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Prospection of Nematotoxic Aqueous Seeds Extracts Derived from the Preserved *Arachis* (Fabaceae) Germplasm Bank

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Abstract: Plant-parasitic nematodes (PPN) are the most damaging plant pathogens all over the world. Root-knot nematodes (RKNs), considered the most important phytonematodes globally, cause significant agricultural losses. Despite the availability of various strategies to manage these pathogenic agents, excessive use of nematicides poses a threat to human health and the environment. Compounds derived from plant sources are proposed as an alternative to new biocides, potentially offering advantages over synthetic components. Several species within the *Fabaceae* family, including those within the *Arachis* genus, have demonstrated potential as sources of nematotoxic compounds. As part of a research program aimed at exploring bioactive compounds and valorizing germplasm banks, this study evaluated the nematicidal and nematostatic effects of aqueous crude extracts (ACEs) obtained from nine *Arachis* species sourced from the Embrapa Active Germplasm Bank against *M. incognita's* second-stage juveniles (J$_2$). The results indicate that *Arachis stenosperma* (ACE1) has promising nematocidal potential, with effectiveness exceeding 95% on dead nematodes for doses above 0.5 mg/mL. ACE1 has also demonstrated thermostability and lower harmful effects on bovine cells. This research provides a fresh outlook on the promising use of preserved germplasms to enhance Germplasm Storage Bank's value, given the underexplored potential of these biological assets.

Keywords: phytonematode; *Meloidogyne incognita*; *Arachis*; aqueous crude extracts; control; sustainability

1. Introduction

Plant-parasitic nematodes (PPNs) are widely considered to be the most harmful plant pathogens globally despite their potential to cause substantial crop losses, posing a significant challenge for contemporary agriculture [1].

Although over 4000 phytonematoid species have been described [2], the *Meloidogyne* genus, commonly referred to as root-knot nematodes (RKNs), has a significant negative impact on agriculture worldwide, particularly in Brazil. These species are considered the most important phytonematodes due to their broad geographic distribution, host range, and efficient parasitic adaptations [3–5]. The damage caused by these nematodes is related to the formation of tumors in plant host roots, which interferes with the soil's nutrient absorption capacity, resulting in a partial loss of efficiency in input use and increased susceptibility to attack by other pathogens [6].

Although some practices such as crop rotation, resistant varieties, biological control, antagonist plants, and integrated pest management can be used to reduce the damage caused by PPN, the main control strategy for this phytopathogen is still based on synthetic nematicides, which can be harmful to human health and the environment [7–11].

Compounds extracted from natural sources, such as plants, have been proposed as an alternative to discover new biocides due to several advantages over synthetic components [12–14]. Compared to chemical products, botanical biocides are typically more easily biodegradable, less concentrated, and less toxic owing to their natural compounds [15].

Over the past two decades, a large number of plant species have been identified as containing a diverse range of extracts, fractions, and compounds exhibiting fungicidal, bactericidal, insecticidal, and nematotoxic properties [11,16–22].

Brazil boasts one of the planet's richest biodiversities, making it a significant source of natural, plant-based products. A variety of plant species have yielded crude aqueous extracts (ACEs) that exhibit noteworthy nematicidal effectiveness against *M. incognita* egg hatchability and juvenile mortality [23–25] Notably, several species within the Fabaceae family, including those within the genus *Arachis*, have shown promise as potential sources of nematotoxic compounds [26,27].

The *Arachis* genus, consisting of perennial angiosperms from the Fabaceae family, is native to South America and contains 81 distinct species. Of these species, 47 can be found exclusively in Brazil [28–30]. *Arachis hypogaea* L. is the most grown member of the peanut family, commonly known as peanuts or groundnuts, and is used both for human consumption in its fresh grain form and for oil extraction. Other species within the *Arachis* genus have utility as fodder and ornamental plants [31].

Embrapa Genetic Resources and Biotechnology maintains a diverse collection of *Arachis* germplasm, consisting of approximately 600 accessions from various regions of Brazil. The integration of genetic resources with research initiatives aims to expand the worth of the preserved *Arachis* germplasm [31]. As part of a research program designed for exploring bioactive compounds and valorizing germplasm banks, this study evaluated the nematostatic and nematicidal effects of ACE of nine *Arachis* species, obtained from the Embrapa Germplasm Bank, against *M. incognita*. The goal was to contribute to the development of new information regarding the control mechanisms targeted towards RKNs.

Given the need for more sustainable and environmentally friendly control strategies, the search for active substances from plants further highlights the wealth deposited in Germplasm Storage Banks (GSBs). Considering the potential of these seldom-explored biological assets, this study offers a new perspective on using preserved germplasms to enhance the value of GSB through targeted applications.

2. Materials and Methods

2.1. Obtaining Eggs and Second Stage Juveniles (J_2) of Meloidogyne incognita from Infected Nicotiana tabacum Plants

Nicotiana tabacum cv. Xanthi (tobacco) seeds were sown in germination tray at greenhouse located at Embrapa Genetic Resources and Biotechnology. Around 20 days after sowing, 30 *N. tabacum* seedlings were individually transferred to plastic bags measuring $20 \times 30 \times 12$ cm, containing around 3100 cm^3 of a sterile mixture of soil, commercial Bioplant$^®$ substrate, and sand, in a ratio of 4:2:4. One plant per plastic bag was inoculated with about 2000 second-stage juveniles (J_2) of *M. incognita*. After 90 days at 26–33 °C and 60% humidity, tobacco plants were removed and the roots were washed and cut into 1–2 cm segments and blended for one minute in a sodium hypochlorite (NaOCl) solution containing 0.5% active chlorine, according to the method described by Hussey and Barker [32], modified by Boneti and Ferraz [33]. For the recovery of eggs, overlapping granulometric sieves of 50, 250, and 500 mesh were used. To obtain the J_2, the eggs were collected in a 500-mesh sieve and transferred to a modified egg incubation chamber at 28 °C. After 48 h, the J_2 were counted using Peters slides and an Olympus BH2 B071 microscope.

2.2. Plant Extracts

Seeds of nine accessions representing different *Arachis* species (Table 1) sourced from the active *Arachis* Germplasm Bank from Embrapa Genetic Resources and Biotechnology, Brasília-DF, Brazil, were kindly provided by the curator of the bank Dr. José F. M. Valls. The seeds were disinfected in a 0.1% NaOCl solution (VV) for 5 min, dried, and weighed on a Shimadzu AUY220$^®$ analytical balance. Following this procedure, the seeds were individually ground using a mortar and pestle with liquid nitrogen and then pulverized by 3 pulses of 30 s each in a Cyclone Sample Mill (UDY Corporation, Fort Collins, CO).

Ten grams of each powdered seed material were suspended in 60 mL of distilled water (dH$_2$O) and gently agitated for 24 h at 4 °C. The resulting solutions were filtered individually through eight layers of cheesecloth, and the aqueous crude seed extracts (ACE) were centrifuged, at 12,000× *g* for 45 min at 4 °C. Approximately 50 mL of supernatant were collected, filter-sterilized (0.22 m pore size; Millipore Corp., Bedford, MA, USA), which were subsequently freeze-dried, weighed, and stored at −80 °C.

Table 1. *Arachis* species name, code, and access bank number.

Species Name	Code BRA	Access Number
Arachis Stenosperma	AM1	V 10309
Arachis triseminata	AM2	V 13080
Arachis retusa	AM4	V 12939
Arachis hassleri	AM5	Sv 3818
Arachis appressipila	AM9	V 9060
Arachis douradiana	AM16	V 14682
Arachis archeri	AM17	V 7614
Arachis gregoryi	AM18	V 14957
Arachis duranensis	AM19	V 14167

The biological materials were freeze-dried in a Savant-Super Modulyo Freeze Dryer (Thermo-Fischer, Waltham, MA, USA) to eliminate all sample moisture. The freeze-dried material was subsequently stored at −80 °C to be used in bioassays at a later stage.

2.3. Viability and Recovery Bioassay: Determine Nematicidal and/or Nematostatic Activity

Viability Bioassay: To perform the bioassay, 60 ± 5 J$_2$ of *M. incognita* were transferred to 1.5 mL microcentrifuge tubes and exposed to aqueous crude seed extracts (ACEs) of *Arachis*, previously prepared at a final concentration of 1 mg.mL^{-1} for 48 h at 27 ± 1 °C. Treatments were conducted in triplicate using distilled water and 70% ethanol (EtOH) as negative and positive controls, respectively [34,35]. After the exposure, nematodes were observed and counted using an optical microscope and a Peters slide, and subsequently categorized as mobile and paralyzed. The paralyzed nematodes were submitted to a recovery assay.

Recovery Bioassay: The J$_2$ displaying a straight/dead posture (paralyzed/stretched) were centrifuged at 700 g for 10 min. The supernatant was discarded, and the J$_2$ were carefully re-suspended in 1.5 mL of distilled water [11,34,35]. The process was repeated three times, and following the last wash, the nematodes were placed in 1.5 mL of distilled water for 24 h. Subsequently, the J$_2$ were counted and classified as either alive (curled/unstretched) or dead (paralyzed/stretched). This approach facilitated the assessment of the nematotoxic activity of each evaluated ACE. The resulting data were statistically analyzed using multiple comparisons between all treatments, with statistical tests for the difference in the proportion of paralyzed/dead nematodes at a significance level of 5% with *p*-value correction using the Holm method.

2.4. Determination of the Lowest Lethal Dose

The determination of the lowest lethal inhibitory dose for *Arachis* ACEs was executed through a dose-response analysis. For this purpose, 60 ± 5 J$_2$ of *M. incognita* were exposed to ACEs at concentrations of 50, 100, 300, 500, and 1000 mg.mL^{-1} for 48 h at 28 ± 1 °C [23]. Afterward, the nematodes were counted using Peters slides and an Olympus BH2 B071 microscope. The nematostatic effect was determined and the paralyzed nematodes were submitted to a recovery assay, as described above. The recovery test certifies the mortality of J$_2$. The bioassay was performed in triplicate, with dH$_2$0 and 70% ethanol (EtOH) as negative and positive controls, respectively. The data generated were statistically analyzed using multiple comparisons between all treatments, with statistical tests for the difference in the proportion of paralyzed/dead nematodes at a significance level of 5% with *p*-value correction using the Holm method.

2.5. Estimate of Arachis ACEs Thermostability

The thermostability evaluation was conducted by heating the ACEs to a temperature of 50 °C for 24 h. Following the heating process, a volume corresponding to a final concentration of 1 mg was used for a subsequent nematotoxicity in vitro bioassay as mentioned above, to confirm whether the nematotoxic property was retained [11]. The bioassay was conducted in triplicate, with dH_2O and 70% ethanol (EtOH) as negative and positive controls, respectively. The statistics were performed using multiple comparisons between all treatments, with statistical tests for the difference in the proportion of paralyzed/dead nematodes at a significance level of 5% with *p*-value correction using the Holm method.

2.6. Hemolysis Assay

Bovine blood samples from the Simental breed (C10) were kindly provided by Sucupira Experimental Farm (Animal Reproduction Laboratory) from Embrapa Genetic Resources and Biotechnology, Brasilia-DF, Brazil. The material was collected in 5 mL tubes containing anticoagulating agent (Heparin) and kept on ice. A total of 10 mL of this sample was centrifuged at $2500\times g$ for 10 min at 4 °C. The supernatant was discarded and the pellet, carrying the red blood cells, was resuspended in saline phosphate buffer (100 µL, PBS–$1\times$, pH 7.4).

The hemolytic effect of active *Arachis* ACEs was tested using both total bovine blood and isolated red blood cells. To this, 30 mg of dried active ACEs were resuspended in phosphate saline buffer and vortexed 5 times for 1 min. Concentrations ranging from 0.125 µg/µL to 16 µg/µL were prepared by conducting serial dilutions. Aliquots (10 µL) of each dilution were individually placed into 96-well microtiter plates containing 190 µL of bovine total blood or isolated red blood cells. The hemolytic effect was monitored using a spectrophotometer at 567 nm using a Benchmark Plus[TM] microplate reader (Biorad). Bioassay was conducted in triplicate with dH_2O as positive control, causing 100% hemolysis, and PBS ($1\times$) as negative control.

The data generated were analyzed using multiple comparisons between all treatments, with statistical tests for the difference in the proportion of dead nematodes at a significance level of 5% with *p*-value correction using the Holm method.

3. Results and Discussion

3.1. Nematotoxic In Vitro Bioassays to Evaluate the Activity of ACEs from Arachis Plant Seeds against J₂ of M. incognita

To preserve biodiversity and maintain *Arachis* genetic variability, Embrapa Genetic Resources and Biotechnology curates a diverse collection of *Arachis* germplasm. This collection contains around 600 accessions from various regions throughout Brazil. Although the biotechnological potential of these accessions exists, it remains largely unexplored. Initiatives aimed at the exploration of bioactive compounds play an important role in the discovery of valuable information on the bioactive available and in the enhancement of the value of the resources within this bank. To contribute to the evaluation of the valuable *Arachis* resources, the nematotoxic effects of nine aqueous crude extracts (ACE) derived from distinct *Arachis* species seeds were estimated in vitro using viability and recovery bioassays.

3.1.1. Viability In Vitro Bioassay

In the viability bioassay, 1 mL of aqueous crude extracts (ACEs) with a final concentration of 1 mg was used to expose approximately 60 ± 5 J₂ of *M. incognita* for 48 h. Figure 1 demonstrates that over 95% of the J₂ of *M. incognita* were paralyzed by the nematostatic action of all ACEs obtained from the nine *Arachis* species.
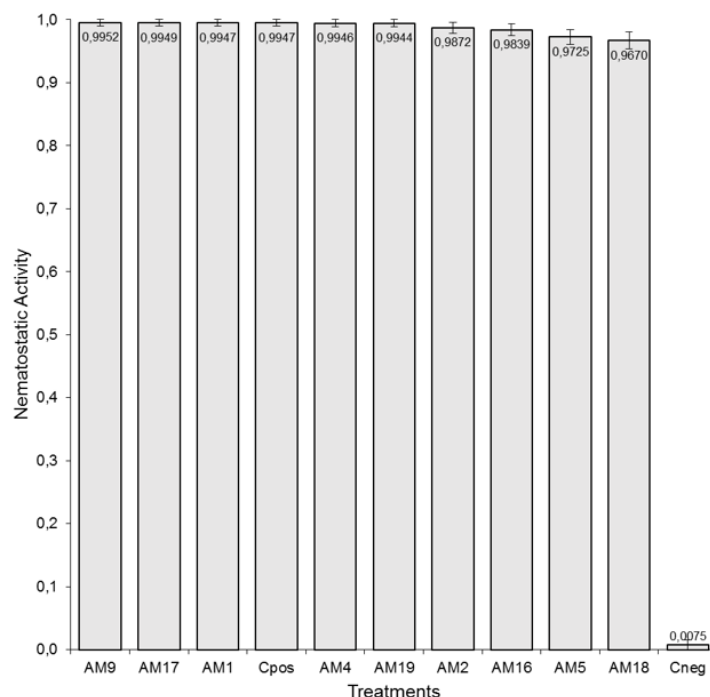
Figure 1. Evaluation of ACEs paralytic activity after 48 h exposure. Bioassay was performed in triplicate. Negative control (C neg) uses dH$_2$O, positive control (C pos) uses 70% EtOH. The data generated were analyzed using multiple comparisons among all treatments, with statistical tests for differences in the proportion of paralyzed nematodes at a 5% significance level, with *p*-value correction using the Holm method.

3.1.2. Recovery In Vitro Bioassay

To confirm the nematocidal and/or nematostatic activity, all ACEs that demonstrated significant ability to paralyze *M. incognita* J$_2$ were tested using an in vitro recovery bioassay. Results showed that ACE from *A. stenosperma* seeds (AM1) did not differ significantly from the positive control, which used 70% alcohol. Both ACE and the positive control showed a maximum nematicide effect with around 100% of juveniles being deceased after being exposed (refer to Figure 2).

All tested ACEs effectively paralyzed the J$_2$ of *M. incognita* (nematostatic effect); however, only AM1 and AM5 extracts of *A. stenosperma* and *A. hassleri*, respectively, demonstrated the ability to kill over 90% of the nematodes (nematicide action). The nematicidal levels exhibited a decrease in the remaining extracts derived from *A. appressipila* (AM9), *A. retusa* (AM4), *A. gregoryi* (AM18), *A. duranensis* (AM19), *A. douradiana* (AM16), and *A. archeri* (AM17), despite being nematostatic. Notably, *A. triseminata* extract (AM2) showed minimal effects, whereas *A. stenosperma* (AM1) had higher levels (Figure 2). Based on the results, *A. stenosperma* (AM1) ACE has a high potential as a nematicidal candidate.

The species *A. stenosperma* contains genes that provide resistance to gall-forming nematodes (RKNs). Studies have been conducted to locate, map, and transfer these genes to the cultivated species through genetic and crossbreeding methods [36–40]. Despite the known resistance of *A. stenoperma*, there is limited information regarding the nematotoxic effects of its aqueous extracts.

Aqueous crude extracts are a valuable source of natural substances that can serve as a substitute for synthetic chemical pesticides [41]. Numerous reports in academic literature demonstrate the efficacy of plant extracts in controlling *M. incognita* and other nematodes. Notably, studies have shown that the aqueous extract of *Canavalia ensiformis* (L.) DC. and *Crotalaria spectabilis* Roth [11,34,42,43], both from Fabaceae family, exhibit promising results. Nematicidal activity has also been reported for compounds derived from botanical species

including *Ricinus communis* L. [44], *Crotalaria juncea* L. [45,46], and *Azadirachta indica* A. Juss. [47], as well as for pyrrolizidine alkaloids that act on *M. hapla* [48].
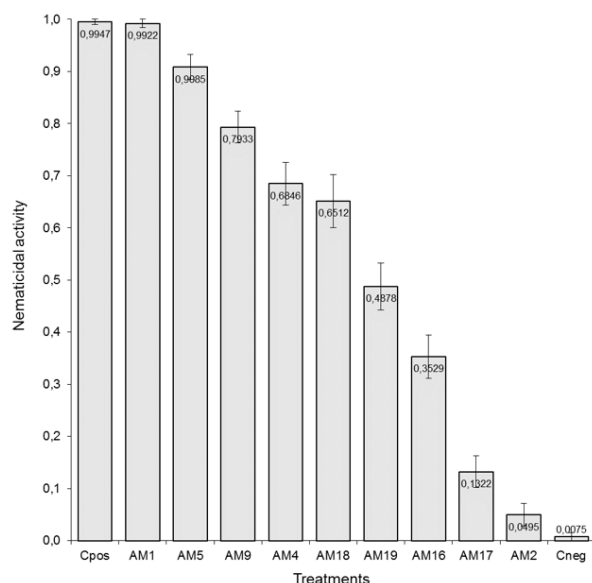

Figure 2. Recovery in vitro bioassay to confirm the nematotoxic activity of *Arachis* ACEs towards J_2 of *Meloidogyne incognita.* The bioassay was performed in triplicate. Negative control (C neg) dH_2O and positive control (C pos) 70% EtOH were used. The data were analyzed using multiple comparisons between all treatments. Statistical tests were conducted to assess the difference in the proportion of dead nematodes at a significance level of 5% with *p*-value correction using the Holm method.

According to the literature, aqueous crude seed extracts may contain chemical components associated with nematotoxic activity, specifically secondary metabolites such as alkaloids, terpenes, terpenoids, tannins, and phenolic compounds [11,42,49,50]. The recorded activity of each ACE may be linked to the presence and concentration of chemical compounds in the sample, which can exert their active potential independently or in synergy with other compounds.

According to Lopes et al. [41], phenylpropanoid derivatives such as stilbenes and flavonoids are the compounds most frequently found in the *Arachis* genus and are involved in defense mechanisms against physical injuries and microbial contamination. Stilbene synthesis in various tissues, including seeds, is associated with resistance to diseases of common peanut (*A. hypogaea*) such as injuries, fungal contamination, insect damage, and other attacks. Furthermore, the nematicidal activity potential of various structure-related flavonoids against J_2 of *M. incognita* has been reported by Bano et al. [51].

Fatty acids are compounds whose presence has been extensively described in the literature for *Arachis* seeds. Zang [52] found that compounds containing linoleic and oleic acids, which are present in several *Arachis* species including *A. durannensis*, *A. monticola*, *A. batizocoi*, *A. cardenasii*, *A. villosa*, *A. stenosperma*, have a high potential for nematicidal activity against second stage juveniles of *M. incognita*.

The results corroborate with the promising potential for the existence of chemical compounds with nematicidal properties in the samples tested, however, additional testing is required to identify and quantify the concentrations of the active chemical compounds that can be present in the samples.

In agreement with analogous experiments, further tests, such as investigating the lowest lethal dose, analyzing thermostability, and conducting cytotoxicity assays, were conducted to investigate the potential of AM1 ACE and provide more data regarding this accession of *Arachis* sp.

3.2. Determination of the Lowest Lethal Dose of Arachis ACEs on J_2 of M. incognita

An in vitro bioassay was conducted to determine the lowest lethal nematotoxic dose using increasing concentrations of 50, 100, 150, 300, 500, and 1000 µg/mL from sample AM1. After 48 h of exposure, nematostatic activity was observed in more than 95% of J_2 in all tested concentrations. However, concentrations below 500 µg/mL in the in vitro recovery bioassay exhibited a significant recovery of J_2, with over 40% of *M. incognita* nematodes regaining mobility. Concentrations of 500 and 1000 µg/mL displayed effective nematicidal activity, with 98.29% and 99.47% of nematodes dead, respectively (Figure 3).
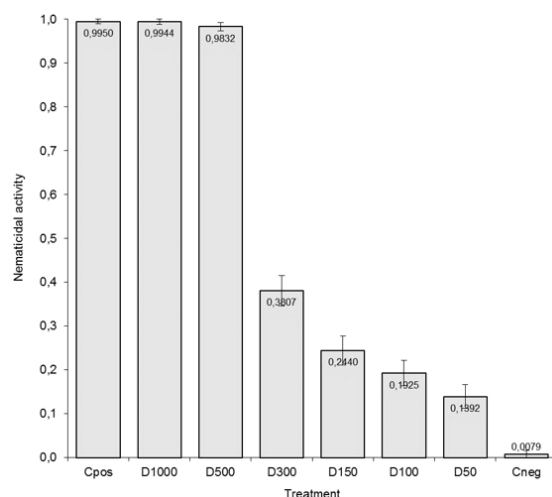

Figure 3. Recovery in vitro bioassay to certify the nematotoxic activity (nematostatic and/or nematicidal) of *Arachis stenosperma* seeds ACE (AM1) at distinct concentrations (50, 100, 150, 300, 500, and 1000 µg.ml^{-1} in 1000 µL/FV). Bioassay was conducted in triplicate, using dH$_2$O and 70% EtOH as negative (Cneg) and positive control (Cpos). The data generated were analyzed using the Holm method.

The concentrations of ACEs have a direct relationship with their nematicidal capacity. Khurma and Singh [53] conducted analogous experiments utilizing seed extracts in different dilutions, noting a gradual decrease in nematicide activity in proportion to reductions in extract concentrations administered during treatments.

3.3. Thermostability Test: Certifying the Thermal Stability of Nematotoxic Arachis AM1 ACE against J_2 of M. incognita

The AM1 ACE heated at 50 °C for 24 h at concentrations of 50, 100, 150, 300, and 500 µg mL^{-1} were used to perform the thermostability in vitro bioassay. Based on the results, all the concentrations effectively paralyzed more than 90% of *M. incognita* J_2 after 48 h regarding the viability bioassay and the thermostability (Figure 4).

On the other hand, concerning the recovery bioassay, the ACE from thermal-treated AM1 showed a nematicidal activity, with nearly 98% of J_2 dead for the concentration of 500 µg mL^{-1}, (Figure 5).

The viability and recovery bioassays were conducted using *A. stenosperma* ACE seeds (AM1) exposed to temperatures higher than those typically found in soil layers containing root systems of several crops, including soybean, coffee, and cotton [54]. Determining the thermal stability of nematicidal compounds is a crucial characteristic, as their effectiveness depends on maintaining stability under various environmental conditions. This result certifies the thermal stability of the ACE analyzed. This aligns with Rocha et al. [11], who found that *Canavalia ensiformis* seed extract retained its nematicidal activity after heat treatment had been conducted on *M. incognita* J_2.
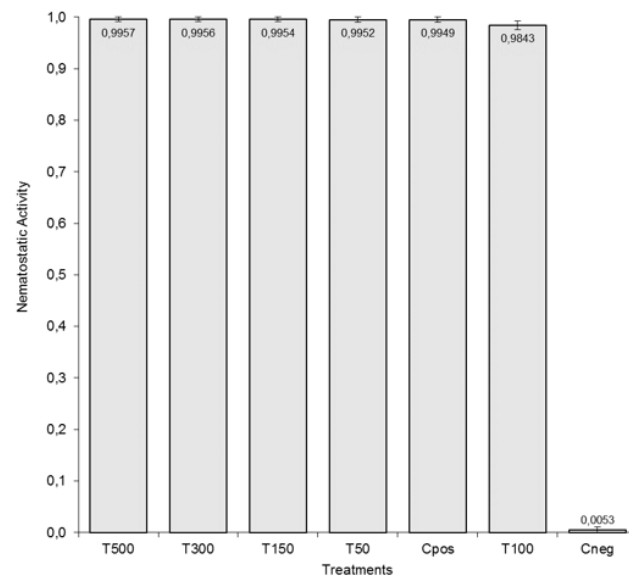
Figure 4. Viability in vitro bioassay of active ACE from *Arachis Stenosperma* seeds (AM1) heated for 24 h at 50 °C. The bioassay was carried out in triplicate, at different concentrations, with dH_2O as a negative control (Cneg); and positive control (Cpos) alcohol—70% EtOH. The data generated were analyzed using the Holm method.
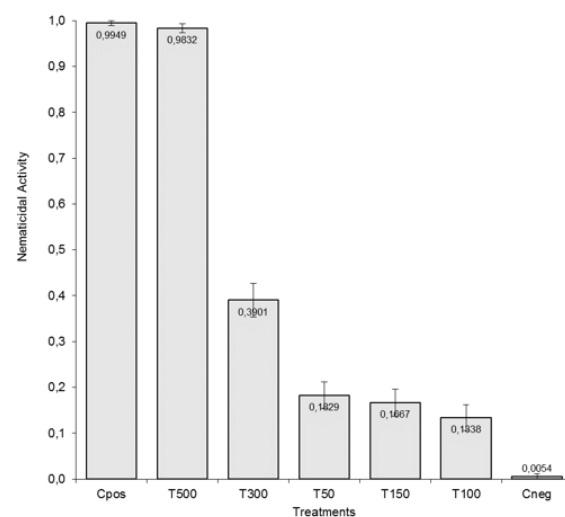

Figure 5. Recovery in vitro bioassay of nematodes treated with ACE from *Arachis Stenosperma* seeds (AM1) heated, to attest the thermal stability of the nematicidal action on J_2 of *Meloidogyne incognita*. Statistical tests for the difference in the proportion of dead nematodes at a significance level of 5% with p-value correction were performed using the Holm method.

Ferris and Zheng [55] suggest that active plant compounds may be sensitive to heat, but Dias et al. [35] observed an increase in nematicidal activity in extracts obtained by infusion of *Tanacetum vulgare* L. and *Momordica charantia* L., concluding that the amount of active compounds present in these plants may have increased with heating in water, which would justify the greater efficiency in controlling *M. incognita*.

3.4. Cytotoxicity: Evaluating the Cytotoxicity of Nematotoxic Arachis AM1 ACE against Bovine Red Cells (Female 3379)

The AM1 ACE from *A. stenosperma* was analyzed for its hemolytic capability using bovine red cells (female 3379) via photometric analysis. The results in Figure 6 demonstrated that AM1 ACE, at concentrations of 150, 300, 500, and 1000 µg/300 µL, did not show a cytolytic effect on bovine red blood cells.
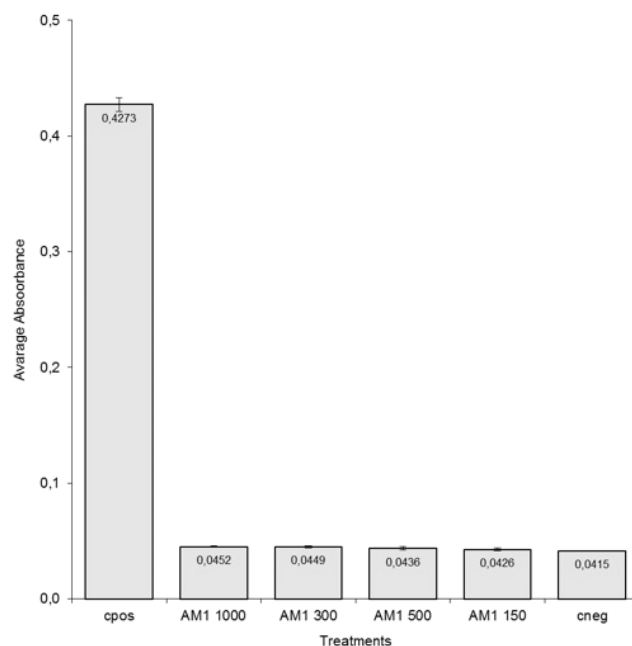
Figure 6. Cytotoxicity in vitro evaluation of *Arachis Stenosperma* seeds ACE (AM1) on bovine red blood cells at different concentrations (150, 300, 500, and 1000 µg/300 µ). The bioassay was carried out in triplicate and the hemolytic activity was monitored by absorbance at 567 nm. dH$_2$O (100% hemolysis) and PBS were used as positive and negative controls, respectively. The data generated were analyzed using the Holm method.

Evaluating the cytotoxic activity of active compounds is a critical step in the biological characterization of bioactive compounds, which could potentially be utilized as a source for developing new products applicable to agriculture [53,55], and this result indicates that ACE AM1 is not toxic to mammalian blood cells.

3.5. Statistical Analysis

The statistical analysis conducted on the data utilized Z-tests to compare the difference in proportions between the treatment pairs. Dawson and Trapp [56] recommend either constructing a confidence interval or conducting a Z-test to determine the difference in proportions between two independent groups.

Among the tested species, *Arachis stenosperma* statistically demonstrated exceptional nematotoxic activity, thermostability, and reduced cytotoxic effects. These findings provide strong support for our hypothesis and emphasize the significance of preserving and characterizing accessible genetic resources.

4. Conclusions

The results suggest that the aqueous crude extract derived from *Arachis stenosperma* (Fabaceae family) displays preliminary indications of nematicidal activity against *M. incognita*. Additionally, the extract is thermally stable and non-cytotoxic to bovine red blood cells. This underscores the significance of plant germplasm banks and their potential to generate novel plant-based technologies.

Further studies are necessary to confirm evidence of nematotoxicity, including the biochemical identification of compounds present in ACE, bioassays on non-target organisms, as well as greenhouse and field bioassays.

This study supports the use of conserved biodiversity as a novel, sustainable biotechnological resource for the development of products for the management of PPNs. These products should be safe for human and animal health and the environment and should reduce reliance on synthetic chemicals while enhancing germplasm banks.

Data Availability Statement: Seeds of different *Arachis* species sourced from the Active *Arachis* Germplasm Bank at Embrapa Genetic Resources and Biotechnology, Brasília-DF, Brazil, were kindly provided by the curator of the bank Dr. José F. M. Valls.

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

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