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Grandisin caused morphological changes larval and toxicity on *Aedes aegypti*

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Abstract: Dengue is a tropical disease caused by an arbovirus transmitted by *Aedes aegypti.* Since no effective vaccine is available for treating dengue, the present study focused on population vector control through investigating the use of the lignan grandisin, isolated from *Piper solmsianum* C. DC., Piperaceae, against the larvae of *A. aegypti.* Grandisin caused larval (L3) mortality at LC50 150 µg/mL. Histological analysis on *A. aegypti* larvae treated with grandisin (LC50 50 µg/mL) showed changes in the anterior-middle midgut, with intense tissue destruction and cell disorganization.

Article

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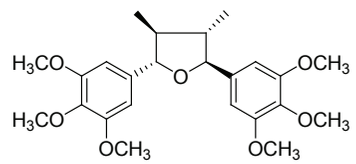
Introduction

The species *Piper solmsianum* C. DC. belongs to the Piperaceae family and is an endemic species from the Atlantic Forest (Mata Atlântica) in Brazil (Quijano-Abril et al., 2006). Secondary metabolism in most of the Piperaceae species appears to be restricted to the production of only a few classes of compounds such as amides, lignans and neolignans (Martins et al., 2000; Kato & Furlan, 2007). Several studies have been carried out on lignans and their effects on insects (Cabral et al., 2000 a,b; Cabral et al. 2001). The tetrahydrofuran lignan grandisin has been described as the major secondary metabolite in the leaves of *P. solmsianum* (Martins et al., 2003) and was first isolated from *Litsea grandis* (Holloway & Scheinmann, 1974). This substance has displayed powerful trypanocidal activity (Lopes et al., 1998; Kato & Furlan 2007) disruption of insect growth activity (Nogueira et al., 2009) and larvicidal activity (Cabral et al., 2009). *Aedes (Stegomyia) aegypti* L., 1762 (Diptera: Culicidae) is a widely distributed Neotropical species and it is the main vector insect for dengue disease (Consoli & Oliveira, 1994). Thus, the aim of this study was to determine the cytotoxicity and digestive system morphological changes of grandisin against immature forms (L3) of *A. aegypti.*

Material and Methods

(-)-Grandisin (**1**) was obtained from dried inflorescences of *P. solmsianum* that was collected from a garden at the Institute of Chemistry, USP, São Paulo (Martins et al., 2000). A sample, isolated as previously described (Martins et al., 2003) was dissolved in acetone and diluted at 1:4 in 0.8% NaCl at concentrations of 1-200 µg/mL. *A. aegypt*i eggs were obtained from the Transmissores de Hematozoários Laboratory, Instituto Oswaldo Cruz, FIOCRUZ, and a colony was maintained in the Diptera Laboratory, Instituto Oswaldo Cruz, Rio de Janeiro. All experiments were carried out on third-instar (L3) larvae (F1-F5).



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The grandisin solution (µg/mL) was applied to the Petri dishes (9.0 x 2.0 cm), which contained dechlorinated water (5 mL) at 5, 30, 50, 100 and 200 µg/mL concentrations, with fish food solution (0.3 mg per

larva) in accordance with the procedure described by Cabral (Cabral et al., 2009). Individualized *A. aegypti* L3 larvae (F1-F5) (20 larvae per group) were evaluated in triplicate. There were again two control groups: one with acetone solution (without grandisin) and another with untreated solution. The larvae were maintained in a climate-controlled chamber at 27±1 °C, 70±10% relative humidity and 12 h photoperiods and LC50 were analyzed using Spearman-Karber analysis (Hamilton et al., 1978).

The digestive system histology were carried out using L3 larvae (treated, control and acetone control) fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.2) for 4 h. They were then dehydrated with increasing concentrations of ethanol (70, 80, 90, 96 and 100%), through immersion in each of these solutions for 15 min. Next, they were embedded in historesin JB4 and the resultant blocks were sliced using a microtome to obtain a series of sections of 3 µm in thickness. These sections were stained using the hematoxylin-eosin technique and the slices thus obtained were examined and photographed using an optical microscope (Bancroft & Stevens, 1996).

The cytotoxicity bioassays were carried out using peritoneal macrophage cells from BALB/c mice. The cells were isolated from the animals' peritoneal and were kept in a culture media consisting of 0.1 mL RPMI 1640 supplemented with 1 mmol 1-1 HEPES, penicillin G (10^5 IU 1:1) and streptomycin sulfate (10 g 1:1). In all the assays, peritoneal macrophage cells were used at a concentration of 2 x 10^5/mL in each well of 96-well plates. After 2 h of incubation at 37 ºC under 5% CO_2, the cells were washed twice with RPMI, to remove non-adhering cells. The grandisin lignan was added to the cell cultures at a standard toxic concentration of 160 µg/mL, corresponding to 200 µM, and then incubated for 24 h at 37 ºC under 5% CO_2, in accordance with the procedure described by Sauvain et al. (1993).

The assays were based on tetrazolium (MTT) [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which is a fast colorimetric assay commonly used to determine cell proliferation and viability (Mosmann, 1983). After 4 h of incubation, the supernatant was discarded and DMSO was added to dissolve the formazan that had been precipitated. The absorbance was read in a spectrophotometer at 490 nm, with untreated cells taken to represent 100% viability (Andrighetti-Frohner et al., 2003). The cytotoxicity of the compound was calculated as follows:

$$\text{Viable cells} = \frac{\text{Average absorbance of each compound concentration}}{\text{Average absorbance of control}} \text{x100}$$

The lethal concentration 50% (LC50) was defined as the concentration causing 50% cell death. The percentage of macrophage lysis was determined in triplicate.

Results

A. aegypti (larvae L3) grandisin treated interfered with the development of larvae at stages L3-L4 and with development from larvae to adults. The grandisin caused 79% (13,6±2,08, $p \leq 0{,}001$) larval (L3) mortality at 200 µg/mL and 50% of the larvae died within 24 h of the treatment, while the remaining larvae survived for up to 192 h, with LC50 150 µg/mL. Mortality was not observed in acetone control solution (without grandisin) and untreated solution. The lignan showed 36% (36±0,009) toxicity on macrophages at LC50/24h 160 µg/mL when compared acetone control solution (1±0,002) and DMSO control solution (1±0,002) at LC50/24 h 190 µg/mL and LC50 32 µg/mL, respectively. Histological analysis on *A. aegypti* larvae treated with grandisin (LC50) showed changes in the intestine, specifically the level in the anterior-middle midgut (Figure 3B), in which intense tissue destruction and cell disorganization were observed. Most cells in this region showed extensive vacuolization in the cytoplasm. Some cells showed absence of cytoplasmic boundaries (Figure 3C). The larvae of the control groups and acetone control had digestive tract cells with a normal appearance and without morphological changes (Figures 1C and 2B). They showed well-preserved epithelium with a basal membrane and a muscle layer (Figure 2D). The epithelial cells were seen to be arranged in a single layer of low cylindrical cells and the apical surface covered with microvilli. Malpighian tubules showing cellular disruption (Figure 3B) and neither was the muscle tissue, in which no effect was observed with grandisin, either in the treated groups or in the control groups (Figures 3B and 2D).

Discussion

The *A. aegypti* mortality after treatment with the lignan grandisin (1) isolated from *P. solmsianum* was corroborated through findings using extracts from *Piper* species against the larvae of Alabama argillacea (Lepidoptera: Noctuidae) (Miranda et al., 2002). Previous studies on treatment of L1 larvae of *A. aegypti* with grandisin showed 100% larval mortality at 100 µg/mL concentration (Cabral et al., 2009). Additionally, 100% mortality was also observed with the lignan, on third-instars larvae (L3) at >200 µg/mL concentrations.

The toxic effect of grandisin on the larvae of *A. aegypti* observed in the anterior-median midgut corroborate the initial observation of changes to the midgut of *A. aegypti* when exposed to treatment with plant extracts (Arruda et al., 2003a). The peritrophic membrane of larvae treated with grandisin continued to present a normal appearance, in contrast to the greater

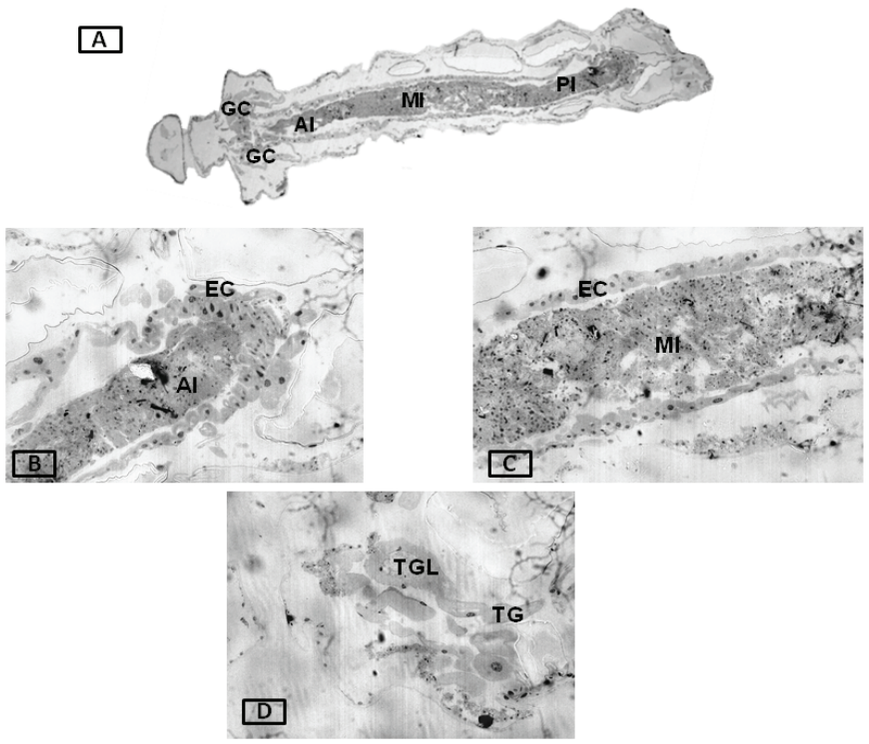

Figure 1. Photomicrographs of the digestive tract of L3 larvae of *A. aegypti* in the control group maintained with food, stained with HE, longitudinal section. A. Overview; gastric caeca (GC), anterior intestine (AI), midgut (MI), hindgut (PI) 10x; B. Details of the anterior intestine (AI) showing intestinal epithelial cells (EC) 20x. C. Midgut (MI) and intestinal epithelial cells (EC) 20x; D. Details of the Malpighian tubules (MT) and lumen of the Malpighian tubule (TGL) 20x.

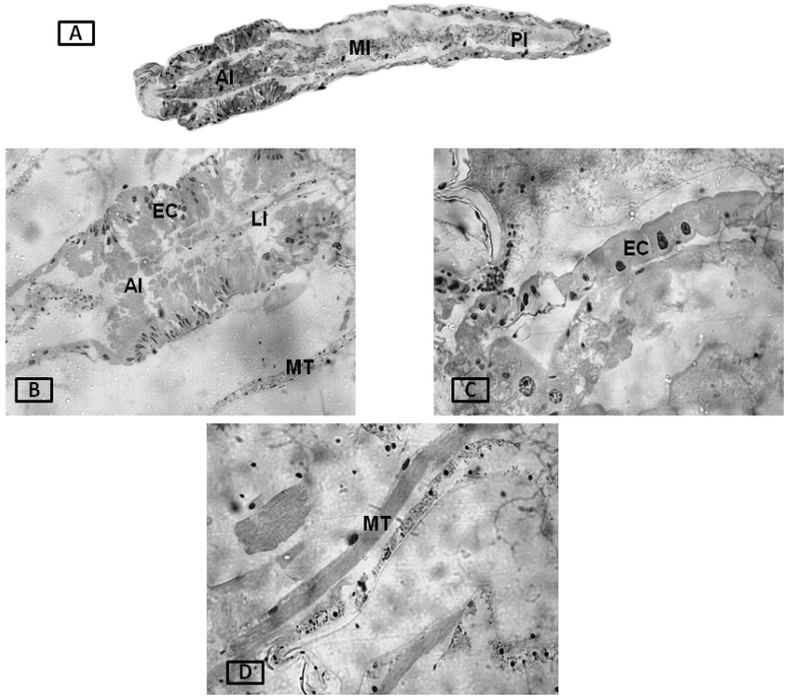

Figure 2. Photomicrographs of the digestive tract of L3 larvae of *A. aegypti* in the control acetone group maintained with food, stained with HE, longitudinal section. A. Overview. Anterior intestine (AI), midgut (MI), hindgut (PI). B. Details of the anterior intestine (AI) showing lumen of the intestine (LI), intestinal epithelial cells (EC), and muscle tissue (MT); C. Details of the posterior intestine cells (EC) 40x. D. Details of the muscle tissue (MT) 4x.

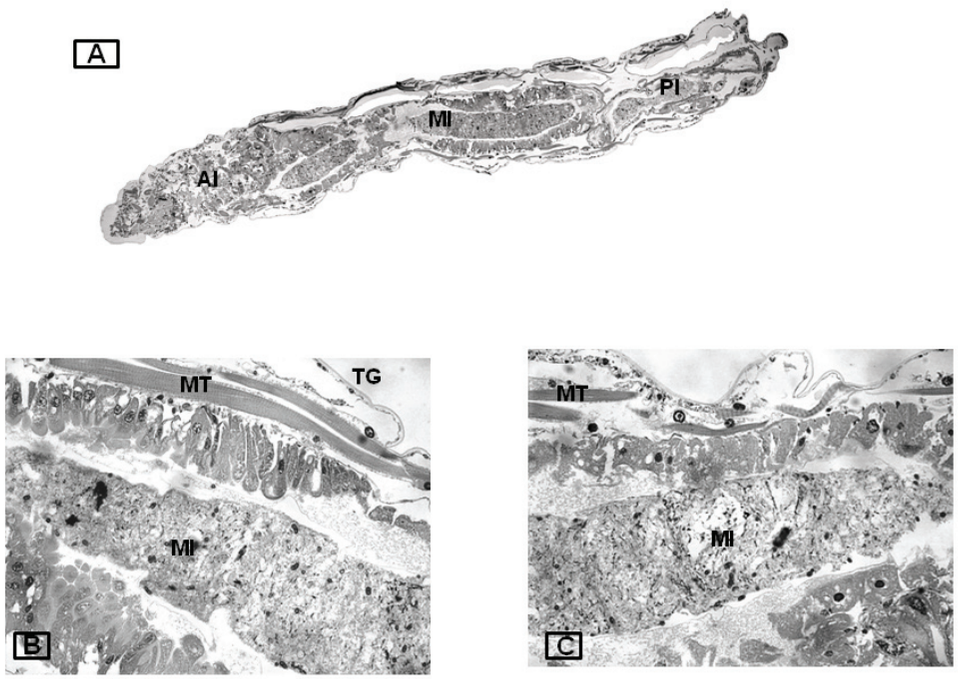

Figure 3. Photomicrographs of the digestive tract of L3 larvae of *A. aegypti* in the grandisin test group maintained with food, stained with HE, longitudinal section. A. Overview. B. Anterior intestine (AI), midgut (MI), hindgut (PI) 10x. C. Details of the midgut (MI) showing muscle tissue (MT) and Malpighian tubules (TG) 20x.

effect on peritrophic membrane thickness in *A. aegypt*i when treated with extracts of *Magonia pubescens* (Arruda et al., 2003b). The cell destruction in *A. aegypti* began in the anterior midgut and ended in the middle, but without reaching the posterior intestine. These data suggest that there is a gradual process of tissue destruction, possibly related to the duration of exposure of larvae to grandisin. In conclusion, this study clearly illustrated the efficacy of the lignan grandisin, with potential for use against *A. aegypti* larvae.

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