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RESEARCH ARTICLE

PHYTOCHEMISTRY AND ANTHELMINTIC EFFICACY OF ACETONIC AND DICLOROMETHANE EXTRACTS OF A. INDICA TWIGS ON FEMALE HAEMONCHUS CONTORTUS EGGS AND WORMS.

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Abstract

Treatments using bioactive plants are increasingly being studied due to their low cost, availability and anthelmintic activities, but also because these plants do not present resistance to parasites. This study evaluated the effect of two extracts, dichloromethane and acetone, on Haemonchus contortus eggs and adult worms. With regard to the inhibition of H. contortus egg hatching, the results showed that the dichloromethane extract was more effective than the acetone extract at doses of 5, 2.5 and 1.25 mg/mL, with rates of 73.93%, 73.03% and 65.24% respectively. However, acetone extract recorded a high inhibition rate at doses of 10 and 20 mg/mL, with 85.59% and 92.04% respectively. Concerning mortality of adult female worms, concentrations of 5, 10 and 20 mg/mL showed the highest mortality rates compared with doses of 2.5 and 1.25 mg/mL. Mortality rates varied with time. The results show that the acetone extract showed the highest mortality rate with the 20 mg/mL dose (77%) and as early as 2^{e} hour of exposure. For the dichloromethane extract, apart from the 0 hour of exposure, all concentrations recorded mortality rates whatever the time. Thus, we can deduce that A. indica could constitute an alternative for gastric nematode control. However, in vivo tests are needed to confirm the results obtained in vitro.

Introduction:-

Agriculture and livestock have always been the main activities of the population, especially in rural areas. In Burkina Faso, livestock farming is the second largest contributor to agricultural value added after cotton (FAO, 2018). It employs more than 86% of the population and alone contributes 10-20% to GDP (FAO, 2018). Livestock provides many goods and services to the population and also supports livelihoods in many ways: income, food and nutrition, insurance, traction, fertilizer, etc (FAO, 2018). In terms of importance, MRAH (2019) showed that the ruminant livestock population is estimated at: 9.84 million cattle; 10.44 million sheep; 15.63 million goats. However, despite the enormous goods and services they provide, the sector faces enormous food and health

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constraints. Food constraints include overcrowding on pastoral land, with risks of soil and vegetation degradation linked to population growth and climate change. These factors affect the quality and quantity of available natural forage (MRA, 2011). Sanitary constraints include the resurgence of certain animal diseases such as avian influenza, pasteurellosis, contagious bovine pleuropneumonia, swine fever, parasitic diseases, etc. (MRA, 2011).

To reduce sanitary constraints, several control methods have been put in place. These include (i) chemical control through the use of antiparasitic agents, including benzimidazoles, imidazothiazoles/tetrahydropyrimidines, macrocyclic lactones, salicylanilides, amino-acetonitrile derivatives, spiro-indoles, etc. (OMSA, 2022). This method has been very effective, but nowadays it is limited by the high cost of the products, their availability and accessibility, coupled with the phenomenon of resistance (Kaboré A, 2009.). Resistance to anthelmintics is a major threat to animal health and welfare and can lead to production losses in food-producing species (OMSA, 2022). (ii) non-chemical control, such as reducing overgrazing, rotating species in a pasture or using bioactive plants with anti-parasitic activity (OMSA, 2022). A number of studies have shown that the use of bioactive plants is highly effective. These include: the bark of Acacia mangium (Oliveira, 2014), Acacia mearrsii (Yoshibara et al., 2014), Calotropis procera (Kanazoé et al., 2017), Acacia nilotica and Acacia raddiana (Zabré et al., 2017), Ceratotheca sesamoïdes (Dicko et al., 2022), Combretum micrathum (Tianhoun et al., 2023). Today, the use of bioactive plants has several advantages in animal husbandry: a) the availability of resources, b) the current absence of resistance to active compounds in worms and c) the low cost compared with synthetic anthelmintics, particularly in developing countries (Kaboré et al., 2007). This work was thus initiated to determine the anthelmintic efficacy of A. indica on the developmental stages of sheep gastrointestinal parasites.

Materials and Methods:-

Study site

The study was carried out in the life and earth sciences laboratory at Normal High School (institute of science and technology) located in the city of Ouagadougou. It is a higher education establishment for training high school and college teachers in scientific disciplines. Phytochemical screening was carried out at the Health Science Research Institute (IRSS/ Burkina Faso).

Material:-

Plant material

The plant material consisted of leafy branches of A. indica harvested in Bangr-weogo Urban Park located in the northern Sudanian region (Guinko 1984), between parallels 12°22'59,4" and 12°23'01,7" north latitude and meridians 1°30'10,00" and 1°37'12,2" west longitude (Guinko 1984). The plants encountered are : Vitellaria paradoxa, Guiera senegalensis, Combretum glutinosum, Cymbopogon schoenanthus, Azadirachta indica, Acacia macrostachya, Cassia sieberiana, Balanites aegyptiaca, Acacia seyal, etc. (Gnoumou et al., 2008).

Animal material

Animal material consisted of ovine abomasum collected at the Saaba cold-storage abattoir. Eggs and adult worms of Haemonchus contortus.

Methods:-

Preparation of plant material

A. indica leafy twigs were harvested early in the morning and placed in a bag for transport to the laboratory. Once in the laboratory, the leafy branches were washed with water and dried dry at room temperature for 14 days. After the 14 days, the leaves were ground by hand using a mortar and pestle, then crushed to powder using an electronic grain blender. The A. indica powder obtained was then placed in plastic bags and stored for extract preparation.

Preparation of animal material

Haemonchus contortus eggs and adult worms were prepared at the institute of Science and Technologyat Normal High School**.** When we collected the abomasum, we made a longitudinal incision to collect the contents, which contained the faeces and female worms. In a porcelain mortar, we put 10 mL of distilled water and then worms, which we crushed to release the eggs.

Preparation of acetonic and dicloromethane extracts

Residual moisture content of plant powders

The method used to determine residual moisture content in samples is the official AOAC thermogravimetri method (1990). Test samples of 2 g of plant powder were, dry, tared crucibles. The whole set was placed in a ventilated oven preset at 105°C for 3 h. The crucibles were then removed, cooled in a desiccator for 30 min, and weighed. The operation was repeated until a constant mass of dry test samples was obtained. The moisture content of each plant powder was determined using this equation.

$$\text{THR}\ (\%) = \frac{(\text{Pe} - \text{Pe}')}{Pe} \text{x}\ 100$$

THR (%) = residual moisture content; Pe (g) = test sample before steaming; Pe' (g) = test sample after steaming - capsule mass.

Dichloromethane macerate

A 200 g mass of leaf powder was placed in a 1,000 mL glass jar fitted with a lid. We added 750 mL of analytical dichloromethane to the test sample. The mixture (plant and solvent) was homogenized by stirring with a glass rod. The mixture was kept in the dark at room temperature for 48 hours.

The extract was filtered through Wattman n°5 pleated paper. After filtration, the residue of plant was exhausted by percolation with small portions of Dichloromethane.

Acetone macerate

A 150 g batch of A. indica plant powder was macerated with 750 mL of acetone/water mixture in the 70:30 v/v (70%) ratio for acetone macerations. The mixture was stirred with a glass rod and maintained for 72 h with the acetone solvent. Extract was then filtered on Wattman paper. The filtrate obtained was concentrated under reduced pressure in a rotavapor at a temperature of 50°C before being packed in opaque vials and stored in a refrigerator at 4°C for biological testing and phytochemical screening.

Phytochemical analysis

Screening was carried out by chemical reactions in solution in test tubes using the method described by Ciulei (1982). This method is based on the ability of chemical groups to react with specific or general reagents to produce characteristic colorations. The biological chemical groups investigated were tannins (ferric chloride reaction), flavonoids (Shibata reaction) and saponosides (foam test).

Identification of tannins by ferric chloride reaction

The ferric chloride ($FeCl_3$) reaction was used to characterize polyphenols. 2 mL of each extract (acetone and dichloromethane) were placed in tubes, followed by a drop of 2% alcoholic ferric chloride solution. The appearance of a blackish-blue coloration for gall tanins (TG). For condensed tanins, the tubes were placed in a water bath (30°) for 15 to 20 minutes to observe the colorations. The formation of a red precipitate indicates the presence of condensed tanins (TC).

Identification of flavonoids by the Shibata reaction

Flavonoids are phenolic or aromatic compounds responsible for the yellow and orange colorations of many flowers, fruits and sometimes young senescent leaves (Koko et al., 2011; Narayan, 2012). Thus, 2 mL of each macerate was evaporated to dryness and the residues obtained were dissolved in 2 mL of 50% methanol and then transferred to a tube. Fragments of magnesium turnings and 4 drops of concentrated HCl were added to the tubes. The appearance of red or orange coloration indicates the presence of flavonoids.

Identification of saponosides by foam test

To a test tube containing 10 mL of distilled water, we added 2 g of extract. The tube was manually shaken for 20-30 seconds, then left to stand for 15 minutes. The persistence of foam indicates the presence of saponins.

Identification of alkaloids

Alkaloids are organic nitrogen compounds of virtually therapeutic interest, Kallo et al., 2018. 1 mL of distilled water was placed in a tube. To this tube, 0.5g of extract, 2-3 drops of 2% sulfuric acid and a few drops of Mayer's reagent were added. The presence of a white precipitate or turbidity indicates the presence of alkaloids.

Biological tests

Preparation of concentrations

Five solutions of concentration (1.25 - 2.5 - 5- 10 and 20 mg/mL) were prepared for the experiment. Firstly, stock solutions of the extracts were prepared with reference to the maximum dose (40mg extract + 2000µL DMSO plus two controls (DMSO and Benzal). From these solutions, cascade dilutions were performed to obtain the concentrations corresponding to the desired doses.

Egg hatching inhibition test

The test was performed according to Coles et al. (1992). In a porcelain mortar, the female worms were lightly crushed to release the eggs. Once crushed, mixture was placed in a beaker and 10 mL of distilled water was added. The resulting solution was filtered to collect the egg solution. 5 µL of this solution was placed on a slide, covered with a coverslip and placed under a microscope at objective 40 for determination of egg number. The solution was adjusted to 200 eggs per mL of solution. To test the direct effect of extracts on nematodes, 4x100 µL of each extract concentration and 100 µL of egg solution were placed in each well of a culture plate (96-Well plate). The plates were covered with parafilm and incubated at 27°C for 48h. After 48 h of incubation, egg hatching was stopped by adding two drops of 10% formalin. The number of L1 larvae and the number of eggs were estimated under the microscope (x 10), and the hatching percentage was calculated using the formula:

$$\text{hatching percentage} = \frac{nomberoflarvaeL1}{(nomberofeggs + nomberoflarvaeL1)}$$

Female worm mortality test

The mortality test was carried in accordance with Jackson and Hoste (2010). Petrie dishes were used, into which 1 mL of each concentration (aqueous and ethanolic extracts) and 3 female worms were added. Two reference controls were used : DMSO as a negative control and benzal as a positive control. Petrie dishes were then incubated at room temperature, and mortalities were observed at 0-2-4 and 6 h after incubation. Worms were declared dead using the technique of Skantar et al. 2005.

Analyses statistiques

The data collected was entered into Excel version 2010 software. GraphPad Prism Version 8.4.3 (686) software was used for one-way analysis of variance (ANOVA 1) using Dunnet test. The significance level was set at 5% for the comparison of means.

Results:-

Residual moisture content

Table 1 shows the residual moisture content of A. indica leaves. The results showed that the yield of the dichloromethane macerate was relatively low compared with the acetonic macerate, with yields of 6.33% and 8.16% respectively.

Table 1:- THR, dry extract masses and extraction yields.

Extracts	Test shots (g)	THR (%)	Dried extract (g)	Rd extraction (%)
Plant powder	1 x 2	5.21 ± 0.24	-	-
Acetonic	188.20	-	15.36	8.16
Dichloromethane macerate	1 x 200.10	-	12.01	6.33

Phytochemical assay

Phytochemical test revealed the presence of certain secondary metabolites in A. indica extracts. In the éthanolic and dichloromethane extract, the tests showed the presence of flavonoids, tannins and alcaloïd. In acetonic extract the test showed the presence of flavonoids, tannins and saponosids. However, only flavonoids and tannins were revealed in the aqueous extract.

Table 2:- Phytochemical assay by text tube.

Métabolites	extract

secondaires	aqueous	ethanolic	Dichloromethane	acetone
Flavonoïd	+	+	+	+
Tannins	+	+	+	+
Saponosid	-	-	-	+
Alcaloïd	-	+	+	-

+ = presence ; - = absence

Inhibition of Haemonchus contortus egg hatching

Table 3 shows the rate of inhibition of H. contortus. Analysis of the results shows that dichloromethane extract was more effective than acetone extract at doses of 5, 2.5 and 1.25mg/mL. However, the acetone extract recorded a high inhibition rate with the highest doses (10 and 20 mg/mL).

Table 3:- Inhibition rate of H. contortus egg hatching as a function of extract and control concentrations.

Concentration (mg/mL)	**extract**	
	dichloromethane	**Acetonic**
20	84,75 ± 7,00 a	92,04 ± 5,71 a
10	75,61 ± 11,55 a	85,59 ± 6,68 a
5	73,92 ± 7,14 a	58,24 ± 25,96 a
2,5	73,03 ± 9,39 a	38,73 ± 5,06 a
1,25	65,24 ± 9,74 a	32,83 ± 5,01 a
Benzal	96,67 ± 5,77 a	96,67 ± 5,77 a
DMSO 2%	9,79 ± 6,11 b	9,79 ± 6,11 b
Probability	P<0.0001	P<0.0001

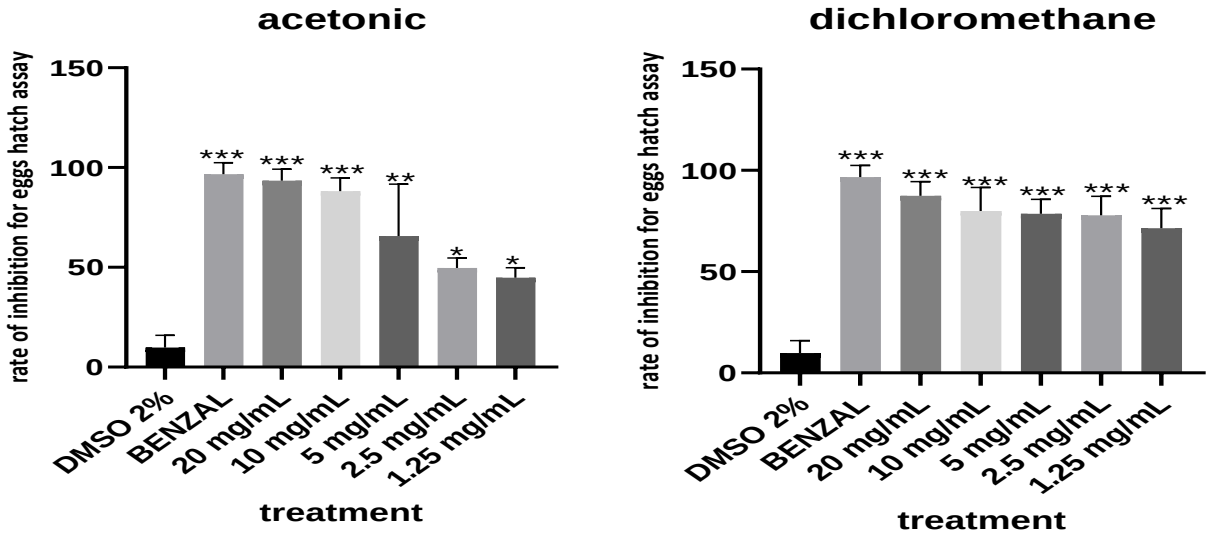

Fig 1:-Dose-response profile for eggs hatch inhibition essay of H. contortus.

Mortality of adult female Haemonchus contortus worms

Table 4 shows the mortality rate of female H. contortus worms as a function of concentration and time for the dichloromethane extract. The analysis showed a highly significant difference (P<0.0001) between concentration and incubation time. In general, the 5, 10 and 20 mg/mL concentrations showed the highest mortality rates compared with the 2.5 and 1.25 mg/mL doses. At 0 h no mortality was recorded. Mortality began after 2 h of incubation.

Table 4:- Mortality rates of female H. contortus worms.

Concentration (mg/ml)	dichloromethane extracts of A. indica.			
	0h	2h	4h	6h
20	0 ± 0 aA	88.89 ± 19.25 bB	88.89 ± 19.25 bB	100.00 ± 0 bB
10	0 ± 0 aA	77.78 ± 19.25 bB	88.89 ± 19.25 bB	100.00 ± 0 bB
5	0 ± 0 aA	55.56 ± 19.25 bB	88.89 ± 19.25 bC	100.00 ± 0 bC
2,5	0 ± 0 aA	55.56 ± 19.25 bB	55.56 ± 19.25 bB	66.67 ± 0 bB
1,25	0 ± 0 aA	33.33 ± 0 bB	33.33 ± 0 bB	33.33 ± 0 aB
Benzal	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

Table 5 shows the mortality rate of female H. contortus worms as a function of concentration and time for the acetonic extract. The analysis showed a highly significant difference (P<0.0001) between concentrations and incubation time. Overall, only the 20 mg/mL concentration showed the highest mortality rate compared with the other doses (77%). At 0 h no mortality was recorded. Mortality began after 2 h of incubation.

Table 5:- Mortality rate of female H. contortus worms.

Concentration (mg/ml)	acetonic extract			
	0h	**2h**	**4h**	**6h**
20	0 ± 0 aA	77.78 ± 38.49 bB	77.78 ± 38.49 bB	77.78 ± 38.49 bB
10	0 ± 0 aA	44.44 ± 19.25 bB	44.44 ± 19.25 bB	55.56 ± 19.25 aB
5	0 ± 0 aA	0 ± 0 aA	44.44 ± 19.25 bB	44.44 ± 19.25 aB
2,5	0 ± 0 aA	0 ± 0 aA	33.33± 0 aA	33.33 ± 0 aA
1,25	0 ± 0 aA	0 ± 0 aA	22.22 ± 19.25 aA	22.22 ± 19.24 aA
Benzal	0±0 aA	33.33 ± 0 aA	100± 0 bB	100± 0 bB
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

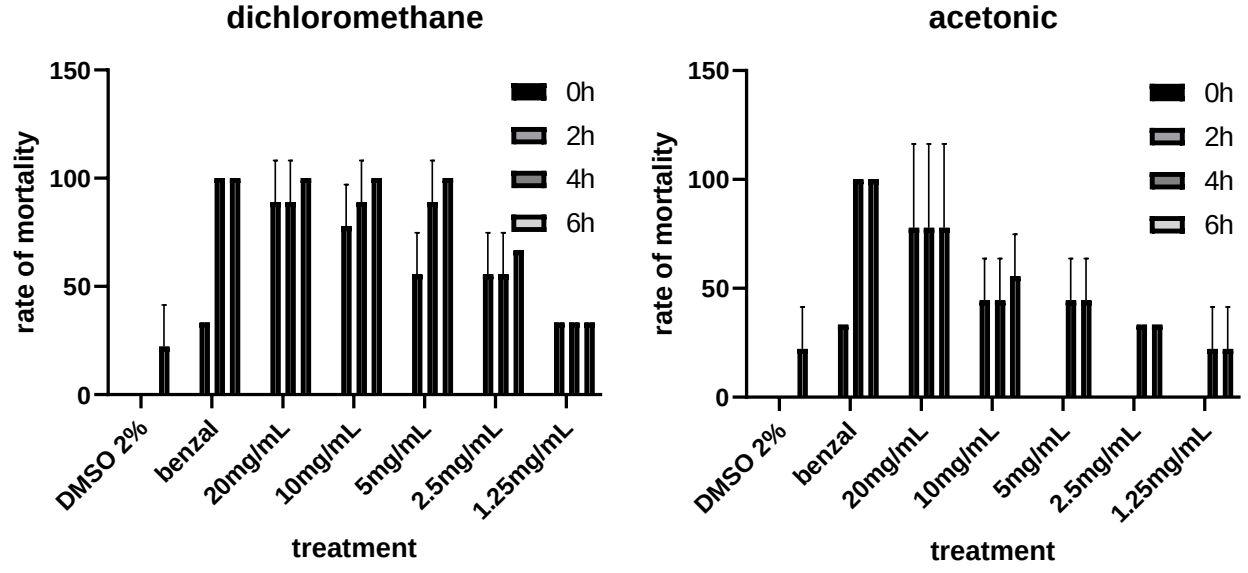

Fig 2:- Dose-response profile of mortality rate of female worms.

Discussion:-

In developing countries, gastrointestinal nematodes are a real concern for low-income rural livestock farmers who cannot afford synthetic anthelmintics. The aim of the present work is to demonstrate the anthelmintic effect of A. indica on the eggs and worms of Haemonchus contortus, the most prevalent and dominant parasite of livestock farming systems in Burkina Faso. Phytochemistry revealed the presence of certain secondary metabolites in both A. indica extracts, such as Flavonoids, Tannins and Saponosides. These metabolites had been identified by other authors as having anthelmintic activities (Barrau et al. (2005), Ayers et al. (2008), Chan-Pérez et al., 2016, Vargas-Magana, 2014).

On the inhibition of H. contortus egg hatching, the results showed that the dichloromethane extract was more effective than the acetone extract at doses of 5, 2.5 and 1.25mg/mL with rates ranging from 73.93%; 73.03%; 65.24% respectively. However, acetone extract recorded a high inhibition rate at doses of 10 and 20 mg/mL with 85.59% and 92.04% respectively. Overall, rates ranged from 65 to 84% for dichloromethane extracts and from 32 to 92% for acetone extracts. The results are slightly similar to those obtained by Zabré et al (2024), who studied the inhibition of H. contortus egg hatching using aqueous and ethanolic extracts of A. indica, with efficacy rates ranging from 18% to 85% for the aqueous extract and 54% to 93% for the ethanolic extract. Authors such as Hounzangbe-Adote et al. (2005) showed that 30% alcoholic extracts of leaves of Zanthoxylum zanthoxiloides, Morinda lucida, Newbouldia levis and seeds of Carica papaya inhibited hatching of Haemonchus contortus eggs only to the level of 40-60% at concentration of 2.4 mg/mL.

For mortality of adult female worms, results showed that concentrations of 5, 10 and 20 mg/mL showed the highest mortality rates compared with doses of 2.5 and 1.25 mg/mL. Mortality rates varied with time. The results show that the highest mortality rate for the acetone extract was recorded with the 20 mg/mL dose, at 77%, from the 2nd hour of exposure. For dichloromethane extract, apart from 0 hours' exposure, all concentrations recorded mortality rates regardless of time. At 6 hours exposure, doses of 5, 10 and 20 recorded 100% mortality. In this study, mortality was recorded after 2 hours of exposure. The same results were found by Zabré et al (2024) with aqueous and ethanolic extracts of A. indica on the same types of parasite.

In general, dichloromethane extract was more effective than acetonic extract in all tests. The dichloromethane extract contained more secondary metabolites than the acetonic extract. This efficiency is due to the chemical compounds contained in the plants. These compounds act either individually or synergistically on nematodes. Several studies have demonstrated the efficacy of secondary metabolites on the developmental stages of gastrointestinal parasites. These include: i) tannins, which are capable of interacting with the parasite's digestive epithelium to inhibit feeding functions and lead to parasite death (Min et al., 2003). Also, Alonso-Díaz et al. (2008a), Molan et al. (2002), Molan and Faraj (2010), Olivera et al. (2011a) suspected tannins to be responsible for the inhibition of L3 larval unsheathing and the mortality of adult C. elegans worms (Katiki et al. (2013); ii) flavonoids Barrau et al. (2005) and Ayers et al. (2008); iii) saponosides, which are said to generate ions and cause membrane peroxidation, thus inhibiting parasite development (Nandi et al., 2004).

Conclusion:-

The present work has enabled us to show that A. indica has anthelmintic properties on H. contortus. Both extracts showed anthelminthic effects on both stages of the parasite. Phytochemical results showed that A. indica contains flavonoids, tannins, saponosids and alkaloidswhich could act either in isolation or synergistically to combat the parasite. So, we can say that A. indica could be an alternative to gastro-intestinal nematodes. However, in vivo trials are needed to confirm the in vitro results

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