Animal and Veterinary Sciences
2022; 10(1): 1-7
http://www.sciencepublishinggroup.com/j/avs
doi: 10.11648/j.avs.20221001.11
ISSN: 2328-5842 (Print); ISSN: 2328-5850 (Online)

In vitro Effects of *Anogeissus leiocarpus* and *Adansonia digitata* on Two Life-cycle Stages of *Haemonchus contortus*, a Gastrointestinal Parasite of Small Ruminants

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To cite this article:

Julienne Kuiseu, Pascal Abiodoun Olounlade, Claude Gbemeho Houssoukpe, Tony Taofick Babalola Abiodoun Sounkere, Frejus Tanguy Ablo Zinsou, Basile Saka Boni Konmy, Christian Cocou Dansou, Ibikounle Moudachirou, Severin Babatounde, Sylvie Mawule Hounzangbe-Adote, Patrick Aleodjrodo Edorh. *In vitro* Effects of *Anogeissus leiocarpus* and *Adansonia digitata* on Two Life-cycle Stages of *Haemonchus contortus*, a Gastrointestinal Parasite of Small Ruminants. *Animal and Veterinary Sciences.* Vol. 10, No. 1, 2022, pp. 1-7. doi: 10.11648/j.avs.20221001.11

Received: October 17, 2021; **Accepted**: December 25, 2021; **Published**: January 8, 2022

Abstract: This study evaluated the *in vitro* anthelmintic activity of aqueous and hydroethanolic extracts of *Anogeissus leiocarpus* and *Adansonia digitata* leaf powder at 75; 150; 300; 600; 1200 and 2400 µg/mL against *H. contortus*, using the egg hatch test and the adult worm motility inhibition test. For egg hatch inhibition, the both plants showed significant concentration-dependent efficacy and were significantly more effective ($p < 0.001$) at the highest concentrations (1200 and 2400 µg/mL) than at the lowest. The hydro ethanolic extract of *A. leiocarpus* showed the highest inhibition ($R^2 = 0.9963$) and the aqueous extract of the same plant showed the lowest inhibition ($R^2 = 0.9742$). The 50% inhibitory concentration (IC50) of the *A. leiocarpus* aqueous and hydro ethanolic extracts (86.19 and 72.5 µg/mL) were 3 times lower than those of the A. digitata extracts (302 and 269.5 µg/mL). Mobility of adult worms was also concentration dependent ($p<0.05$); and also dependent on incubation time ($p<0.01$). At 2400 µg/mL, all adult worms were immobile after 6 h of exposure, whereas at the lowest concentration (75 µg/mL), all adult worms were immobile after 36 h of exposure. Extracts from both plants had similar activity. Finally, the results of the present study suggest that the both plants studied have anthelmintic properties *in vitro* which would explain their use by livestock farmers. However, *in vivo* tests should be performed to confirm these properties in vivo as well.

Keywords: *Haemonchus contortus*, Two Life-cycle Stages, *Anogeissus leiocarpus*, *Adansonia digitate*, Anthelmintic, In *vitro* Methods

1. Introduction

In developing countries, livestock in general and small ruminant livestock in particular is one of the important sectors of the economy [1]. The demand for meat products is multiplying rapidly due to rapid population growth [2]. However, the availability of sheep meat is decreasing as a result of the natural exposure of grazing animals to infestation of several gastrointestinal parasites that inhibit their development [3, 4]. Infections caused by gastrointestinal nematodes remain a major health problem that cause production losses and disrupt the welfare of small ruminants [5, 6]. Among these parasites, *Haemonchus contortus* is the most prevalent species that causes a drastic decrease in production. *H. contortus* is one of the most economically important parasites infecting small ruminants in the world. It is a hematophagous nematode that feeds on blood in the capillaries of the abomasum of sheep and goats in particular [7]. *H. contortus* haemonchosis causes anemia, weight loss, and even death in animals.

Over the past few decades, the primary means of controlling this parasite has been the use of chemical anthelmintics [6]. These chemical anthelmintics have shown their limitations. In most farms there are recurrent problems of chemoresistance coupled with the presence of toxic residues from these molecules in the milk and meat of these animals. In this context, the search for new alternative methods of controlling ovine haemonchosis becomes an emergency [8, 9]. Several alternative strategies are under investigation. Among these strategies, the search for effective plant extracts is of particular and considerable interest [9, 10]. These plants possess active molecules such as tannins, flavonoids and phenols that have been associated with effective anthelmintic activities in small ruminants [11-16]. Therefore, the use of natural local plant resources, which are cheaper, effective and safe for consumer health, remains the most relevant alternative to synthetic molecules. Among these plants include *Adansonia digitata* and *Anogeissus leiocarpus*.

Adansonia digitata, also known as baobab, is a tree of recent interest, particularly due to the high nutritional value of its fruit pulp [17]. Baobab pulp and leaves are rich in procyanidins and flavonol glycosides [17]. *A. digitata* possesses antimalarial activity and modulates biochemical and hematological changes in malaria infection [18]. In West Africa, *Anogeissus leiocarpa* leaves are used against zoonotic diseases in traditional medicine [19]. *A. leiocarpa* exhibits growth inhibitory activity against Mycobacterium smegmatis [20]. Previous studies have focused on investigating the nutritional and microbial properties of *A. digitata* and *A. leiocarpus*. Therefore, the present study was conducted to evaluate the *in vitro* anthelmintic potential of the leaves of both plants on *H. contortus*, a nematode parasite of small ruminants.

2. Material and Method

2.1. Collection and Preparation of Plant Material

The plant material consisted of *A. digitata* and *A. leiocarpus* leaves harvested in the rainy season in the Commune of Kétou located in the Plateau department of South Benin. These leaves were certified at the National Herbarium of the University of Abomey-Calavi under the identification numbers: YH 481/HNB for *A. leiocarpus* and YH 482/HNB for *A. digitata*. They were dried for 14 days at room temperature, in the shade, away from the sun to preserve sensitive phytochemicals. The dry leaves were crushed using an electric mill (of Flour MILLS NIGERIA EI MOTOR No 1827) with a sieve of 710 micrometers mesh. The powders obtained were stored in airtight sealed.

2.2. Plant Extract Preparation

The extraction was done by decoction for aqueous extracts and by maceration for hydroethanolic extracts. After drying the leaves of the two plants for 14 days in the laboratory, they were crushed with an electric grinder. The resulting powders were stored in airtight boxes. One hundred grams (100 g) of each species powders were introduced respectively into 1000 mL of distilled water and a hydroethanolic mixture (700 mL of ethanol and 300 mL of distilled water). The decoction is made for 30 min while the maceration was done for 72 hours. After filtration with Whatman Paper No.1, each filtrate was transferred to a 1000 mL flask and then evaporated at 57°C by using a rotavapor (Heidolph Laborota 4000 efficient) coupled to a water cooler (Julabo FL 300). The concentrated extracts were dried in the oven (40°C) and then weighed to determine the extraction yields.

2.3. Egg Hatching Test

2.3.1. Collection and Extraction of Eggs

The number of eggs in freshly collected fecal material was determined by the classical Mc Master method modified by Raynaud [30]. The initial amount of eggs (N) was calculated as N1 times the total weight of feces (P) to be extracted. This amount of feces was put under continuous agitation for dissolution in 5 times its volume. The mixture was poured onto a series of decreasing mesh sieves (125 µm-40 µm). The residue retained by the 40 µm sieve was recovered in a small volume of water and centrifuged (1000 RPM, 5 min, 20°C). The pellet was recovered in 95 saturated NaCl solution and centrifuged. The supernatant was removed and the residue washed 3 times with PBS by centrifugation (1000 RPM, 5 min, 20°C). The pellet was recovered in a known volume of water.

The number of eggs extracted was determined according to the formula:

$$C = \frac{nxdxV}{3}$$

C = egg concentration in the solution; n = number of eggs in the cell; d = Dilution factor;

V = Final volume.

2.3.2. Technique for Inhibiting Egg Hatching

The egg solution was adjusted to 100 eggs per mL. Thus 100 µl of the egg solution was deposited per well of plates (NUNC, 96 wells) and contacted with 100 µl of plant extracts at different concentrations (75, 150, 300, 600, 1200, 2400

µg/mL), prepared with PBS at a rate of 5 replicates per concentration of extract tested. A negative control (PBS buffer) and a positive control (Thiabendazole) at different concentrations (125, 250, 500 µg/mL) prepared with PBS were also included. The eggs were incubated at 24°C for 48 hours after which the hatched eggs were counted under a light microscope. Percent inhibition was calculated according to the modified formula of [21].

$$\text{Percentage of inhibition (\%)} = \left(1 - \frac{X1}{X2}\right) x100$$

Where X1 is the number of eggs hatched in the tested extracts and X2 is the number of eggs hatched in the negative control.

2.4. Test on the Motility of Adult Worms

2.4.1. Collection of Adult Worms

After slaughtering animals artificially infested with *H. contortus*, the quail were collected and the contents poured into physiological fluid previously prepared by dissolving 9 g of NaCl in 1 L of water (NaCl at 9‰). The worms were then recovered and placed in physiological fluid at 37°C.

2.4.2. Adult Worm Motility Inhibition Technique

The test extract solutions are prepared with PBS at six different concentrations (75, 150, 300, 600, 1200 and 2400 µg/mL). Collected worms with good motility are put each in 1 ml of physiological fluid in wells of NUNC plates, 24 wells and placed in the oven at 37°C. After one hour, 800 µL of physiological fluid is removed and replaced with the extracts to be tested. A negative control (PBS buffer) and a positive control (Levamisole at 250 µg/mL in PBS) are also made up. The test is repeated six times for each concentration and for the controls. The inhibition of adult worm motility in the treatments performed is used as a criterion for anthelmintic activity. After the worms are put in contact with the extracts, the motility is observed with a magnifying glass every 6 hours. The observation stops when all worms in the PBS are found to be immobile.

2.5. Statistical Analyses

The different values of the measured parameters were integrated into a two-criteria repeated measures analysis of variance model run in R software [22]. Since the responses obtained are proportions, we used a binomial distribution with a "logit" link in the syntax. Prioritization of means was done with Tukey's multiple comparison of means test with HSD procedure, test from the agricolae package [23]. Differences are considered significant at the 5% level.

3. Results

3.1. Effects of Plant Extracts on Egg Hatching

Figure 1 shows the effect of aqueous and hydroethanol extracts of *A. digitata* and A. leiocarpus on the hatching of *Haemonchus contortus* eggs at different concentrations. We note that the inhibition rate of egg hatching of the negative control (7.25 ± 0.85%) is significantly lower than that of the positive control (87.75 ± 1.03%) at the concentration of 125 µg/mL ($p<0.001$).

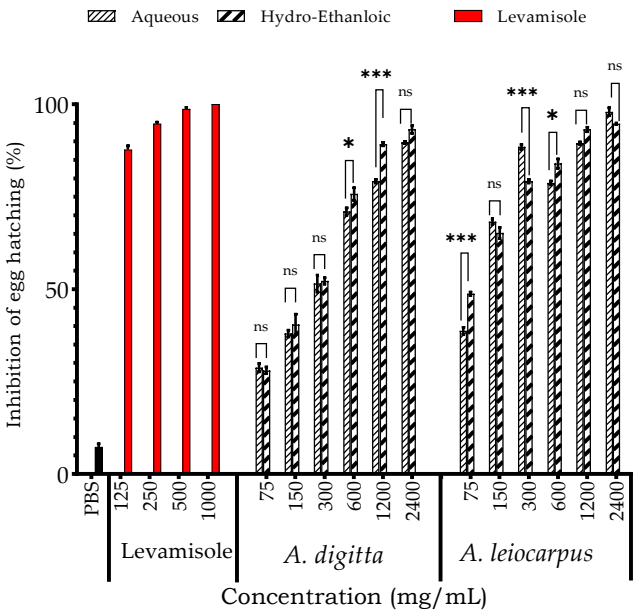

Figure 1. *Effects of the two plant extracts at concentrations of 75, 150, 300, 600, 1200, and 2400 µg/mL and the positive control, levamisole (125 µg/mL), on the hatching of H. contortus eggs. Each bar in the graph represents the mean ± SEM of the number of eggs that failed to hatch. Tukey's HSD multiple comparison test was performed. ns =not significant; * = p<0.05; *** = p<0.001.*

Similarly, both extracts of each plant showed significant inhibitory effects on egg hatching rates ($P < 0.01$). The efficacy of the lowest concentrations (28.75 ± 1.11% and 28.00 ± 0.91%) recorded with ADAQ and ADHE, respectively, were significantly different from the highest (98.00 ± 1.08% and 94.75 ± 0.25%) recorded with ALAQ and ALHE, respectively. *A. leiocarpus* extracts showed better efficacy against egg development compared to A. digitata by an average of 10% (aqueous extract vs. aqueous extracts) and 1% (hydro-ethanolic extract vs. hydro-ethanolic extract).

Both plants showed a significantly concentration dependent anthelmintic effect with $R^2 = 0.992$; $R^2 = 0.988$ for ADAQ and ADHE respectively and $R^2 = 0.974$ and $R^2 = 0.996$ for ALAQ and ALHE respectively (Table 1).

Table 1. *Concentrations of extracts required to inhibit 50% (IC50) of egg hatching.*

Extraits	CI50 (µg/mL)	IC50 (µg/mL)		R^2
		Lower (µg/mL)	Upper (µg/mL)	
ADAQ	302	230.8	475.2	0.9922
ADHE	269.5	213.6	380.1	0.9879
ALAQ	86.19	74.81	98.32	0.9742
ALHE	72.5	65.32	79.77	0.9963
Levamisole	25.44	8.495	40.87	0.9994

ADAQ = Aqueous extract of *A. digitata*; ADHE = Hydro-ethanolic Extract of *A. digitata*; ALAQ = Aqueous extract of *A. leiocarpus*; ALHE = Hydro-ethanolic Extract of *A. leiocarpus;* CI50 = Inhibitory Concentration 50; CI50 = Confidence Interval 50; R^2 = Pearson's coefficient of determination.

In addition, the inhibitory concentration 50 (IC50) was different the values were 302 µg/mL; 269.5 µg/mL; 86.19 µg/mL and 72.5 µg/mL for ADAQ, ADHE; ALAQ and ALHE respectively (Figure 2).

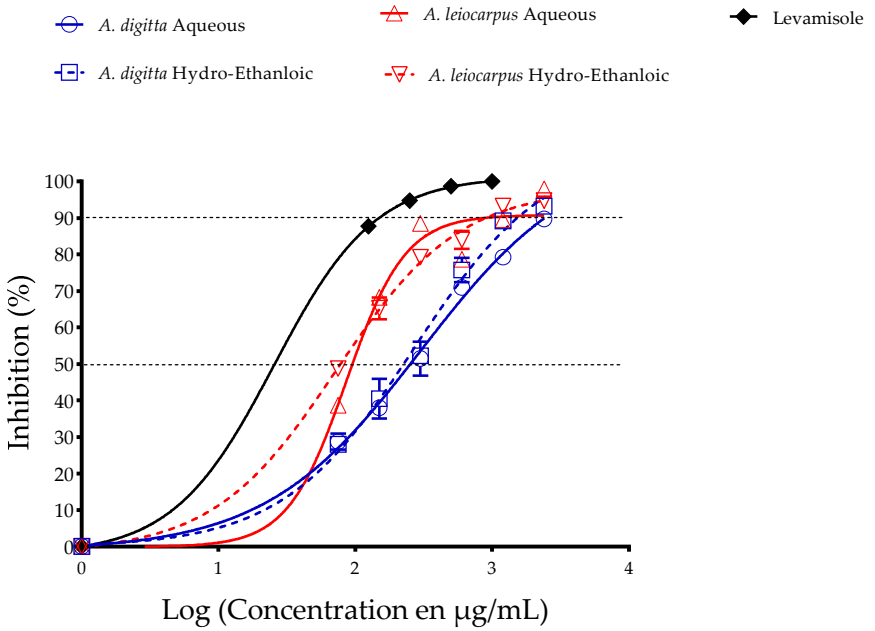

Figure 2. *Efficacy (%) of different concentrations (log-1) (µg/mL) of aqueous and hydro-ethanolic extracts of A. digitata and A. leiocarpus leaf powders on egg hatching.*

ADAQ = Aqueous extract of *A. digitata*; ADHE = Hydroethanol extract of *A. digitata*; ALAQ = Aqueous extract of *A. leiocarpus*; ALHE = Hydroethanol extract of *A. leiocarpus.*

Table 2. *Effects of various concentrations of extracts of two plants on the motility of adult H. contortus worms as a function of time.*

	Concentration (µg/ml)	6h	12h	24h	36h	48h
PBS	0	100	100	75	50	0
Levamisol	125	0	0	0	0	0
ADAQ	75	50	50	25	0	0
	150	50	25	25	0	0
	300	25	25	0	0	0
	600	0	0	0	0	0
	1200	0	0	0	0	0
	2400	0	0	0	0	0
ADHE	75	75	75	25	0	0
	150	75	75	25	0	0
	300	50	50	0	0	0
	600	25	0	0	0	0
	1200	0	0	0	0	0
	2400	0	0	0	0	0
ALAQ	75	75	75	25	0	0
	150	75	50	25	0	0
	300	50	50	0	0	0
	600	25	0	0	0	0
	1200	0	0	0	0	0
	2400	0	0	0	0	0
ALHE	75	50	50	25	0	0
	150	50	50	25	0	0
	300	50	25	0	0	0
	600	25	0	0	0	0
	1200	0	0	0	0	0
	2400	0	0	0	0	0

ADAQ = Aqueous extract of *A. digitata*; ADHE = Hydroethanolic extract of *A. digitata*; ALAQ = Aqueous extract of *A. leiocarpus*; ALHE = Hydroethanolic extract of *A. leiocarpus.*

3.2. Effects of Plant Extracts on the Motility of Adult Worms

The positive control and plant extracts significantly ($p < 0.001$) reduced the motility of *Haemonchus contortus* adult worms *in vitro* compared to the negative control (PBS). This reduction was dependent on concentration and incubation time ($p < 0.01$) (Table 2).

After 6 h of exposure, complete inhibition was observed in wells treated with the lowest concentration (125 µg/mL) positive control. While complete inhibition was observed in the wells treated with the extracts of both plants from the concentration (600 µg/mL) after 12h. During the same period, in the negative treatment, 75% of the worms remained alive. After 24 hours of exposure, motility of live worms was 100% inhibited with plant extract concentrations of 300, 600, 1200 and 2400 µg/mL and for this motility was 75% inhibited with concentrations of 75 and 150 µg/mL (Table 2). Total inhibition (100%) of motility of adult *H. contortus* worms was observed after 36h of exposure with each plant extract, regardless of concentration (Table 2) whereas motility in the negative control was 50%.

Extracts of *A. digitata* and *A. leicarpus* had significant and rapid effects on adult worm survival at higher concentrations (600, 1200 and 2400 µg/mL). However, low concentrations of both plants were also effective over a longer period (Table 2).

4. Discussion

The present study evaluated the *in vitro* capacity of aqueous and hydro-ethanolic extracts of *A. digitata* and *A. leiocarpus* leaf powders against the hatching of *H. contortus* eggs in sheep. Indeed, the study focused on the evaluation of the anthelmintic activity of the extracts of these two plants through *in vitro* biological tests on the eggs and the adult worms of *H. contortus* which constitute two stages of the development cycle of this parasite. The methods of inhibition of egg hatching and adult worm motility were used for these *in vitro* tests according to several previous works [6, 14, 24-26].

The egg hatch inhibition method relies on the ability of the active ingredients to annihilate eggs and neutralize egg hatch [24]. In this study, egg paralysis was demonstrated with the aqueous and hydroethanol extracts of *A. digitata* and *A. leiocarpus*. Indeed, the extracts of these plants showed significant ($p< 0.001$) total inhibition of *H. contortus* egg hatching at high doses. Several authors have found satisfactory results similar to those of this study. For example, Maestrini et al. [26] showed that aqueous extract of licorice root (Glycyrrhiza glabra) and glycyrrhetinic acid showed significant concentration-dependent efficacy at (30 and 10 mg/mL) against gastrointestinal nematode egg hatching in sheep. Methanolic extract of *B. ferruginea* and acetone extracts of *C. glutinosum* and *M. inermis* significantly reduced the number of hatched eggs ($p < 0.01$) of *H. contortus* at concentrations of 1200 and 2400 µg/mL [6]. According to previous works, Castagna et al. [27] demonstrated high efficiency (>82%) of *in vitro* hatching of gastrointestinal nematode eggs after 48 h of exposure to all doses tested (1 to 0.005 mg/mL) of *P. granatum*. These lower inhibition rates than those found in the present study would be related to the doses used (1 to 0.005 mg/mL) in contrast to those in the present study (2400 to 75 µg/mL). The results of the *H. contortus* egg hatch tests with the aqueous and hydroethanolic extracts of A. digitata and *A. leiocarpus* indicated that the extracts of both plants had ovicidal activity *in vitro*. The inhibition of *H. contortus* egg hatch rate observed in the *in vitro* tests indicates that these plants would disrupt or even interrupt the developmental cycle of *H. contortus*. The plants studied, once in contact with the eggs of this parasite, would affect the physiology of the eggs with the consequence of blocking the passage from the egg stage to the larva stage and thus leading to the death of the egg. According to Zangueu et al. [28], herbal treatments reduce the hatching of eggs excreted in the feces, leading to both a reduction in the risk of reinfection. These plants also reduce the worm burden of sheep while reducing pasture contamination.

The pathogenic stage of gastrointestinal nematodes in sheep is marked by the adult worm stage. In these adult worms, partial or total disruption of motility would induce feeding and reproductive difficulties. This would lead to the death of the worms and a drastic reduction of the pathogenic flora in sheep. The objective of inhibiting the motility of adult *H. contortus* worms in the present work was achieved with the aqueous and hydro ethanolic extracts of the leaf powder of *A. digitata* and *A. leiocarpus*. After exposure of the worms to the different extracts, both plants induced a strong and interesting inhibition of the motility of these adult worms. The inhibition was total (100%) after 6h of exposure to the concentrations of 1200 and 2400 µg/mL of the two extracts of the two plants. Weaker results (63%) were obtained *in vitro* by [29] with *Moringa oleifera* proteins on the motility of adult male and female worms of *H. contortus*. The differences in concentration used in the two studies and the difference in plant material are thought to be responsible for the variation in motility inhibition rates observed. According to previous studies, Medeiros et al. [29] detect in their study a higher proteolytic activity in extracts of adult male and female worms after incubation with lectins. In these male and female worms, changes in cuticle ridges, longitudinal striations, and vulva marked lectin-induced morphological changes Medeiros et al. [27]. Interesting and satisfactory results were obtained by Goel et al. [30] on albendazole-resistant *H. contortus* using silver nanoparticles protected by an aqueous extract of *Lansium parasiticum*. The effectiveness of this treatment is due to the oxidative stress generated by these nanoparticles that causes physical damage in the tissues of these worms. According to previous studies, Zabré et al. [31] shows that both *Acacia nilotica* and *Acacia raddiana* extracts were effective but *A. raddiana* was more effective with 100% mortality at 2.5 mg/mL with an LD50 = 0.84 mg/mL (acetone extract). These results are similar to those found in the present study.

5. Conclusion

In conclusion, the aqueous and hydro-ethanolic extracts of *A. digitata* and *A. leiocarpus* showed effective anthelmintic activity *in vitro* on eggs and adult worms of *H. contortus*, although the leaf extracts of *A. leiocarpus* were more effective on inhibition of egg hatching than the extracts of *A. digitata*. However, both plants showed similar anthelmintic activities against the motility of adult worms of the parasite. Further studies to evaluate the in vivo efficacy of the aqueous and hydro ethanolic extracts of the two plants studied on artificially or naturally infected sheep are to be considered.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thanks the members of the research team of the Zootechnics and Livestock System Research Unit (URZoSE) of the Animal and Fisheries Sciences Laboratory (LaSAH) of the National University of Agriculture (UNA), Benin.

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