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Quantitative analyses of polyphenol extracts from inflorescences of *Musa x paradisiaca* against free-living stages of *Haemonchus contortus*

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Abstract: Extracts from Musa x paradisiaca inflorescences were chemically characterized and tested *in vitro* on ruminant parasite egg hatch (EHT) and larval migration (LMT) tests. The aqueous (EHW-I) and hydroalcoholic (EOH-I) extracts of the plant were analyzed by using colorimetric assays, high-performance liquid chromatography (HPLC), and electrospray tandem mass spectrometry (ESI-MS/MS). EHW-I and EOH-I had similar phenolic contents. However, EOH-I had a higher flavonoid content than EHW-I. Both extracts presented low contents of condensed tannins. HPLC analysis showed that EHW-I contained phenolic acids (chlorogenic acid > coumaric acid > ferulic acid), other than rutin (glycosylated flavonoid). EOH-I contained rutin as the predominant compound. EHW-I and EOH-I showed an inhibitory effect on EHT, reaching almost 100% at a concentration of 1000 µg/mL. The inhibitory concentration of 50% (IC50) of egg hatching was 62.58 and 137.79 µg/mL of EHW-I and EOH-I, respectively. Based on the quantification data of phenolic and flavonoid compounds in the extracts, chlorogenic acid and rutin were selected for the EHT. Chlorogenic acid presented high activity, with an IC50 of 17.55 µg/mL. Rutin, however, did not affect egg hatching under the conditions tested. It can be inferred that chlorogenic acid may be a candidate with ovicidal activity. EHW-I and EOH-I did not affect the migration of third-stage nematode larvae, which may be related to the low tannin content. The inflorescences of Musa x paradisiaca can be further explored to develop a plant-derived anthelmintic product for ruminants and other animals.
Keywords: Phytotherapy, Biofriendly products, principal components, gastrointestinal parasites, *in vitro* assays.

1. Introduction

World ruminant production is economically affected by gastrointestinal parasite infections. One of the most important gastrointestinal nematode parasites affecting livestock is *Haemonchus contortus*. This hematophagous parasite can cause severe anemia, which reduces weight gain, and, in more severe infections, can lead to animal death (Kumarasingha et al., 2016). Conventional parasite control relies almost exclusively on the regular use of broad-spectrum anthelmintics. However, such treatments present major disadvantages, including the selection of resistant populations, the risk of environmental pollution, high costs, and, in addition, the unavailability of these medicines in some developing countries (Fortes and Molento, 2013).

The use of natural plant extracts to treat parasitic infections in livestock and humans is a long-standing practice. This therapeutic approach may offer sustainable and environmentally acceptable alternatives (Athanasiadou et al., 2007; Molento, 2009; Pavela and Benelli, 2016). Classes of secondary metabolites, such as terpenoids (essential oils) and polyphenols (phenolic acids, flavonoids, and condensed tannins), are sources of chemical components responsible for the broad therapeutic effects of several medicinal plants, including anthelmintic activity (Molan et al., 2002; Hoste et al., 2015). *In vitro* tests serve as a preliminary step for validating novel extracts, as they enable the characterization of active compounds present in plants and the evaluation of their biological properties (Pavela and Benelli, 2016).

Musa paradisiaca L., a plant in the family Musaceae, is a large shrub that grows in tropical regions, and its fruit is known as a banana (Padam et al., 2012; Jaleel et al., 2024). This plant is a functional and medicinal plant of high therapeutic potential, and its parts, including peels, leaves, pseudostem, and inflorescence, have various applications (Padam et al., 2012; Jaleel et al., 2024). They serve as alternative sources of macronutrients and micronutrients, are also used in nutraceuticals and livestock feed, and function as natural fibers and sources of bioactive compounds (Padam et al., 2012; Jaleel et al., 2024; Senevirathna et al., 2024). Studies on the phytochemical composition of banana (*Musa* spp.) inflorescences have increased due to the growing interest in

utilizing agricultural by-products as sustainable sources of bioactive compounds (Padam et al., 2012; Senevirathna et al., 2024). Many studies have identified and characterized metabolites in *M. paradisiaca* inflorescences, including phenolic compounds such as flavonoid-like quercetin, epicatechin (Bashkar et al., 2012, Bovo et al., 2013, Nisha and Mini, 2013, Amornlerdpison et al., 2021, Ramírez-Bolaños et al., 2021), anthocyanins (Pazmiño-Duran et al., 2001, Senevirathna et al. 2024), tannins (Mahmood et al., 2011, Amornlerdpison et al., 2021), terpenoids, such as tetracyclic triterpenoids (Dutta et al., 1983), saponins, steroids (Mahmood et al., 2011), alkaloids (Mahmood et al., 2011), and phenolic acids, such as gallic acid (Nisha and Mini, 2013, Amornlerdpison et al., 2021).

Most studies have evaluated the anthelmintic activity of plant extracts based on qualitative data from various classes of metabolites, using techniques such as phytochemical screening. Quantitative studies on the components, however, are lacking (Athanasiadou et al., 2007; Kumarasingha et al., 2016; Pavela and Benelli, 2016). For example, the aqueous extracts of the leaves, stems, and inflorescences of *Musa* spp. showed high efficacy in the inhibition of egg hatching (IC_{50} =190 µg/ml) (Nogueira et al., 2012). In another study, the hydroalcoholic extract of *M. paradisiaca* (banana bracts), characterized by the presence of polyphenols, condensed tannins, and flavonoids, exhibited *in vitro* anthelmintic activity against bovine nematodes, inhibiting 88% of larval hatching at 2.5 mg/mL and 67.56% of larval migration at 5 mg/mL, while showing no effect in sheep nematodes (Kakimori et al., 2019). However, the study did not provide detailed chemical profiling of the phenolic compounds nor establish which compounds were associated with the anthelmintic effects (Kakimori et al., 2019). Díaz et al. (2017) demonstrated that chlorogenic acid, present in the extracts of *T. filifolia*, is one of the phenolic compounds responsible for the ovicidal activity against important sheep parasites. The present study aimed to analyze the anthelmintic effects of chemically characterized polyphenol-rich extracts from *M. paradisiaca* inflorescences in *in vitro* egg-hatch and larval-migration tests. We have also tested the isolated compounds, chlorogenic acid and rutin, using the same methodology.

2. Materials e Methods

Chemicals and reagents

Coumaric acid, chlorogenic acid, ferulic acid, gallic acid, sinapic acid, syringic acid, epicatechin, quercetin, and rutin were obtained from Sigma-Aldrich (St. Louis, USA). Methanol-grade HPLC was obtained from JT Baker (Xalostoc, Mexico). Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). Water was purified using an ultra-purifier model MS 2000 from Gehaka (São Paulo, Brazil). Ivermectin (Ivomec 1 % w/v, Merial Limited, Duluth, USA) and albendazole sulfoxide (Ricoben 13.6 % w/v, Noxon Limited, Cravinho, Brazil). Folin-Ciocalteu reagent was obtained from Chromate (São Paulo, Brazil). All other reagents were of analytical grade.

Plant material and obtention of the aqueous and hydroalcoholic extracts of the inflorescences

The *M. paradisiaca* L. inflorescences were collected in Maringa, in the state of Paraná, Brazil. The plant was botanically identified, and a voucher specimen (number: 367379) was retained at the Municipal Botanical Museum of Curitiba. Fresh inflorescences (40 g) were cut into small pieces and ground in a blender (Philips Walita Co, Varginha, MG, Brazil) with water (100 mL) for 10 min. The mixture was then placed in a water bath at 70 °C for 60 min. The aqueous extract, designated EHW-I, was vacuum-filtered, concentrated in a rotary evaporator under reduced pressure at 40 °C, freeze-dried, and stored at -4 °C in the dark. For preparing the hydroalcoholic extract, fresh plant material (40 g) was mixed with 70% aqueous ethanol (v/v) (100 mL). After 10 min of grinding and stirring, the mixture was poured into glass beakers and stored at 25 °C, in the dark, for 10 days. The extract, designated EOH-I, was vacuum-filtered, concentrated in a rotary evaporator under reduced pressure at 40 °C, and then freeze-dried. It was stored at -4 °C in the dark. Yields, calculated relative to plant fresh mass, were 1.8% and 2%, respectively, for EHW-I and EOH-I.

Colorimetric methods

The total phenol content was determined using a microassay with the Folin–Ciocalteu reagent, adapted from Singleton and Rossi Jr. (1965). The calibration curve was prepared using gallic acid (GA) as a standard. The flavonoid content was determined using the aluminum chloride method (Vennat et al., 1992), with rutin (RUT) as the standard. Condensed tannin (CT) content was determined by the sulfuric-vanillin method (Queiroz et al., 2002). The results are expressed as µg epicathecin equivalents (EPI) per mg dry extract.

High-performance liquid chromatography analysis

The phenolic analysis was conducted using an Agilent 1200 Series high-performance liquid chromatography (HPLC) system (Agilent Co., St. Clara, USA) equipped with a vacuum degasser (G1322A), quaternary pump (G1311A), manual injector (Rheodyne, 7725i), and a multi-UV-VIS wavelength detector (G1365D) operating at a wavelength of 254, 280, 300, 325, and 375 nm, using an Agilent Eclipse XDB-C-18 column (150 mm × 4.6 mm, 5 µm particle size). The mobile phase used was water:trifluoroacetic acid (TFA; 0.1 %) (A) and methanol:TFA (0.1 %) (B) set with the following gradient: 0–10 min 40 % B, 10–22 min 60 % B, 22–28 min 30 % B, 28–30 min 40 % B at a flow rate of 1 mL/min (Pavei et al., 2010). The standards were prepared individually in methanol at a concentration of 100 µg/mL. Twenty microliters of the sample were injected through a manual injector. The samples were diluted in a methanol:water (1:9 v/v) solution to a concentration of 5 mg/mL, then filtered through a 0.22-µm membrane filter (JetBiofil, Guangzhou, China) before injection. The phenolic compounds were identified by comparing their retention times with

standards. Sensitivity parameters of the analysis, including limits of detection (LOD) and limits of quantification (LOQ), were calculated for rutin and chlorogenic acid based on the standard deviation of the areas in sextuplicate at the minimum detectable concentration. The EZChrom Elite program, running on Windows 7, was used for system control and data analysis.

Mass spectrometry analysis

The tandem mass spectrometry (MS/MS) system used for quantitative analysis was a Quattro LC triple-quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with a Z-electrospray (ESI) interface and operated in positive-ion mode. The temperatures of the source block and the desolvation gas were set at 100°C and 350°C, respectively. Nitrogen was used as both the desolvation gas (approx. 360 L/h) and the nebulizer gas (approx. 40 L/h), while argon was used as the collision gas. The voltages used in the ESI source during the analysis were 20 V for the cone, 3 kV for the capillary, and 4 V for the extractor. Ion detection was performed in the multiple reaction monitoring (MRM) mode, utilizing a collision energy range of 15–25 eV. The analytical data were processed by MassLynx software (Micromass, Manchester, UK). The target analytes were chlorogenic acid, ferulic acid, and rutin. The identification of these compounds was carried out based on mass spectra and by comparing the results with reference compounds from the literature (Jong et al., 2006; Horai et al*.,* 2010).

Fecal culture analysis

The presence of *Haemonchus contortus* was monitored using fecal samples that were obtained from naturally infected sheep with gastrointestinal nematodes. Feces were collected directly from the rectum of the animals and maintained under 4º C. Samples were used to determine the fecal egg count per gram of feces (Gordon and Whitlock, 1939). To analyze which species was predominant, the fecal culture analysis was performed from a pool of feces. The data revealed that *H. contortus* (strain HcUFPR2015) was the most abundant (95%) nematode species, followed by *Trichostrongylus* spp. (4%).

Egg Hatch Test

The egg hatch test (EHT) was performed using the methodology described by Coles et al. (1992), adapted by Bizimenyera et al. (2006). Eggs were recovered from the feces of sheep naturally infected with gastrointestinal nematodes. The feces were homogenized in water at 30 °C and then filtered successively through sieves with mesh sizes of 1 mm, 150 µm, 75 µm, 56 µm, and 25 µm. The eggs retained in the final filter were collected and centrifuged for 2 min at 140 x g. The supernatant was removed, and sucrose solution (0.9 M, d = 0.375 g/mL, 25 °C) was added. In this condition, the eggs floated, and any unwanted waste precipitated in the tube, allowing their easy collection using a Pasteur pipette. The eggs obtained from this process were transferred to a 12-mL plastic tube, washed again with warm distilled water to remove sucrose, and then re-suspended in distilled water. The material was then centrifuged (1×) for 2 min at 140x g. Supernatants (eggs) were put together and subsequently washed with distilled water (2 ×) and centrifuged under the same conditions, as described before, to collect the clean eggs. The egg concentration was counted via an optical microscope (SM-LUX model, Ernst Leitz GmbH, Wetzlar, Germany). The egg suspension was distributed into 24-well plates (100 eggs/well) and then mixed with the plant extract prepared in distilled water at final concentrations of 25, 50, 100, 500, 1000, 1500, and 2000 µg/mL, in a total volume of 1 mL. Test samples for the negative control (distilled H_2O) and a positive control (albendazole sulfoxide, at final concentrations of 10 to 1000 µg/mL) were obtained in parallel. Following this, the plates were maintained in a BOD-type incubator (Biological Oxygen Demand, Eletrolab, São Paulo, Brazil) at 25 °C for 24 h. Egg hatch was blocked by the addition of Lugol's iodine solution, which also facilitated the readings using an inverted microscope (INV 100 model, Bel Engineering, Monza, Italy). The number of eggs and first-stage larvae per well was determined. Three experiments, each with three replicates for the control group and each concentration, were performed. The results are expressed as percentages of inhibition of egg hatch, determined by the formula: (number of eggs / (number of eggs + number of L1) × 100, considering (L1) = L1 larvae; (eggs) = eggs not hatched. Photos of eggs and larvae were taken using an inverted microscope (Axiovert 40 CLF, Carl Zeiss, Jena, Germany).

Larval migration test (LMT)

The effect of plant fractions on the migration of third-stage larvae (L3) of helminths was determined using the method described by Molento and Prichard (2001), modified by Demeler et al. (2010). For this experiment, L3 (30-day-old) were incubated in a 0.3% (v/v) sodium hypochlorite solution for 1 h at 25 °C to remove the cuticle. After that, they were washed (3 x) with distilled water and were quantified and distributed into 24-well plates (200 larvae/well). The plant extracts, with final concentrations of 25, 50, 100, 500, 1000, 1500, and 2000 µg/mL, were prepared as previously described and added to the plates, which were then incubated in a BOD at 25 ºC for 6 h. Tests using a negative control (distilled water) and a positive control (Ivermectin at a final concentration of 100 µg/mL) were performed in parallel. After the first incubation, the material was transferred to plates with an apparatus containing a mesh size 22 µm nylon membrane, and incubated under the same conditions, but with a source of light (150 Watts) situated below the plates to stimulate L3 migration. After 18 h, the apparatus was removed, and the L3 that had migrated were counted using an inverted microscope. The L3 that did not migrate was also included in the count. Three experiments, each with three replicates for the control group and each concentration, were performed. The average number of L3 that migrated at each tested concentration was transformed into a percentage.

Statistical Analysis

The results of the quantitative phytochemical measurements were subjected to One-Way analysis of variance (ANOVA), followed by post-hoc Fisher's LSD multiple comparison tests to compare means. Results are expressed as mean ± standard deviation of at least three experiments. The values were considered statistically significant if the p-value was ≤ 0.05. The results of the EHT and LMT tests were analyzed using ANOVA followed by Tukey's multiple comparison test, as performed with GraphPad Prism 5. The differences were considered statistically significant at $p \leq 0.05$. The concentrations of the extract that inhibited 20, 50, and 90% of egg hatching, expressed as IC_{20}, IC_{50}, and IC_{90}, were calculated using linear equations ($R^2 \geq 0.95$) obtained by linearizing the activity curves with the GraphPad Prism 5 program.

3. Results and Discussion

Chemical characterization of *M. paradisiaca* inflorescence extracts

Table 1 presents the results of the quantitative analysis of phenolics, flavonoids, and CT for the EHW-I and EOH-I. The phenolic contents of the *M. paradisiaca* inflorescence extracts (35 and 26 mg GA equivalent/g dry sample for EHW-I and EOH-I, respectively) were higher than those reported for the extracts of the other parts of the species, such as the fruit aqueous extract (0.94 mg GA equivalent/g dry extract) (Shodehinde and Oboh, 2013) and the pseudostem methanolic extract (12 mg GA equivalent/g dry extract) (Loganayaki et al., 2010). Studies have demonstrated that inflorescence extracts are rich in phenolic acids and flavonoids, with total polyphenol contents typically between 10 and 30 mg GAE/g dry weight (Bashkar et al., 2012; Ramírez-Bolaños et al., 2021; Senevirathna et al., 2024; Gayathry et al., 2024). The content of flavonoids in EOH-I (25 mg RUT equivalent/g dry sample) was significantly higher than that in EHW-I (3 mg RUT equivalent/g dry sample), as expected, since the mixture of ethanol and water is more efficient in extracting flavonoids. The content of flavonoids, as described in the literature for *Musa* spp., varies according to the type of extraction and the type of plant material used. For example, the flavonoid contents were 0.73 mg quercetin equivalent/g dry extract and 75 mg rutin equivalent/g dry extract for the aqueous and methanolic extracts from the fruit pulp and peel, respectively (Shodehinde and Oboh 2013; Singhal and Ratra 2013). The content of CT was 0.8 mg EPI equivalent/g dry extract for EOH-I and 2 mg EPI equivalent/g dry extract for EHW-I, respectively. By comparing the values described for other parts of the plant (*Musa* spp.), such as fruit (3.7 to 25.9 %) and leaves (2.54 %), it can be inferred that EHW-I (0.08 %) and EOH-I (0.2 %) had a low content of CT (Bennett et al., 2010; Marie-Magdeleine et al., 2014).

	EHW-I	EOH-I
Yield (%) [1]	1.9	1.8
Phenolic (mg GA/g dry extract) [2]	35 ± 6.9^{a}	26 ± 5.4^{a}
Flavonoid (mg RUT/g dry extract) [3]	3 ± 0.9^{b}	25 ± 0.3^{c}
Condensed tannins (mg EPI per g dry extract) [4]	0.8 ± 0.005^{c}	2 ± 0.004^{e}

Table 1 – Chemical data of the aqueous (EHW-I) and hydroalcoholic (EOH-I) extracts obtained from the inflorescences of *Musa x. paradisiaca*. [1]Calculated as dried weight in relation to the original material. [2] Determined according to Singleton and Rossi Jr. (1966); expressed as gallic acid equivalents (GA). [3] Determined according to Vennat et al. (1992); expressed as rutin acid equivalents (RUT). [4] Determined according to Queiroz et al. (2002); expressed as epicathecin equivalents (EPI). Values are expressed as mean ± standard deviation of three experiments. Different letters in each column represent a significant difference between the samples, as determined by the Fisher LSD test ($p \leq 0.05$).

Figure 1 shows the HPLC chromatogram profile of the phenolics in *M. paradisiaca* inflorescence extracts. Comparison between the retention times of the standard peaks with the retention times of the peaks of the extracts led to the identification of phenolic acids: chlorogenic acid (present in EHW-I), coumaric acid (present in EHW-I), ferulic acid (present in EHW-I), and sinapic acid (present in EOH-I), and flavonoids: rutin (present in both extracts). The identification of other unnamed peaks was not possible because their retention points did not match any of the standards used. LOD and LOQ values were 0.55 and 1.67 µg/mL for rutin and 0.61 and 1.85 µg/mL for chlorogenic acid. The concentrations of phenolic and flavonoid compounds in the extracts were calculated using the equations obtained from the standard curves for rutin and chlorogenic acid (Table 2). The identification of phenolic acids and flavonoids was based on ESI-MS/MS analysis, using data such as the molecular ion (MI) and fragment ions (FIs) observed in the MS/MS spectra reported in the literature (Jong et al., 2006; Horai et al., 2010). The presence of chlorogenic acid ($[M + H]^+ = 355$, for MI), rutin ($[M + H]^+ = 611$ and 303, for MI and FI, respectively), and ferulic acid ($[M + H]^+ = 195$ and 177, for MI and FI respectively) was also suggested by ESI-MS/MS analysis.

HPLC analysis of the inflorescence extract demonstrated the presence of phenolic compounds, such as gallic acid, chlorogenic, ferulic, gallic, and p-coumaric acids, and rutin, quercetin, isoquercetin, catechin, and epicatechin (Bashkar et al., 2012; Ramírez-Bolaños et al., 2021; Amornlerdpison et al., 2021; Senevirathna et al., 2024; Gayathry et al., 2024). The cultivar and solvent polarity strongly influence differences in chemical composition, and the extraction methodology applied (Senevirathna et al., 2024). The phenolic compounds identified in *M. paradisiaca* inflorescences, such as rutin, chlorogenic acid, ferulic acid, coumaric acid, and

sinapic acid, are commonly found in fruits, vegetables, and cereals and have numerous beneficial health effects (Yang et al., 2008; Tajik et al., 2017). These beneficial health effects are associated with their antioxidant properties (Crozier 2010). The consumption of plant-based foods rich in nutrients and bioactive compounds has been linked to a lower incidence of chronic diseases in humans (Crozier 2010). In livestock nutrition, it has been proposed that consuming plant-based foods or adding bioactive compounds to the animal diet can be an alternative approach to improving animal health and enhancing anti-parasitic activity in the gastrointestinal system (Hoste et al., 2015). In addition, it has been speculated that the anthelmintic activity of plant extracts against nematodes may be attributed to phenolic compounds, particularly CT (Molan et al., 2002; Athanasiadou et al., 2007). The overall action of the extracts is a sum of the activities of their constituents (Adedapo et al., 2005). Díaz et al. (2017) have demonstrated that chlorogenic acid, present in the extracts of *T. filifolia*, is one of the phenolic compounds responsible for the ovicidal activity against *H. contortus*.

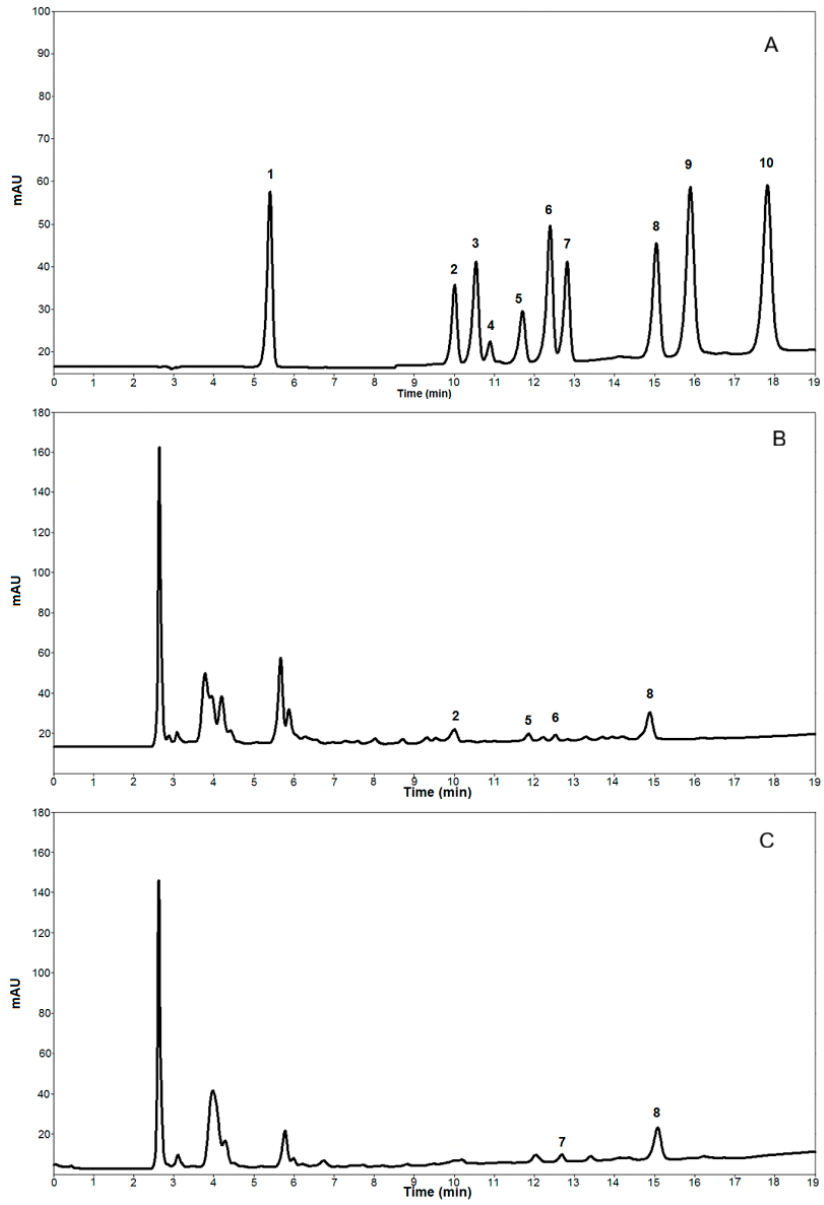

Figure 1 – HPLC Chromatogram of phenolic standards (A), of the aqueous (EHW-I) (B) and hydroalcoholic (EOH-I) (C) extracts obtained from the inflorescences of *Musa x. paradisiaca*.

The UV detector was set to an absorbance wavelength of 254 nm. Numbers in the chromatogram refer to the phenolic standards compounds: (1) gallic acid; (2) chlorogenic acid; (3) syringic acid; (4) epicatechin; (5) coumaric acid; (6) ferulic acid; (7) sinapic acid; (8) rutin; (9) myricetin; (10) quercetin. The phenolic compounds of EHW-I (B) and EOH-I (C) were identified by comparing their retention times with standards.

Phenolic acids or flavonoids (µg/mg dry sample)	EHW-I	EOH-I
Chlorogenic acid	0.8 ± 0.04	-
Coumaric acid	0.17 ± 0.63	-
Ferulic acid	0.16 ± 0.58	-
Sinapic acid	-	0.21 ±0.14
Rutin	1.00 ± 0.067[a]	2.35 ± 0.08[a]

Table 2 – Quantification of phenolic and flavonoid compounds in the aqueous (EHW-I) and hydroalcoholic (EOH-I) extracts obtained from the inflorescences of *Musa paradisiaca*.

Data are presented as mean ± standard deviation of three analyses. Different letters in each column represent statistically significant differences between samples, as determined by the Fisher LSD test ($p \leq 0.05$). (-) = not detected. The quantification of chlorogenic acid, coumaric acid, ferulic acid, and synaptic acid was calculated in relation to the standard curve of chlorogenic acid, and the concentration of rutin was calculated in relation to the curve of rutin.

Egg hatch activity

EHW-I and EOH-I extracts showed a dose-dependent inhibitory action (Fig. 2A and 2B). Egg hatch inhibition increased to close to 100 % at the highest concentrations tested (1000 to 2000 µg/mL). The control groups, negative (H_2O) (7.4%) and positive control (Albendazole sulfoxide, 10 to 1000 µg/mL), presented different results with respect to egg hatch inhibition. In the case of the group treated with a commercial anti-anthelmintic, which was used as the positive control, at concentration tested the egg hatch inhibition was higher than 99.9% (Fig. 2D). The concentrations of EHW-1 that could inhibit the hatching of eggs of *H. contortus* by 20, 50, and 90 % (IC_{20}, IC_{50}, and IC_{90}) were 7.99, 62.58, and 972.98 µg/mL, respectively (Fig. 2A and 2B). EOH-I exhibited IC_{20}, IC_{50}, and IC_{90} values of 34.89, 137.79, and 860.26 µg/mL, respectively. Figure 3 shows the general aspects of nematode eggs and/or larvae after 24 h of treatment in the Egg Hatch Test (EHT). Increasing concentrations of the plant extracts (EHW-I and EOH-I) resulted in a higher number of eggs that did not hatch after 24 h of incubation (Figure 3A–D). At the concentration of 1000 µg/mL of EHW-I, most eggs remained at the morula stage (Figure 3C). Figures 3E and 3F show the inhibition of egg hatching by albendazole sulfoxide (60 µg/mL), used as the positive control, in which eggs were mainly observed at the morula or larvated stages. In contrast, the negative control (H_2O) did not affect egg hatching, as shown in Figures 3G and 3H, where hatched L1 larvae were observed. The literature data concerning the effect of *Musa* spp. extracts on egg hatch inhibition are contradictory. Some authors reported no significant activity (Marie-Magdeleine et al., 2014), which differs from the results reported by others, who described high efficacy for the aqueous extracts in egg hatch inhibition, with IC50 and IC_{90} values of 190 µg/mL and 840 µg/mL, respectively (Nogueira et al., 2012). Another study, which presented the hydroalcoholic extract of *M. paradisiaca* bracts, showed 88% inhibition of hatching at 2.5 mg/mL against bovine nematodes (Kakimori et al., 2019). However, the quantitative information concerning the bioactive compounds was also insufficient to identify the compound(s) responsible for the tested activity.

Considering the data on phenolic and flavonoid compounds in EHW-I and EOH-I, chlorogenic acid and rutin were selected for testing in the *in vitro* anthelmintic assays (Fig. 2C). Figure 2C shows the efficacy of chlorogenic acid against egg hatch inhibition, with IC_{20} = 3.55 µg/mL, IC_{50} = 17.55 µg/mL, and IC_{90} = 147.52 µg/mL. In contrast, rutin (a glycosylated flavonoid) did not show any effect in the EHT under the tested conditions. Our results were similar to those published by Seyfried et al. (2022), which also demonstrated the efficacy of chlorogenic acid against egg hatch inhibition, with IC values as IC_{20} = 1.81 µg/mL, IC_{50} = 8.66 µg/mL, and IC_{90} = 69.70 µg/mL, and no effect on the EHT by rutin.

Several reports suggest that the anthelmintic activity of plant extracts, such as quebracho (*Schinopsis* sp.) and various forage species (*Lotus pedunculatus*, *Lotus corniculatus*, *Onobrychus viciifolia*, *Rumex obtusifolius*), is related to their content of CT (Molan et al. 2002; Athanasiadou et al. 2007). In the present study, the tested extracts, EHW-I and EOH-I, presented 0.08 and 2 % of CT, respectively, which was considerably less in comparison to the results reported for the aqueous extracts of quebracho bark, which contained 73 % of CT (Athanasiadou et al., 2007), suggesting that other chemical constituents were responsible for the ovicidal activity of *M. paradisiaca*. The ovicidal activity of plant extract with low-tannin content has been demonstrated (Díaz et al., 2017; Seyfried et al., 2022). The methanolic extract of *T. filifolia* exhibited significant ovicidal activity against *H. contortus*, reaching 99% efficacy at its highest concentration (10 mg/mL) after 48 h of incubation. For the extract of *Polygonum acre* (EHW-PA and ETOH-PA), the presence of phenolic acids (ferulic acid, rosmarinic acid, and sinapic acid) and aglycone flavonoid (quercetin) was speculated as relevant for influencing the observed egg hatch activity (Seyfried et al., 2022). It had been demonstrated that the chlorogenic acid presented a relevant activity against egg hatch inhibition, and in contrast, the glycosylated flavonoids (quercetagitrin and rutin) showed no effect (Díaz et al., 2017; Seyfried et al., 2022).

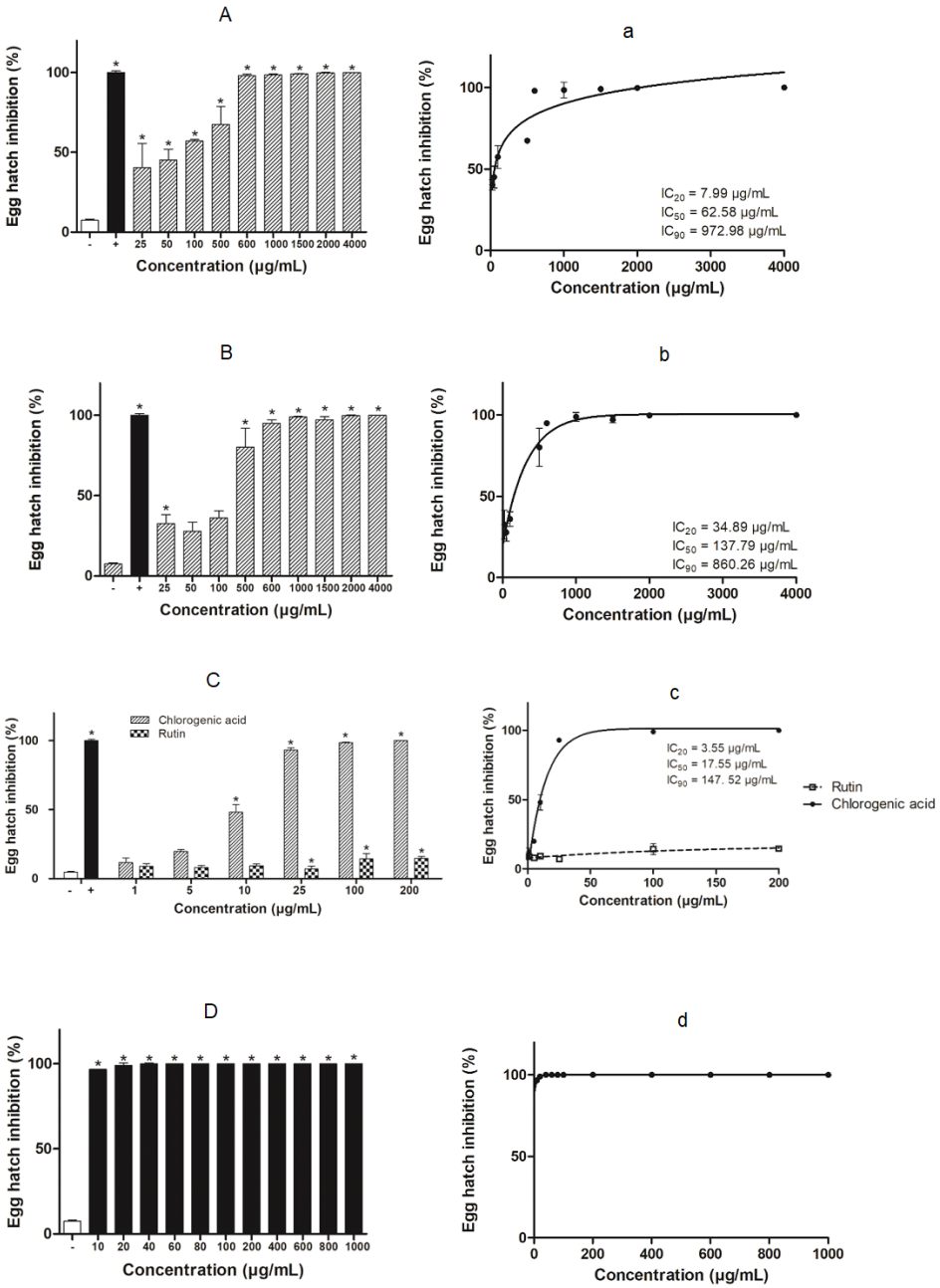

Figure 2 – Inhibition of the nematode egg hatch and the inhibition concentration (IC) determination of the aqueous (EHW-I) and hydroalcoholic (EOH-I) extracts obtained from the inflorescences of *Musa paradisiaca*, phenolic commercial standards and albendazole sulfoxide using the Egg Hatching Test. (A,a) EHW-I; (B, b) EOH-I; (C, c) Chlorogenic acid and rutin); (D, d) Albendazole sulfoxide; (-) H_2O; (+) Albendazole sulfoxide (60 µg/mL). Different letters in each column represent significant difference compared to negative control (H_2O) determined using Tukey's and t test ($p \leq 0.05$).

The nematocidal activity of chlorogenic acid has been described against *Pratylenchus scribneri* (grass nematode) and *Caenorhabditis elegans* (free-living nematode). However, the mechanism of the nematocidal or ovicidal activities needs to be elucidated. Some possibilities could be inferred from consideration of its properties described in other biological tests, for example, as an enzyme inhibitor (Tajik et al., 2017). Thus, the mechanism could involve inhibition of the so-called "hatching enzymes," including proteases, lipases, glycosidases, and leucine aminopeptidases, as well as cyclic AMP-dependent protein kinase (Rogers and Brooks 1977).

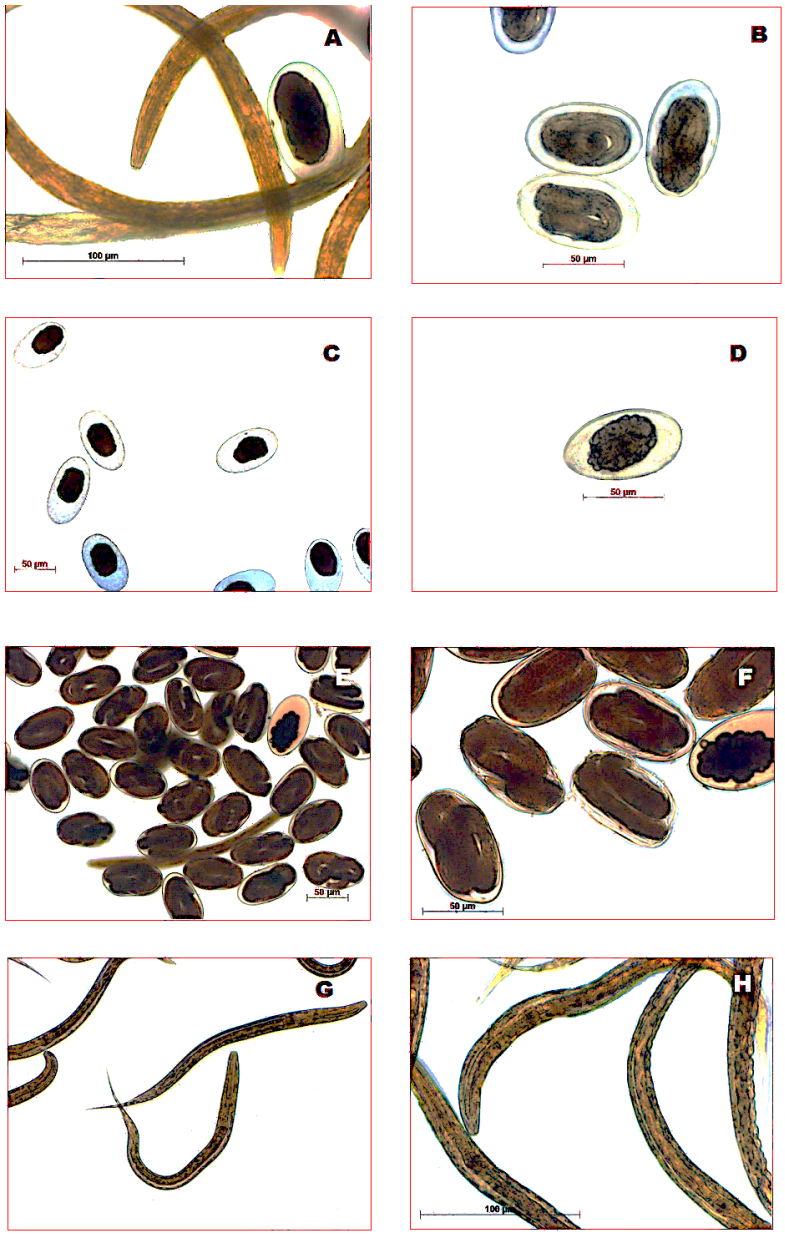

Figure 3 – The general appearance of nematode eggs and/or larvae from the Egg Hatching Test groups after 24 h of treatment with of the aqueous (EHW-I) and hydroalcoholic (EOH-I) extracts obtained from the inflorescences of *Musa paradisiaca*, Albendazole sulfoxide (positive control) and H_2O (negative control). Images: A: Group test with EHW-I at 200 µg/mL (40x magnification), B: Group test with EHW-I at 600 µg/mL (40x magnification), C: Group test with EHW-I at 1000 µg/mL (40x magnification), D: Group test with EOH-I at 1000 µg/mL (20x magnification), E: Group test with albendazole sulfoxide (positive control) (60 µg/mL) (20x magnification), F: Group test with albendazole sulfoxide (positive control) (60 µg/mL) (40x magnification), G: Group test with H_2O (negative control) (20x magnification), H: Group test with H_2O (negative control) (40x magnification).

Larval migration test

EHW-I and EOH-I did not affect L3 motility at the concentrations tested (Fig. 4). The percentages of L3 migration calculated for the tested extracts were similar to those calculated for the negative control (H_2O). As expected, in the negative control, 92.27 % of the larvae migrated (Fig. 4). However, the same observation was made for the positive control group (Ivermectin, 100 µg/mL), where the migration of approximately 82 % of the larvae occurred. In theory, this would not be expected for ivermectin, since its mechanism of action is related to a change in the motility of larvae, by acting as an agonist of the neurotransmitter, GABA, in the nervous system cells and of ligands of the glutamate-chlorine channels in neuron and muscle cells (Laing et al., 2017). The results suggested that the parasitic population was resistant to the drug at the concentration tested (Campos et al., 2017). Several authors have reported the current drug resistance situation, relating to the macrocyclic lactone family of anthelmintics worldwide (Gaudin et al., 2016).

The hydroalcoholic extract of *M. paradisiaca* bracts presented 67.56% of inhibition of larval migration at 5 mg/mL against bovine nematodes, but no effect was found against sheep nematodes (Kakimori et al., 2019). The extracts of the leaves and stems of *M. paradisiaca*, with low tannin content, also showed no significant effect on the migration of L3 from *H. contortus*, exhibiting activity similar to the negative control. However, these fractions could suppress the development of L3 of *H. contortus* from L1 to L3, in a dose-dependent manner (300 and 2400 µg/mL) (Marie-Magdeleine et al., 2014).

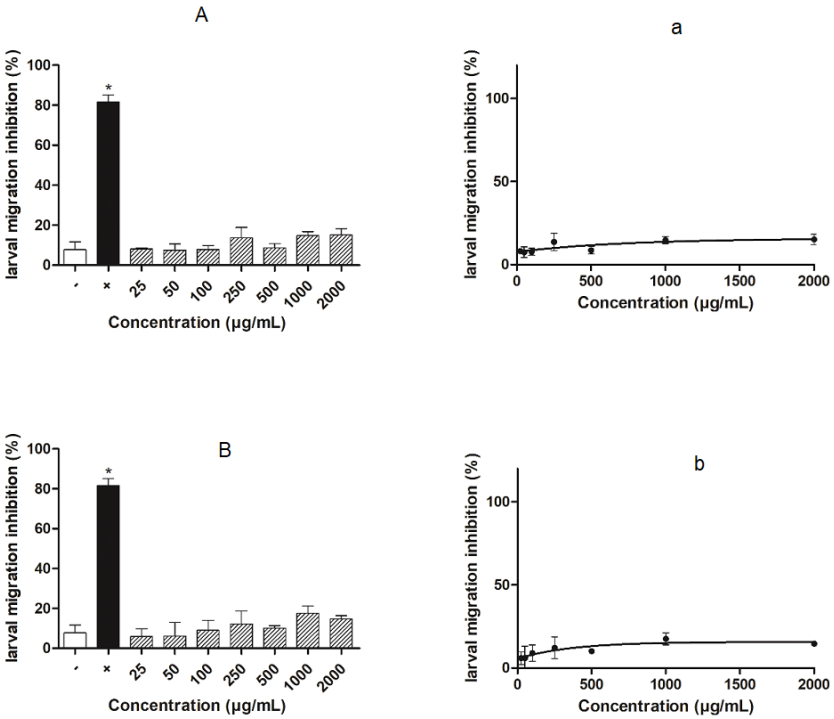

Figure 4 – Larval migration inhibition and the inhibition concentration (IC) determination of the aqueous (EHW-I) (A, a) and hydroalcoholic (EOH-I) (B, b) extracts obtained from the inflorescences of Musa paradisiaca on the larval migration test.
(-) H2O; (+) Ivermectin (100 µg/mL). Different letters in each column represent a significant difference compared to the negative control (H2O) determined using Tukey's test ($p \leq 0.05$).

The results presented in this study indicate that extracts obtained from the inflorescences of *Musa paradisiaca* L. show promising potential for further exploration as plant-derived anthelmintic agents. Although the activity was evaluated through *in vitro* assays, these approaches represent an important first step in the investigation of new bioactive products. Despite their inherent methodological limitations, *in vitro* assays offer several advantages, as they provide rapid, reproducible, and cost-effective tools for the initial screening and validation of plant extracts, allowing the identification of promising candidates for subsequent *in vivo* studies. The *in vitro* assays allow the preliminary identification of promising extracts and enable the evaluation of biological activity under controlled laboratory conditions (Pavela & Benelli, 2016). In recent years, *in vitro* studies investigating plant-derived products against *Haemonchus contortus* have increased considerably, reinforcing the interest in alternative strategies for controlling gastrointestinal nematodes in livestock (Molento et al., 2020). Several studies have shown that plant extracts can affect different developmental stages of the parasite, including egg hatching and larval viability (Molento et al., 2020; Davuluri et al., 2020; Rojas-Morales et al., 2021; Chishti et al., 2024). Among the available methods, the Egg Hatch Test (EHT) is widely considered the standard approach for initial screening, while the Larval Migration Test (LMT) evaluates larval motility and viability. These assays are particularly relevant for medicinal plants, whose activity often results from the combined effects of compounds within the phytochemical biocomplex (Athanasiadou et al., 2007). In the present study, plant extracts were chemically standardized to obtain reliable and reproducible data for future investigations by using other biological approaches.

4. Conclusion

It has been demonstrated that the ovicidal activity of extracts with low CT content. This is related to the presence of phenolic acids, whereas the reported larvicidal activity is associated with tannin content, which appears to be a relevant factor. Additionally, the glycosylated flavonoids did not affect egg hatching. However, it is interesting to note that using different models of anthelmintic activity, such as migration and development of L3 and adult larvae, may provide a better understanding of the effect of isolated plant compounds. Therefore, parts of the plant may act in different sites in the parasite stages. The inflorescences of *Musa paradisiaca* L. represent a promising banana by-product for the development of plant-derived anthelmintic agents, particularly when supported using chemically characterized extracts associated with active phytochemicals.

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