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Evaluation of Anthelmintic Potential of Ethanolic Plant Extracts from Northern Cameroon Against Eggs and Infective Larvae of *Haemonchus contortus*

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Abstract: A commercial anthelmintic, Febendazole (Panacur) and ethanolic leaf extracts from five herbal plants (*Annona senegalensis*, *Anogeissus leiocarpus*, *Lippia rugosa*, *Stereospermum kunthianum* and *Vernonia tonoreana*), collected in the northern region of Cameroon in September 2001, were assessed for egg-inhibition ability as well as toxicity against the infective larval stage (third-instar) of *Haemonchus contortus* between January and March of 2002. The eggs and larvae were exposed to different concentrations (0, 0.1, 0.2, 0.4 and 0.8% (w/v)) of the test substances for 7 days and 1-48 h, respectively. Seven day EC₅₀ and 6 and 48 h LC₅₀ values were also determined. All the test substances induced a significant (p<0.01) dose-dependent inhibition of egg-hatch and larval mortality. Complete egg-hatch inhibition was recorded only for *V. tonoreana*, while the highest larval mortality of 98.33% was accomplished by *A. senegalensis* for the 0.8% level. Seven day EC₅₀ and 48 h LC₅₀ values were respectively 0.220 and 0.090% for Febendazole, 0.108 and 0.070% for *A. senegalensis*, 0.140 and 0.061% for *A. leiocarpus*, 0.299 and 0.129 for *L. rugosa*, 0.200 and 0.152% for *S. kunthianum* and 0.174 and 0.078% for *V. tonoreana*. Overall, the potency of the plant extracts was comparable to that of Febendazole, although the extracts from *L. rugosa* and *S. kunthianum* tended to be less active. The findings suggest these plants could yield natural alternative treatment products for *H. contortus*. However, further studies are required to determine their *in vivo* nematocidal properties.

Key words: *Haemonchus contortus*, plant extracts, anthelmintic properties, alternative control methods, Cameroon

INTRODUCTION

Infectious diseases due to parasitic nematodes remain a major problem to humans and food animals especially in developing countries. In grazing livestock, gastrointestinal parasites constitute a major constraint to animal production throughout the world particularly in the tropics and subtropics (Perry *et al.*, 2002). Control of parasitic nematode infection has been largely based on repeated use of synthetic anthelmintic drugs. However, in Cameroon and other developing countries, commercially available anthelmintic drugs are expensive and unaffordable to small livestock farmers and pastoralists (IRRI, 1994; Ketzis, 1999; Ketzis *et al.*, 2002). Intensive and often indiscriminate use of sometimes adulterated anthelmintic drugs (Monteiro *et al.*, 1998) has led to resistance in nematode parasites, particularly *Haemonchus contortus* to currently available drugs

(Prichard, 1994; Waller 1997; Coles *et al.*, 2005). Besides the problem of developing resistance to chemotherapy, there is an increasing global consumer concern over drug residues in meat and milk (WHO, 1996; Gasbarre *et al.*, 2001) as well as the environmental impact of drug residues in animal faeces (Herd and Wardhaugh, 1993). Control is further compounded by the complexity of parasites that make vaccine development difficult (Ivory and Chadee, 2004). These factors or a combination of them have necessitated the search for alternative or complimentary solutions for the control of parasitic diseases in livestock (Waller, 1997; Paolini *et al.*, 2003). Amongst the alternative strategies, research into biomedical and ethnoveterinary medicine is gaining substantial interest. In many regions and countries of the world, plants purportedly used in the treatment of livestock diseases are being screened for nematocidal activity for the development of new and effective anthelmintic drugs

(Githiori *et al.*, 2002; Ketzis *et al.*, 2002; Alawa *et al.*, 2003). Plant-derived anthelmintic products have the advantages of less toxicity, more biodegradability and environmental friendliness (Hammond *et al.*, 1997). Besides, the use of locally available plants with established chemotherapeutic activity would be accessible and affordable to small farmers and pastoralists in developing countries for effective control of livestock parasitic diseases.

In Cameroon, particularly the northern region (Adamawa and North Provinces) with the largest cattle population in Central Africa, there is a long tradition of using medicinal plants for the treatment of livestock diseases by herdsmen and small cattle farmers as an integral part of their nomadic culture (Nfi *et al.*, 1997). Some of these plants have been claimed, on empirical grounds to have some anthelmintic properties. Unlike in many developing nations where the anthelmintic properties of such plants have been investigated, there is paucity of published scientific information on the biological activity of plants commonly used by livestock farmers in Cameroon. However, Musongong *et al.* (2004) recorded high *in vitro* acute toxicity of three plants from the region to the infective larval stage (third-instar) of *strongyloides papillosus*.

Haemonchus contortus is a parasitic nematode with high pathogenicity and prevalence in Sub-saharan Africa, causing high economic losses due to low productivity, weight loss and mortality in food animals (Anene *et al.*, 1994; Fakae *et al.*, 2000; Ngambia *et al.*, 2000). In addition, *H. contortus* has developed resistance even to the macrocyclic lactones to which some nematodes are still susceptible (Coles *et al.*, 2005). In the temperate countries, cattle develop some immunity and become refractory to infection within 12 months of age under rotational grazing management (Scouthcott and Barger, 1975). Rotational grazing cannot be easily practiced in developing countries due to the limited land size and communal ownership of grazing pastures, thus limiting the acquisition of immunity in calves even at the age of 2 years (Benitez-Usher *et al.*, 1984).

Based on information from the herdsmen, the present study investigated the *in vitro* anthelmintic efficacy of the crude ethanolic leaf extracts of five plants (*Annona senegalensis* Pers. (Annonaceae), *Anogeissus leiocarpus* (DC) Guil and Perrott, (Combretaceae), *Lippia rugosa* A. Chev. (Verbenaceae), *Stereospermum kunthianum* Champ (Bignoniaceae) and *Vernonia tonoreana* Linn. (Asteraceae)) from northern Cameroon relative to that of the commercial anthelmintic, Febendazole (Panacur), against eggs and infective larvae of *H. contortus*.

MATERIALS AND METHODS

Plant materials: Leaves were collected from the five plant species in the northern region of Cameroon in September 2001. The plants were identified at the National Herbarium in Yaounde, where Voucher samples were deposited. Ethanolic extracts were prepared according to Musongong *et al.* (2004) in the Applied Chemistry Laboratory of the University of Ngaoundere. The viscous brown liquid extract was stored at 4°C until used between January and March of 2002 for egg-hatch inhibition and larval mortality bioassays. Working aqueous solutions were prepared by emulsifying each plant extract and Febendazole (check) in 0.1% Tween-80 (Garg and Rajshree, 1992) to give concentrations of 0.1, 0.2, 0.4 and 0.8%. Tween-80 diluted in distilled water was used as the control.

***Haemonchus contortus* eggs and infective larvae:** Two Gudali calves (*Bos indicus*) were housed in the animal facilities at the Agricultural Research Institute for Development (IRAD), Wakwa and screened for nematode infection over a 2-month period until confirmed to be worm-free (Soulsby, 1982). They were infected orally with 3000 *H. contortus* infective larvae, a dose that had been shown to establish infection (Musongong *et al.*, 2004). The infected calves served as sources for fresh eggs throughout the experiment. To obtain eggs and third-stage larvae for the assay, faecal samples were collected from the rectum of calves 30 days after oral infection and used directly in the assays after some washes or cultured in sterile Petri dishes over one week for their development to infective larvae (Hansen and Perry, 1990; Thienpont *et al.*, 1986). A Baermanns apparatus was then used to recover the larvae which were placed in sterile test tubes and stored at 7°C for 12 h to immobilize the larvae according to standard procedures (MAFF, 1977). The larvae were concentrated by extracting the supernatant with a pipette and the process was repeated until all the larvae were recovered.

Egg-hatch assay: This was carried out according to standard guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) for determining anthelmintic resistance (Coles *et al.*, 1992), in the Parasitology Laboratory of IRAD Wakwa. Concentrated *H. contortus* eggs (2000 eggs mL) were diluted in various concentrations of test substances to get approximately 40 eggs per 0.1 mL test solution in 0.5 mL final volume in test tubes, incubated at room temperature and sampled at intervals up to 7 days post

incubation. At each sampling time-point, a tube was agitated, 0.1 mL pipetted onto a slide and repeated three times, then observed under a microscope with the 20×objective. Dead or embryonating eggs were scored according to standard procedures (Coles *et al.*, 1992).

Infective larvae culture assay: A concentrated stock of *H. contortus* larvae was diluted in distilled water to give approximately 25 larvae/0.1 mL of water, in the Parasitology Laboratory of IRAD Wakwa. Plant extracts and Febendazole (check) were emulsified in 0.1% Tween 80 to give aqueous solutions with doubling concentrations of 0.1, 0.2, 0.4 and 0.8% (w/v), while Tween 80 diluted in water was used as control solution. A total volume of 2 mL test solution plus larvae in a test tube was incubated at room temperature and sampling for larval motility was made at 0, 1, 6, 12, 24 and 48 h post-incubation. During each sampling time-point, 0.1 mL solution was pipetted from the 2 mL test tube after agitation and placed on a microscope slide and the number of dead and live larvae counted under a stereoscope microscope. Mortality was determined according to Musongong *et al.* (2004). The percent mortality was calculated from an average of three replicates.

Data analysis: Data on % egg-hatch inhibition and % larval mortality were arcsine [(square root(x/100))] transformed, then subjected to the analysis of variance

(ANOVA) procedure of the Statistical Analysis System (SAS Institute, 2003) to check for differences among test substances (Plant extracts plus Febendazole), concentrations and time post-exposure. Probit analysis (Finney, 1971; SAS Institute, 2003) was applied to determine lethal concentrations causing 50% (LC₅₀) larval mortality of *H. contortus* at 6 and 48 h exposure periods for each test substance. Probit analysis was also used to compute the EC₅₀ values (effective concentrations of plant extract or Febendazole required to inhibit egg-hatching by 50%). Control mortality was taken into consideration (Abott, 1925) before probit analysis. R² values were estimated using the regression of mortality on log10 (concn. + 1) (Prog Reg: SAS Institute, 2003).

RESULTS

Egg-hatch inhibition: There were significant (p<0.01) differences in egg-hatch inhibition (% unhatched eggs) among concentrations and test substances. Egg-hatch inhibition was significantly (p<0.01) lower for the control (2.7%) as compared with that for any of the test concentrations. This parameter was dose-dependent irrespective of test substance (Fig. 1). At the lowest tested concentration of 0.1%, the plant extracts generally inhibited egg-hatching more than Febendazole, which recorded less than 20% unhatched eggs, while only *A. senegalensis* inhibited more than 50% egg-hatching at this concentration. Percentage inhibition for the other

Table 1: Relationship between *H. contortus* egg-hatch inhibition and concentration of five crude plant extracts and Febendazole 7 days post-exposure

Plants	Slope±SE	R ²	EC ₅₀ ^a (95% FL) ^b	χ ²	p>χ ²
<i>A. senegalensis</i>	1.732±0.413	0.77	0.108 ^e	6.26	0.0438 ^d
<i>A. leiocarpus</i>	2.156±0.240	0.81	0.140 (0.113-0.164)	1.00	0.6078
<i>L. rugosa</i>	1.982±0.332	0.96	0.299 (0.146-0.683)	4.94	0.0846
<i>S. kunthianum</i>	1.494±0.314	0.81	0.200 (0.007-0.460)	4.79	0.0911
<i>V. tonoreana</i>	2.301±0.719	0.94	0.174 ^e	23.96	<0.0001 ^d
Febendazole	3.002±0.549	0.86	0.220 (0.074-0.447)	9.00	0.0112 ^d

^aEC₅₀ in % (wt:vol.), ^b considered significantly different when 95% fiducial limits (FL) fail to overlap, ^c Poor fit of probit model prevented estimation of 95% fiducial limits, ^d Variances and covariances have been multiplied by the heterogeneity factor in computing fiducial limits of lethal concentration because the probability of χ² value is p<0.05

Table 2: Relationship between *H. contortus* larval mortality and concentration of five crude plant extracts and Febendazole, 6 and 48 h post-exposure

Plants	Slope±SE	R ²	LC ₅₀ ^a (95% FL) ^b	χ ²	p>χ ²
6 h					
<i>A. senegalensis</i>	0.800±0.190	0.79	0.341 (0.235-0.545)	0.77	0.6818
<i>A. leiocarpus</i>	0.354±0.188	0.55	0.644 ^e	0.07	0.9647
<i>L. rugosa</i>	0.897±0.191	0.82	0.340 (0.245-0.507)	0.83	0.6604
<i>S. kunthianum</i>	0.665±0.191	0.75	0.620 (0.390-1.980)	1.14	0.5661
<i>V. tonoreana</i>	0.565±0.188	0.70	0.417 (0.250-1.231)	3.06	0.2168
Febendazole	0.952±0.192	0.82	0.261 (0.185-0.358)	1.48	0.4772
48 h					
<i>A. senegalensis</i>	1.875±0.277	0.64	0.070 (0.043-0.093)	0.43	0.8054
<i>A. leiocarpus</i>	1.170±0.365	0.59	0.061 ^e	5.84	0.0540
<i>L. rugosa</i>	1.384±0.208	0.78	0.129 (0.088-0.165)	1.42	0.4912
<i>S. kunthianum</i>	1.053 ±0.196	0.74	0.152 (0.095-0.205)	1.23	0.5405
<i>V. tonoreana</i>	1.271±0.219	0.66	0.078 (0.040-0.110)	1.82	0.4016
Febendazole	1.554±0.534	0.70	0.090 ^e	11.39	0.0039 ^d

^aLC₅₀ in % (wt:vol.), ^b considered significantly different when 95% fiducial limits (FL) fail to overlap, ^c Poor fit of probit model prevented estimation of 95% fiducial limits, ^d Variances and covariances have been multiplied by the heterogeneity factor (5.47) in computing fiducial limits of lethal concentration because the probability of χ² value is p<0.05

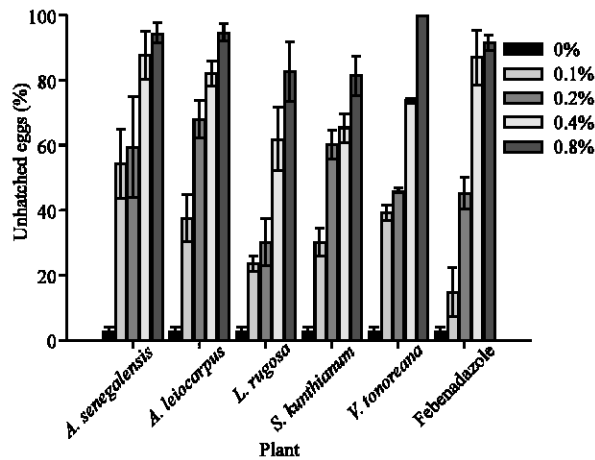


Fig. 1: Inhibition of *H. contortus* egg-hatch (mean±SE) 7 days after exposure to different concentrations (0, 0.1, 0.2, 0.4 and 0.8% (w/v)) of Febendazole and crude extracts from five plants

plants was in the range 20-40%. At the 0.8% concentration level, the egg-hatch inhibition potential of Febendazole was generally comparable with those of the plant extracts, but only *V. tonoreana* among the test substances, accomplished complete egg-hatch inhibition. Following the 95% fiducial limits, EC_{50} values appeared to be comparable among test substances (Table 1). However, Payton *et al.* (2003) stated that checking for overlap of fiducial limits to compare EC values is much too conservative. *A. senegalensis* (0.108%) presented the lowest EC_{50} value. The EC_{50} values for Febendazole (0.220%) and *S. kunthianum* (0.200%) were approximately twice that of *A. senegalensis*. *L. rugosa* (0.299%) recorded the highest EC_{50} value, which was roughly three times that of *A. senegalensis*. However, Febendazole recorded the highest slope, although it was different only from that of *S. kunthianum*.

Larval mortality: Similarly to egg-hatch inhibition, larval mortality varied significantly ($p < 0.01$) among concentrations and test substances and also among exposure periods. Control mortality was significantly lower ($p < 0.01$) than that recorded for any of the test concentrations (Fig. 2). Mortality increased over time post-exposure and ascending concentrations. By the 6th h after exposure and with the highest tested concentration of 0.8%, all the test substances recorded greater than 50% mortality, with Febendazole (72.00%) having the highest value. For the same time, but with the lowest concentration (0.1%), the plants recorded greater than 30% mortality, with *A. leiocarpus* (40.33%) and *V. tonoreana* (41.00%) recording the highest values. With

the highest tested concentration of 0.8%, *A. senegalensis* (98.33%) achieved the highest mortality after 48 h exposure, although all the test substances recorded greater than 85% mortality. For the same exposure period, but concentration of 0.1%, the order of decreasing effects on larval mortality was *A. senegalensis* (63.67%), Febendazole (61.00%), *V. tonoreana* (58.33%), *A. leiocarpus* (57.00%), *L. rugosa* (50.00%) and *S. kunthianum* (47.00%). LC_{50} values were generally lower at 6 h than at 48 h post exposure (Table 2). However, the trends among the test substances were generally similar for the two time-points. Apart from *S. kunthianum* (0.620) and *A. leiocarpus* (0.644) with the highest values at the 6 h exposure period, LC_{50} s were comparable between each plant extract and Febendazole and among plant extracts. Febendazole and *A. senegalensis* had the highest slopes, 6 and 48 h post-exposure, respectively. Nonetheless, slopes were generally comparable among test substances.

DISCUSSION

The study was carried out to determine the *in vitro* toxicity of plant extracts from Northern Cameroon to eggs and infective third-stage larvae, two important life cycle stages of *H. contortus*. The plants were chosen based on their wide distribution in this part of the country and on information of their regular use by herdsman and pastoralists for the treatment of human and livestock parasitism. Besides, *H. contortus* being a highly pathogenic and dominant parasite of the tropics and subtropics (Nginyi *et al.*, 2001) is developing serious drug resistance to current anthelmintics (Coles *et al.*, 2005), thus necessitating the search for alternative and effective drugs that would be environmentally friendly and affordable to resource poor farmers in developing countries.

Ethanollic extracts from the five plants studied demonstrated significant negative effects on *H. contortus* depending on the life cycle stage. Overall, the effectiveness of these plant extracts was comparable to the commonly used orthodox anthelmintic, Febendazole (Panacur). At concentrations of 0.2% or higher, the plants generally inhibited 45-100% egg-hatch, suggesting that they could be useful in the control of egg shedding into the environment. This could interrupt the parasite life cycle and prevent infection of new hosts during grazing. *V. tonoreana* was more potent than Febendazole and 100% effective in inhibiting egg-hatching at the highest tested concentration of 0.8% after 7 days.

Although results from the egg-hatch inhibition assays are encouraging, the *in vivo* effects of these plant extracts on egg shedding merits further investigation

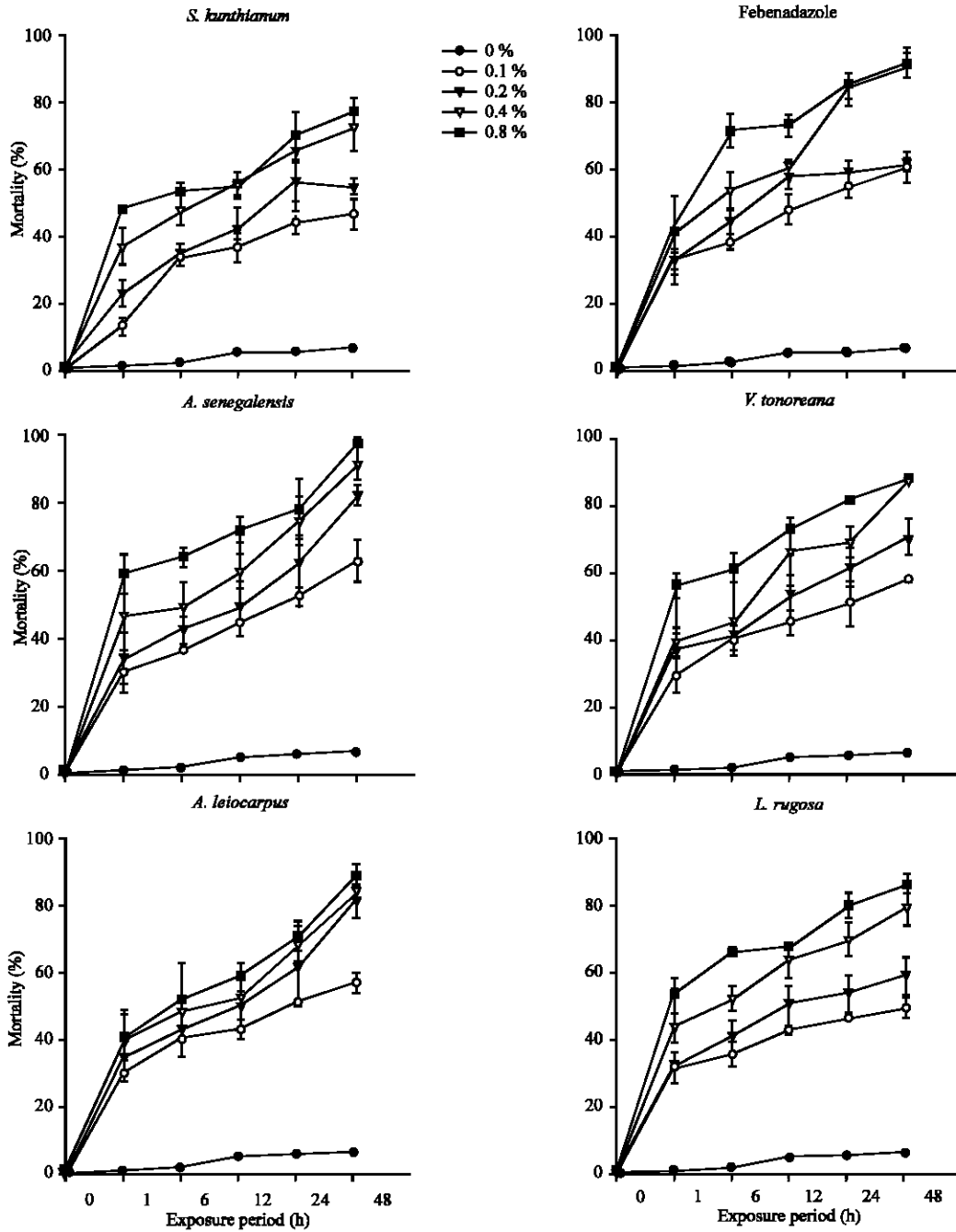


Fig. 2: Mortality of *H. contortus* larvae (mean±SE) over 48 h when exposed to different concentrations (0, 0.1, 0.2, 0.4 and 0.8% (w/v)) of Febendazole and crude extracts from five plants

since previous studies had observed contrasting *in vitro* and *in vivo* efficacy of extracts from the same plant against nematode parasites. Ketzis *et al.* (2002) for example, reported significant *in vitro* reduction in *H. contortus* egg-hatch with the plant *Chenopodium ambrosioides* and its essential oils but no *in vivo* effects in reducing fecal egg counts in experimentally infected

goats. The study of Ketzis *et al.* (2002) however is not directly comparable to ours, given the differences in plant materials and bio-active components. A parallel *in vivo* studies to ours though using a different *Vernonia* species had demonstrated efficacy against nematode and protozoan parasites (Nfi *et al.*, 1999). According to Nfi *et al.* (1999) *Vernonia amygdalina* reportedly reduced

fecal egg counts by 52.4% 21 days post treatment in a natural parasite mixed infection including *H. contortus* in cattle. In another study, Musongong *et al.* (2004) showed significant *in vitro* toxicity of *V. amygladina* to the highly pathogenic parasite, *Strongyloides papillosus*. Other studies on the contrary did not demonstrate any *in vitro* nor *in vivo* effects of *V. amygladina* on *H. contortus* and other nematode parasites (Paolini *et al.*, 2003). Possible explanations for such observed discrepancies in reported results include variation between individual plants due to genetic or environmental differences and variation in procedures of harvesting, drying, processing and storage of plant materials (Croom, 1983). There is therefore a need for standardizing the collection and processing of plant materials for determining their anti-parasitic properties. Our results with *A. senegalensis* parallel that of Igweh and Onabanjo (1989) and Alawa *et al.* (2003) which equally demonstrated potent *in vitro* and *in vivo* effects of *V. tonoreana* against protozoans and nematodes.

The significant effects of the plant extracts in our study on *H. contortus* infective third-stage larvae suggest that these plants may be useful in controlling parasite transmission under natural field grazing conditions. All the plants were significantly effective in a dose-dependent manner against *H. contortus* third-stage larvae with maximal killing generally in the range of 80-100% for the highest dosage over a 48 h-period. The results were highly comparable to that of the commercial anthelmintic, Febendazole. Interestingly, some plant extracts showed differences in potency between the eggs and larvae of *H. contortus*. For example, at the highest concentration of 0.8%, extracts of *V. tonoreana* completely inhibited egg-hatch, but not larval mortality. This may not be surprising however, as even commercial anthelmintics are known to be effective against different parasite stages. The drug Ivermectin (Mectizan) currently used for the treatment of human onchocerciasis kills the third-stage larvae and microfilariae of *Onchocerca volvulus* and its related cattle nematode *O. ochengi* but is not effective against the adult worms (Borsboom *et al.*, 2003; Njongmeta *et al.*, 2004).

Although the bio-active components responsible for the toxicity of the plants used in this study are under investigation, Alawa *et al.* (2003) attributed the anthelmintic properties of *A. senegalensis* to ene-Kaurene Diterpenoids. *L. rugosa* and *S. kunthianum* contain alkaloids. Studies with another botanical species of the genus *Lippia* (Verbenaceae) (*L. multiflora* Moldenke) showed that its toxicity against lice and scabies are likened to the terpenoid compounds, α - and β -pinene (Oladimeji *et al.*, 2000). It is possible that these chemical constituents may also poses anthelmintic properties responsible for the toxicity of *L. rugosa* in this study. *A. leiocarpus* exhibited high anti-fungal (Batawila *et al.*,

2005) and nematocidal (Okpekon *et al.*, 2004) activity due to the presence of tannins, flavonoids and saponins. The biological activity of *V. tonoreana* is relatively unknown but this study confirms our previous observation (Musongong *et al.*, 2004) that this plant is highly toxic against nematode parasites. Overall, how the bio-active composition of each plant compares with the chemical structure of Febendazole that blocks mitochondrial function (Prichard, 1970) as mode of action merits investigation. Previous studies with tanniferous legume extracts from Benin Republic (Hounzangbe-Adote *et al.*, 2005) and New Zealand (Molan *et al.*, 2000) equally demonstrated significant inhibitory effects on *H. contortus* infective third-stage larvae based on the larvae migration inhibition test. The authors of the above studies however could only speculate the mechanism of plant action on the larvae. On the contrary, complete larval killing in our study suggests a direct effect of the plant extracts on the integrity of *H. contortus* larvae.

There has been considerable interest in targeting the third-stage larvae of parasites for vaccines and new therapeutic development for the simple reason that curtailing the development of the infective stage reduces the risks of parasitism (Devaney and Osborne, 2000). The third-stage larva is the first life cycle stage exposed to the host, thus interfering with it is likely to play a central role in the establishment of the parasite and/or development of immunity. Demonstration of larval killing in our study has significant implications for the use of these plants both for prophylactic and curative purposes as *in vivo* exposure to chemically attenuated *Dirofilaria immitis* in ferrets (Grieve *et al.*, 1988) and *Onchocerca ochengi* in cattle (Njongmeta *et al.*, 2004) were shown to prevent the development of adult parasites. It is therefore probable that *in vivo* exposure of *H. contortus* larvae to the plants in this study may also arrest development of adult parasites which could validate both their prophylactic and therapeutic uses.

In conclusion, the present study has demonstrated that indigenous plant extracts commonly used as decoctions by herdsmen and small livestock farmers for the treatment of parasitic diseases are highly toxic *in vitro* against eggs and infective third-stage larvae of *H. contortus*. Therefore, substances from these plants could be implicated in the development of anthelmintic drugs against *H. contortus*. Nevertheless, *in vivo* studies may be needed to ratify these findings.

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