# MOLECULAR EVIDENCE OF PHOSPHOLIPASE $A_2$ ($PLA_2$) IN THE VENOM OF *Loxosceles similis*

# *EVIDÊNCIA MOLECULAR DE FOSFOLIPASE $A_2$ ($PLA_2$) NO VENENO DE Loxosceles similis*

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**ABSTRACT**

Brown spiders of the genus *Loxosceles* (Araneae, Sicariidae) occur in temperate and tropical regions. Their bites can cause loxoscelism, a condition classified by the WHO as a neglected public health problem. In Brazil, where species of medical importance are concentrated in the South and Southeast regions, cases have been increasing. Although *Loxosceles similis* is associated with underground environments, it also occurs in surface areas and even in homes, suggesting potential endemicity. Caves, especially tourist caves, can harbor the species, posing a risk of accidents. The bite is usually painless at first, and pain arises after 2 to 4 hours, which often delays treatment. In this study, with the aim of locating the main components of *L. similis* venom, we analyzed the transcriptome of the venom gland and identified a Phospholipase $A_2$ ($PLA_2$) coding sequence, the first record of this type in *Loxosceles*. The presence of this sequence indicates that the venom may contain additional enzymes besides Loxtox family, potentially involved in tissue damage, inflammation, and systemic manifestations. These results expand our knowledge about the composition of the venom and the molecular mechanisms of loxoscelism.

**KEYWORDS:** *Loxosceles similis*, Brown Spider, Phospholipase $A_2$, $PLA_2$, $LsPLA_2$-1.

**RESUMO**

As aranhas marrons do gênero *Loxosceles* (Araneae, Sicariidae) ocorrem em regiões temperadas e tropicais. Suas picadas podem causar o loxoscelismo, uma condição classificada pela OMS como problema de saúde pública negligenciado. No Brasil, onde as espécies de importância médica se concentram nas regiões Sul e Sudeste, os casos têm aumentado. Embora *Loxosceles similis* seja associada a ambientes subterrâneos, também ocorre em áreas de superfície e até em residências, sugerindo potencial endemicidade. Cavernas, especialmente as turísticas, podem abrigar a espécie, representando risco de acidentes. A picada é geralmente indolor no início, e a dor surge após 2 a 4 horas, o que frequentemente atrasa o tratamento. Neste estudo, com o objetivo de localizar os principais componentes do veneno de *L. similis*, analisamos o transcriptoma da glândula de veneno e identificamos uma sequência codificadora de Fosfolipase $A_2$ ($PLA_2$), sendo o primeiro registro desse tipo em *Loxosceles*. A presença dessa sequência indica que o veneno pode conter

enzimas adicionais além das Loxtox, potencialmente envolvidas em danos teciduais, inflamação e manifestações sistêmicas. Esses resultados ampliam o conhecimento sobre a composição do veneno e os mecanismos moleculares do loxoscelismo.
**PALAVRAS-CHAVE:** *Loxosceles similis*, Aranha marrom, Fosfolipase $A_2$, $PLA_2$, $LsPLA_2$.

## INTRODUCTION

Spiders of the genus *Loxosceles*, popularly known as brown spiders, belong to the family Sicariidae and form a group comprise approximately 148 accepted species[1] found in regions with tropical and temperate climates in the America, Oceania, Asia, Africa and Europe. *Loxosceles* spiders are considered one of the most dangerous groups of spiders worldwide[2], and accidents involving this genus are considered a public health problem in South America, including Brazil, with the South and Southeast regions considered endemic for loxoscelism[3].

In Brazil, the number of accidents involving spiders of the genus *Loxosceles* has increased over the years, despite being underestimated because of flaws in the notification system[4], owing to a lack of information and awareness among the population and health professionals. In 2024, 9,657 accidents involving spiders of this genus were reported, and the fatality rate, calculated as the ratio of deaths to notifications, increased from 0.08% in 2014 to 0.39% in 2024[5]. Loxoscelism is a clinical condition resulting from poisoning by spiders of the genus *Loxosceles*. It can lead to serious clinical symptoms, mainly due to dermonecrotic lesions arising from the disruption of connective tissue by venom components that affect homeostasis, as has already been demonstrated for *Loxosceles similis*[6,7] and other *Loxosceles* species[8–10].

Systemic loxoscelism is characterized by intravascular hemolysis, platelet aggregation, and acute kidney injury[11]. Clinical manifestations such as oliguria and dark urine indicate extensive hemolysis and often precede acute renal failure, which is the main cause of loxoscelism-related mortality[4]. Kidney injury arises from the rupture of red blood cells induced by toxins from the Loxtox family, releasing

hemoglobin that is filtered by the renal glomeruli and precipitates within the tubules, leading to obstruction of urinary flow[12–14].

*L. similis* is one of the 21 species of *Loxosceles* that occur in Brazil[1], commonly found inhabiting caves, including tourist-accessible sites such as the 'Gruta da Lapinha' in Minas Gerais State. In 2001, Andrade *et al*. warned about the risk of loxoscelism in tourist caves in Mato Grosso do Sul, owing to the presence of *L. similis*[15]. In human dwellings, a study published by our group in 2005, documented the presence of *L. similis* inside residences in Belo Horizonte[16], located in the southeastern region of Brazil. The variety of habitats in which these species live, contributes to this expansion and makes them potentially synanthropic, increasing the risk of loxoscelism.

*Loxosceles* spider venom contains bioactive components such as Loxtox, metalloproteinases, hyaluronidases, and insecticidal toxins[17]. The presence and significant role of secreted phospholipase 2 (sPLA2) in other arthropod venoms, such as those of scorpions and honeybees[18,19], underscores the potential biological importance of characterizing these enzymes in spider venoms. The venom of *L. similis* has hemolytic activity, causing the direct lysis of human erythrocytes in a concentration-dependent manner[20]. Although enzymes from the Loxtox family are recognized as the primary toxins responsible for hemolysis in *Loxosceles* venom, the role of sPLA2 remains unclear. Given the established importance of sPLA2 in the venom of bees and scorpions, where it contributes to membrane disruption and hemolysis, it is plausible that sPLA2 participate in the hemolytic mechanisms of *L. similis* venom, either independently or synergistically with Loxtox, warranting further investigation.

Secreted isoform hvPLA$_2$ is abundant in honeybee venom (hv) and contributes to membrane disruption, inflammatory responses, and allergenic effects[19,21]. Similarly, scorpion sPLA$_2$, often exhibit unique structural adaptations, as observed in Phaiodactylipin (*Anuroctonus phaiodactylus*)[22], HmTx (*Heterometrus laoticus*)[23] and Sm-PLGV (*Scorpio maurus*)[24]. PLA$_2$ displays diverse biological activities, including potent hemolytic effects on mammalian

erythrocytes, membrane disruption through phospholipid hydrolysis, induction of muscular inflammation, anticoagulant properties, high toxicity toward arthropods, and modulation of muscle excitation and calcium signaling via the inhibition of ryanodine binding to skeletal muscle $Ca^{2+}$ channels[25]. The ryanodine receptor (RyR) plays a crucial role in neurons and, is being essential for calcium-dependent signaling, synaptic plasticity, and neuronal development. Toxins that inhibit ryanodine binding to RyRs can modulate muscle and neural excitation, impair signal transmission, and elicit neurotoxic effects. These enzymes are crucial for the effectiveness and potency of the venom in these species.

The large subunit structure of HmTx is highly similar to *Pandinus imperator* Imperatoxin I (IpTx(i)) and to *Mesobuthus tamulus* $PLA_2$ ($MtPLA_2$). The three-dimensional structure of HmTx consists of three conserved alpha-helices: h1 (Lys24-His34), h2 (Cys59-Asp71), and h3 (Ala80-Phe89). The beta-sheet consisted of a single-stranded anti-parallel beta-sheet (b1.1 at Glu43-Lys45 and b1.2 at Lys48-Asn50) that was highly similar to the conserved sequences (-CGXG-, -CCXXHDXC- and -CXCEXXXXXC-) of *Apis mellifera* (bee) phospholipases.

Partial characterization of the venom from *Phoneutria boliviensis* was conducted, with emphasis on the detection of $PLA_2$ activity, which has not previously been described for this genus. The presence of $PLA_2$ was confirmed through specific enzymatic assays, in which the venom and two fractions obtained using Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) demonstrated the ability to hydrolyze the substrate 4-nitro-3-octanoyloxy-benzoic acid, indicating significant $PLA_2$ activity. This finding was further supported by the pronounced calcium-dependent indirect hemolysis observed in laboratory tests, a classic indicator of $PLA_2$ presence in animal venom[26]. In this context, the role of $PLA_2$ in envenomation was confirmed by the indirect hemolytic activity of the venom, specific hydrolysis of the synthetic $PLA_2$ substrate, and identification of active fractions using RP-HPLC.

In this study, we explored the transcriptome of the L. similis venom gland and identified a sequence that potentially encodes $PLA_2$. To the best of our

knowledge, this is the first report of a $PLA_2$ transcript from the *Loxosceles* genus. This discovery indicates that the venom of *L. similis* may contain enzymatic components beyond the well-known Loxtox family[7,27], which could play a significant role in exacerbating local tissue injury, driving inflammatory responses, and contributing to the systemic symptoms associated with loxoscelism. These insights enrich the current understanding of the molecular diversity in *Loxosceles* venom glands and open new possibilities for elucidating the complex processes underlying envenomation, ultimately guiding the development of improved therapeutic strategies.

## METHODOLOGY

### NGS SEQUENCING AND BIOINFORMATIC ANALYSIS

*L. similis* specimens were collected from the touristic cave "Gruta da Lapinha", located in the Sumidouro State Park, Lagoa Santa, Minas Gerais, Brazil (43°57′W, 19°33′S), as previously described[27]. The collection was performed under a license from the "Instituto Estadual de Florestas de Minas Gerais" (IEF/MG; license number 047.2012). Spiders were identified using a previously described method[28].

The venom glands of 35 spiders were removed as described previously[29]. Total RNA was extracted from a pool of venom glands using TRI Reagent (Sigma-Aldrich, MO, USA), following the manufacturer's instructions. After RNA purification, quantification was performed using a Qubit 2.0 Fluorometer (Life Technologies, MD, USA), and the RNA was stored at -80°C until cDNA library construction. The quality and integrity of the obtained RNA were evaluated using agarose gel electrophoresis (1%).

Next Generation Sequencing (NGS) was performed using an automated MiSeq sequencer (NextSeq 550 System - Illumina®). The cDNA library was prepared with 1 μg total RNA, using the TruSeq™ RNA Sample Set Kit, Version2 (KR045) and TruSeq™ RNA Sample Preparation Kit, Version 2, according to the

manufacturer's instructions (Illumina®, CA, USA). The KAPA library quantification kit (Illumina®) was used to quantify cDNA libraries. Sequencing was performed using and automated MiSeq sequencer with MiSeq Reagent Kit (v.3; 300 cycles) using a paired-end strategy.

Trimming, filtration and *de novo* assembly methodology were performed using the Geneious Prime 2022.1.1 software, as described[30].

Raw reads were trimmed and filtered with the quality parameter Phred ≥30, generating a file that was used to assemble the contigs, and Illumina® adapters were removed. After trimming and filtering, 14.3 million reads were retained. De novo assembly was then performed using a k-mer value of 24 and a bubble size of 150, resulting in 685,366 contigs.

Annotations were performed to identify $PLA_2$. We search in the PHTK software using known protein patterns found in the consensus sequence of known $PLA_2$ from the database[31].

To accelerate and improve the identification of novel protein sequences, specific sequence motifs were searched against contigs generated via de novo assembly. For this purpose, our group developed the PHTK program[32], a tool designed to detect predefined patterns within nucleotide or amino acid sequences. PHTK program, provides a folder of query reads or contigs and a motif or sequence of interest, that scans the input to identify regions that match the specified pattern. For example, in searches aimed at identifying conserved cysteine arrangements that characterize specific toxin families, sequence motifs such as C20CC5C can be used. This notation specifies the spacing of cysteine residues, where the numbers represent the exact number of amino acid residues separating consecutive cysteines. Using these motifs, sequences that share distinctive structural features can be efficiently retrieved and compared.

This strategy aimed to identify the conserved cysteine pattern observed in the sequence alignment of $PLA_2$ from scorpions and bees (C20CC5C23C1C6C23C10C)[19,25], as well as the consensus sequence of the

catalytic sites characteristic of Group IA PLA2s (Y28G30G32)[33], Group IVA (R57K58R59)[34] and Group VIA (G-X-S519-X-G)[35].

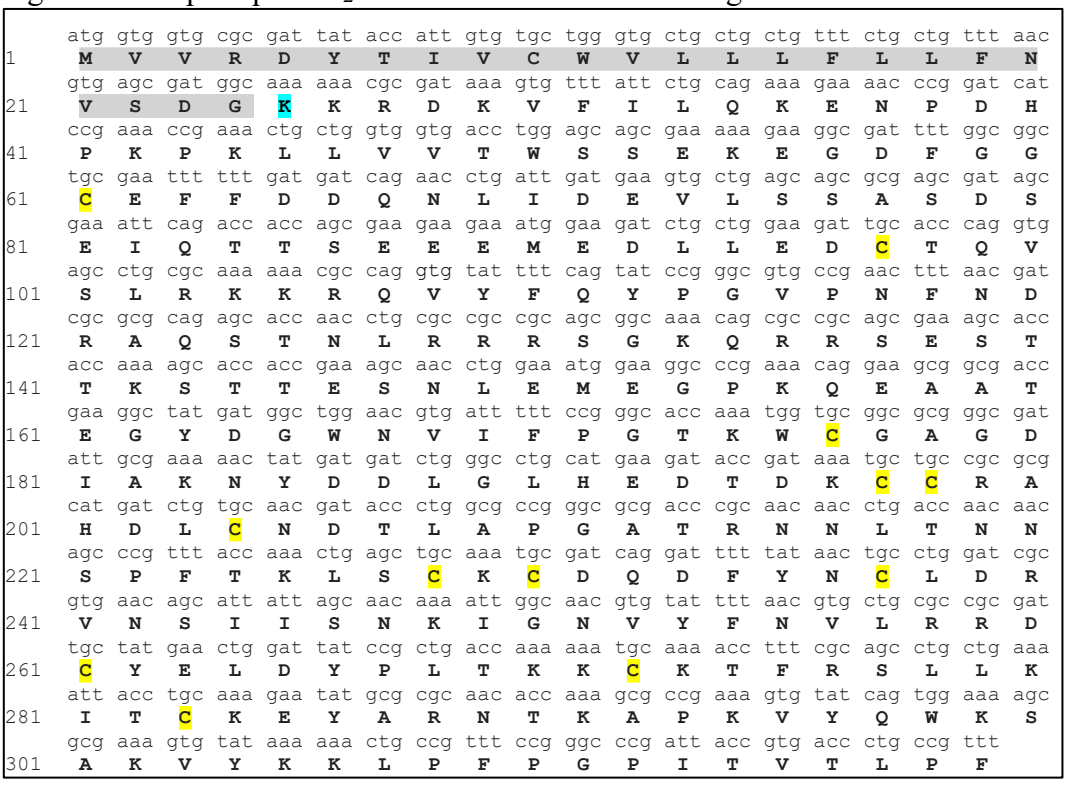
## RESULTS AND DISCUSSION

For the first time in the literature, a sequence showing similarity to the $PLA_2$ group of enzymes was found among the transcripts of *L. similis* and subsequently designated as $LsPLA_2$-1 (Figure 1), where "Ls" refers to *Loxosceles similis*, "$PLA_2$" denotes phospholipase $A_2$, and the suffix "-1" indicates that this is the first sequence of this enzyme family identified in this species. $LsPLA_2$-1 is a high-molecular-weight protein with putative enzymatic activity that degrades the extracellular matrix. Table 1 presents the identified sequence, highlighting the signal peptide and cysteine residues in the catalytic site[25] as well as its affiliation with the primary sequence and mature protein. Sequences similar to $PLA_2$ have been reported in transcriptomic analyses of other spider species, such as *Pamphobeteus verdolaga*[36] and *Phoneutria boliviensis*[26].

Analysis using EXPASY ProtParam indicated that the full-length primary sequence of $LsPLA_2$-1 encodes a protein of 319 amino acids, with a predicted molecular weight of approximately 38 kDa and a theoretical pI of 7.4. Following signal peptide cleavage and post-translational processing, the mature form consists of 295 amino acids with an estimated molecular weight of 33 kDa and a theoretical pI of 7.94.

$PLA_2$, also known as phosphatidylcholine-2-acyl hydrolase, is a widespread superfamily of enzymes found in various organisms, including vertebrates and invertebrates[19,37]. This superfamily is classified into 15 distinct groups, organized into five main types: $sPLA_2$, cytosolic $PLA_2$ ($cPLA_2$), calcium-independent $PLA_2$ ($iPLA_2$), platelet-activating factor acetylhydrolase (PAF-AH) and lysosomal $PLA_2$[38]. Despite their diversity, these proteins exhibit remarkably conserved structural and functional properties.

Figure 1. Phospholipase-$A_2$ toxin from *L. similis* venom gland.




The nucleotide and deduced amino acid sequences of LsPLA$_2$-1 (GenBank PX396025) are shown. In the protein sequence, the predicted signal peptide using Signal IP 5.0 is highlighted in gray. Lysin (K) in cyan indicates the first amino acid of the mature protein with 295 amino acids.

Table 1. Sequence similarity of LsPLA$_2$-1 to homologous proteins identified using BLASTP.

| PHOSPHOLIPASE $A_2$ | | | | | | |
|---|---|---|---|---|---|---|
| Toxin | Amino acid sequence | Affiliation | E-value | Similarity | ID | QC |
| LsPLA$_2$-1 | MVVRDYTIVCWVLLLFLLFNVSDGKKRDKV FILQKENPDHPKPKLLVVTWSSEKEGDFGG CEFFDDQNLIDEVLSSASDSEIQTTSEEEM EDLLEDCTQVSLRKKRQVYFQYPGVPNFND RAQSTNLRRRSGKQRRSESTTKSTTESNLE MEGPKQEAATEGYDGWNVIFPGTKWCGAGD IAKNYDDLGLHEDTDKCCRAHDLCNDTLAP GATRNNLTNNSPFTKLSCKCDQDFYNCLDR VNSIISNKIGNVYFNVLRRDCYELDYPLTK KCKTFRSLLKITCKEYARNTKAPKVYQWKS AKVYKKLPFPGPITVTLPF | (P) Phospholipase $A_2$-like (KFM82483.1) *Stegodyphus mimosarum* | $9e^{-117}$ | 64% | 49.72% | 99% |
| | | (M) Phospholipase $A_2$-like (KFM82483.1) *Stegodyphus mimosarum* | $1e^{-113}$ | 66% | 52.66% | 99% |

The signal peptide, predicted using SIGNALP 5.0, is indicated by underlining, with the first amino acid of the mature protein in cyan. Twelve cysteine residues are highlighted in yellow, with 10 located within the catalytic domain and emphasized in bold. The catalytic site, according to InterPro, is highlighted in gray. The sequence identity (ID) and query coverage (QC) with reference proteins are presented in the last two columns. P indicates the primary sequence, and M indicates the mature protein.

Animal venom is a rich source of structurally diverse secreted $sPLA_2$, which is subdivided into 16 subgroups: IA, IB, IIA, IIB, IIC, IID, IIE, IIF, III, V, X, XIIA, XIIB, XIII, XIV, and XV. The most extensively studied venom $sPLA_2$ is derived from Elapidae snakes (group IA), Viperidae snakes (groups IIA and IIB), and bees (group III), each characterized by unique structural motifs and disulfide bond arrangements that underpin their diverse toxicological and pharmacological functions. Group IA enzymes contain a conserved calcium-binding loop featuring a critical tyrosine residue (Y28), an elapid-specific loop insertion between the second α-helix and β-strands, and a catalytic network centered around histidine, aspartic acid, and tyrosine residues. Group IIA and IIB enzymes share a calcium-binding motif and catalytic residues, but diverge in their disulfide bond patterns, with the absence of the Cys61–Cys91 bond in group IIB. Group III enzymes, exemplified by bee venom $PLA_2$, display a distinct calcium-binding loop in which tryptophan replaces the conserved tyrosine, shorter α-helices, and increased β-sheet content. Additionally, $PLA_2$-like proteins, although catalytically inactive, possess unique membrane interaction motifs including cationic residues that form membrane docking sites and hydrophobic residues that constitute membrane disruption sites, which are integral to their cytotoxic activities. Although other $PLA_2$ types, such as $cPLA_2$ and $iPLA_2$, are present in venom glands, their roles in venom pathophysiology are less well defined than those of $sPLA_2$. This structural and functional diversity among the $sPLA_2$ subgroups equips animal venoms with a broad array of biological activities and mechanisms of action[39].

$LsPLA_2$-1 is a full-length sequence that retain cysteine residues essential for the formation of characteristic disulfide bridges[19,31]. The primary sequence and mature protein exhibited 64% and 65% similarity, respectively, to $PLA_2$ from the spider *Stegodyphus mimosarum* (UniProt A0A087U096) (Table 1), which belongs to Group XV of $PLA_2$, a lysosomal, calcium-independent enzyme that degrades glycerophospholipids and transfers acyl groups to ceramides. This class of phospholipase $A_2$ is well characterized in mammals, where it plays a central role in lysosomal phospholipid degradation, functioning optimally at an acidic pH and

independently of calcium ions. In spider venom, sPLA$_2$ is expected to facilitate the disruption of prey cell membranes and promote tissue digestion, thereby contributing to cytotoxicity, rapid prey immobilization, and synergistic interactions with other venom components that amplify the overall toxic effects. In a study by Estrada-Gómez *et al*., one of the contigs obtained from the transcriptome analysis of the spider *P. verdolaga* exhibited approximately 80% sequence similarity to *S. mimosarum* PLA$_2$ and showed similarity to PLA$_2$ sequences from other taxa, such as insects, lizards, and ticks[40].

Although relatively few studies have focused on spider venom PLA$_2$ enzymes, several sequences have been identified and deposited in the public databases. Refinement of the BLASTP parameters for the alignment of LsPLA$_2$-1 with the spider isoforms, revealed significant sequence similarities (Table 2). According to UniProt, most of these sequences correspond to cPLA$_2$, with the exception of XP_054716960.1 from *Uloborus diversus*, which is annotated as a putative sPLA$_2$. In UniProt, the biological activity of proteins is classified based on curated annotations derived from experimental evidence, literature and computational predictions. This information is integrated into functional descriptions, including molecular functions, biological processes, and pathway involvement, providing a standardized framework for protein characterization. The alignment results are shown in Figure 2, highlighting the key catalytic motifs IFPGTKWCG (residues 145–153), DLGXXXDTDXCCRAHDXC (residues 163–180), and LTNXSPFTXLSCKCDXDFYXC (residues 193–213). A conserved set of 12 cysteine residues was identified, with 10 localized within the catalytic domain, as indicated in bold in Figure 2: C153C173C174C180C204C206C213C237C248C259. The numbering of amino acid residues corresponds to that of the LsPLA$_2$-1 mature protein.

Although current knowledge of PLA$_2$ in spiders is still scarce, more comprehensive insights are available for scorpions and the LsPLA$_2$-1 sequence can display substantial similarity to PLA$_2$ isoforms identified in scorpion venoms, suggesting possible structural or functional conservation across distant arachnid

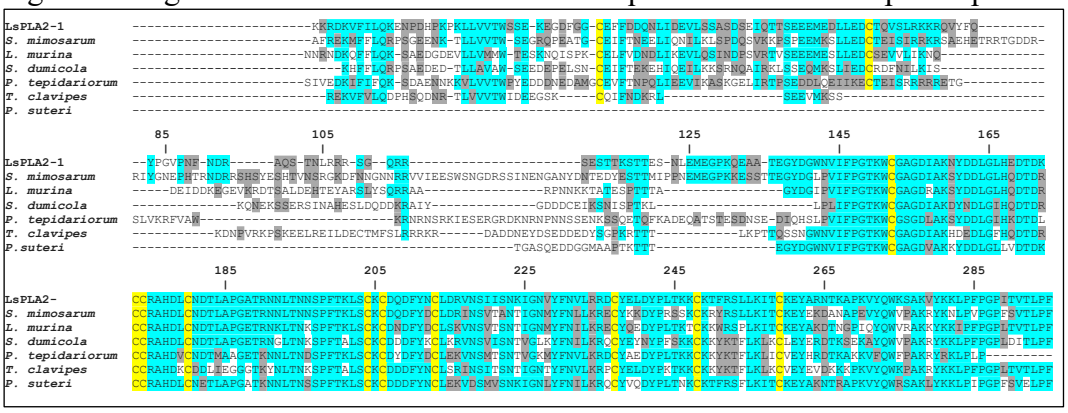
lineages. In the context of the scorpion clade, a previous study by our group, provided the first evidence of functional $PLA_2$ activity in *Tityus melici* venom, as confirmed using an indirect hemolytic assay. Transcriptomic analysis identified an $sPLA_2$ gene with 98% sequence similarity to its homolog in *Tityus serrulatus*[30,41]. Figure 3 shows the alignment of $LsPLA_2$-1 with $PLA_2$ sequences from *T. serrulatus* and *T. melici*. Although $sPLA_2$ enzymatic activity has not yet been demonstrated in *Loxosceles* venom, their proposed contribution to loxoscelism underscores the importance of further investigation into their potential functional role in spider venoms.

Table 2. Alignment parameters of $LsPLA_2$-1 against $PLA_2$ sequences from other spider species, obtained using BLASTP analysis.

| Organism | Accession Number | Total Score | QC | E-value | ID | Acc Lenght |
|---|---|---|---|---|---|---|
| ***Periegops suteri*** | QHA25180.1 | 295 | 56% | 2e-100 | 75.42% | 18 |
| ***Stegodyphus mimeosarum*** | KFM82482.1 | 181 | 55% | 5e-32 | 73.33% | 370 |
| ***Caerostris dawrini*** | GIY79658.1 | 178 | 45% | 4e-52 | 55.63% | 34 |
| ***Uloborus diversus****** | XP_054716960.1 | 278 | 86% | 4e-92 | 51.46% | 272 |
| ***Trichonephila inaurata madagascariensis*** | GFY62303.1 | 268 | 92% | 2e-87 | 51.02% | 335 |
| ***Argiope bruennichi*** | KAF8783068.1 | 158 | 48% | 1e-45 | 50.65% | 255 |
| ***Trichonephila clavipes*** | GFX25397.1 | 282 | 92% | 2e-92 | 50.51% | 378 |
| ***Nephila pilipes*** | GFS29729.1 | 278 | 92% | 2e-91 | 50.34% | 315 |
| ***Trichonephila clavata*** | GFR30777.1 | 270 | 92% | 1e-87 | 50.17% | 388 |
| ***Stegodyphus mimosarum*** | KFM82483.1 | 345 | 99% | 4e-117 | 49.72% | 360 |
| ***Trichonephila clavipes*** | GFX25412.1 | 179 | 53% | 1e-53 | 49.41% | 238 |
| ***Argipe bruennichi*** | KAF8783069.1 | 152 | 46% | 1e-41 | 49.01% | 429 |
| ***Nephila pilipes*** | GFS75130.1 | 181 | 54% | 3e-54 | 48.26% | 283 |
| ***Stegodyphus mimosarum*** | KFM82478.1 | 279 | 92% | 5e-92 | 47.95% | 314 |
| ***Nephila pilipes*** | GFT65360.1 | 122 | 34% | 4e-32 | 47.71% | 212 |
| ***Araneus ventricosus*** | GBM90199.1 | 277 | 98% | 2e-91 | 46.82% | 284 |
| ***Carostris extrusa*** | GIY90557.1 | 228 | 86% | 1e-71 | 45.82% | 336 |
| ***Araneus ventricosus*** | GBN62881.1 | 132 | 43% | 4e-35 | 45.26% | 302 |
| ***Caerostris extrusa*** | GIY41771.1 | 148 | 54% | 2e-42 | 44.83% | 191 |
| ***Nephila pilipes*** | GFS39049.1 | 159 | 59% | 3e-47 | 44.68% | 157 |
| ***Stegodyphus mimosarum*** | KFM72530.1 | 104 | 32% | 6e-25 | 44.66% | 273 |
| ***Argiope bruennichi*** | XP_055939774.1 | 137 | 41% | 2e-37 | 44.62% | 249 |
| ***Parasteatoda tepidariorum*** | XP_015925461.2 | 141 | 43% | 2e-38 | 44.20% | 307 |
| ***Trichonephila clavipes*** | GFX25390.1 | 141 | 43% | 1e-38 | 43.80% | 283 |
| ***Caerostris darwini*** | GIY23761.1 | 201 | 85% | 1e-62 | 43.38% | 232 |
| ***Stegodyphus dumicola*** | XP_035226339.1 | 91.7 | 30% | 2e-21 | 42.71% | 132 |
| ***Argiope bruenichi*** | XP_055933415.1 | 135 | 43% | 8e-37 | 42.65% | 256 |
| ***Araneus ventricosus*** | GBL82754.1 | 136 | 43% | 2e-36 | 42.65% | 312 |
| ***Trichonephila inaurata madagascariensis*** | GFY71282.1 | 134 | 43% | 3e-36 | 42.65% | 250 |
| ***Nephila pilipes*** | GFS57439.1 | 130 | 43% | 5e-35 | 42.65% | 251 |
| ***Sregodyphus dumicola*** | XP_035213673.1 | 135 | 41% | 1e-36 | 42.42% | 264 |
| ***Argiope bruennichi*** | KAF8783066.1 | 136 | 43% | 8e-37 | 42.03% | 292 |
| ***Stegodypchus dumicoola*** | XP_035205653.1 | 133 | 43% | 3e-36 | 42.03% | 229 |
| ***Trichinephila clavata*** | GFR30780.1 | 131 | 43% | 8e-35 | 42.03% | 280 |
| ***Parasteatoda tepidariorum*** | XP_015911956.1 | 130 | 43% | 8e-35 | 41.91% | 251 |
| ***Caerostris extrusa*** | GIY36382.1 | 129 | 43% | 9e-35 | 41.91% | 212 |
| ***Stegodyphus mimosarum*** | KFM82479.1 | 221 | 87% | 2e-69 | 41.43% | 288 |
| ***Araneus ventricosus*** | GBN03458.1 | 162 | 69% | 3e-47 | 41.18% | 243 |

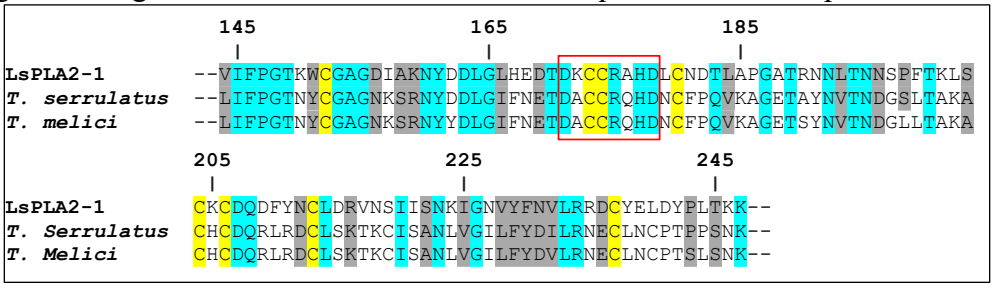
Presents metrics including sequence identity (ID), query coverage (QC), and E-values, highlighting the degree of conservation and divergence among spider PLA2s as revealed by comparative alignment. Nine sequences with identity ≤40% were identified but not included in Table 2. *** indicates a $sPLA_2$. Acc Length indicates the total length of the sequence deposited in the database.

Figure 2. Alignment of LsPLA2-1 with PLA2 sequences from other spider species.




Conserved cysteine residues are highlighted in yellow, identical residues are shown in cyan blue and conserved residues are indicated in light gray. The alignment was performed using the mature protein sequences, revealing patterns of conservation and divergence among PLA2s from *S. mimosarum* (KFM82483.1), *L. murina* (WBW70078.1), *S. dumicola* (XP_035222170.1), *P. tepidariorum* (XP_071035760.1), *T. clavipes* (GFX25397.1) and *P. suteri* (QHA25180.1). Residue numbering was assigned according to that of LsPLA2-1 (PX396025)

Figure 3. Alignment of LsPLA2-1 with PLA2 sequences from scorpion.




Cysteine residues are marked in yellow, identical amino acids in cyan and conserved residues in gray. Alignment was performed using the catalytic domain sequences, highlighting conservation patterns and sequence divergence between LsPLA2-1 and $PLA_2$ from *T. melici* (WLF82745.1) and *T. serrulatus* (JAW07019.1). The red square highlights the amino acids of the catalytic site (DXCCXXHD). Residue numbering was assigned according to LsPLA2-1 mature protein.

A minireview by Krayem and Gargouri (2020) summarizes the biochemical properties, structural characteristics, and diverse biological activities of $sPLA_2$ identified in scorpion venoms[25]. These enzymes predominantly belong to Group III and exhibit a distinctive heterodimeric structure composed of a long enzymatic chain linked to a short chain via a disulfide bond. Despite limited structural and functional studies compared with other venom $sPLA_2$, scorpion venom $sPLA_2$ have demonstrated a range of potent pharmacological effects including neurotoxic[42],

hemolytic[43], pro-inflammatory[44] and immunogenic effects[45]. This review highlights the importance of the short chain (a smaller polypeptide subunit covalently linked via a disulfide bond to the longer enzymatic chain of the heterodimeric enzyme) in catalytic efficiency and biological function, including $PLA_2$ Sm-PLGV from *S. maurus*, whose catalytic activity and kinetic parameters significantly decrease in the absence of the short chain, and its involvement in biological effects such as anti-angiogenic, anti-tumoral, and hemolytic activities. Additionally, this review discusses recombinant expression strategies and underscores the potential of these enzymes as structural templates for the development of novel therapeutic agents with significant biotechnological and pharmacological relevance, as exemplified by their demonstrated anti-angiogenic, anti-tumor, hemolytic, and anticoagulant activities, which highlight their promise as leads for drug discovery and biomedical applications[25].

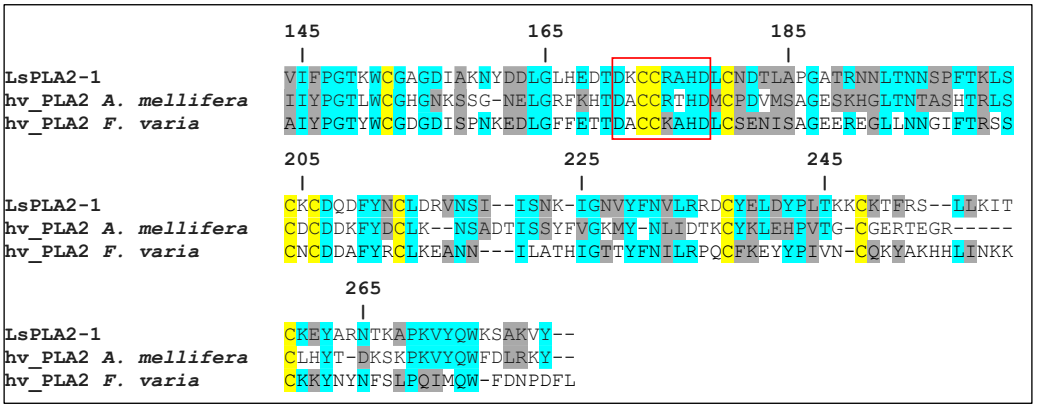
Further analysis using multiple sequence alignments of $LsPLA_2$-1 against database-deposited sequences revealed that this enzyme shares numerous conserved residues within its catalytic site with the well-characterized isoforms identified in bees ($hvPLA_2$) (Figure 4), indicating a high degree of conservation of cysteine residues within the catalytic site (DXCCXXHD), strong preservation of amino acids in the consensus sequence[19,37]. Bee $PLA_2$ ($hvPLA_2$) is classified as Goup IIIA phospholipase. Figure 4 illustrates the alignment of the catalytic domain of $LsPLA_2$-1 identified in *L. similis* against $hvPLA_2$ sequences: *A. mellifera* (Q7M4I5.1) and *Frieseomelitta varia* (XP_043509018.1), revealing several highly conserved residues, including cysteine residues, which are critical for structural stability and enzymatic function.

Group III $PLA_2$ enzymes are secreted proteins characterized by a conserved globular structure featuring three α-helices, two β-wings, a calcium-binding loop at the N-terminus, and an extended C-terminal domain. They possess a catalytic histidine and a specific calcium-binding motif essential for calcium-dependent enzymatic activity. Structural variations in the active site, hydrophobic substrate-binding channel, and C-terminal region distinguish species-specific adaptations

across diverse organisms including insects, scorpions, reptiles, mammals, and aquatic species. Phylogenetic analyses have classified Group III $PLA_2$ into distinct subgroups reflecting their evolutionary divergence and functional specialization[46].

A recent study extensively reviewed $hvPLA_2$ from the venom *A. mellifera*, and highlighted its significant biological and pharmacological importance. $PLA_2$ in honeybee venom is responsible for multiple toxic and immunological effects, including hydrolysis of phospholipids in cell membranes, release of fatty acids and lysophospholipids that cause cellular damage and inflammation, and act as a bioactive mediator. This enzyme is the primary allergen in the venom, triggering IgE- and IgG-mediated immune responses in humans and serving as a key target for specific immunotherapies[19,21]. Considering that $LsPLA_2$-1 from *L. similis* shares several structural characteristics with $hvPLA_2$ from *A. mellifera*, especially with Group IIIA, it represents a promising molecule for further biochemical and toxicological studies. In contrast, no significant similarity was observed between $LsPLA_2$-1 and XV $PLA_2$ isoforms X1 (XP_624472.3) and X2 (XP_006570673.1) from *Apis mellifera*.

Figure 4. Multiple sequence alignment of the catalytic domain of LsPLA2-1 and two hvPLA2 isoforms.




Identical residues are highlighted in cyan blue, and similar amino acids are shown in gray. The conserved cysteine residues are marked in yellow, emphasizing their role in maintaining structural stability. The red square highlights the amino acids of the catalytic site (DXCCXXHD). Residue numbering was assigned according to the LsPLA2-1 mature protein. Protein ID: NP_001011614.1 (*A. mellifera*) and XP_043509018.1 (*F. varia*).

$PLA_2$ enzymes from animal venoms have not necessarily evolved to exclusively target mammals; their biological roles often focus on invertebrate taxa, such as insects and isopods. A notable example is the heterodimeric $PLA_2$ Phaiodactylipin, purified from the venom of *A. phaiodactylus*, which exhibits high toxicity to arthropods, causing lethality at doses as low as 5 μg per cricket (~120 mg) and 3 μg per isopod (~50 mg). In contrast, equivalent or higher doses (up to 20 μg per 20 g mouse) induce only mild local inflammation in mammalian models, without systemic toxicity or disruption of muscle cell basal membranes[22]. These findings emphasize that many conclusions of "no toxic activity" often apply specifically to mammals and do not preclude potent biological effects on insects and other invertebrates, highlighting the functional specialization toward arthropod prey or competitors rather than mammalian targets.

Accurate classification of $PLA_2$ into specific group (or groups) requires detailed biochemical characterization. Colorimetric assays, enzymatic inhibition, and physiological testing are essential for distinguish endogenous $PLA_2$, which is involved in normal cellular functions, from venom-derived $PLA_2$, which serves adaptive roles in predation and defense, such as prey digestion and modulation of physiological responses. Therefore, integrating structural, enzymatic, and functional analyses is fundamental to clarify the biological significance of $PLA_2$ and their contribution to venom-induced toxicity.

The identification of $LsPLA_2$-1 in *L. similis* significantly advances the current understanding of the venom composition of this species, given that earlier transcriptomic analyses detected only phospholipase D enzymes, such as the Loxtox family and PLD3-like proteins, as the primary contributors to venom toxicity[27]. Thus, the detection of $LsPLA_2$-1 thus introduces a novel enzymatic class into the constellation of *L. similis* venom components, revealing a broader diversity of phospholipase types among *Loxosceles* spiders than previously recognized.

Although $PLA_2$-like transcripts have been identified in the venom gland transcriptomes of other spider, such as *P. verdolaga* and *P. boliviensis*, their precise biological contributions remain unclear. The discovery of $PLA_2$ in *Loxosceles*

suggests a more intricate evolutionary history of venom proteins and raises important new questions for functional and pharmacological research on brown spider toxicity.

Given these findings, it remains unclear whether LsPLA$_2$-1 from *L. similis* acts as a venom-secreted toxin, an endogenous enzyme, or both. Typically, secreted PLA$_2$ is implicated in envenomation mechanisms, contributing to prey immobilization and tissue damage, whereas endogenous PLA$_2$ participates primarily in cellular processes such as membrane remodeling, signal transduction, and eicosanoid production, and is not directly linked to venom toxicity. Determining the biological context of LsPLA$_2$-1 is essential for understanding its functional significance in *L. similis* venom.

## CONCLUSION

In this study, we described the presence of a PLA$_2$ in the venom glands of *L. similis*, designated as LsPLA$_2$-1. This enzyme corresponds to the first PLA$_2$-like sequence reported in this species and encodes a full-length protein of 319 amino acids, with a predicted mature form of 295 residues. LsPLA$_2$-1 retains conserved cysteine residues critical for disulfide bridge formation and displays catalytic motifs characteristic of the PLA$_2$ family.

LsPLA$_2$-1, classified as either secreted or endogenous PLA$_2$, holds significant biological importance due to its distinct physiological roles. Secreted PLA$_2$ enzymes primarily participate in extracellular processes, such as inflammation and venom toxicity, where they hydrolyze membrane phospholipids to release bioactive lipid mediators that modulate immune responses and contribute to tissue remodeling. In contrast, endogenous (cytosolic) PLA$_2$ acts within cells to regulate membrane homeostasis, signal transduction, and lipid metabolism, and plays crucial role in cellular function and physiological balance.

Understanding the potential role of LsPLA$_2$-1 is essential for elucidating its involvement in normal physiological processes and pathological conditions. These

include its function in venom-induced pathologies and broader biological contexts, highlighting its significance as a prospective therapeutic target for envenomation. Moreover, studying $LsPLA_2$-1 structural characteristics provides insight into its enzyme specificity and interactions with cellular membranes. Its role in modulating local and systemic inflammatory responses after venom inoculation underscores its impact on clinical outcomes. The evolutionary conservation and homology of $LsPLA_2$-1 across species revealed its essential biological functions and adaptive significance in predation and defense mechanisms. Finally, understanding the possible synergistic interactions between $LsPLA_2$-1 and other venom components may lead to development of targeted antivenoms and novel therapies.

## CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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