Comparison of the \textit{in vitro} anthelmintic effects of \textit{Acacia nilotica} and \textit{Acacia raddiana}

Geneviève Zabré\textsuperscript{a,b}, Adama Kabore\textsuperscript{a,*}, Balé Bayala\textsuperscript{b}, Luciana M. Katiki\textsuperscript{c}, Lívio Martins Costa-Júnior\textsuperscript{d}, Hamidou H. Tamboura\textsuperscript{a}, Adrien M.G. Belem\textsuperscript{e}, Adibe L. Abdalla\textsuperscript{f}, Vincent Niderkorn\textsuperscript{e}, Hervé Hoste\textsuperscript{b,*}, and Helder Louvandini\textsuperscript{f}

\begin{itemize}
  \item \textsuperscript{a} Laboratoire de Biologie et Santé Animaux-DPA/INERA, 04 BP 8645 Ouagadougou 04, Burkina Faso
  \item \textsuperscript{b} Université de Ouagadougou / UFR-SVT, 03 BP 7021 Ouagadougou 03, Burkina Faso
  \item \textsuperscript{c} Instituto de Zootecnia (SAA, APTA), Rua Heitor Penteado 56, Nova Odessa, SP, cep 13469-000, Brazil
  \item \textsuperscript{d} Universidade Federal do Maranhão – UFMA, Campus do Bacanga CEP 65080-805 São Luis- MA, Brazil
  \item \textsuperscript{e} Université Nazi Boni, Bobo-Dioulasso, 01 BP 3770 Ouagadougou 01, Burkina Faso
  \item \textsuperscript{f} Universidade de São Paulo, Centro de Energia Nuclear na Agricultura, NAPTSA, CP 96, CEP 13.400-970, Piracicaba, SP, Brazil
  \item \textsuperscript{g} UMR1213 Herbivores, INRA – Clermont-Ferrand, Vetagro Sup, 63122 Saint-Genès-Champanelle, France
  \item \textsuperscript{h} UMR IHAP 1225 INRA/ENVT, 23 Chemin des Capelles, 31076 Toulouse Cedex, France
\end{itemize}

Received 7 May 2017, Accepted 20 October 2017, Published online 27 November 2017

\textbf{Abstract} – Gastrointestinal nematodes are a major threat to small ruminant rearing in the Sahelian area, where farmers traditionally use bioactive plants to control these worms, including \textit{Acacia nilotica} and \textit{Acacia raddiana}. The main aim of this study was to screen the potential anthelmintic properties of aqueous and acetone extracts of leaves of these two plants based on three \textit{in vitro} assays: (1) the egg hatch inhibition assay (EHA); (2) the larvae exsheathment inhibition assay (LEIA) using \textit{Haemonchus contortus} as a model; and (3) an adult mortality test (AMT) applied on \textit{Caenorhabditis elegans}. For the EHA, only \textit{A. raddiana} was effective with IC\textsubscript{50} = 1.58 mg/mL for aqueous extract, and IC\textsubscript{50} = 0.58 mg/mL for acetic extract. For the LEIA, all extracts inhibited the exsheathment of larvae compared to the controls, and the aqueous extract of \textit{A. nilotica} was more larvicidal with IC\textsubscript{50} = 0.195 mg/mL. In general, all responses to the substances were dose-dependent and were significantly different from the control group (\(p<0.05\)). For the AMT, the extracts of the two \textit{Acacia} species were effective but \textit{A. raddiana} showed greater efficacy with 100% mortality at 2.5 mg/mL and LC\textsubscript{50} = 0.84 mg/mL (acetic extract). The addition of polyvinyl polypyrrolidone (PVPP) to the extracts suggested that tannins were responsible for blocking egg eclosion and inducing adult mortality but were not responsible for exsheathment inhibition. These results suggest that the leaves of these \textit{Acacia} species possess ovicidal and larvicidal activities \textit{in vitro} against \textit{H. contortus}, and adulticidal effects against \textit{C. elegans}.

\textbf{Keywords:} Natural products, tannins, secondary plant metabolites, nematodes, anthelmintics

\textbf{Résumé – Comparaison des effets anthelminthiques \textit{in vitro} d’\textit{Acacia nilotica} et \textit{Acacia raddiana}.} Les nématodes gastro-intestinaux demeurent une menace majeure de l’éleveage des petits ruminants en zone sahélienne, où les éleveurs utilisent traditionnellement des plantes bioactives pour maîtriser ces parasites, notamment \textit{Acacia nilotica} et \textit{Acacia raddiana}. L’objectif de ce travail était de vérifier l’efficacité anthelmintique d’extraits aqueux et acétoniques de feuilles de ces deux plantes sur la base de 3 tests \textit{in vitro} : 1) l’inhibition de l’éclosion des œufs (EHA), 2) l’inhibition du dégainement des larves (LEA) appliquée au modèle \textit{Haemonchus contortus} et 3) la mortalité des \textit{Caenorhabditis elegans} adultes (AMT). Pour l’EHA, seul \textit{A. raddiana} a été efficace avec IC\textsubscript{50} = 1.58 mg/mL pour l’extrait aqueux et IC\textsubscript{50} = 0.58 mg/mL pour l’extrait acétonique. Pour l’LEA, tous les extraits ont inhibé le dégainement des larves par rapport aux témoins et l’extrait aqueux d’\textit{A. nilotica} a été plus larvicidal avec un IC\textsubscript{50} = 0.195 mg/mL. De façon générale, toutes les concentrations agissent de façon dose-dépendante et sont significativement différentes du groupe témoin (\(P<0.05\)). Pour l’AMT, les extraits des deux \textit{Acacia} ont été efficaces mais \textit{A. raddiana} a été plus efficace avec 100% de mortalité à 2.5 mg/mL avec une DL\textsubscript{50} = 0.84 mg/mL (extrait acétonique). L’addition de

\textsuperscript{*Corresponding author: adel\_bf@yahoo.fr

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polyvinylpolypyrrolidone (PVPP) aux extraits a montré que les tannins seraient responsables de l’hibition de l’élosion des œufs et de la mortalité chez les vers adultes, mais ne seraient pas responsables de l’hibition du dégénèrancement des larves. Ces résultats suggèrent que les feuilles de ces deux Acacia possèdent des activités ovicidés et larvicidés in vitro contre H. contortus et des effets sur les C. elegans adultes.

Introduction

Small ruminant rearing contributes strongly to human livelihoods in developing countries, especially for rural farmers [65]. In Sahelian countries, such as Burkina Faso, Mali and Niger, livestock play an important role in the economy [5]. In Burkina Faso, the livestock sector alone contributes over USD 51.3 million annually to the national economy, of which 32% is from small ruminants [49]. Despite the importance of this sector, small ruminant rearing faces feeding and health constraints that limit animal production. Among the health constraints, gastrointestinal nematodes (GINs), and Haemonchus contortus in particular (a highly prevalent and pathogenic species of GIN [15,56]), lead to low performances and higher animal mortality [35,58,67]. Like in many developing countries, the majority of livestock owners in rural areas do not use synthetic anthelmintic (AH) drugs because they are not affordable [31,55]. Therefore, they usually rely on practices related to ethnoveterinary medicine to control health problems in livestock [26]. In the Sahel, many natural fodder plants such as different species of Acacia, are used to control disease in ruminants, especially GINs. The use of such bioactive plants has several advantages for local farmers: (a) they are locally available; (b) they cost less compared to synthetic AH drugs; and (c) they are well accepted and consumed by sheep and goats.

An ethnoveterinary survey performed in this area of the Sahel showed that among Acacia species targeted for ruminants, *Acacia nilotica* var *adansonii* (Guill. & Perr.) O. Ktze and *Acacia raddiana* (Savi) are commonly used because of their high nutritional value and their potential AH effects [70]. All of the breeders investigated used *A. raddiana* essentially as feed for cattle. However, 57% and 43% of the breeders questioned the use of *A. nilotica* to treat and to feed the cattle, respectively. For example, decoctions prepared from the pods and dried leaves of *A. nilotica* are used to treat foot-and-mouth disease (98%), diarrhoea (73%), leg ulcers (51%), loss of appetite (36%) and gingivitis (32%) in small ruminants [70]. In Mauritania, the powdered seed of *A. nilotica* macerated in fresh water is used to treat diarrhoea. The fruit, leaves and bark extracts of *A. nilotica* have been examined in *in vitro* studies [11,12,34,45] and their effects confirmed in *in vivo* studies [11,12,46]. However, information on *A. raddiana* is sparse.

Both *A. nilotica* and *A. raddiana* species are assumed to contain tannins [61]. Many *in vitro* and *in vivo* studies have now provided consistent evidence to support the AH effect of feed containing tannins and other polyphenols against abomasal and intestinal parasitic nematodes [27,29]. These previous investigations have shown that some local plants that contain tannins can impair different key biological processes of the parasitic nematode life cycle: (i) the establishment of the infective third-stage larvae [18,20,47]; (ii) the excretion of eggs by adult worms [41,47,66] and (iii) the development of nematode eggs into larvae [51], and can therefore be used as nutraceuticals [30].

The objectives of this study were therefore two-fold: (1) to evaluate, based on *in vitro* assays, the anthelmintic effects of both aqueous and acetonic extracts of the two *Acacia* plants on two different development stages of *H. contortus* and on adult nematode mortality of members of *Caenorhabditis elegans*; (2) to confirm the possible role of tannins and related polyphenols on *in vivo* AH effects using the tannin inhibitor polyvinyl polypyrrolidone (PVPP).

Materials and methods

Plant materials

Fresh leaves of *A. nilotica* and *A. raddiana* were collected in December 2014 at Dori (14° and 15° N; 0° and 3° W), located in north-eastern Burkina Faso. The climate of the region, classified as Sahelian, is marked by a long dry season from November to June and a short rainy season from July to September. The specimens were identified by reference to the herbarium of the French National Centre of Scientific and Technological Research in Ouagadougou, Burkina Faso. Harvested leaves were then cleaned with water and dried at room temperature for a week according to the procedure used by traditional healers in the country.

Dried leaves were first transformed into powders using a blade grinder (basic IKA A 11) and the water content was determined according to the Association of Analytical Communities (AOAC) [6]. Two grams of each powder were macerated with 750 mL of distilled water for 24 h. The specimens were identified by reference to the herbarium of the French National Centre of Scientific and Technological Research in Ouagadougou, Burkina Faso. Harvested leaves were then cleaned with water and dried at room temperature for a week according to the procedure used by traditional healers in the country.

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\[
\text{water content} = \left( \frac{(\text{powders before drying (g)} - \text{powders after drying (g)})}{\text{powders before drying (g)}} \right) \times 100.
\]

Preparation of extracts

Two extracts were prepared for each *Acacia* species: one aqueous and one water/acetonic extract. For aqueous extracts, 150 g of each powdered material were macerated with 750 mL of distilled water for 24 h. Macerated extracts were then filtered over cotton wool and concentrated under reduced pressure in a rotary evaporator at 40-50°C before being stored at 4°C. For water/acetonic extracts, 150 g of each powder were macerated with 750 mL of mixed acetone/water (70/30) (v/v) for 72 h. Macerated extracts were filtered over Whatman paper before being concentrated under reduced pressure.
in a rotary evaporator at 40-50°C and stored at 4°C. Dry extracts were subjected to phytochemical screening in order to identify the main phytochemical groups.

Quantification of tannins and polyphenolic compounds

Total tannins (TT), total phenols (TP) and condensed tannins (CT) were determined according to Makkar [39] and Makkar et al. [38]. TP and TT were determined by adding 250 μL of Folin-Ciocalteu reagents (1N) and 1.25 mL sodium carbonate solution (20% Na₂CO₃) to an aliquot of the supernatant and then taking absorbance readings at 725 nm. To determine TT, a binding tannin agent, 100 mg of insoluble polyvinyl polypyrrolidone (PVPP), was added to the extract and the measurement was repeated. A calibration curve was prepared from aliquots of the solution of tannic acid. The difference between measurements of TP and TP + PVPP extract readings was an estimate of TT. The concentrations of TP and TT were calculated as tannic acid equivalents (eq) and expressed as g/kg DM. CT were expressed as leucocyanidin equivalents (% of DM) and were determined using 3 mL butanol-HCl reagent and 0.1 mL ferric reagent. The absorbance was read at 550 nm.

Materials for in vitro assays

The faecal matter of a donor goat experimentally infected with H. contortus was collected at the Department of Pathology at the Federal University of Maranhão (UFMA) (Brazil) to obtain the eggs and L₃ larvae of H. contortus. C. elegans adult nematodes were obtained by culture according to the method of Chitwood and Feldlaufer [22]. All procedures were approved by the Ethics Committee for Animal Experimentation of the Federal University of Maranhão under number 23115018061.

In vitro assays

Three different tests were performed: the egg hatching assay (EHA), the L₃ larval exsheathment assay (LEIA) of H. contortus, and the C. elegans adult mortality assay (AMT).

Egg hatching assay

This assay was performed according to the method described by Coles et al. [23]. The fresh faecal matter was recovered, crushed and filtered four times on 1-mm, 105-μm, 55-μm and 25-μm sieves. Eggs collected from the 25-μm sieve were centrifuged at 450 g /20°C for 10 min and the supernatant was removed and replaced by an NaCl solution (density: 1.2), mixed and centrifuged at 1012 g/20°C for 5 min. The recovered supernatant was filtered with a 25-μm sieve, washed and centrifuged three times at 450 g /20°C for 10 min. The quantity was adjusted to reach a concentration of 100 eggs/mL. Five increasing concentrations of solutions were prepared for each extract: 0.3, 0.6, 1.25, 2.5 and 5.0 mg/mL diluted in PBS (0.1 M phosphate, 0.05 M NaCl, pH 7.2) for the two aqueous extracts, and in 2% methanol for the acetonic extracts. To test the direct effect of the extracts on nematodes, 100 μL of each concentration + 100 μL of eggs were placed in each well (96-well plate). Four replicates were performed per concentration. The plates were covered with Parafilm and incubated at 27°C for 48 h. After 48 h of incubation, hatching was stopped by adding lugol iodine solution, and the number of L₁ larvae and eggs per well was counted using a reverse microscope (magnification x 10). Thereafter, the percentage of hatched eggs was determined using the following ratio: number of L₁/(number of eggs + number of L₁).

Larval exsheathment inhibition assay

The assay on larval exsheathment inhibition was performed as previously described by Bahuaud et al. [13]. Larvae were obtained by larval culture from goat faecal matter incubated at room temperature for 14 days to obtain L₁ larvae at the concentration of 2000 larvae/mL. Five increasing concentrations were prepared for each of the two extracts: 2.4, 1.2, 0.6, 0.3 and 0.15 mg/mL, all diluted in phosphate-buffered saline (PBS) (0.1 M phosphate, 0.05 M NaCl, pH 7.2) for aqueous extracts and 1% methanol for acetonic extracts. PBS and 1% methanol were used as a negative control. Briefly, 1 mL of L₁ larvae solution was incubated with 1 mL of extract at 20°C for 3 h, and then washed with PBS and centrifuged three times at 1380 g for 3 min. Then, 200 μL of the larvae was subjected to the artificial exsheathment process by adding 200 μL of a Milton solution (2% w/v sodium hypochlorite and 16.5% sodium chloride) diluted to 1/300 with PBS. Four replicates were performed per concentration. The kinetics of larval exsheathment in the different experimental treatments were then monitored at 0, 20, 40 and 60 min intervals by microscopic observations (40×).

Mortality assay of adult C. elegans

Adult C. elegans were isolated according to Katiki et al. [36]. The test was performed with young adults and adults with intact cuticles. Five concentrations were prepared for the assay: 0.6, 1.25, 2.5, 5 and 10 mg/mL and two negative controls: salt solution M-9 (1.5 g KH₂PO₄; 3 g Na₂HPO₄; 2.5 g NaCl; 0.5 mL 1M MgSO₄; final volume of 500 mL) for aqueous extracts, and 2% dimethyl sulfoxide (DMSO) for acetonic extracts used as a solvent. The test was performed using 48-well plates and a nematode stock of 50 larvae/100 μL. In each well, nematodes were placed in contact with each concentration to a final volume of 500 μL/well with four replicates. The plates were then incubated at 25°C for 20 h. After incubation, plates were read using a microscope and all adult worms were counted and determined as dead or alive, according to Skantar et al. [63]. They were considered dead when they did not show any movement and as alive when there were at least
some tails, head or pharyngeal movements (during 10 s of observation). The negative control consistently showed 95-100% live adults at 20 h after incubation.

**Verification of the role of tannins by use of PVPP**

In order to confirm the role of tannins on the AH effect, another series of EHA, LEIA and AMT was performed with both extracts of each Acacia species. However, for EHA, only A. raddiana extracts were used for this test because A. nilotica was not effective. Thus, the best concentrations of each extract (5 mg/mL + 100 mg of PVPP for EHA, 10 mg/mL + 200 mg of PVPP for AMT and 1.2 mg/mL + 250 mg of PVPP for LEIA) were pre-incubated with PVPP for 2 h, centrifuged at 1012 g / 10 min at 20°C before being used for the assay. After centrifugation, all supernatants were removed to perform the three assays according to the procedure previously described and the results compared to an assay without the addition of PVPP. The ratio of pre-incubated PVPP was 1/10 for EHA and AMT. The rate for LEIA was 1/40.

**Statistical analyses**

Data from parasite tests were recorded on Excel 2010 (Microsoft corporation) and transformed to log (x + 1) before being subjected to a variance analysis with SAS software 2010, Version 6.20.4. IC50 and LC50 were calculated by probit analysis. IC50 and LC50 were considered significantly different when the 95% LC fails to overlap [60]. Comparison of averages (expressed as mean ± standard error of mean) was performed using the Kruskal-Wallis test at 0.05.

**Results**

**Quantification of tannins and polyphenolic compounds**

Table 1 presents the content of total phenol (TP), condensed tannins (CT) and total tannins (TT) for both Acacia species. A. nilotica was rich in CT compared to A. raddiana which was rich in TT and TP.

**Egg hatch inhibition assay**

Tables 2 and 4 show the percentage of inhibition of egg hatching at different concentrations and 50% inhibition concentration for *H. contortus*. In general, the two controls recorded the lowest percentages of inhibition (4.3 and 9.8%, respectively, for PBS and 2% methanol) and were significantly different for all extracts (p < 0.05). The inhibition of egg hatching increased with increasing concentrations. However, A. nilotica was not effective and recorded a percentage of inhibition of less than 22% and an IC50>5 mg/mL. The percentage of egg inhibition of A. raddiana ranged from 12.67% to 92.76% with an IC50 = 1.58 mg/mL for the aqueous extract, and from 27.34% to 91.45% for acetonic extract with an IC50 = 0.58 mg/mL.

**Table 1.** Quantity of condensed tannin (CT), total tannin (TT) and total polyphenols (TP) in Acacia species.

| Samples       | CT     | TT     | TP     |
|---------------|--------|--------|--------|
| A. nilotica   | 53.60  | 54.23  | 76.66  |
| A. raddiana   | 3.41   | 391.56 | 401.28 |

CT: g of leucoeyandin/kg dried mass (DM) TT and TP: g of tannic acid/kg DM.

**Larval exsheathment inhibition essay**

Both extracts revealed inhibition activity on L3 larvae exsheathment of *H. contortus*. The aqueous extract of A. nilotica showed the lowest concentration of inhibition (IC50) compared to all of the extracts (Table 4).

**Mortality of adult *Caenorhabditis elegans***

Tables 3 and 4 present the efficacy of the extracts on adult *C. elegans* mortality and the lethal concentration. In general, the two controls recorded the lowest mortality rate (4.69% and 0.84%, respectively, for M-9 and 2% DMSO). The differences between concentrations and controls were highly significant (p < 0.001). Moreover, both extracts induced adult *C. elegans* mortality. When the concentration increased, the percentage of adult mortality increased only for the acetonic extract of A. raddiana, which reached 100% mortality at 2.5 mg/mL with the lowest lethal concentrations: LC50 = 0.84 mg/mL compared to the other extracts.

**Using the inhibitor PVPP to assess the role of tannins and polyphenols**

The addition of PVPP to the two extracts of each species significantly affected (p < 0.05) egg hatching of *H. contortus* (Table 5) and adult *C. elegans* mortality (Table 6). In contrast, for larval exsheathment, the results seem to indicate that incubation of extracts with PVPP did not change the exsheathment process when compared to non-treated extracts (p > 0.05) (Fig. 1).

**Discussion**

The use of tannin-rich plants as an alternative treatment to chemical anthelmintics is one approach that could reduce the development of parasite resistance [32,34,57]. The objective of this study was to evaluate and compare the *in vitro* ovicidal and larvicidal efficacy of aqueous and acetonic leaf extracts of *A. nilotica* and *A. raddiana* against *H. contortus*, because these two Acacia species are widely used by Sahelian breeders in ethnoveterinary medicine. According to the literature, many species of *Acacia* have been reported to have anthelmintic activities: the leaves of *Acacia cyanophylla* [1], *Acacia karoo* [34], *Acacia nilotica* [34,45], *Acacia pennatula* [2,3] and *Acacia polyacantha* [42], and the bark of *Acacia mangium* [54] and *Acacia mearnsii* [69].
In our studies, three in vitro assays were performed with two extracts (aqueous and acetonic extracts) at different concentrations. These types of extracts have been commonly used in many in vitro tests. Moreover, solvents and protocols used for extraction caused variations in concentrations and the classes of metabolites present in extracts [40].

The quantification of tannins revealed that *A. nilotica* was 18 times richer in CT than *A. raddiana*. However, *A. raddiana* was seven times richer in TT and five times richer in TP than *A. nilotica*. According to Hoste et al. [27], tannin-rich plants could interact with the proteins of the cuticle, oral cavity, oesophagus, cloaca and vulva of nematodes, modifying their chemical and physical prop-

Table 2. Percentages of egg hatch of *H. contortus* after 48 h incubation with *Acacia nilotica* and/or *Acacia raddiana* extract at different concentrations.

| Concentration (mg/mL) | *A. nilotica* | *A. raddiana* |
|-----------------------|---------------|---------------|
| Aqueous extract       |               |               |
| 5                     | 21.14 ± 2.34 a| 18.72 ± 3.88 a|
| 2.5                   | 15.56 ± 3.34 b| 17.23 ± 1.61 a|
| 1.25                  | 7.51 ± 1.71 c | 10.95 ± 1.24 ab|
| 0.625                 | 6.48 ± 1.97 c | 10.33 ± 1.63 ab|
| 0.3125                | 3.42 ± 1.94 d | 9.85 ± 3.42 abB|
| PBS                   | -             | 9.81 ± 9.98 ab|
| Methanol 2%           | -             | 9.81 ± 9.94 e |
| P                     | 0.0024        | 0.0067        |

- : no administered

(a,b,c,d,e) are compared means within the columns and (A,B) indicate differences within the lines (different extracts) for the two *Acacia* species for each plant. Different letters indicate significant differences (p < 0.05).

Table 3. Percentage of adult *Caenorhabditis elegans* mortality after 20 h of incubation with *Acacia nilotica* and *Acacia raddiana* extract at different concentrations.

| Concentration (mg/mL) | *A. nilotica* | *A. raddiana* |
|-----------------------|---------------|---------------|
| Aqueous extract       |               |               |
| 10                    | 94.97 ± 3.9aA | 85.89 ± 3.0 aB|
| 5                     | 87.73 ± 2.9 bA| 79.61 ± 2.2 bB|
| 2.5                   | 81.78 ± 5.2 bA| 80.32 ± 4.8 bA|
| 1.25                  | 51.42 ± 2.8 aC| 11.25 ± 1.6 aCa|
| 0.625                 | 34.31 ± 5.5 a da| 11.16 ± 1.4 aCe |
| M9                    | 4.69 ± 1.8 e | -             |
| DMSO 2%               | -             | 0.84 ± 0.8 d |
| p                     | <0.001        | <0.001        |

- : not administered.

(a,b,c,d,e) are compared means in the columns and (AB) in the lines. Different letters indicate significantly values (p < 0.05).

Table 4. Inhibitory concentrations (mg/mL) in the EHA and LEIA (mg/mL) with *Haemonchus contortus* (IC50) and lethal concentrations (mg/mL) for the AMT with *Caenorhabditis elegans* (LC50) with respective 95% confidence intervals for the different plant extracts.

| Plant          | Extract | Assay  | EHA        | LEIA        | AMT          |
|----------------|---------|--------|------------|-------------|--------------|
| *A. nilotica*  | Aqueous | >5     | 0.195 (0.180 - 0.211)a | 1.01 (0.78 - 1.23)a,b |
| Acetonic       | >5      | 0.224 (0.226 - 0.265)b,c | 5.39 (4.24 - 7.42)c |
| *A. raddiana*  | Aqueous | 1.36 (1.16-1.59)b | 0.331 (0.252 - 0.408)c | 1.43 (1.07 - 1.84)b |
| Acetonic       | 0.68 (0.51-0.87)a | 0.207 (0.173 - 0.246)a,b | 0.84 (0.79 - 0.89)a |

(a,b,c) are compared between lines (different extracts) for the two *Acacia* species for each plant. Different letters indicate significant differences.
Table 5. Inhibition of egg hatching after 48 h incubation with extracts treated or not treated with polyvinyl polypyrrolidone (PVPP) for Acacia raddiana aqueous and acetic extracts at a concentration of 5 mg/mL.

| Extracts | Control without PVPP (%) | with PVPP (%) |
|----------|---------------------------|---------------|
| aqueous  | 89.2 ± 6.5
          | 2.4 ± 2.9 |
| acetic   | 6.2 ± 1.7
          | 98.6 ± 2.7 |

(a,b): significant difference (p < 0.001) between columns.

Table 6. Adult Caenorhabditis elegans mortality percentage, after incubation for 20 h with Acacia raddiana and Acacia nilotica aqueous and acetic extracts previously treated or not with polyvinyl polypyrrolidone (PVPP).

| Extract [10 mg/mL] | Control Without PVPP (%) | with PVPP (%) |
|--------------------|---------------------------|---------------|
| A. raddiana aqueous | 0.69 ± 0.0
          | 100.0 ± b
          | 14.91 ± c |
| A. raddiana acetic  | 0.96 ± 0.0
          | 100.0 ± b
          | 13.26 ± c |
| A. nilotica aqueous | 0.69 ± 0.0
          | 94.97 ± b
          | 0.00 ± ac |
| A. nilotica acetic  | 0.96 ± 0.0
          | 85.89 ± b
          | 4.31 ± ac |

(a,b,c): significant difference (p < 0.05) between values in rows.

The anthelmintic effects of tannins may be attributed to their capacity to bind free protein available in the tubes for larval nutrition, and this reduced nutrient availability could therefore have resulted in larval starvation or decrease in gastrointestinal metabolism directly through the inhibition of oxidative phosphorylation, causing larval death [7].

Our studies conducted in vitro with A. nilotica and A. raddiana extracts showed the inhibition action on egg hatching and larval exsheathment of H. contortus and mortality of C. elegans adults. Extracts acted in a dose-dependent manner and their efficacy differed depending on the organ. This anthelmintic efficacy may be attributed to an individual or a combined effect of the bioactive compounds.

In the EHA, A. nilotica extracts were not effective compared to A. raddiana. A. nilotica inhibited 22% of egg hatching for the most effective concentration (5 mg/mL) with IC₅₀ > 5 mg/mL. However, the two extracts of A. raddiana presented a high oviductal activity (more than 90% at 5 mg/mL) with different concentrations of inhibition: IC₅₀ = 0.68 mg/mL for acetic extract and IC₅₀ = 1.36 mg/mL for aqueous extract. Aqueous extracts were more oviductal than aqueous extracts, but no significant difference was recorded between the extracts in our study. Thus, the results obtained for A. nilotica contrasted with those obtained by Eguale et al. [24] who reported significant egg hatching inhibition for aqueous extracts for the same plant. Likewise, Badar et al. [12] found that crude aqueous methanol extracts of A. nilotica bark (LC₅₀ = 0.201 mg/mL) had higher inhibitory effects compared with leaves (LC₅₀ = 0.769 mg/mL). Other results have confirmed the anthelmintic effect of different Acacia species on EHA. Thus, Oliveira [54] reported IC₅₀ = 1.35 mg/mL and 4.66 mg/mL with A. mangium extract on two H. contortus (White River: a strain resistant to ivermectin, benzimidazole and closantel; and the Juan strain: susceptible to all synthetic anthelmintics, respectively). Secondary plant metabolites (1) might bind the lipoproteins responsible for eggshell membrane permeability [59], and (2) tannins might inactivate enzymes responsible for the hatching process [43].

In the LEIA, both Acacia extracts were effective against larval exsheathment. A. nilotica revealed the lowest concentration of inhibition (IC₅₀ = 0.195 mg/mL) for the aqueous extract compared to the aqueous extract of A. raddiana (IC₅₀ = 0.331 mg/mL). However, the acetic extract of A. nilotica was numerically higher compared to A. raddiana. Moreover, all extracts at 600 and 1200 μg/mL blocked the exsheathment process. Previous in vitro results indicated that extracts of various tannin-rich woody plants inhibited the exsheathment of nematode L₃ [13]. Alonso-Díaz et al. [23] tested Acacia pennatula and A. ganameri extracts and found an inhibition of 51% at 1200 μg/mL, 93.5% at 600 μg/mL, and a total exsheathment process after 70 min at 1.2 mg/mL, respectively. The exsheathment process in trichostrongyle nematodes is a crucial step that represents the transition from the free-living to the parasitic stages.

In the AMT, the free-living soil nematode C. elegans was used to test the efficacy of the extracts. C. elegans is a system to screen products for their potential anthelmintic effect against small ruminant gastrointestinal nematodes, including H. contortus [36]. The two Acacia extracts tested were effective against adult C. elegans and their lethal concentrations were less than LC₅₀ < 1.5 mg/mL, except for the acetic extract of A. nilotica (LC₅₀ = 5.4 mg/mL). For the same concentration (2.5 mg/mL), the acetic extract of A. raddiana revealed 100% adult mortality compared to 19% for A. nilotica. However, their two aqueous extracts revealed the same percentage of mortality (80.32 and 81.78%, respectively). In general, A. raddiana was more larvicidal than A. nilotica. A. raddiana contained high total tannins (TT). Plant extracts containing hydrolysable tannins (HT) such as gallic and/or ellagic acid or containing prodelphinidin CT (with gallic acid units) had higher levels of anthelmintic activity in vitro than the proanthocyanidin CT, which lack gallic acid units [17]. According to Katiki et al. [37] plants containing both CT and HT of the gallotannin and ellagitannin types were more lethal to C. elegans than plants containing CT lacking gallic acid units.

The results with PVPP for all assays showed that both extracts of Acacia species may have variability in the roles of tannins for EHA, LEIA and AMT. For our results, the addition of PVPP in Acacia extracts was associated with a significant decrease (restoration of control values) of EHA and AMT, but, did not seem to influence LEIA.

In the AMT, tannins and the associated metabolites seemed responsible for the mortality of adult C. elegans for A. raddiana extracts compared to A. nilotica extracts. Thus, tannins would be responsible for the efficacy of the extracts for the inhibition of egg hatching, but were not the
only metabolites involved in adult mortality for *A. raddiana* extracts. These results suggest that a possible synergistic relationship of tannins and other compounds may be involved in killing the worms. For tannins, including condensed tannins, their anthelmintic activities have been reported by Athanasiadou et al. [8], Paolini et al. [57], Min et al. [48], Hoste et al. [27] and Gertrude et al. [25]. Tannins would appear to be able to bind to proteins and glycoproteins of the adult cuticle (a structure rich in proline and hydroxyproline), to the enzymes secreted by worms and involved in various essential functions [27], or to interact with their digestive epithelium to inhibit nutrition and cause the death of the parasite. Similar deductions were made with aqueous and ethanolic extracts of *Moringa oleifera* [25], aqueous extracts of *Anogeissus leiocarpus* and *Daniellia oliveri* [32], *Leucaena leucocephala* and *Gliricidia sepium* [33]. In our study, the inefficacy of PVPP to restore control values of larval exsheathment suggested that tannins were not the metabolites responsible for anthelmintic activity on *H. contortus*. According to Chan-Pérez et al. [21] and Vargas-Magana [68], tannins were not the sole secondary plant metabolites responsible for the AH effects. Our results contrasted with many previous studies that suspected tannins of being responsive to inhibit exsheathment [2,43,44,52]. Thus, Brunet et al. [18] and Hoste et al. [28] observed a structural lesion on *H. contortus* L3 larvae (*in vitro* and *in vivo*) in contact with sainfoin extracts, a tannin-rich plant. Oliveira et al. [53], showed the inhibition of larval exsheathment in *H. contortus* subject to *A. mangium* extract. Son-de Fernex et al. [64] observed an inhibition effect on exsheathment of *H. contortus* larvae subjected to five plants containing CTs. Consequently, we think that other metabolites besides tannins would be able to affect the exsheathment kinetics of L3 larvae by binding to proteins and glycoproteins of the sheath to prevent the formation of the indented ring in the anterior part.

![Figure 1](image.png)

**Figure 1.** Larval exsheathment of *H. contortus* in the presence of acacia extracts at a concentration of 1.2 mg/mL, and its combination with polyvinyl polypyrrolidone (PVPP).
A. nilotica and A. raddiana extracts both have in vitro anthelmintic activities against H. contortus and C. elegans. The two species of Acacia had completely different tannin contents. A. nilotica, rich in CT, was (1) not effective in inhibiting egg hatching; (2) partially effective for adult mortality; and (3) effective in inhibiting larval exsheathment. However, A. raddiana, rich in TT and TP, was highly effective in the three assays. Thus, in our study, TT-rich plants showed higher and more diverse anthelmintic activities against H. contortus compared to CT-rich plants. HT and CT are found in TT. It is therefore possible that HT are responsible for the efficacy of A. raddiana extracts. Moreover, HT are known to show some toxicity for the ruminants, but the leaves of this plant were consumed by small ruminants in the Sahelian region of Burkina Faso. It is possible that the types and the quantity of HT contained in the leaves were not sufficient to cause ruminal toxicity but could affect the parasites. Future studies are required to ascertain which types or HT (gallotannins or ellagitannins) are contained in A. raddiana extract and underlie the anthelmintic activities. In general, the two species of Acacia used in our study were effective against H. contortus. Therefore, the association of these Acacia in the treatment of gastrointestinal parasites in a traditional environment might be a valuable approach. However, it would be necessary to conduct in vivo parasitological studies to consider the metabolism of extracts in the digestive tract of ruminants.

Acknowledgements. The authors thank the Tripartite Research Project (Africa – Brazil – France), “Fight against desertification in Africa”, funded by INRA/IRD-AIRD and CNPq 457559/2012-8, for supporting the study, as well as the translator of the manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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