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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;

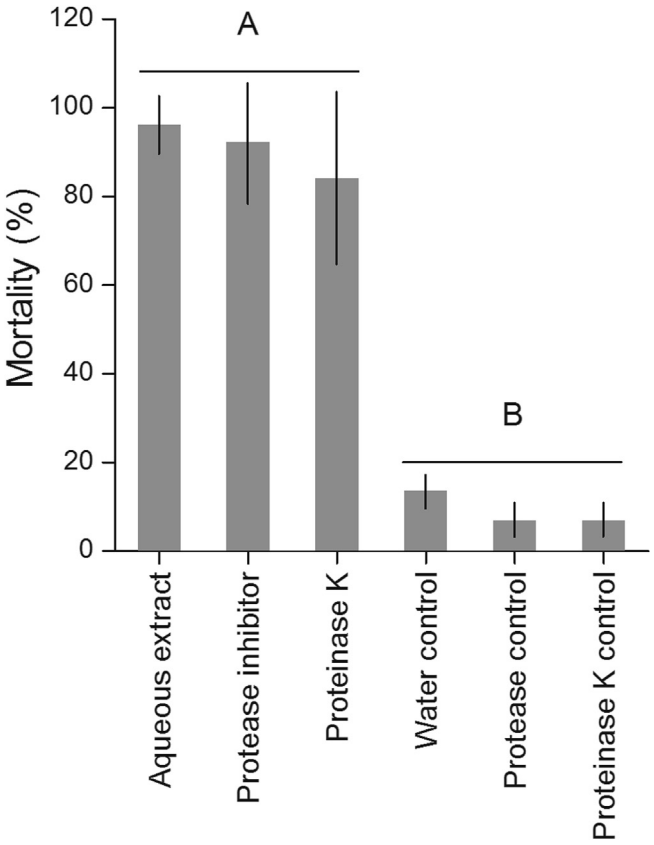

Fig. 1. Mortality (Mean ± SE) of *Anticarsia gemmatalis* larvae caused by the saliva of *Podisus nigrispinus* in aqueous extract with protease inhibitor and proteinase K. Letters in columns indicate significant differences by Tukey's HSD test (P < 0.05).