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)

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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−1) 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−1). Both types of extracts killed larvae. Mebendazole proved more lethal (EC50 of 0.745 and 0.323 mg·mL−1, resp., for L1 and L2 larvae). The aqueous extracts were the least lethal (EC50 of 4.76 and 2.29 mg·mL−1, resp., for L1 and L2 larvae). The ethanolic extracts showed intermediate activity (EC50 of 1.323 and 1.511 mg·mL−1, resp., for L1 and L2 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

Helminthiases are worldwide and constitute a major health issue. More than 1 billion people are infected with gastrointestinal helminths. Helminthiases 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 anthelmintics 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 Gulei [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⁻¹ from which a series of dilutions were made to obtain solutions of 1.250, 2.5, 5.0, and 7.5 mg·mL⁻¹ concentrations. The final tested concentrations were 0.625, 1.25, 2.5, and 3.75 mg·mL⁻¹.

**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 Nematodiroidea 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]:

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

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 prehatch 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 (L₁) were counted under a microscope (at 4x magnification). The hatching rate (HR%) was computed as follows [6]:

\[
HR(\%) = \frac{\text{Number of L₁ larvae}}{\text{Number of embryonated eggs in culture}} \times 100. \tag{2}
\]

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, L₁ and L₂ 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 L₁ and L₂ larvae, 1 mL of a solution containing about 30–40 parasite larvae was distributed in
each of 12 Petri dishes (35 mm × 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 (Mc%) was
determined using Abbott’s formula for corrected mortality
[7]:
\[ Mc(\%) = \frac{Mce - Mt}{100 - Mt} \times 100, \]  
(3)
where Mce is the mortality obtained during the test and Mt
the mortality registered in the negative control dishes. It is
considered that when the mortality rate in the letor dishes is
less than 5%, Mc = Mce [17].

2.7. Data Analysis. At equal concentration, the mean emby-
ronation 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 mg·mL\(^{-1}\),
the mean embryonation rates were 75.7 ± 5.7% and 77.7
± 13.3% for aqueous and ethanolic extract, respectively.
At 3.75 mg·mL\(^{-1}\), the embryonation rates were moderate
and similar in solution containing both extracts. The mean
embryonation rate was maximum (100 ± 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 mg·mL\(^{-1}\), mean hatching rate
was 48.7 ± 27.2%. The negative controls did not affect the
hatching rate (97.9 ± 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
mg·mL\(^{-1}\). The mean mortality rate due to ethanolic extract
was 96.3 ± 4.8 and 99.4 ± 1.2% and MBZ (89.4 ± 7.4 and
84.35 ± 3.83%) was similar (\( P > 0.05 \)) and higher (\( P < 0.05 \))
than that attributed to aqueous extract (32.3 ± 12.4 and
55.0 ± 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 ± 5.0% and 3.0 ± 4.8%, respectively.
The EC\(_{50}\) obtained were 2.14 mg·mL\(^{-1}\), 0.66 mg·mL\(^{-1}\), and
0.37 mg·mL\(^{-1}\) for aqueous, ethanolic extracts, and mebend-
azole 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 ± 3.5%, 86.7
± 5.2%, 96.0 ± 0.8%) was higher (\( P > 0.05 \)) than that
due to aqueous extract (32.6 ± 20.3%, 40.8 ± 15.0%, 50.1
± 13.1%) and to ethanolic extract (13.6 ± 7.0%, 32.5 ±
4.2%, 35.4 ± 29.0%). With the highest concentration, the
mortalities were similar (\( P > 0.05 \)) and higher (\( P < 0.05 \))
in ethanolic extract (95.48 ± 5.2%) and MBZ (100 ± 0.0%)
than in aqueous extract (58.2 ± 20.0%). Distilled water and
5% tween did not affect \( H. \) bakeri larval survival with a
mortality rate of 3.5 ± 5.0% and 3.0 ± 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 mg·mL\(^{-1}\), 1.51 mg·mL\(^{-1}\), and 0.32 mg·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
\( C. \) mammii. 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 hydro-soluble 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
oil aspect in this work. Extracts of A. conyzoides likewise showed larvicidal activity, and L₁ larvae were more sensible to extract than the L₂ 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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Table 2: Mean mortality rate of Heligmosomoides bakeri L₁ and L₂ larvae incubated in increasing concentrations (mg·mL⁻¹) of aqueous and ethanolic extracts of Ageratum conyzoides compared to Mebendazole.

| Larval stages | Treatment       | Tested concentrations (mg·mL⁻¹) | 0.625 | 1.25 | 2.5  | 3.75 |
|--------------|----------------|---------------------------------|-------|------|------|------|
| L₁ larvae    | Aqueous extract| NA, ±                      | 6.0 ± 5.1 | 8.1 ± 1.0 | 32.3 ± 12.4 | 55.0 ± 22.3 |
|              | Ethanolic extract| NA, ±                  | 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  | 94.4 ± 3.8  |
| L₂ 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| 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.
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