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Agave sisalana extract induces cell death in *Aedes aegypti* hemocytes increasing nitric oxide production

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ABSTRACT

Objective: To investigate the effects of *Agave sisalana* (*A. sisalana*) extract on *Aedes aegypti* (*Ae. aegypti*) primary cell culture.
Methods: Cells of *Ae. aegypti* were exposed to different concentrations of *A. sisalana* crude extract (0.18–6.00 mg/mL) for 24 h. Then, the cells were labeled with propidium iodide and subjected to fluorescence microscopy to verify cell viability. In addition, nitric oxide production was measured.
Results: Results showed that cells exposed to 6 mg/mL of the crude extract presented a greater percentage of death when compared to control (73.8% ± 9.6% *vs*. 34.6% ± 9.6%). Furthermore, there was an increase in the nitric oxide production in cells exposed to 6 mg/mL of *A. sisalana* crude extract [(0.81 ± 0.08) μmol/L] compared to control group [(0.41 ± 0.18) μmol/L].
Conclusions: The results show that *A. sisalana* is cytotoxic to *Ae. aegypti* and may be used as raw material for new eco-friendly and inexpensive insecticides, since sisal industry discards the liquid waste for the extraction of plant fiber.

1. Introduction

Aedes aegypti (*Ae. aegypti*) is a mosquito that is responsible for transmitting many diseases such as dengue fever, Chikungunya fever, yellow fever and Zika fever [1–3]. These diseases cause significant morbidity and mortality in developing countries [3]. Recently, dengue transmission has increased in urban and semi-urban areas of tropical countries around the world, becoming a major global public health problem. The World Health Organization estimates that there may be 50–100 million dengue infections worldwide every year [4,5].

Considering that there is no specific treatment or vaccine for dengue fever, its control is performed by combating with the mosquito vector [6–9].

Usually, mosquito control is done with chemical insecticides [9]. Mosquito larvae are usually targeted using organophosphates, insect growth regulators, and microbial control agents. Indoor residual spraying and insecticide-treated bed nets are also employed to reduce transmission of mosquito-borne disease in tropical countries [10,11]. However, studies have shown the development of resistance to the most common insecticides in many countries [9,12]. Therefore, the seek for new insecticides is needed, and natural products can be a reliable and eco-friendly source of raw materials for that purpose [13].

Agave sisalana (*A. sisalana*) is a monocotyledonous plant from the Agavaceae family, originally from Mexico. The plant, commonly known as sisal, is cultivated mainly in tropical and semi-arid areas around the world [14–16]. Several studies have been conducted to explore the possible biological activities of *A. sisalana*. Researchers have described anti-inflammatory, analgesic, anthelmintic and bactericidal activities of *A. sisalana* [17–19]. In addition, a recent study from our laboratory reported that *A. sisalana* possessed larvicidal activity against *Ae. aegypti* [20].

Therefore, the aim of this study was to investigate the effects of *A. sisalana* crude extract on *Ae. aegypti* hemocytes death,

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exploring the possible mechanisms involved, especially as regards the nitric oxide production, which is an important substance involved in the insects immune system.

2. Materials and methods

2.1. Plant material

A. sisalana was cultivated in the city of Pocinhos (7°24′54″ S, 39°24′36″ W, 624 m above sea level) in Paraiba State, Brazil. The crude extract was obtained by grinding the leaves in a manual grinder until complete extraction of the crude extract. Then, the crude extract was strained and placed in a plastic container, protected from light, in a freezer at −10 °C until use. The crude extract was characterized regarding its physical and chemical composition, for determination of soluble solids (°Brix), total solids, saponins, reducing sugars and pH. Soluble solids (°Brix) were determined by manual refractometer. Total solids were determined according to the method described by Carle and Reinhardt [21], and saponins were determined by gas chromatography. Reducing sugars were determined by the dinitrosalicylic acid method [22], and pH was measured using a digital pH meter.

The Brazilian Agricultural Research Corporation (Embrapa), located in Campina Grande, Paraiba, was responsible for harvesting the plant and extracting the crude extract.

2.2. Primary cell culture

Ae. aegypti third instar larvae were sanitized with 70% alcohol and rinsed in phosphate buffer solution, and then they had their hemolymph collected and placed in tube containing phosphate buffer solution. The pool of hemolymph was centrifuged at 1 500 r/min for 7 min in a refrigerated centrifuge at 4 °C and the supernatant was discarded. The hemocytes were placed in cell culture vials containing Leibovitz medium (Himedia), supplemented with 10% of fetal bovine serum, penicillin-streptomycin (300 IU/mL), gentamicin (100 μg/mL), and fungizone (2.5 mg/mL). The culture was incubated at 28 °C for 5 days.

2.3. Evaluation of cell viability by fluorescence microscopy

For the assay, 1×10^5 cells from the primary cell culture were plated in 12-well plates and exposed to different concentrations of *A. sisalana* crude extract (0.18–6.00 mg/mL) for 24 h. Cells were centrifuged at 1 500 r/min for 7 min in a refrigerated centrifuge at 4 °C and the supernatant was discarded. The final volume was supplemented with 2 mL of Leibovitz medium (Himedia) and transferred to 15 mL tubes containing 15 μL of propidium iodide to differentiate intact hemocyte from those necrosis. After 15 min of incubation in the dark, the cells were counted using a fluorescence microscope (Motic, Xiamen). In the control groups cells were exposed only to PBS. Assay was performed in triplicate.

2.4. Measurement of NO

For this assay, 1×10^5 cells were plated in 12-well plates and exposed to *A. sisalana* crude extract (0.18–6.00 mg/mL) for 24 h. The NO production was evaluated in the cultures exposed to different concentrations of the *A. sisalana* crude extract (0.18–6.00 mg/mL), according to method of Green *et al.* [23]. For this purpose, 50 μL of the cell supernatant was collected and placed in 96-well plates containing 50 μL of Griess reagent and incubated for 15 min. In the control groups cells were exposed only to PBS. The assay was performed in triplicate. The absorbance was measured using a microplate reader under 562 nm and the NO was quantified using a standard curve of $NaNO_2$ as a reference.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). Significant differences between groups were analyzed by ANOVA followed by Tukey's HSD test ($P < 0.05$).

3. Results

The composition of the crude extract included: soluble solids (7.70 ± 0.09) °Brix, pH 4.82, phenolics (1.93 ± 0.02) mg/L, nitrogen 980 mg/L, protein 6.13 mg/L, glucose 12.4 g/L, fructose 1.2 g/L and saponins 2.4 mg/mL.

In order to evaluate the cytotoxicity of the *A. sisalana* crude extract, hemocytes were exposed to different concentrations of the plant extract (0.18–6.00 mg/mL) for 24 h. Fluorescence microscopy showed that the cells exposed to concentrations below 6 mg/mL had no significant difference in cell death compared to the control group. The cells exposed to 6 mg/mL of the crude extract of *A. sisalana* showed (73.8 ± 9.6)% of necrotic cells *vs.* (34.6 ± 9.6)% in the control group, after 24 h of exposure (Figure 1). It is worth mentioning that it presented more 39.2% necrotic cells than that in the control group.

The NO is involved in immunological reactions from the insect's metabolism, and for this reason it was measured in this study. The results showed that the cells exposed to concentrations below 6 mg/mL had no significant difference in the NO production compared to the control group. After 24 h of exposure to the 6 mg/mL of *A. sisalana* crude extract, the NO concentration in the cell supernatant was (0.81 ± 0.08) μmol/L, which was twice as much as that in the control group [(0.41 ± 0.18) μmol/L] (Figure 2).

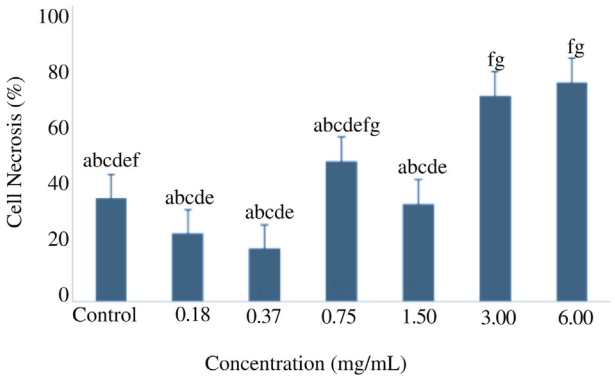

Figure 1. Percentage of cell necrosis of *Ae. aegypti* hemocytes exposed to different concentrations of crude extract of *A. sisalana*.
Bars with the same letter are not significantly different by Tukey test, 5%.

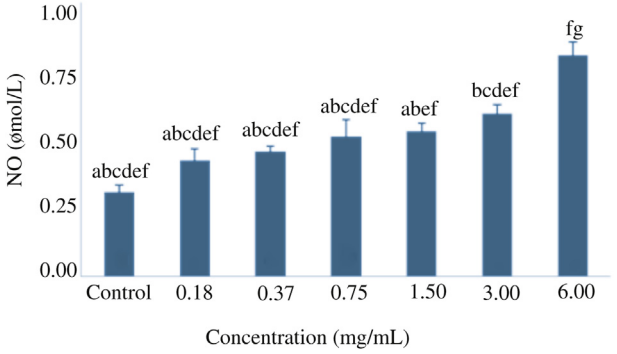

Figure 2. NO production in *Ae. aegypti* hemocytes exposed to different concentrations of the *A. sisalana* crude extract.
Bars with the same letter are not significantly different by Tukey test, 5%.

4. Discussion

Our research highlighted that the crude extract of *A. sisalana* has cytotoxic activity, killing 73.8% of the *Ae. aegypti* cells after 24 h of exposure to the extract at concentration of 6 mg/mL. The experimental group showed more 39.2% hemocytes necrosis, when compared to the control group. In addition, the results also showed that the *A. sisalana* crude extract led to increased production of NO by *Ae. aegypti*.

In order to evaluate the cytotoxicity of *A. sisalana* to the hemocytes, the cells were labeled with the fluorochrome propidium iodide and observed in a fluorescence microscope. The propidium iodide binds to DNA of cells that have damaged cellular membrane. External stimuli can trigger cell death by necrosis or apoptosis [24]. In our study, the *A. sisalana* crude extract was responsible for causing this deleterious effect on hemocytes of *Ae. aegypti*, killing more than 70% of the cells in 24 h.

In a previous study, Nunes *et al.* tested the larvicidal activity of *A. sisalana* against *Ae. aegypti* [20]. They tested the concentration of (4.50 ± 0.07) mg/mL for 3, 6, 12 and 24 h. The flow cytometry of the hemolymph from larvae showed that the experimental group presented more 16.5% necrotic cells than the control group. In the present study, the cells exposed to 6 mg/mL of extract presented more 39.2% necrotic cells than the control groups, which represents more than twice of cellular necrosis as that of the study performed by Nunes *et al.* [20]. The difference in the results may be explained by the fact that our study was done *in vitro* while Nunes *et al.* conducted the experiments *in vivo* [20]. In addition, the concentration of *A. sisalana* crude extract used in our study was higher than that used in the previous study (6.0 *vs.* 4.5 mg/mL). Pizarro *et al.* tested the effect of the liquid waste from *A. sisalana* on *Ae. aegypti* and *Culex quinquefasciatus* [25]. The researchers reported that the LC_{50} for *Ae. aegypti* was 322 ppm and the LC_{50} for *Culex quinquefasciatus* was 183 ppm. Dharmshaktu *et al.* reported the larvicidal properties of leaf and seed extract of *Agave americana* against *Anopheles*, *Aedes* and *Culex* larvae [26]. After 24 h, dilution of the seed extract of 1:200 produced a larval mortality of 100% for *Anopheles* and *Aedes*, and 56% for *Culex* spp.

Aguiar *et al.* tested the cytotoxicity of essential oils isolated from *Siparuna guianensis* to cells (C6/36) from *Aedes albopictus* [27]. They found that the cells from the experimental groups, which were exposed to 0.86 μg/mL of *Siparuna guianensis* essential oil lowered cell viability to less than 20%, compared with 90% in the control group.

Our study also aimed to verify the production of NO by the cells exposed to the *A. sisalana* crude extract. NO is an important cellular mediator capable of destroying pathogens and tumor cells. It also has a role as a modulator in several biological essential processes. On the other hand, NO is potentially toxic, particularly in oxidative stress [28,29]. The increase in the NO production in insects is related to the immune response against foreign agents [30]. In 1998, Luckhart *et al.* reported the involvement of reactive nitrogen species in the mosquito immune system [31]. They found that *Anopheles stephensi* fed with NO synthesis inhibitors facilitated infection by *Plasmodium berghei*. Gupta *et al.* observed that the NO production was also able to combat infection by *Plasmodium* sp. after the passage of the parasite from the intestine, possibly acting directly on the oocysts [32].

Guimarães *et al.* evaluated the NO production in *Diatraea flavipennella* exposed to sublethal concentrations of *Metarhizium anisopliae* (Metsch.) Sorok [33]. They reported that NO production was higher in the experimental group than that in the control group. Chavez *et al.* shown that intoxication of *Manduca sexta* larvae with *Bacillus thuringiensis* Cry1Ab activates expression of NO synthase with a corresponding increase in NO production [34]. These findings indicate that the NO has been implicated in immune response against microbial pathogens.

Overall, in our study, we observed that the concentration of NO in the cell supernatant of hemocytes exposed to crude extract of *A. sisalana* was higher than that in the cells from the control group. These findings indicate that the cells that were exposed to *A. sisalana* crude extract responsed to the aggression, increasing the NO production in an attempt to combat the suffered injury.

The results of this study show that the *A. sisalana* crude extract has cytotoxic effect on hemocytes of *Ae. aegypti*. In addition, *A. sisalana* crude extract increased the NO production in the cell supernatant. In conclusion, *A. sisalana* may serve as raw material for the production of a new insecticide against *Ae. aegypti*. Furthermore, only the fiber of this plant is used by industry, and the liquid part is completely wasted. Thus, the liquid plant extract becomes a low cost raw material.

Conflict of interest statement

We declare that we have no conflict of interest.

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