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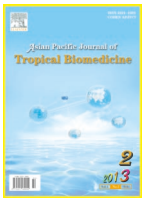

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In vitro anthelminthic efficacy of *Dichrocephala integrifolia* (Asteraceae) extracts on the gastro-intestinal nematode parasite of mice: *Heligmosomoides bakeri* (Nematoda, Heligmosomatidae)

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PEER REVIEW

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Comments

This is a good study in which the authors evaluated the *in vitro* anthelmintic efficacy of *D. Integrifolia* leaf for the control of gastro-intestinal parasitism. The three different leaf extracts were compared for their efficacy and were found to have different levels of efficacy depending on the development stage of the parasite and on the nature of the extract. This may lead to agropedoclimatic parameters. The results are interesting and suggest that anthelmintic substances are present in *D. Integrifolia* leaf.
(Details on Page 103)

ABSTRACT

Objective: To evaluate the ovicidal and larvicidal activities of aqueous and ethanolic extracts of leaves of *Dichrocephala integrifolia* (*D. integrifolia*) against the eggs (fresh and embryonnated), the first and second larval stages of *Heligmosomoides bakeri*. In order to verify if this medicinal plant possesses active compounds capable of inhibiting the embryonation and hatching of eggs or to induce the mortality of larvae (L1 and L2). **Methods:** Dried extracts were diluted in distilled water to obtain five different concentrations: 625, 1250, 2500, 3750 and 5000 μg/mL. Fresh eggs obtained from artificially infected mice feces were exposed to these different concentrations for 48 h. Time of contact for embryonated eggs was 6 h while L1 and L2 larvae were exposed for 24 h. Distilled water (placebo) and 1.5% DMSO were used as negative controls. **Results:** Distilled water, and 1.5% DMSO had no effect on embryonation, hatching and larval survival. Aqueous extracts of *D. integrifolia* showed a weak activity against all stages of the parasite at all concentrations tested. On the contrary, the ethanolic extract of *D. integrifolia* inhibited the embryonation of 87.5% of fresh eggs, the hatching of 81.1% of embryonated eggs and induced the mortality of 98.1% and 98% of L1 and L2 larvae respectively at 5000 μg/mL. **Conclusions:** The results of the present study indicate that the ethanolic extracts of *D. integrifolia* contained compounds with ovicidal and larvicidal properties. In spite of these results, *in vivo* tests, studies on toxicity and mechanism of action of active compounds are also needed to validate the utilisation of this medicinal plant by population of Dschang-Cameroon to treat gastro-intestinal parasites.

KEYWORDS

Dichrocephala integrifolia, *Heligmosomoides bakeri*, *In vitro*, Eggs, Larvae

1. Introduction

Gastro-intestinal parasite infections are a world-wide problem for both domesticated animals and humans. Helminthiasis has a crucial impact on small ruminant production, leading to enormous economic losses particularly in areas where extensive grazing is practiced[1]. Gastro-intestinal parasites cause economic losses in different ways such as lowered fertility, reduced work capacity, reduction in food intake and low weight gain,

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decreased in milk production as well as mortality in heavily parasitized animals[2]. In humans, intestinal nematodes are also important pathogens, with a range of pathologies and consequences for human health. Actually, Ascaris lumbricoides, Trichuris trichura, Ancylostoma duodenalis and Necator americanus are four species dominating in humans, and it is estimated that three billion people around the world are believed to carry these species[3,4]. Control of gastro-intestinal nematodes has long been performed almost exclusively with conventional anthelmintics in developing countries. However, the rapid development of resistance to these drugs by nematodes associated with high cost, food residues and environmental pollution have limited the success of gastro-intestinal parasites control and thus, led to the screening and proper evaluation of medicinal plants which could offer possible alternative that may both be sustainable and environmentally acceptable[5]. Medicinal plants have been used by indigenous peoples for centuries as sources of extracts used to treat infectious diseases and those caused by parasites in livestock and humans[6]. *Dichrocephala integrifolia* (*D. integrifolia*), an annual herb of the Asteraceae family is used by the population of Dschang-Cameroon to treat intestinal worms and other discomforts. It has been the subject of *in vitro* studies that have noted its anticancer, antimicrobial, anti-inflammatory and anti-oxydant activities[7]. The anthelmintic activity, however, has not been scientifically tested. The aim of this study was to investigate the *in vitro* anthelmintic activity of the leaves of *D. integrifolia*. The tests were performed using the infused, macerated and ethanolic extracts and testing was done using fresh and embryonated eggs, first and second larval stages of *Heligmosomoides bakeri* (*H. bakeri*), a gastro-intestinal nematode parasite of rodents commonly used as a model to test new antiparasitic substances since it is resistant to a number of available anthelmintics.

2. Materials and methods

The leaves of *D. integrifolia* used in this work were harvested in Dschang, Menoua Division and West Region of Cameroon. They were dried in the shade for 1 to 4 h per day, ground and stored in airtight plastic bags in the laboratory for further use.

2.1. Preparartion of extracts

Three types of extracts (infused, macerated and ethanolic) were prepared to compare their activities. The procedure described by Wabo Pone *et al*. were used to obtain five different solutions of concentrations 1 250, 2 500, 5 000, 7 500 and 10 000 μg/mL[8,9]. The final tested concentrations were 625, 1 250, 2 500, 3 750 and 5 000 μg/mL.

2.2. Recovery of nematode eggs

Fresh eggs of *H. bakeri*, (previously known as *Nematospiroides dubius* and *Heligmosomoides polygyrus*) were obtained from the faeces of experimentally infected mice according to Ngangout *et al*[10].

2.3. Recovery of the Nematode larvae

Eggs obtained from the above were allowed in a beaker with water for 2 d and 4 to 5 d to obtain L1 and L2 larvae respectively. The solution containing the larvae was then distributed in test tubes and allowed for 10 min. The supernatant was decanted and Ringer solution was added in the test tubes to optimize the survival of larvae.

2.4. Evaluation of ovicidal and larvicidal activity

Fresh and embryonated eggs were used to evaluate the ovicidal efficacy, while L1 and L2 larvae were used for larvicidal one according to Wabo Poné *et al*[11,12].

2.5. Statistical analysis

At equal concentration, the mean embryonation rates, hatching rates and larval mortality rates due to the extracts were compared using the chi-square at the $P<0.05$ significance level. The IC_{50} and LC_{50} were 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 [distilled water (DW) and 1.5% DMSO].

3. Results

The yield obtained after extraction with ethanol, hot and cold water solvents from 100 g of *D. integrifolia* leaf powder was 7.30 g, 8.67 g and 11.31 g, respectively.

The variation of the mean embryonation rate of *H. bakeri* according to the different concentrations of *D. integrifolia* extracts is shown in Figure 1. It showed that DW and 1.5% DMSO allowed the normal embryonation of the parasite eggs, with the mean embryonation rates of 98.1% and 100%, respectively. The aqueous extracts of *D. integrifolia* presented a weak activity with the mean embryonation rates which remained higher than 50% in all concentrations tested. On the contrary, ethanolic extract inhibited 69%-0.6% and 87.9%-0.2% embryonation of eggs at the concentrations of 3 750 and 5 000 μg/mL, respectively, with a significant difference ($P<0.05$). The IC_{50} obtained on fresh eggs after transformation of the embryonation rate to probit (not illustrated) were 11 402.1, 20 000.4 and 1 967.1 μg/mL for infused, macerated and ethanolic extracts respectively.

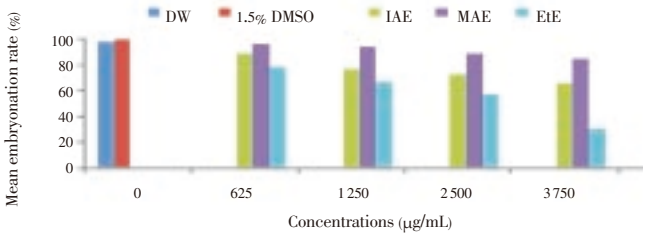

Figure 1. Variation of the mean embryonation rate of *H. bakeri* eggs according to the concentrations of the extracts of *D. integrifolia*.
DW: Distilled water; DMSO: Dimethylsulfoxide; IAE: Infused aqueous extract; MAE: Macerated aqueous extract; Et E: Ethanolic extract.

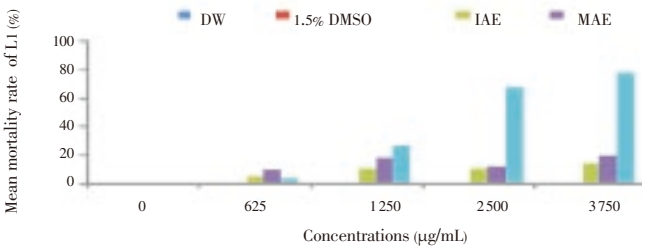

Figure 3. Variation of the mean mortality rate of L1 larvae of *H. bakeri* according to the concentrations of the extracts of *D. integrifolia*.
DW: Distilled water; DMSO: Dimethylsulfoxide; IAE: Infused aqueous extract; MAE: Macerated aqueous extract; Et E: Ethanolic extract.

The variation of the mean hatching rate of embryonated eggs of *H. bakeri* according to the concentrations of the extracts is illustrated in Figure 2. As on fresh eggs, both DW and 1.5% DMSO had no effect on embryonated eggs, with mean hatching rate of 90%. In Petri dishes treated with extracts, the mean hatching rate of the eggs of *H. bakeri* reduced with the increase in concentration of extracts. At the concentration less or equal to 2500 μg/mL, the hatching rate remained higher than 80%, with aqueous extracts. At 3750 and 5000 μg/mL, ethanolic extract presented the lowest mean hatching rate (18.9%−0.5% and 9.4%−0.3%), followed by the macerated extract (47.3%−2.5% and 30.8%−0.9%) respectively, with a significant difference (P<0.05). The IC_{50} obtained from embryonated eggs were 7.9×10^6, 4 141 and 1734.6 μg/mL for infused, macerated and ethanolic extracts respectively. These results showed that the ethanolic was the most efficient on eggs.

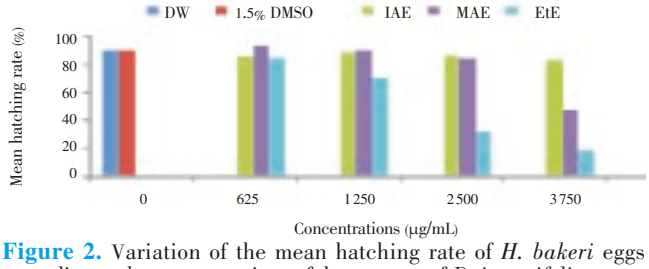

Figure 2. Variation of the mean hatching rate of *H. bakeri* eggs according to the concentrations of the extracts of *D. integrifolia*.
DW: Distilled water; DMSO: Dimethylsulfoxide; IAE: Infused aqueous extract; MAE: Macerated aqueous extract; Et E: Ethanolic extract.

Figure 3 shows the effects of different extracts of *D. integrifolia* on L1 larvae of *H. bakeri* after 24 h of contact. DW and 1.5% DMSO allowed the normal development of the L1 larvae. The aqueous extracts of *D. integrifolia* showed weak activity on these larvae, with mortality rates less than 50% in all concentrations tested. Ethanolic extract showed a concentration-dependent activity, with mortality rates going from 67.7%−1.1% at 2500 μg/mL to 98.1%−0.9% at 5000 μg/mL. The LC_{50} obtained with L1 larvae were 16714.4, 46819.1 and 2120.2 μg/mL for infused, macerated and ethanolic extracts respectively.

The effect of different extracts of *D. integrifolia* on L2 larvae of *H. bakeri* after 24 h of contact is presented in Figure 4. As on L1 larvae, DW and 1.5% DMSO had no effect on L2 larvae, with the mortality rate of 0%. Larval mortality was concentration dependent in dishes treated with extracts, even though activity of aqueous extracts remained relatively low. This result is significatively different (P<0.05) from that obtained with ethanolic extract which registered mean mortality rates increasing from 74.6%−0.9% at 1250 μg/mL to 98%−0.9% at 5000 μg/mL. The LC_{50} obtained with L2 larvae were 2.9×10^5, 5918.4 and 981.9 μg/mL for infused, macerated and ethanolic extracts respectively. These results indicate that ethanolic extract of *D. integrifolia* is the most efficient on all the stages of the parasites.

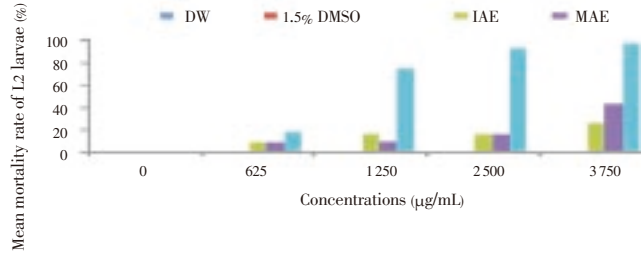

Figure 4. Variation of the mean mortality rate of L2 larvae of *H. bakeri* according to the concentrations of the extracts of *D. integrifolia*.
DW: Distilled water; DMSO: Dimethylsulfoxide; IAE: Infused aqueous extract; MAE: Macerated aqueous extract; Et E: Ethanolic extract.

4. Discussion

In vitro tests using free stages of parasitic nematodes offer a mean to rapidly screen for potential anthelmintic activity of new plant compounds as already reported by various authors[13]. These *in vitro* tests measure the effects of anthelmintics directly on physiological processes such as embryonation, egg hatch and larval survival. The high rates of embryonation (98.1% and 100%) egg hatch (90%) and low mortality rates (0%) observed in the absence of the extract showed that the negative controls (DW and 1.5% DMSO) did not affect the natural development of eggs and larvae of *H. bakeri*. Aqueous extracts of *D. integrifolia* were either ineffective or had a weak activity against all stages

of the parasite at all tested concentrations. Camila *et al*. also reported no ovicidal or larvicidal activity with all the concentrations tested with oil extracted from seeds of Carapa guianensis on Haemonchus contortus[14]. It's known that the lack of efficacy of a plant extract could be due to: (i) the locality of collection of the plant; (ii) the age of the plant and (iii) the species of parasites[15]. According to Diehl *et al.*, a plant extract showing no anthelmintic activity cannot be considered as being completely inactive[16]. Since activity can result from a special preparation procedure. Therefore, it is imperative to test once again aqueous extracts studied in this work using samples obtained from the same species, collected from other regions at different seasonal periods.

The results of this study also revealed that the ethanolic leaf extract of *D. integrifolia* inhibit embryonation, egg hatch and larval survival at various concentrations when compared with the control. In general, the extract with higher concentrations showed more activity when compared to extract with lower concentration. This observation showed that, an increase in concentration represent a supplementary input of different active compound[12]. Ethanolic extract of *D. integrifoila* inhibited the embryonation of 87.5% of fresh eggs and the hatching of 81.1% of embryonated eggs at 5 000 μg/mL. Taylor *et al.* showed that Benzimidazole anthelmintics prevent embryonation and hatching of nematode eggs[17]; therefore, this suggests that active substances contained in ethanolic extract of *D. integrifolia* may have similar mechanism of action as Benzimidazole and can be useful for further evaluation as a possible anthelmintic. Egg inhibition, will reduce contamination of pasture by nematode larvae during grazing by livestock, and this is an important practice in overall helminth control[18]. Further evaluation of ethanolic extract of the leaves of our plant on larvae revealed that this extract induced mortality at various concentrations, especially at 5 000 μg/mL where we registered very high mortality rates of 98.1% for L1 and 98% for L2 larvae. Here again, activity was concentration dependent, suggesting a pharmacological basis. As we can see, ethanol extract was more potent against L2 larvae than L1 larvae; this result is in agreement with the one obtained by Wabo Poné *et al*. confirming the literature findings[19] and could be explained by the fact that since L2 are just from the process of molting, they are still weak and thus, more vulnerable to the active compounds[11]. We also observed that ethanolic extract was more efficient against the larvae than eggs whereas, Adama *et al*. observed the contrary with extracts of *Anogeissus leiocarpus* and *Daniellia oliveri* on *Haemonchus contortus*[20]. Mortality of rhabditoid larvae will reduce the burden of infective larvae and therefore will prevent contamination of grazing animals in pastures. The activity of *D. integrifolia* may be related to active substances such as sesquiterpens previously reported from the plant[21]. However, due to biotransformation, interaction with feed materials and absorption encountered *in vivo*, the results obtained by the *in vitro* method could not be extrapolated for *in vivo* activity. Hence, the results should be ascertained by *in vivo* evaluation[22].

Based on results presented in this work, *D. integrifolia* offers an opportunity for a new source of control of gastro-intestinal nematodes. However, more studies on mechanism of action of active compounds, toxicity and *in vivo* evaluation are needed to justify the utilization of this medicinal plant as anthelmintic by populace.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Helminthiasis has become a worldwide problem for animals and humans. The intensive use of chemical anthelmintics has led to the development of parasite resistance. To try and solve this problem several alternative methods are studied such as phytotherapy, which is used by traditional healers or by indigenous people for humans or animals.

Research frontiers

Studies are being performed in order to scientifically evaluate the traditional anthelmintic use of *D. Integrifolia*, a plant used by Cameroon population to treat intestinal worms. The *in vitro* anthelmintic efficacy of three leaf extracts (infused, macerated and ethanolic) was tested against the nematode *H. Bakeri* parasites were isolated from mice.

Related reports

The lack of effect of some extracts (the water extract here) has to be reconsidered as it was demonstrated in other studies (Raskin *et al.*, 2002) that it could vary depending on several parameters in the plant (origin, soil, age, seaso, *etc.*) and also the type of parasite tested.

Innovations and breakthroughs

An innovation in the paper is that several development stages of the parasite were tested and the choice of the parasite was based as it is a model for multidrug resistance.

Applications

The results of this study suggest that the traditional anthelmintic use of *D. Integrifolia* is worth. Thus it is important, to better utilization of the plant, to evaluate the extracts on several parasite species and to test the *in vivo* efficacy and toxicity.

Peer review

This is a good study in which the authors evaluated the *in vitro* anthelmintic efficacy of *D. Integrifolia* leaf for the control of gastro-intestinal parasitism. The three different leaf extracts were compared for their efficacy and were found to have different levels of efficacy depending on the development stage of the parasite and on the nature of the extract. This may lead to agropedoclimatic parameters. The results are interesting and suggest that anthelmintic substances are present in *D. Integrifolia* leaf.

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