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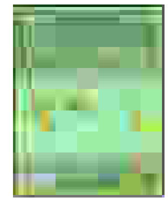

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In vitro activities of acetonic extracts from leaves of three forage legumes (*Calliandra calotyrsus, Gliricidia sepium and Leucaena diversifolia*) on *Haemonchus contortus*

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ABSTRACT

Objective: To assess ovicidal activity of three acetonic extracts from the leaves of three forage legume, *Calliandra calotyrsus (C. calotyrsus)*, *Gliricidia sepium (G. sepium)* and *Leucaena diversifolia (L. diversifolia) in vitro* on *Haemonchus contortus (H. contortus)*. **Methods:** Eggs were exposed for 24 hours to five different concentrations (0.075, 0.15, 0.3, 0.6 and 1.25 mg/mL) of acetonic extracts at room temperature (24 ℃). Distilled water and 0.4% Tween were used in the bioassay as negative controls. **Results:** The later did not affect embryonation and egg hatching of *H. contortus*. Conversively, significant effects were obtained with the acetonic extracts of leaves of all three plants and the maximum activity was observed with the highest concentration (1.25 mg/mL). The acetonic extract of *G. sepium* was found to be more active (2.9% and 0.0% for embryonation and egg hatching, respectively) than the other substances 16.5% and 33.5%, respectively for *C. calothyrsus*, 33.7% and 33.3%, respectively for *L. diversifolia*. **Conclusions:** These results suggest that the three forage legumes do possess ovicidal properties and further studies on larvae should be carried out.

1. Introduction

Small ruminants play an important role in food production for developing countries. Sheep or goat meat contributes 3.5% of animal proteins in these countries compared to 0.2% in the developed world[1]. In Africa where climatic factors such as humidity and temperature favour the development of parasites, 97% of small ruminants suffer from gastro-intestinal helminthiases. Among these diseases, haemonchosis is the one which has a significant impact on productivity and leads to serious economic losses for small holders. In Kenya for example, the loss to the agricultural sector was recently estimated at US$ 26 million per year[2] .Commercial anthelmintics have been mainly used for some decades for the control of intestinal helminths. However, these synthetic compounds are on one hand not available in the tropics and sub-tropics and on the other hand their costs in the small local market are relatively high. In addition, the misuse of these substances by the farmers has led to the development of resistant organisms in many developed countries[3,4]. Therefore new approaches for helminth control are needed especially in countries of the South. Works carried out in this field to assese the *in vitro* anthelmintic activities of extracts of: *Vernonia amygdalina* and *Annona senegalensis* in Nigeria, *Canthium mannii (C. mannii)* in Cameroon, *Melia azedarach* in Brazil and *Cucurbita moschata* in Guadeloupe, respectively[5-9]. The discovery of natural new cheaper drugs with relatively low toxicity seems to be a desirable solution[10,11]. The present study aimed to evaluate *in vitro* the activities of acetonic extracts of leaves of three forage legumes namely *Calliandra calotyrsus (C. calotyrsus)*, *Gliricidia sepium (G. sepium)* and *Leucaena diversifolia (L. diversifolia)* on *Haemonchus contortus (H. contortus)*, one of the most pathogenic grastrointestinal nematode of small ruminants. These forages are used to feed animals.

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2. Materials and methods

2.1. Plant materials

Leaves of *C. calotyrsus*, *G. sepium* and *L. diversifolia* were collected in the experimental field of the Faculty of Agronomy and Agricultural Sciences of the University of Dschang. These specimens were dried separately in a ventilate oven at a maximum temperature of 50 ℃ for two days, ground and stored in airtight plastic bags for about one week, at room temperature (25 ℃) and relative humidity 67% for subsequent use in the laboratory.

2.2. Preparation of acetonic extracts of leaves

Five hundred grams of each leaf powder were macerated in 1.5 L of acetone which removed phenolic compounds from the leaves[12]. Each mixture was daily stirred, and 72 hours later the acetonic extracts were obtained[13]. A total of 100 mg of a given concentrated extract was diluted with 0.4 mL of 0.4% tween. Then distilled water was added to the solution to obtain a total volume of 20 mL. This resulted to a 5 mg/mL stock solution from which a series of dilutions were made to obtain different final tested concentrations: 0.075, 0.15, 0.3, 0.6 and 1.25 mg/mL.

2.3. Reference drug

The reference drug, Mebendazole (MBZ), use in larvicidal test was bought in a local pharmacy (Mebencol N. D., Colorama pharmaceutical Ltd). The product was diluted in distilled water to obtain a stock solution with the same concentration as with organic extracts. The controls used for the bioassay were 0.4% tween and distilled water.

2.4. Recovery of nematode eggs

Fresh eggs of *H. contortus* were obtained from the faeces of sheep experimentally infected[14]. Briefly, 3 g of faeces were collected, homogenised in a mortar, suspended in saturated salt solution (0.4% NaCl), and cleaned of organic debris by filtration through sieves (1 mm and 150 μm) into a 100 ml beaker. The contents of the latter were poured into four tubes and centrifuged at 1 000 *g* for 5 min. The supernatant was poured through a 45 μm sieve then, the retained material on the sieve containing eggs was washed with tap water to remove the salt solution. The sieve was then turned, the opposite side was washed with tap water and eggs were collected in a Petri dish (Ø=16 cm)[15].

2.5. Evaluation of ovicidal activity

The ovicidal efficacy test of the different acetonic extracts was performed using two different procedures. To assess the effects of the extracts on fresh eggs, 1 mL of a diluted solution containing about 48 parasite eggs was distributed in each of 12 Petri dishes (35 mm Ø × 10 mm) and mixed with the same volume of a specific plant extract at different concentrations. The dishes were covered and the eggs incubated at room temperature for 24 hours, after which the number of embryonated eggs per Petri dish was counted using a microscope (at 4× magnification). The percentage of embryonation (EM%) was determined using the formula below[7]:

$$E(\%) = \frac{\text{Number of embryonated eggs}}{\text{Number of eggs in culture}} \times 100$$

In the second experiment, the hatching rate or eclodibility (E%) was calculated by the formula below[6]:

$$E(\%) = \frac{\text{Number of } L_1 \text{ larvae}}{\text{Number of embryonated eggs in culture}} \times 100$$

2.6. Recovery of nematode larvae

3 mL of the egg suspension were poured on a filter paper covering the bottom of two Petri dishes. The dishes were then covered to maintain a high relative humidity (65%–67%) to prevent from drying out, and stored at 24℃. After 3 and 4–5 days of incubation, L_1 and L_2 larvae were observable in Petri dishes, respectively. They were differentiated using their morphological features[16] and were concentrated with a Baermann apparatus[17,18].

2.7. Evaluation of larvicidal activity

To test the effects of the extracts on L_1 and L_2 larvae, 1 mL of a solution containing about 30 parasite larvae was distributed in each of 12 Petri dishes (35 mm Ø × 10 mm) and mixed with the same volume of a specific plant extract or MBZ at different concentrations. The dishes were covered and kept at room temperature for 24 hours, after which the number of dead or immobilize larvae was determined under a microscope (at 4× magnification). The percent mortality (Mc%) was calculated using Abott's formula[5]:

$$Mc(\%) = \frac{Mce-Mt}{100-Mt} \times 100$$

Where Mc, Mce and Mt correspond to the corrected mortality; the ones which were obtained during the test, and registered in the negative control dishes, respectively. When the rate in the latter dishes is less than 5%, Mc=Mce[19].

The larvicidal concentration 50 (LC_{50}) was determined using the regression line of the mortality expressed in probit according to the decimal logarithm of the concentration. All tests were repeated six times for each treatment and control.

2.8. Data analysis

The mean percentage of embryonation, hatching and the mean mortality rates were compared using the *chi*-square test at the $P<0.05$ significance level.

3. Results

The effects of different acetonic extracts of the leaves of three forage legumes (*C. calotyrsus*, *G. sepium* and *L. diversifolia*) were given in Table 1. The mean embryonation rate of fresh eggs of *H. contortus* were all 96.97±2.62 and the hatching rate were 100% in 0.4% tween and distilled water. The values obtained in both embryonation and egg hatch tests with various concentrations of determined extracts differed significantly from negative controls. In general, the mean embryonation rates decreased gradually with the concentration of different acetonic extracts. At 0.075 mg/mL, the effect of *C. calothyrus* leaf extracts was similar to that of the negative controls. Conversely, at the concentration higher or equal to 0.3 mg/mL, the acetonic extract of *C. calothyrsus*, *G. sepium*, *L. diversifolia* significantly reduced ($P<0.05$) the embryonation of *H. contortus* and this activity was stronger at the highest concentration. Thus, at 1.25 mg/mL the mean inhibition rate of embryonation was 100%. Concerning the effects of various extracts on egg hatching (Table 2), it was observed that, the hatching rate of L_1 larvae decreased with increasing concentrations of extracts as in the first experiment. *G. sepium* extract at concentration of 0.075 mg/mL inhibited eclodibility for about 20% and this rate fell to 0% at concentration equal to or higher than 1.5 mg/mL. The extract of the later forage legume seem to be more active in this second experiment than the other two extracts. 0.4% tween and distilled water did not provoke mortality of L_1 and L_2 larvae of *H. contortus*. Conversively, with the mebendazole and the different acetonic extracts the mortality rates increased in concentration dependence. The later seems to be more efficient producing mortality rates of 44%-97%. Mortality due to the acetonic extracts of *C. colotyrsus* and *L. diversifolia* were similar and higher than that due to *G. sepium* extracts at concentration less or equal to 0.6 mg/mL. At the highest tested concentration (1.25 mg/mL), the activity of the extracts of *G. sepium* and *L. diversifolia* was stronger than that of *C. calotyrsus*. For concentrations higher than or equal to 0.3 mg/mL, these three extracts inhibited significantly (P<0.05) larval survival of *H. contortus*. Meanwhile, the larvicidal activity of different extracts was similar to that of the positive control at 1.25 mg/mL. After the transformation of the mortality rate to probit according to the decimal logarithm of the concentration (mg/mL), we observe that there was a concentration dependent relationship with all the tested substances and the larvicidal concentrations 50 (LC_{50}) were 0.177, 0.463, 0.840 and 0.373 mg/mL for MBZ, *C. colotyrsus*, *G. sepium* and *L. diversifolia* respectively.

Table 1

Effects of different concentrations of acetonic extracts on embryonation and hatching of eggs of of *H. contortus* (%).

Group		Embryonation	Hatching of eggs
TW (0.4 %)		96.97±2.62	100.00±0.00
DW		96.97±2.62	100.00±0.00
Me	0.075 mg/mL	56.05±8.00	66.99±12.46
	0.15 mg/mL	40.82±11.08	59.77±16.95
	0.30 mg/mL	40.69±17.34	60.61±9.46
	0.60 mg/mL	21.22±15.79	41.28±35.75
	1.25 mg/mL	20.88±8.78	10.00±17.32
Cc	0.075 mg/mL	58.67±33.01	68.98±21.62
	0.15 mg/mL	42.87±9.12	55.37±5.57
	0.30 mg/mL	29.89±26.91	55.97±17.02
	0.60 mg/mL	22.13±20.06	52.36±11.33
	1.25 mg/mL	16.49±14.51	33.54±6.37
Gs	0.075 mg/mL	46.65±8.33	19.95±7.56
	0.15 mg/mL	33.44±11.01	14.17±7.00
	0.30 mg/mL	30.21±12.19	0.00±0.00
	0.60 mg/mL	15.79±6.14	0.00±0.00
	1.25 mg/mL	2.09±3.61	0.00±0.00
Ld	0.075 mg/mL	62.07±29.58	70.75±4.17
	0.15 mg/mL	56.83±15.04	71.98±11.34
	0.30 mg/mL	54.93±7.45	63.69±21.45
	0.60 mg/mL	41.83±12.19	49.80±19.03
	1.25 mg/mL	33.72±15.59	33.29±13.97

Legend: TW (0.4%) = tween, DW= Distilled water, Me = mebendazole, Cc = *Calliandra calotyrsus*, Gs = *Gliricidia sepium*, Ld = *Leucaena diversifolia*.

On the other hand, the larvicidal effect on L_2 larvae increases proportionally with the increased of the concentration. At all tested concentrations, the acetonic extract inhibited significantly (P<0.05) the survival of L_2 larvae but, no statistical difference was observed between the activity of the extracts and that of MBZ. After the transformation of the mortality rate to probit, the regression lines obtained showed a dose dependent response according to the decimal logarithm of the concentrations, and the LC_{50} were 0.218, 0.226, 0.286 and 0.117 mg/mL for MBZ, *C. calotyrsus, L. diversifolia* and *G. sepium*. These findings showed that, the different substances seem to be more active on the L_1 larvae than on the L_2 one. It was also noticed that, the extract of *G. sepium* was more efficient on L_2.

Table 2

Effects of different concentrations of acetonic extracts on the L_1 and L_2 larvae of *H. contortus* (%).

Group		L_1 larvae	L_2 larvae
TW (0.4%)		0.00±0.00	0.00±0.00
DW		0.00±0.00	0.00±0.00
Me	0.075 mg/mL	44.93±35.54	50.66±18.13
	0.15 mg/mL	66.33±13.08	53.65±5.82
	0.30 mg/mL	80.76±12.23	83.65±26.91
	0.60 mg/mL	87.28±4.73	97.53±4.28
	1.25 mg/mL	96.56±5.96	100.00±0.00
Cc	0.075 mg/mL	37.05±21.27	40.75±26.13
	0.15 mg/mL	40.32±27.95	49.11±20.68
	0.30 mg/mL	49.91±3.71	83.99±22.95
	0.60 mg/mL	65.65±17.44	100.00±0.00
	1.25 mg/mL	79.08±18.29	100.00±0.00
Gs	0.075 mg/mL	8.60±2.74	59.91±11.67
	0.15 mg/mL	13.15±11.39	72.78±15.69
	0.30 mg/mL	39.52±30.78	81.49±15.66
	0.60 mg/mL	43.11±8.48	94.44±9.62
	1.25 mg/mL	92.06±4.46	98.04±3.39
Ld	0.075 mg/mL	29.66±17.35	41.33±34.88
	0.15 mg/mL	39.87±31.02	41.11±30.97
	0.30 mg/mL	63.92±12.11	66.39±8.99
	0.60 mg/mL	72.83±15.03	94.50±4.97
	1.25 mg/mL	94.50±4.97	90.96±8.32

Legend: TW (0.4%) = tween, DW= Distilled water, Me = mebendazole, Cc = *Calliandra calotyrsus*, Gs = *Gliricidia sepium*, Ld = *Leucaena diversifolia*.

4. Discussion

Results in the present study are similar to the report from Hounzangbe-Adote *et al*[20]. It indicates that, the active compounds in the extract penetrates the egg shell and prevents egg development or paralyse the first stage larvae within the egg as is the case with levamisol. The unidentified mass in un-embryonated egg may be as a result of segmentation inhibition of the egg. Overall, the leaf extracts of the three forage legumes negatively affected the egg of *H. contortus*. It was shown that, the acetonic extract of *G. sepium* was more active on the embryogenic development than the extracts of the other leaves. In the same manner, this effect was observed on embryonated egg compared to the effect of the other extracts and MBZ. The same findings were observed on *H. polygyrus* and *Ancylostoma caninum* with the extract of the stem bark of the shrub named *C. mannii*, a medicinal plant used traditionally by the people of Dschang, Western Region of Cameroon, Central Africa[6,7].

These observations were also shown in Guadeloupe[9]. The activity of the three extracts may be attributed to phenolic compounds and particularly to tannin present in the leaves. These substances were identified in the leaves of the three forage legumes.

At higher concentration, the extracts significantly inhibited the development of L_1 and L_2 larvae. Similar findings were reported that *Hedysanium coronarium* extracts which inhibited the migration of *H. contortus* larvae[21,22]. They also reported the activity of tannins on *Strongylus colubriformis* larvae. The larvicidal activity observed in this work with different acetonic extracts may therefore be attributed to phenolic compounds in general or to tannins in particular. These substances are able to penetrate the cuticle of the nematode and to prevent the absorption of the glucose, or block post synaptic receptors thus paralysing the larvae. Tannins may also induce the release of gamma-aminobutyric acid (GABA) which could block transmission of nerve impulses or could act in decoupling the phosphorylation oxydative reaction which led to the energy exhortion of the larvae[23]. In the same manner, tannins absorbed by insects bind on the intestinal mucosa and led to autolysis[24]. These findings can be extrapolated on nematode larvae. Also, tannins may bind on the cuticle of the larvae and led to dead. Extracts were more active on the L_1 larvae. These findings confirm the fact that, these life cycle-stages are more sensible than the L_2 one[25]. In conclusion, the different acetonic extracts tested in this work exhibited *in vitro* negative effects on larvae of *H. contortus* parasite of small ruminants. The extracts were more active on L_1 larvae and the LC_{50} obtained in this study were very low compared to that obtained with the extracts of *C. mannii* on *Heligmosomoides polygyrus* larvae[7].

In conclusion, acetonic extract of *C. calothyrsus, G. sepium* and *L. diversifolia* exhibited in vitro negative effects on eggs and larvae of *H. contortus* parasite of small ruminants. In addition to their high nutritive value on animal feeding, the leaves of the three forage legume can be use for the control of intestinal nematode of ruminants. However, further experiment incorporating *in vivo* and toxicological studies should be carried out.

Conflict of interest statement

We declare that we have no conflict of interest.

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