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# Spider venom neurotoxin based bioinsecticides: A novel bioactive for the control of the Asian citrus psyllid *Diaphorina citri* (Hemiptera)

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ABSTRACT

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is a key vector of the phloem-limited bacteria *Candidatus* Liberibacter asiaticus (CLas) associated with huanglongbing (HLB), the most serious and currently incurable disease of citrus worldwide. Here we report the first investigation into the potential use of a spider venom-derived recombinant neurotoxin, ω/κ-HxTx-Hv1h (hereafter HxTx-Hv1h) when delivered alone or when fused to snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) to control *D. citri*. Proteins, including GNA alone, were purified from fermented transformed yeast *Pichia pastoris* cultures. Recombinant HxTx-Hv1h, HxTx-Hv1h/GNA and GNA were all orally toxic to *D. citri*, with Day 5 median lethal concentrations ($LC_{50}$) derived from dose-response artificial diet assays of 27, 20 and 52 μM, respectively. Western analysis of whole insect protein extracts confirmed that psyllid mortality was attributable to protein ingestion and that the fusion protein was stable to cleavage by *D. citri* proteases. When applied topically (either via droplet or spray) HxTx-Hv1h/GNA was the most effective of the proteins causing >70 % mortality 5 days post treatment, some 2 to 3-fold higher levels of mortality as compared to the toxin alone. By contrast, no significant mortality or phenotypic effects were observed for bumble bees (*Bombus terrestris* L.) fed on the recombinant proteins in acute toxicity assays. This suggests that HxTx-Hv1h/GNA has potential as a novel bioinsecticide for the management of *D. citri* offering both enhanced target specificity as compared to chemical pesticides and compatibility with integrated pest management (IPM) strategies.

## 1. Introduction

Huanglongbing (HLB) is considered the most serious citrus disease worldwide and is associated with the gram-negative phloem-limited bacteria *Candidatus* Liberibacter asiaticus (CLas), *Candidatus* Liberibacter americanus, and *Candidatus* Liberibacter africanus (CLaf) (Bové, 2006). The Asian form (CLas) is the most widespread and it is currently present in the Americas, Asia, and Africa (Boa, 2023). Some *Citrus* relatives, without economic importance, have been reported to be resistant to CLas (Alves et al., 2021; Ramadugu et al., 2016). However, to date, there is neither commercial citrus varieties resistant to *Candidatus* Liberibacter spp., nor curative measures (Alquézar et al., 2022). HLB results in significant economic losses due to reduced fruit quality, premature fruit drop, and tree dieback (Bassanezi et al., 2011; Gottwald, 2010). Therefore, its management is based on preventative tactics, such as the planting of healthy nursery trees from insect-proof nurseries, the eradication of symptomatic trees, and the application of insecticides to suppress insect vector populations (Alquézar et al., 2022; Bassanezi et al., 2020).

*Candidatus* Liberibacter spp. are transmitted by the African citrus psyllid, *Trioza erytreae* (Del Guercio) (Hemiptera: Triozidae), and the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) (Capoor et al., 1967; McClean and Oberholzer, 1965; Reynaud et al., 2022; Yamamoto et al., 2006). *Trioza erytreae* is present in different regions of Africa and, in 2014 it was reported in the Iberian Peninsula (Arengo, 2013; Pérez-Otero et al., 2015). *Diaphorina citri* is disseminated in the main citrus-growing regions worldwide (Asia, Americas, and Africa) except for the Iberian Peninsula (Monzó Ferrer and Vanaclocha, 2023). Recently, *D. citri* was reported in Israel and Cyprus, representing a threat to the citrus industry in Europe (EPPO, 2023, 2022). The ideal

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temperature range for *D. citri* development is between 25 °C and 28 °C (Ying Hong Liu and Tsai, 2000). However, adult psyllids can survive at higher (>40 °C) and lower (<0 °C) temperatures and this may explain why *D. citri* is present in most continents (Aubert, 1990; Hall et al., 2011). The *D. citri* life cycle is closely related to the presence of young citrus flushes (Cifuentes-Arenas et al., 2018; Sétamou et al., 2016), and consequently, population peaks occur mainly during spring (Hall and Hentz, 2011; Yamamoto et al., 2001; Zorzenon et al., 2021).

Chemical control is currently the most common and efficient tactic for reducing *D. citri* populations (Boina and Bloomquist, 2015; Miranda and Ayres, 2020). However, frequent insecticide application may cause environmental contamination, affect beneficial insects, and might be incompatible with the use of biological control agents such as entomopathogenic fungi (Cisneros et al., 2022; Conceschi et al., 2016; Saldarriaga Ausique et al., 2017). In addition, several studies from different countries have reported the resistance of *D. citri* populations to the main insecticide chemical groups used in citrus orchards (pyrethroids, neonicotinoids, organophosphates, diamides) (Chen et al., 2022; Naeem et al., 2019; Pardo et al., 2018; Tiwari et al., 2011; Vázquez-García et al., 2013). Thus, the development of environmentally sustainable approaches and the identification of novel actives with new target sites of insecticidal action that are effective in controlling *D. citri* is of critical importance.

The selectivity and potency of many small cysteine-rich (30–40 amino acid) spider venom peptides towards insects, along with the development of scaleable recombinant production methods, makes them promising candidates for commercialisation as bioinsecticides. Indeed, the venom derived neurotoxin HxTx-Hv1h has been commercialised (designated as GS-omega/kappa HxTx-Hv1a by Vestaron) and the Vestaron product Spear®-T is sold as a contact foliar spray for the control of glasshouse pests such as aphids, thrips, spider mites and whiteflies (www.vestaron.com). Non toxicity towards biological control agents (rove beetle *Dalotia coriaria* and flower bug *Orius insidiosus*) commercially used in glasshouses has been reported suggesting use is compatible with Integrated Pest Management (IPM) strategies (Cloyd and Herrick, 2018). The HxTx-Hv1h toxin belongs to the well-characterised hexatoxin family of peptides originally isolated from funnel web spiders that also includes ω-hexatoxin Hv1a which is insecticidal when injected into a range of different insect pests but is non-toxic to honeybees (Atkinson et al., 1998; Bloomquist, 2003; Fitches et al., 2012; Nakasu et al., 2014; Powell et al., 2020; Tedford et al., 2004). Whilst ω/κ-HxTx-Hv1h and ω-hexatoxin Hv1a are structurally similar (both are knottins with mature 37 amino acid peptide sequences), they have distinct amino acid sequences and act principally as allosteric modulators of insect nicotinic Acetylcholine receptors (nAChRs) but have secondary sites of neuronal action (Chambers et al., 2019). HxTx-Hv1h is more generally toxic than ω-hexatoxin Hv1a towards insects and this has been attributed to its ability to target nAChRs, voltage-activated $Ca^{2+}$ ($Ca_v$) and $Ca^{2+}$ activated $K^+$ ($K_{ca}$) channels, whereas Hv1a is only active against nAChRs and $Ca_v$ channels (Chambers et al., 2019; Chong et al., 2007; Fletcher et al., 1997; Sollod, 2006; Tedford et al., 2004).

Whilst some spider venom peptides show oral activity towards insects, they are most potent when injected, mimicking natural envenomation by spiders to deliver paralysing peptides to the haemocoel, and thereby access to the central nervous system of target prey (Guo et al., 2018). The fusion of several venom derived neurotoxins, including HxTx-Hv1h to the mannose-specific snowdrop lectin *Galanthus nivalis* agglutinin (GNA) "carrier" protein has been shown to potentiate oral, and more recently, contact insecticidal toxicity of linked peptides (Fitches et al., 2004, 2012; Sukiran et al., 2022; Trung et al., 2006; Yang et al., 2014). Enhanced oral activity is attributed to GNA-mediated transport of fused toxins across the gut epithelium to the haemocoel following ingestion. Mannosylated proteins are abundant within insects and GNA has been shown to bind to the nerve chord of lepidopteran larvae (Fitches et al., 2012; Vandenborre et al., 2011). Thus, enhanced contact activity of GNA-based fusion proteins is thought to be due to the ability of the lectin to "deliver" linked toxins to their target sites of action in the insect nervous system.

The aim of this study was to investigate the oral and contact efficacy of HxTx-Hv1h when administered alone, or when linked to GNA and delivered as fusion proteins towards adult *D. citri*. We tested the hypothesis that fusion to GNA would enhance the insecticidal efficacy of HxTx-Hv1h. Furthermore, oral toxicity towards worker bumble bees, *Bombus terrestris* (L.), was assessed to inform the potential for off-target effects of the fusion protein upon beneficial pollinator species.

## 2. Material and methods

### 2.1. Materials

Anti-GNA antibodies were prepared by Genosys Biotechnologies, Cambridge, UK. Monoclonal 6x-His Tag Antibodies were from Fisher Scientific, UK. Secondary IgG horseradish peroxidase antibodies were from Biorad. Chemicals for chemiluminescence, buffer salts and Dimethoate were supplied by Sigma.

### 2.2. Recombinant protein production

The generation of constructs encoding for the expression of HxTx-Hv1h (ω/κ-hexatoxin-Hv1h; NCBI accession no. S0F209; residues 38–76)GNA, and HxTx-Hv1h/GNA and protein production by bench-top fermentation of transformed yeast clones has previously been reported (Powell et al., 2020; Sukiran et al., 2022). In brief, *P. pastoris* cells expressing HxTx-Hv1h, GNA, or HxTx-Hv1h/GNA were cultured in a bench-top fermenter (ez-control Applikon 7.5 L vessel) as previously described (Fitches et al., 2012). Following fermentation, proteins were separated from cells by centrifugation (20 min at 7000 *g*, 4 °C) and purified via nickel affinity chromatography as previously described (Pyati et al., 2014). Pooled fractions containing purified proteins were dialysed against distilled water and lyophilized. Protein purities and quantities in lyophilized samples were determined from SDS-PAGE gels stained for total proteins with Coomassie blue using known concentrations of GNA as a protein standard with visualisation and analysis carried out using iBrightTM software (Thermo Fisher). The solubility of recombinant proteins in diluents (sucrose or surfactant, respectively) was assessed by SDS-PAGE prior to insect bioassays.

### 2.3. Insects

Psyllids were from a free *Ca.* Liberibacter spp. colony maintained for several generations on orange jasmine *Murraya paniculata* (L.) Jack in a climate-controlled room (25 ± 2 °C, 60 ± 10% R.H., and 14 L:10D h photoperiod] at Fund for Citrus Protection (Fundecitrus). *Diaphorina citri* adults (5–7 days old) of both sexes were used in the experiments.

Honey combs containing *B. terrestris* were purchased from Agralan Ltd. (Swindon, UK) and were maintained in continuous darkness at 25 °C, 50 % RH and fed *ad libitum* on 50 % (w/v) aqueous sucrose solution. Bees for bioassays were collected within 24 h of receiving the hives. Combs were exposed to $CO_2$ until sufficiently immobilised to safely select female workers. Workers of similar size were selected and checked for weight (150–250 mg). Selected bees were placed into individual nicot queen cages, fed *ad libitum* on 50 % (w/v) aqueous sucrose solution from disposable plastic syringes and allowed to acclimatize for ≥16 h (O/N) prior to bioassays.

### 2.4. Diaphorina citri *feeding experiments*

The artificial diet used in the experiments was a sucrose solution (15 % [w/v]) plus yellow (0.4 % [v/v]) and green (0.1 % [v/v]) food dyes following the procedures described by Hall et al. (2010). Stock solutions of lyophilized purified proteins (HxTx-Hv1h, HxTx-Hv1h/GNA, or GNA)

at different concentrations (treatments) were solubilized in the artificial diet. The preparation of protein stocks and diets were performed in a laminar flow hood to ensure sterile conditions. An artificial diet with no added protein was also included in the experiments as a control. Feeding chambers consisted of plastic tubes (3 cm high and 2 cm diameter) with a hole (0.5 cm diameter) on the side to release the psyllids, and a group of four small holes (0.1 cm each) on the opposite side to provide ventilation. A membrane (Parafilm® M) was stretched on the top of the plastic tube, 200 μl of the artificial diet was dispensed onto the membrane and covered with another stretched membrane. Ten adult psyllids were released into each feeding chamber (replicate) and 20 replicates per treatment were used. Psyllid mortality was assessed daily for five days and the diets were changed after the first 48 h. Psyllid samples were collected after 48 h (dead psyllids) and 120 h (dead and live psyllids) after psyllid release and kept at −20 °C for Western blot analysis. The experiments were conducted in a completely randomized design at the same environmental conditions described for the psyllid rearing.

### 2.5. *Preparation of* Diaphorina citri *samples for western analysis*

Psyllids from feeding assays (10 psyllids/replicate; three replicates with highest mortality rates from the highest protein doses) were lyophilized (LJJ02, JJ Cientifica equipment, São Carlos, Brazil) for 48 h and provided to Durham University for western analysis. Lyophilized samples were each homogenized using an eppendorf micropestle in 25 μL sample buffer (0.1 M Tris-HCl, pH 6.8, 2 % [w/v] glycerol, 5 % [v/v] β-mercaptoethanol, 0.01 % bromophenol blue), boiled for 5 min and then centrifuged (12 000 rpm) for 5 min at room temperature. Aliquots of 15–30 μL were loaded onto SDS-PAGE gels and electroblotted as previously described (Fitches et al., 2012) to determine if the proteins could be detected in the psyllids and if any degradation had occurred.

### 2.6. Diaphorina citri *topical application experiments*

Proteins were re-suspended in water containing 0.1% (v/v) of Polyether-Polymethylsiloxan-Copolymer (Break-thru® S 240, Evonic, Guaratinguetá, SP, Brazil). Break-thru is an adjuvant used in agriculture, which breaks the surface tension of the water resulting in a better protein solution spread and penetration into the psyllid body. Preliminary experiments showed that Break-thru 0.1 % (v/v) was not toxic to *D. citri* adults (results not shown). Psyllids were individually anesthetized with $CO_2$ for 3 s and two droplets of 0.2 μL (0.4 μL per insect) of protein solution were applied to the insect's body surface (one on the pronotum and another on the abdomen) (Supplementary material, Fig. S1) using an analytical syringe (SGE Analytical Science, Ringwood, Australia). Thereafter, a group of 10 treated-psyllids was confined on a seedling of 'Valencia' sweet orange [*Citrus* × *sinensis* (L.) Osbeck] (replicate) and 20 replicates per treatment were used. Psyllids treated only with a solution containing 0.1% (v/v) of Break-thru were used as a control. Psyllid mortality was assessed daily for five days. The experiments were conducted in a completely randomized design under the same environmental conditions described for psyllid rearing.

### 2.7. Diaphorina citri *topical spray experiments*

Proteins were re-suspended in water containing 0.1% (v/v) of Break-thru and applied at equimolar concentrations as follows: Hxtx-Hv1h 1.11 mM, GNA 1.12 mM, and Hxtx-Hv1h/GNA 1.12 mM (treatments). As described in item 2.6, break-thru was not toxic to *D. citri* adults. A group of 10 psyllids (replicate) were anesthetized with $CO_2$ for 3 s and sprayed with protein solution using an airbrush (250μL/replicate). Thereafter, treated-psyllids were confined on a seedling of Rangpur lime (*Citrus limonia* Osbeck) using a sleeve cage made with tulle fabric and transferred to a screenhouse (Supplementary material, Fig. S2). Psyllids treated only with a solution containing 0.1% (v/v) of Break-thru were used as a control. Psyllid mortality was assessed daily for five days. The experiments were conducted in a completely randomized design using 20 replicates per treatment, totaling 200 psyllids per treatment. During the experiment there was an average temperature of 22.4 °C (max. 30.4 and min. 13.6 °C) and a relative humidity of 60.6 % (max. 70.8 and min. 44.9 %) in the screenhouse.

### 2.8. Bombus terrestris *acute oral toxicity assay*

Acute oral toxicity towards adult bumble bees (*B. terrestris*) was investigated to assess potential for off-target effects of HxTx-Hv1h, GNA, or HxTx-Hv1h/GNA. Methods were based upon OECD guidelines (OECD, 2017). Total protein doses for the assays were >6 × the $LC_{50}$ values derived from *D. citri* feeding assays. Worker bees (150 mg average wt.) were maintained at 25 °C, 60 % RH for 2–3 h, briefly anesthetized, and transferred to individual nicot cages (queen rearing devices) and provided with a 50 % (w/v) sucrose solution for approx. 16 h. Following acclimatisation, the bees (10 per treatment) were starved for 4 h to encourage feeding and then each provided with 80 μL of sucrose solution containing 32 nmols each of HxTx-Hv1h (200 μg/bee), GNA (320 μg/bee), HxTx-Hv1h/GNA (560 μg/bee) or Dimethoate (4 μg/bee) as a positive control, and sucrose solution only as a negative control treatment. All diet was consumed during the 4 h treatment period, after which the bees were fed *ad libitum* on sucrose solution. Thereafter mortality and phenotypic effects were monitored for a period of 6 days.

### 2.9. *Statistical analysis*

Survival data for the experiments of *D. citri* feeding, topical application, and topical spray were analyzed using Kaplan-Meier survival analysis. The $LC_{50}$ values for the feeding experiments were calculated using the log-logistic model fixed with the 'drm' function of the 'drc' package. Based on Akaike information criterion estimates, a three-parameter log-logistic model (LL.3u function in 'drc' package) with a fixed upper limit at 1 was used. In order to assess the effects of the topical application and spray of lyophilized proteins on psyllid mortality, generalized linear models (GLMs) with Quasi-Binomial distribution were used. Goodness of fit was assessed using half-normal plots with a simulation envelope of the 'hnp' package. In the case of significant differences among treatments, post-hoc multiple comparisons were performed with Tukey's test using the 'glht' function of the 'multcomp' package with adjusted *P*-values. All statistical analysis were performed by R statistical software version 4.1.2. (R Foundation for Statistical Computing, Vienna, Austria), except for the survival analysis, performed by Prism software version 8.4.3 (GraphPad, San Diego, CA, USA).

## 3. Results

### 3.1. *Recombinant protein production in the yeast* P. pastoris

The generation of constructs in the yeast expression vector pGAPZαB and production of recombinant HxTx-Hv1h, GNA and the HxTx-Hv1h/GNA fusion protein have previously been reported (Powell et al., 2020; Sukiran et al., 2022). As previously described by Sukiran et al. (2022) the mature HxTx-Hv1h 39 residue sequence is fused to the N-terminus of GNA via an 8 amino acid linker sequence (Gly-Gly-Gly--Gly-Ser-Ala-Ala-Ala). All proteins were expressed as intact full-length products at > 30 mg/L culture supernatant, and purified to >95% protein purity by nickel-affinity chromatography (Sukiran et al., 2022).

### 3.2. Diaphorina citri *feeding experiments*

Oral toxicity of the purified proteins was determined by feeding adult *D. citri* with artificial diets containing a range of concentrations (0.1–1.0 mg/mL) of HxTx-Hv1h, GNA or HxTx-Hv1h/GNA. As shown in Fig. 1, significant dose dependent reductions in the survival of psyllids fed on all of the protein containing diets were observed, whereas control

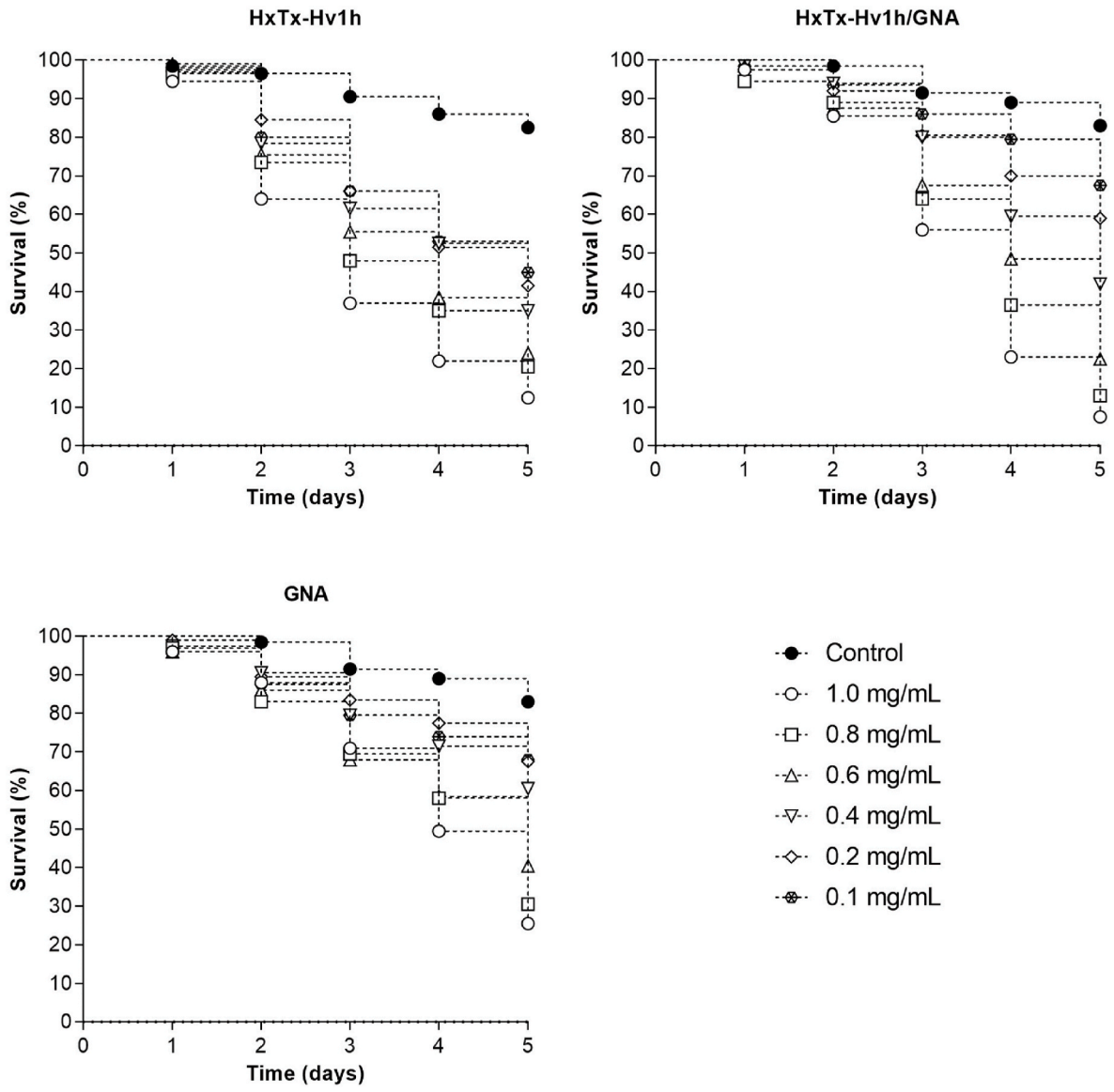


**Fig. 1.** Kaplan–Meier survival plots for *D. citri* adults fed on artificial diets containing different concentrations of HxTx-Hv1h, HxTx-Hv1h/GNA, GNA or artificial diet alone (control).

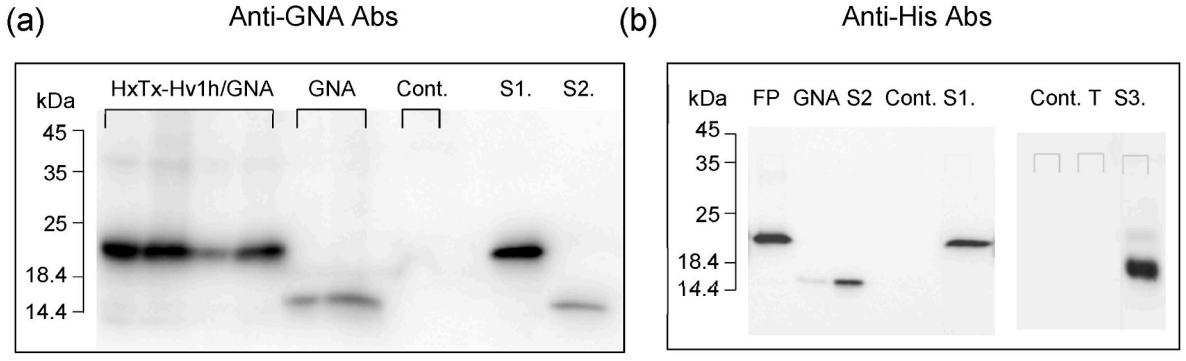


**Fig. 2.** (a) Immunoblot analysis of protein extracts from insects fed on 1 mg/mL diet treatments. In all cases Cont. denotes extracts from control (no added protein) diets, the proteins that insects were fed on are depicted above the lanes and the location of mass markers run on the same gels are shown. (a) Two samples (5 and 10 μL of a 25 μL extract from 10 insects) from two fusion proteins and GNA replicates are loaded. S1 and S2 (in both [a] and [b]) depict 200 ng standards of fusion protein and GNA, respectively. (b) Composite of two immunoblots; FP, GNA, and T depict samples extracted from insects fed on HxTx-Hv1h/GNA, GNA, and HxTx-Hv1h, respectively (15 μL for FP and 30 μL for toxin extracts loaded). S3 is HxTx-Hv1h standard (800 ng).

(no added protein diet) survival was >80 % (HxTx-Hv1h: $\chi^2 = 275.50$, df = 6, $p < 0.0001$; GNA: $\chi^2 = 205.40$, df = 6, $p < 0.0001$; and HxTx-Hv1h/GNA: $\chi^2 = 419.5$, df = 6, $p < 0.0001$). On the third day of assessment, the highest protein concentration (1 mg/mL) caused a reduction in psyllid survival of 59, 22, and 39 %, for HxTx-Hv1h, GNA, and HxTx-Hv1h/GNA respectively. At the final assessment (5 days after feeding), psyllid survival was <25 % for HxTx-Hv1h and HxTx-Hv1h/GNA at doses of ≥0.6 mg/mL, whereas GNA and control survival was respectively, >40 % and >80%.

As shown in Table 1, on a total protein basis, the HxTx-Hv1h toxin (Day 5 $LC_{50}$ 0.17 mg/mL) was 2.2 and 3.9-fold more effective as compared to HxTx-Hv1h/GNA (Day 5 $LC_{50}$ 0.37 mg/mL) or GNA (Day 5 $LC_{50}$ 0.66 mg/mL). On a molar basis, HxTx-Hv1h/GNA (Day 5 $LC_{50}$ 20 μM) showed comparable oral efficacy to HxTx-Hv1h (Day 5 $LC_{50}$ 27 μM) but was 2.6-fold more orally effective as compared to GNA (Day 5 $LC_{50}$ 52 μM).

### 3.3. *Detection of proteins in insects fed on recombinant proteins*

To investigate if the proteins were prone to proteolysis following internalization, pooled samples of protein extracts prepared from lyophilized whole insects (from the highest protein dose of 1.0 mg/mL) were analyzed by Western blotting. As shown in Fig. 2 positive immunoreactivity with ant-GNA and anti-His antibodies was observed in extracts prepared from insects fed on HxTxh-Hv1h/GNA or GNA. Mass alignment of the immunoreactive proteins with standards run on the same gel indicates that both proteins are resistant to proteolysis following ingestion by *D. citri* proteases. No immunoreactivity with anti-His antibodies was detectable in extracts prepared from insects fed on HxTx-Hv1h, despite maximal sample loading on gels. Maximal sample loading results in the presence of one major immunoreactive band, which corresponds to the HxTx-Hv1h toxin (as described in full by Sukiran et al., 2022), and the higher mass band is thought to be a dimeric form of the recombinant toxin. This suggests that HxTx-Hv1h may be prone to proteolytic degradation following ingestion either via removal of the N-terminal histidine tag or degradation within the toxin itself. However, of note is the relatively poor sensitivity of the anti-His antibodies with the toxin samples, and as such, the lack of immunoreactivity may be due to insufficient protein being present in the samples.

### 3.4. Diaphorina citri *topical application experiments*

The efficacy of topically applied HxTx-Hv1h, HxTx-Hv1h/GNA, or GNA was evaluated by applying a total of 0.4 μL droplets (containing 0.1% (v/v) Break-thru) at two different concentrations to the body surface of adult *D. citri*. As shown in Fig. 3a, topically applied proteins caused significant dose dependent reductions in psyllid survival whereas control survival was 95 % after five days ($\chi^2 = 388.8$, df = 6, $p < 0.0001$). HxTx-Hv1h was the least effective of the proteins, causing a gradual reduction to 80 % and 60 % survival after 5 days for the 450 pmol and 800 pmol treatments, respectively. By contrast, the highest doses of HxTx-Hv1h/GNA fusion protein and GNA reduced psyllid survival to less than 40% and 25% after 2 and 5 days of application, respectively.

At the end of the experiment (5 days after topical application), psyllid mortality was significantly greater for all protein treatments as compared to the control group. For both concentrations (450 and 800 pmol), the fusion protein and GNA treatments caused significantly higher psyllid mortality rates (F = 49.46, df = 6, 133, $p < 0.0001$) as compared to HxTx-Hv1h, but the fusion protein and GNA treatments did not differ from each other (Fig. 3b).

**Table 1**
Day 5 $LC_{50}$ values (mg/mL and μM) derived from *D. citri* bioassay data presented in Fig. 2. C.I. depicts confidence intervals.

| Treatment | mg/mL | | μM | |
|---|---|---|---|---|
| | $LC_{50}$ | 95 % C.I. | $LC_{50}$ | 95 % C.I. |
| HxTx-Hv1h | 0.17 | 0.09–0.26 | 27 | 14–40 |
| HxTx-Hv1h/GNA | 0.37 | 0.29–0.45 | 20 | 16–25 |
| GNA | 0.66 | 0.50–0.82 | 52 | 39–64 |

### 3.5. Diaphorina citri *topical spray experiments*

As shown in Fig. 4a, when applied as a spray the proteins caused a significant reduction in psyllid survival whereas control survival was 89.5 % after five days ($\chi 2 = 238.5$, df = 3, $p < 0.0001$). HxTx-Hv1h and GNA reduced psyllid survival by 27.4% and 41.3% after 5 days, respectively. By contrast, the HxTx-Hv1h/GNA fusion protein was the most effective treatment reducing psyllid survival by 78.2% five days after spraying.

At the end of the experiment, psyllid mortality was significantly greater for all protein treatments as compared to the control (Fig. 4b). The fusion protein caused the highest psyllid mortality rate which was significantly greater than either the HxTx-Hv1h toxin or GNA treatments (F = 34.95, df = 3, 76, $p < 0.0001$).

### 3.6. Bombus terrestris *acute oral toxicity assay*

An acute oral toxicity assay was conducted to evaluate the potential for non-target effects of HxTx-Hv1h, GNA, or HxTx-Hv1h/GNA upon the pollinator species *B. terrestris.* Bumble bee workers were fed on sucrose solutions containing recombinant proteins at > 6 × the $LC_{50}$ values derived from *D. citri* feeding assays. The organophosphate Dimethoate which was used as a positive control caused 100 % mortality 24 h after each bee had consumed a total of 4 μg of product. By contrast, 100 % survival was recorded after 6 days for bees that had consumed 32 nmols of either GNA (320 μg protein/bee) or HxTx/Hv1h/GNA (570 μg protein/bee) or control (no added protein) and survival for the HxTx-Hv1h treatment (32 nmols, 200 μg protein/bee) was 90 %. No phenotypic effects were observed suggesting that all proteins were effectively non-toxic to adult bumble bees.

## 4. Discussion

The management of *D. citri* is currently focused on chemical control (Bassanezi et al., 2020; Miranda and Ayres, 2020). However, the frequent use of chemical pesticides can result in negative environmental effects, and has led to the selection of psyllid populations that are resistant to most classes of synthetic insecticides (Chen et al., 2022; Pardo et al., 2018; Tiwari et al., 2011). Consequently, approaches such as the use of kaolin as a physical repellent barrier, trap crops, botanical insecticides, *Bacillus thuringiensis* (*Bt*), and biological control have been studied as alternative environmentally benign insect control strategies (Diniz et al., 2020; Miranda et al., 2018; Saldarriaga Ausique et al., 2017; Tomaseto et al., 2019; Fernandez-Luna et al., 2019; Volpe et al., 2016). Protein-based bioinsecticides that exploit naturally derived plant lectins and insect-selective toxins have also been studied as alternative biological control agents to control sap-sucking insects such as aphids and planthopper species (De-Thier et al., 2023; Down et al., 2006; Sukiran et al., 2022). In the present study, we report for the first time an evaluation of the oral and topical insecticidal activity of the snowdrop lectin GNA, the spider venom derived peptide HxTx-Hv1h alone, and when fused to GNA, towards *D. citri* adults. All proteins were affinity purified from fermented cultures of transformed yeast clones as previously described (Sukiran et al., 2022).

Overall, >60 % mortality was recorded for adult psyllids fed for 5 days on artificial diets containing of ≥0.6 mg/mL of recombinant HxTx-Hv1h, HxTx-Hv1h/GNA, or GNA, whereas control (artificial diet alone) survival was >80%. Recombinant HxTx-Hv1h has previously been shown to be orally toxic to both pea (*Acyrthosiphon pisum* Harris) and peach potato (*Myzus persicae* Sulzer) aphids where survival was similarly

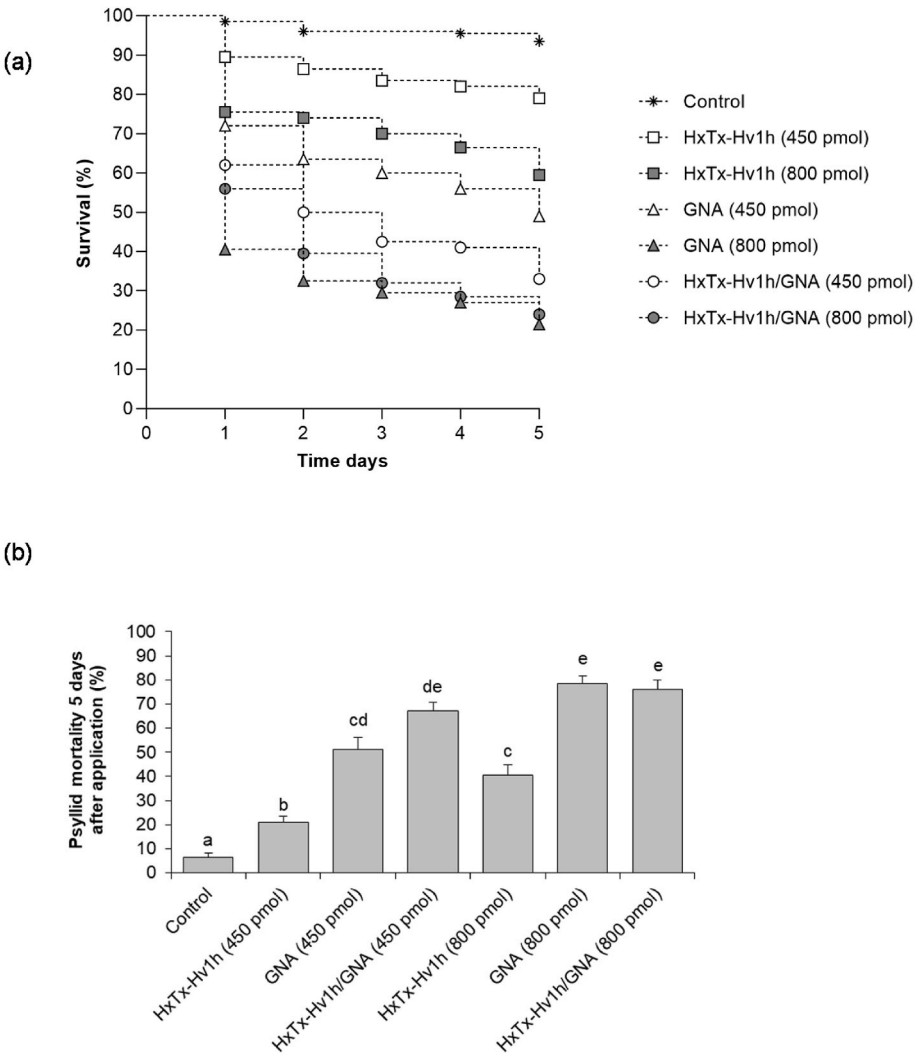


**Fig. 3.** (a) Kaplan–Meier survival plots of *Diaphorina citri* adults topically treated with solutions containing two concentrations (450 and 800 pmol) of HxTx-Hv1h, GNA, HxTx-Hv1h/GNA, or water + 0.1% (v/v) Break-thru (control no added protein). (b) Mean (±SEM) mortality of *Diaphorina citri* adults 5 days after topical treatments as described in (a). Means capped with different letters are significantly different (Tukey's test: $p < 0.05$).

reduced by > 80% after 4 days of feeding on artificial diets containing >0.6 mg/mL of protein as compared to > 90 % survival for control aphids (Sukiran et al., 2022). The derived $LC_{50}$ value of 27 μM for HxTx-Hv1h against *D. citri* is somewhat lower than the ca. 100 μM $LC_{50}$ value reported for aphids but the dose-responses are comparable and the difference largely attributable to the choice of data (5 days versus 2 days) used to derive median lethal doses. Overall, HxTx-Hv1h shows similar levels of oral toxicity against *D. citri, A. pisum*, and *M. persicae.*

GNA alone also showed significant effects on *D. citri* survival which was reduced to 25–40 % at dietary concentrations ≥0.6 mg/mL. The GNA day 5 $LC_{50}$ value of 52 μM for *D. citri* is comparable to the respective day 5 $LC_{50}$ values of 62 and 78 μM previously reported for GNA against pea and peach potato aphids (De-Thier et al., 2023). The insecticidal effects of mannose binding lectins and particularly GNA against homopteran pests are well-established and studies have focused principally on aphids, whiteflies, and plant-hoppers (reviewed by Macedo et al., 2015). Here we report the first evaluation of GNA against a species of psyllid and results confirm that the lectin is also chronically toxic to *D. citri.* Chronic insecticidal effects are attributed to the abundance of mannosylated proteins in insects (Vandenborre et al., 2011) that provide multiple binding possibilities for GNA and potential for the disruption of various physiological processes following internalization.

Whilst HxTx-Hv1h/GNA was orally toxic towards *D. citri,* fusion of HxTx-Hv1h to GNA did not significantly enhance toxin efficacy. This is in contrast to previous reports that have demonstrated the benefit of using GNA to transport fused insecticidal peptides to the haemolymph of phloem sap-sucking insects after ingestion (De-Thier et al., 2023; Down et al., 2006; Nakasu et al., 2014; Sukiran et al., 2022; Yang et al., 2014). Sukiran et al. (2022) reported HxTx-Hv1h/GNA to be approximately 3-fold more orally effective against pea (*A. pisum*) and peach potato (*M. persicae*) aphids as compared to HxTx-Hv1h alone. In chase-feeding experiments, enhanced persistence of fluorescence in the body cavities of pea aphids fed on HxTx-Hv1h/GNA or GNA as compared to HxTx-Hv1h alone was also reported. This provided evidence to support the hypothesis that GNA transports linked toxins across the gut epithelium allowing toxin access to target sites in the nervous system. In this study we were able to detect intact HxTx-Hv1h/GNA and GNA but not HxTx-Hv1h in western blots of extracts prepared from psyllids fed on protein containing diets. This suggests that the toxin, when not fused to GNA, is prone to degradation by *D. citri* proteases and, as occurs in *A. pisum*, that GNA may act to enhance the stability and persistence of HxTx-Hv1h following ingestion. Nevertheless, in contrast to aphid studies, enhanced stability of the fusion protein in *D. citri* did not correlate with significant effects on mortality and we suggest that this may be attributable to reduced GNA-mediated transport of linked HxTx-Hv1h across the psyllid gut.

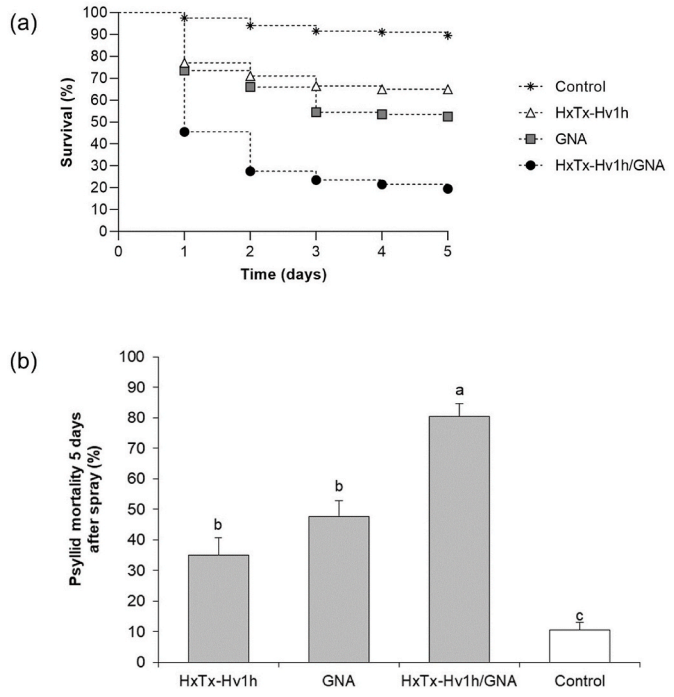


**Fig. 4.** (a) Kaplan–Meier survival plots of *Diaphorina citri* adults topically sprayed with HxTx-Hv1h, GNA, HxTx-Hv1h/GNA, proteins or water + Break-thru (control, no added protein). (b) Mean (±SEM) mortality of *D. citri* adults topically sprayed with solutions containing HxTx-Hv1h, GNA, HxTx-Hv1h/GNA, and water + Break-thru (control) after 5 days. Means capped with different letters are significantly different (Tukey's test: $p < 0.05$).

When topically applied (either directly to the body surface or as a spray application) recombinant HxTx-Hv1h, GNA and HxTx-Hv1h/GNA all caused significant reductions in the survival of *D. citri* as compared to a no added protein control treatment. By contrast to the relatively small difference in oral efficacy between the HxTx-Hv1h and HxTx-Hv1h/GNA treatments, the fusion protein when topically applied caused significantly greater levels of mortality as compared to the toxin only treatments. Topical assay results are broadly comparable to previously reported contact efficacy against adult pea aphids where HxTx-Hv1h/GNA was found to be significantly more toxic as compared to either HxTx-Hv1h or GNA alone (Sukiran et al., 2022). However, in this study, direct application of 800 pmols GNA itself caused comparable levels of mortality to the fusion protein treatment, but was less effective than HxTx-Hv1h/GNA at the lower concentration of 450 pmols. By contrast, when applied as a spray the results are more comparable to Sukiran et al. (2022) as significant mortality differences between the fusion protein and either GNA or HxTx-Hv1h treatments were observed. Interestingly, direct application of GNA caused a more rapid onset of psyllid mortality as compared to the fusion protein treatment, whereas the opposite was observed when the treatments were applied as a spray. This difference may refect the more rapid penetration of the proteins into the psyllid when applied directly to the body surface as compared to a spray application and highlights the influence of assay methodologies upon efficacy evaluation. Nevertheless, the results obtained from the most commercially relevant screenhouse assays do suggest that fusion of HxTx-Hv1h to GNA significantly enhances toxin efficacy with levels of psyllid mortality caused by the fusion protein under variable environmental conditions similar to those observed in field trials for some chemical insecticides used to control *D. citri* (Qureshi et al., 2014).

The reason for the disparity between the feeding and topical assay data is not clear but may reflect differences in the abundance of mannosylated proteins as targets for GNA binding in the psyllid gut as compared to the body cavity. That GNA is considerably more topically effective against psyllids as compared to pea aphids (Sukiran et al., 2022) may be indicative of a greater abundance of targets for lectin binding within the body cavity of *D. citri*. This could provide greater opportunities for GNA-mediated disruption of physiological processes in *D. citri* as compared to *A. pisum*. Further research to elucidate differences in the abundance of GNA-binding glycoproteins in the gut and body cavity of the two species would be required to support this hypothesis.

HxTx-Hv1h is the active ingredient in the contact foliar spray Spear®-T and is sold for the control of greenhouse and nursery pests (aphids, spider mites, spotted-winged drosophila, thrips, and whiteflies) (vestaron.com). Published literature describing the efficacy of this product towards insect pests under laboratory or field conditions is limited to a single study investigating the pesticidal activity towards *Drosophila suzukii* (Fanning et al., 2018). Whilst spray applications of the toxin alone (at concentrations of 1–5 ppt) resulted in less than 10 % mortality of *D. suzukii* adults 24 h after treatment, survival was reduced to 0 % when the peptide was delivered in combination with surfactant adjuvants. Unfortunately, Fanning et al. (2018) did not report the effects of the adjuvants alone but this study did highlight the need to use a penetrating adjuvant to aid transport of the active ingredient into the haemolymph. In this study Break-thru was used to enhance the spreadability of the recombinant proteins and thereby encourage penetration across the cuticle and was shown to have no toxic effect on *D. citri* when applied as a control (no added protein) treatment.

Contrary to the insecticidal effects of the recombinant proteins against *D. citri* none were shown to negatively impact the survival or health of worker bumble bees (*B. terrestris*) in acute oral toxicity assays. The transport of GNA across the gut to the haemolymph of adult and larval honeybees (*Apis mellifera*) following ingestion has previously been reported (Nakasu et al., 2014). Furthermore, Nakasu et al. (2014), reported no toxicity towards honey bees following injection of the closely related Hv1a toxin when fused to GNA. Whilst not directly comparable, this suggests that orally delivered GNA may also enter the circulatory system of *B. terrestris*, and if so, that the HxTx-Hv1h may not bind to neuronal targets in adult bumble bees. Further research is required to understand the observed lack of oral toxicity to *B. terrestris*. Cloyd and Herrick (2018) also reported the Vestaron peptide to be non-toxic towards two biological control agents (rove beetle *Dalotia coriara* and flower bug *Oriius insidiosus*). This suggests that HxTx-Hv1h, whether delivered alone or when fused to GNA is unlikely to exert negative effects upon beneficial insects in glasshouse or field conditions, although further studies would be required to substantiate this supposition.

Commercialisation of the Vestaron Spear®-T product provides evidence that recombinant toxin production is scaleable and it is anticipated that fusion proteins incorporating venom-derived peptides such as HxTx-Hv1h could similarly be developed for application as contact foliar sprays. As for *Bt* (Jurat-Fernandez et al., 2021) insect pests may well be able to adapt to fusion-protein based actives; although beyond behavioral adaptations, pests would need to evolve resistance to both GNA binding glycoproteins and to target sites of toxin action within the insect nervous system. In conclusion, this study highlights significant potential for the development of protein-based bioinsecticides incorporating venom-derived insecticidal peptides as an IPM compatible strategy to control *D. citri*. and thereby contribute to combating HLB, the most serious citrus disease worldwide.

## Ethical statement

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. All authors have contributed significantly in this study and approved the manuscript. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these.

## CRediT authorship contribution statement

**Marcelo P. Miranda:** Writing – original draft, Supervision, Investigation, Conceptualization. **Elaine C. Fitches:** Writing – review & editing, Supervision, Conceptualization. **Nur Afiqah Sukiran:** Investigation. **Wellington I. Eduardo:** Investigation, Formal analysis, Data curation. **Rafael B. Garcia:** Investigation. **Fabrício J. Jaciani:** Investigation. **Jennifer J. Readshaw:** Investigation. **Jack Bell:** Investigation. **Leandro Peña:** Writing – review & editing, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.toxicon.2024.107616.

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