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Review Article

JOURNAL OF BIOTECHNOLOGY AND BIOMEDICINE

ISSN: 2642-9128

Proteomic Analysis of the Hemolymph of the *Lonomia Obliqua* Caterpillar and its Protective Effect Against Hippocampal Neuronal Death Induced by Oxidative Stress

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Abstract

Investigation of substances with potentially neuroprotective effects has been one of the focuses of drug development studies. The hemolymph of *Lonomia obliqua* caterpillars, which have caused several accidents in southern Brazil, contains several proteins with anti-apoptotic activity. This study aims to investigate the potential protective effects of caterpillar hemolymph and its fractions in primary rat hippocampal neurons subjected to oxidative stress. Semi-quantitative shotgun proteomics was used to evaluate the protein profile. A total of 71 proteins in the hemolymph of *Lonomia obliqua* crude were identified. In purified fractions were identified serine proteases, protease inhibitors, and hemolins. The treatment of primary cultured hippocampal neurons with the chromatographic fraction at concentrations of 0.05 and 0.10% (v/v) for 24 hours, followed by apoptosis induction, was able to maintain cell viability significantly higher than the positive control. The results shown in this study may contribute to the identification of proteins with potential neuroprotective activity.

Keywords: Hippocampal culture cells, cell death, neuroprotector agents. Lonomia obliqua, hemolymph, shotgun, proteomic

Introduction

Insects represent about 75% of all invertebrate animals, are among the most adapted to life on earth. These organisms are capable of producing a number of chemicals that help them survive environmental attacks during their evolution (Ratcliffe *et al*., 2011). These animal poisons and venoms were developed for the purpose of defense against predators or prey capture, causing physiological changes in natural enemies. These substances have a potential application as new therapeutic drugs and have been the subject of research (Calvete, 2009).

Several accidents involving the larval form of the *Lonomia obliqua* Walker, 1855 (Lepidoptera, Saturniidae) have been reported in southern Brazil since the 80s (Duarte *et al.*, 1990; Duarte *et al.*, 1994; Kelen *et al.*, 1995; Duarte *et al.*, 1996; Zannin *et al.*, 2003). Individuals who had contact with the bristles of *L. obliqua* caterpillars can manifest a hemorrhagic syndrome associated with consumption coagulopathy, which may include intravascular hemolysis and acute renal failure. Individual characteristics of the victims, intensity of exposure to poison, and the number of animals

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Citation: Silviane Maggi, Antônio Frederico Michel Pinto, Mariana Sayuri Berto Udo, Mariana Aguilera Alencar da Silva, Raphael Caio Tamborelli Garcia, Luciane Minetto, Leandro Tasso, Pablo Machado, Diógenes Santiago Santos, Paula Eichler, Thiago Barcellos, Pedro Ismael da Silva Junior, Tania Marcourakis, Ronaldo Zucatelli Mendonça, Sidnei Moura. Proteomic Analysis of the Hemolymph of the *Lonomia Obliqua* Caterpillar and its Protective Effect Against Hippocampal Neuronal Death Induced by Oxidative Stress. Journal of Biotechnology and Biomedicine. 9 (2026): 126-139.

Received: April 29, 2026
Accepted: May 05, 2026
Published: May 25, 2026

Maggi S, et al., J Biotechnol Biomed 2026
DOI:10.26502/jbb.2642-91280217

involved may determine slight, serious, or even fatal accidents (Duarte *et al*., 1996; Fan *et al*., 1998; Gamborgi *et al*., 2006; Malaque *et al*., 2006; Riella *et al*., 2008; Basulado *et al*., 2008). Physiological activity presented by the venom in their victims initiated several studies on its composition, in order to develop therapy for poisoning and identify potential resources for the treatment of various conditions. Among the compounds identified in hemolymph, a potent antiapoptotic protein has been identified in the caterpillar of *Lonomia obliqua* (Souza et al. 2005, Vieira et al, 2010, Carmo et al., 2012, Mendonça and Martins, 2022) and in the hemolymph of caterpillars of the Megalopygidae family (Carvalho et al, 2025). Studies have shown the potential of caterpillar hemolymph to maintain the electrical potential of mitochondrial membranes (Mendonça and Martins, 2022) and the structure of the cytoskeleton (Carvalho et al, 2025), thereby preventing cell death by apoptosis. Therefore, the literature still lacks studies to elucidate the overall protein content of hemolymph composition and biological caterpillar fluids, which remains incomplete.

Proteomes are highly dynamic and may change with development and insect environment (Wilkins *et al*., 1997). Among the wide possibilities of bioactive molecules that insects can provide protein compounds with anti-apoptotic activity have been described (Rhee *et al.*, 2013; Choi *et al.*, 2002; Kim *et al*., 2001; Rhee and Park, 2000; Souza et al. 2005; Vieira et al, 2010; Carmo et al., 2011; Mendonça and Martins, 2022 and Carvalho et al, 2025). This activity may be interesting in pathologies that develop with cell loss by apoptosis, such as neurodegenerative diseases. Anti-apoptotic activity has been observed in *L. obliqua* hemolymph in insect cells and mammals forward physical inducers, biological and chemical (Vieira *et al*., 2010; Mendonça *et al*., 2008; Souza *et al*., 2005; Maranga *et al*., 2003). However, this has not been described in studies conducted with nerve cells.

Apoptosis is a central mechanism in neurodegenerative diseases as Alzheimer's, Parkinson's, and Huntington's. In these diseases the self-destruction of neurons occurs due to factors like oxidative stress, mitochondrial dysfunction and protein aggregation (amyloid-beta, tau, alpha-synuclein). So, apoptosis induces to the neuronal loss, highlighting that this death pathway could be a key therapeutic target for neuroprotection. Consequently, this study aimed to analyze the proteomic composition of *Lonomia obliqua h*emolymph and to evaluated its anti-apoptotic action in primary cultures of rat hippocampal cell.

Materials and Methods

Hemolymph Collection

The hemolymph of *Lonomia obliqua* used in this work was collected from three caterpillar colonies, obtained on different cities and from different plants (Monte Belo do Sul - 41 animals (from *Cupania vernalis*), Passo Fundo - 52 animals (from *Platanus acerifolia*) and Bento Gonçalves - 55 animals (from *Platanus acerifolia*) from State of Rio Grande do Sul, South of Brazil). Caterpillars from the sixth larval instar, had their pseudo-feet cut and the extravasated hemolymph was collected with a Pasteur pipette. After collection, the hemolymph was centrifuged at 1000 x g for 10 minutes and the supernatant was inactivated at 56 ºC for 30 minutes, filtered through a 0.22µm membrane, and stored at –20º C until use.

Hemolymph Semi-Purified Fraction

Semi-purified fraction with anti-apoptotic activity was obtained from Butantan Institute (São Paulo, Brazil) (Souza et al., 2005). Briefly 0.5 mL of total hemolymph was fractionated on an AKTA Purifier chromatography system equipped with a Resource Q ion exchange column (Amersham Pharmacia Biotech, USA) at a rate of 0.5 mL min-1 and eluted at a linear gradient (0-100%), TrisHCl 20 mmol L-1/ Tris HCl – NaCl 1 mol L-1, pH 8.0. The eluate was monitored at 280, 254 and 214 nm and harvested in fractions of 1 mL.

Animals

Male (weighing 200 g, 100 days old) and female (weighing 190g, 90 day old) Wistar rats were obtained from the School of Pharmaceutical Sciences/Chemistry Institute, at the University of São Paulo, São Paulo, Brazil. Female rats were caged overnight with males, in a ratio of 3:1, for copulation. Vaginal plug was verified the next morning to confirm the first day of pregnancy. All animals were housed under controlled temperature (22±1 °C) on a 12:12 hours light/dark cycle. On the embryonic day 18-19, pregnant rats were anesthetized with pentobarbital (45 mg Kg-1) and the fetuses were rapidly euthanized by decapitation to collect their hippocampi. All experiments were conducted according to the guidelines issued by the National Council for Animal Experimentation and was approved by the Animal Uses Ethic Committee of the Pharmaceutical Sciences School of University of São Paulo.

Primary Cell Culture.

The primary hippocampal cultures were obtained according Garcia *et al.* (2012) with slightly modifications. Primary cultures were obtained by hippocampal neurons dissociation from hippocampi of E18-E19 Wistar rat embryos. Hippocampi were maintained in solution containing cooled neurobasal medium (Gibco, USA) with 100 U mL-1 penicillin and 100 µg mL-1 streptomycin. Tissues were washed with *Hank's Balanced Salt Solution* (HBSS; Gibco, USA) and fragmented mechanically. Cell isolation was obtained by proteolytic digestion with trypsin according Jahr and Stevens (1987) and Silva *et al.* (2006). Hippocampi fragments were incubated with trypsin, 0.25%, pH 7.2-7.4, at 37 °C for 10 min. Reaction was stopped with HBSS containing 277.5 U mL-1

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DNAse (Sigma, USA) and 10% fetal bovine serum (Gibco, USA), pH 7.2-7.4. Cells were then dispersed mechanically with Pasteur pipettes and resuspended in neurobasal medium (Gibco, USA) supplemented with 0.5 mmol L-1 L-glutamine (Gibco, USA), 25 µmol L-1 L-glutamic acid (Sigma, USA), 100 U mL-1 penicillin: 100 µg mL-1 streptomycin (Sigma, USA) and 2% B27 supplement (Gibco, USA), to reduce glial cell proliferation (Brewer *et al*., 1993; Silva *et al*., 2006). Cell suspensions were plated into 0.01% poly-L-lysine-coated (Sigma, USA) 96-well culture plates at a density of $5x10^4$ cells per well. Neuron´s maturation was achieved after 7-8 days of incubation, at 37°C, 5% CO_2 Half of the complete medium was replaced every 48 hours. Cell treatments occurred between 6 and 8 day. Previous immunohistochemistry study (Garcia *et al.* 2012) showed 92% predominance of neurons and 8% of astrocytes in this methodology.

Cell death induction

Hippocampal cells $5x10^4$ cells/well were treated for 1 or 2 hours with 0.05, 0.1, 0.25, 0.5, 1.0, 2.0% of hemolymph or it fractions. After this, the supernatant was removed and 10 or 30 µmol L-1 H_2O_2 solutions (Merck, Germany), were added into the supplemented neurobasal medium, for 30 min (37°C, 5% CO H_2O_2). After this, the supernatant was removed, and MTT solution was added for viability assay..

MTT ASSAY. Cells viability was evaluated by MTT reduction assay (Mosman, 1983; Liu et al., 1997). For the assessment of cell viability, cells were plated into flat-bottoned 96 wells plates ($5x10^4$ cell/well) and cultured in supplemented neurobasal medium. They were pre incubated with hemolymph or fraction at 0.05 and 0.1% (into supplemented neurobasal medium) for 1 and 24 h (37°C, 5% CO2), and then 10 µmol. L^{-1} or 30 µmol. L^{-1} of H_2O_2 for 30 min (37ºC, 5% CO_2). The medium was then replaced with 100 µL of 0.5 mg/mL MTT (Sigma-Aldrich Co., USA) solution and incubated for 3 hours at (37 °C, 5 % CO_2). The MTT solution was removed and 200 µL of dimethyl sulfoxide (DMSO, Synth, Brazil) was added to each well. Pates were shaking for 30 min and the absorbance was measured at 570 nm in the Synergy H1 Hybrid Reader (Biotek Instruments Inc., USA).

Protein determination

Protein concentration of the samples was determined using a Bradford assay (Bradford,1976), with bovine serum albumin (BSA) as a standard. Proteins were concentrated according to Wessel and Flügge (1984). Briefly, 20 µg (total proteins) of each sample were submitted to protein precipitation with chloroform and methanol (1:3:1:3, sample: methanol: chloroform: destilated water) followed by centrifugation at 17,000 rpm/ 10 min. Supernatant was removed, and three volumes of methanol, were added. After, the liquid phase was discarded and the precipitate was maintained at room temperature to dry. Precipitates of concentrated proteins were resuspended in buffer urea (8 mol.L-1), tris (0.1 mol.L-1), and followed the digestion protocol adapted of Klammer and MacCoss (2006). Briefly, disulfide bonds were reduced by DTT 10 nmol L^{-1} addition (37ºC/20 min) followed by alkylation by IAA 50 nmol L^{-1} addition at room temperature, in the dark, 20 min. After, urea was diluted with 60µl of Tris 0.1 mol L-1 to a final concentration of 2 mol L-1. Then, proteins were digested with trypsin (1:50, enzyme: substrate) containing 1 mmol L^{-1} of calcium chloride ($CaCl_{2)}$) for 18 hours 37°C. The reaction was stopped using formic acid (5% v/v).

NanoLC LTQ-XL Orbitrap MS/MS.

Chromatography separations of tryptic peptide mixture were obtained by nanoLC Ultra (nanoLC Ultra 1D plus, Eksigent, USA) equipped with autosampler nanoLC AS-2 (Eksigent, USA) and connected to LTQ-XL Orbitrap Discovery MS/MS (Thermo Fischer Scientific, USA), containing a nano- electrospray ionization source (Thermo Fischer Scientific, USA). Analytical capillary columns (100 µm × 20 cm) and pre-columns (150 µm × 2 cm) was packaged *in house* with phase-reversed C18 (5 µm ODS-AQ C18, Yamamura Chemical Lab).

Hemolymph and chromatography fractions were loaded in an auto sampler with an injection volume of 10 µL and at a flow rate of 1 µL per mL for 15 min. Step gradient mobile phase A (5 % acetonitrile, 0.1 % formic acid in water) was used to chromatography separations of 120 min (fractions) or 360 min (hemolymph) to mobile phase B (90% acetonitrile, 0.1% formic acid): 0-5% B in 5 min; 5-25% B in 60 min; 25-50% B in 20 min; 50-80% B in 15 min; 80 % isocratic B for 5 min; 5 % B in 1 min; 5 % isocratic B for 14 min at a flow rate of 400 nL per min).

Positive ion mode was employed, and the normalized collision energy was set to 35 %. Full scan MS spectra were acquired from *m/z* 400 - 1,600 at a resolving power of 30,000, followed by acquisition of 8 MS2 mode spectra of the most abundant ions. Fragmentation was obtained by dissociation-induced collision (CID), with *Q* activation = 0.250, time of activation = 30 ms, and isolation amplitude = 1 Da. Acquired peptide masses were added to the dynamic exclusion list with a size of 100 ions by 30 s during acquisition of MS2 spectra. Spray voltage used was 2.2 kV, capillary temperature of 275 °C, capillary voltage of 34 V, and the collision gas used was helium.

Data analysis

For protein identification, a no-redundant sequences data bank referred to entries of individual proteins for "*Lonomia*" presents in NCBI data bank (http://www.ncbi.nlm.nih.gov) was constructed. From this bank, RAW files of MS-2 spectra were searched using *Comet* search software (Eng *et al.*,

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DOI:10.26502/jbb.2642-91280217

2013). Identification of proteins and peptides was obtained using *Pattern Lab for Proteomics* software from homology with data bank sequences (Carvalho *et al*., 2012). Candidate peptides were considered those containing one or two tryptic ends. Cysteine carbamido methylation was adopted as fixed modification. Tolerance of 50 ppm to precursors ions and 1 Da to fragment ions were employed on the data search. *Search Engine Processor* software (Carvalho *et al.,* 2012) was used to filter identified spectra. Parameters *Xcorr*, *DeltaCN*, *DeltaMass*, *Peaks Matched,* and *Spec Count Score* were used to generate a Bayesian score. Cut-off was established to accept 1% false-positive, based on reversed database identifications. Furthermore, 6 residues were adopted as the minimum sequence length. Results were post processed to set identifications less than 10 ppm of mass variation.

Statistical analysis.

Data were reported as means ± SD (standard deviation), and statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test ($p < 0.05$ was accepted as statistically significant). Experiments were conducted in quadruplicate from at least three independent experiments. Assays were analyzed by Prism 5 software (Graph Pad Software, USA).

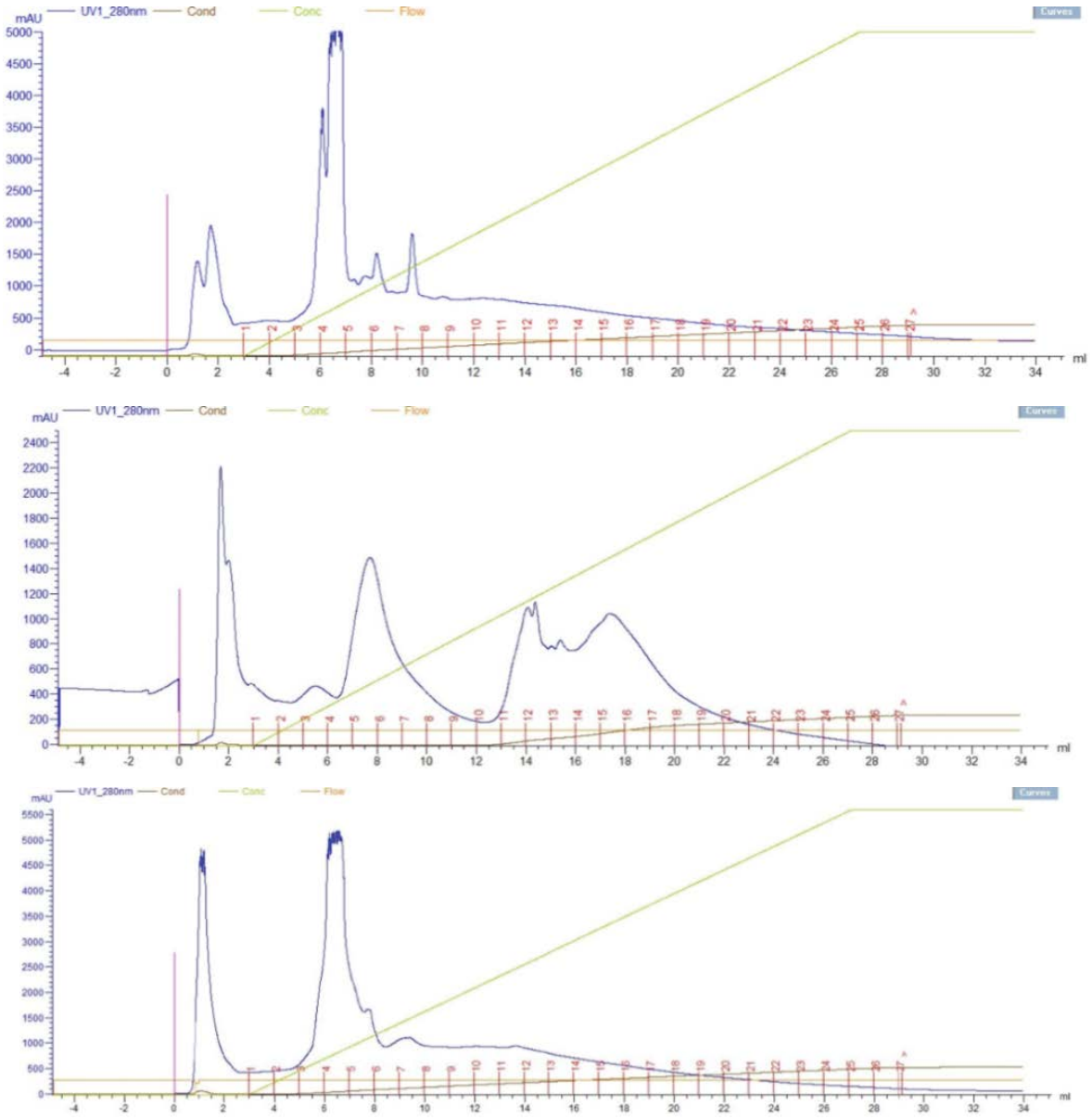

Figure 1: High-performance liquid chromatography (HPLC) with Akta Purifier; Resource Q anionic column of L. obliqua caterpillar hemolymph from 3 different lots (A: Lot 1; B: Lot 2; C: Lot 3). The column was eluted with 20 mmol L^{-1} Tris-HCl, 1 mol L^{-1} NaCl (linear gradient 0-100%) and a flow rate of 0.5 mL min^{-1}. The regions indicated in red show the peaks containing the fractions with anti-apoptotic activity.

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Results and Discussion

Hemolymph fractionation

Hemolymph from 3 different origins was fractionated on an AKTA Purifier chromatography system equipped with a Resource Q ion exchange column. **Figure 1** shows the electrophoretic profile obtained.

As can be observed, a significant qualitative and quantitative variations in electrophoretic profile was observed between the 3 different lot of hemolymph. As described by Whitmore and Gilbert (1974), and Roman and Jegorov (1991) the amino acids concentration and protein composition are related to insect age and stage of development, due the diet (Ortel, 1995) and ambient temperature (Roman and Jegorov, (1991) and Cui *et al.*, 2011). Thus, this can have determined differences in protein composition observed in the three lots of hemolymph.

The anti-apoptotic action was identified by us in the first region, similar that observed by Souza et al. (2005). This fraction has a pI of 6.7 (Martins 2011) and, due to its nearly neutral charge, does not exhibit binding affinity for the chromatographic column, causing it to elute earlier than other electrically charged proteins in the sample. This anti-apoptotic protein obtained by us (51kJ) differs in molecular mass from the anti-apoptotic protein found in others silkworms (*Bombyx mori Linnaeus,* 1758), which have a molecular mass of 30kJ (Choi et al., 2002; Kim et al., 2001; Rhee et al., 2013).

The fraction from batch 3 of hemolymph was chosen for testing in cell cultures because it showed the highest mAU.

Identification of proteins in the hemolymph and chromatographic fraction by NanoLC MS/MS

To Identification of proteins, studies were conducted using a gel-free proteomic in a nanoLC/LTQ- Orbitrap system to identify the proteins in the hemolymph. A total of 71 distinct proteins were identified in the caterpillars' hemolymph. In purified fractions, we found total 40 distinct proteins, being 9 shared between the 3 lots. 34 common proteins were observed between crude and fractions. The protein identified showed a molecular weights ranging from 2,107.1 to 71,540.4 Da. The results is showed at **Fig. 2**.

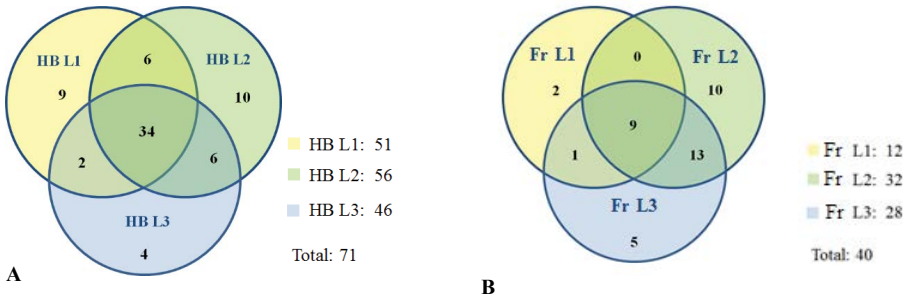

Figure 2: Venn diagram depicting the total number of proteins identified in 3 lots of hemolymph (HB) and chromatographic fractions (Fr), as well as the number of overlapped proteins.

Compared to the PF, a proportionately larger number of peptides was generated by crude hemolymph. The number of peptides produced by proteolytic digestion is proportional to the abundance of the protein in a sample (Liu *et al.,* 2004). Thus, peptides in larger quantities were selected for MS2 more frequently, generating a larger number of spectral counts, which is proportional to the abundance of each protein in the sample. Ideally, there should be more proteins in a crude fraction compared to a purified fraction, but in the case of Fl3 and the intersection of Fl2 and Fl3, the number was higher. This may have occurred due to protein breakdown during proteolytic digestion. Protein identification in *L. obliqua* samples is impaired by the limited range of data contained in the database for this species. Protein amino acid sequences found in databases are mainly from transcriptomic studies (Veiga *et al.*, 2005).

Serine proteases (45%), hemolins (38%), protease inhibitors (7%) and antiviral protein (5%) were found in PF, as well as sensory proteins, heat shock proteins, cysteine proteases and lectins. In hemolymph were found predominantly antiviral protein (24 %), serine proteases (23 %), hemolins (16 %) and protease inhibitors (10 %). Heat shock proteins, serpins, sensory proteins, oxidoreductases, lectins, lyases and ribonucleases also were identified. Other 20% proteins were identified as structural proteins or proteins involved in metabolic functions **(Table 1)**.

Table 1: Identified proteins on hemolymph and chromatographic fractions samples by *shotgun*. Based on spectral counts, the quantity of these proteins is presented on a color scale ranging from green to red.

Proteín	F L1	F L2	F L3	HB L1	HB L2	HB L3
Putative antiviral proteín	10	487	154	575	2.694	913
Putative defense proteín 1	0	141	28	11	81	15

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Heat shock protein 1	0	0	0	16	0	0
Heat shock protein 3	0	0	0	28	0	0
Heat shock protein 4	0	0	74	368	97	54
Sensory protein 1	0	131	187	67	96	36
Sensory protein 2	0	7	18	0	8	5
Hemolin (ABF21073.1)	204	569	601	61	423	154
Hemolin (ABF21071.1)	176	733	482	78	589	204
Hemolin (ABF21070.1)	196	604	571	98	402	153
Hemolin (ABF21072.1)	0	561	595	97	409	0
Actin 1	0	0	0	84	85	32
Myosin light chain 1	0	0	0	4	4	0
Tropomiosin 1	0	0	6	0	10	3
Stretch factor 1	5	0	0	59	34	5
Ferritin 1	0	5	0	0	6	20
Ribosomal protein 4	0	0	0	3	0	0
Ribosomal protein 5	0	0	0	3	0	0
Ribosomal protein 7	0	0	0	13	4	5
Ribosomal protein 11 (subunit larger)	0	0	0	2	0	0
Ribosomal protein (subunit S19)	0	0	0	5	0	0
Cuticle protein 1	0	0	0	0	4	0
Cuticle protein 4	0	0	0	0	7	0
Proteín LIM 1	0	0	0	3	0	3
Serine protease 1	0	0	0	5	0	0
Putative serine protease-like proteín 2	0	27	0	22	34	50
Serine protease 4 (partial)	0	0	0	0	9	20
Serine protease 6	0	0	0	0	3	0
Serine protease 7	0	4	0	0	8	0
Prophenoloxidase activating fator 1 (partial)	0	12	172	100	123	189
Protease inhibitor 1	0	0	0	0	2	8
Serine protease inhibitor 3/4 (Serpin - 3/4)	13	246	106	140	359	154
Serine protease inhibitor 4 (partial)	0	241	156	136	363	160
Putative protease inhibitor 4	0	18	0	0	11	0
Protease inhibitor 5 (partial)	0	0	0	0	1	0
Protease inhibitor 6	0	0	1	0	0	0
Protease inhibitor 7	13	13	42	6	33	9
Serpin 1	0	0	0	33	54	34
Serpin 2	0	0	0	32	47	33
Putative cystatin precursor 1	2	58	40	36	124	106
Lipocalin 1	6	1.148	1.026	574	914	424
Lipocalin 4	0	909	808	0	757	0
Lopap (Lipocalin-1/4; Prothrombin activator)	0	0	1.025	0	0	415
Lectin 1 putative imunolectin	0	0	0	3	3	0
Lectin 3	0	0	0	2	3	12
Lectin 4 type C	0	0	0	6	0	0

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Lectin 5	0	10	0	9	7	6
Hypothetical protein	0	0	0	0	4	0
Hypothetical protein 2	0	0	0	38	10	3
Hypothetical protein 3	0	0	0	32	33	46
Hypothetical protein 4	0	4	0	0	0	0
Hypothetical protein 7 (partial)	0	0	0	3	0	0
Hypothetical protein 9 (partial)	0	0	0	15	6	0
Hypothetical protein 10 (partial)	0	0	0	33	65	1
Hypothetical protein 11 (partial)	0	0	0	44	12	2
Hypothetical protein 12 (partial)	0	3	7	9	18	10
Hypothetical protein 13	0	2	0	333	103	46
Hypothetical protein 21 (partial)	0	3	0	0	0	0
Hypothetical protein 22 (partial)	2	0	0	39	745	51
Hypothetical protein 23 (partial)	0	0	0	9	9	0
Hypothetical protein 31	0	49	0	307	502	421
Hypothetical protein 38	0	3	7	0	11	0
Unknown proteín 3	0	2	3	3	7	7
Unknown proteín 4	0	4	0	1	3	0
Unknown proteín 6	0	1	0	0	0	0
Prothrombin activator	0	0	890	0	0	0
Putative secreted peptíde 30	0	0	0	0	28	0
Translarionally controlled tumor protein homologue TCTP)	0	0	0	72	19	38
Esterase-type protein 1	0	5	11	4	7	8
Cysteíne peptidase 1 type catepsin-L (partial)	0	0	0	6	6	4
Cysteín peptidase 2 type catepsin-B	0	7	25	0	15	12

F L1-3: Fractions from batches 1, 2, or 3. **HB L1-3:** Crude hemolymph from batches 1, 2, or 3.

The values marked in green represent the absent proteins, in yellow the least abundant, in red the most abundant, and in shades of increasing intensity between yellow, orange, and red those of intermediate abundance, from the least to the most abundant, respectively.

The greater the abundance of a protein in a sample, the greater the number of peptides of that protein that will be produced from proteolytic digestion. Therefore, peptides present in greater quantities in the sample were selected for MS2 more frequently, generating a greater number of spectral counts. In this way, spectral counts allow for label-free quantification, and the number of spectral counts for each protein is proportional to its abundance in the sample (LIU et al., 2004). In the label-free approach, each sample is analyzed in individual LC-MS/MS acquisitions, and the abundance of peptides is then aligned between different chromatographic runs, since the same peptide may not be present in the same MS1 scan in different runs, producing discrepancies in retention times. In the present study, serine proteases (45%) and hemolins (38%) were predominantly identified in the chromatographic fraction, followed by protease inhibitors (7%) and antiviral protein (5%). Sensory proteins, heat shock proteins, cysteine proteases, and lectins, among others, were also identified, albeit less expressively.

Furthermore, other proteins together represented 20% of the total, and generally refer to structural proteins or those involved in metabolic functions. The distribution of protein groups identified in hemolymph and fractions is presented in the **Figure 3**

In this study, the number of hemolins stands out, which are related to the immune system and the development of lepidopterans. These proteins are capable of binding lipopolysaccharides, being upregulated in the hemolymph during insect infections, as well as during metamorphosis (Alvares-Flores et al., 2011). Serine proteases and protease inhibitors are among the major proteins, agreeing with the findings of Terra (2010). The creation of the database was a strategy used to reduce the search space and optimize the protein identification process; thus, it was built from the entries for the genus "Lonomia" in the NCBI (Resende, 2013).

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DOI:10.26502/jbb.2642-91280217

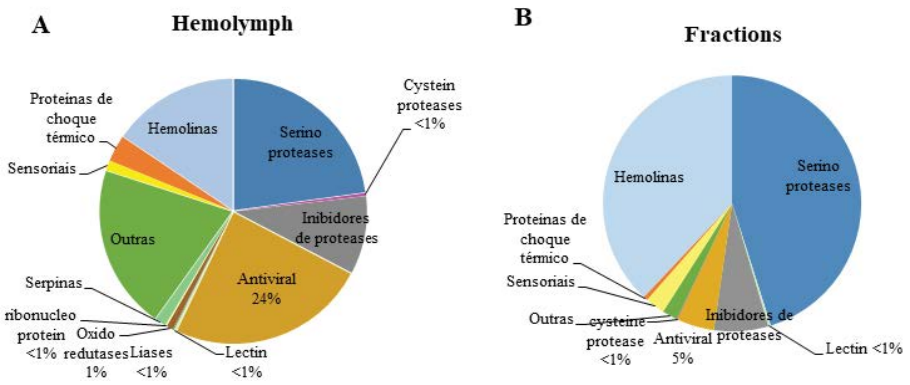

Figure 3: Distribution of proteins identified in samples A: **Hemolymph** and B: **Fractions**, by shotgun. Semi-quantification based on the number of spectral counts.

In quantification using a label-free approach, data normalization is important so that interferences arising from technical variabilities such as electrospray instability, ionic suppression of samples, changes in orbitrap ion filling, among others, are minimized and do not significantly affect the result. *Lonomia obliqua* does not have a sequenced genome, and the amino acid sequence data for proteins found in databases are mainly derived from transcriptomic studies (Veiga et al., 2005). Therefore, the identification of proteins in insect samples using a shotgun approach is hampered by the limited scope of data contained in databases for this species. There is no formal definition or consensus that clarifies the differences between hypothetical, putative, and unknown proteins. Still, it is assumed that the former represent amino acid sequences for which there is no experimental proof that they actually constitute proteins, while the latter are recognized as proteins, but for which experimental proof of function is lacking. Finally, unknown proteins are those proteins for which no function is known (Liberman, 2004)

Hemolymph

Cytotoxicity of hemolymph and fraction in primary hippocampal embryonic culture

In this study, primary hippocampal cell cultures were exposed to different concentrations of crude hemolymph and its fraction for different periods. The result obtained allowed us to establish the concentrations and exposure times that did not reduce viability in the cell type under study regarding the anti-apoptotic effect. Crude hemolymph produced a significant reduction ($p<0.05$) in mitochondrial metabolism compared to the control after exposure for 1 h at a concentration of 5%, (**Figure 4 A).** This reduction was greater ($p<0.001$) for concentrations of 0.25, 0.5, 1.0 and 2.0% when the exposure time was 2 h (**Figure 4 B).** The purified fraction caused a significant reduction ($p<0.05$) in cell viability compared to the control at concentrations of 0.5, 1.0, and 2.0% after 1 h of exposure (**Figure 4 C).** After 2 h of exposure, concentrations of 1.0 and 2.0% caused a significant reduction in viability ($p<0.05$ and $p<0.01$, respectively) (**Figure 4 D).**

Based on the results obtained, the concentrations chosen for the treatment of primary hippocampal embryonic cell cultures in this work were 0.05 and 0.1% (v/v) for both crude hemolymph and the purified fraction, since these did not produce a significant difference in cell viability compared to the control. The 1% concentration described in previous studies with other cell types proved to be toxic to primary hippocampal cells, with a significant reduction in cell viability compared to the control.

Figure 4 shows the results of cell viability, by MTT reduction method assays, using cells treated with whole hemolymph or its fractions, for 1 or 2 hours of contact.

In this study, primary cell cultures were used. These cells were removed from their physiological environment and adapted for survival in culture for short periods, thus making them more sensitive than immortalized cell lines. For this reason, in this work, we tested lower concentrations than those previously described regarding hemolymph toxicity. Maranga et al. (2003) obtained maximum viable cell concentrations 2 to 3 times higher than the control when Sf-9 insect cell cultures were supplemented with 1% hemolymph (v/v). The authors tested concentrations of 1, 2, and 5% (v/v), with the 5% concentration showing a toxic effect. The anti-apoptotic effect in Sf-9 cell cultures obtained by Souza et al. (2005) also used a 1% hemolymph concentration. A 1% concentration was also used in a study of CHO-K1 cell supplementation, where hemolymph similarly provided positive effects to the culture, such as increased maximum cell concentration and maximum productivity.

Therefore, as can be observed in **Figure 4,** when 5% of hemolymph was used, a higher cell growth was obtained. The mechanism by which hemolymph exerts its protective effect on increasing and prolonging cell viability in culture has not yet been fully elucidated. Its interaction with cellular

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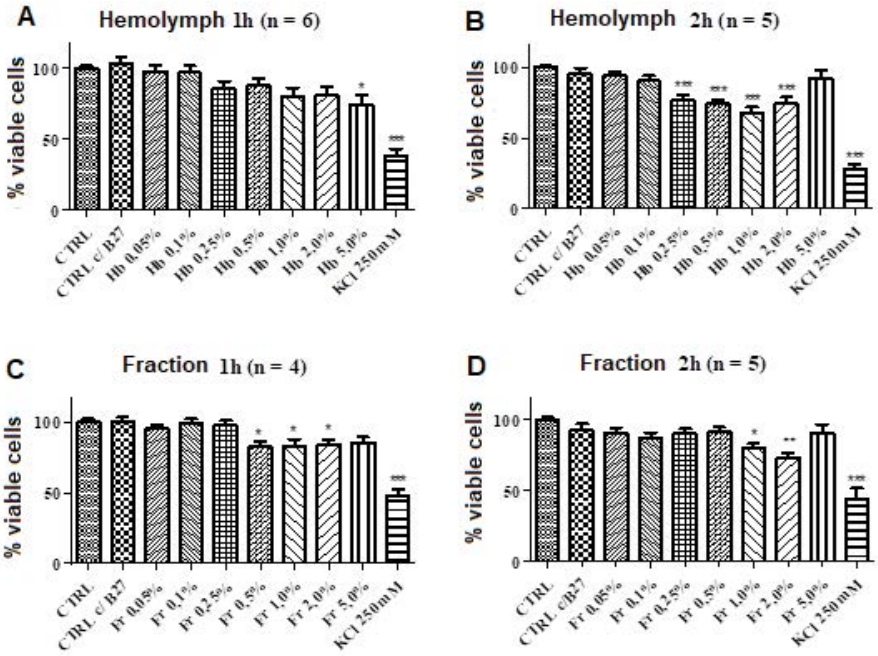

Figure 4: Cell viability at different concentrations of crude hemolymph and fraction purified by the MTT reduction method in primary embryonic hippocampal culture. CTRL: Control; Hb: Crude Hemolymph; Fr: Fraction (ANOVA and Newman-Keuls multiple comparison, *p< 0.05; **p<0.01 and ***p<0.001 in relation to the control).

receptors, which can vary qualitatively and quantitatively under numerous conditions, including tissue of origin, species, and stimuli from the cellular environment, remains unknown.

Isolated neurons in culture have been used as a model for investigating the pharmacological mechanisms of toxin action in vivo (Silva et al., 2006). Carvalho et al. (2014 b) used primary hippocampal cultures from rats to evaluate the neurotoxicity produced by phospholipases from snake venom and found that it produces cell death with mixed characteristics of apoptosis, necrosis, and autophagy. Neurotoxicity in hippocampal neurons in culture was also investigated in a study by Garcia et al. (2012). The authors verified neurotoxicity in cells treated with cocaine and a product of its pyrolysis, with the neurotoxic potential being greater for the latter. The neuroprotective action of molecules can also be evaluated in hippocampal neuronal cultures, as in the study developed by He et al. (2014), which verified this effect with ginsenoside Rg1. In our studies, primary embryonic hippocampal cultures showed a normal aspect, even after 7th day of culture, with the presence of neuritic branching. This data is important to demonstrate that there are no deleterious effects on the cells even after 7 days of cultivation.

Figure 5 shows microscopic images at different stages of differentiation. Cell viability assays were performed on the 7th day of culture, where the presence of neuritic branching could be observed.

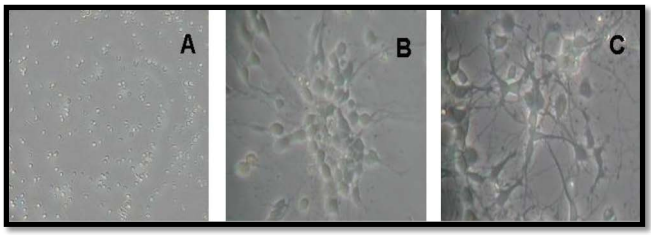

Figure 5: Embryonic hippocampal cells from rats in culture, with different stages of differentiation. A: day 0 of culture; B: 3rd day of culture; C: 7th day of culture

Protector effect of crude hemolymph and its purified fraction

Earlier, we showed a potent anti-apoptotic effect in Sf-9 cell cultures at 1% hemolymph concentration Souza et al., 2005. Here, the protective effect against cell death of crude hemolymph was tested in hippocampal neurons in culture using 0.05% or 0.1% of the hemolymph against apoptosis inducer (H_2O_2 10 µmol L^{-1} or 30 µmol L^{-1}, for 1 or 24 h). The results obtained show that the mitochondrial metabolism was reduced (p < 0.01-0.001) when the cells were treated with 10 µmol L^{-1} or 30 µmol L^{-1} of H_2O_2. A slight reduction in the apoptotic effect (induced by H_2O_2 10 µmol L^{-1}) was observed when cultures were pre-treated for 1 hour with 0.05 or 0.1% crude hemolymph (**Figure 6A**). This reduction was not observed after 24 hours of treatment with hemolymph **(Figure 6B).**

Treated cells with 0.05% and 0.1% of purified fraction (PF) for 1 h or 24 hours, respectively, did not produce a

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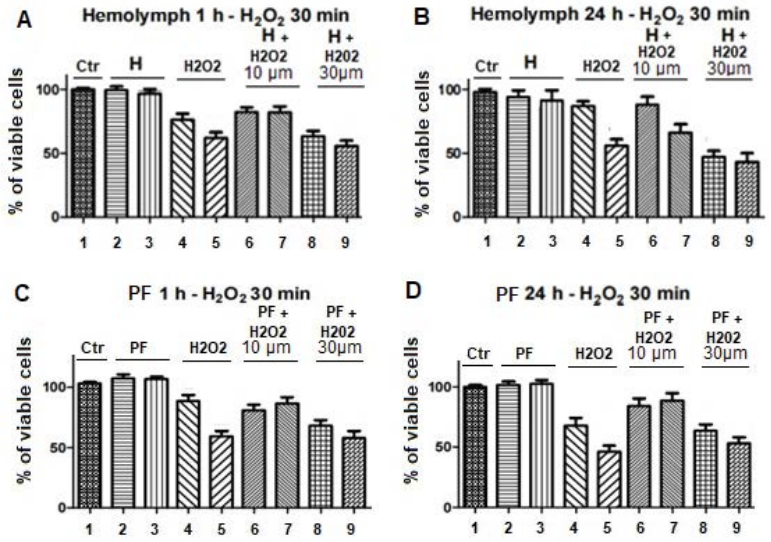

Figure 6: Cell viability by MTT assay. (n= 5 wells/group and three independent assay) **CTR** (control cells), **H** (hemolymph at concentration of 0.05 or 0.1 % v/v), **H_2O_2** (Positive control at concentration of 10 or 30 µM), **PF** (purified fraction). mmm

A (1 hour) and **B** (24 hours). 1) Control; 2) hemolymph 0.05%; 3) hemolymph 0.1%; 4) H_2O_2 10µm 5) H_2O_2 30µm 6) hemolymph 0.05% + H_2O_2 10µm; 7) hemolymph 0.1% + H_2O_2 10µm 8) hemolymph 0.05% + H_2O_2 30µm; 9) hemolymph 0.1% + H_2O_2 30µm.

C (1 hour) and **D** (24 hours). **1)** Control; **2)** PF 0.05%; **3)** PF 0.1%; **4)** H_2O_2 10µm **5)** H_2O_2 30µm **6)** PF 0.05% + H_2O_2 10µm; **7)** PF 0.1% + H_2O_2 10µm **8)** PF 0.05% + H_2O_2 30µm; **9)** PF 0.1% + H_2O_2 30µm ANOVA AND Newman-Keuls multiple comparison (p* <0.05, p <0.01 compared with CTRT and intergroup comparison).

reduction in cell viability ($p < 0.001$ and $p < 0.01$, respectively) when compared to untreated control. This demonstrates that 0.05% and 0.1% PF for 1 or 24 hours are not toxic to cells. As observed with crude hemolymph, no significant difference was obtained regarding the protective effect of hemolymph when cultures were pretreated with hemolymph for 1 hour (**Figure 6C**). However, a statistically significant protective effect was observed when cultures were pre-treated for 24 hours with purified hemolymph fractions (with 10 µmol L-1 or 30 µmol L-1 H_2O_2. ($p < 0.05$ and $p < 0.01$, respectively). This demonstrates that, under these conditions, PF is not toxic and protects rat hippocampal neuron culture from cell death by H_2O_2 (10 µmol L-1 or 30 µmol L-1) **(Figure 6D).**

Several studies have shown the property of substances from insect hemolymph in the inhibition of apoptosis promoted by different inducers (physical, chemical and biological), in insect and mammal cell cultures (Rhee *et al.*, 2013; Vieira *et al.*, 2010; Mendonça *et al.*, 2008; Souza *et al.*, 2005; Maranga *et al.*, 2003; Choi *et al.*, 2002; Kim *et al.*, 2001; Rhee and Park, 2000).

The effect in a variety of cells, opposite to the apoptosis induction by multiple mechanisms, suggests that it may act in some apoptosis-conserved way (Heinen *et al.*, 2014).

Earlier, we observe an intensive anti-apoptotic activity in Sf-9 insect cells treated with *Lonomia obliqua* hemolymph (Souza *et al.*, 2005; Maranga *et al.*, 2003; Vieira *et al.*, 2010), in HEK-293 human cells (Mendonça *et al.*, 2007), and in mammalian cells V-79 (Heinen *et al.*, 2014). Mendonça *et al.* (2007) also observed that exposure to hemolymph and chromatographic fractions produced high electrochemical potential in mitochondrial membrane maintenance.

Recently, Mendonça and Martins **(2022)** showed that the antiapoptotic effect of *Lonomia obliqua* hemolymph is associated with the mitochondria patway by preventing the release of proteins like cytochrome c from the mitochondria into the cytosol. This action is a key part of the intrinsic apoptotic pathway, which is often triggered by cell stress and leads to cell death if not regulated. Complementing this information, Carvalho et al (2025) demonstrate that, a protein with anti-apoptotic action in the hemolymph of caterpillars of the megalopygidae family, acts by maintaining the structure of the cellular cytoskeleton. This allows for the maintenance of the cellular structure and its functionality.

In vitro studies, treatment of neuronal cell cultures with low concentrations of H_2O_2 produces cell death by apoptosis, which allows its use as an experimental model for studying mechanisms of neuronal toxicity and protective strategies against oxidative damage involved in neurodegenerative conditions (Whittemore et al., 1995; Chen et al., 2009). This study describes for the first time the evaluation of the effect of hemolymph and *L. obliqua* fraction on the cell viability of primary cultured neuronal cells. Preliminary results reveal that treatment of primary embryonic cultures of hippocampal neurons from wistar rats with chromatographic fraction for 24 h, subsequently exposed to H_2O_2 for 30 min, showed greater

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cell viability compared to the positive control, treated only with the inducing agent H_2O_2, but had a significant reduction in mitochondrial metabolism when compared to the control without oxidative induction. The other treatment conditions and concentrations tested did not produce evidence that hemolymph and its fractions had a neuroprotective effect.

The death of neuronal cells observed in neurodegenerative conditions has been associated with damage from oxidative stress, resulting from the observation of high concentrations of H_2O_2 in brain regions involved in these conditions, such as in Alzheimer's and Parkinson's diseases, in which neurons of the hippocampus, cerebral and frontal cortex, and dopaminergic neurons of the substantia nigra are affected, respectively (Whittemore et al., 1995; Mattson, 2000; Giasson et al., 2002).

In the hemolymph of *L. obliqua*, the presence of an anti-apoptotic protein has been described, which promoted increased cell viability in Sf-9 cultures treated with 1% (v/v) hemolymph or chromatographic fraction, subsequently subjected to nutrient deprivation and treatment with the apoptosis-inducing agent actinomycin D (Souza et al., 2005). The same behavior was observed when Maranga et al. (2003) used hemolymph and chromatographic fractions (1% v/v) as a supplement to culture media in Sf-9 cell cultures. Supplementation of the culture medium with hemolymph or semi-purified fractions (1% v/v) for 1 h also promoted protection against cell death induced by oxidative stress with tert-butyl hydroperoxide (t-BHP), H_2O_2 and baculovirus infection in Sf-9 cell culture (Vieira et al., 2010).

Mendonça et al. (2007) verified the anti-apoptotic effect of hemolymph and fractions (1% v/v) in insect and human cell cultures (Sf-9 and HEK-293, respectively) after treatment with the apoptosis inducers t-BHP, H_2O_2, and baculovirus. In the same study, the authors showed that treatment with hemolymph and fractions maintained a high electrochemical potential in the mitochondrial membrane of the cells, thus preventing an increase in its permeability and the consequent release of cytochrome c into the cytosol, inhibiting signaling for this apoptosis pathway and resulting in the prolongation of cell life. Therefore, this study suggests that the anti-apoptotic action of hemolymph and fraction may be associated with a direct action on mitochondrial membranes.

Heinen et al. (2014) observed that the aqueous extract of *Lonomia obliqua* bristles promoted an increase in cell viability and proliferation of U-138MG (human glioma) and HT-29 (human colorectal cancer) cell lines in culture. On the other hand, they observed a drastic decrease in the viability of the V-79 cell line (Chinese hamster fibroblasts). Furthermore, the same authors found that the purified anti-apoptotic protein, as described by Souza et al. (2005), produced a strong protective effect on V-79 cells, which was not observed in U-138MG and HT-29 cells exposed to the same treatment. This study highlights that cell behavior differs depending on the cell type exposed to the same treatment with hemolymph and anti-apoptotic protein, suggesting that this is attributed to different receptors and/or cell signaling pathways involved.

The protein composition of *Lonomia obliqua* hemolymph from different regions or diets can present qualitative and quantitative variations. The concentration of free ions naturally present in the hemolymph can similarly differ between animals and cause shifts in the elution of specific proteins during ion-exchange chromatography, resulting in non-reproducible elution patterns in the fractions. Therefore, the proteins present in the fraction used in this study may differ from those described in the literature using the same fractionation strategy. Furthermore, there may be synergistic interaction between substances present in the hemolymph, which can contribute to a greater or lesser effect.

Recently, we have showed that proteins with anti-apoptotic action in the hemolymph of caterpillars of the *Megalopygidae* family acts by maintaining the structure of the cellular cytoskeleton. They observed that supplementation of cell cultures with hemolymph was sufficient to inhibit cell death by apoptosis induced by different inducers such as terbutyl, actinomycin D, hydrogen peroxide, or even by nutrient depletion. The protein responsible for anti-apoptotic action was isolated and the anti-apoptotic effect was tested in VERO and SF-9 cells. The protector effect blocked and attenuated the disruption of the cytoskeleton (actin filaments), being that the protective effect also was observed on the integrity of the mitochondrial membrane of SF-9 cells pre-treated with both hemolymphs and treated with the apoptosis inducer by acting on the mitochondrial pathway of death by apoptosis, and by maintaining the structure of the cytoskeleton and cellular functions, pathway that can cause disorders and diseases neurodegenerative, the substances present in the hemolymph of these and other caterpillars could be good candidates in studies for the treatment of neurodegenerative diseases such as Parkinson's disease and Alzheimer's (Carvalho et al., 2025).

Insects undergo a metamorphosis process where the combination of growth signaling, activation, differentiation, and physiological apoptosis in specific tissues is highly regulated. Substances responsible for this signaling likely circulate in their hemolymph, making their study interesting from the perspective of identifying bioactive compounds with biotechnological potential. In this context, different stages of animal development may present biological fluids with different properties (Maranga et al., 2003).

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Total hemolymph contains proteins with both, anti-apoptotic and pro-apoptotic activity. This balance between the quantities of the two, depends on the caterpillar's developmental stage. The possible presence of a pro-apoptotic protein in the total hemolymph under test could explain the weaker effect observed when crude hemolymph was used. When the hemolymph was purified, the presence of this probable protein was eliminated, allowing a more intense anti-apoptotic effect to be observed.

One point to highlight is that most studies with *Lonomia obliqua* hemolymph have been conducted at concentrations of 1 to 5% (v/v). In our study, the concentration was 0.05 or 0.1% (v/v). Even at this lower quantity, the hemolymph was able to inhibit cell death in neural cells, demonstrating the potential of these molecules against neurodegenerative diseases. These low concentrations were used because neural cells are more sensitive than established mammalian or insect cell lines. Further studies are being conducted to explore higher concentrations of these proteins, allowing for a more intense protective effect. As already mentioned above, in previous studies, we have demonstrated that the hemolymph of *Lonomia obliqua* possesses proteins with potent anti-apoptotic activity. Although the MTT assay used in this study is a standard technique for determining cell death, it does not necessarily indicate that the death observed in the test is due to apoptosis. Therefore, tests with specific apoptosis markers will have to be performed to confirm that the protection observed in this study in the neural cells is indeed due to the anti-apoptotic proteins present in the hemolymph of *Lonomia obliqua.* In any case, the important thing is that hemolymph does indeed have a protective effect, regardless of the type of death in question.

Conclusion

As demonstrated in this study, the composition of insect hemolymph such as that of *L. obliqua,* can exhibit both qualitative and quantitative intraspecific variations. These differences are influenced by the geographic origins of the animals and the environmental factors to which they are exposed. So, proteins present in the hemolymph used in this study may differ over those described in the literature using the same fractionation strategy. This study is the first to report the potential neuroprotective effect of *Lonomia obliqua* hemolymph on the viability of primary neuronal cell cultures subjected to oxidative stress for 30minutes. This effect was effective when purified fraction was used, after 24 hours of pre-treatment. Further studies are needed to reduce the sensitivity of neural cells to hemolymph, in order to allow the use of higher concentrations and thereby increase the protective effect.

Conflict of interest statement

The authors have declared that there is no conflict of interest.

Funding

This work was supported by Coordination for the Improvement of

Higher Education Personne (CAPES)

Acknowledgments

The authors gratefully aknowlege the support of Dr. Lisete M. Lorini, from University of Passo Fundo, Brazil, the Rio Grande do Sul Toxicology information Centre, Porto Alegre, Brazil, Dr. Mariana R. Ely and

Dr. Edegar Fronza, from University of Caxias do Sul, Brazil.

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Citation: Silviane Maggi, Antônio Frederico Michel Pinto, Mariana Sayuri Berto Udo, Mariana Aguilera Alencar da Silva, Raphael Caio Tamborelli Garcia, Luciane Minetto, Leandro Tasso, Pablo Machado, Diógenes Santiago Santos, Paula Eichler, Thiago Barcellos, Pedro Ismael da Silva Junior, Tania Marcourakis, Ronaldo Zucatelli Mendonça, Sidnei Moura. Proteomic Analysis of the Hemolymph of the *Lonomia Obliqua* Caterpillar and its Protective Effect Against Hippocampal Neuronal Death Induced by Oxidative Stress. Journal of Biotechnology and Biomedicine. 9 (2026): 126-139.

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