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Stink bug predator kills prey with salivary non-proteinaceous compounds

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ABSTRACT

Podisus nigrispinus Dallas (Hemiptera: Pentatomidae) is a predator insect with potential applications in biological control because both nymphs and adults have been shown to prey on other insect pests by injection of toxic salivary gland contents. This study identified non-proteinaceous compounds with insecticidal activity from the saliva of *P. nigrispinus* in *Anticarsia gemmatalis*. In particular, the ether extract from *P. nigrispinus* saliva led to mortality in *A. gemmatalis* larvae, with a LC_{50} = 2.04 μL and LC_{90} = 3.27 μL. *N,N*-dimethylaniline and 1,2,5-trithiepane fractions were identified as non-proteinaceous extract components. *N,N*-dimethylaniline had a LC_{50} = 136.1 nL and LC_{90} = 413.8 nL, suggesting that it could be responsible for toxicity in *P. nigrispinus* saliva.

1. Introduction

Arthropod venoms have insecticidal activities against various pests of economic importance (Escoubas et al., 1995; Parkinson et al., 2002; Zhang et al., 2005; Baek et al., 2011). Venom is a toxin produced by glands and injected into another organism through a specialized apparatus, which can immobilize or kill the prey (Blum, 1978; Schmidt, 1982). Venoms from several Hemiptera, Hymenoptera, and Lepidoptera species have been isolated, identified, and evaluated against other insects and vertebrates (Ramos et al., 2004; Zhang et al., 2005; Sahayaraj and Muthukumar, 2011). Venom transfer can be active, such as a sting or injection, or passive as a defense mechanism, such as transfer through bristles, spines, or hairs after contact (Schmidt, 1982). Insect venoms have been chemically described to contain alkaloids, terpenes, polysaccharides, biogenic amines, organic acids, and amino acids (Blum, 1978), although primary components may also include peptides, oligopeptides, and proteins (Schmidt, 1982; Calvete et al., 2009).

Podisus nigrispinus Dallas (Hemiptera: Pentatomidae) is a predator insect used in biological control in the agriculture and forestry in the United States (Cohen, 1990; Medeiros et al., 2000; Lemos et al., 2001; Mohaghegh et al., 2001). *P. nigrispinus* has been used against defoliating pests, such as *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae), *Alabama argillacea* Hübner, *Spodoptera exigua* Hübner, and *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) (Lemos et al., 2001; Mohaghegh et al., 2001; Medeiros et al., 2003; Ferreira et al., 2008; Neves et al., 2010; De Bortoli et al., 2011).

The feeding strategies and extra-oral digestion of several predatory Hemiptera species have been studied, including *Belostoma lutarium* Stal (Belostomatidae), *Deraeocoris nebulosus* Uhler (Miridae), and *Podisus maculiventris* Say (Pentatomidae) (Cohen, 1995; Boyd et al., 2002; Bell et al., 2005; Swart et al., 2006). Pentatomidae predators insert their mouthparts into the body of the prey and inject saliva, causing rapid paralysis and death (Cohen, 1990), followed by ingestion of body contents (Lemos et al., 2005a,b;

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Azevedo et al., 2007).

Although the key salivary gland compounds responsible for prey death are unknown in *P. nigrispinus* and other predatory hemipterans, paralysis and death have been attributed to the action of digestive enzymes produced by the salivary glands and released within the prey (Schmidt, 1982; Cohen, 1990; Ferreira et al., 2008). Cytochemical and ultrastructural analysis of the principal and accessory salivary glands in *P. nigrispinus* revealed high levels of secretory proteins and other compounds (Martínez et al., 2014). However, Fialho et al. (2012) reported that the salivary glands do not contain digestive enzymes except for collagenase, which might rupture the internal organs in prey to facilitate ingestion.

Identification of venomous compounds in *P. nigrispinus* saliva is important in understanding toxicity as it relates to predation. In this study, we hypothesized that *P. nigrispinus* salivary glands contain non-proteinaceous compounds and evaluated the effects of salivary gland extracts in *A. gemmatalis* larvae to elucidate the toxic components.

2. Materials and methods

2.1. Insects

P. nigrispinus adults were obtained from the Laboratório de Controle Biológico do Instituto de Biologia Aplicada à Agricultura e Pecuária (BIOAGRO, Universidade Federal de Viçosa, Minas Gerais, Brazil). Insects were maintained at 25 ± 2 °C in 75 ± 5% relative humidity with a 12-h photophase in wooden cages (30 × 30 × 30 cm) coated with nylon and glass. They fed on *Tenebrio molitor* (L.) pupae (Coleoptera: Tenebrionidae), *Eucalyptus grandis* (W. Hill ex. Maiden) leaves and water *ad libitum* (Lemos et al., 2001). *A. gemmatalis* larvae obtained from a laboratory colony that had been reared at a temperature of 26 ± 1 °C in 75 ± 5% relative humidity with a 12-h photophase were placed in polystyrene boxes (15 × 9 cm). Larvae were fed an artificial diet containing 10 g agar, 15.6 g brewer's yeast, 25 g wheat germ, 25 g soy protein, 31.2 g minced beans, 12.5 g casein, and a 2.5 mL vitamin solution (1.2% ascorbic acid, 0.03% calcium pantothenate, 0.015% niacin, 0.008% riboflavin, 0.004% thiamin, and 0.004% HCl) (Greene et al., 1976). Adult *P. nigrispinus* males and *A. gemmatalis* fifth instar larvae without amputations or malformations were used in the bioassays regardless of size and weight.

2.2. Preparation of salivary gland extracts

P. nigrispinus males (n = 800) were anesthetized at −4 °C. Salivary glands were dissected in insect saline solution (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4) and washed with distiller water to remove the hemolymph, extraneous tissue and cuticle. Salivary glands were transferred to four glass vials containing 200 μL distilled water, macerated, and centrifuged at 10000× *g* at 4 °C for 20 min. The supernatant, consisting of the aqueous saliva extract, was removed and stored at −18 °C for use in bioassays.

The *P. nigrispinus* aqueous saliva extract was further separated into ether and aqueous + ether phases. Aqueous extract (200 μL) was diluted in 200 μL of petroleum ether. The petroleum ether phase was transferred to a glass vial, air dried, and re-suspended in 200 μL distilled water.

2.3. Inhibitors

To inhibit salivary proteases, a protease inhibitor cocktail (P2714; Sigma–Aldrich, St. Louis, MO, USA) was added to the aqueous extract at a 1:1 ratio (v/v). For complete protein and peptide degradation, proteinase K was also mixed with the aqueous extract at a 1:1 ratio (v/v).

2.4. Toxicity studies using P. nigrispinus extracts

Extracts from the aqueous, ether and aqueous + ether phases (2.7 μL) were injected into *A. gemmatalis* larvae using a micropipette. In addition, aqueous extracts (2.7 μL) containing the protease inhibitor and proteinase K were injected into *A. gemmatalis* larvae. An equal volume of distilled water was injected as a control. Fifteen *A. gemmatalis* larvae were used for each treatment. Larvae were individualized in Petri dishes (9 cm diameter) with an artificial diet, and mortality was evaluated for 72 h.

A non-proteinaceous extract (200 μL) was obtained from the *P. nigrispinus* saliva ether phase as described. Saliva extracts at volumes of 1, 2, 3, 4, and 5 μL (adjusted to a 5 μL final volume) and 5 μL distilled water (control) were used to determine lethal LC_{50} and LC_{90} concentrations. Solutions of varying concentrations were injected into *A. gemmatalis* larvae using a micropipette, and insects were individualized in Petri dishes with an artificial diet. Forty larvae per concentration were treated, and mortality was recorded for 72 h after injection. Rates were calculated with a correction for natural mortality (Abbott, 1925).

2.5. Purification of non-proteinaceous compounds

The ether phase (200 μL) was air dried, re-suspended in 10 mL of 0.05% (v/v) aqueous formic acid, and fractioned by reverse phase high-performance liquid chromatography (RP/HPLC; Shimadzu, LC20A) using a Vydac® C18-218TP54 column (5 × 250 mm;

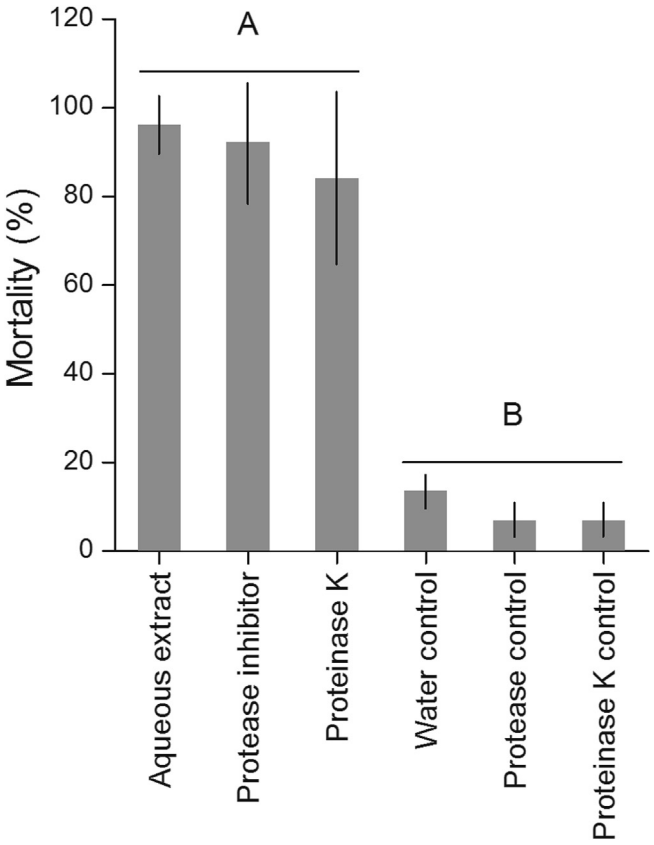

Fig. 1. Mortality (Mean ± SE) of *Anticarsia gemmatalis* larvae caused by the saliva of *Podisus nigrispinus* in aqueous extract with protease inhibitor and proteinase K. Letters in columns indicate significant differences by Tukey's HSD test (P < 0.05).

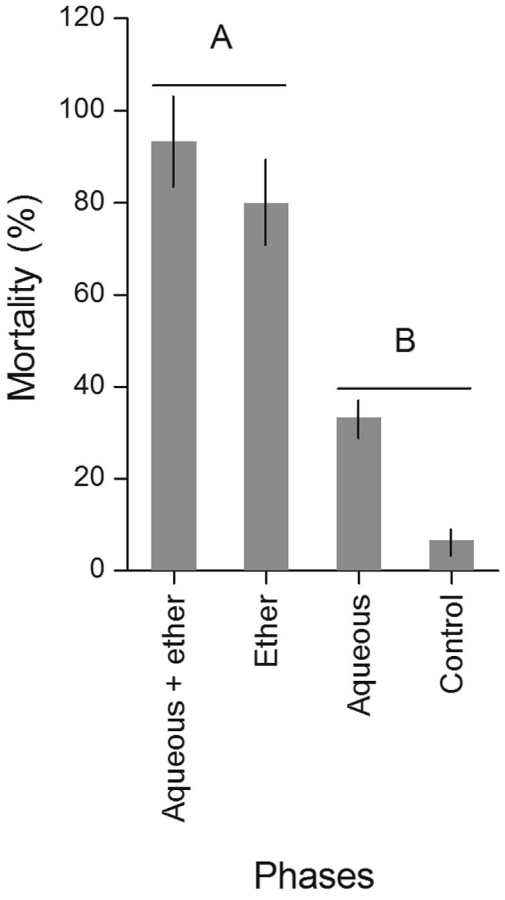

Fig. 2. Mortality (Mean ± SE) of *Anticarsia gemmatalis* larvae caused by salivary compounds of *Podisus nigrispinus* in water, ether phase and aqueous + ether. Letters in the columns indicate significant differences by Tukey's HSD test ($P < 0.05$).

Deerfield, IL, USA) equilibrated with 0.05% (v/v) aqueous formic acid. The concentration of acetonitrile in the eluting solvent was raised from 5% to 50% over 30 min using a linear gradient at a flow rate of 1 mL/min. Absorbance of the column effluent was monitored at $\lambda = 214$ nm and fractions (1 mL) were collected at 1-min intervals. Fractions were collected at their retention peaks and used as test solutions in the toxic activity test; distilled water was used as control. A 2.7 μL aliquot was injected into *A. gemmatalis* larvae using a micropipette. Fifteen larvae were used per concentration and were maintained individually in glass vials (2.5 × 15 cm) with 10 g of artificial diet. Mortality was recorded every 24 h for 10 days or up until changes from the larval to pupal stage occurred. Rates were corrected for natural mortality (Abbott, 1925).

2.6. Identification of toxic non-proteinaceous compounds in *P. nigrispinus* saliva

The toxic fractions identified in *A. gemmatalis* larvae were lyophilized and re-suspended in 10 μL dichloromethane at a 9:1 ratio (v/v). Analyses were performed using a gas chromatograph coupled with a mass detector CG/MS (CGMS-QP 5050A; Shimadzu). For each fraction, a 1 μL aliquot was injected in the splitless mode. Helium was used as the carrier gas with a flow rate constant of 1.7 mL^{-1} on a Rtx®-5MS capillary column (30 m, 0.25 mm × 0.25 μm; Bellefonte, PA, USA) using the Crossbond® stationary phase (35% diphenyl—65% dimethyl polysiloxane). The initial temperature of the injector and detector was 25 °C for 2 min, with a programed temperature of 1–230 °C/min, increasing by 3 °C every 60 min. The mass spectrometer was programmed to detect masses in the range of 29–600 Da with 70 eV ionization energy. Compounds were identified by comparing the mass spectra with those available in the Wiley Spectroteca database (7th edition), NISTCH05 library, diagnostic ions and retention indices. The identification of some compounds such as *N,N*-dimethylaniline was conducted by comparison with synthetic standard product (Sigma–Aldrich, São Paulo, Brazil).

2.7. Toxicity of commercial N,N-dimethylaniline in *A. gemmatalis* larvae

N,N-dimethylaniline, identified as a toxic compound in *P. nigrispinus* saliva, was purchased from Sigma–Aldrich. Six different concentrations of the commercial compound were used to calculate the lethal LC_{50} and LC_{90} concentrations: 18.4, 36.8, 69, 138, 276, and 552 nL. Distilled water was used as a control. Solutions were injected into *A. gemmatalis* larvae using a microinjector (Nanoject II auto nanoliter, Drummond Scientific Co., USA), and larvae were individualized in Petri dishes with an artificial diet. Ninety larvae were used for each concentration and mortality was evaluated for 72 h after injection, followed by rate corrections according to Abbott (1925).

2.8. Lethal time of *P. nigrispinus* saliva extracts and N,N-dimethylaniline

Toxicity of salivary extracts and commercially obtained *N,N*-dimethylaniline at the calculated LC_{50} and LC_{90} concentrations were compared. Distilled water was used as a control. The LC_{50} and LC_{90} of each compound were injected into *A. gemmatalis* larvae. Larvae were individualized in Petri dishes with an artificial diet. A total of 30 *A. gemmatalis* larvae were used for each treatment, with a total of three replicates. Mortality was recorded every 6 h for 72 h, and estimated lethal time values for 50% mortality (LT_{50}) were compared.

2.9. Statistical analyses

Mortality data for inhibitor and water controls, extract phases (aqueous, ether, and aqueous + ether), and saliva fractions were transformed using the formula $\sqrt{x + 0.05}$ and analyzed by one-way analysis of variance (ANOVA). Tukey's HSD (honest significant difference) was used to compare the mean at a 5% significance level

Table 1
Lethal Concentration of the extract in petroleum ether phase of the saliva of *Podisus nigrispinus* on *Anticarsia gemmatalis* larvae at 72 h after injection and estimated in probit values.

Concentration (df = 3)	Estimated value (μL)	95% confidence limits		X^2	P > F
		Lower	Upper		
LC_{50}	2.04	0.67	2.59	98.85	<0.0001
LC_{90}	3.27	2.56	4.97		
LC_{95}	3.74	3.02	4.97		
LC_{99}	4.81	3.71	17.3		

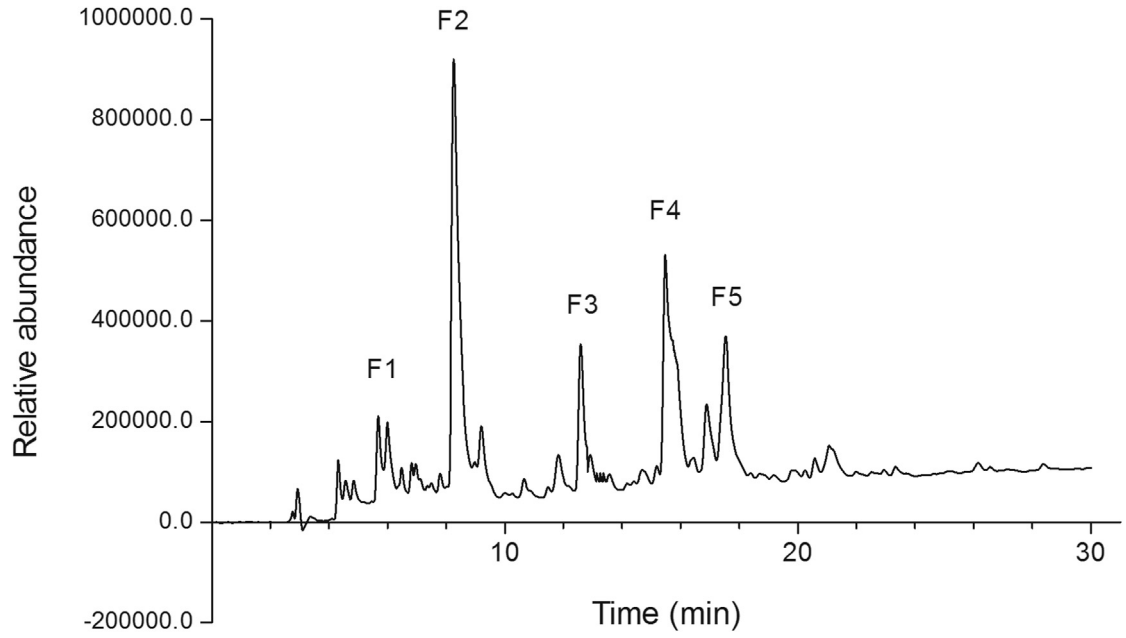

Fig. 3. HPLC chromatogram profile of peak retention of fractions and compounds of the venom of *Podisus nigrispinus*.

using SAS User software (v. 9.1) for Windows (SAS, 2002). The LC_{50} or LC_{90} and their confidence limits were determined by logistic regression in dose–response assays based on concentration probit–mortality (Finney, 1971) using XLSTAT-PRO (v. 7.5) for Windows (XLSTAT, 2004). Student's *t* test was used for pairwise comparisons regarding lethal effects in *A. gemmatalis* using SAS User software.

3. Results

Aqueous *P.* nigrispinus saliva extracts combined with protease inhibitors and proteinase K had significant toxic effects on A. gemmatalis larvae compared to controls ($F_{1,17} = 33.68$, $p < 0.05$). Extracts containing a protease inhibitor cocktail to block enzyme activity showed a 92.29% mortality rate, while extracts with proteinase K to digest proteins and peptides had a 84.11% mortality rate. Crude aqueous extract without protease inhibitors or proteinase K showed a 96.25% mortality rate. Mortality rates were 9.71, 7.03, and 7.03% for larvae injected with distilled water, protease inhibitors, and proteinase K controls, respectively (Fig. 1).

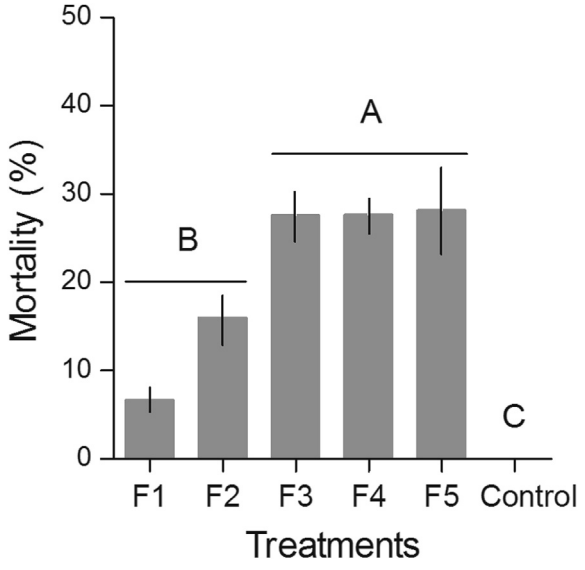

Fig. 4. Mortality (% ± SE) of *Anticarsia gemmatalis* larvae after 10 days injected with fractions that compose the venom of *Podisus nigrispinus*. Means followed by different letters in the column differ by LSD Tukey's HSD test ($P < 0.05$).

The crude aqueous extracts of *P. nigrispinus* saliva were further separated into the aqueous, ether, and aqueous + ether phases and also had significant effects on mortality in *A. gemmatalis* larvae ($F_{1,14} = 16.02$, $p < 0.05$). Ether and aqueous + ether phases resulted in mortality rates of 80.02% and 93.32%, respectively; both values were higher than rates for the aqueous phase and distilled water control, with 33.32% and 6.60%, respectively (Fig. 2).

Dose–response bioassays of different concentrations of the ether phase extract showed optimal results at a concentration of 4 μL ($R^2 = 0.94$, $p < 0.001$), with a $LC_{50} = 2.04$ μL at 24 h and $LC_{90} = 3.27$ μL after 36 h (Table 1). Concentrations below 4 μL induced symptoms of intoxication in *A. gemmatalis* larvae, such as progressive paralysis, reduced food consumption, regurgitation, and necrosis in the area injected with the ether extract.

The ether phase was separated into five fractions (F1–F5) (Fig. 3) and injected in *A. gemmatalis* larvae, showing significant mortality responses ($F_{1,17} = 6.94$, $p < 0.05$). The F3 (33.82%), F4 (33.79%), and F5 (34.14%) fractions showed more toxic effects than the F1 (6.6%) and F2 fractions (18.1%) (Fig. 4). The mortality rate of the distilled water control was <0.1%.

The F3, F4, and F5 fractions from the ether phase were identified as hydrocarbons. F3 and F4 corresponded to *N,N*-dimethylaniline ($C_8H_{11}N$) and 1,2,5-trithiepane ($C_4H_8S_3$), respectively (Table 2;

Table 2
Chemical composition of the fractions of extract of salivary glands *Podisus nigrispinus* toxic to *Anticarsia gemmatalis*. MM – Molecular mass, RI – Relative intensity, Ri – Retention indices, Rt – Retention time, *m/z* – Molecular weight.

Fraction	Compound	MM	Formula	RI	Ri	Rt	*m/z*
F3	*N,N*-dimethylaniline	121	$C_8H_{11}N$	12.5	942	16.61	120.15
F4	1,2,5-trithiepane	152	$C_4H_8S_3$	17.2	1263	29.49	152
F5	n.s	–	–	1.60	641	39.90	31

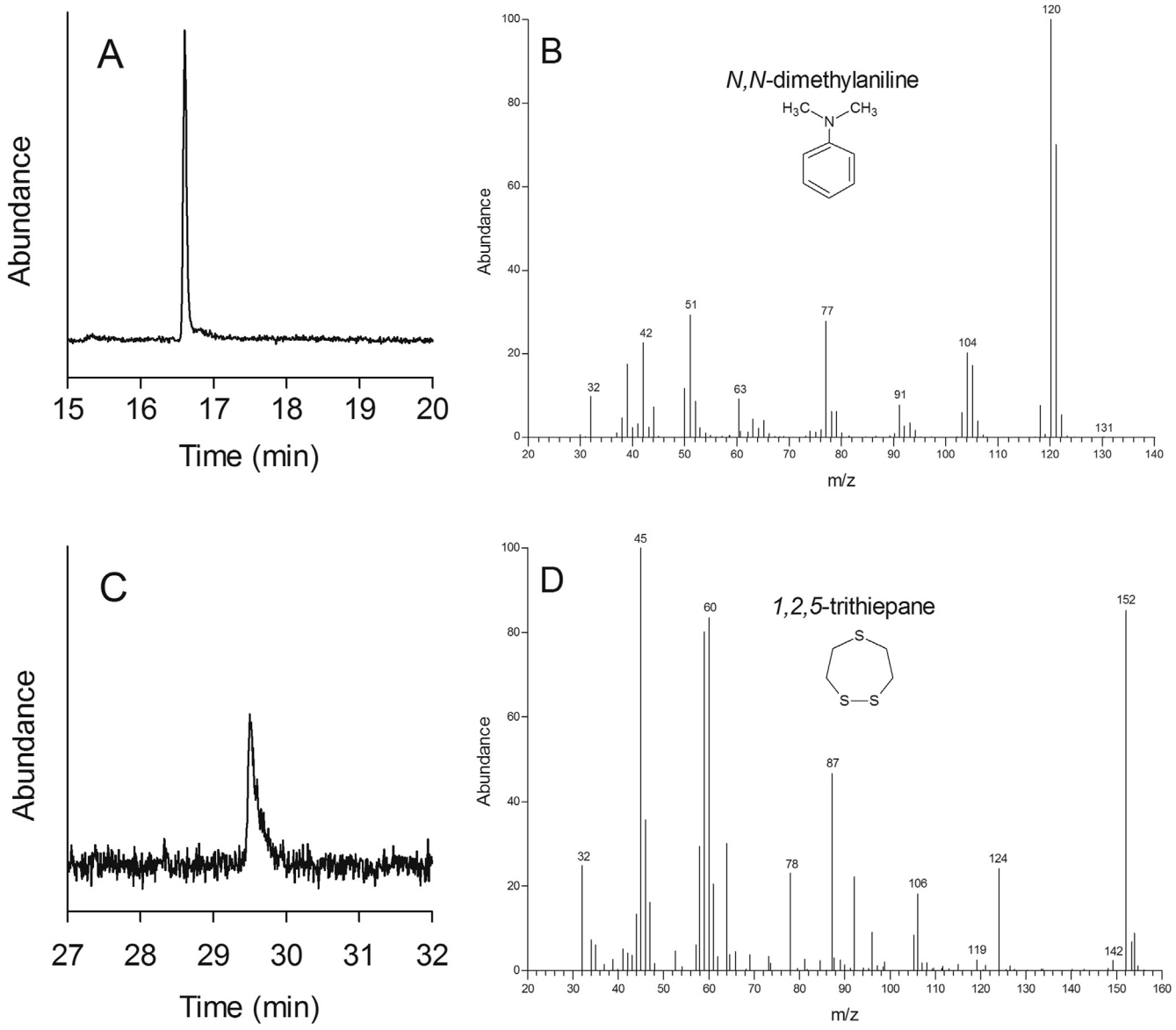

Fig. 5. Non-proteinaceous compounds identified of the salivary glands of *Podisus nigrispinus*. *N,N*-dimethylaniline chromatogram peak (A), mass spectra (B), 1,2,5-trithiepane chromatogram peak (C) and mass spectra (D).

Fig. 5, Supplementary Fig. 1). Compounds in the F5 fraction, with a retention time of 39.90 min, were not identified.

The toxicity of commercially obtained *N,N*-dimethylaniline in *A. gemmatalis* was evaluated at different concentrations (Table 3). Increased mortality was observed following injection of 552 nL of *N,N*-dimethylaniline ($R2 = 0.84$, $p < 0.001$), with an estimated LC_{50} and LC_{90} of 136.1 nL and 413.8 nL, respectively. *A. gemmatalis* larvae showed symptoms of intoxication and necrosis at concentrations below 552 nL (Fig. 6). The lethal times calculated for 36.8, 69, 138, and 276 nL concentrations were 36 h for the LC_{50} and 48 h for the LC_{90}. *A. gemmatalis* mortality was 99% for all *N,N*-dimethylaniline concentrations after 72 h.

A. gemmatalis larvae injected with lethal concentrations (LC_{50} and LC_{90}) of salivary extracts from *P. nigrispinus* and *N,N*-dimethylaniline showed lethal effects at different time points. However, LT_{50} values from injection assays showed that *N,N*-dimethylaniline took longer to kill insects than the *P. nigrispinus* saliva extract (Fig. 7). At a high concentration (LC_{90}), *N,N*-dimethylaniline took longer to kill the larvae than the salivary extract, with LT_{50} values of 19.2 ± 0.85 h and 16.6 ± 0.75 h, respectively ($t = 10.49$, $p < 0.00126$) (Fig. 7A). At LC_{50} levels, the LT_{50} was 66.1 ± 0.21 h for *N,N*-dimethylaniline and 51.1 ± 0.63 h for the salivary extract ($t = 4.22$, $p < 0.00621$) (Fig. 7B).

Table 3
Lethal concentration of *N,N*-dimethylaniline in *Anticarsia gemmatalis* larvae at 72 h after injection and estimated in probit values.

Concentration	Estimated value (nL)	95% confidence limits		X^2	Pr > F
		Lower	Upper		
LC_{50}	136.1	3.28	234.4	136.55	<0.0001
LC_{90}	413.89	343.64	520.25		
LC_{95}	531.23	441.06	520.25		
LC_{99}	751.33	623.80	944.38		

Fig. 6. Time-course of *N,N*-dimethylaniline on *Anticarsia gemmatalis* larvae after injection. Sequential necrosis effects at 2 h (A), 36 h at LC_{50} (B), and 48 h at LC_{90} (C). Injection point (arrows).

4. Discussion

Aqueous *P. nigrispinus* saliva extract treated with protease inhibitors and proteinase K showed toxic activity in *A. gemmatalis* larvae, suggesting that the compounds responsible death of prey during *P. nigrispinus* feeding are not proteins. Saliva from predatory stink bugs has been reported to contain enzymes required for extra-oral digestion (Terra and Ferreira, 1994; Swart et al., 2006; Azevedo et al., 2007) and other proteins and peptides that induce paralysis and death in prey (Silva-Cardoso et al., 2010; Sahayaraj and Muthukumar, 2011). Although pioneering studies reported protein involvement in prey death based on identification of protein profiles present in the saliva, data from the present study indicates that the toxic compounds in *P. nigrispinus* saliva are non-proteinaceous.

A. gemmatalis mortality was highest following treatment with *P. nigrispinus* saliva ether and aqueous + ether extracts, which contain low molecular weight substances such as aromatic compounds and hydrocarbons. *A. gemmatalis* larvae injected with the salivary aqueous phase containing only proteins had a lower mortality compared to the ether and aqueous + ether extracts, which suggests that the venomous effects of *P. nigrispinus* saliva are not due to proteins and peptides. Venom components containing low molecular weight and short carbon chains have been reported to induce lethal and sublethal effects from several insect species, including *Apis cerana* Fabricius (Hymenoptera: Apidae) (Schmidt et al., 1997), *Galerita lecontei* Dejean (Coleoptera: Carabidae) (Rossini et al., 1997), and *Polistes sulcifer* Zimmermann (Hymenoptera: Vespidae) (Bruschini and Cervo, 2011). However, venom is produced by specialized venom glands in these insects versus the salivary glands in *P. nigrispinus*.

Different concentrations of *P. nigrispinus* saliva ether extracts showed toxic effects on *A. gemmatalis* larvae between 12 and 36 h after injection. A dose–response bioassay confirmed toxicity against *A. gemmatalis*, reaching a 90% mortality rate. Increasing concentrations of substances isolated and injected into *A. gemmatalis* larvae have shown immediate toxic responses within 12 h of application (Beard, 1963; Boyd et al., 2002; Baek et al., 2011; Sahayaraj and Muthukumar, 2011). The LC_{50} (2.04 μL) and LC_{90} (3.27 μL) of the *P. nigrispinus* saliva ether extract indicates the presence of small quantities of toxic substances.

In this study, three fractions of *P. nigrispinus* saliva demonstrated toxic activity against *A. gemmatalis*, and two compounds were identified as *N,N*-dimethylaniline and 1,2,5-trithiepane. Injection of *N,N*-dimethylaniline caused mortality in *A. gemmatalis* larvae (LC_{50} = 136.1 nL). *N,N*-dimethylaniline, a substituted derivative of aniline, is an organic chemical compound consisting of a tertiary amine group and a dimethylamine group attached to a phenyl group (Krieger, 2010). *N,N*-dimethylaniline can modify enzymes by N-demethylation, N-oxidation, and N-hydroxylation as pathways for formation of toxic compounds in mammalian cells (Gorrod and Gooderham, 1981). *N,N*-dimethylaniline and xenobiotic

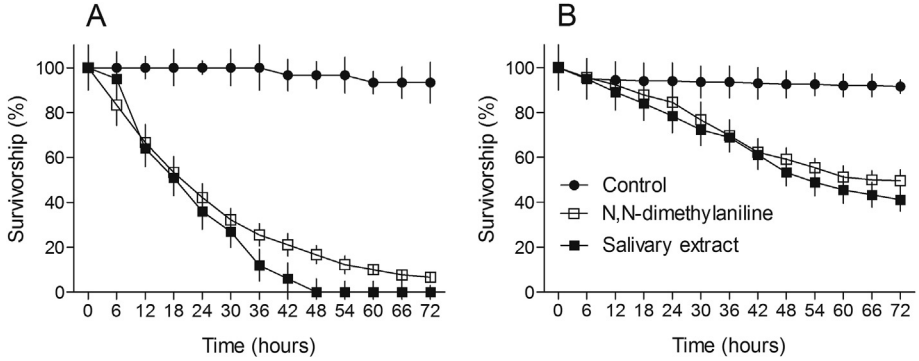

Fig. 7. (A) Survivorship of *Anticarsia gemmatalis* larvae after 72 h injected with a LC_{90} salivary extract of *Podisus nigrispinus*, *N,N*-dimethylaniline and control insects (injected with water). (B) Survivorship of *Anticarsia gemmatalis* larvae after 72 h injected with a LC_{50} salivary extract of *P. nigrispinus* and *N,N*-dimethylaniline (control insects were injected with water).

compounds derived from these pathways have been implicated in degradation of enzymes and other genotoxic, teratogenic and carcinogenic activities (Hlavica and Kiinzel-Mulas, 1993; Hover and Kulkarni, 2000; Taupp et al., 2006). *N,N*-dimethylaniline has been reported to act as a phagostimulant in *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) and as a potent toxin in *Melophagus ovinus* L. (Diptera: Hippoboscidae) by increasing the penetration rate through the body cuticle (Webb, 1946; Hedin et al., 1968).

1,2,5-trithiepane is an organic chemical compound synthesized from 2-chlorethyl disulfide by reacting with sodium sulfide and a heterocyclic closed chain compound (Windhager et al., 2007). Synthesis of 1,2,5-trithiepane in living organisms has reportedly occurred via metabolism of proteins rich in sulfide and disulfide (Field and Foster, 1970) in *Allium* (Alliaceae) and *Brassica* (Brassicaceae) plant species (Kubec et al., 1997). However, this is the first report of 1,2,5-trithiepane in insects. Unfortunately we did not found a commercial version of this compound to be tested in larvae.

P. nigrispinus saliva and *N,N*-dimethylaniline induced mortality in *A. gemmatalis* larvae within a short period of time. The LT_{50} of *A. gemmatalis* injected with LC_{90} saliva and *N,N*-dimethylaniline were approximately 16 and 19 h, respectively. One possible explanation for the time difference is that saliva contains a mixture of substances that act synergistically to potentiate lethal effects on prey. Lethal times are similar to those of *Rhynocoris fuscipes* preying on *Helicoverpa armigera* (Sahayaraj and Vinothkanna, 2011) and *Rhynocoris marginatus* preying on *Spodoptera litura* (Sahayaraj and Muthukumar, 2011). Predatory *Podisus* species attack multiple regions of the prey body over a period of time, ranging from one to 13 min. Partial prey paralysis occurs in two hours, allowing the predator to possibly suck the prey while it is still alive (Lemos et al., 2005a,b), although almost 50% of predators fail at prey paralysis after one hour of multiple attacks (Saavedra et al., 1997). The longer time period (16 h) for prey death following injection of saliva extracts compared to field observations may be due to the ability of the predator to attack multiple times, resulting in injection of a significant amount of saliva associated with tissue digestion by collagenase (Fialho et al., 2012). This may also explain the LT_{50} for *N,N*-dimethylaniline (19 h) as being due to a single injection of toxin without any co-factors.

Overall, the present study characterizes the toxic substances present in *P. nigrispinus* saliva that are responsible for prey paralysis and death. These toxic substances are likely non-proteinaceous compounds, including *N,N*-dimethylaniline and 1,2,5-trithiepane. This is the first study to report the presence of such compounds in insect saliva. These findings show that the salivary glands of predatory insects are a potential source of insecticidal compounds and warrants further investigation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.11.006.

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