

## Research Article

# The *In Vitro* Effects of Aqueous and Ethanolic Extracts of the Leaves of *Ageratum conyzoides* (Asteraceae) on Three Life Cycle Stages of the Parasitic Nematode *Heligmosomoides bakeri* (Nematoda: Heligmosomatidae)

J. Wabo Poné,<sup>1</sup> Olivia Fossi Tankoua,<sup>1</sup> Jeannette Yondo,<sup>1</sup> Marie Claire Komtangi,<sup>1</sup> Mpoame Mbida,<sup>1</sup> and C. F. Bilong Bilong<sup>2</sup>

<sup>1</sup> Department of Animal Biology, Faculty of Science, University of Dschang, P.O. Box 067, Dschang, Cameroon

<sup>2</sup> Department of Animal Biology and Physiology, Faculty of Science, University of Yaounde I, P.O. Box 812, Yaounde, Cameroon

Correspondence should be addressed to J. Wabo Poné, waboponejosue@yahoo.fr

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A comparative *in vitro* study was carried out to determine the ovicidal and larvicidal activity of aqueous and ethanolic extracts of *Ageratum conyzoides* (Asteraceae) leaves on the eggs (unembryonated and embryonated), first and second larval stages of *Heligmosomoides bakeri*. Four different concentrations (0.625, 1.25, 2.5, and 3.75 mg·mL<sup>-1</sup>) of both aqueous and ethanolic extracts were tested. Distilled water and 5% tween were used as negative controls in the bioassay. In fact, they did not affect development of eggs, hatching, and larval survival. The extract activities were dose dependent. The ethanolic extract was more potent against embryonation (39.6 ± 2.9%) than the aqueous extract (53.3 ± 10.9%) at the highest concentration (3.75 mg·mL<sup>-1</sup>). Both types of extracts killed larvae. Mebendazole proved more lethal (EC<sub>50</sub> of 0.745 and 0.323 mg·mL<sup>-1</sup>, resp., for L<sub>1</sub> and L<sub>2</sub> larvae). The aqueous extracts were the least lethal (EC<sub>50</sub> of 4.76 and 2.29 mg·mL<sup>-1</sup>, resp., for L<sub>1</sub> and L<sub>2</sub> larvae). The ethanolic extracts showed intermediate activity (EC<sub>50</sub> of 1.323 and 1.511 mg·mL<sup>-1</sup>, resp., for L<sub>1</sub> and L<sub>2</sub> larvae). It is concluded that the ovicidal and larvicidal properties of aqueous and ethanolic extracts of *Ageratum conyzoides* leaves are demonstrated in this work.

## 1. Introduction

Helminthiasis are worldwide and constitute a major health issue. More than 1 billion people are infected with gastrointestinal helminths. Helminthiasis are more common in the tropics where poor hygienic conditions and poverty increase the risk of infection. In addition, these diseases are responsible for mortality in human beings and affect especially children of school age, thus, compromising their growth, intellectual, development and their school performance as well as increasing their vulnerability to other infections. On the other hand, in domesticated animals, helminth infections impair health, welfare, and productivity. Worm infections result in increased death rate and poor growth and reproduction. To control verminosis, patients and likewise farmers use synthetic anthelmintic drugs. Unfortunately, these substances are not only less available in the countries of

the South but also their cost in local markets is relatively high. Moreover, for more than two decades now, new anthelmintic molecules have not been discovered. Also, the misuse of these drugs for many years had led to the development of resistant worm strains. Some side effects are noted and the use of disinfectants to control free stage of parasites is harmful to the environment [1]. This has motivated investigation into alternative approaches. Phytotherapy based on the use of preparation of medicinal plant, with low cost and efficiency, remains the most serious alternative to modern anthelmintic drugs. The discovery of new natural, less expensive, and nontoxic products remains a continued quest in the control of helminths worldwide [2, 3]. Studies carried out in that direction include those of Alawa et al. [4], Wabo Poné et al. [5–7], Maciel et al. [8], and M.-M. Claire et al. [9] who assessed the *in vitro* anthelmintic activities of extracts of: *Vernonia amygdalina* and *Annona senegalensis* in

Nigeria, *Canthium mannii* in Cameroon, *Melia azedarach* in Brazil, and *Cucurbita moschata* in Guadeloupe, respectively. In Cameroon, *Ageratum conyzoides* commonly called “king of herbs” is used to treat different human ailments and as worm medicine in some countries. In the present study, a comparative assay was performed to evaluate the ovicidal and larvicidal properties of aqueous and ethanolic extracts of the leaves of *Ageratum conyzoides* against *Heligmosomoides bakeri*, a gastrointestinal nematode parasite of rodents.

## 2. Materials and Methods

The *A. conyzoides* leaves used in this study were harvested in Dschang, Menoua division, West Region of Cameroon. They were then dried in an oven at 50°C for 7 days, ground, and stored in airtight plastic bags in the laboratory for further use.

**2.1. Preparation of Extracts.** Two types of extracts (aqueous and ethanolic) were prepared to compare their activities.

**Ethanolic Extract.** The procedure used is as described by Wabo Poné et al. [5, 6]. Briefly, 445 grams of stored powder were macerated in 4 liters of ethanolic 95%. The mixture was stirred daily and 72 hours later filtered through a filter paper of pore size 2.5 µm. The ethanolic extract was obtained using the procedure described by Ciulei [10]. This was followed by the dilution of 100 mg of the extract (concentrated using a rotary evaporator) with 1 ml of 5% tween which helped dilute the extract and to facilitate the mixing with water. After 5–10 min, distilled water was added to obtain a total volume of 10 mL which produced a stock solution of 10 mg·mL<sup>-1</sup> from which a series of dilutions were made to obtain solutions of 1.250, 2.5, 5.0, and 7.5 mg·mL<sup>-1</sup> concentrations. The final tested concentrations were 0.625, 1.25, 2.5, and 3.75 mg·mL<sup>-1</sup>.

**Aqueous Extract.** The above procedure was used for the aqueous extract except that distilled water was used and the maceration took 48 hours (to avoid fungal growth). Also, the solvent was dried for 24 hours in an oven heated to 50°C.

**2.2. Reference Drug.** The reference drug, Mebendazole (MBZ), was used only for the larvicidal tests. The drug was bought in a local pharmacy and diluted with distilled water (DW) to obtain a stock solution with the same concentration as with organic extracts. The negative controls used for the bioassay were 5% tween and DW.

**2.3. Recovery of Nematode Eggs.** *Heligmosomoides bakeri* (previously known as *Nematospiroides dubius* and *H. polygyrus*; see [11, 12]) fresh eggs were obtained from the faeces of experimentally infected mice according to Michael et al. [13]. 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 later were poured into four tubes and centrifuged

at 1000 g for 5 min. The supernatant containing eggs was poured through a 45 µm mesh sieve. The material retained on the sieve and containing eggs was washed with tap water to remove the salt solution. The sieve was then turned over, the opposite side was washed with tap water and eggs were thus collected in a Petri dish (Ø = 16 cm) [14].

**2.4. Evaluation of Ovicidal Activity.** The ovicidal efficacy test of the different extracts was performed using two different procedures. To assess the effects of the extracts on fresh eggs, 1 mL of suspension containing 30 parasite eggs was distributed in each of 12 Petri dishes (35 mm Ø × 10 mm) and mixed with the same volume of a specific concentration of a given extract. The Petri dishes were covered and the eggs incubated at room temperature for 24 hours, after which 2 to 3 drops of Lugol’s iodine were added in each Petri dish to fix the different life cycle stages and the number of embryonated eggs per Petri dish was counted under a microscope (at 4x magnification). The percentage of embryonation (EM%) was determined as follows [5]:

$$EM(\%) = \frac{\text{Number of embryonated eggs}}{\text{Number of eggs in culture}} \times 100. \quad (1)$$

To assess the effects of the extracts on the hatching mechanism, the same number of unembryonated eggs, distributed in Petri dishes as above, was allowed at room temperature for about 24 hours until the eggs had developed to the fully embryonated pre-hatch stage. When the first stage larvae became transparent and were actively moving within the egg envelope (>90% in the control Petri dish), 1 ml of a range of each concentration of extract was added to Petri dishes. The Petri dishes were then covered and incubated for a further 6 hours at room temperature (24°C) to allow hatching to be completed in the control dish. Thereafter, 2 to 3 drops of Lugol’s iodine were added to each Petri dish to fix the different life cycle stages and all embryonated eggs. The first-stage larvae (L<sub>1</sub>) were counted under a microscope (at 4x magnification). The hatching rate (HR%) was computed as follows [6]:

$$HR(\%) = \frac{\text{Number of L}_1 \text{ larvae}}{\text{Number of embryonated eggs in culture}} \times 100. \quad (2)$$

**2.5. Recovery of Nematode Larvae.** Eggs were cultured using the technique described by Smyth [15]. Briefly, 3 ml of the egg suspension was poured on filter paper at the bottoms of two Petri dishes, which were then covered to maintain a high relative humidity (65–67%) to prevent the dishes from drying out, and was stored at 24°C. After 3 and 4–5 days of incubation, L<sub>1</sub> and L<sub>2</sub> larvae, respectively, were observable in Petri dishes and were concentrated with a Baermann apparatus [15, 16].

**2.6. Evaluation of Larvicidal Activity.** To assess the effects of the extracts on L<sub>1</sub> and L<sub>2</sub> larvae, 1 mL of a solution containing about 30–40 parasite larvae was distributed in

TABLE 1: Variation of the mean embryonation and hatching rates of *Heligmosomoides bakeri* eggs according to the concentration ( $\text{mg}\cdot\text{mL}^{-1}$ ) of aqueous and ethanolic extract of leaves of *Ageratum conyzoides*.

	Treatment	Negative controls		Tested concentrations ( $\text{mg}\cdot\text{mL}^{-1}$ )			
		Distilled water	5% tween	0.625	1.25	2.5	3.75
Embryonation	Aqueous extract	100.0 $\pm$ 0.0	NA	94.5 $\pm$ 7.8	85.1 $\pm$ 6.2	75.7 $\pm$ 5.7	53.3 $\pm$ 10.9
	Ethanolic extract	NA	100.0 $\pm$ 0.0	96.1 $\pm$ 2.9	91.5 $\pm$ 6.0	77.7 $\pm$ 13.3	39.6 $\pm$ 2.9
hatching	Aqueous extract	97.9 $\pm$ 2.6	NA	97.0 $\pm$ 6.1	85.2 $\pm$ 13.8	59.1 $\pm$ 26.2	48.7 $\pm$ 27.2
	Ethanolic extract	NA	100.0 $\pm$ 0.0	61.4 $\pm$ 8.4	47.5 $\pm$ 9.6	26.4 $\pm$ 12.5	26.7 $\pm$ 7.3

NA: not applicable.

each of 12 Petri dishes (35 mm  $\varnothing$   $\times$  10 mm) and mixed with the same volume of a specific concentration of each extract. The dishes were covered and the larvae incubated at room temperature for 24 hours, after which the number of dead or immobilised larvae was counted under a microscope (at 4x magnification). The percent mortality ( $M_c\%$ ) was determined using Abbott's formula for corrected mortality [7]:

$$M_c(\%) = \frac{M_{ce} - M_t}{100 - M_t} \times 100, \quad (3)$$

where  $M_{ce}$  is the mortality obtained during the test and  $M_t$  the mortality registered in the negative control dishes. It is considered that when the mortality rate in the letter dishes is less than 5%,  $M_c = M_{ce}$  [17].

**2.7. Data Analysis.** At equal concentration, the mean embryonation rates, hatching rates, and larval mortality rates due to the two types of extracts were compared using the chi-square at the  $P < 0.05$  significance level. The 50 per cent effective concentration ( $EC_{50}$ ) was determined using the regression lines of the probit according to the decimal logarithm of the concentration. All tests were repeated four times for each treatment and control.

### 3. Results

The variation of mean embryonation rate of *H. bakeri* eggs according to the concentration of extracts of *A. conyzoides* leaves is shown in Table 1. Overall, the effect was dose dependent. At a concentration of  $2.5 \text{ mg}\cdot\text{mL}^{-1}$ , the mean embryonation rates were  $75.7 \pm 5.7\%$  and  $77.7 \pm 13.3\%$  for aqueous and ethanolic extract, respectively. At  $3.75 \text{ mg}\cdot\text{mL}^{-1}$ , the embryonation rates were moderate and similar in solution containing both extracts. The mean embryonation rate was maximum ( $100 \pm 0.0\%$ ) in distilled water and 5% tween.

The variation of the mean hatching rate of  $L_1$  larvae of *H. bakeri* according to the concentration of extract is illustrated in Table 1. The aqueous extracts did not affect egg hatching. At a concentration of  $3.75 \text{ mg}\cdot\text{mL}^{-1}$ , mean hatching rate was  $48.7 \pm 27.2\%$ . The negative controls did not affect the hatching rate ( $97.9 \pm 2.6$  and 100% hatching rate for 5% tween and DW, resp.).

The effects of different extracts and Mebendazole (MBZ) on  $L_1$  larvae of *H. bakeri* after 24 h of contact are shown

in Table 2. Larval mortality rate was highest at 2.5 and 3.75  $\text{mg}\cdot\text{mL}^{-1}$ . The mean mortality rate due to ethanolic extract was  $96.3 \pm 4.8$  and  $99.4 \pm 1.2\%$  and MBZ ( $89.4 \pm 7.4$  and  $84.35 \pm 3.83\%$ ) was similar ( $P > 0.05$ ) and higher ( $P < 0.05$ ) than that attributed to aqueous extract ( $32.3 \pm 12.4$  and  $55.0 \pm 22.3\%$ ) with the same concentrations. Distilled water and 5% tween did not affect the survival of the larvae with mortality rate of  $3.5 \pm 5.0\%$  and  $3.0 \pm 4.8\%$ , respectively. The  $EC_{50}$  obtained were  $2.14 \text{ mg}\cdot\text{mL}^{-1}$ ,  $0.66 \text{ mg}\cdot\text{mL}^{-1}$ , and  $0.37 \text{ mg}\cdot\text{mL}^{-1}$  for aqueous, ethanolic extracts, and mebendazole respectively. Twenty-four (24) hours after incubation of  $L_2$  larvae in three treatments, the larval mortality increases with that of the concentrations (Table 2). With the three first concentration, the mortality due to MBZ ( $77.0 \pm 3.5\%$ ,  $86.7 \pm 5.2\%$ ,  $96.0 \pm 0.8\%$ ) was higher ( $P > 0.05$ ) than that due to aqueous extract ( $32.6 \pm 20.3\%$ ,  $40.8 \pm 15.0\%$ ,  $50.1 \pm 13.1\%$ ) and to ethanolic extract ( $13.6 \pm 7.0\%$ ,  $32.5 \pm 4.2\%$ ,  $35.4 \pm 29.0\%$ ). With the highest concentration, the mortalities were similar ( $P > 0.05$ ) and higher ( $P < 0.05$ ) in ethanolic extract ( $95.48 \pm 5.2\%$ ) and MBZ ( $100 \pm 0.0\%$ ) than in aqueous extract ( $58.2 \pm 20.0\%$ ). Distilled water and 5% tween did not affect *H. bakeri* larval survival with a mortality rate of  $3.5 \pm 5.0\%$  and  $3.0 \pm 4.8\%$  for DW and 5% tween, respectively. Regression analysis indicates that there was a dose-dependent relationship and the  $EC_{50}$  obtained were  $2.29 \text{ mg}\cdot\text{mL}^{-1}$ ,  $1.51 \text{ mg}\cdot\text{mL}^{-1}$ , and  $0.32 \text{ mg}\cdot\text{mL}^{-1}$  for aqueous, ethanolic extracts, and mebendazole, respectively.

### 4. Discussion

Generally, the extracts of *A. conyzoides* affect embryonic development and hatching rate of *H. bakeri*. Similar findings were shown by Wabo Poné et al [5] with the extracts of *Canthium manni*. The ovicidal activities of the extracts of *A. conyzoides* could be due to the fact that the active compounds penetrated the egg shell and stopped the segmentation of blastomeres or paralysed the larvae inside embryonated egg. The fact that aqueous solution seems to be more efficient against embryonation of eggs than ethanolic could be due to the composition of each extract. In fact, Ciulei [10] stated that aqueous extract contained hydro-soluble compounds compared to ethanolic extract which possesses, in addition to hydrosoluble substances, lipid substances, alkaloids, and polyphenols. However, since the layers of the egg shell are hydrophobic, the aqueous extract has the ability to penetrate the layers than the ethanolic extract which has an

TABLE 2: Mean mortality rate of *Heligmosomoides bakeri* L<sub>1</sub> and L<sub>2</sub> larvae incubated in increasing concentrations (mg·mL<sup>-1</sup>) of aqueous and ethanolic extracts of *Ageratum conyzoides* compared to Mebendazole.

Larval stages	Treatment	Negative controls		Tested concentrations (mg·mL <sup>-1</sup> )			
		Distilled water	5% tween	0.625	1.25	2.5	3.75
L <sub>1</sub> larvae	Aqueous extract	3.5 ± 5.0	NA	6.0 ± 5.1	8.1 ± 1.0	32.3 ± 12.4	55.0 ± 22.3
	Ethanolic extract	NA	3.0 ± 4.8	6.7 ± 4.5	60.7 ± 6.8	96.3 ± 4.8	99.4 ± 1.2
	Mebendazole	3.5 ± 5.0	NA	33.8 ± 21.1	32.2 ± 12.4	89.4 ± 7.4	84.4 ± 3.8
L <sub>2</sub> larvae	Aqueous extract	3.5 ± 5.0	NA	32.6 ± 20.34	40.8 ± 15.0	50.1 ± 13.1	58.2 ± 20.0
	Ethanolic extract	NA	3.0 ± 4.8	13.6 ± 7.0	32.5 ± 4.2	35.4 ± 29.0	95.5 ± 5.2
	Mebendazole	3.5 ± 5.0	NA	77.0 ± 3.5	86.7 ± 5.2	96.0 ± 0.8	100.0 ± 0.0

NA: not applicable.

oil aspect in this work. Extracts of *A. conyzoides* likewise showed larvicidal activity, and L<sub>1</sub> larvae were more sensible to extract than the L<sub>2</sub> confirming the literature findings [18]. The higher larvicidal activity of ethanolic extract could be due to secondary metabolites such as tannins, flavonoids, polyphenol, coumarins, or alkaloids isolated in this medicinal plant [19]. These compounds may create unfavorable conditions to the survival of the larvae.

## 5. Conclusion

From the above results, it is concluded that aqueous and ethanolic extracts of *A. conyzoides* have potent anthelmintic activity. Further experiment incorporating *in vivo* model studies are required to find out and to establish effectiveness and pharmacological rationale for the use of *A. conyzoides* extracts as anthelmintic drug. Further studies to isolate active constituent from extracts to establish mechanism of action are required. In addition, toxicological studies should be carried out.

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