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Molecular Pharmacology and Toxinology of Venom from Ants

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1. Introduction

In the last decades, poisonous animals have gained notoriety since their venoms (secreted or injected) contain several of potentially useful bioactive substances (polypeptide toxins), which are mostly codified by a single gene or, in the case of venom organic compounds, by a given enzymatic route presented in a specialized tissue where the biosynthesis occur – the venom gland.

In this context, in the age of genomic sciences, sequencing the entire genome or portion of it, can be thought as the straightforward step to understand a given venom composition. Particularly because, in many cases, the venom is produced in so small quantities, requiring great challenge (natural and bureaucratic) to obtain biological material for its investigation or the necessity of sacrifice the animal to get samples for analysis by conventional biochemical methods. Genome sequencing allows us the identification of mRNAs, as well as prediction of protein structure and function. In addition, the construction of cDNA libraries is useful to clone, catalog and identify genes, and subsequently express the proteins of interest from these libraries. By this approach, we can have adequate amounts of polypeptide toxins for functional analysis and application, by which otherwise would be difficult to isolate.

According to [1], venoms' complexity in terms of peptide and protein contents, together with the number of venomous species indicate that only a small proportion (less than 1%) of the all bioactive molecules has been identified and characterized to date, and little is known about the genomic background of the venomous organisms. Consequently, if we take into account that nature, operated by evolutionary processes, is the most efficient source of new functional molecules and drug candidates, the study of all species of venomous animals, including small

insects, such as those belonging to the order Hymenoptera [2] will be crucial and timely for basic and applied research.

2. Ants biology: Subfamily Ponerinae

Ants (Vespoidea: Formicidae) belong to the insect order Hymenoptera, which includes other important families like Apidae (bees) and Vespidae (wasps) [3]. The family Formicidae consists of approximately 13.000 species of ants, most of them exhibiting an advanced and sophisticated social life. With colonies ranging from tens to millions of individuals, a high diversity as well as numerical and biomass dominance in almost every habitat throughout the world, ants form an important component of terrestrial biodiversity, especially in the Neotropical Region, where about 30% of all known ant species are found [4,5]. All ant species possess eusocial habits, the most conspicuous one being the reproductive division of labor, with one to many queens specialized in reproduction, while the more and less sterile, and nonreproductive workers, help the queen(s) reproduction, tending the brood and dealing with all other tasks of the colony like food collection, nest repair, nest and/territory defense [6].

With more than 1000 species distributed in 28 genera, like *Dinoponera* and *Paraponera*, the Ponerinae subfamily is a primitive group of ants mainly found in tropical habitats [4]. It is also one of the four major ant groups (Myrmicinae, Formicinae, Ponerinae and Dolichoderinae), all characterized by high species diversity and widespread geographic distribution [4]. *Dinoponera* Roger, 1861 [7] is a strictly Neotropical genus with six known species [5] that are considered the largest ants of the world (3-4 cm in length): *D. australis* Emery, 1910; *D. gigantea* (Perty, 1833); *D. longipes* Emery, 1901; *D. lucida* Emery, 1901; *D. mutica* Emery, 1901; and *D. quadriceps* Santschi, 1921 (Figure 1). Like in other ponerine ants, *Dinoponera* colonies have a poor social organization, with small colonies that are queenless [9, 10]. Contrary to most ant species, all workers of the *Dinoponera* colony are potential reproductives with functional spermatheca. However, only one (sometimes more) worker mate and become the dominant worker with reproductive function that is regularly disputed by subdominant workers [9, 10]. Like most Ponerinae, *Dinoponera* are mostly predatory ants: their common prey are medium size to large arthropods (mainly insects) that they subdue with their sting [11, 12]

Like all Aculeata hymenopterans (Chrysidoidea, Apoidea, Vespoidea), *Dinoponera* ants have a sting apparatus that is located in the last portion of the gaster, and is formed by the sting itself (derived from the ovipositor of more basal hymenopteran groups) along with two associated glands: the Dufour's gland and the venom gland [4,13]. In all ants, the venom gland apparatus typically consists of paired venom secreting tubules that converge into a single convoluted gland (an elongated continuation of the secretory tubule into the venom gland reservoir), which in turn empties into a sac-like reservoir that leads into the sting (in ants with sting) [4](Figure 2). In *D. australis*, it was shown that the convoluted gland has, like the free tubules, a secretory function [14]. The free tubules and convoluted gland are responsible for toxin production [14], which seems to be composed mainly of proteins [4,15]. Furthermore, it was also shown that its morphology and ultrastructural organization presents simi-

ilarity with the convoluted gland of vespine wasps (Vespinae), a fact that supports the hypothesis of a phylogenetic origin of ants from wasp-like ancestors [14].



Figure 1. *Dinoponera quadriceps* (Quinet, Y.P. 2011)

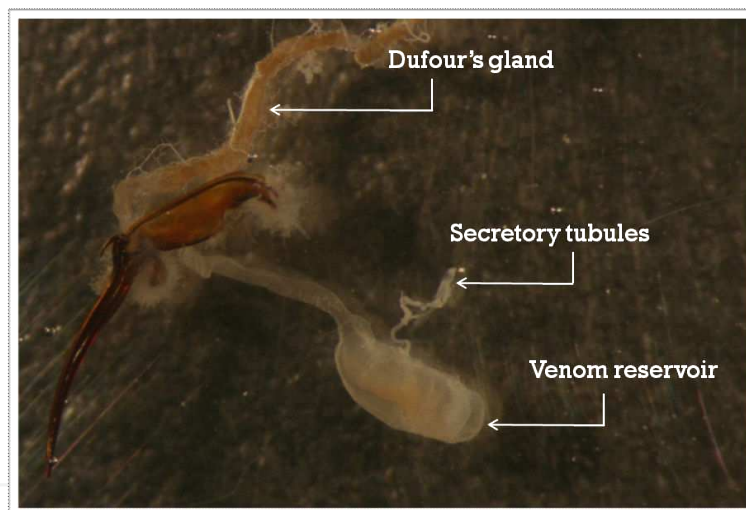


Figure 2. Secretory apparatus from *D. quadriceps* (Quinet, Y.P. 2010)

In solitary Aculeata hymenopterans, and in social bees and wasps, the venom has two main functions: prey capture and defense, respectively [13,16]. In ants, the products from the venom exhibit much higher diversity of biological roles. Particularly In stinging ants, particularly in primitive groups like Ponerinae, the primary function of venom gland products is to serve as injectable offensive or/and defensive agents (to capture prey, fight with competitors or against predators, for example) [13,16]. In more derived functions, the venom gland products are used as defensive (toxic and/or repellent) agents by non-stinging ants that topically apply them on the cuticle of enemies, as in *Crematogaster* or *Monomorium* ants for example. Venom gland products can also serve as chemical communication agents (alarm and recruitment pheromones, for example) [16,17].

3. Clinical aspects of ants' stings

Many insect stings are associated with local pathophysiological events, characterized by pain, swelling and redness at the sting site for about 1-2 days [18]. The most severe reactions are associated with allergic disorders, presenting neutrophilic and eosinophilic infiltration and specific IgE production [19]. These manifestations are common in accidents with Hymenoptera insects. Most studies that describe the clinical aspects of ant stings reported accidents with ants of the genus *Solenopsis* (Myrmicinae), known as fire ants [20,21,22]. In most serious cases, these accidental encounter with fire ants can promote multiple body rash, seizures, heart failure, and serum sickness nephritis and, more rarely, acute renal failure [23,24].

Accidents with ants of the Ponerinae subfamily are rare or rarely reported. In fact, several concomitant or sequential stings are necessary in order to produce significant clinical symptoms of envenomation, in giant ants, multiple attacks are less probable, since workers have a solitary foraging behavior. However, some of the accidents with giants ants may have medical importance, such as the ones produced by the genus *Paraponera* and *Dinoponera*, popularly known as "true tocandira" and "false tocandira", respectively. Their stings are extremely painful and can cause potentially systemic manifestations such as fever, cold sweats, nausea, vomiting, lymphadenopathy and cardiac arrhythmias [8,25,26]. According to [27,28] the venom of these ants may be neurotoxic for other insects.

4. Venom composition and pharmacological properties

The ant's venoms have been investigated in a relatively small number of species. In the group of stinging ants, the most investigated species belong to the Myrmeciinae, Ponerinae, Pseudomyrmecinae and Myrmicinae subfamilies. They produce aqueous solutions of proteinaceous venoms containing enzymatic and non-enzymatic proteins, free amino-acids and small biologically active compounds like histamine, 5-hydroxytryptamine, acetylcholine, norepinephrine, and dopamine [16,17]. Venoms with proteinaceous components are considered as most primitive and are consequently found in other aculeate hymenopterans like wasps and bees [4,16]. A notable exception to this proteinaceous nature of the venom in ants with sting is found in ants of the genera *Solenopsis* (fire ants) and *Monomorium* (Myrmicinae) that produce alkaloid-rich venoms with few proteins. In the Formicinae ants (ex: *Camponotus*, *Formica*), the sting is no more presented, but the poison gland produces a mixtures of simple organic acids an aqueous solution. Formic acid is presented in concentrations up to 65% along with some peptides and free amino-acids [16,17].

As a member of a group of predatory ants (Ponerinae), it is expected that *Dinoponera* would produces such a kind of proteinaceous venom. However, until now few studies have been done with *Dinoponera* venoms. In two of these studies, which compared venoms of a variety of hymenopterans, the presence of proteins, some with enzyme activities (phospholipase A, hyaluronidase, and lipase), was shown for *D. grandis* (in fact, *D. gigantea*) venom [16,29]. In a more recent study, in which the peptide components from the venom of *D. australis* was

investigated, over 75 unique protein components were found with a large diversity of properties ranging in size, hydrophobicity, and overall abundance [30]. The biological effects of several ants' venoms have been attributed to their protein repertoire. As showed by [31] high molecular weight proteins are present in the venom of *Dinoponera australis*. In a comparative evaluation of protein composition of hymenopteran venom reservoirs, proteins with molecular weight ranging from 24 to 75kDa were evidenced [29]. Additionally, two peptides with less than 10 kDa, as well as proteins with molecular weight ranging from 26-90 kDa were also found in the venom of *Myrmecia pilosula* [32]. The electrophoretic profile of wasps also shows variation in the protein molecular weight, ranging from 5 to 200kDa [33,34], whereas the venoms of bees was shown to range from 2 to 108 kDa [35].

5. Pharmacology and therapeutic uses of venom form ants

The first reported case about the therapeutic use of venoms from ants were to treat rheumatoid arthritis. In fact, insects might have components that justify its use in traditional medicine in countries of East Asia, Africa and South America [36]. Lately, several studies of ant venom aimed to demonstrate their beneficial intrinsic properties such as reduction of inflammation, pain relief, improved function of the immune system and liver [37,38].

As the venom from Ponerinae subfamily is composed of a complex mixtures of proteins and neurotoxins [39] we would expected to have several pharmacological properties. Small peptides isolated from *Paraponera clavata* venom, called poneratoxin (PoTx) interfere with sodium channels function and have potential use as a biological insecticide [40,41].

Several distinctive pharmacological activities were demonstrated with peptides isolated from *Pachycondyla goeldii* and *Myrmecia* sp. In one of these works, antimicrobial activity against both Gram positive and Gram negative bacteria was observed [42, 43]. In a recent study [44], it was reported that the venom from *Pachycondyla sennaarensis* has a significant antitumor effect on breast cancer cells in a dose and time dependent manner without affecting the viability of non tumor cells. In addition, some studies have also shown the renal effects of Hymenoptera venoms. In fact, in more serious accidents with venoms from wasps and bees acute renal failure generally occurs [45,46, 47, 48].

6. Genomic study of ant venom composition

Since the description of DNA double helix by Francis Crick and James Watson (1953), recombinant DNA technology and genomics revolutionized numerous areas of life science. The comprehension of the biochemical and molecular basis of inheritance had been improved our knowledge about the complexity of all forms of life and the manner how genes and proteins interact to create diversity. The genomic revolution was additionally expanded with the advent of bioinformatic, the 'omic' science (transcriptomic, proteomic, peptidome, metabolomic, glycome) and, presently, system biology.

Collective efforts have been joined to annotate the gene composition of insects. The first complete sequenced genome of insect was from the fruit fly *Drosophila melanogaster*, in 2000, followed by a flurry of activities aimed at sequencing the genomes of several additional insect species. In the field of toxinology, the hymenopterans are receiving special attention due to their behavior and the ability to produce venom.

Up to now, at least 10 ant species had their genomes analyzed and published. The ants whose genomes were sequenced include: the fire ant *Solenopsis invicta* found in South America, United States, China, Taiwan, Australia [49]; the Argentine ant *Linepithema humile* [50], the leaf-cutting ant *Acromyrmex echinator* [51] and *Atta cephalote* [52] found in South America; the red harvester *Pogonomyrmex barbatus* found in North and South America [53], the florida carpenter ant *Camponotus floridanus* from United States; and, the jumper ant *Harpegnatos saltator* from India, Sri Lanka and Southeast Asia [54]. Those ant genomes have provided hundreds of new available nucleotide data.

Apart of a detailed genome analysis, the construction of cDNA libraries from ants' venom glands is an important tool in order to analyze venom composition and discover new molecules that could have biological and pharmacological properties. But an important question arises: why hymenopteran venoms? As we pointed at the beginning of this chapter, there are several reports that hymenopteran venom could have biological properties useful for medical purposes. In this scope, from traditional and modern medicine reports, description can be found not only about clinical manifestation caused by hymenopterans venom, as allergic response, but also the benefits of ant venom to treat disease like rheumatoid arthritis and pain [36].

Genomic and transcriptomic studies of hymenopteran cDNA libraries would provide useful information about their protein constituents. Some of these informations would include signal peptide sequences and the presence of post-translational modifications, which cannot be predicted by the studies of mature proteins. Ants genomic studies have shown a number of substances involved in the biology of these insects, such as: vitellogenins, gustatory and odorant receptors, molecules involved in immune response, as well as metabolic and structural proteins like cytochrome P450.

7. Molecular pharmacology and toxinology of *D. quadriceps* venom

Recently, we have initiated a research project dedicated to investigate the composition, the pharmacological properties, and the transcripts from the venom gland components of *Dinoponera quadriceps*.

Using one-dimensional (SDS-PAGE) electrophoresis (1-DE) to resolve *Dinoponera quadriceps* venom proteins, only eight major large polypeptides (ranging from 15 to 100 kDa) were visualized by Coomassie Brilliant Blue (CBB) Staining. The 1-DE and the insensitive method of staining with CBB was not adequate to separate small proteins below 15 kDa and peptides (Figure 3)

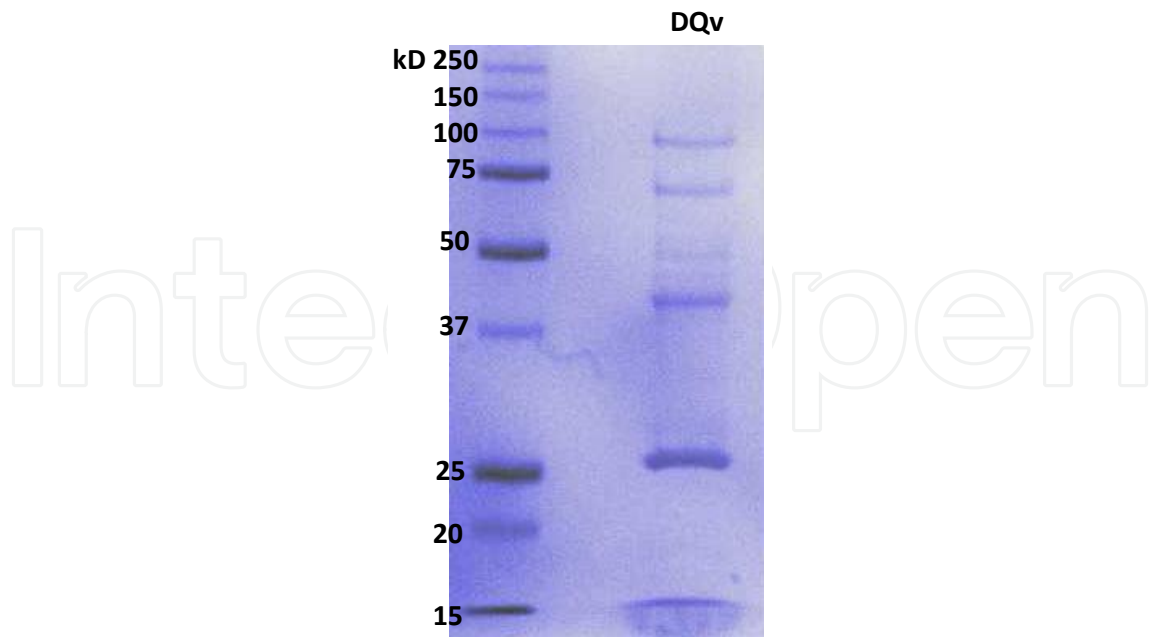


Figure 3. Electrophoretic profile of *Dinoponera quadriceps* total venom (DQv) in one-dimensional SDS-PAGE gel electrophoresis visualized with Coomassie Brilliant Blue.

The peptide mass fingerprint (PMF), as well as other proteomic analysis is being conducted and a report will be published elsewhere.

Pharmacological studies have been realized with *Dinoponera quadriceps* venom, particularly, in a system of isolated perfused rat kidney. We now know that at concentrations of approximately 10 μ g/mL increased urinary flow, glomerular filtration rate and decreased vascular resistance and sodium tubular transport, suggesting a natriuretic and diuretic effect. Furthermore, in studies with renal tubule cells (MDCK - Madin-Darbin Canine Kidney) the same venom induced cell cytotoxicity, on MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at a dose and time dependent manner. Interestingly, greater cytotoxicity was observed in the shorter incubation periods, suggesting that the cell culture could recover after a given exposure time. Additional assays have been designed to evaluate the biological and pharmacological activity of purified component of this venom, as well as highlighting the mechanisms related to the observed effects.

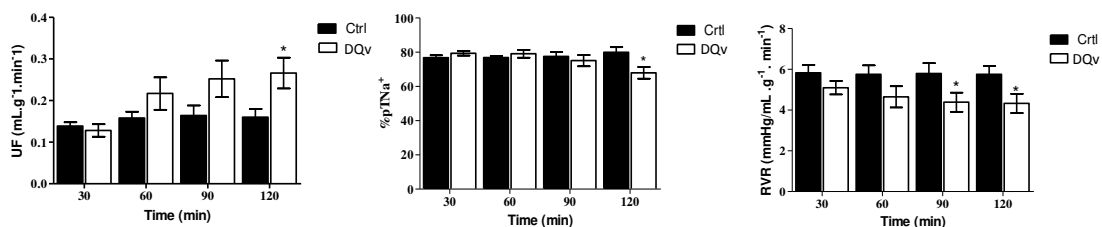


Figure 4. Effect of *D. quadriceps* total venom (DQv) on Urinary flow (UF; A), sodium tubular transport percent(%pTNa; B) and renal vascular resistance (RVR; C). Ctrl=control. Results are expressed as means \pm S.E.M., * $p < 0.05$ (ANOVA).

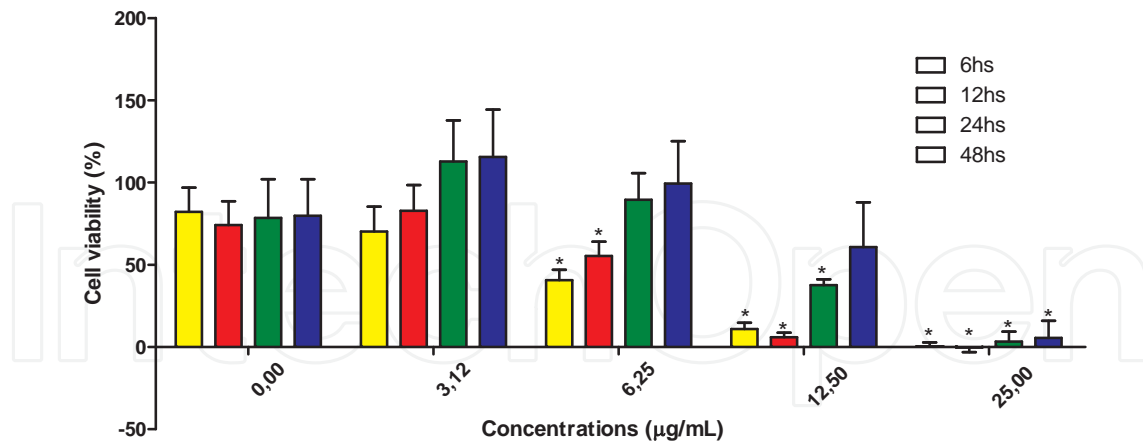


Figure 5. Citotoxicity of *D. quadriceps* total venom on MDCK (Madin-Darbin Canine Kidney) cells culture on MTT assay. Results are expressed as means \pm S.E.M., * $p < 0,05$ (ANOVA).

Recently we also demonstrated the neuroprotective activity of *D. quadriceps* venom in models of seizures induced by pentylenetetrazol (PTZ), when administered intraperitoneally. The effect was an increase in latency to first seizure and a tendency to increased latency of death, as well as reduction of lipid peroxidation in the prefrontal cortex of mice [55].

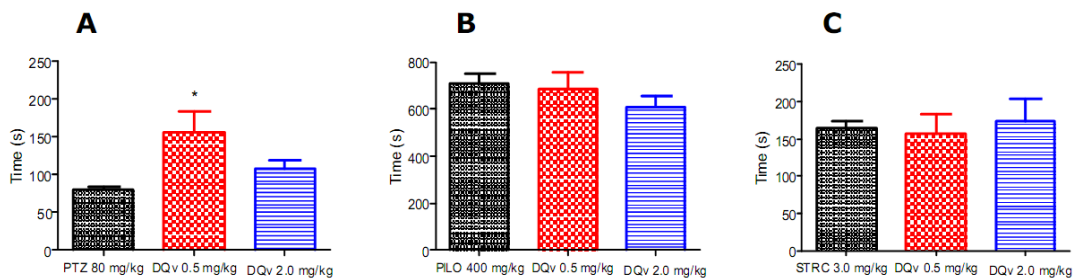


Figure 6. Effects of *D. quadriceps* venom (DQv) on latency of the first seizure in the models of seizure of pentylenetetrazol (PTZ) (A), pilocarpine (PILO) (B) and strychnine (STRC) (C). Results are expressed as means \pm S.E.M., * $p < 0.05$ (ANOVA).

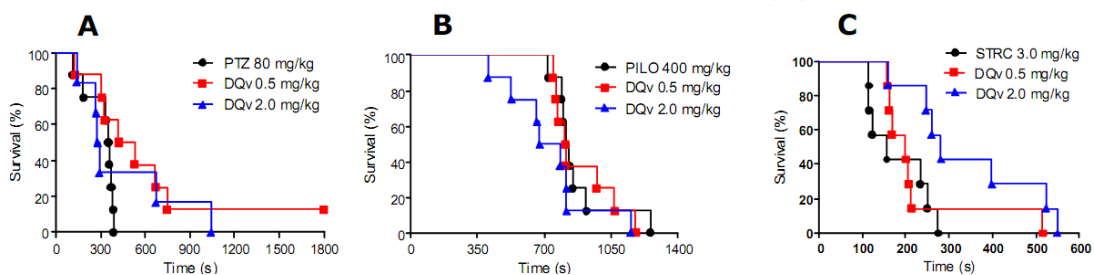


Figure 7. Effects of *D. Quadriceps* total venom (DQv) on latency of death in models of seizure of pentylenetetrazol (PTZ) (A), pilocarpine (PILO) (B) and strychnine (STRC) (C). Results are expressed as means \pm S.E.M (n=8), * $p < 0.05$.

A part of proteomic and pharmacological studies, we prepared a *D. quadriceps* venom gland cDNA library to use an EST-strategy to identify the major transcripts expressed in the giant ant venom. We successfully constructed a full-length cDNA library of approximately 20 venom glands from *D. quadriceps*, using In-Fusion SMARTer kit (Clontech, USA). We obtained an efficiency of 1×10^5 cfu/ μ g of DNA, our medium insert was 700bp and the library was amplified and stored at -80°C . A total of 432 individual ESTs were sequenced by the dideoxy chain termination (Sanger) method. Of these, 125 were undergone to a preliminary analysis through BLASTx. The Tabel 1 and Figure 8(A) shows an overview of the relative abundance of the protein groups. Most of the transcripts represent proteins involved in the whole metabolism as transferases, ATP synthase, dehydrogenases, ribosomal proteins, cytocrome c. Those sequences are being annotated for deposit in DNA and protein data bank. A note of caution is that, as in most transcriptome project, a significant number of transcripts showed no similarities with well-known sequences in data bank. These ESTs presents a typical structure of true ORFs (Open Reading Frame), that is start and stop codons, in addition a poly A tail. They were classified as (1) hypothetical proteins with unknown function and (2) cDNA precursors with no hits found. However, by comparing against DNA and protein data the hypothetical proteins showed high similarities with proteins from scorpions (*Opisthacanthus cayaporum*) and others ants, as *Harpegnatos saltator*, *Solenopsis invicta* and *Camponotus floriandus*. The Figure 8(B) represents the percentage of three classification of hits over the total clones analyzed, were probable toxins comprises a significant percentage of ESTs, representing about 34% of messages. Other 37% represents no-significant hits, which give us a number of perspectives to analyze several novel proteins.

Class	Function	% Clones
No hit	Typical ORF with no hits	40.8
DnTx	Mast cell degranulation	28.8
Hypothetical protein	Unknown function	12.0
Antigen like	Allergenic	9.6
Cytocrome c oxidase	Metabolism	1.6
Cytocrome b	Metabolism	1.6
Transferase	Metabolism	2.4
Ionic channel blocker	Toxin	1.6
Ribosomal protein	Structural protein	1.6
Chymotripsin inhibitor	Metabolism	0.8
Dehydrogenase	Metabolism	0.8
ATP synthase	Metabolism	0.8
Phospholipase A1	Enzyme/Toxin	0.8
Bacterial ESTs	Symbionts (?)	4.0
Mitochondrial protein	Metabiolism	0.8

Table 1. Classification of ESTs from *D. quadriceps* venom gland cDNA library on their putative functions.

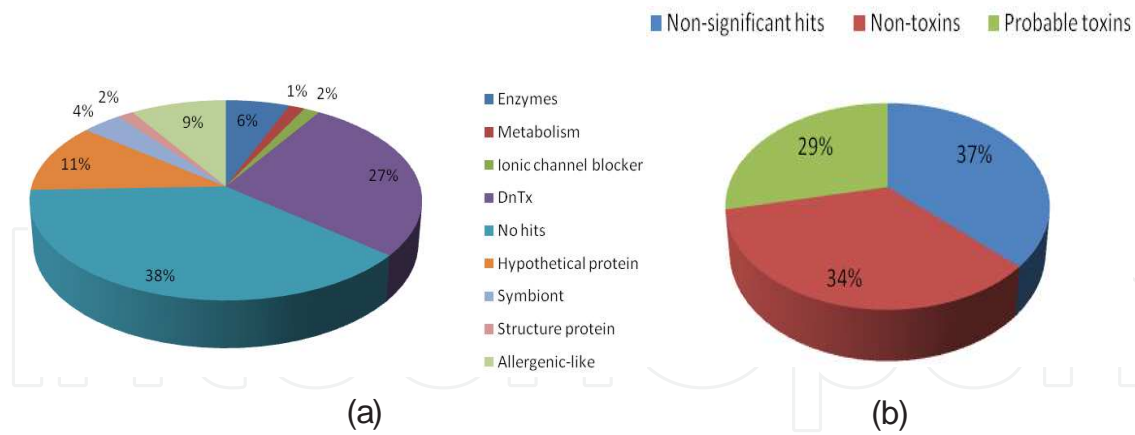


Figure 8. Classification of ESTs from *D. quadricipes* venom gland cDNA library on their putative functions (A). Relative proportion of toxin-encoding, non-toxin encoding and no significant hit ESTs (B).

As a matter of example, the most abundant toxin was dinoponera toxin (DnTx). The dinoponeratoxin whole sequence (accounting for 27% of the total clones analysed) was identified in this cDNA library. Deduced aminoacid sequences (DnTx01 and DnTx02), corresponding to two cDNA isoform precursors, from *D. quadricipes* transcriptome (this work) and three mature venom peptides (DnTx_Da-3105, DnTx_Da-3177 and TX01_DINAS - GenBank accession numbers GI:294863162, GI:294863159 and GI:294863158, respectively) from *D. australis* [30] were aligned with ClustalW software using default parameters (<http://www.ebi.ac.uk>). DnTx01 and DnTx02 are represented with their respective signal peptides and pro-peptides, in which putative cleavage sites are shown in green and blue, respectively, according to SignalP software (<http://www.cbs.dtu.dk/services/SignalP>) and proteomic data. In the alignment A is clearly observed that DnTx01 shares high similarity with DnTx_Da-3105 and DnTx_Da-3177, whereas the mature DnTx02 and TX01_DINAS are highly similar to each other (part B).

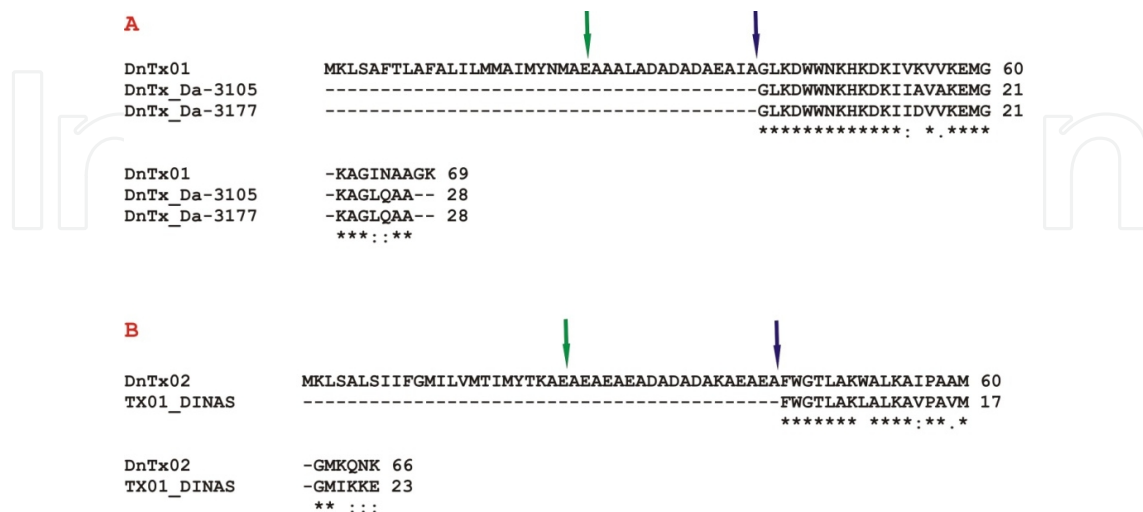


Figure 9. Alignment of dinoponeratoxin precursors and mature peptides from *D. quadricipes* and *D. australis* using ClustalW software (<http://www.ebi.ac.uk>).

8. Conclusion

Taking into account the information presented in this chapter, a second question arises and should be answered in the near future: "Is there any hymenopteran venom component that could be used as a biotechnological tool?" The majority of works done to discover new biotechnological tools from hymenopteran venoms were performed using proteomic science analysis, probably because ant venom is so hard to identify and dissect. Nevertheless, the size of some poneromorph primitive ants may permit subdue these difficulties allowing us to construct a cDNA library and thus opening new perspectives to better understand the biology of ants as well as to analyze the properties of the venom in the search for new molecules with pharmacological and / or biotechnological potential.

Thus, it's clear that further work is necessary to understand ant venom, as well as venoms from hymenopteran, since several precursors comprise hypothetical and predicted toxins/polypeptides with unknown function. Moreover, a deep functional analysis in the coming period will be made to comprehend the effects presented by total venom and peptides isolated from it.

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