Molecules with antimicrobial activity in the secretion of the arthrodial membrane gland of a harvester (Arachnida, Opiliones)

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Short Report

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Abstract

Because of the exoskeleton, arthropods must have flexible areas to be able to move. Such regions are called arthrodial membranes and are particularly vulnerable to bacteria and fungi. Here, we analyzed the secretion in the glands underneath it in a Neotropical harvester (Arachnida, Opiliones) and tested whether it has antiseptical properties. We punctured the membrane, collected and diluted the secretion and quantified proteins and peptides in a spectrophotometer. We also fractionated and analyzed the samples in reversed-phase high-performance liquid chromatography (RP-HPLC) and then incubated the treated fractions and determined growth inhibition by measuring absorbance. The secretions resulted in 42 fractions, among which two had activity against the Gram-positive bacteria *Micrococcus luteus* and against the yeast *Candida albicans*. The low concentrations at which the secretions were active are relevant from a biotechnological point of view. For the animals, the secretions possibly prevent infections, including when they are attacked in these regions by predators that pick that spot to bite.

Introduction

The cuticle of arthropods has several functions, such as a protective barrier against predators, preventing water loss, barrier against ingress of water, ions or environmental chemicals, maintaining body shape, structural support and physical barrier against parasites and microorganisms [Charnley & Leger 1991, Ortiz-Urquiza & Keyhani 2013]. Fungi, for example, have strategies that allow them to cross arthropod cuticles, including adhesion mechanisms and the production of enzymes and other metabolites that facilitate infection [Ortiz-Urquiza & Keyhani 2013]. If the physical barriers are crossed, an immune response mediated by compounds in the hemolymph, which contain hemocytes and plasma, comes into play [Loker et al 2004].

The main sites of infection by microorganisms in arthropods are wounds, sense organs and the arthrodial membrane (AM) at joints and between segments [Klowden 2013]. The AM are particularly great sites for infection since they are generally thinner due to the reduction or absence of the exocuticle [Leger RJ 1990, Klowden 2013]. In a recent work, we demonstrated that the arthrodial membrane of the harvester *Mischonyx squalidus* (Arachnida, Opiliones) shows features of secretory activity such as pores and cuticular canals, secretory cells with mitochondria, smooth endoplasmic reticulum and secretory granules [Silva et al 2021, unpublished data]. Furthermore, we demonstrated that the secretions of these secretory cells include proteins and oils [Silva et al 2021]. In view of this evidence and based on the literature, we suggest that the arthrodial membrane has a gland with a lubricating function at least in the studied leg joint of leg IV [Silva et al 2021, unpublished data]. Since arthrodial membranes are easily accessible sites for invasion by microorganisms, we can expect that these animals have ways of avoiding infections. We therefore intended to test whether the secretion released by the secretory cells below the arthrodial membrane of *M. cuspidatus* inhibits the growth of pathogenic microorganisms, looking at specific fractions of molecules with antimicrobial activity.

Methods

Mischonyx squalidus (Roewer, 1913) appears as *Mischonyx cuspidatus* or *Ilhaia cuspidata* in previous papers (see Gueratto et al. 2021). We manually collected male individuals of *M. squalidus* (August 2018 and January 2020) under SISBIO/ICMBio license number 61431-1- 2018. We found the animals under trunks at the Parque Ecológico do Tietê, São Paulo city, São Paulo State, Brazil. We fed the animals twice a week with moist dog food. We extracted the secretions from prechilled animals (-20 °C for 15 min) by puncturing the dorsal region of the arthrodial membrane gland with a pyrogenic syringe. We used a total of 100 µL of secretion extracted from 35 animals. We diluted the secretion in ultrapure water (50 µL) and 0.05% trifluoroacetic acid (TFA) (50 µL). To quantify the gland secretion molecules, we quantified proteins and peptides by reading the absorbance at 280 nm and 205 nm using 1 µL of sample in the spectrophotometer NanoDrop 2000 model (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA) full spectrum (190 to 940 nm). To fractionate the secretion from the arthrodial membrane gland in *M. squalidus* (Fig. 1), we injected the secretion dilution (200 µL) in 0.05% TFA (18000 µL). We fractionated and analyzed the samples in a reversed-phase high-performance liquid chromatography (RP-HPLC) instrument (Shimadzu LC-8A). We ran the analysis in a 60 min gradient at a 1 mL/min flow rate with a C18 analytical column (Jupiter, 4.6 mm × 250 mm) equilibrated with 0.05% trifluoroacetic acid (TFA) at ambient temperature. The elution gradient for the sample was 0–80% of solution B (acetonitrile acidified with 0.05% trifluoroacetic acid) in solution A (0.05% trifluoroacetic acid). We monitored the effluent absorbance at 225 nm, and the fractions were hand collected, concentrated under vacuum, and reconstituted in ultrapure water. To assess whether AM secretion has the ability to inhibit the growth of microorganisms, we conducted a liquid microbial growth inhibition assay in 96-well microplates. We concentrated each fraction of secretions obtained via RP-HPLC in 100 µL of ultrapure water. **Microbial Strains** – We obtained bacterial and yeast strains from the collection of microorganisms of the Laboratory for Applied Toxinology (LETA) of the Butantan Institute (São Paulo, Brazil). We performed bioassays with *Micrococcus luteus* A270, *Escherichia coli* SBS363 and *Candida albicans* MDM8. **Antimicrobial Assays** – We evaluated the antimicrobial effects by liquid growth inhibition assays as described in Hayashida and Silva Junior (2021). We cultured bacteria in poor nutrient broth (PB) (1.0 g of peptone in 100 mL of water containing 86 mM NaCl at pH 7.4; 217 mOsm) and yeast in poor potato dextrose broth (1/2 PDB: 1.2 g of potato dextrose in 100 mL of H2O at pH 5.0; 79 mOsm). We determined antimicrobial activity using a fivefold microtiter broth dilution assay in 96-well sterile microplates at a final volume of 100 µL. We diluted the mid-log phase culture to a final concentration of 5×10^4 CFU/mL for bacteria and 5×10^5 CFU/mL for yeast (Hayashida and Silva Junior 2021). We dissolved dried fractions in 100 µL of ultrapure water and placed 20 µL aliquots in each well with 80 µL of the microbial dilution. We incubated the microplates for 18 h at 30 °C; we determined growth inhibition by measuring absorbance at 595 nm. As a positive control for microbial growth inhibition, we used 10 mg/mL antibiotics (ampicillin, streptomycin and tetracycline).

Results

The secretions resulted in 42 fractions via HPLC (we tested all fractions for antimicrobial activity), among which two had antimicrobial activity (Fig 2). Fraction 59 was active against the gram-positive bacteria

Micrococcus luteus, and fraction 71 was active against the yeast *Candida albicans* (Table 1). None of the molecules were active against the gram-negative bacteria *Escherichia coli* (Table 1). Fraction 71 was more concentrated than fraction 59 (Table 2).

Discussion

A number of papers describe antimicrobial molecules in setae, venom, secretions, hemolymph (hemocytes and plasma) and body extracts in arthropods, including arachnids (Riciluca et al 2021; Chaparro & Silva-Junior 2016; Diaz-Roa et al 2018; Segura-Ramírez & Silva-Junior 2018). In harvesters, Sayegh et al. (2016) found an AMP (longipin) in the hemolymph of *Acutisoma longipes* (Opiliones, Gonyleptidae) that has antifungal activity. However, to our knowledge, ours is the first study to find antimicrobial molecules beneath the arthrodial membranes in arachnids.

Harvesters in the suborder Laniatores are known for their thick integument that protects them against predators (Souza and Willemart 2011; Dias and Willemart 2013). One of the vulnerable areas is precisely the arthrodial membranes of legs, which are used by specific predators to bite (Segovia et al 2015). In such cases, should the harvester escape the attack, it will have a wound that gives free access to bacteria and fungi (Leger 1990; Charnley & Leger 1991). Therefore, the antimicrobial molecules we found are probably of importance in this context.

The proteomic analysis of the content of the secretory cells in the AM revealed similarities with proteins, specifically with peptides with antimicrobial activity [Silva et al 2021], whose activity has now been demonstrated. Moreover, both fractions have antimicrobial activity at low concentrations, which is important from a pharmacological point of view (Nascimento et al 2016; Diniz et al 2018; Diaz-Roa et al 2018, 2019).

The function of the other proteins remains unknown, but Silva et al. (2021) found molecules putatively homologous to peptides and/or proteins with functions such as cellular metabolism, signaling and binding and defense. We also do not know the mechanisms through which the peptides act, but they usually either break the cell membrane or interact with internal components of the bacterium cell (Benfield and Henriques 2020).

In conclusion, we found an antiseptic function of the secretions in the arthrodial membrane of leg IV of a harvester, a region particularly vulnerable to infections. Because of its low concentration and efficiency against a bacterium and a fungus, there is also potential pharmacological interest.

Declarations

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Tables

Table 1 and 2 are available in the Supplementary Files section.

Figures

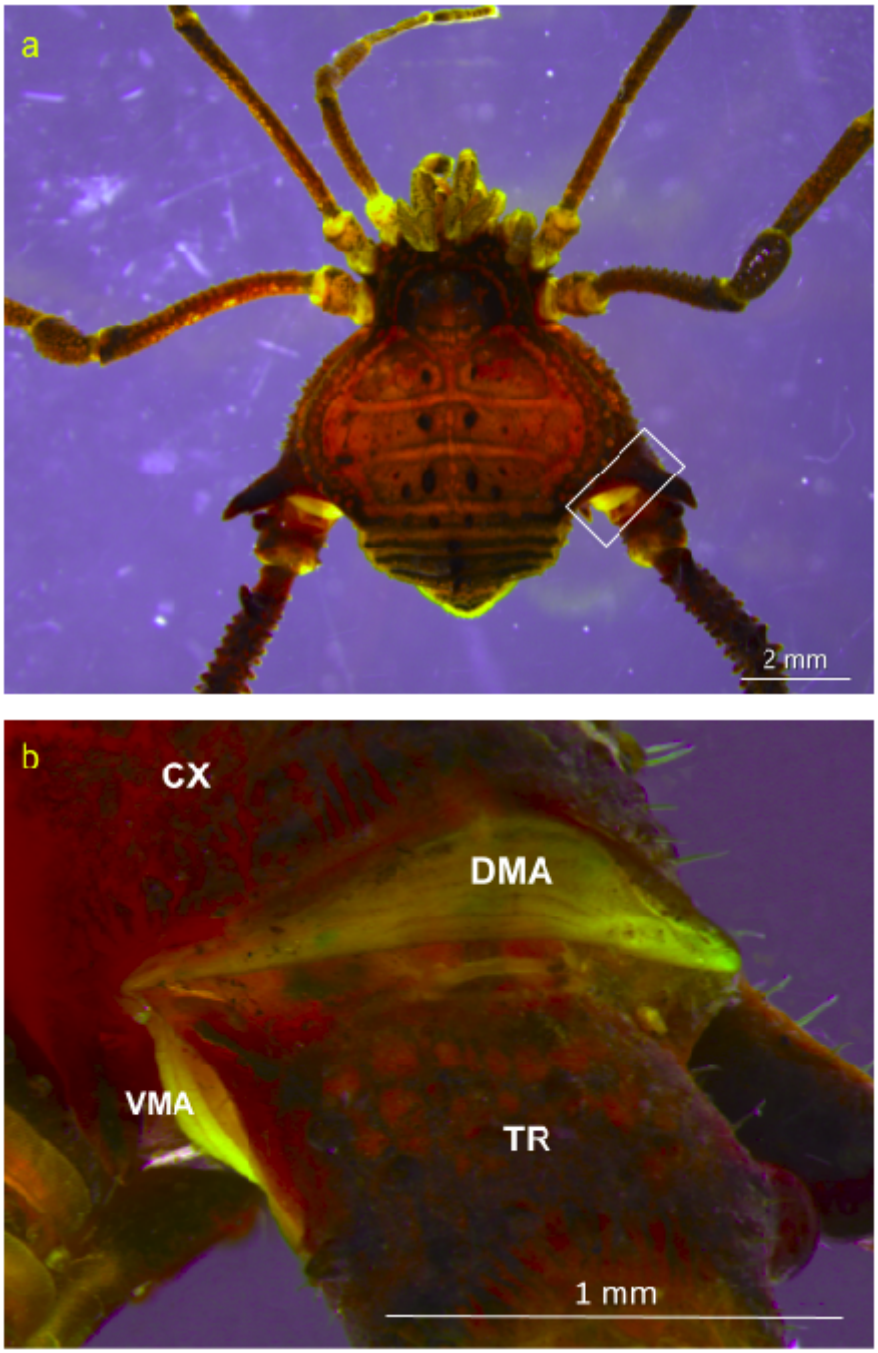

Figure 1

a) Dorsal view of a male in the harvester *Mischonyx squalidus*(Arachnida, Opiliones). The white square shows the arthrodial membrane on leg IV. b) Detail of the arthrodial membrane in the ventral and dorsal regions. cx = coxa, tr = trochanter, dma = dorsal arthrodial membrane, vma = ventral arthrodial membrane.

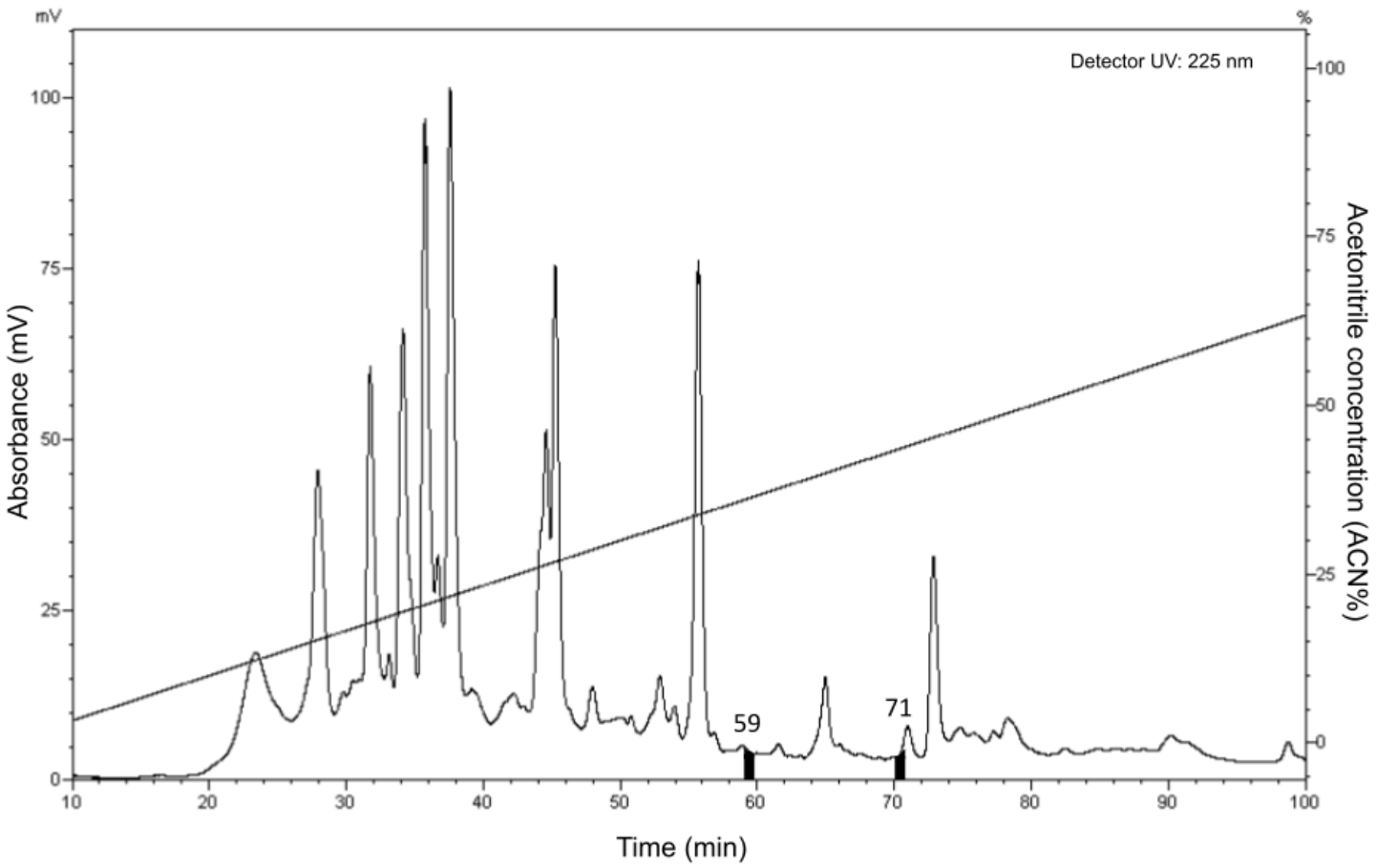

Figure 2

Chromatographic profile of the purification step of the harvester *Mischonyx squalidus* (Arachnida, Opiliones) arthrodial membrane gland secretion. High-performance liquid chromatography in reversed-phase (RP-HPLC) analysis was performed in a 60 min gradient at a 1 mL/min flow rate (Jupiter C18 analytical column, 4.6 mm × 250 mm) equilibrated with 0.05% trifluoroacetic acid (TFA). The enumerated peaks correspond to the fractions with antimicrobial activity.

Supplementary Files

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- Table11.png
- Table21.png