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Comparative study of aqueous and alcoholic extract of *Azadirachta indica* A. Juss. (Meliaceae) leaves on two life stages of *Haemonchus contortus*

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Abstract

In Burkina Faso, the production of small ruminants is confronted with many constraints, including gastrointestinal parasitosis. These parasitic diseases causes many mortality in small ruminant breeding therefore considerable economic losses. Thus, the present study was carried out with the aim of evaluating the i*n vitro* anthelmintic effectiveness of *Azadiracta indica* (*A. indica*) leaves on two life stages of *Haemonchus contortus* (*H. contortus*): eggs and adult worms. To do this, an aqueous and ethanolic extract of *A. indica* leaves was carried out. From these extracts, five (5) increasing concentrations were performed: 1.25; 2.5; 5 ; 10 and 20 mg/mL and three (3) controls (distilled water, DMSO 2% and albendazol). These concentrations were brought into contact with i) eggs for Eggs Hatch inhibition Assay st (EHA) and ii) Adult Mortality Test (AMT). Three replicates were performed for each experimental preparations. In vitro observations showed that ethanolic extract of A. indica caused high mortalities ($p < 0.0001$) ranging from 44.44 to 100% at 2 hours and 6 hours of exposure respectively at 20 mg/mL. In the same time interval, albendazol showed 100% of mortality in H. contortus. For EHA, the inhibition rate varied from 18% to 85% for the aqueous extract and from 54% to 93% for ethanolic extract. We can therefore say that A. indica extracts have nematicidal and ovicidal effect.

Keywords: *Azadirachta indica; Haemonchus contortus*; Small ruminants; Gastrointestinal; **A**nthelmintic

1. Introduction

In order to achieve food and nutritional security for its population, Burkina Faso, a Sahelian country, must review one of the sectors which constitutes the support for animal protein production. One of the components of this sector, which is livestock breeding, plays an important role in its economy and constitutes a factor of intensification due to the rapid rate of population growth. Also, it is a source of income for more than 86% of active population [1]. In the same context, it constitutes an important lever in the fight against poverty and makes an essential contribution to food security.

However, despite this importance, the breeding of small ruminants (PR), which is one of the sub-components of this sector, remains faced with constraints which limit its development. Note that systematic prevention measures for major infectious and parasitic diseases, vaccinations, treatments and prophylaxis in the field of livestock farming remain a real challenge to be taken up [2]. Thus, to minimize these effects, alternative endogenous solutions are increasingly explored by breeders to combat gastrointestinal parasites [2-4]. Among these endogenous methods, the traditional veterinary

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constitutes a very important recourse to fight against these animal diseases [5,6]. Indeed, much work has been carried out with medicinal plants with antiparasitic properties. Among these plants we have the family of Asteraceae, Fabaceae and Euphorbiaceae which are recognized in other African countries as anthelmintic plants [7,8]. It is in this mind that the present study entitled: Comparative study of the aqueous and alcoholic extract of *Azadirachta indica* A. Juss. (Meliaceae) leaves on two life stages of the parasite *H. contortus* was initiated with the aim of evaluating the ovicidal and vermicidal effectiveness of *A. indica*. To do this, aqueous extract and ethanolic leaves of *A. indica* were used on eggs and female worms of *H. contortus*.

2. Material and methods

2.1. Study sites

The study was carried out in the laboratory of institute of science and technology at Normale High Scool. The institute is located in 46e sector in the city of Ouagadougou. The institute is interested in scientific research, particularly in educational sciences

2.2. Plant material

The plant material consists of *A. indica* leaves. The leaves of *A. indica* were collected from September 15 to 18, 2023 in the city of Ouagadougou. They were then washed in water then dried in the shade away from the sun for one to two weeks. The dry leaves were ground into a fine powder using a grinder before being used for the preparation of the extracts.

2.3. Biological material

The biological material consists of sheep abomasum containing adult *H. contortus* worms. The abomasum was collected at the slaughterhouse located in Saaba rural commune.

2.4. Applied methodology

2.4.1. Residual humidity rate of vegetable powders

The residual humidity rate (RHR) of plant material was determined by thermodesiccation. Operating mode:

A triplicate test portion of 1 g of each vegetable powder was taken and put into porcelain capsules previously tared. The capsules and their contents were placed in a ventilated oven preset at 105°C for 3 hours. After steaming, the capsules and their dried contents were cooled in a desiccator for 30 min and weighed using an analytical balance. The residual moisture content of each vegetable powder was determined with the following relationship.

RHR (%)=((Pe-Pe))/Pe x 100

RHR (%) = residual humidity rate; Pe (g) = test portion before steaming; Pe' (g) = Test portion after baking - mass of the capsule.

2.4.2. Extract preparation

Preparation of aqueous extract

50 grams of this powder were dissolved into 500 mL of distilled water. The mixture was macerated for 24 hours using a rotary shaker then filtered through hydrophilic cotton. The filtrate was evaporated using an evaporator. After complete evaporation, we obtain a powder used as an aqueous extract. The dry extract obtained was weighed and then stored in the refrigerator until use

Preparation of ethanolic extract

For ethanolic extract, a mass of 2 x 200 g of leaf powder was placed in 1000 mL glass with a cover. 750 mL of 96% ethanol was added to each test portion. The vegetable powder and extractor solvent mixture was homogenized by stirring using a glass rod. The mixture was kept away from daylight and at room temperature in the laboratory (approximately 25°C) for 48 h. The mixture was filtered through Wattman paper. The plant residue after filtration was percolated with small portions of extractant solvent until exhausted. The filtrate obtained was concentrated under reduced pressure on a rotary evaporator (BÜCHI) then the concentrated extract obtained was transferred to tared

crystallizers and then dried in a ventilated oven at 50°C. The mass of dry extract obtained was determined as well as the extraction yield.

2.4.3. Preparation of concentrations

For the preparation of stock solutions, 2 mg of each dry extract was dissolved by vortex in 10 mL of dimethyl sulfoxide (DMSO 2%) for ethanolic extract and distilled water for aqueous extract for a final concentration of 20 mg/mL. From this stock solution, cascade dilutions made it possible to obtain concentrations of 10 mg/mL, 5 mg/mL, 2.5 mg/mL and 1.25 mg/mL

2.4.4. Collection of adult H. contortus worms

A longitudinal cut was made on the abomasum in order to empty its contents. Subsequently, after successive cleanings, the female worms were identified and isolated in Petrie dish containing water.

2.4.5. Collection of H. contortus eggs

Females worms collected after longitudinal were placed in a porcelain mortar. Using a pestle, the worms were lightly crushed to release the eggs. The obtained ground was diluted with distilled water and filtered through sieves of 1 mm and 100 µm. The eggs were collected in a 38 µm sieve, rinsed several times with distilled water before being recovered into a 15 mL tube. Ten (10) µL of the eggs solution were placed on a slide and observed under a microscope (x40) to count the number of eggs. Then, eggs solution was adjusted to approximately 200 eggs/mL solution.

2.4.6. Biological tests

Egg hatch inhibition assay

Egg hatch inhibition assay was performed according to the method described by Coles et al. [9]. 100 µL of each concentration and 100 µL of egg solution were put in 96-well Petri plate plus two controls (DMSO 2% and Albendazole). The test was repeated three times for each extracts as well as the controls. The plates were covered and placed in the incubator at 27°C for 48 hours. After 48 hours of incubation, the test was subsequently stopped by adding 2 to 3 drops of 10% formalin. The inhibitory activity of the extracts was determined by calculating the egg hatch inhibition rate (IEO) using the formula below: number of L1/(number of eggs +number of L1)*100.

Adult worm mortality test

The mortality test was carried out according to Jackson and Hoste [10]. 1 mL of each concentration (aqueous and ethanolic extracts) were placed in Petrie dishes. In each Petri dish, 3 female worms were placed in the extract solution and three controls (distilled water, DMSO and Albendazole as positive control). the test was repeated three times for each extracts as well as the controls. Subsequently, the plates were incubated at room temperature for 6 h. mortalities were observed at 0-2-4 and 6 hours of incubation. The worms were declared dead according to the technique of Skantar et al. [11].

2.5. Statistical analyzes

The data collected was entered into Excel version 2010 software before being used to do the analyses. GraphPad Prism Version 5.03 software was used for one-way analysis of variance (ANOVA 1) followed by Dunnet test. The significance level was set at 5% for the comparison of means.

3. Results

3.1. Egg hatch Inhibition Assay (EHA)

Table 1 presents the hatching inhibition rates of *H. contortus* eggs subjected to increasing concentrations of aqueous extract of *A. indica*. Statistical analyzes showed that there is a significant difference ($P<0.0001$) between the control (distilled water) and the treatments applied. The inhibition rate of control was 17.84% compared to the extract which varied from 18% to 85%. Albendazole obtained the highest inhibition rate (96.67%).

Table 1 Hatching inhibition rate of *H. contortus* eggs subjected to the aqueous extract of *A. indica*.

Concentration (mg/mL)	aqueous extract
20	85.69 ± 5.86 a
10	78.14 ± 8.72 a
5	38.42 ± 7.74 a
2.5	29.92 ± 21.21 a
1.25	18.15 ± 3.93 b
Albendazole	96.67 ± 5.77 a
Distilled water	17.84 ± 3.38 b
Probability	P<0.0001

a, b = difference between treatments

Performing Dunnet test noted a very highly significant difference (p <0.0001) for albendazole, the dose of 20 and 10 mg/mL, highly significant (p <0.001) for the dose of 5 mg/mL and significant for the dose of 2.5 mg/mL. The dose of 1.25 mg/mL did not record any significant difference (p>0.05) compared to the control (ED)

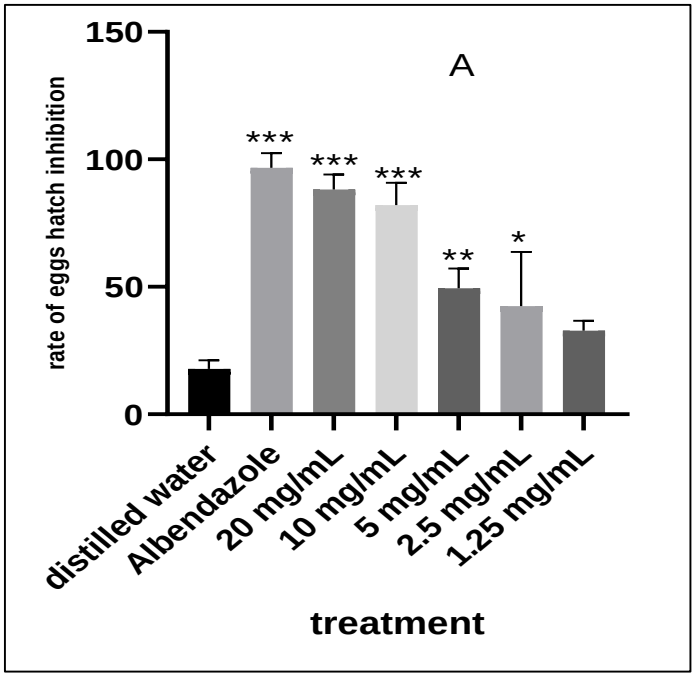

Figure 1 Profile of dose-response effect for eggs hatch inhibition assay subjected to concentrations of aqueous extracts of *A. indica*

***= p<0.0001 ; **= p<0.001 ; *= p<0.05

Table 2 presents eggs hatch inhibition rates of *H. contortus* eggs subjected to increasing concentrations of *A. indica* ethanolic extract. Statistical analyzes showed that there is a significant difference (P<0.0001) between the control and the treatments applied. The inhibition rate of control (DMSO 2%) was 9.79% compared to the extract which varied from 54% to 93%. Albendazole obtained the highest inhibition rate (96.67%).

Table 2 Rate of eggs hatch inhibition assay of *H. contortus* eggs subjected to ethanolic extract of *A. indica*

Concentration (mg/mL)	Ethanolic extract
20	93.60 ± 5.77 a
10	69.49 ± 15.50 a
5	59.43 ± 20.82 a
2.5	55.27 ± 7.13 a
1.25	54.15 ± 22.09 a
Albendazole	96.67 ± 5.77 a
DMSO 2%	9.79 ± 6.11 b
Probability	P<0.0001

a , b = difference between treatments

Performing Dunnet test noted a very highly significant difference (p <0.0001) for albendazole and the dose of 20mg/mL, highly significant (p <0.001) for the doses of 10 and 5mg/mL and significant for doses of 2.5 and 1.25 mg/mL (figure 2).

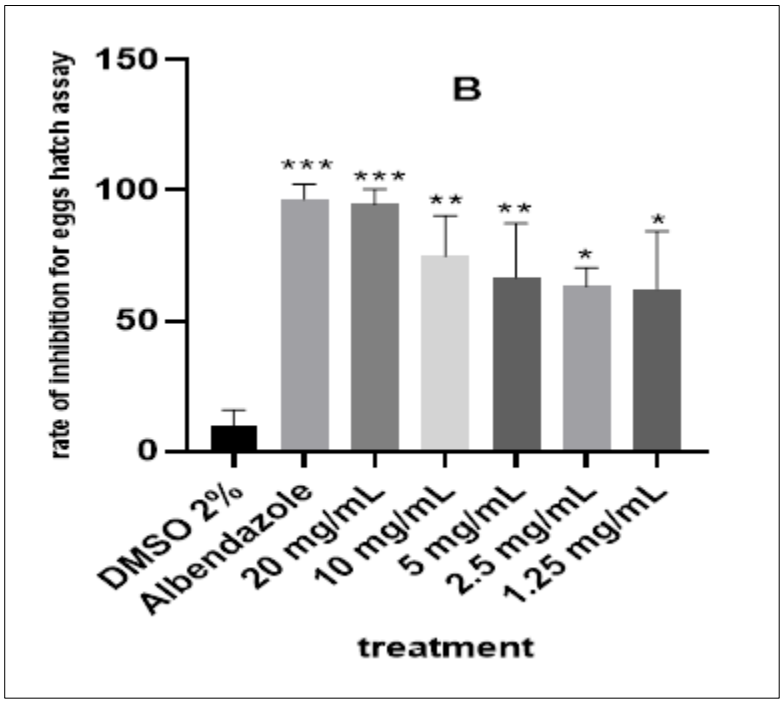

Figure 2 Profile of dose-response effect for eggs hatch inhibition assay subjected to concentrations of ethanolic extracts of A. indica

***= p<0,0001 ; **= p<0,001 ; *= p<0,05 ;

3.2. Adult worm mortality test (AMT)

Table 3 presents the results of mortality test of female adult worms subjected to increasing concentrations of *A. indica* aqueous extract. No mortality was recorded at 0h and 2h in all treated group. Likewise, no mortality was recorded at concentrations 1.25 and 2.5 mg/mL at the 4th hour. Mortalities were recorded at high concentrations (5, 10 and 20 mg/mL) at 4th hour of incubation with mortality rates of 22.22%, 44.44% and 77.78% respectively. At 6 th hour of incubation, mortalities were recorded in all treated group with rates varying from 11% to 66%. Albendazole, presented mortalities of 33.33% to 100% after 2 hours of exposure.

Table 3 Mortality rate of adult *H. contortus* worms subjected to *A. indica* aqueous extract

Concentration (mg/mL)	***A. indica* aqueous extract**			
	0h	2h	4h	6h
20	0 ± 0 aA	0±0 aA	55.56 ± 19.25 aB	66.67± 19.25 aB
10	0±0 aA	0±0 aA	44.44± 19.25 aB	55.56± 1925 aB
5	0±0 aA	0±0 aA	22.22± 19.25 bA	33.33±0 aB
2.5	0±0 aA	0±0 aA	0 ± 0 bA	22.22± 19.25 aA
1.25	0±0 aA	0±0 aA	0± 0 bA	11.11± 19.25 aA
Albendazole	0±0 aA	33.33 ± 0 bB	100± 0 aB	100± 0 bB
Distilled water	0±0 aA	0±0 aA	0±0 bA	22.22±19.24 aA
Probability	P>0.05	P<0.0001	P<0.0001	P<0.0001

a,b= significant difference between concentrations; A,B and C= significant difference between columns

Figure 3 and table 4 presents the profile of dose-response effect of adult *H. contortus* mortality subjected to *A. indica* aqueous extract. Dunnet test noted a difference between concentrations and incubation times.

Table 4 Result of Dunnett test depending concentrations and times

times	**Control 1**	**Control 2**	**Treatments (mg/mL)**				
	ED	Albendazole	20	10	5	2,5	1,25
0h vs. 2h	>0.9999	<0.0001	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
0h vs. 4h	>0.9999	<0.0001	0.0006	0.0078	0.3474	>0.9999	>0.9999
0h vs. 6h	0.3473	<0.0001	<0.0001	0.0006	0.0693	0.3474	0.8368
2h vs. 4h	>0.9999	>0.9999	0.0006	0.0078	0.3474	>0.9999	>0.9999
2h vs. 6h	0.3473	>0.9999	<0.0001	0.0006	0.0693	0.3474	0.8368
4h vs. 6h	0.3473	>0.9999	0.3472	0.8367	0.8368	0.3474	0.8368

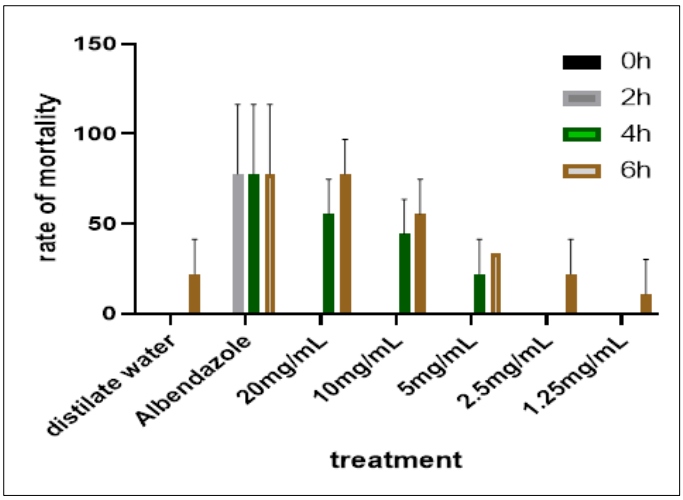

Figure 3 Profile of dose-response effect of adult *H. contortus* mortality subjected to aqueous extract of *A. indica*

Table 5 shows the effect of ethanolic extract of *A. indica* on adult worm mortality. No mortality was recorded at 0 hour. Similarly, no mortality was recorded at 1.25; 2.5 and 5mg/mL at 2 hour of incubation. At 2 hours of incubation mortality varied of 33.33 to 44.44 at 10 an 20mg/ml respectively. Mortalities were recorded progressively at all concentrations from the 4rd to the 6th hour of incubation.

Table 5 Adult worms mortality rate of *H. contortus* subjected to ethanolic extract of *A. indica*

Concentration (mg/mL)	**Ethanolic extract of *A. indica***			
	0h	2h	4h	6h
20	0 ± 0 aA	44.44 ± 19,24 bB	77.77 ± 19.24 bC	100 ± 0 bC
10	0 ± 0 aA	33.33 ± 0 bB	55.55 ± 19.24 bB	88.88 ± 19.24 bC
5	0 ± 0 aA	0 ± 0 aA	55.55 ± 19.24 bB	88.88 ± 19.24 bC
2.5	0 ± 0 aA	0 ± 0 aA	33.33 ± 0 bB	44.44 ± 19.24 aB
1.25	0 ± 0 aA	0 ± 0 aA	22.22 ± 19.24 aA	33.33 ± 0 aA
Albendazole	0±0 aA	33.33 ± 0 bB	100± 0 bC	100± 0 bC
DMSO 2%	0 ± 0 aA	0 ± 0 aA	0 ± 0 aA	22.22 ± 19.25 aA
Probability	P>0.05	P<0.0001	P<0.0001	P<0.0001

a,b= significant difference between concentrations; A,B and C= significant difference between columns

Figure 4 and table 6 presents the profile of dose-response effect of adult worms mortality of *H. contortus* subjected to ethanolic extract of *A. indica*. Dunnet test noted a difference between concentrations and incubation times. Statistical analysis applied to these data shows that there is a significant difference between the mortality rates obtained with the concentrations of the extracts.

Table 6 Result of Dunnett test depending concentrations and times

Times	**Control 1**	**Control 2**	**treatments (mg/mL)**				
	DMSO 2%	Albendazol	20	10	5	2,5	1,25
0h vs. 2h	>0.9999	0.0024	<0.0001	0,0024	>0.9999	>0.9999	>0.9999
0h vs. 4h	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	0.0024	0.0718
0h vs. 6h	0.0718	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0024
2h vs. 4h	>0.9999	<0.0001	0.0024	0.0718	<0.0001	0.0024	0.0718
2h vs. 6h	0.0718	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0024
4h vs. 6h	0.0718	>0.9999	0.0718	0.0024	0.0024	0.5999	0.5999

Table 7 Inhibition concentrations (IC50) of *A. indica* extracts on adult worm mortality (AMT) and egg hatch inhibition (IEO)

Extraits de *A. indica*	**Assay**	**IC $_{50}$ (mg/mL)**		
		Minimum	Mean	Maximum
aqueous	EHA	0.001	2.006	4.104
	AMT	3.438	3.986	4.868
Ethanolic	IEO	0.325	0.761	2.655
	AMT	0.274	1.588	2.523

MVA = Adult worm mortality ; EHA = Eggs Hatch inhibition

Table 7 presents the inhibitory concentrations 50 (IC50) obtained by probit analysis method of *A. indica* extracts on eggs (IEO) and adult worms (MVA). Generally, ethanolic extract recorded low IC50s on eggs and adult worms of *H. contortus* (IC50 = 0.761 mg/mL for IEO and IC50 = 1.588 mg/mL for MVA) compared to aqueous extract (IC50 = 2.006 mg/mL for IEO and IC50 = 3.986 mg/mL for MVA)

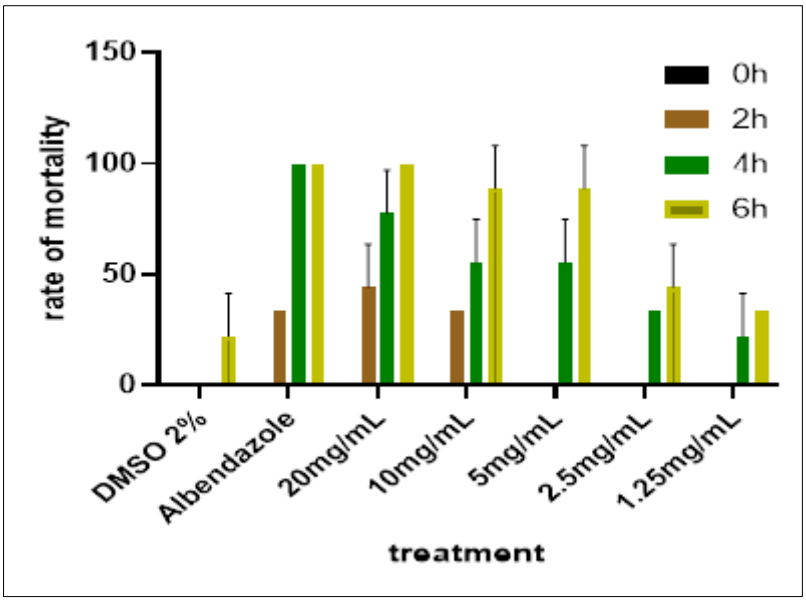

Figure 4 Profile of dose-response effect of adult worms mortality of H. contortus subjected to ethanolic extract of *A. indica.*

4. Discussion

The high costs of synthetic anthelmintics coupled with the phenomenon of parasite resistance have prompted the search for alternative solutions that are available, accessible, inexpensive and have a low level of resistance. Thus, many authors have evaluated the anthelmintic effect of several bioactive plants against gastrointestinal nematodes of small ruminants. It is in this mind that our study was carried out to see the anthelmintic effectiveness of aqueous and ethanolic extracts of A. indica leaves on gastrointestinal parasite, *H. contortus*, prevalent in sheep farming system. Our *in vitro* results showed that the extracts obtained from A. indica leaves have anthelmintic effectiveness. The aqueous and ethanolic extract of *A. indica* exhibited vermicidal and ovicidal properties on *H. contortus* adult worms and eggs.

For egg hatch inhibition assay, the two extracts of *A. indica* had an inhibitory effect against *H. contortus*. Similar results were reported by Hounyovi and Lokossou [12] who showed that aqueous extract of *A. indica* exhibits nematicidal properties. Also, Costa et al. [13] evaluated the effect of ethanolic extracts of *A. indica* on eggs and larvae of *H. contortus*. The results showed inhibition rates of 99.77% for egg hatching at 3.12 mg/mL and 87.11 mg/mL for larval development at a dose of 50 mg/mL. In our study, we noticed that for ethanolic extract the rate of egg hatch inhibition was higher compared to aqueous extract (93% compared to 85% at a dose of 20 mg/ mL). This result is similar to that of Hounzangbe-Adote et al. [14] who showed a high anthelmintic effect of ethanolic extracts of four plants from Benin (*Zanthoxylum zanthoxyloides*, *Newbouldia laevis*, *Morinda lucida* and *Carica papaya*) on eggs and adult worms of *H. contortus*. Also, Ahmed et al., [15] recorded low effectiveness of aqueous extracts of *A. indica* seeds against gastrointestinal nematodes of sheep.

For adult worm mortality, no mortality was recorded at 0h and 2h of incubation with *A. indica* extracts in general. Most mortalities were recorded from the 4th hour of incubation and those at the highest doses 10 and 20 mg/mL which shows vermicidal activity of the extracts. Similar results were obtained by Zabré et al., [16] with aqueous and acetone extract of *A. nilotica* pod.

The inhibition concentration (IC50) of ethanolic extract (IC50=0.761 mg/mL and IC50=1.588 mg/mL) were low compared to aqueous extract (IC50= 2.006 mg/mL and IC50=3.986 mg/mL). when we compared both assay, the IC50 of egg hatch inhibition were relatively lower compare to adult worm mortality. Thus, we can said that ethanolic extract and aqueous extract are ovicid and vermicid effect on *H. contortur*.

Previous studies have shown vermicidal effects of bioactive plants. Thus, Mishra et al., [17] showed that alcoholic and aqueous extracts of *A. indica* flowers had anthelmintic activity *In vitro* against Setaria cervi worms. Just like egg hatching, ethanolic extract had a more pronounced and rapid effect on *H. contortus* unlike aqueous extract. The worm mortalities obtained with these extracts could be linked to the secondary metabolites contained in the plant. Neem owes its vermicidal activity essentially to limonoid compounds, mainly azadirachtin, salanin, tannin and nimbin and their analogues [18].

In the literature, phytochemical screening reveals that the leaves of *A. indica* contain chemical compounds such as: alkaloids, flavonoids, tannins, terpenoids and saponins which can have repellent, insecticidal and ovicidal properties [19]. Isoprenoids which are subdivided into Diterpenoids and Triterpenoids which contain limonoids, constituting the bitter principle [20]. Among these bitter principles, we find the groups of azadirone and its derivatives, nimbin, salannin and azadirachtin [21]. According to several authors, the molecules suspected of being responsible for antiparasitic properties of plants belong to different biochemical classes, such as proteinases [22,23], alkaloids [24], saponins [25], flavonoids [26] or condensed tannins [27]. Thus, numerous studies have shown that plants containing condensed tannins represent an alternative to the use of synthetic anthelmintics [28-30].

5. Conclusion

The study carried out with aqueous and ethanolic extracts of *A. indica* showed ovicidal and vermicidal effects on the parasite *H. contortus*. Ethanolic extract has been shown to be more effective than aqueous extract even at low doses. Thus, we can say that the leaves of *A. indica* can have anthelmintic effectiveness on *H. contortus* gastrointestinal nematodes of small ruminants. However, these effects still do not correspond to in vivo conditions because plants are metabolized. As a result, a plant active In vitro can become inactive in vivo. So, it is important to note that these studies are limited and more research is needed to confirm these In vitro study. Therefore, it would be wise to realized phytochemical analyzes in order to see the secondary metabolites contained in the plant and involved in the effectiveness of the extracts. Make in vivo tests with sheep or goats in natural infestation

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare that they have no competing interests.

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