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In vitro Anthelmintic Activity of *Bidens pilosa* Linn. (Asteraceae) Leaf Extracts against *Haemonchus contortus* Eggs and Larvae

Mbogning Tayo Gertrude[1], J. Wabo Poné[1*], Komtangi Marie Claire[1], Yondo Jeannette[1], M. Ngangout Alidou[1] and Mpoame Mbida[1]

[1]Department of Animal Biology, Faculty of Science, University of Dschang, P.O. Box 067 Dschang, Cameroon.

Authors' contributions

This work was carried out in collaboration between all authors. Authors MTG, JWP and MM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors KMC and YJ managed the analyses of the study. Author MNA managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Haemonchus contortus is a major health and welfare problem for small ruminants, responsible for economic losses through reduced productivity and increased mortality. The *in vitro* efficacy of *Bidens pilosa* was determined against this gastrointestinal nematode (GIN). Fresh eggs, embryonated eggs and larvae (L_1 and L_2) were incubated at room temperature in infused aqueous extract, macerated aqueous and ethanolic leaf extract of *B. pilosa* at concentrations of 0.625, 1.25, 2.5, 3.75 and 5 mg/ml for 48, 6 and 24 hours, respectively. Distilled water and 1.5% Tween 80 were used as negative controls. They did not affect development of eggs and larvae whereas extracts showed a concentration dependent activity eventhough aqueous extracts exhibited a weak activity on the different developmental stages of *H. contortus* compared to ethanolic extract. Ethanolic extract was more potent on larvae than on eggs. It inhibited 92.5±7.5% and 67.4±7.4% egg embryonation and egg hatch at 5 mg/ml, with IC_{50} values of 2.1 mg/ml and 3.3 mg/ml respectively and induced 100±0% and 89.8±3.2% L_1 and L_2 larvae mortality at 5 mg/ml with LC_{50} values of 1.8 and 1.96 mg/ml respectively. The overall findings of the current study indicated that the evaluated medicinal plant in occurrence *B.*

**Corresponding author: Email: waboponejosue@yahoo.fr;*

pilosa possess potential anthelmintic effect and further *in vivo* and toxicity evaluation are indispensable to validate its use as anthelmintic for the control of GIN.

Keywords: Anthlemintic; Bidens pilosa; eggs; Haemonchus contortus; In vitro; larvae.

1. INTRODUCTION

Gastrointestinal nematode (GIN) infections remain one of the greatest limiting factors to successful and sustainable livestock production worldwide [1]. Among different types of parasitic infections, GIN infections are the most important as far as their adverse effects and prevalence are concerned. They cause weight loss, reduced feed intake, impaired fertility, lowered immunity, damaged gastric function and high mortality rate [2], leading to enormous economic losses. In Cameroon, Awa et al. [3] demonstrated that GINs are responsible for high mortality rate in goats and sheep. Moreover, a post mortem examination of sheep revealed that more than 75% of mortality was attributable to nematodiasis [4]. During decades, commercial anthemintics have been used effectively to control infections. However, indiscriminate administration of the drug has lead to development of a wide range of resistant strain of nematodes [5]. Commercial drugs are unavailable and unaffordable to farmers of developing countries with poor socio-economic status since in most of the cases these drugs are imported, hence are expensive and inaccessible as they are sold at the agricultural offices for safety and control purposes [6]. Therefore, an integrated approach is required for the effective control of helminths. World Health Organization (WHO) estimated that 80% of the world's population, mostly in developing countries depends on traditional medicine for their primary healthcare requirements [7]. These estimates are likely to be the same for livestock. The plant kingdom is known to provide numerous species of plants capable of providing bioactive compounds as a chemical defense mechanism against pathogens [8]. A number of medicinal plants have been used to treat parasitic infection in man and animal [9]. However, evidence of their effectiveness and safety is not always scientifically proved. *Bidens pilosa*, commonly called "*Tseneug* in *yemba*" is a medicinal plant used in Dschang-Cameroon to treat intestinal worms and gastrointestinal tract related complications. The literature review revealed that, leaves of *B. pilosa* contains secondary metabolites such as saponins, alkaloids, steroids, flavonoïdes, tannins, sesquitrepenes, chalcones, phytosterols, diterpenes, esculin and flavones [10,11,12]. The objective of this study was to evaluate the *in vitro* ovicidal and larvicidal activities of *B. pilosa* (Asteraceae) leaf extracts on the GIN of small ruminants (*Haemonchus contortus*) using egg embryonation, egg hatch and larval mortality assays.

2. MATERIALS AND METHODS

2.1 Collection and Storage of Plant Material

Leaves of *Bidens pilosa* were collected at the teaching and research farm of the University of Dschang-Cameroon. An entire plant was taken to the National Herbarium of Cameroon where it was identified under the reference number 32987/HNC as *Bidens pilosa* Linn. The collected plant material was dried in shade, at ambient temperature for three weeks [13]. Dried leaves were ground to powder and stored in airtight plastic bags in the Laboratory of Biology and Applied Ecology of the University of Dschang.

2.2. Plant Extracts Preparation

The infused and macerated aqueous extracts as well as ethanolic extract were prepared to compare their activities on eggs and larvae (L_1 and L_2) of *H. contortus*. Extraction was done according to the procedure described by Wabo Pone et al. [14,18] Briefly, for ethanolic extract, 100 g of stored powder were macerated in 1.5 l of ethanol 95%. The mixture was daily stirred to permit better extraction of the active ingredients. Seventy-two (72) hours later, this solution was sieved and filtered through a cotton layer and a filter paper of pore size 2.5 µm. The filtrate was evaporated in a rota vapor at 82ºC for 8 hours. The extract obtained was poured in a large Petri dish and allowed at room temperature for two days, at the end of which a completely dried ethanolic extract was obtained. For aqueous extracts a similar procedure was carried out except that, hot (at 100ºC) and cold distilled water were used as solvent for infusion and maceration aqueous extract, respectively. Infusion took 3 hrs and maceration 48 hrs to avoid growth of fungi. Also dried aqueous extracts were obtained after 7 days of filtrates in a ventilated oven heated at 50ºC. Each dried extract was used to prepare a stock solution which was then diluted with distilled water to obtain five different solutions of concentrations 1.25, 2.5, 5, 7.5 and 10 mg/ml. The final tested concentrations were 0.625, 1.25, 2.5, 3.75 and 5 mg/ml.

2.3 Parasites Donor Goat

Goats' abomasums were obtained from the abattoir of the "Marché B" of Dschang town after necropsy of animals. Adult female worms were identified using morphometric and morphologic characteristics according to Soulsby [14]. These female worms were crushed to liberate eggs [15]. The eggs were then cultured *in vitro* in Petri dishes at room temperature for seven days [16]. The culture media was 3 ml of sterile liquid of faeces prepared from 3 g of faeces removed in the rectum of parasites free goat, to which was added charcoal. At the end of the 7^{th} day, infective larvae were harvested. About 2500 larvae were estimated by counting the number of larvae contained in 0.1 ml of a well homogenized solution of infective larvae. After five repetitions of counting, the mean number of larvae in 0.1 ml of solution was determined and the volume containing 2500 larvae were deduced, measured and inoculated into a worm-free goat kept indoors in a separate house at the teaching and research farm of the University of Dschang throughout the study period. This goat served as *H. contortus* egg donor for subsequent *in vitro* trials.

2.4 Recovery of Nematode Eggs

After the pre-patent period of 18 to 21 days [16], three (3) g of faeces directly collected (each day) from the rectum of the donor goat mentioned above were used in recovering eggs according to the procedure carried out by Wabo Poné et al. [17]. Briefly, collected faeces were homogenized in a motar, adding 60 ml of salt solution (NaCl 40% W/V). The solution was then cleared of organic debris by filtration through a 250 µm mesh-size sieve into a beaker and finally poured into four conical tubes until the formation of a meniscus at the top. Then, a cover slide was deposited on each tube, in direct contact with the solution. Three (3) minutes later, the cover slides were removed and put on a slide for examination under an optical microscope at 4x objective to look for the presence of eggs. Slides and cover slides containing the eggs were rinsed with distilled water into a 100 ml beaker. The beaker was allowed to stand for 30 minutes for the sedimentation of the eggs at the bottom. To completely remove the salt solution, eggs were washed three times by siphoning out 90 ml of solution and replacing with the same amount of distilled water each 30 minutes. Finally,

the supernatant was removed and the remaining solution containing eggs was used in the assay.

2.5 Evaluation of Ovicidal Activity

Fresh eggs and embryonated eggs of *H. contortus* were used to evaluate the ovicidal efficacy of different extracts. To assess the effects of the extracts on embryonation, 1 ml of suspension containing 30 to 40 fresh eggs was distributed in each of 15 Petri dishes (35 mm ⌀ x 10 mm) and mixed with the same volume of each concentration of a given extract. Two (2) other Petri dishes were used as negative control and were mixed with 1 ml of distilled water and 1 ml of 1.5% Polyoxyethylene Sorbitan Monooleate (Tween 80). The dishes were covered and eggs incubated at room temperature for 48 hours, after which the number of L_1 larvae per Petri dish was counted under a microscope (at 4x magnification). The percentage of embryonation inhibition (EI%) was calculated using the formula:

$$\text{EI}\ (\%) = 100 - [\left(\frac{\text{Number of L1 larvae}}{\text{Number of fresh eggs in culture}}\right)\ \text{x}\ 100]$$

To test the effect of plant extracts on hatching process, the same number of embryonated eggs were distributed in Petri dishes as above and allowed at room temperature for 6 h. After this time, when there were at least 90% of hatching in the control Petri dishes, 2 to 3 drops of Lugol's iodine were added to each Petri dish to fix the different life cycle stages of the parasite. The first stage larvae (L_1) were counted under a microscope (at 4x magnification). The percentage of hatching inhibition (HI%) was compute as follow:

$$\text{HI}\ (\%) = 100 - [\left(\frac{\text{Number of L1 larvae}}{\text{Number of embryonated eggs in culture}}\right)\ \text{x}\ 100]$$

2.6. Recovery of Nematode Larvae

To eggs solution obtained above, a solution of faeces prepared from the goat free of parasite was added and the mixture was allowed at room temperature (24 – 25ºC) in a beaker for 2 days and 4 to 5 days to obtain L_1 and L_2 larvae, respectively. The solution containing the larvae was then distributed in conical tubes and allowed for 10 minutes. The supernatant was carefully siphoned using a pipette and Ringer solution was added in tubes to optimize the survival of larvae [18].

2.7. Evaluation of Larvicidal Activity

Larval mortality assay using L_1 and L_2 larvae were performed according to Wabo Poné et al. [19,20]. Briefly**,** 1ml of a solution containing about 15 to 20 parasite larvae (L_1 or L_2) was distributed in each of 15 Petri dishes and mixed with the same volume of a plant extract at different concentration. The dishes were covered and kept at room temperature for 24 h, after which the number of dead larvae was counted under a microscope (at 4x magnification) based on their straight shaped, their immobility and the presence of holes and irregularities on their external layer. The percentage of mortality (Mt%) was determined using the following formula:

$$\text{Mt}\ (\%) = \left(\frac{\text{Number of dead larvae}}{\text{Number of larvae in culture}}\right)\ \text{x}\ 100$$

2.8 Statistical Analysis

The inhibitory 50% concentrations (IC_{50}) for embryonation and hatching rates were calculated using equations of regression lines drawn after transformation of the embryonation inhibiting rate and hatching inhibiting rate to probit according to the decimal logarithm of concentrations. While the lethal 50% concentrations (LC_{50}) for L_1 and L_2 larvae were determined using equations of regression lines drawn after transformation of larval mortality rate to probit according to the decimal logarithm of concentrations.

Comparison of the mean inhibition percentage of egg embryonation, mean inhibition percentage of egg hatch and mean percentage of larval mortality at different concentrations with control was performed by two-way analysis of variance (ANOVA). Statistical analyses were performed using the software SPSS version 17.0. The post hoc statistical significance test employed was Duncan, differences between the means were considered significant at $P < 0.05$. The 50% inhibitory concentration (IC_{50}) and lethal concentration (LC_{50}), i.e., effective concentration to inhibit 50% of the eggs and to kill 50% of larvae were determined using the regression lines of the probit according to decimal logarithm of the concentrations.

3. RESULTS

Table 1 presents the variation of the mean egg embryonation inhibition and egg hatch inhibition of *H. contortus* at different concentrations. For egg embryonation, distilled water and 1.5 % Tween 80 allowed the normal embryonation of the eggs with mean inhibition rate of 0.0±0.0% and 2±2.3% respectively. Aqueous extract presented a weak activity with mean inhibition rate less than 50% at concentrations less or equal to 2.5 mg/ml. However, at 3.75 mg/ml and 5 mg/ml, infused aqueous extract inhibited 55.8±17.7% and 69.2±7.2% egg embryonation respectively with no significant difference ($P < 0.05$). While at these same concentrations, ethanolic extract inhibited 74.7±4.1% and 92.5±7.5% egg embryonation respectively with a significant difference ($P < 0.05$). The IC_{50} values obtained on fresh eggs after transformation of the egg embryonation inhibition rate to probit (not illustrated) were 31 mg/ml, 3.1 mg/ml and 2.1 mg/ml for macerated, infused and ethanolic extracts respectively. The gradient of inhibition of embryonation is as follows: EthE > IAE > MAE.

The variation of the mean egg hatch inhibition rate of *H. contortus* revealed that as on fresh eggs, both distilled water and 1.5% Tween 80 had no effect on embryonated eggs with mean egg hatch inhibition rate of 2±2.3%. Aqueous extracts also exhibited weak activity on egg hatch with inhibition rates which remain less than 40% at all the concentrations tested. While ethanolic extract of *B. pilosa* inhibited 61.7±11.2% and 67.4±7.3% egg hatch at 3.75 and 5 mg/ml respectively with no significant difference ($P < 0.05$). The IC_{50} values obtained from inhibition of embryonated eggs were 25.7 mg/ml, 23.3 mg/ml and 3.3 mg/ml for infused, macerated and ethanolic extracts respectively. The gradient of egg inhibition hatch is as follows: EthE > MAE > IAE.

Table 1. Effect of *Bidens pilosa* leaf extracts on mean egg embryonation and mean egg hatch inhibition of *Haemonchus contortus*

Concentrations (mg/ml)	Egg embryonation inhibition (mean ± Sd)			Egg hatch inhibition (mean ± Sd)		
	IAE	MAE	EthE	IAE	MAE	EthE
DW	0 ± 0^{Aa}	0 ± 0^{Aa}	NA	2 ± 2.3^{Aa}	2 ± 2.3^{Aa}	NA
1.5 % Tween 80	NA	NA	2 ± 2.3^{a}	NA	NA	2 ± 2.3^{a}
0.625	0 ± 0^{Aa}	1.9 ± 3.8^{Aa}	5.1 ± 6.5^{Ba}	5 ± 7^{Aa}	5.1 ± 4.2^{Aab}	5.8 ± 4.2^{Aa}
1.25	23.3 ± 20.4^{Bb}	10.4 ± 10.1^{Aab}	28.1 ± 23.1^{Bb}	9.9 ± 17.3^{Aa}	8.8 ± 10.7^{Aab}	9.2 ± 8.3^{Aa}
2.50	42.3 ± 7.5^{Bbc}	18.7 ± 11.5^{Abc}	55.8 ± 10.7^{Cc}	11.5 ± 19.9^{Aab}	12 ± 6.3^{Abc}	38.9 ± 12.7^{Bb}
3.75	55.8 ± 17.7^{Bcd}	18.2 ± 5.4^{Abc}	74.7 ± 4.1^{Cd}	14.3 ± 13.3^{Aab}	21.4 ± 5.9^{Bcd}	61.7 ± 11.2^{Cc}
5.00	69.2 ± 7.2^{Bd}	24.2 ± 10.7^{Ac}	92.5 ± 7.5^{Ce}	32.4 ± 17^{Bb}	25.9 ± 11.4^{Ad}	67.4 ± 7.3^{Cc}

Small letters compare means in a column and capital letters means in a row. Different letters indicate significant difference (P < 0.05)
Legend: DW = Distilled water; IAE = Infused aqueous extract; MAE = Macerated aqueous extract; EthE = Ethanolic extract; Sd = Standard deviation; NA = Not applicable

Table 2. Effect of *Bidens pilosa* leaf extracts on mean L_1 and L_2 larval mortality of *Haemonchus contortus*

Concentrations (mg/ml)	L_1 larval mortality (mean ± Sd)			L_2 larval mortality (mean ± Sd)		
	IAE	MAE	EthE	IAE	MAE	EthE
DW	0 ± 0^{Aa}	0 ± 0^{Aa}	NA	0 ± 0^{Aa}	0 ± 0^{Aa}	NA
1.5 % Tween 80	NA	NA	0 ± 0^{a}	NA	NA	0 ± 0^{a}
0.625	2.8 ± 5.6^{Aa}	0 ± 0^{Aa}	5.2 ± 6.3^{Bab}	0 ± 0^{Aa}	0 ± 0^{Aa}	7.7 ± 9^{Ba}
1.25	7.3 ± 10.5^{Bab}	0 ± 0^{Aa}	16.3 ± 6.6^{Cab}	5.6 ± 6.6^{Ba}	0 ± 0^{Aa}	20.6 ± 12.5^{Cb}
2.50	17.5 ± 12.6^{Bb}	4.6 ± 5.3^{Aa}	20.1 ± 25.8^{Bb}	37 ± 24.2^{Bb}	4.4 ± 5.2^{Aab}	65.2 ± 4.2^{Cc}
3.75	31.8 ± 4.7^{Bc}	11.8 ± 13.7^{Aa}	87.4 ± 4.3^{Cc}	60.6 ± 21.3^{Bc}	12.1 ± 10.7^{Abc}	84.4 ± 10.4^{Cd}
5.00	55.5 ± 13.2^{Bd}	33.1 ± 10.7^{Ab}	100 ± 0^{Cc}	63.4 ± 10.1^{Bc}	18.8 ± 9^{Ac}	89.8 ± 3.2^{Cd}

Small letters compare means in a column and capital letters means in a row. Different letters indicate significant difference (P < 0.05)
Legend: DW = Distilled water; IAE = Infused aqueous extract; MAE = Macerated aqueous extract; EthE = Ethanolic extract; Sd = Standard deviation; NA = Not applicable

Table 2 (above) shows the effects of different extracts of *B. pilosa* on L_1 and L_2 larvae of *H. contortus* after 24 hours of contact. Distilled water and 1.5% Tween 80 allowed the normal development of the larvae, with mean mortality rate of 0.0±0.0%. The aqueous extracts showed weak activity on L_1 larvae by inducing less than 40 % larval mortality at concentrations less or equal to 3.75 mg/ml. However, at 3.75 mg/ml, ethanolic extract caused mortality of 87.4±4.3% and 100±0.0% L_1 larvae with a significant difference ($P < 0.05$). The LC_{50} values obtained from L_1 mortality rate were 6.2 mg/ml, 5.7 mg/ml and 1.8 mg/ml for macerated, infused and ethanolic leaf extracts of *B. pilosa* respectively. The gradient of L_1 larval mortality is as follows EthE > IAE > MAE. As on L_1 larvae, macerated aqueous extract has a weak activity on L_2 larvae by inducing less than 20% larval mortality at all the concentrations tested while, infused aqueous extract induced 60.6±21.3% and 63.4±10.1% L_2 larval mortality at 3.75 and 5 mg/ml respectively, with no significant difference ($P < 0.05$).

Concerning ethanolic extract, it induced 65.2±4.2% L_2 mortality at 2.5 mg/ml which reached 89.8±3.2% at 5 mg/ml with a significant difference ($P < 0.05$). The LC_{50} values obtained with L_2 larvae were 7 mg/ml, 3.5 mg/ml and 1.96 mg/ml for macerated, infused and ethanolic extracts respectively. The gradient of L_2 larval mortality was the same as for L_1 larval mortality i.e EthE > IAE > MAE.

4. DISCUSSION

In this study, we deal with goat experimentally infected with *H. contortus*, a species of GIN which have been documentated as veterinary important worldwide [21]. The anthelmintic activity of three extracts (infused aqueous extract, macerated aqueous extract and ethanolic extract) of *B. pilosa* leaves were therefore evaluated on the eggs and the larvae of this GIN of small ruminants (sheep and goats). The major finding of this study was the high efficacy of ethanolic extract as compare to aqueous extracts on the egg embryonation and larval mortality assays. In fact, ethanolic extract of *B. pilosa* inhibited 92.5±7.5% and 67.4±7.3% egg embryonation and egg hatch at 5 mg/ml and induced 100±0% and 89.8±3.2 % L_1 and L_2 larvae mortality at this same concentration. While on one hand, infused aqueous extract inhibited 69.2±7.5% egg embryonation, 32.4±17.1% egg hatch, and induced 55.5±13.2% L_1 larvae mortality and 63.4±10.1% L_2 larvae mortality. On the other hand, macerated aqueous extract inhibited only 24.2±10.7% egg embryonation, 25.9±11.4% egg hatch and induced 33.1±10.7% and 18.8±9% L_1 and L_2 larval mortality respectively, at 5 mg/ml. Almost similar results have been validated by Wabo Poné et al. [22] and Payne et al. [23] where water extracts of *Erythrina sigmoidea* and *Dichrocephala integrifolia* presented a weak activity on eggs and larvae of *H. bakeri* as compared to ethanolic extract. The variation in activity of the extract types of the plant might be due to difference in the proportion of the active components responsible for the tested anthelmintic activity resulting from the difference in solubility in solvent [24]. It could also be probably related to the different chemical ingredients extracted in different solvents and their biological effects on parasites [25]. The possible explanation for better anthelmintic activity of ethanolic extract compared to aqeous extract on larvae could be due to easier transcuticular absorption of the ethanolic extract into body of parasites than the aqueous extracts [26], since transcuticular diffusion is a common mean of entry of non-nutrient and non-electrolyte substances into helminth parasites. As recorded in the present study, ovicidal and larvicidal effects of some plants have also been reported earlier against *H. contortus* eggs and larvae [27,28,29,24,30,31,32]. Even though egg inhibition activity was observed in the current study, not all eggs were inhibited and some managed to hatch. This might be because the egg is the stage disseminated into the

environment and is protected with a thick wall making it resistant to various environmental conditions [33]. However 100% larval mortality obtained in this work could be explained by the fact that the most susceptible stage of the larvae is the pre-infective one, that is the feeding stage and they are much exposed to medicinal components than eggs [34]. The study showed that, efficacy of extracts increased with increasing concentration of extracts, which could be due to the saturation of target receptors. Lullman et al. [35] observed that, the receptors get saturated with increasing dose of active ingredients that increases with incubation period. The anthelmintic activity of *B. pilosa* extracts could be attributed to the variety of secondary metabolites present. Okoli et al. [11] revealed presence of saponins, alkaloid and steroids in that plant and Khemraj et al. [12] demonstrated the presence of flavonoids, tannins, sesquiterpenes, esculin, diterpenes, chalcones and phytosterols. These plant metabolites may have worked in combination or singly to cause inhibition of eggs or mortality of larvae that was observed in this work. According to Schoenian [36]**,** anthelmintic drugs like the benzimidazoles kill the parasites by binding to the beta-tubulin and prevent its incorporation into micro-tubules which are essential for energy metabolism. Paralysis of parasites tissues makes them unable to feed, leading to death as results of lack of energy. Thus, may be our compounds caused their effect through the same mechanism. Moreover chemically, tannins are polyphenolic compounds and synthetic phenolic anthelmintic such as niclosamide and oxyclozanide are said to interfere with energy generation in helminths parasites by uncoupling oxidative phosphorylation [37]. Tanins contained in *B. pilosa* could have produced similar effect. It was also reported that tanins, which are rich in glycoproteins connect to free proteins or to the larval cuticle, reducing the availability of nutrients which in turn results in the death of the larvae due to starvation [38]. Saponnins present in *B. pilosa* are also known to cause feed refusal and starvation of the parasites, leading to their death due to lack of energy [25]. The anthelmintic activity of *B. pilosa* may also be due to the presence of alkaloids which have ability to intercalate with DNA synthesis of parasites [26]. According to Tarnopolsky and Beal [39], alkaloids are competitive antagonist at muscarinic acethylcholine receptor preventing the binding of acethycholine and are report physiologically active with sedative and analgesic properties in addition to leading to excitation of cells and neurological dysfunction.

5. CONCLUSION

In conclusion, findings of the present study showed that ethanolic and aqueous extract of *B. pilosa* exhibited evidence of *in vitro* anthelmintic activity against eggs and larvae of *H. contortus*. However, further *in vivo* studies are needed to evaluate bioactivity of the extracts of the above plant and, to investigate the potential presence of toxic effects in order to determine the minimum non-lethal doses for the treatment of GIN.

CONSENT

Not applicable.

ETHICAL APPROVAL

This work was carried out in accordance with the Animal Ethical Committee of the Animal Biology Department of the University of Dschang, Cameroon.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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