermore, for providing knowledge as explained above, studies like this one also contribute to the identification of substances as possible prototypes for synthesis of macromolecules for new purposes, from their use as molecular markers to the manufacture of drugs and insecticides.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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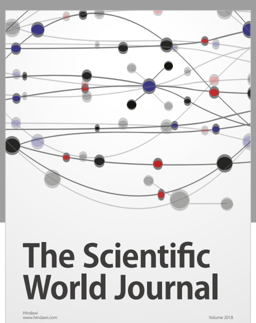

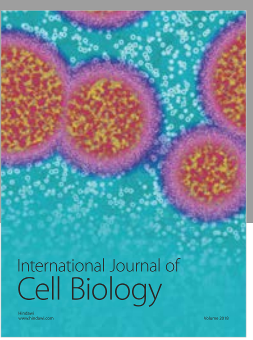

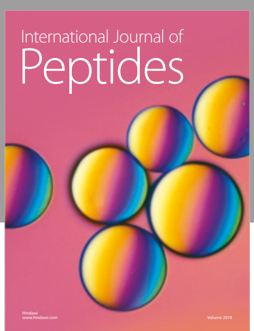

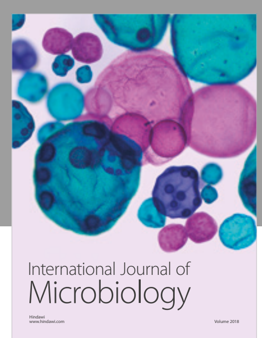

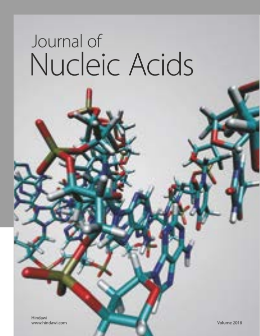

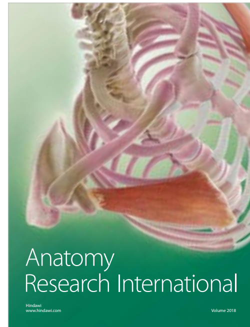

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