Vol.67: e24230057, 2024
https://doi.org/10.1590/1678-4324-2024230057
ISSN 1678-4324 Online Edition

BABT
Brazilian Archives of Biology and Technology

Article - Biological and Applied Sciences

The Polypeptides/Proteins Exuded from *Mimosa caesalpiniifolia* Seeds Induce Ultrastructural and Biomechanical Modifications of the *Caenorhabditis elegans* Surface and Inhibit the Nematode Motility.

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Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Jane Manfron Budel

Received: 19-Jan-2023; Accepted: 07-Sep-2023.

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HIGHLIGHTS

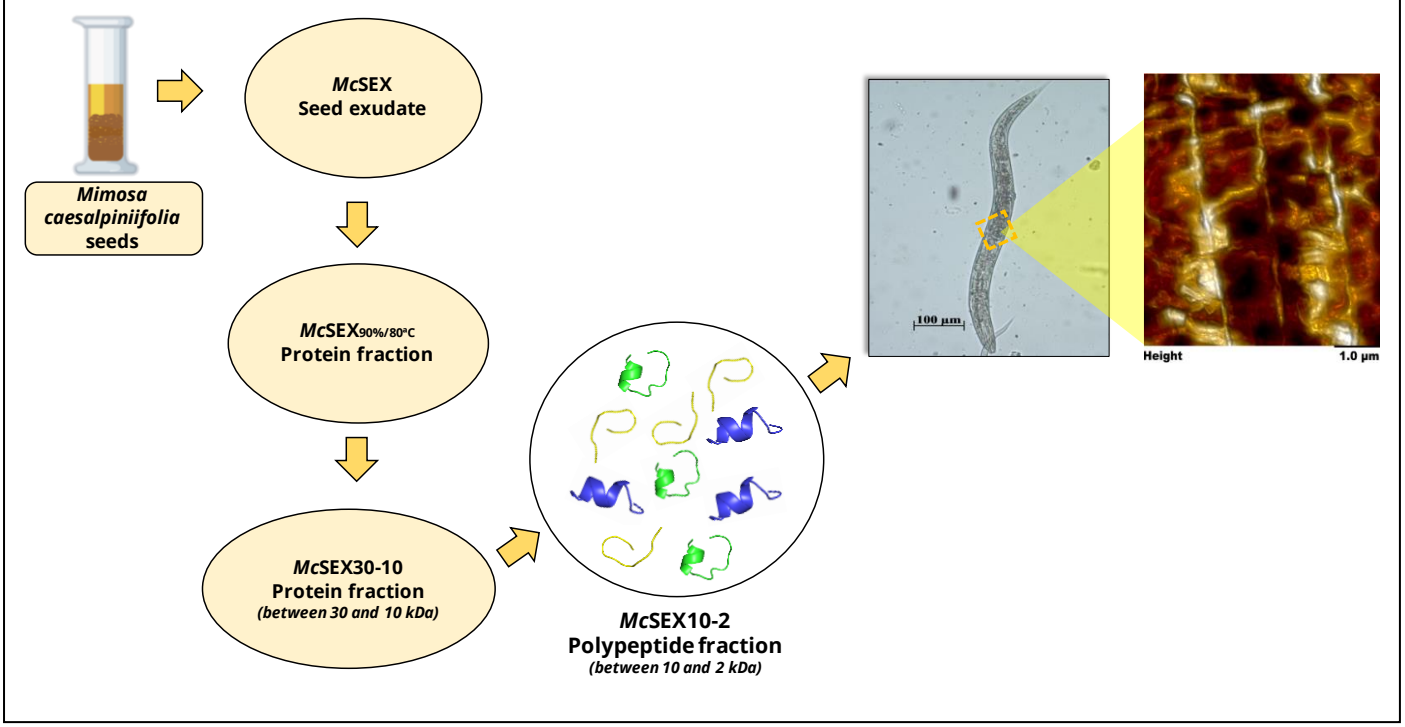
- Polypeptides/proteins exuded from *Mimosa caesalpiniifolia* mature seeds have anthelmintic effects.
- The Polypeptides fraction (*Mc*.PEP) of *M. caesalpiniifolia* have anthelmintic activity.
- *Mc*.PEP altered the ultrastructure and mechanical properties of nematode.
- The polypeptides fraction not is hemolytic or toxic to human cells.

Abstract: Natural products have been studied as potential anthelmintic drugs. This study aimed to evaluate the effects of the polypeptides/proteins exuded from *Mimosa caesalpiniifolia* mature seeds on the model nematode *Caenorhabditis elegans*. After seed immersion for 24 h at 25 ºC in sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl and 1.0 M $MgSO_4$, the constituent polypeptides/proteins were fractionated using ammonium sulfate precipitation followed by ultrafiltration and their effects on *C. elegans* motility were assessed. Moreover, we evaluated by Atomic Force Microscopy (AFM) whether exposition of the nematode to the polypeptide fraction *Mc*SEX10-2 could cause any alteration on the animal's external surface (cuticle).

The whole seed exudates (SEX) from the *M. caesalpiniifolia* mature seeds (named *Mc*SEX) and its polypeptide/protein derived fractions, termed *Mc*SEX$_{90\%/80°C}$, *Mc*SEX30-10, and *Mc*SEX10-2, inhibited the *C. elegans* motility with an IC_{50} of 15.18, 12.59, 9.74 and 1.34 µg mL^{-1}.The *Mc*SEX10-2 fraction, which encompassed the polypeptides with the molecular mass varying from 10 kDa to 2 kDa, was the most effective. In addition, *Mc*SEX10-2 altered the ultrastructure and the mechanical properties of the *C. elegans* cuticle. *Mc*SEX10-2, in particular, can be used as a model to bioinspire *structure-based design of new peptides* that could control, alone or in combination with other drugs, economically important parasitic nematodes.

Keywords: *Mimosa caesalpiniifolia;* seed exudation; anthelmintic protein/peptides; *Caenorhabditis elegans*; nematodes.

GRAPHICAL ABSTRACT



INTRODUCTION

Infections caused by parasitic nematodes constitute one of the major public health problems. Although of common occurrence, as they reach more than 24% of the world population, helminth infections are amongst the neglected tropical diseases (NTDs) [1-3]. To further complicate the issue, NTDs occur mostly in the world's poor populations of low and middle-income countries, which partly explain the limited interests of the pharmaceutical industries on such investments to develop new drugs and diagnoses for these diseases, as they represent low-profit markets [4, 5]. Less than 1% of global health research is directed to diseases caused by nematodes and to the production and development of new drugs for that purpose [6]. Estimates indicate productivity losses of up to US$ 138.9 billion annually due to nematode infection diseases [7]. Nematode infection is also a problem in the cattle and small ruminant industry worldwide. For example, *Haemonchus contortus* is recognized as the most economically important parasitic nematode at endemic zones, chiefly due to its common occurrence and potential to provoke high rates of mortality in small ruminants [8, 9]. Importantly, the indiscriminate use of anthelmintic drugs has contributed to the selection and establishment of resistant parasites towards the current anthelmintic agents. Therefore, the discovery of novel drugs and the development of alternative therapies are urgently needed [10, 11].

Caenorhabditis elegans is a free-living nematode that belongs to the order Rhabditida, naturally found in temperate climate soils [12]. It shares many similarities with other parasitic nematodes. As such, it has been used as a model in experimental studies to assess the potential of various substances to act as anthelmintic drugs [13, 14], because the animal is readily available, it can be easily handled, and the *in vitro* assay is inexpensive [15-17].

Mature seeds, under specific conditions (germination, for example), exude diverse chemical compounds. Amongst them, some polypeptides and proteins that eventually possess relevant biological activities against

bacteria, fungi, and plant and animal parasitic nematodes [18-24]. For instance, the proteins exuded from *Myracrodruon urundeuva* mature seeds prevent the larval development of *H. contortus* [25].

Isolation of natural polypeptides/proteins with relevant biological properties is challenging due to their low yield and the presence of contaminants. The use of synthetic bioinspired compounds is becoming more popular [26, 27]. They can be designed based on the primary structure of previously purified and characterized bioactive polypeptides/proteins, their associated biological and/or toxic activities can be simulated *in silico* employing publicly available prediction algorithms, and they can be synthesized with a high degree of purity [28].

Mimosa caesalpiniifolia Benth. belongs to the family Leguminosae, subfamily Mimosoideae. It is a native, fast-growing tree of northeastern Brazil, used as forage among other utilities [29-31]. Encouraged by the need to seek new bioactive compounds against nematodes and considering the attractive and constant growth of the protein and peptide markets due to their lower price, non-toxic nature, and fewer adverse side effects in the treatment of diseases [32], we, recently, studied and reported the anthelminthic activity of the seed exudates of *M. caesalpiniifolia* against *H. contortus* [33].

In this work, we fractionated the *M. caesalpiniifolia* seed exudates into their constituent polypeptides/proteins and evaluated their effects on *C. elegans* motility. In addition, we assessed by Atomic Force Microscopy (AFM) whether exposition of the nematode to the polypeptide fraction *Mc*SEX10-2 could cause any alteration on the animal's external surface (cuticle).

MATERIALS AND METHODS

Biological Material

Plant material

Mature seeds of *Mimosa caesalpiniifolia*, free from chemical or physical treatments, were commercially purchased from Arbocenter (Birigui, São Paulo, Brazil).

Cultivation of Caenorhabditis elegans

Cultivation of the *Caenorhabditis elegans* wild-type N2 was done according to the methodology described by Katiki [12]. Cultures were maintained in the Nematode Growth Medium (NGM) at the Laboratory of Parasite Control of the Center for Biological and Health Sciences, UFMA. Briefly, *C. elegans* was kept in Petri dishes that contained NGM seeded with 50 µL of 1% (w/w) *Escherichia coli* (NA22 strain) suspension in Luria Bertani-LB medium (10 g Bacto-tryptone, 5 g Bactoyeast, 5 g NaCl, H_2O to 1 liter, and pH adjusted to 7.0 with 1 M NaOH) [34, 35]. Nematodes were identified as been at the L_1–L_3 juvenile larval stages, and adults (L_4), based on their size and the presence and extent of vulvar development [36].

To select L_4 animals, four 4-7-day old cultures of *C. elegans* were transferred to a tube and the desired volume completed with the M9 buffer, pH 7.0, prepared with 3.0 g KH_2PO_4, 6.0 g Na_2HPO_4, 5.0 g NaCl, and 1.0 mL 1 M $MgSO_4$ dissolved to 1.0 L distilled H_2O. The suspension obtained, which contained animals at all stages of the nematode life cycle, was laid in a rack or 3 min to allow settling of the largest nematodes. The nematodes were separated according to their size using a 32 µm sieve. This procedure permitted to retain the largest nematodes, whereas the smaller ones passed through the lattice. The retained animals, which represented young adults, adults, and large dead nematodes, were recovered in the M9 buffer and the suspension obtained passed through a 53 µm sieve. The living, active young adult and adult nematodes were not retained, whereas the inactive, dead ones were trapped by the sieve.

Collection of the *Mimosa caesalpiniifolia* mature seed exudates (*Mc*SEX) and preparation of the constituent polypeptide/protein fractions

Preparation and collection of exudates

The visually healthy *M. caesalpiniifolia* mature seeds were surface sterilized with 70% (v/v) ethanol for 5 min, rinsed three times with distilled water, and air-dried at room temperature [33]. To collect the exudates, selected seeds with no visible cracks were distributed in 32 groups, each weighting on average 3 g, and transferred to 50 mL Erlenmeyer flasks. Then, the seeds were immersed in 12.0 mL of the M9 buffer and maintained at 25 °C for 24 h. The choice of the M9 buffer was because it was used in the assays with the nematode. The *M. caesalpiniifolia* seed exudates (*Mc*SEX) samples were collected, and the volumes recorded.

Preparation of the constituent polypeptide/protein fractions and protein determination.

The polypeptides/proteins present in the *Mc*SEX fraction were precipitated by addition of an amount of crystalline ammonium sulfate required to reach 90% saturation [37], while kept in an ice bath under continuous agitation. Next, the suspension was left standing for 4 h at 10 °C. The precipitated polypeptides/proteins were recovered by centrifugation at 15,000 x *g*, 4 °C, 30 min (Thermo scientific Heraeus Megafuge 16R centrifuge, DJB Labcare Limited, UK) and resuspended with and dialyzed (2 kDa cut off) against the M9 buffer for 24 h (with three buffer changes), at 10 °C. Then, the suspension obtained was heated to 80ºC in a water bath for 15 min and centrifuged at 15,000 x *g* for 10 min, at 4 ºC [38]. The supernatant obtained, denominated *Mc*SEX$_{90\%/80°C}$, was recovered. The *Mc*SEX$_{90\%/80°C}$ fraction was further fractionated using tangential flow filtration (TFF) coupled with a 30 kDa cut-off ultrafiltration membrane and centrifugation at 5,5 *g* for 2 h at 4 ºC. The material that passed through the 30 kDa cut-off ultrafiltration membrane was then forced to pass, by centrifugation, through a 10 kDa cut-off ultrafiltration membrane. The retentate, which did not pass through the 10 kDa cut-off ultrafiltration membrane, was denominated *Mc*SEX30-10 fraction and comprised the proteins with molecular mass between 30 and 10 kDa. The permeate, which passed through the 10 kDa cut-off ultrafiltration membrane, was denominated *Mc*SEX10-2 fraction and encompassed the polypeptides with the molecular mass varying from 10 kDa to 2 kDa. The *Mc*SEX10-2 fraction was dialyzed (2 kDa cut-off membrane) for 24 h against the M9 buffer (with three buffer changes) at 10 °C. The protein/polypeptide contents of the *Mc*SEX, *Mc*SEX$_{90\%}$, *Mc*SEX$_{90\%/80^oC}$, *Mc*SEX/30-10, and *Mc*SEX/10-2 fractions were determined according to the Bradford's method [39], using bovine serum albumin (BSA) as the standard protein.

In vitro assay to assess the anthelmintic properties of *Mc*SEX, *Mc*SEX$_{90\%}$, *Mc*SEX$_{90\%/80°C}$, *Mc*SEX/30-10, and *Mc*SEX/10-2 fractions against *C. elegans*

C. elegans motility was evaluated in 96-well culture plates, in which 100 µL adult nematodes, suspended in 10 mL M9 buffer, were dropped in each well. Next, 100 µL of the samples, at concentrations varying from 163.75 to 1.25 µg mL^{-1}, were added. In the controls, 100 µL of the M9 buffer was added instead of fractions. The 96-well culture plates were incubated, for 24 h, in a BOD-type greenhouse regulated to operate at 24 ± 1 ºC, RH ≥ 80 % [12]. Next, motile and non-motile nematodes were observed for 5 s using an inverted microscope (Zeiss Lab A1, AXIO). The nematodes were considered alive when they showed some type of motility. Animals were considered dead when they did not present tail, head, or pharyngeal movements.

Assessment of the effects of the *Mc*SEX/10-2 fraction on the cuticle structure of *C. elegans*

To assess the possible alterations on the cuticle structure of *C. elegans* after treatment with *Mc*SEX/10-2, optical microscopy and atomic force microscopy (AFM) were employed. Adult nematodes, incubated for 24 h in the M9 buffer (control) or in the *Mc*SEX/10-2 fraction prepared in M9, at 1.34 µg mL^{-1} final concentration, were fixed in 5% formalin for 24 h before being transferred to glass slides, for AFM observations.

Atomic force microscopy analysis

AFM measurements were performed using a Multimode 8 microscope (Bruker, Santa Barbara, CA) in PeakForce Tapping Quantitative Nanomechanics mode, in air (23 ºC temperature and 44% air humidity), for the acquisition of topography, adhesion, roughness, and stiffness data. Silicon-made probes, with a nominal spring constant of 0.4 N/m and tip radius of 2 nm, were used. The images were obtained approximately in the central region of the adult *C. elegans*, with a scan size of 5 x 5 µm and of 256 x 256 force curves. The mean square roughness (R_q) values were obtained from the height maps, according to the methodology used in [40]. The adhesion and stiffness data were obtained from the 65,536 force curves produced on the nematode cuticle. The control and *Mc*SEX/10-2-treated nematodes were compared. For the adhesion calculation, the maximum negative deflection values for each force curve were evaluated. For stiffness data, the values were obtained from the slope of the force curve in the sample contact portion.

The hemolysis assay of *Mc*SEX/10-2 against bovine erythrocytes

The *in vitro* hemolysis assay was carried out by measuring the lysis of bovine erythrocytes as previously described [41], with minor modifications. Bovine whole blood was collected from the jugular vein of calves in

a glass bottle containing heparin (5 IU mL^{-1}) as anticoagulant. The red blood cells (RBC) were recovered and washed three times with 0.15 M NaCl. They were recovered by centrifugation at 300 x *g* (centrifuge Mikro 200R, Hettich, Germany), 5 min, 4 °C, followed by resuspension in 0.15 M NaCl. To carry out the hemolysis assay, the *Mc*SEX/10-2 fraction was dialyzed (2 kDa cut off) for 24 h, at 10 °C, against 0.05 M Na-phosphate buffer, pH 7.2, containing 0.15 M NaCl *(PBS).* Then, the protein content of the dialysate was quantified [39]. Aliquots (100 µL) of a 2.5% RBC suspension were transferred to microtubes (1.5 mL) and incubated for 30 min at 37 °C with 100 µL of the *Mc*SEX/10-2 fraction prepared in PBS at 45.00, 22.50, 11.25, 5.63, 2.81, and 1.41 µg mL^{-1} final concentrations followed by centrifugation at 300 x *g*, 5 min, 4 °C. The supernatant was collected and transferred to flat bottom wells of 96-well culture plates and hemolysis determined by measuring the absorbance at 450 nm using an automated microplate reader (Epoch, BioTek Instruments Inc., USA). Negative (0%) and positive (100%) hemolysis was achieved by treatment of RBC with PBS and 0.1% (v/v) Triton X-100, respectively, in the place of *Mc*SEX/10-2. The percentage of hemolysis was calculated using the following equation: [(Abs_{450} nm of RBC treated with *Mc*SEX/10-2 – Abs_{450} nm of RBC treated with PBS) / [(Abs_{450} nm of RBC treated with 0.1 % Triton X-100 – Abs_{450} nm of RBC treated with PBS)] x 100.

Statistical Analysis

Data from protein content were compared using ANOVA followed by the Tukey test ($P<0.05$). Inhibitory Concentration (IC_{50}) of samples against nematodes was calculated using GraphPad Prism 8.0 (San Diego, CA, USA). The data of adult *C. elegans* observed in AFM and FIJI/IMAGEJ were analyzed using D'Agostino & Pearson for normality [42], and the means were compared using the Kolmogorov-Smirnov test ($P<0.05$).

RESULTS

The protein content of the seed exudates and fractions

The protein content of *Mc*SEX, *Mc*$SEX_{90\%/80^{\circ}C}$, *Mc*SEX/30-10, and *Mc*SEX/10-2 was respectively, 2.34, 0.33, 0.00109, and 0.000765 milligrams of protein per gram of mature seed (mgP g^{-1} seed).

Effect of *Mc*SEX, *Mc*$SEX_{90\%/80^{\circ}C}$, *Mc*SEX/30-10, and *Mc*SEX/10-2 on *C. elegans* motility

*Mc*SEX inhibited *C. elegans* motility (Table 1). Its fractionation in *Mc*$SEX_{90\%/80^{\circ}C}$, *Mc*SEX/30-10, and *Mc*SEX/10-2 progressively increased the inhibitory effect by 17.06%, 35.83%, and 91.17%, respectively, in relation to the exudates (*Mc*SEX). The highest bioactive fraction was *Mc*SEX/10-2, with an IC_{50} of 1.34 µg protein mL^{-1} (Table 1).

Table 1. Effect of *Mc*SEX, *Mc*$SEX_{90\%/80^{\circ}C}$, *Mc*SEX/30-10, and *Mc*SEX/10-2 on *Caenorhabditis elegans* motility

Samples	IC_{50}* ± SD (µgP mL^{-1})	95% CL**
*Mc*SEX	15.18 ± 6.27^{b}	12.32 – 18.73
Mc$SEX_{90\%/80^{\circ}C}$	12.59 ± 7.39^{b}	9.97 – 16.06
*Mc*SEX/30-10	9.74 ± 4.74^{b}	7.94 – 12.05
*Mc*SEX/10-2	1.34 ± 0.92^{a}	0.96 – 1.81

*IC_{50} denotes the protein concentration that inhibited the motility of 50% of the nematode population tested. The values represent the mean ± standard deviation of three independent experiments. ** 95% CL means the average of inferior and superior confidence limits at 95 % probability.

Ultrastructural and nanomechanical characteristics of *C. elegans* treated with *Mc*SEX/10-2

Analyses by Atomic force microscopy analysis revealed (Figures 1A and 1B) the ultrastructural morphology and biomechanical properties of the nematode were altered, as revealed by AFM data. Figures 1A (control) and 1B (treated), in combination with the corresponding three-dimensional maps (Figures 1C and 1D), revealed a significant ($p<0.05$) decrease in the cuticle height in adult *C. elegans* (571.0 ± 185.9 nm) incubated with *Mc*SEX/10-2 (1.34 µg mL^{-1}), in comparison with that of control animals (734.5 ± 244.7 nm). Moreover, the *AFM* images showed *Rq values* of 39.7 nm for the *Mc*SEX/10-2-treated nematodes and 68.9 nm for the control animals. Such a significant ($p<0.05$) difference denotes a decrease in the surface roughness of the *Mc*SEX/10-2-treated animals. The AFM results also revealed differences in the biomechanical properties of the animals treated with *Mc*SEX/10-2 in comparison with controls (Figure 2).

*Mc*SEX/10-2 promoted a decrease in the nematode cuticle stiffness to 8.586 ± 0.558 N/m in relation to 11.11 ± 0.863 N/m observed in the control animals (p<0.05). Contrarily, the cuticle surface adhesion increased to 6.693 ± 3.537 nN in the *Mc*SEX/10-2-treated *C. elegans* in comparison to 5.029 ± 1.499 nN verified for the control animals (Figures 2A and 2B).

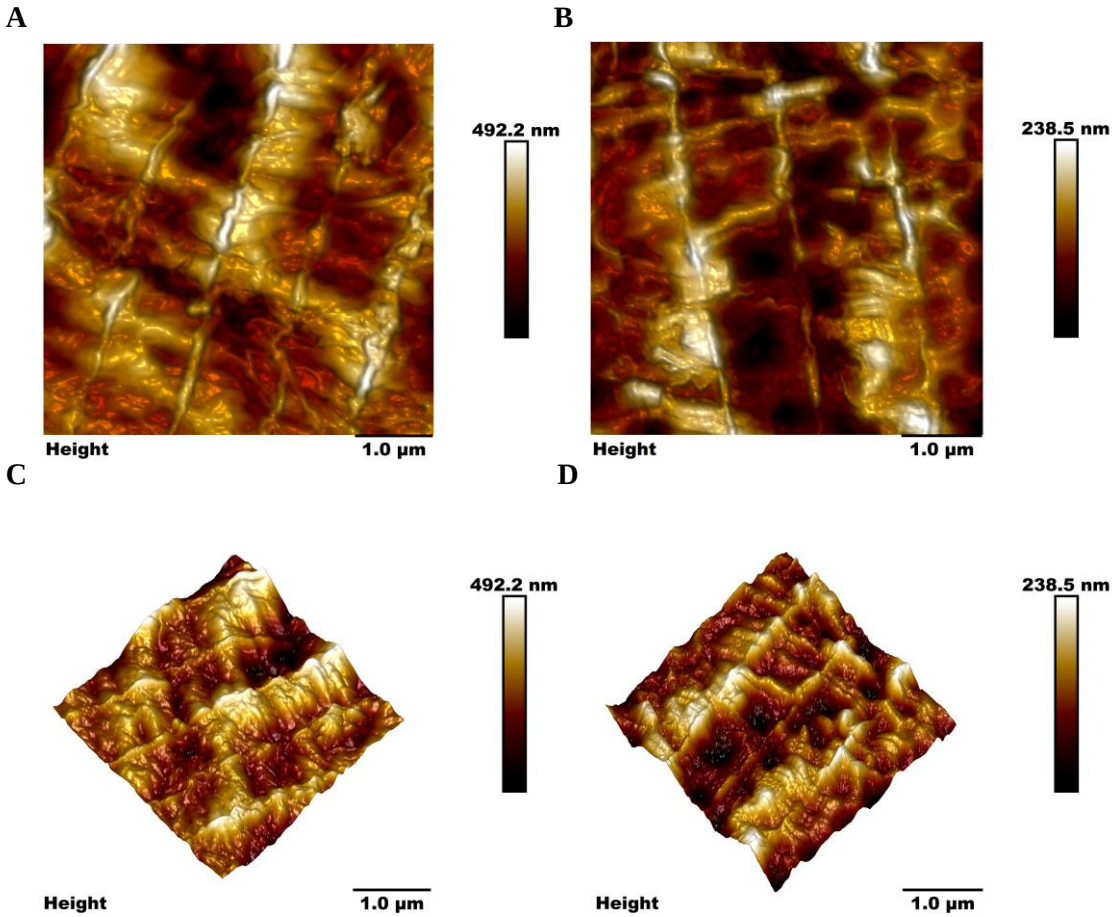


Figure 1. Structure of *Caenorhabditis elegans* cuticle. (A) Atomic force microscopy images of *C. elegans* treated with the M9 buffer (control) and with (B) the *Mc*SEX/10-2 fraction (1.34 µg mL^{-1} final concentration), showing its respective three-dimensional maps (C and D).

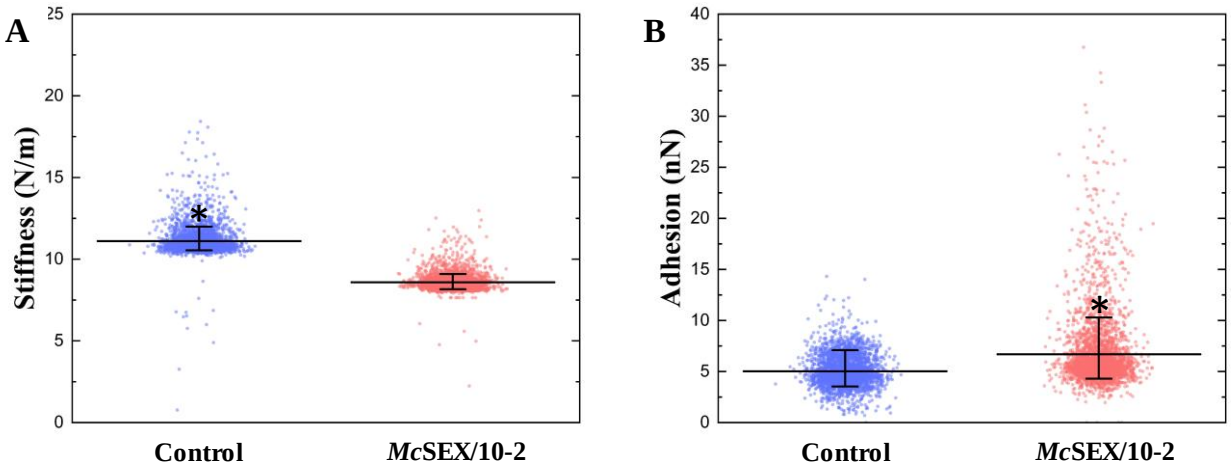


Figure 2. Cuticle stiffness (A) and adhesion (B) data of adult *Caenorhabditis elegans* incubated with the M9 buffer (Control) and with the *Mc*SEX/10-2 fraction (1.34 µg mL^{-1} final concentration). *Denotes significant (p<0.05) difference.

Assessment of the cytotoxic activity of *Mc*SEX/10-2 against mammalian cells

The McSEX/10-2 fraction exhibited low hemolytic activity on animal red blood cells, resulting in only 1.8% hemolysis at a protein concentration of 45.00 µg mL^{-1} (Table 2).

Table 2. Hemolytic activity of *Mc*SEX/10-2 against bovine erythrocytes.

Samples	µg of protein mL^{-1}	Hemolysis (%)
*Mc*SEX/10-2	45.00	1.8
	22.50	1.3
	11.25	0.5
	2.81	0.0

DISCUSSION

During evolution, plants were exposed to a variety of abiotic and/or biotic factors, which, under certain circumstances, may have been detrimental to their growth, development and productivity [43, 44]. Seed exudates represent a useful source of defense molecules, which are mainly polypeptides/proteins and secondary metabolites, some of which have the capacity to specifically inhibit the development of certain pathogens [20, 22, 45-48].

The exudates from *M. caesalpiniifolia* mature seeds obtained at 25 ºC comprised, on average, 2.34 mgP g^{-1} seed. The precipitated and heated fraction (*Mc*$SEX_{90\%/80^{\circ}C}$) comprised 0.33 mgP g^{-1} seed. At 10 °C, the precipitated polypeptide/protein fraction from the *M. caesalpiniifolia* seed exudates, without heating, comprised 0.49 mgP g^{-1} seed [33]. Nevertheless, the protein content of exudates from seeds varies when obtained under different conditions. For example, very much lower protein contents were found in the mature seed exudates of *Myracrodruon urundeuva* (0.24 mgP g^{-1} seed), after immersion in 0.1 M sodium acetate buffer, pH 5.0, at 5 °C [25], and in *Moringa oleifera* (0.57 ± 0.02 mg^{-1} g^{-1} seed) by seed immersion in distilled water, at 28 °C [22].

The use of *C. elegans* as an experimental model for anthelmintic testing of plant substances is of great importance for the development of new products [17, 49]. The experimental inhibition of the motility of adult *C elegans* observed herein, promoted by the *Mc*SEX, *Mc*$SEX_{90\%/80^{\circ}C}$, *Mc*SEX/30-10, and *Mc*SEX/10-2 fractions, may be a consequence of some deleterious effects that these products exerted on the animal, which could, eventually, influence survival [50]. Several proteins exuded from seeds, such as proteases, chitinases, and lectins may interfere with the general physiology of nematodes, for example [22, 25, 51-53]. Nevertheless, our work shows that the *M. caesalpiniifolia* mature seed exudates and their constituent polypeptide/protein fractions presented anthelmintic potential (Table 1), as their concentrations to inhibit the motility of the exposed adult *C. elegans* by 50% were lower than those reported by other studies [25, 54, 55].

There is a plethora of published works that report the anthelminthic activity of plant peptides, highlighting the importance of these molecules as potential agents to combat nematodes. For instance, the peptides extracted from the leaves of *Oldenlandia affinis* display activity against *Schistosoma* [56]. The nematicidal activity of cyclotids, namely kalata B1, cycloviolacin O2, and hyen D, which were isolated from plants, was assessed on *C. elegans* larvae [17]. In addition, the bioactive proteins exuded by *M. caesalpiniifolia* mature seeds showed stage-specific inhibition of *H. contortus* [25]. These anthelmintic molecules may have access to the nematode target tissues after oral ingestion or diffusion across the animal cuticle, which although a highly impermeable barrier between the animal and its environment, it is a permeable exoskeleton for several drugs that can diffuse across this extracellular matrix and reach the plasma membrane [57, 58], to which they can exert local deleterios effects or internalized. Indeed, a strong example is that regarless the nematodes behave as a free-living, animal or plant parasites, they are sensitive to the crystal (Cry) protein Cry5B. It belongs to a family of membrane pore-forming proteins synthetized by the Gram-positive bacterium, *Bacillus thuringiensis*. The constituent monomers of the Cry protein monomers interact with the intestinal cell receptors, oligomerize, get inserted into the plasma membrane and form pores that disrupt the cell integrity, ionic balance, and membrane potentials, causing death or severe disruption of the cell activity [58]. Thus, besides to be essential for maintenance of the body morphology and integrity, this exoskeleton has a critical role in locomotion via attachments to body-wall muscles, confers environmental protection, and allows growth by molting [57].

To verify whether the *Mc*SEX/10-2 fraction would alter the nematode's motility by inducing modifications of its cuticle, we used AFM. This constituted a valuable approach since *Mc*SEX/10-2 promoted remarkable ultrastructural and nanomechanical alterations on the *C. elegans* cuticle (Figures 1 and 2). The cuticle surface is especially important for the host-parasite interaction. For instance, when the parasitic nematode *H. contortus* physically interacts with the host tissue, changes in the lipids of its surface might establish the onset of the infection process [59, 60].

To the best of our knowledge, for the first time, this study shows that the constituent polypeptides/proteins from *M. caesalpiniifolia* mature seed exudates promote the reduction of the height and roughness of the *C. elegans* cuticle (Figures 1B and 1D). The *C. elegans* cuticle consists mainly of cross-linked collagens associated with the insoluble proteins named cuticlins and with glycoproteins and lipids [57]. Previously, it was shown that residues of cyclotides, a class of disulfide-rich cyclic peptides from plants [61], bind to the constituent lipids of the cuticle surface by hydrophobic interactions [62]. Nevertheless, further studies are needed to define the responsible agent(s) and elucidate the interaction (mechanisms) of the constituent polypeptides/proteins of the *M. caesalpiniifolia* exudates with the nematode cuticle and the importance of the cuticle height and roughness reduction on the mechanism of action of these molecules. As depicted in Figure 2, the AFM approach used allowed us to detect nanoscale alterations on the *C. elegans* cuticle stiffness and adhesion, after treatment with *Mc*SEX/10-2. As the control and the *Mc*SEX/10-2-treated nematodes were analyzed at the same life cycle stage, the alterations observed may be related to the interaction of the yet unknown polypeptide(s)/protein(s) of the *M. caesalpiniifolia* exudates with the nematode cuticle. We also observed increased adhesion forces on the *C. elegans* cuticle after incubation of the nematode with *Mc*SEX/10-2. It is important to notice that adhesion forces are a combination of electrostatic, van der Waals and capillary forces promoted by chemical bonds [63]. Especially in the cases of non-functionalized probes (as used in this research), the adhesion forces are taken as nonspecific interactions and it is not possible to separate individually the contribution of each one of these forces. However, as the probes used in the AFM analyses were made of the same material (Si) and the experiments and observations were performed under the same conditions (temperature and air humidity), differences in the adhesion forces between the controls and the *Mc*SEX/10-2-treated animals are plausible. The scatter plots (Figure 2B) show values that suggest a trend of increasing adhesion force after the *Mc*SEX/10-2 treatment of *C. elegans*. As reported by Essmann and coworkers [64], in their Young's modulus results of the *C. elegans* cuticle over 18 days, the scattering direction suggests the trend of the kinetic events.

To treat nematode infection orally with any drug, one should be aware of the possible side effects it can cause in the host. The *Mc*SEX/10-2 fraction tested caused very low hemolysis of bovine blood cells (Table 2), even at concentrations higher than the IC_{50} values needed to arrest *C. elegans motility. Nevertheless,* as a perspective, aiming at achieving higher anthelmintic efficacy of the synergistic effect of *Mc*SEX/10-2, when used in combination or together with other anthelmintic drugs, and at different nematode life-stage, must be addressed, as they furnish directions for the bio-inspired design of novel medicines.

CONCLUSION

The results presented herein indicate that the polypeptides/proteins present in the exudates from *M. caesalpiniifolia* mature seeds caused harmful effects against the nematode *C. elegans*. Accordingly, they have the potential to bioinspire the design of new synthetic peptides that could control, alone or in combination with other drugs, parasitic nematodes.

Acknowledgment: This research was partially supported by IECT Biotechnology through grants from FAPEMA (Maranhão State Research Foundation, Brazil) and CNPq (The Brazilian National Council for Scientific and Technological Development) process 407460/2018-7. The authors thank Dr. Paul Michels from The University of Edinburgh, for his valuable suggestions, assistance and critical review of this manuscript. We also thank FAPEMA for awarding a fellowship to W.A.S Bezerra. This study was financed in part by the Coordination for the Improvement of Higher Education Personnel (CAPES/Brazil) – Finance Code 001.

Conflicts of Interest: The authors have no conflict of interest to declare.

REFERENCES

1. Dudlová A, Juriš P, Jurišová S, Jarčuška P, Krčméry V. Epidemiology and geographical distribution of gastrointestinal parasitic infection in humans in Slovakia. Helminthologia. 2016;53(4):309-17.

2. Ong YC, Roy S, Andrews PC, Gasser G. Metal compounds against neglected tropical diseases. Chem. Rev. 2018;119(2):730-96.
3. Flammer PG, Ryan H, Preston SG, Warren S, Přichystalová R, Weiss R, et al. Epidemiological insights from a large-scale investigation of intestinal helminths in Medieval Europe. PLoS Negl Trop Dis. 2020;14(8):1-20.
4. Barda BD, Keiser J, Albonico M. Human trichuriasis: diagnostics update. Curr. Trop. Med. 2015;2:201-8.
5. Fonseca BP, Albuquerque PC, Zicker F. Neglected tropical diseases in Brazil: lack of correlation between disease burden, research funding and output. Trop. Med. Int. Health. 2020;25(11):1373-84.
6. Wexler A, Valentine A. The US Global Health Budget: Analysis of the Fiscal Year 2016 Budget Request. Menlo Park: KFF. 2015;22(1):1-10.
7. Bartsch SM, Hotez PJ, Asti L, Zapf KM, Bottazzi ME, Diemert DJ, Lee BY. The global economic and health burden of human hookworm infection. PLoS Negl Trop Dis. 2016;10(9):1-17.
8. Besier R, Kahn L, Sargison N, Van Wyk JA. The pathophysiology, ecology and epidemiology of *Haemonchus contortus* infection in small ruminants. J. Adv. Parasitol. 2016;93:95-143.
9. Julienne K, Fréjus T, Pascal AO, Géorcelin GA, Adam DA, Christian CD, Sylvie HA, Olaniyi JB, Patrick AE. Prevalence, effects and alternative control methods of *Haemonchus contortus* in small ruminants: A review. J. Vet. Med. Anim. Health. 2021;13(2):84-97.
10. Gillan V, Maitland K, Laing R, Gu H, Marks ND, Winter AD, et al. Increased expression of a microRNA correlates with anthelmintic resistance in parasitic nematodes. Front. cell. infect. microbiol. 2017;7:1-15.
11. Figueiredo LA, Reboucas TF, Ferreira SR, Rodrigues-Luiz GF, Miranda RC, Araujo RN, Fujiwara RT. Dominance of P-glycoprotein 12 in phenotypic resistance conversion against ivermectin in *Caenorhabditis elegans*. PloS one. 2018;13(2):1-14.
12. Katiki LM, Ferreira JF, Zajac AM, Masler C, Lindsay DS, Chagas ACS, et al. *Caenorhabditis elegans* as a model to screen plant extracts and compounds as natural anthelmintics for veterinary use. Vet. Parasitol. 2011;182(2-4):264-8.
13. Blaxter M. Nematodes: the worm and its relatives. PLoS Biol. 2011;9(4):1-9.
14. Liu M, Kipanga P, Mai AH, Dhondt I, Braeckman BP, De Borggraeve W, Luyten W. Bioassay-guided isolation of three anthelmintic compounds from *Warburgia ugandensis* Sprague subspecies *ugandensis*, and the mechanism of action of polygodial. Int J Parasitol. 2018;48(11):833-44.
15. Liao VH-C. Use of *Caenorhabditis elegans* to study the potential bioactivity of natural compounds. J. Agric. Food Chem. 2018;66(8):1737-42.
16. Hahnel SR, Dilks CM, Heisler I, Andersen EC, Kulke D. *Caenorhabditis elegans* in anthelmintic research–Old model, new perspectives. Int. J. Parasitol.: Drugs Drug Resist. 2020;14:237-48.
17. Bajpai A, Jackson MA, Huang Y-H, Yap K, Du Q, Chau TC-Y, et al. Nematicidal Activity of Cyclotides: Toxicity Against *Caenorhabditis elegans*. J. Nat. Prod. 2023;86(5):1222-9.
18. Nóbrega F, Santos I, Cunha MD, Carvalho A, Gomes V. Antimicrobial proteins from cowpea root exudates: inhibitory activity against *Fusarium oxysporum* and purification of a chitinase-like protein. Plant Soil. 2005;272:223-32.
19. Lanoue A, Burlat V, Henkes GJ, Koch I, Schurr U, Röse US. De novo biosynthesis of defense root exudates in response to *Fusarium* attack in barley. New Phytol. 2010;185(2):577-88.
20. Rocha RO, Morais JK, Oliveira JT, Oliveira HD, Sousa DO, Souza CEA, et al. Proteome of soybean seed exudates contains plant defense-related proteins active against the root-knot nematode *Meloidogyne incognita*. J. Agric. Food Chem. 2015;63(22):5335-43.
21. Licá ICL, Santos Soares AM, Mesquita LSS, Malik S. Biological properties and pharmacological potential of plant exudates. Food Res Int. 2018;105:1039-53.
22. Sousa AJ, Souza PF, Gifoni JM, Dias LP, Freitas CD, Oliveira JT, et al. Scanning electron microscopy reveals deleterious effects of *Moringa oleifera* seed exuded proteins on root-knot nematode *Meloidogyne incognita* eggs. Int. J. Biol. Macromol. 2020;154:1237-44.
23. Shao H, Zhang P, Peng D, Huang W, Kong LA, Li C, et al. Current advances in the identification of plant nematode diseases: From lab assays to in-field diagnostics. Front. Plant Sci. 2023;14:1-12.
24. Fan Z, Wang L, Qin Y, Li P. Activity of chitin/chitosan/chitosan oligosaccharide against plant pathogenic nematodes and potential modes of application in agriculture: A review. Carbohydrate Polymers. 2023:1-8.
25. Soares AM, Oliveira JT, Rocha CQ, Ferreira AT, Perales J, Zanatta AC, et al. *Myracrodruon urundeuva* seed exudates proteome and anthelmintic activity against *Haemonchus contortus*. PLoS One. 2018;13(7):1-12.
26. Vlieghe P, Lisowski V, Martinez J, Khrestchatisky M. Synthetic therapeutic peptides: science and market. Drug Discov. Today. 2010;15(1-2):40-56.
27. Sachdeva S. Peptides as ‘drugs’: the journey so far. Int. J. Pept. Res. Ther. 2017;23(1):49-60.
28. Souza PF, Lima PG, Freitas CD, Sousa DO, Neto NA, Dias LP, et al. Antidermatophytic activity of synthetic peptides: Action mechanisms and clinical application as adjuvants to enhance the activity and decrease the toxicity of Griseofulvin. Mycoses. 2020;63(9):979-92.
29. Tavares RC, Mendes Filho PF, Lacerda CF, Silva J. Mycorrhizal colonization and root nodulation in “sabia” seedlings (*Mimosa caesalpiniaefolia* Benth.) at different salinity levels. Rev. Cienc. Agron. 2012;43(3):409-16.
30. Abreu MG, Garcia KG, da Silva Junior JM, Albuquerque GHdS, Araujo JM, Tavella LB. Effect of manganese on growth and nodulation of *Mimosa caesalpiniaefolia* (Benth.). J. Agric. Sci. 2019;11(2):339-52.

31. Herrera AM, de Mello ACL, Oliveira Apolinário VX, Dubeux Júnior JCB, da Cunha MV, Santos MVF. Potential of *Gliricidia sepium* (jacq.) Kunth ex Walp. and *Mimosa caesalpiniifolia* Benth. in silvopastoral systems intercropped with signalgrass [*Urochloa decumbens* (Stapf) RD Webster]. Agrofor. Syst. 2021;95:1061-72.
32. Hernández-Ledesma B, Hsieh C-C. Chemopreventive role of food-derived proteins and peptides: A review. Crit. Rev. Food Sci. Nutr. 2017;57(11):2358-76.
33. Licá ICL, Soares AMS, Ferreira ATS, Perales J, Cunha IAL, Costa-Júnior LM. *In vitro* assessment of the efficacy of protein exudates from seeds against *Haemonchus contortus*. Vet. Parasitol. 2021;292:1-5.
34. Brenner S. The genetics of *Caenorhabditis elegans*. Genet. 1974;77(1):71-94.
35. Stiernagle T. The *C. elegans* Research Community. WormBook. 2006;1(1):1551-8507.
36. Wood WB. The Nematode *Caenorhabditis elegans*. Edited by Wood, W.B. and the Community of *C. elegans* Researchers. CSHL. 1988;1:667.
37. Scopes RK, Scopes RK. Making an extract. Protein purification: Principles practice. 1994:22-43.
38. Santos LA, Taveira GB, Ribeiro SFF, Silva Pereira L, Oliveira Carvalho A, Rodrigues R, et al. Purification and characterization of peptides from *Capsicum annuum* fruits which are α-amylase inhibitors and exhibit high antimicrobial activity against fungi of agronomic importance. Protein Expr. Purif. 2017;132:97-107.
39. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976;72(1-2):248-54.
40. Costa-Junior LM, Silva CR, Soares AM, Menezes AS, Silva MR, Amarante AF, et al. Assessment of biophysical properties of *Haemonchus contortus* from different life cycle stages with atomic force microscopy. Ultramicroscopy. 2020;209:1-21.
41. Oliveira JT, Souza PF, Vasconcelos IM, Dias LP, Martins TF, Van Tilburg MF, et al. Mo-CBP3-PepI, Mo-CBP3-PepII, and Mo-CBP3-PepIII are synthetic antimicrobial peptides active against human pathogens by stimulating ROS generation and increasing plasma membrane permeability. Biochim. 2019;157:10-21.
42. D'agostino RB, Belanger A, D'Agostino Jr RB. A suggestion for using powerful and informative tests of normality. Am Stat. 1990;44(4):316-21.
43. Demirevska K, Simova-Stoilova L, Fedina I, Georgieva K, Kunert K. Response of oryzacystatin I transformed tobacco plants to drought, heat and light stress. J. Agron. Crop Sci. 2010;196(2):90-9.
44. Nadeem SM, Ahmad M, Zahir ZA, Javaid A, Ashraf M. The role of mycorrhizae and plant growth promoting rhizobacteria (PGPR) in improving crop productivity under stressful environments. Biotechnol. Adv. 2014;32(2):429-48.
45. Scarafoni A, Ronchi A, Prinsi B, Espen L, Assante G, Venturini G, et al. The proteome of exudates from germinating *Lupinus albus* seeds is secreted through a selective dual-step process and contains proteins involved in plant defence. FEBS J. 2013;280(6):1443-59.
46. Yang G, Zhou B, Zhang X, Zhang Z, Wu Y, Zhang Y, et al. Effects of tomato root exudates on *Meloidogyne incognita*. PLoS one. 2016;11(4):1-16.
47. Ribeiro HS, Soares AMS, Jesus Castro Brito D, Oliveira JTA, Costa-Junior LM. Inhibition of Protease and Egg Hatching of *Haemonchus contortus* by Soybean Seed Exudates. J. Parasitol. 2021;107(1):23-8.
48. Ahmed H, Kilinc SG, Celik F, Kesik HK, Simsek S, Ahmad KS, et al. An inventory of anthelmintic plants across the globe. Pathogens. 2023;12(1):1-26.
49. Ayuda-Durán B, González-Manzano S, González-Paramás AM, Santos-Buelga C. *Caenorhabditis elegans* as a model organism to evaluate the antioxidant effects of phytochemicals. Molecules. 2020;25(14):1-23.
50. Weeks JC, Roberts WM, Leasure C, Suzuki BM, Robinson KJ, Currey H, et al. Sertraline, paroxetine, and chlorpromazine are rapidly acting anthelmintic drugs capable of clinical repurposing. Sci. Rep. 2018;8(1):1-17.
51. Salles HO, Braga ACL, Nascimento MTSC, Sousa AMP, Lima AR, Vieira LS, et al. [Lectin, hemolysin, and protease inhibitors in seed fractions with ovicidal activity against *Haemonchus contortus*]. Rev. Bras. Parasitol. Vet. 2014;23:136-43.
52. Soares AMS, Araújo SA, Lopes SG, Costa Junior LM. Anthelmintic activity of *Leucaena leucocephala* protein extracts on *Haemonchus contortus*. Rev. Bras. Parasitol. Vet. 2015;24:396-401.
53. Soares AMS, Wanderley LF, Costa LM. The potential of plant and fungal proteins in the control of gastrointestinal nematodes from animals. Rev. Bras. Parasitol. Vet. 2019;28:339-45.
54. Araujo SA, Soares AMS, Silva CR, Almeida Junior EB, Rocha CQ, Ferreira ATS, et al. *In vitro* anthelmintic effects of *Spigelia anthelmia* protein fractions against *Haemonchus contortus*. PloS one. 2017;12(12):1-11.
55. Wanderley LF, Soares AMS, Figueiredo IM, Ferreira ATS, Perales J, Mota HRO, et al. A cysteine protease from the latex of *Ficus benjamina* has *in vitro* anthelmintic activity against *Haemonchus contortus*. Rev. Bras. Parasitol. Vet. 2018;27:473-80.
56. Malagón D, Botterill B, Gray DJ, Lovas E, Duke M, Gray C, et al. Anthelminthic activity of the cyclotides (kalata B1 and B2) against *Schistosome* parasites. Pept. Sci. 2013;100(5):461-70.
57. Page AP, Johnstone I. The cuticle. WormBook. 2007:1-16.
58. Holden-Dye L, Walker R. Anthelmintic drugs and nematocides: studies in *Caenorhabditis elegans*. WormBook: the online review of *C. elegans* biology. 2014:1-29.
59. Proudfoot L, Kusel J, Smith H, Kennedy M. External stimuli and intracellular signalling in the modification of the nematode surface during transition to the mammalian host environment. Parasitol. 1993;107(5):559-66.

60. Mendoza ML, Modha J, Roberts M, Curtis R, Kusel J. Changes in the lipophilicity of the surfaces of *Meloidogyne incognita* and *Haemonchus contortus* during exposure to host signals. Parasitol. 2000;120(2):203-9.
61. Narayani M, Babu R, Chadha A, Srivastava S. Production of bioactive cyclotides: A comprehensive overview. Phytochem. Rev. 2020;19:787-825.
62. Kamimori H, Hall K, Craik DJ, Aguilar M-I. Studies on the membrane interactions of the cyclotides kalata B1 and kalata B6 on model membrane systems by surface plasmon resonance. Anal. Biochem. 2005;337(1):149-53.
63. Butt H-J, Cappella B, Kappl M. Force measurements with the atomic force microscope: Technique, interpretation and applications. Surf. Sci. Rep. 2005;59(1-6):1-152.
64. Essmann CL, Martinez-Martinez D, Pryor R, Leung K-Y, Krishnan KB, Lui PP, et al. Mechanical properties measured by atomic force microscopy define health biomarkers in ageing C. elegans. Nat. Commun. 2020;11(1):1-16.