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## Honeybee venom: influence of collection on quality and cytotoxicity

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**ABSTRACT:** Apitoxin is the venom produced by bees. It is a complex chemical compound, rich in protein substances and with pharmacological effects. This study was carried out with the objective of comparing the quality of apitoxin extracted in an apiary in different parts of the collector in relation to moisture content, protein analysis and cytotoxicity assay with *Artemia salina* L. Type 1 apitoxin was collected from glass slabs at the entrance to the hive, while type 2 apitoxin was collected from the waste accumulated in the collection rods and treated by rinsing in distilled water. Both apitoxins presented significant differences ( $P > 0.05$ ) in relation to protein profile, with type 1 showing a higher content (77.8%) than type 2 (51.9%), and presented polypeptide bands with more than 50% of their nitrogenous components having molecular weight below 10KDa. Regarding cytotoxicity assays, type 1 apitoxin had LD50 of  $71.5 \mu\text{g mL}^{-1}$ , while type 2 had LD50 of  $191.6 \mu\text{g mL}^{-1}$ . Thus, the region where apitoxin accumulates in the collector does influence the product quality if moisture and protein contents are in accordance with the standards recommended in specific legislation, and so it can be commercialized by the beekeeper.

**Key words:** bees, beekeeping, bee venom, *Artemia salina* L.

## Veneno de abelha: influência da coleta em qualidade e citotoxicidade

**RESUMO:** A apitoxina é o veneno produzido pelas abelhas, um composto químico complexo, rico em substâncias proteicas e com efeito farmacológico. Com objetivo de comparar a qualidade da apitoxina extraída em apiário em relação ao teor de umidade, análise das proteínas e ensaio de citotoxicidade frente *Artemia salina* L. este trabalho foi realizado. A apitoxina denominada neste estudo como tipo 1 foi coletada de placas de vidro na entrada da colmeia, enquanto que a denominada tipo 2, coletada a partir dos resíduos acumulados nas varetas coletoras e tratada com lavagem em água destilada. As apitoxinas apresentaram diferenças significativas ( $P > 0,05$ ) em relação ao perfil proteico, sendo a tipo 1 com maior teor (77,8%) que a tipo 2 (51,9%) e apresentou bandas de polipeptídeos reveladas possuindo mais de 50% de seus componentes nitrogenados com peso molecular abaixo de 10KDa. Quanto aos ensaios de citotoxicidade, a apitoxina tipo 1 apresentou DL50 de  $71,5 \mu\text{g mL}^{-1}$  e a tipo 2 DL50  $191,6 \mu\text{g mL}^{-1}$ . Assim, a região onde se acumula a apitoxina no coletor influencia na qualidade do produto, mas apresenta teores de umidade e proteína de acordo com padrões preconizados em legislação específica, podendo ser comercializada pelo apicultor.

**Palavras-chave:** abelhas, apicultura, veneno, *Artemia salina* L.

## INTRODUCTION

Animal venoms and toxins consist of proteins and peptides with potential medical and biotechnological use. Apitoxins are produced and secreted through a gland (acid gland) located in the posterior region of the *Apis mellifera* bee's abdomen, containing a complex mixture of substances with biological activity (ORŠOLIĆ, 2012; DANNEELS et al., 2015; SOBRAL et al., 2016) composed of enzymes, peptides, amino acids and other compounds (SCIANI et al., 2010; FERREIRA-JUNIOR et al., 2010). Apitoxin

constituents have been used as analgesic, anticoagulant and anti-inflammatory agents for treating chronic diseases such as arthritis, rheumatism, tendinitis, bursitis, fibrosis, multiple sclerosis (SOBRAL et al., 2016), and antineoplastic (ORŠOLIĆ, 2012), and has also been recently studied for its anti-acne (HAN et al., 2016) and antifungal actions (LEE, 2016).

Melittin is the main constituent of apitoxin (SOBRAL et al., 2016). It has a low molecular weight (2.85kDa) representing about 40-50% of apitoxin dry weight (GAJSKI et al., 2013; YILMAZ et al., 2016), where it synergistically acts with phospholipase A2

(molecular weight 11kDa) which represents 11% of apitoxin dry weight (FERREIRA JUNIOR et al., 2010). Furthermore, apamines are also present in a smaller proportion (2-3%), being a low molecular weight peptide (2kDa). Concentration of constituents present in apitoxin may vary depending on seasonality, influencing the protein content of the venom (FERREIRA-JUNIOR et al., 2010; SILVEIRA et al., 2015), and also in relation to bees age (DONG et al., 2015).

Bee venom is extracted using a collector composed of plates and a pulse generator, which induces the bees to sting the electric collector plate resting on a glass plate (DANTAS et al., 2013). Volatile phase of the venom evaporates onto the glass plate, from where the apitoxin is then collected by scraping. However, brown-color venom which is also deposited in the collector filaments requires a treatment in order for the product to obtain the characteristic white powder of the apitoxin.

Brazil has legislation for all bee products derived from beekeeping by Normative Instruction No. 3 of the Ministry of Agriculture Livestock and Supply (BRAZIL, 2001). Technical regulation for identity and quality of apitoxin determines requirements of 3% maximum moisture of the product and protein content ranging from 50 to 85%. It is also indicated that the product packaging must be made with materials suitable for storage conditions and that they provide appropriate protection against contamination; and no additives to the product are authorized.

From the above, this study aimed to analyze the quality of collected apitoxin from the collector filaments after beneficiation and to compare it with the apitoxin collected directly from collector's glass plate, considering moisture content, protein and toxicity through *Artemia salina* L. bioassay, seeking to establish product quality.

## MATERIALS AND METHODS

Apitoxin used in this study was collected from an apiary located in the municipality of Ceará Mirim, Rio Grande do Norte, Brazil (longitude 36° 25' 32" and latitude 5° 38' 04"), in the months of January and February of 2015. Samples used in this study were obtained from three different lots, lot contained about 10 swarms, and sent to the Laboratory of Technology of Animal Origin of the Agro-Food Science and Technology Center at the Universidade Federal de Campina Grande.

Collection procedure used was the electric collector method, composed of stainless steel electrodes connected to a battery and glass slide positioned at the

hive entrance. When the bees land on the collector, they received a shock and react by stinging the electric collector plate, depositing a load of venom on the glass, as well as on the equipment filaments, where it dries and is subsequently scraped and commercialized.

The apitoxin in this study was denominated as type 1 apitoxin. It is the material collected directly from the glass plates scraped with steel blades after natural dehydration and marketed directly in this form. Type 2 apitoxin is removed from the material accumulated in the equipment filaments (collecting ditches), which is scraped with steel sheets after its natural dehydration, washed in distilled water (1:3 w/v), filtered on qualitative filter paper (125mm) and then dried at a temperature of 30°C (±5°C) in an air circulating oven.

Apitoxin was analyzed for its moisture content using a moisture-determining scale (MARTE brand) at a temperature of 105°C until constant weight of the sample (in triplicate). Protein content was determined by the Micro-Kjeldahl method in triplicate, using a factor of 6.25 for transformation of total nitrogen into proteins as recommended by the AOAC (2010).

Moisture content and protein analyzes were submitted to statistical analysis for mean comparison using t student test, considering a level of probability error (p) less than 5% to determine significance using the Assistat 7.6 free statistical program (SILVA, 2014).

PAGE-SDS-2βMe system polyacrylamide gel electrophoresis was carried out according to the methodology described by LAEMMLI (1970), adapted for the use of plaque separation gels (10x10x0.1cm) (Kasvi brand), where application gel containing 6% polyacrylamide was prepared in 0.6Mol L<sup>-1</sup> Tris-HCl buffer, 6.8 pH and 1.0% SDS (w/v) (sodium dodecyl sulfate). The 15% polyacrylamide gradient separation gel was prepared in 3.7Mol L<sup>-1</sup> Tris-HCl buffer, 8.9 pH containing 1.0% SDS. Electrophoresis was run at a constant voltage of 200V with gel stained for 12 hours with Coomassie Blue Brilliant R-250 0.05% prepared in methanol, acetic acid, water (1:3.5:8, v/v/v) and gel decolorization using methanol, acetic acid and water (1:3.5:8, v/v/v). Molecular weight markers used were 17, 14.2, 6.5, 3.49 and 1.06kDa (M 3546, SIGMA – Molecular Weight Marker, Ultra Low Range for SDS-PAGE).

The stained gel was photographed in a scanner and the obtained image was processed by Image J software (IMAGEJ, 2015), where the visible bands were related through densitometric analysis. *Artemia salina* L. bioassay was performed according to the technique described by MEYER et al. (1982), using a mixture of seawater and distilled

water as the saline solution (1: 1, v/v), where the microcrustacean eggs (0.2g) were incubated for 48 hours in a cage built with screens to prevent insect entry, aeration with a specific pump and direct incidence of light (60W lamp). After hatching, 10 nauplii/larvae were transferred to test tubes containing 5.0mL of saline water with the apitoxin concentrations varying from 0 to 500 $\mu\text{g mL}^{-1}$ . Each concentration tested was performed in triplicate. Dead larvae count was performed after 24 hours and used for calculating LD 50, a lethal dose to 50% of the larval population, determined from PROBIT analysis (FINNEY, 1962) using BioStat® 2009 software, with 95% confidence.

## RESULTS AND DISCUSSION

The apitoxin accumulated and removed from the collector equipment's protective material (type 2) has a brown color, and it becomes white after the beneficiation process with visible diminution of impurities.

Regarding the moisture content, type 1 and type 2 apitoxin presented values of 2.5% ( $\pm 0.15$ ) and 3.0% ( $\pm 0.15$ ), respectively, not being statistically different ( $P > 0.05$ ) and complying with the legislation enforced in Brazil (BRAZIL, 2001).

Apitoxin processed using the analyzed methods showed different results regarding protein content; however, it was within the recommended legislation with protein contents between 50 and 85% (BRAZIL, 2001). The beneficiation process through rinsing and filtration methods (type 2 apitoxin) led to a decrease in the sample's protein content through solubilization, differing statistically ( $p > 0.05$ ) with protein content at 51.9% ( $\pm 0.56$ ), while apitoxin collected directly from the collector glass plates (type 1 apitoxin) had a higher protein content at 77.8% ( $\pm 0.93$ ).

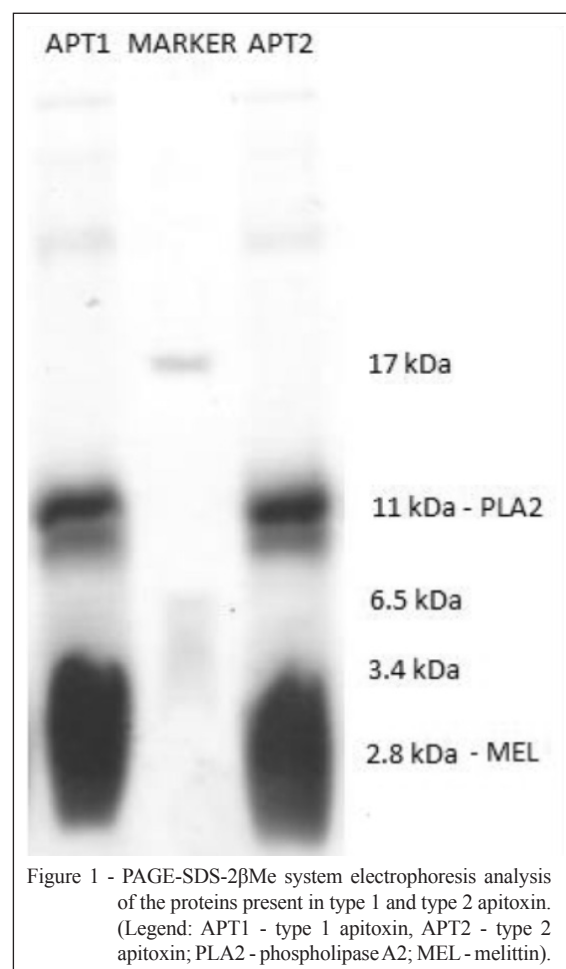
In analyzing polypeptide bands revealed by electrophoretic analysis (Figure 1) through densitometry analysis, we observed that both apitoxins presented a similar protein profile independent of collection site, with well-pronounced lower molecular weight fractions below 10kDa. The analysis also indicated the presence of melittin (2.8kDa), equivalent to 53.5% melittin for type 1 apitoxin, and 50.2% melittin for type 2 apitoxin. Fraction between 15 and 10kDa indicated A2 phospholipase content (11kDa), presenting around 32% for type 1 and 2. Low molecular weight peptides such as melittin, considered a quality marker of apitoxin (SOBRAL et al., 2016; GAJSKI et al., 2013;

YILMAZ et al., 2016) is present in similar amounts in both forms of analyzed beneficinations.

Both type 1 and type 2 apitoxin presented reduced *Artemia salina* L. naupili/larvae viability depending on the concentration tested, with LD50 of 71.5 $\mu\text{g mL}^{-1}$  and 191.6 $\mu\text{g mL}^{-1}$  respectively, with bioactive action being considered for presenting LD50 values lower than 250 $\mu\text{g mL}^{-1}$  (SIQUEIRA et al., 1998; DANTAS et al., 2013). Apitoxin biological activity is related to the bioactive action of its chemical constituents (GAJSKI et al., 2013).

Thus, type 1 apitoxin had greater bioactivity than type 2 apitoxin, possibly due to its higher protein content. Such a fact may be related to protein loss during the beneficiation process in type 2 apitoxin.

From the proposed processing technique, the material previously discarded by beekeepers leads to an increase in apitoxin yield from collection of around 40%, which in practice represents a substantial financial gain for beekeepers.





## CONCLUSION

The apitoxin presented low molecular weight peptides in similar amounts and bioactive action. Thus, the region where apitoxin accumulates in the collector does influence the product quality and biological activity if moisture and protein contents are in accordance with the standards recommended in specific legislation, and so it can be commercialized by the beekeeper. Finally, we suggested beekeepers implementing the collection of apitoxin from collector filaments (on its own or mixed with samples obtained from the glass plates) since they have similar characteristics.

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