In vitro anthelmintic activity of *Combretum molle* (R. Br. ex G. Don) (Combretaceae) against *Haemonchus contortus* ova and larvae

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Abstract

Parasitic nematodes, especially *Haemonchus contortus* (Rudolphi), are among the most common and economically important causes of disease in sheep and goats owned by pastoralists and small holder farmers in Africa. The control of these infections relies mainly on the use of anthelmintic drugs. However, herbal preparations are widely used by pastoralists and small holder farmers for the treatment of their livestock against helminth parasites. The anthelmintic effect of acetone leaf extract and fractions of *Combretum molle* was investigated to determine the relative efficacy of the components against gastrointestinal sheep nematodes. The fractions were obtained by solvent:solvent extraction from the acetone extract. These were evaluated for nematocidal activity by means of an egg hatch (EHA) and larval a development and viability assay (LDVA) *in vitro*. The effect of the test extracts on the hatchability of eggs and development of first to third stage larvae and the survival rate of the third stage larvae. *H. contortus*, were used to determine the relative bioactivities. Best-fit LC_{50} values were computed using global model of nonlinear regression curve-fitting. The extracts inhibited egg hatching and development of the larvae of *H. contortus* in a concentration-dependent manner. Best-fit LC_{50} values for the egg hatch test were 0.866, 0.333, 0.833, 0.747, and 0.065 mg/mL for acetone extract, n-butanol, hexane, chloroform, and 35% water in methanol fractions, respectively. The best-fit LC_{50} values for the LDVA were 0.604, 0.362, 1.077, 0.131 and 0.318 mg/mL for the acetone extract, butanol, hexane, chloroform, and 35% water in methanol fractions, respectively. In the EHA the 35% water in methanol fraction was significantly more active than all the other fractions ($p < 0.05$);

however the activity was not significantly different with the LDVA. *C. molle* leaf could find application in anthelmintic therapy in veterinary practice.

Keywords: Anthelmintic activity; *Combretum molle*; *Haemonchus contortus*; Solvent:solvent fractions

1. Introduction

Gastrointestinal parasites are of major economic importance in livestock because they cause clinical and sub clinical infections that reduce animal survival and depress growth rates, wool and milk production, and reproductive performance. Animal deaths due to nematode infections are very common in tropical and subtropical regions, where marginal levels of nutrition exacerbate the detrimental effects of infection (Waller, 1997). Control programs based solely on the use of synthetic anthelmintics are no longer considered sustainable because of an increased prevalence of gastointestinal nematode resistance (Barry and Mc Nabb, 1999), the slow development of new anthelmintics, high costs to poor farmers and concerns regarding residue in food and the environment. Alternative methods of control are thus required that are both practical and realistic for introduction into farm production systems.

Traditional health practitioners in many parts of Africa usually employ the leaves and barks of *Combretum* species as remedies for a variety of human ailments, including abdominal discomfort, body pains, respiratory disorders, colds and fevers, ear and eye ailments, schistosomiasis, hookworms, dysmenorrhoea and infertility in women, leprosy, syphilis, microbial infections and general body weakness (Hutchings et al., 1996). It is regarded as a medicine for both humans and animals. The leaves of *Combretum molle* contain steroidal acids and saponins (Pegel and Rogers, 1985). Many triterpenoids and their glycosides have been isolated from the leaves of South African *Combretum* species (Pegel and Rogers, 1985 and Rogers and Verotta, 1996).

The aim of this study was to investigate the anthelmintic effect of acetone leaf extract and fractions of *C. molle* and the relative efficacy of the components against gastrointestinal sheep nematodes.

2. Materials and methods

2.1. Preparation plant extracts

The leaf of the plant *C. molle* was collected in Zaria, Nigeria. Voucher specimens (No. 900191) were identified and deposited by the Herbarium Section of the Biological Sciences Department, Ahmadu Bello University, Zaria. The plant material was air dried and ground to a powder (170 g) using a Macsalab Model 200 LAB grinder. The extract was prepared by maceration with shaking (Labotec Model 20.2 shaker) for 24 h in 70% acetone (A) with a 10:1 solvent to dry weight ratio (Eloff, 1998) and the extract was filtered through Whatman No. 1 filter paper using a Buchner funnel, and the acetone removed by air drying.

A solvent:solvent group separation procedure used by the USA National Cancer Institute as described by Suffness and Douros (1979) was adopted to fractionate the acetone extract with a slight variation. The acetone extract (15.80 g) was dried in a rotary evaporator under reduced pressure and this extract was dissolved in a 1:1 mixture of chloroform and water. The water fraction was extracted with an equal volume of butanol in a separating funnel to yield the water and butanol fractions. The chloroform fraction was dried in a rotary evaporator under reduced pressure and extracted with a 1:1 mixture of hexane and 10% water in methanol. The hexane fraction was recovered with a separating funnel. The 10% water in methanol extract was diluted to 35% water in methanol and extracted by adding equal volume of chloroform to yield the chloroform fraction and the 35% water in methanol fractions.

2.2. *Haemonchus contortus* egg recovery

H. contortus eggs were recovered from faeces according to Hubert and Kerboeuf (1992). Sample of faeces (10–15 g) were collected from sheep experimentally infected with mono-specific larval suspensions of fresh *H. contortus*. The faecal samples were suspended in water and cleared of organic debris by filtration through 1 mm and 150 μm sieves. Eggs were collected on a 25 μm sieve and further cleared of organic debris by centrifugation in magnesium sulphate (density 1.10) for five minutes at 1000 × *g*. The supernatant was filtered through 100 μm and 63 μm sieves and the eggs were washed in water and collected on a 25 μm sieve. The concentration of eggs was estimated in 200 μL samples and adjusted to 500 eggs/mL. 5 μg/mL amphotericin B solution (Sigma, Germany) was added to the egg suspension to avoid fungal development.

2.3. Egg hatch assay

The *in vitro* egg hatch assay was based on the method described by Coles et al. (1992). Egg suspension (0.2 mL) was distributed in a 48-flat-bottomed microplate so that each well contain100 fresh eggs and mixed with the same volume of plant extract dissolved in acetone at concentrations of 10 mg/mL in 8 serial dilutions. Albendazole (99.8% pure standard reference) (Sigma, USA) was used as a positive control. The albendazole was dissolved in dimethyl sulfoxide (DMSO) and diluted at concentrations between 1 µg/mL and 0.0075 µg/mL. The control plates contained the diluents water and acetone or 0.3% DMSO and the egg solution. The eggs were incubated in this mixture for 48 h at 27 °C and 70% relative humidity. After this time a drop of Lugol's iodine solution (Reidel de Hae, Germany) was added to stop the eggs from hatching. All the eggs and first-stage larvae (L_1) in each plate were counted. There were three replicates for each concentration and control.

2.4. Larval development and viability assay

The procedures used were a modification of the technique described by Hubert and Kerboeuf (1992). 150 µL aliquots of egg suspension which contained approximately 100 eggs and 20 µL of filtrate obtained by faecal washing during egg recovering were distributed to wells of a 48-well flat-bottomed microtiter plate. This suspension was supplemented with 30 µL of the nutritive medium described by Hubert and Kerboeuf (1984) and comprised of Earle's balanced salt solution (Sigma, Germany) plus yeast extract (Sigma, Germany) in saline solution (1 g of yeast extract/90 mL of saline solution) at a ratio of 1:9 (v/v). The plates were incubated at 27 °C and 70% relative humidity. After 48 h, 200 µL of the extract, albendazole (Sigma, USA) or diluent (control) were added. The third stage larvae were recovered six days later. At this time the parasites were counted by separating the larvae into two classes, third-stage larvae (L_3) and other developmental stage larvae (L_1 and L_2). There were three replicates for each concentration and control.

2.5. Statistical analysis

The LC_{50} was determined by calculating the concentration of extract that gave a response halfway between the minimum and maximum responses in a concentration–response sigmoid curve. The relation below gives the egg hatch and larval viability parameter respectively:

$$\frac{\text{Number of larvae/total number of larvae and eggs in wells with plant extract}}{\text{Number of larvae/total number of larvae and eggs in control well (water)}}$$

$$\frac{\text{Number of larvae L3/total number of nematode in wells with plant extract}}{\text{Number of larvae L3/total number of nematode in control well (water)}}$$

Determination of LC_{50} of a sigmoidal concentration response (variable slope) curve was performed using GraphPad Prism version 4.01 for Windows (GraphPad, San Diego, CA, USA). The analysis of the family of data sets generated by four solvent:solvent fractions tested was performed by the global curve-fitting model of nonlinear regression analysis with top and bottom shared among the data sets. In addition, the bottom of the curve was constrained as >0 and the top was constrained as <1.0. A (global) best-fit value that applies to the family of data sets was calculated for each of these shared parameters, while the best-fit LC_{50} value (unshared parameter) was calculated with a 95% confidence interval for each of the data sets (fractions). The relative bioactivities of the fractions was further assessed by comparing the best-fit LC_{50} value of the various fractions by one-way ANOVA and Tukey's multiple comparison test, which was performed using GraphPad Prism version 4.01 for Windows (Ademola et al., 2005).

3. Results

3.1. Yields of extracts and fractions

The acetone extract gave a yield of 20.23 g (11.90%, w/w), whereas the hexane, chloroform, butanol and 35% water in methanol fractions of the acetone extract gave yields of 6.01 g (38.05%), 6.00 g (38.04%), 1.08 g (6.86%) and 1.28 g (8.11%), respectively. The water fraction was contaminated with fungi while drying, and was therefore excluded from the study.

3.2. Egg hatch assay

C. molle acetone extract and its fractions inhibited hatching of eggs in a concentration-dependent manner (Fig. 1). The shared statistical parameters of the curve fitting analysis and the best-fit LC_{50} values for the acetone extract and its fractions are shown in Table 1. Tukey's multiple

comparison (post-ANOVA) test shows that the chloroform fraction is significantly more active than all the other fractions ($p < 0.05$) Table 2. Albendazole produced an LC_{50} for egg the hatch inhibition assay at a low concentration (0.164 μg/mL), indicating that the strain of *H. contortus* used in the current study was susceptibility.

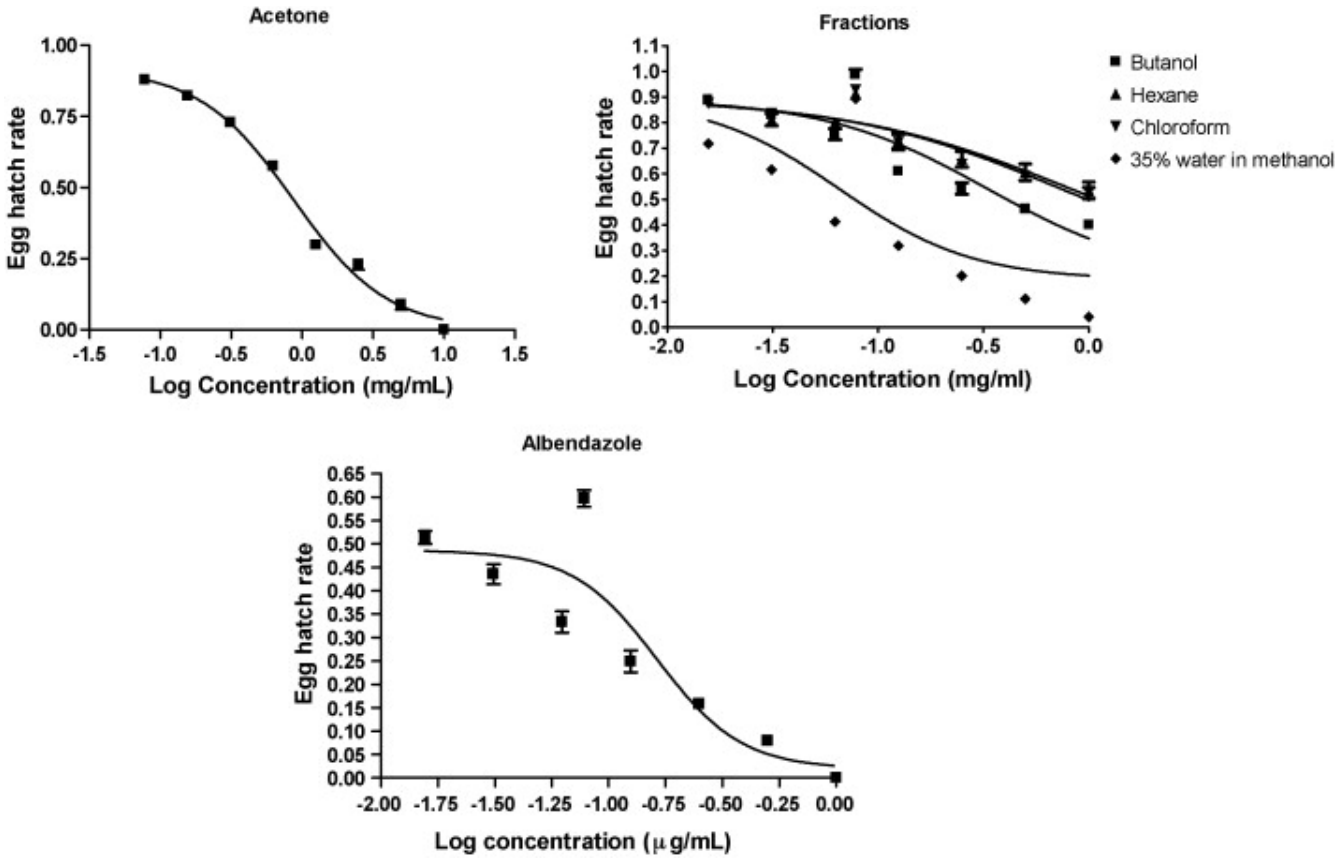

Fig. 1. *Egg hatch assay concentration–response curve of acetone extract and fractions of C. molle, and albendazole against eggs of H. contortus using global sigmoidal model of curve fitting.*

Table 1: *Egg hatch assay LC_{50} of C. molle acetone extract and its fractions using global sigmoidal (four-parameter logistic) model of curve-fitting.*

Fractions	**log LC_{50}**		**LC_{50} (mg/mL)[a]**		***R^2***
	Best-fit	**Std. error**	**Best-fit**	**95% CI**	
Acetone	−0.0624	0.03771	0.8661	0.7226–1.038	0.9878
Butanol	−0.4770	0.1256	0.3334	0.1874–0.5932	0.7866
Hexane	−0.0803	0.1773	0.8311	0.3686–1.874	0.7942
Chloroform	−0.1267	0.1668	0.7470	0.3477–1.605	0.8127
35% water in methanol	−1.182	0.1010	0.06576	0.04138–0.1045	0.6204
Albendazole	−0.7865	0.09927	0.1635[b]	0.1015–0.2634	0.8157

[a] Global shared parameters for acetone extract and fractions.

[b] μg/mL.

Table 2: *Tukey's multiple comparison test comparing the LC_{50} values of fractions of C. molle.*

Egg hatch assay		**Larval dev. and viability assay**	
Comparisons	***p*-Value**	**Comparisons**	***p*-Value**
Butanol vs hexane	$p > 0.05$	Hexane vs 35% water in methanol	$p > 0.05$
Butanol vs chloroform	$p > 0.05$	Hexane vs butanol	$p > 0.05$
Butanol vs 35% water in methanol	$p > 0.05$	Hexane vs chloroform	$p > 0.05$
Hexane vs chloroform	$p > 0.05$	35% water in methanol vs butanol	$p > 0.05$
Hexane vs 35% water in methanol	$p < 0.05$	35% water in methanol vs chloroform	$p > 0.05$
Chloroform vs 35% water in methanol	$p < 0.05$	Butanol vs chloroform	$p > 0.05$

3.3. Larval development and viability assay

The *C. molle* extracts killed the nematode larvae in a concentration-dependent manner (Fig. 2). The shared statistical parameters of the curve fitting analysis and the best-fit LC_{50} values for the acetone extract and the fractions are shown in Table 3. There was no significant difference in the activity of the fractions on the larvae ($p > 0.05$). Albendazole produced an LC_{50} at a low concentration (0.144 μg/mL), indicating that the strain of *H. contortus* used in the current study was susceptible.

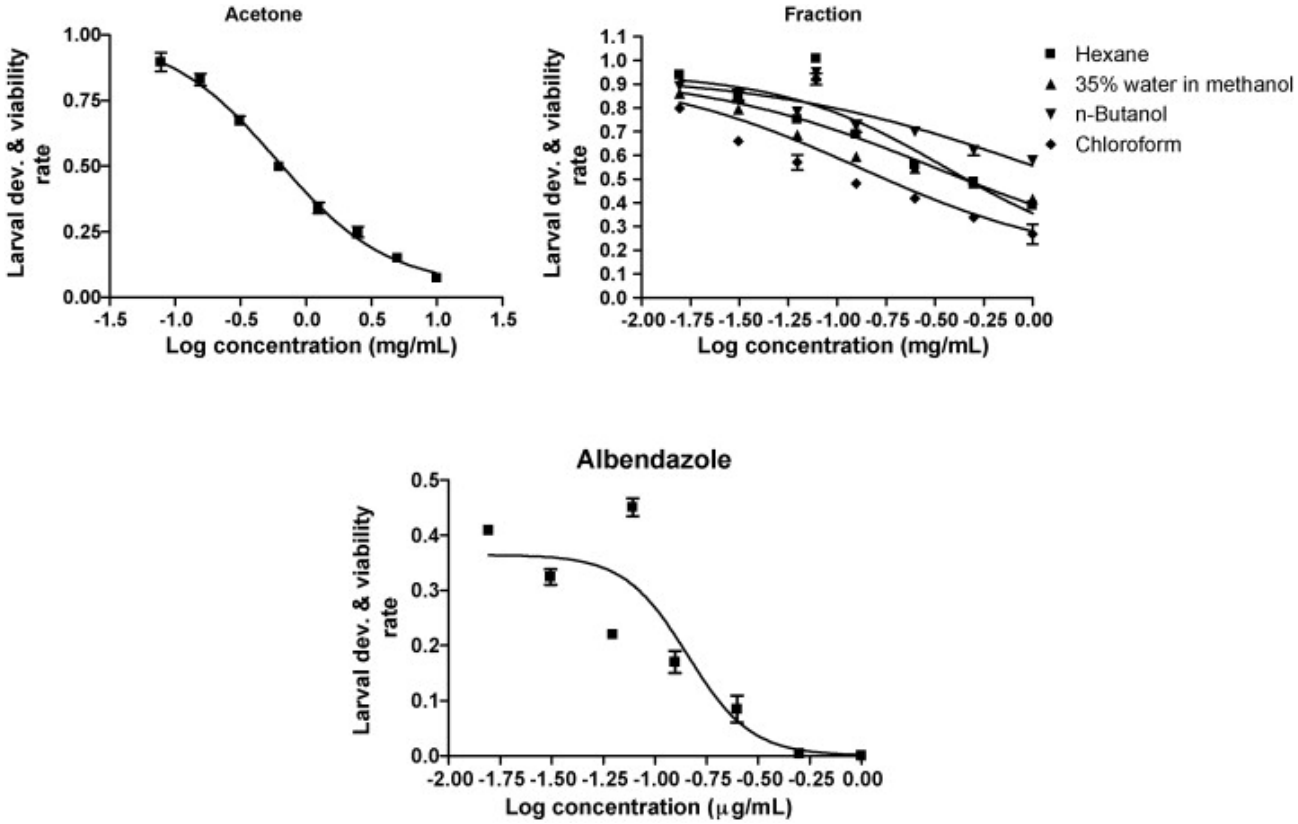

Fig. 2. *Larval development and viability assay concentration–response curve of acetone extract and fractions of C. molle, and albendazole against larvae of H. contortus using global sigmoidal model of curve fitting.*

Table 3: *Larval development and viability assay LC_{50} of C. molle acetone extract and its fractions using global sigmoidal (four-parameter logistic) model of curve-fitting.*

Fractions	**log LC_{50}**		**LC_{50} (mg/mL)[a]**		***R^2***
	Best-fit	**Std. error**	**Best-fit**	**95% CI**	
Acetone	−0.2192	0.05611	0.6037	0.4611–0.7904	0.9872
Butanol	−0.4418	0.3175	0.3616	0.1417–0.9226	0.8264
Hexane	0.03218	0.2043	1.077	0.2511–4.619	0.7512
Chloroform	−0.8832	0.2659	0.1308	0.03866–0.4429	0.6785
35% water in methanol	−0.4970	0.2750	0.3184	0.09023–1.124	0.7631
Albendazole	−0.8422	0.08774	0.1438[b]	0.09436–0.2192	0.8076

[a] Global shared parameters for acetone extract and fractions.

[b] μg/mL.

4. Discussion

The problem of anthelmintic resistance in nematodes and an increasing concern over the presence of drug residues in animal products, when pure compounds are administered, has led to a resurgence of interest in the use of phytomedicines, in the form of extracts containing a mixture of compounds (Athanasiadou et al., 2001).

The LC_{50} values for the hexane and chloroform fractions for egg hatch inhibition were close to each other (Table 1), which explains why only three distinct curves are seen in Fig. 1. The acetone extract and solvent:solvent fractions affected the hatchability of *H. contortus* eggs in a concentration-dependent manner. The LC_{50} obtained for *C. molle* acetone extract was 0.866 mg/mL. On the other hand, the solvent:solvent fractions of *C. molle* were more potent. The 35% water in methanol fraction gave the lowest LC_{50}, which is about one-tenth of the value obtained for the hexane fraction which had the highest LC_{50} and suggest a significant difference ($p < 0.05$). The LDVA results suggest that the *C. molle* extracts affected the larval development from L_1 to the infective stage (L_3) in a concentration-dependent manner. The LC_{50} value obtained for the *C. molle* acetone extract was 0.604 mg/mL. On the other hand, the

solvent:solvent fractions of *C. molle* were more active except for the hexane fraction which had a higher LC_{50} (1.077 mg/mL) than the acetone extract. The statistical comparisons made by the one-way ANOVA were computed with associated standard error from the curve-fitting analysis and suggested absence of significant difference ($p > 0.05$). The *in vitro* activity of pure anthelmintic compounds is usually higher. The LC_{50} values for albendazole were 0.164 µg/mL and 0.144 µg/mL for EHA and LDVA, respectively. However, there was no anthelmintic activity with aqueous extract of *C. molle* at 0.5 mg/mL and 1 mg/mL, when tested on *Caenorhabditis elegans* (McGaw et al., 2001). Each fraction represents a mixture of chemical compounds with similarity in chemical structure or other physicochemical properties. According to Eloff (1998) acetone dissolves many hydrophilic and lipophylic components from *Combretum erythrophyllum* and the antimicrobial components in the plant are more lipophylic. It could be that the polar constituents of the plant were responsible for the anthelmintic activity observed in the present study.

The anthelmintic effect of *C. molle* against nematode may be attributed to its saponin (Mc Allister et al., 2001). Monodesmoside saponins destabilize membranes and increase cell permeability by combining with membrane-associated sterols (Price et al., 1987 and Gee and Johnson, 1988). Other plant extracts with *in vitro* anthelmintic activity are *Peltophorum africanum* and *Leucaena leucocephala* (Bizimenyenra et al., 2006 and Ademola and Idowu, 2006).

5. Conclusions

The use of the crude methanol or chloroform extract of the powdered leaf of *C. molle* may therefore be useful and effective product for the control of gastrointestinal nematode in livestock production. However further spectroscopic studies on the active principles and the development of quality assurance protocols involving the use of reference substance of plant origin for this extract is warranted. Unambiguous structure elucidation of the active principles could provide leads for drug discovery and suitable bioactive marker compounds for standardization of the extract as a phytomedicine.

Acknowledgements

The authors are grateful to the National Research foundation South Africa for funding the work. The University of Ibadan, Nigeria is thanked for granting the main author staff development leave. We thank Onderstepoort Veterinary Institute, South Africa and Dr. A.F. Vatta for providing access to facility and technical assistance. The authors are grateful to F. van Schalkwyk, Biozetica Agri-source (Pty) Ltd. Ondersteeport, Pretoria, for providing *H. contortus* larvae.

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