



In vitro Anthelmintic Activity of *Euphorbia hirta* L Aqueous Extracts on Small Ruminant's Gastrointestinal Parasites Evaluation

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Abstract

Purpose: With the aim of proposing an effective and accessible alternative for small ruminant gastrointestinal parasitosis control, the *in vitro* anthelmintic activity of *Euphorbia hirta* on *Haemonchus contortus* biology was evaluated.

Methods: Aqueous extracts were used to determine phytochemistry and for the biological tests of adult worm mortality as well as egg hatching and L3 larval migration inhibition. Aqueous extract concentrations of 6.25, 12.5, 25, 50 and 100 mg/mL were used for the adult worm mortality test, while concentrations of 0.31, 0.62, 1.25, 2.5 and 5 mg/mL were used for the egg hatching and L3 larval migration inhibition tests.

Results: The results show a total polyphenol content of 17.5 mgEAG/100 mg, total flavonoids of 0.24 mgEQ/100 mg and condensed tannins of 0.17 mgEAT/100 mg. Adult worm mortality was concentration dependent, reaching 100% at 50 and 100 mg/mL concentrations, from the sixth hour, with a lethal concentration 50 (LC₅₀) of 21.09 mg/mL. The 50% inhibitory concentration of egg hatching (IC₅₀) was 1.7 mg/mL, while the IC₅₀ of larval migration recorded was 0.78 mg/mL.

Conclusion: In view of the results obtained, the *in vitro* anthelmintic activity of the aqueous extract of *E. hirta* was confirmed. However, further biological studies will be needed to validate these results, with a view to their wider use.

Keywords: Anthelmintic plant; *Euphorbia hirta*; Gastrointestinal nematodes; *Haemonchus contortus*; *In vitro* tests

Introduction

Gastrointestinal parasites remain one of the major constraints to small ruminant farming development. These parasitic worms affecting pasture-raised animals cause countless economic losses for livestock farmers [1]. Several gastrointestinal parasites species are known, but the most widespread and dangerous

remains the species *Haemonchus contortus*. Indeed, *H. contortus* is a hematophagous parasite that adapts to several types of climate and causes severe anemia that can lead to small ruminant death [2].

Management of these parasites is usually based on the use of anthelmintic molecules. However, the massive and sometimes inappropriate use of these chemical molecules has led to the emergence of resistant nematode strains in many livestock farms around the world [3,4]. In addition, consumer concern over the possible presence of these chemical molecule residues in food product, and the environmental risks inherent in their use are increasingly expressed [5].

In the face of this problem, the search for alternative solutions to the use of chemotherapy is becoming essential to improve the productivity of small ruminants. Among the alternative solutions developed is the use of bioactive plants. Indeed, certain herbaceous plant species, such as *Euphorbia hirta* L, could provide a sustainable solution for effective control that is easily accessible to livestock farmers.

E. hirta is an herbaceous plant commonly known in the African local language as "Daba dablé" in Bamankan, Dioula;

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“Wallé-bisum” in Mooré; and “Dabbirteeki” in Fulani, belonging to the Euphorbiaceae family. Many studies have demonstrated the chemical and pharmacological properties of *E. hirta*. This herbaceous plant possesses several chemical compounds such as alkanes, terpene compounds, tannins, polyphenols, organic acids, and flavonoids [6,7].

Additionally, *E. hirta* is traditionally used to treat respiratory conditions and has multiple pharmacological properties, including antidiarrheal, antimicrobial, anti-inflammatory, antiviral, and antifungal properties [8]. Thus, the particularity of this plant due to its chemical and medicinal properties and especially its ease of access has led our research team to take a particular interest in considering a better use of these properties in the treatment of small ruminant gastrointestinal parasitosis.

This study aimed to evaluate the *in vitro* anthelmintic activity of *E. hirta* aqueous extracts.

Materials and Methods

Plant material

Whole plant samples of *E. hirta* were collected early in the morning in the urban communities of Dori and Ouagadougou between the end of September and the middle of October. Plant samples were washed with water, then dried to the ambient temperature away from sunlight and dust. Samples were ground into powder after drying. The plant species were identified by a botanist, and voucher specimen number 8760 was preserved at the Herbarium of the National Center of Technological and Scientific Research (CNRST) in Burkina Faso.

Animal material

Adult worms, infesting L3 larvae and eggs of *H. contortus* were used as animal material for this study.

Methods

Preparation of aqueous extraction: One hundred grams (100 g) of *E. hirta* whole plant powder was added to 1 L of distilled water for 24 hours. The macerate was filtered three (3) times and freeze-dried (marque ALPHA 1-2 LD).

Phytochemical studies

Total polyphenol content: The method described by Meda NTR, et al. [9], was used to determine the total polyphenol content. One hundred and twenty-five microliters (125 μ L) of plant aqueous extract (at 0.1 mg/mL) was mixed with 625 μ L of Folin-Ciocalteu reagent (0.2 N). After 5 min incubation in the dark, 500 μ L sodium carbonate (Na₂CO₃, 75 g/L) was added to the mixture. The resulting solution was incubated for an additional 2 h in the dark before determination of the total polyphenol content at 760 nm against a gallic acid calibration curve ($y = 4668e-3 * x - 0.034$, $r^2 = 0.9991$). Each test was repeated 3 times, and the results were expressed as mg gallic acid equivalent per 100 mg extract (mg GAE/100 mg extract).

Total flavonoid content

Six hundred and twenty-five microliters of methanolic solution of each extract (at 0.1 mg/mL) was mixed with 625 μ L of aluminum trichloride (AlCl₃, 2%). After 10 min of incubation in the dark, the flavonoid content was determined at 415 nm, using a quercetin calibration curve ($Y = 1.259e-2 * x$; $r^2 = 0.9990$). Each test was repeated 3 times and the results were expressed in milligram of quercetin equivalent per 100 mg of extract (mg EQ/100 mg of extract) [9].

Condensed tannin content

A sulfuric vanillin solution was prepared by dissolving 1 g of vanillin in 100 mL of sulfuric acid (70%).

Half a milliliter (0.5 mL) of plant sample diluted at 1/100 in ethanol was then mixed with 1 mL of the sulfuric vanillin solution. After 15 min of mixture incubation in the dark in 30°C water bath, the condensed tannin content of the extracts was measured at 500 nm against a tannic acid calibration curve. The tests were repeated 3 times, and the results were expressed in milligrams tannic acid equivalent per gram of extract (mg ATE/g extract) [10].

Biological tests

Adult worm mortality tests: The test was performed according to the modified method of Akouedegni CG, et al. [11].

H. contortus adult worms were collected with forceps from freshly slaughtered sheep, incised longitudinally and emptied of their contents. The harvested worms were placed in a Petri dish (80 x 15 cm) containing a physiological solution: phosphate-buffered saline (PBS 1x).

Aqueous extract concentrations were prepared by diluting 1 g of extract in 10 mL of PBS 1x to obtain a concentration of 100 mg/mL. This was followed by cascade dilution to produce 4 other aqueous extract concentrations: 50 mg/mL, 25 mg/mL, 12.5 mg/mL and 6.25 mg/mL. A negative control, PBS 1x, and a positive control, levamisole 2.5 mg/mL, were used.

The test consisted of contacting five perennial adult worms of both sexes with 1.5 mL of each *E. hirta* extract concentration test in 24-well culture microplates. After the worms were placed in contact with the extract concentrations, the whole set was incubated for 20 h at 27° C. Observations were made at 1 h, 2 h, 4 h, 6 h and 20 h. The number of dead adult worms was assessed 20 hours later. When a worm remains in continuous immobility for 30 seconds, with no return of vitality 30 minutes after immersion in PBS, it is declared dead.

The Mortality Rate (MR %) was calculated using the following formula:

$$MR (\%) = (DAWN / IAWN) * 100$$

DAWN: Adult Worm Number of Death

IAWN: Incubated Adult Worm Number.



Egg hatching inhibition tests

Eggs were obtained using the modified method of Hussain A, et al. [12].

The females were sorted and lightly crushed in a mortar using a porcelain pestle to obtain the eggs. The crushed eggs were filtered through sieves of decreasing mesh size (100, 50 and 38 μm) and the egg solution was readjusted to 100 eggs per mL.

Five aqueous extract concentrations were prepared. Zero point one (0.1) gram extracts were diluted in 10 mL of PBS 1x to obtain a stock solution of 10 mg/mL. Next, a cascade dilution was performed to obtain five other concentrations to be used for testing: 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 0.62 mg/mL, 0.31 mg/mL. A negative control, PBS 1x, and a positive control, levamisole 2.5 mg/mL, were used.

The test was carried out according to the modified method of Coles GC, et al. [13]. One milliliter of egg suspension at 100 eggs per mL was placed in contact with 1 mL of each of the test concentrations in Petri dishes (60 x 15 cm) and incubated for 48 hours at 27 °C. After 48 hours, two drops of Lugol's solution were placed in each Petri dish to halt egg development. Next, 40 μL were placed between the slide and coverslip, and the number of L1 larvae and unhatched eggs were assessed under a light microscope (10x). The test was repeated three times, with three replicates of each concentration in each run. The Percentage inhibition of egg hatching (EHI %) was calculated using the following formula:

$$\text{EHI (\%)} = (1 - \text{EHTC}/\text{EHNC}) \times 100$$

EHTC: Number of eggs hatched in the tested concentration

EHNC: Number of eggs hatched in the negative control.

L3 larval migration inhibition tests

L3 larvae were obtained using the modified method of Olounladé PA, et al. [14] after culturing fresh eggs of *H. contortus* for 14 days at 31°C and then harvested using the Baermann device based on the positive hygrotopism of the larvae. The larval solution was readjusted to 1000 larvae/mL.

The test was carried out using the modified method of Hernandez-Villegas MM, et al. [15]. Five concentrations of aqueous extracts were made as previously described for the egg hatch inhibition test. A negative control, PBS1x, and a positive control, levamisole 2.5 mg/mL, were used.

One milliliter of larvae solution at 1000 larvae/mL was placed in contact with 4 mL of each test concentration in Petri dishes (60 x 15 cm) for 3 hours at 27° C. After 3 hours, the larvae were rinsed by centrifugation at 2000 rpm for 10 minutes and then allowed to migrate through a 20 μm diameter membrane for 3 hours. The number of migrated larvae was assessed under a light microscope, and the percentage inhibition of larval migration (IML %) was calculated using the modified formula of Rabel B, et al. [16]:

$$\text{IML (\%)} = (X1 - X2/X1) * 100$$

X1: Number of larvae that migrated in the negative control

X2: Number of larvae that migrated into the test concentration.

Data analysis

Excel software 2016 was used for data entry and calculation of means, standard deviations and percentages. Chemical composition data were expressed as mean \pm standard deviation. The data collected for each test were subjected to a one-factor analysis of variance (ANOVA 1) followed by multiple comparison of means using Tukey's method at the 5% significance level using R software version 4.2.1, the Rcmdr version 2.8-0 package and the R studio version 4.2.1 interface.

Prism software version 5.00.288 was used to produce the graphic and calculate the lethal and inhibitory concentrations 50.

Results

Phenolic compound content

E. hirta aqueous extract contains a wide range of phenolic compounds. Total polyphenols (TP) are well represented in the aqueous extracts, while total flavonoids (TF) and condensed tannins (CT) are present at slightly lower levels (Table 1).

Adult worm mortality assay

A high and significant mortality ($p < 0.05$) compared to the negative control was obtained with the aqueous extracts. However, the difference was not significant ($P > 0.05$) compared with the positive control. A mortality rate of 100% was noted at a concentration of 50 mg/mL from the sixth hour (Table 2).

Egg hatching inhibition assay

Aqueous extracts of *E. hirta* produced a high and significant ($p < 0.05$) egg hatching inhibition compared with negative controls. A hatching inhibition rate of 76.67% at 5 mg/mL was recorded (Table 3).

Inhibition of L3 larval migration assay

A high and significant ($p < 0.05$) L3 larval migration inhibition was obtained. *E. hirta* aqueous extract showed concentration dependent migration inhibition that reached 94% at a concentration of 5 mg/mL (Figure 1).

Discussion

Parasitic diseases caused by gastrointestinal nematodes severely limit sheep production. The emergence of parasite strains resistant to the synthetic anthelmintics usually used to treat these parasites means that we need to find alternative, sustainable solutions to improve the productivity of sheep farms.

Quantification of the secondary metabolites responsible for the medicinal plant properties in aqueous extracts of *E. hirta* shows a wide range of levels of total polyphenols, total flavonoids and condensed tannins. In contrast to our present



Table 1: Phenolic compound content of *E. hirta* aqueous extracts.

Extract	Total Polyphenols (mgEAG/100 mg)	Total Flavonoids (mgEQ/100 mg)	Condensed Tannins (mgEAT/100 mg)
Aqueous extract	17.5 ± 0.49	0.24 ± 00	0.17 ± 0.01

Table 2: Effect of *E. hirta* aqueous extracts on *H. contortus* adult worms.

Dose	Mortality Rate (%)				
	1 h	2 h	4 h	6 h	20 h
PBS	0 ^a ± 0	0 ^a ± 0	0 ^a ± 0	0 ^a ± 0	0 ^a ± 0
6.25 mg/mL	0 ^b ± 0	0 ^a ± 0	0 ^a ± 0	4.44 ^a ± 3.84	21.45 ^b ± 0
12.5 mg/mL	0 ^{ab} ± 0	0 ^a ± 0	18.88 ^b ± 6.77	26.11 ^b ± 7.69	46 ^c ± 0
25 mg/mL	0 ^b ± 0	11.11 ^b ± 3.87	28.88 ^c ± 3.84	42.44 ^c ± 0	61.33 ^d ± 0
50 mg/mL	0 ^{ab} ± 0	57.77 ^d ± 3.84	77 ^d ± 0	100 ^d ± 0	100 ^e ± 0
100 mg/mL	48.88 ^d ± 0	80 ^e ± 6.66	100 ^e ± 0	100 ^d ± 0	100 ^e ± 0
Levamisole (2.5 mg/mL)	20 ^c ± 0	40 ^c ± 0	76.66 ^d ± 5.77	100 ^d ± 0	100 ^e ± 0

p-value = 0.00212

a, b, c, d, e: Difference between columns, p-value : Comparison between concentration.

Table 3: Effect of *E. hirta* aqueous extracts on *H. contortus* hatching eggs.

Dose	Egg Hatch Inhibition Rate (%)			
	R1	R2	R3	Mean ± SD
PBS	0 ^a ± 0	0 ^a ± 0	0 ^a ± 0	0 ^a ± 0
0.31 mg/mL	4 ^b ± 1	3 ^b ± 1	3 ^b ± 1	3,33 ^b ± 0,58
0.62 mg/mL	30 ^c ± 6,66	10 ^c ± 3,29	20 ^c ± 5	20 ^c ± 4
1.25 mg/mL	50 ^d ± 10	50 ^d ± 14,06	30 ^d ± 8,03	43,33 ^d ± 4,54
2.5 mg/mL	70 ^e ± 4,81	60 ^e ± 12,05	50 ^e ± 6,67	60 ^e ± 10
5 mg/mL	80 ^f ± 17,34	80 ^f ± 19,25	70 ^f ± 12,79	76,67 ^f ± 5,77
Levamisole 2.5 mg/mL	80 ^f ± 19,24	80 ^f ± 19,25	70 ^f ± 4,81	76,67 ^f ± 5,77

p-value= 0.003411

a, b, c, d, e, f: Difference between columns, p-value : Comparison between concentration

study, numerous studies have qualitatively shown the presence of these secondary metabolites within different plant extracts [17,18]. Phytochemical screening studies carried out in Niger, Senegal and Côte d'Ivoire showed the presence of these various secondary metabolites in various *E. hirta* extracts, similar to our present study [19-21].

The vermicial activity of *E. hirta* aqueous extracts was high and significant compared with PBS taken as a negative control. Dose-dependent vermicial activity was observed, with total mortality of *H. contortus* adult worms at the 6th hour, starting at a concentration of 50 mg/mL. In contrast to our present study, *Hedera helix* aqueous extracts obtained a lower mortality rate of *H. contortus* adult worms [22]. The difference in results found may be due to the content of secondary metabolites in the plants, as well as the nature of the plants and the packaging used. However, aqueous extracts of *Artemisia absinthium* obtained a high mortality rate of *Haemonchus contortus* adult worms at the 8th hour after exposure to 25 mg/mL concentration, similar to our present study [23]. The similarity of the results obtained is inherent in the anthelmintic activity. The presence of secondary metabolites in *E. hirta* aqueous extracts would explain its vermicial activity. Indeed, Githiori JB, et al. [3] report that the presence of secondary metabolites such as condensed tannins and polyphenols are responsible for the plants' anthelmintic properties.

The ovicidal activity of *E. hirta* aqueous extracts was high compared with PBS taken as a negative control. Costa CTC, et al. [24] obtained similar results to our present study, with high inhibition of *H. contortus* egg hatching using extracts from *Azadirachta indica* leaves in Brazil. Different *Phytolacca icosandra* extracts produced high *H. contortus* egg hatching inhibition

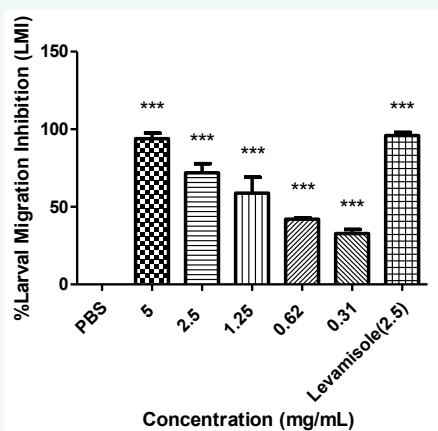


Figure 1 Effects of *E. hirta* aqueous extracts on the L3 larvae migration of *H. contortus*.

*** : significant difference compared to negative control



in a dose-dependent manner identical to the results of our present study [15]. Similarly, the *Melia azedarach* aqueous and hydroalcoholic leaf extracts significantly inhibited the hatching of *H. contortus* eggs, corroborating the results of our study [25]. The ovicidal activity similarity observed is due to the presence of secondary metabolites in the different extracts tested, as emphasized by many authors [26].

The L3 larval migration inhibition by *E. hirta* aqueous extracts was high and significant compared with that of the negative control. This result shows that *E. hirta* aqueous extracts could prevent or reduce the settlement of infective larvae in the mucosal wall of the digestive tract. Our results are identical to those obtained using *Phytolacca icosandra* extracts with high inhibition of *H. contortus* L3 larvae migration [15]. Additionally, the hydroalcoholic grape pomace fractions resulted in total *H. contortus* L3 larval migration inhibition [27]. The different extracts used all possess secondary metabolites that would be at the origin of the anthelmintic activity of plants, which would explain the similarity of the results obtained.

The anthelmintic activity of *E. hirta* aqueous extracts is related to the presence of total polyphenols, total flavonoids and condensed tannins, as emphasized by some authors [28]. These secondary metabolites act specifically on egg cell membranes and larvae cuticle collagen proteins to modify membrane permeability and reduce membrane cholesterol levels. These modifications will allow the passage of these metabolites inside eggs and larvae, inhibiting blastomere segmentation in the case of eggs damaging the cuticle and digestive system of larvae or binding to glycoproteins on the nematode cuticle, leading to worm death [29-31]. Further studies will enable us to validate the results obtained *in vitro* and confirm the *E. hirta* anthelmintic activity.

Conclusion

The present study demonstrates the *in vitro* anthelmintic efficacy of *E. hirta* aqueous extracts. Phytochemical assays indicate the presence, at variable levels, of the secondary metabolites responsible for the plants' anthelmintic activity in the aqueous extracts tested. Biological tests show high adult worm mortality, as well as strong inhibition of egg hatching and L3 larval migration of *H. contortus*. These results confirm some of the data in the literature attributing anthelmintic activity to *E. hirta*, justifying its use in traditional veterinary medicine.

These results are promising, but it would be essential to carry out additional biological tests, in particular safety tests, as well as *in vivo* station tests, to validate the results obtained in this study.

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Authors' contributions

Amadou Dicko, Almamy Konaté, Sami Eric Kam, Hadidjatou Belem, Basile Tindano; Abdoul Aziz Almoustapha Cissé: Have contributed to the realization of the biological tests in laboratory. Collected and analyzed data. Writing of the article.

Gaoussou Keïta; Roland Nâg-Tiéro Meda; Adama Kaboré, Amadou Traoré, Balé Bayala, Hamidou Hamadou Tamboura: Have contributed to the realization of the biological tests by validating the protocols. Followed the work in the laboratory. Amendment and validation of the article. All authors reviewed the manuscript.

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Conflict of interests

Authors declare that there is no conflict of interest regarding these results.

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