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In vitro Activity of Ethanol, Cold Water and Hot Water Extracts of the Bark of *Canthium mannii* (Rubiaceae) Stem on *Ancylostoma caninum* Eggs

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An *in vitro* evaluation was performed to determine the efficacy of ethanol, cold water and hot water extracts of the stem bark of *Canthium mannii* (Rubiaceae) on un-embryonated and embryonated eggs of *Ancylostoma caninum* obtained from the faeces of naturally infected local dogs. The extracts were diluted in distilled water to obtain five concentrations namely 125, 250, 500, 750 and 1000 µg/ml. Mebendazole, similarly diluted, distilled water and 0.02 % ethanol were used in the bioassay as the standard reference drug, placebo and ethanol control respectively. One milliliter portions of the extracts and controls at the different concentrations were added to 1 ml solutions containing 30 to 40 of the parasite eggs distributed in different Petri dishes followed by incubation at 24 °C for 48 h in the case of un-embryonated eggs and 6 h for the embryonated eggs, after which first stage larvae and eggs were counted. The 1000 µg/ml ethanol extract produced a 90 % reduction in the number of eggs that hatched after treatment for 48 h. This effect was similar to that produced by mebendazole. The cold water and hot water extracts showed lower eclodibility inhibition (< 50 %) at all the concentrations tested. These results support the possible use of the ethanol extract of *C. mannii* in the control of gastrointestinal helminthiasis. These effects remain to be studied under *in vivo* conditions.

Key words: ***Canthium mannii*, anthelmintic action, *Ancylostoma caninum*, Cameroon.**

INTRODUCTION

Gastrointestinal helmintiasis of domesticated animals constitutes a major preoccupation of small-scale local farmers in tropical countries [1]. The associated parasites cause excessive morbidity and often, mortality in animals of all age groups thus resulting in significant economic losses [2]. To date, the principal mode of control of parasites in the digestive tract has been based on chemical treatment with anthelmintics. Because of the increasing development of anthelmintic resistance within worm populations and the high cost of chemical products in developing countries, there is currently an emerging interest for alternative approaches to helminth therapy [3-7]. In developing nations, traditional methods of controlling helminths, used by small-scale farmers, remain largely dependent on medicinal plants.

Research has been directed towards medicinal plants, which are apparently beneficial, less expensive and less toxic to man, animals and the environment as compared to the synthetic anthelmintics [8-9]. Many recent studies conducted in various parts of the world have revealed the anthelmintic properties of extracts and/or essential oils of some medicinal plants [10-14].

The present study is a report on an evaluation of ethanol, cold and hot water extracts of the powder of the stem bark of *Canthium mannii,* used by

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traditional doctors in Western Cameroon to cure gastro-intestinal helminthiasis, on un-embryonated and embryonated eggs of *Ancylostoma caninum,* a parasite of dogs, cats and wild carnivores.

MATERIALS AND METHODS

The plant used in this study is a shrub, *Canthium mannii*, belonging to the family Rubiaceae. Botanists at the National Herbarium of Cameroon carried out the identification of the plant. The fresh bark of the stem of *C. mannii,* collected in Fondonera (Dschang), West Cameroon, was dried, ground and stored in plastic bags for further use in the Applied Ecology Laboratory in the Department of Animal Biology of the University of Dschang.

Five hundred grams of the powder from the stem bark of *C. mannii* was used to prepare the ethanol extract [15]. This was followed by the dilution of 50 mg of the concentrated extract with 0.5 ml of 95 % ethanol to form a gel. After 5 to 10 minutes, 25 ml of distilled water was added to the gel according to the method of Ibarra and Jenkins [16]. This resulted in a 2000 µg/ml ethanol extract stock solution from which a series of dilutions was made to obtain solutions with final test concentrations of 1000, 750, 500, 250 and 125 µg/ml.

A further 500 g of the powder was macerated in 3 litres of distilled water for 72 hours, accompanied by a daily stir, to obtain the cold water extract. This solution was filtered using a 2.5 µm pore size filter paper. A 250 ml aliquot of the filtrate was distributed in five beakers, which were then placed in a desiccator at 50 °C for one week. About 50 mg of the dried extract obtained was diluted with 25 ml of distilled water to get the stock solution, which was serially diluted to obtain the five different concentrations as with the ethanol extract. For the hot water extract, a procedure similar to that for the cold water extract was employed except for the fact that the water was heated to 100 °C and the infusion (500 g of powder in hot water) was allowed to stand at room temperature for approximately 3 h for the water to cool before preparing and serially diluting the stock solution.

The reference drug, mebendazole, was diluted in distilled water to obtain a stock solution with a concentration of 2000 µg/ml which was serially diluted to obtain the same concentrations as with the extracts.

Evaluation of anthelmintic activity

The fresh eggs of *A. caninum* were obtained from the faeces of naturally infected local dogs [17]. To evaluate the effect of the various extracts on non-embryonated eggs, 1 ml of the solution containing 30 to 40 eggs of the parasite was distributed in Petri dishes measuring 35 mm by 10 mm and mixed with the same volume of the extracts at the different concentrations already prepared. In this experiment, embryonation and hatching occurred in constant contact with the test products. The Petri dishes were covered and the eggs were incubated at room temperature (24° C) for 48 h, after which all the first-stage larvae (L_1) were counted.

The same number of un-embryonated eggs distributed in Petri dishes as above, were allowed to stand at room temperature for about 24 h until the eggs developed to the fully embryonated pre-hatch stage. When the first stage larvae became transparent and started moving actively within the egg envelope (>90 % in the control Petri dish), 1 ml of the prepared range of product concentrations was added to each Petri dish according to the method of Dobson *et al.* [18]. The Petri dishes were then covered and incubated for a further 6 h at room temperature to allow for almost complete hatching in the control dish. In this part of the study, only hatching occurred in contact with the test products. When the eclodibility in the distilled water and 0.02 % ethanol controls was higher than 90 %, 2 to 3 drops of Lugol's iodine (5 %) were added to each Petri dish to stop egg hatching as recommended by Pessoa *et al.* [19]. All the embryonated eggs and first-stage larvae (L_1) were counted.

$$E\ (\%) = \frac{\text{No. of } L_1 \text{ larvae}}{\text{No. of embryonated eggs in culture}} \times 100$$

Where E is Eclodibility.

The test was repeated five times for each

treatment and control. The values obtained were compared using the Chi-square test at the 0.05 significance level. The controls used for the bioassay were 0.02 % ethanol (the maximum concentration of ethanol in test dishes) and distilled water.

RESULTS

The effects of the different products on the fresh eggs are displayed in figure 1. The mean hatching rate measured was 98.8 % and 96.8 % for distilled water (placebo) and 0.02 % ethanol (negative control) respectively. The values obtained with the various concentrations of mebendazole differed significantly from those obtained with the placebo and negative control ($P< 0.05$). Similarly, the ethanol and cold water extracts of *C. mannii* showed significant effect on the egg hatching rates ($P<0.05$). In contrast, no statistical difference ($P>0.05$) was detected between distilled water and 0.02 % ethanol. A significant difference ($P<0.05$) was observed between the ethanol extract and the other two extracts for all the concentrations tested. There was also a significant difference ($P<0.05$) between the cold water and the hot water extracts.

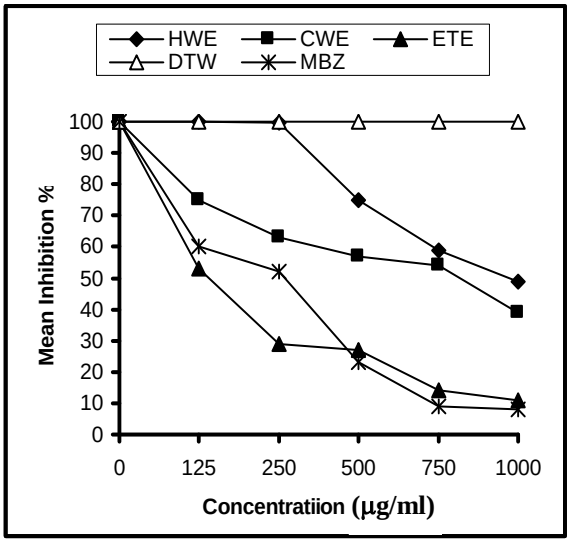

Figure 1. Effect of different concentrations of extracts of *C. mannii* and mebendazole on the hatching of un-embryonated eggs of *Ancylostoma caninum*. HWE-Hot Water Extract, CWE-Cold Water Extract, ETE-Ethanol Extract, DTW-Distilled water, MBZ-Mebendazole

However no significant difference ($P>0.05$) was observed at the concentrations of 500 and 1000 µg/ml.

For all the extracts tested, a linear relationship was observed between the probit of egg hatch and the logarithm of the concentrations. The LC_{50} values were 136, 673 and 836 µg/ml for the ethanol, cold water and hot water extracts respectively.

The mean inhibition percentage on egg hatch of *A. caninum* using the different extracts is shown in figure 2. The value obtained was 10 % for distilled water and 0.02 % ethanol. For all the extracts, the inhibition percentage was higher than 49 %. Only the 1000 µg/ml concentration of the CWE and HWE showed eclodibility values below 50 % (39 % and 46 % respectively). An increase in concentration was characterized by a decrease in eclodibility rate, and no significant difference ($P>0.05$) was observed between the different concentrations tested. Although a linear concentration-effect relationship was also established as with the un-embryonated eggs the LC_{50} values were higher for all the extracts. Thus, the extracts seemed to be more active on un-embryonated eggs. The reference drug, mebendazole, was more active than the extracts.

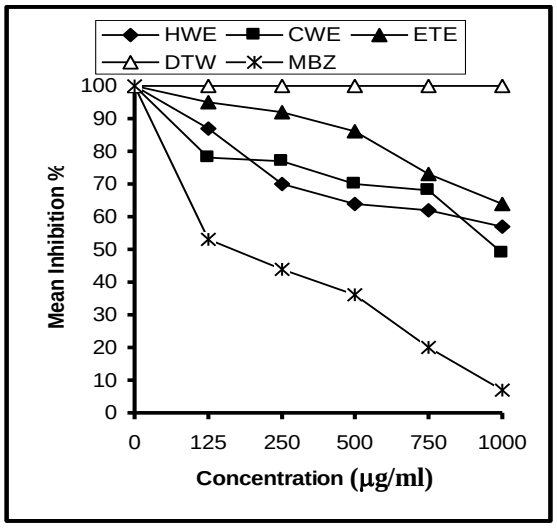

Figure 2. Effect of different concentrations of extracts of *C. mannii* and mebendazole on the hatching of embryonated eggs of *Ancylostoma caninum*. HWE-Hot Water Extract, CWE-Cold Water Extract, ETE-Ethanol Extract, DTW-Distilled water, MBZ-Mebendazole

DISCUSSION AD CONCLUSION

Overall, the extracts of the shrub *C. mannii* which were examined in the present study had *in vitro* effects on the eggs of the nematode *A. caninum* which is the stage disseminated into the environment. Dose-dependent effects on egg hatching were observed with the extracts of the plant. These results are similar to those obtained by Dobson *et al*. [18] in their studies on egg-hatch assay for resistance to levamisole in trichostrongyloid nematode parasites and those obtained by Pessoa *et al.* [19] on the anthelmintic activity of the essential oil of *Ocimum gratissimum* against *Haemonchus contortus.* These observations confirmed the results obtained previously on rhabditiform larvae of *A. caninum* by Wabo Poné *et al.* [13].

The ethanol extract was more active on the eggs when compared to the other two extracts. This assay corroborates an earlier *in vitro* trial [20] in which the extract of *Anonna senegalensis* was found to be effective against the eggs of *Haemonchus contortus.* The hot water extract at concentrations of 125 and 250 µg/ml was ineffective on egg hatch, a finding similar to that obtained in the study on the effect of *Vernonia amygdalina* on *H. contortus* [20]. The LC_{50} value of the ethanol extract of *C. mannii* in this study (136 µg/ml) was relatively low, indicating that this extract is more active.

Except for the ethanol extract at concentrations of 125 and 250 µg/ml, all extracts tested in this study inhibited the hatching of embryonated eggs to a larger extent when compared to the controls and had higher IC_{50} values as well. It is thought that these extracts penetrate the eggshell and stop the development of un-embryonated eggs in the same way as mebendazole [21] and paralyse the first stage larvae similarly to levamisole [18]. In fact, Dobson *et al*. developed a levamisole egg hatch assay based on the paralysis of the first stage larvae within the eggs [18]. However, the assay is complex to perform, requiring close monitoring of egg development so that addition of the anthelmintic can be made close to the point of hatch. Furthermore, eggs are required to be at similar stages of development. The variation of eclodibility in the present bioassay can be attributed to the action of the extracts since the egg hatch was high in the controls (>97 %). These results suggest that the extracts of *C. mannii* could contain different active compounds with different modes of action.

In conclusion, the present *in vitro* results suggest that the plant extracts could affect the biology of the parasitic egg when sprayed on soil. By itself, a reduction in egg hatching can help to modulate the risk of parasitism by limiting the infectivity of soil to animals and man. Nonetheless, further, experiments incorporating *in vivo* and toxicological investigations are necessary.

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