African Journal of Biotechnology Vol. 10(47), pp. 9700-9705, 24 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1706
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Anthelmintic efficacy of cashew (*Anarcadium occidentale* L.) on *in vitro* susceptibility of the ova and larvae of *Haemonchus contortus*

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Accepted 16 April, 2010

The use of plants for the treatment of human and animal diseases continues to rise although there are few studies providing proof of these effects. Among them is the *Anacardium occidentale* L., popularly known as cashew. *In vitro* egg hatch and larval development and viability assays was conducted to determine possible direct anthelmintic effect of acetone extract and fractions of *A. occidentale* against nematode of sheep, predominantly, *Haemonchus contortus*. The effect of the extracts on hatching of eggs and development and survival of infective larvae (L_3) was assessed. The best-fit LC_{50} values were computed by global model of non-linear regression curve fitting (95% confidence interval). The presence of *A. occidentale* extracts in the cultures decreased the hatchability of eggs and survival of L_3 larvae in a concentration dependent manner. The LC_{50} values of acetone extract was 0.311 and 1.72 mg/ml for egg hatch and larval viability test, respectively. The fractions of *A. occidentale* were more active, demonstrating a lower LC_{50} compare with the acetone extract. The activities of the fractions were not significantly different against the eggs and larvae of *H. contortus* ($p > 0.05$). Further studies are required to identify the compound(s) responsible for activity and more clearly comprehend the anthelmintic mechanism detected in this study.

Key words: *Anacardium occidentale*, anthelmintic, *Haemonchus contortus*, *in vitro* detection, larvae, ova.

INTRODUCTION

The control of helminth parasite infections is necessary for the maintenance of healthy, productive livestock. Nematodes damage the gastrointestinal (GI) tract, decrease feed intake, decrease nutrient absorption, alter feed utilization and, in some cases, can lead to livestock death (Parkins and Holmes, 1989). Helminth parasite control methods rely on a combination of management methods and chemotherapeutics (anthelmintics). Alternatives to the commonly used chemotherapeutics are needed for several reasons. Firstly, many of the available treatments for helminth parasites are becoming less effective. Helminth parasites are becoming resistant to almost every chemical class of available anthelmintics (Prichard, 1994). Secondly, there are environmental pollution and human health concerns with both types of treatments. For example, ivermectin, which is one of the most commonly used anthelmintics, can potentially kill beneficial soil microorganisms (Pfeiffer et al., 1998). Thirdly, there is a growing desire among the general population for more natural and environmentally friendly treatments (example, the increase in the organic food market). Fourthly, in many parts of the world, synthetic anthelmintics are either unavailable or are not cost-effective (Hammond et al., 1997).

Plants with bioactive compounds are a potential alternative to the chemotherapeutics currently used to control parasite infections. Plant treatments for helminth can be given as single oral doses, daily doses mixed with feeds and planted in pastures. *Anacardium occidentale* is yellowish-pink with 5-petalled flowers borne in 6 to 10-in

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(15 - 25 cm) terminal panicles of mixed male, female and bisexual. The true fruit of the tree is the cashew nut resembling a miniature boxing-glove; consisting of a double shell containing a caustic phenolic resin in honeycomb-like cells, enclosing the edible kidney-shaped kernel. The oil of *A. occidentale* is active against *Ascaridia galli* in chicken (Varghese et al., 1971) and against hookworms in dogs and man (Cavier, 1973). Commercially available cashew nut shell liquid (CNSL) mainly contains the phenolic constituents, anacardic acid, cardol and cardanol. These phenolic constituents are themselves heterogeneous and each of them contains saturated, monoene, diene and trienes in the fifteen-carbon side chain. The objective of the present work was to assess the ovicidal and larvicidal effects of cashew leaf acetone extract and its fractions on *Haemonchus contortus* eggs and larvae.

MATERIALS AND METHODS

Plant extracts preparation

The leaf *A. occidentale* was collected in Zaria, Nigeria. Voucher specimens (No: 184) were identified and deposited with the Herbarium Section of the Department of Biological Sciences, Ahmadu Bello University, Zaria. 120 g of the plant material was air dried and ground to powder using a Macsalab Model 200 LAB grinder. The acetone, extract was prepared by maceration with continuous shaking (Labotec Model 20.2 shaker) for 24 h in 70% acetone with a 10:1 solvent to dry weight ratio (Eloff, 1998). The extract was then filtered through Whatman No 1 filter paper using a Buchner funnel and the acetone removed under stream of air.

The solvent: solvent group separation procedure used by the USA National Cancer Institute as described by Suffness and Douros (1979) was used to fractionate the acetone extract with a slight modification. The acetone extract 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 with chloroform to yield the chloroform fraction and the 35% water in methanol fractions.

Nematode egg recovery

Nematode eggs predominantly *H. contortus* (87%) were recovered according to the method described by Hubert and Kerboeuf (1992). A sample of faeces (10 to 15 g) from sheep experimentally infected with mono-specific larval suspensions of fresh *H. contortus* was suspended in water and cleared of organic debris by filtration through 1 mm and 150 µm sieves. The eggs were collected on a 25 µm sieve and further cleared of organic debris by centrifugation in magnesium sulphate (density 1.10) for 5 min at 1000 g. The super-natant was filtered through 100 and 63 µm sieves. The eggs in the filtrate were washed in water and collected on a 25 µm sieve. The concentration of eggs was estimated in 200 µl samples and then adjusted to 500 eggs/ml. To avoid the proliferation of fungi, 5 µg amphotericin B solution (Sigma, Germany) was added per millilitre of egg suspension.

Egg hatch test

The *in vitro* egg hatching test was based on the method described by Coles et al. (1992). 0.2 ml of the egg suspension containing approximately 100 fresh eggs was distributed in a 48-flat-bottomed microtitre plate and mixed with the same volume of plant extract dissolved in acetone at concentrations of 10 mg/ml in 8 serial dilutions. Albendazole (Sigma, USA) (99.8% pure standard reference) was used as a positive control. Albendazole was dissolved in dimethyl sulfoxide (0.3% DMSO) and diluted at concentrations between 1 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 unhatched eggs from hatching. All the eggs and first-stage larvae (L_1) in each plate were counted under an inverted microscope. There were three replicates for each concentration and control.

Larval viability test

The method adopted was a modification of the technique described by Hubert and Kerbouef (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 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). There were three replicates for each concentration and control. The plates were incubated at 27°C and 70% relative humidity for 48 h. Thereafter, 200 µl of the extract or diluents (control) were added for further six hours to obtain the third stage larvae. The parasites were then counted by separating the larvae into two classes, third-stage larvae (L_3) and other developmental stages larvae (L_1 and L_2).

Statistical analysis

The LC_{50} was determined by computing 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 parameters, respectively:

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

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

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, California, USA). The family of data sets generated by the four solvent: solvent fractions tested was analysed 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 computed for each of these shared parameters, while the best-fit LC_{50} value (unshared parameter) was calculated with 95% confidence interval for each of the data sets (fractions). The relative bioactivity of the fractions was further assessed by comparing the best-fit LC_{50} value of the various

fractions by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test, which was performed using GraphPad Prism version 4.01 for Windows (Ademola et al., 2005).

RESULTS

Yield of extract and fractions

The acetone extract gave a yield of 15.83 g (13.19%), whereas the hexane, chloroform, butanol and 35% water in methanol fractions of the acetone extract gave a yield of 2.99 g (27.18%), 2.95 g (26.73%), 1.25 g (11.33%) and 0.79 g (7.15%), respectively. Water fraction was excluded from the study.

Egg hatch assay

The acetone extract and the fractions of *A. occidentale* killed the eggs in a concentration-dependent manner (Figure 1). 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 1. The best-fit LC_{50} values were calculated with reasonable precision (95% CI). Acetone extract produced LC_{50} value of 0.311 mg/ml while butanol fraction was the most active compared to the other fractions (LC_{50} of 0.074 mg/ml). But the LC_{50} obtained for each of the fractions did not differ significantly ($p > 0.05$). Albendazole produced LC_{50} at low concentration (0.083 µg/ml), indicating susceptibility of the strain of *H. contortus* used in the current study.

Larval development viability assay

Acetone extract and the solvent: solvent fractions of *A. occidentale* affected larval development and killed the transformed infective stage (L_3) of the *H. contortus* larvae (Figure 2). The best-fit LC_{50} values were calculated with reasonably narrow precision (95% CI) (Table 2). The hexane and 35% water in methanol fraction were the most active fractions with LC_{50} values of 0.142 and 0.143 mg/ml, respectively. However the acetone extract showed a much higher LC_{50} of 1.572 mg/ml. Table 3 shows Tukey's multiple comparison (post ANOVA) test, the mean LC_{50} of the fractions were not significantly different for egg hatch inhibition and larval viability tests ($p > 0.05$). Albendazole produced LC_{50} at a low concentration (0.061 µg/ml), indicating susceptibility of the strain of *H. contortus* used in the current study.

DISCUSSION

The use of plants with medicinal properties for the treatment, cure and prevention of diseases is one of the oldest medicinal methods known in history. At the beginning of the 1990s, the World Health Organization stated that 65 – 80% of the population of developing countries depended on medicinal plants as their only form of basic health care (Akerele, 1993). The present study evaluated the possible ovicidal and larvicidal effects of the plant extract and fractions using egg hatch inhibition and larval development and viability tests as parameters. The results of the egg hatch inhibition test showed that the LC_{50} values for butanol fraction was the lowest (Table 1), which means that it is the most active fraction. Statistically, Tukey's multiple comparison (post

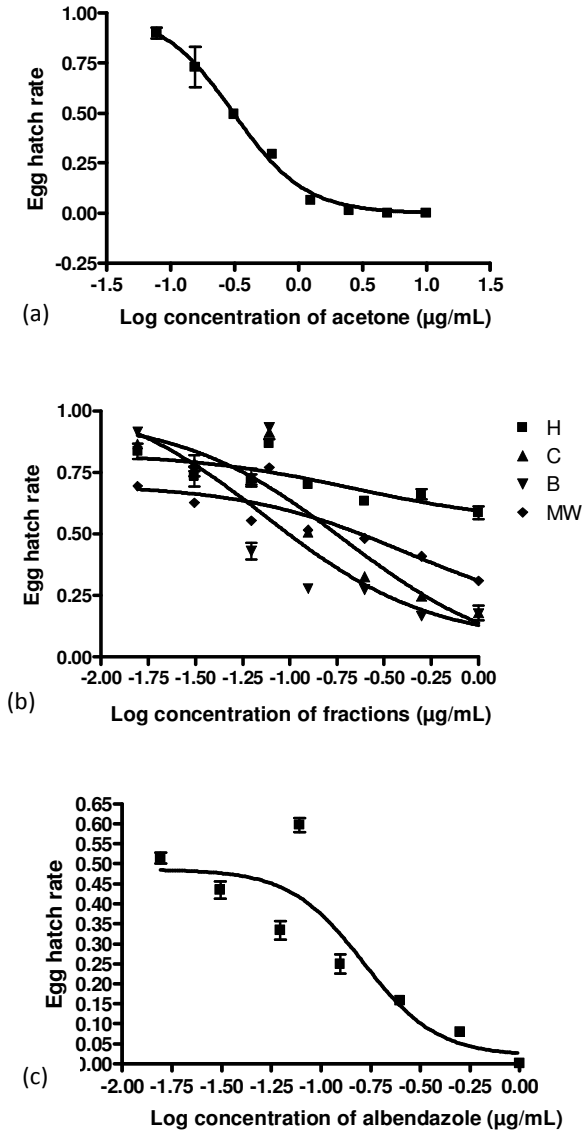

Figure 1. Egg hatch assay concentration–response curve of acetone extract and fractions of *A. occidentale*, and Albendazole against eggs of *H. contortus* using global sigmoidal model of curve fitting (A: acetone; C: chloroform; H: hexane; B: butanol; MW: 35% water in methanol).

Table 1. Egg hatched assay LC_{50} of *A. occidentale* acetone extract and fractions using global sigmoidal (4-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[b]	
Acetone	-0.5069	0.06819	0.3113	0.2243 to 0.4319	0.9707
Hexane	-0.6725	0.4097	0.2126	0.02988 to 1.512	0.5658
Chloroform	-0.7097	0.1774	0.1951	0.08344 to 0.4562	0.8712
Butanol	-1.130	0.2702	0.07420	0.02035 to 0.2706	0.7215
35% water in methanol	-0.3843	0.2916	0.4128	0.1022 to 1.668	0.7716
Albendazole	-0.7865	0.09927	0.1635[c]	0.1015 to 0.2634 [c]	0.8157

[a]Global shared parameters for acetone extract and fractions; [b]CI: confidence interval and [c]µg/ml.

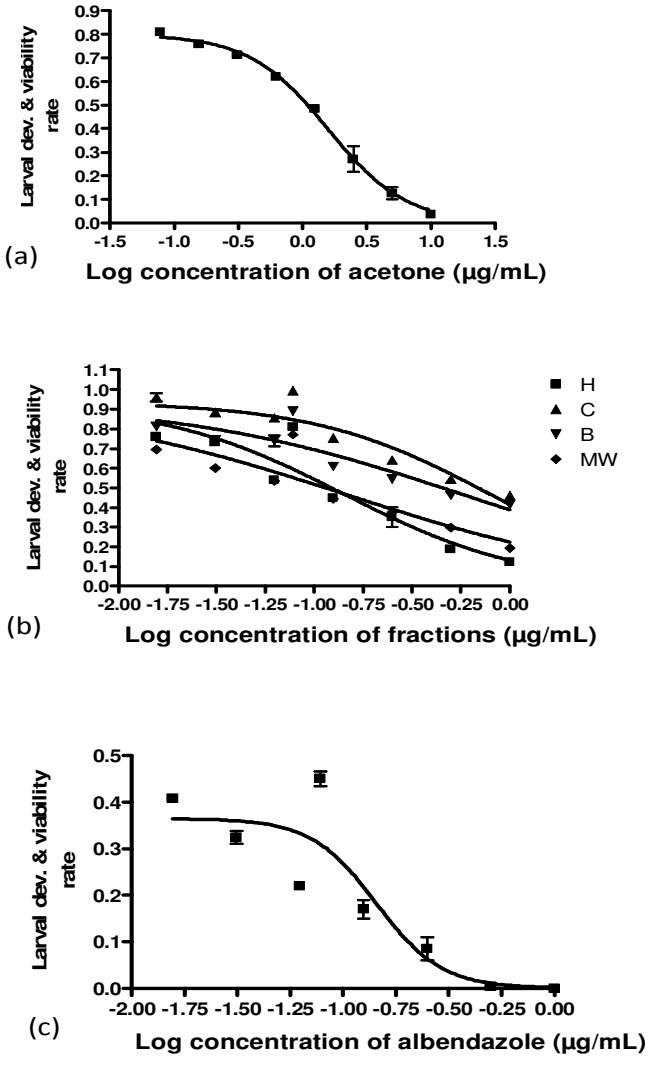

Figure 2. Larval development and viability assay concentration–response curve of acetone extract and fractions of *A. occidentale*, and Albendazole against larvae of *H. contortus* using global sigmoidal model of curve fitting (A: acetone; C: chloroform; H: hexane; B: butanol; MW: 35% water in methanol).

ANOVA) suggested absence of significant difference when fractions were tested on nematode eggs and larvae. The effect of acetone extract and solvent: solvent fractions on egg hatching were comparatively close. The hexane and 35% water in methanol had a comparable effect on *H. contortus* larvae with of an LC_{50} value of 0.142 and 0.143 mg/ml, respectively. It could be that the active principles of the plants act synergistically against the stages of the nematode. However, the mechanism of the ovicidal and larvicidal actions still needs to be explained. The acetone extract was less effective than the fractions. But the synthetic anthelmintic albendazole indicated a much lower LC_{50} values. Albendazole is a pure active substance, while acetone extract and fractions contains several chemical compounds, among them the active ingredient with ovicidal and larvicidal actions, in small amounts. In general, the extract of a plant has small concentrations of active compounds and a great number of promising properties.

Cavalcante et al. (2003) evaluated fresh and processed cashew juice on *Salmonella typhimurium* and indicated that *A. occidentale* presented antimutagenic activity. According to the authors, this property could be related to the chemical compounds of the juice, such as high concentrations of vitamin C, various carotenoids and phenolics compounds. Severine and Herve (2006) reported that monomers of condensed tannins interact with the exsheathment of nematode third-stage larvae. Bahuaud et al. (2003) had earlier indicated that condensed tannins of whole extracts of four tannin-rich plants interact with the exsheathment of nematode third-stage larvae. It is possible that tannins contained in the extract of *A. occidentale* produced similar effects. In another study, polyphenols from bryophytes were demonstrated to have anthelmintic activity against *Nippostrongylus brasiliensis* (Gamenara et al., 2001). Some synthetic phenolic anthelmintics, example, niclosamide, oxyclozanide, bithionol and nitroxynil are found to interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation (Martin, 1997).

Table 2. Larval development and viability assay LC_{50} of *A. occidentale* acetone extract and fractions using global sigmoidal (4-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[b]	
Acetone	0.1964	0.04608	1.572	1.260 to 1.961	0.9830
Hexane	-0.8492	0.1605	0.1415	0.06779 to 0.2955	0.8542
Chloroform	-0.1026	0.1377	0.7896	0.4200 to 1.484	0.8681
Butanol	-0.2492	0.2115	0.5634	0.2136 to 1.486	0.7921
35% water in methanol	-0.8446	0.2251	0.1430	0.05096 to 0.4014	0.7795
Albendazole[c]	-0.8422	0.08774	0.1438[c]	0.09436 to 0.2192[c]	0.8076

[a]Global shared parameters for acetone extract and fractions, [b]CI: confidence interval and [c]µg/ml.

Table 3. Tukey's multiple comparison test comparing the LC_{50} values of fractions of *A. occidentale*.

Egg hatch inhibition assay		Larval dev. and viability assay	
Comparisons	p value	Comparisons	p value
H vs C	P > 0.05	H vs C	P > 0.05
H vs B	P > 0.05	H vs B	P > 0.05
H vs MW	P > 0.05	H vs MW	P > 0.05
C vs B	P > 0.05	C vs B	P > 0.05
C vs MW	P > 0.05	C vs MW	P > 0.05
B vs MW	P > 0.05	B vs MW	P > 0.05

C: Chloroform; H: hexane; b: butanol; mw: 35% water in methanol.

Another possible anthelmintic effect of tannins is that they can bind to free proteins in the gastrointestinal tract of host animal (Athanasiadou et al., 2001) or glycoprotein on the cuticle of the parasite (Thompson and Geary, 1995) and cause death. However, the anthelmintic effect of plants containing tannins actually depends on the type and content of tannins in the plant (Niezen et al., 1998; Athanasiadou et al., 2001). Some anthelmintics act by paralysing the worms, which then may have to be expelled by a purge; others destroy the parasite through lysis, because they contain proteolytic enzymes such as bromelain (*Ananas comosus* (L.) Merr.), calotropain (*Calotropis procera* (Aiton) W.T. Aiton) and pawpaw (*Carica papaya* L.) (Stepek et al., 2004). Other active anthelmintics are found amongst the flavonoids, terpenoids, mustard-oil heterosides and plants containing proteolytic enzymes (Boreham, 1995).

Although the use of plant extracts as phytomedicines is becoming increasingly popular as alternative to the use of single molecule synthetic drugs, accurate knowledge of the composition of phytomedicines is still warranted.

Conclusion

The present results indicate that cashew leaf acetone extract could be useful in the control of helminth. Future studies *in vitro* and *in vivo* are required to develop a clearer understanding of the anthelmintic properties attributed to cashew leaf extract and its components, as well as regarding its safe use in ethno-veterinary medicine.

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