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In vitro effects of four tropical plants on the activity and development of the parasitic nematode, *Trichostrongylus colubriformis*

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Abstract

The *in vitro* effects of extracts of four tropical plants (*Zanthoxylum zanthoxyloides*, *Newbouldia laevis, Morinda lucida* and *Carica papaya*) on the egg, infective larvae and adult worms of *Trichostrongylus colubriformis* were screened for potential anthelmintic properties. Significant effects were observed with the four plants on *T. colubriformis* but they differed depending on the stage of the parasite. Extracts of each plant induced a dose-dependent inhibition of egg hatching. Using a larval inhibition migration test, the effects on the infective larvae were also detected with the four plant extracts. In contrast, for adult worms, the effects were statistically significant only for *N. laevis* and *C. papaya*. No significant activity was shown for *M. lucida* and *Z. zanthoxyloides*. These *in vitro* results suggest the presence of some anthelmintic properties associated with these four plants, which are traditionally used by small farmers in western Africa. These effects need to be studied under *in vivo* conditions.

Introduction

Parasitic infections of the gastrointestinal tract of ruminants with nematodes remain a main issue in developing countries since these worms are responsible for major production losses in livestock. To date, the principal mode of control of parasites in the digestive tract has been based on chemical treatments with anthelmintics. Because of the increasing development of anthelmintic resistance within worm populations (Jackson & Coop, 2000) and also of the cost of such treatments in developing countries, there is currently an emerging interest for an ethnoveterinary approach to examine the anthelmintic properties of plants which are traditionnally used by local farmers (Hammond *et al.*, 1997; Waller, 1997; Akhtar *et al.*, 2000). Because *Haemonchus contortus* is one of the most prevalent and pathogenic nematode species in Africa, most previous studies have examined the effects of plant or plant extracts on this abomasal species (Al Qarawi *et al.*, 2001; Ketzis *et al.*, 2002; Alawa *et al.*, 2003). However, the intestinal nematode *Trichostrongylus colubriformis* is also widespread in the tropics (Fritsche *et al.*, 1993; Pandey *et al.*, 1994; Salifou, 1996; Rugutt, 1999; Nginyi *et al.*, 2001) and can provoke important losses of productivity (Rugutt, 1999; Kagira & Kanyari, 2001).

Recently, Hounzangbe-Adote *et al.* (2004) assessed the *in vitro* effects of extracts from four tropical plants collected in western Africa on different stages of *Haemonchus contortus*. Extracts were from leaves of fagara, *Zanthoxylum zanthoxyloides* (Rutaceae), *Morinda lucida* (Rubiaceae) and *Newbouldia laevis* (Bignoniaceae) and from seeds of *Carica papaya* (Caricaceae). Current studies were performed to complete the information acquired from this first study, by examining the anthelmintic properties of the same plants on the main parasitic stages of *Trichostrongylus colubriformis*. The plants were chosen on the basis of results of a recent survey in Benin which indicated that they were frequently used by farmers against nematode infections and/or their associated symptoms (Hounzangbe-Adote, 2000).

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Materials and methods

Plant extracts

The four plants examined were leaves of *Zanthoxylum zanthoxyloides* (Rutaceae), *Morinda lucida* (Rubiaceae), *Newbouldia laevis* (Bignoniaceae) and seeds of *Carica papaya* (Caricaceae). Plants were collected in the field in the southern part of Benin and dried indoors at room temperature. Thereafter, 10 g of ground, dried plant material were extracted in 100 ml of 30% ethyl alcohol for 2 h at 60°C. After filtration on filter paper (Whatmann Ltd), filtrates were concentrated under low pressure at 40°C and lyophilized to obtain dry powder extract. The overall rate of the extraction procedure was calculated to be approximately 12%.

Biological assays

The effects of the four plant extracts on the three main stages of the parasite cycle, i.e. the egg, the third-stage larvae (L3) and the adult worms were measured using different laboratory procedures.

Nematode eggs

The method was based on a modification of the egg hatch assay performed to measure anthelmintic resistance (WAAVP recommendations, Coles *et al.*, 1992). Eggs were freshly obtained from donor sheep experimentally infected with *Trichostrongylus colubriformis*. Eggs were extracted, washed repeatedly and distributed in 96-multiwell plates at a density of 100 eggs per well. Increasing concentrations of plant extracts (75, 150, 300, 600, 1200 and 2500 μg dry matter (DM) per ml) were obtained from dry ground extracts dissolved in phosphate buffered saline (PBS 0.1 M, pH = 7.2) and then added to each well. Each concentration was tested on six replicates. In addition, a positive (oxfendazole at 0.5, 1, 5 and 10 $\mu g\,ml^{-1}$) and negative (extracts from rye grass hay) control were included in the assay. Eggs were incubated for 48 h at 24°C. Thereafter, the number of larvae present per well was counted and the percentage hatched determined as the ratio between the number of larvae to the number of eggs deposited per well. A mean percentage of hatching was calculated for each concentration of the different plant extracts.

Infective larvae

A larval migration inhibition (LMI) bioassay was used as described by Rabel *et al.* (1994) to measure the inhibiting effects of different concentrations of plant extracts on the infective larvae. Briefly, larvae were incubated for 3 h at 20°C with increasing concentrations of plant extracts, i.e. 150, 300 or 600 $\mu g\,ml^{-1}$. Larvae were then washed 3 times in PBS (pH = 7.2, 0.15 M) and centrifuged. After the final washing, 1 ml of larvae at the concentration of 1000 L3 ml^{-1} was added to inserts equipped with a 20 μm mesh. Sieves were then placed in a conical tube, with the mesh just above the PBS. Three replicates were run for each plant concentration at room temperature. In addition, negative (larvae in PBS) and positive (levamisole at 15, 30 and 60 $\mu g\,ml^{-1}$) controls were also run in parallel. After 3 h, each insert was retrieved and the number of larvae present in the PBS, i.e. those which had actively migrated through the mesh, were counted under a stereomicroscope at magnification × 20.

The percentage of LMI was calculated as

$$\frac{T - M}{T} \times 100$$

where T is the total number of L3 deposited in the sieve and M the number of L3 present in the PBS.

Adult worms

Adult worms were obtained from naive sheep which were experimentally infected *per os* with a pure strain of *T. colubriformis* infective larvae. Four weeks after infection, the sheep were euthanized and immediately after death, the small intestine was collected, opened, briefly washed and placed in a Baermann apparatus with saline at 37°C. After 2 h, worms which had migrated to the saline were collected and immediately placed in a 48-multiwell plate at a concentration of seven to eight worms per well. The plates were maintained at 37°C throughout. Worms were first washed for 1 h in PBS with penicillin and streptomycin at concentration of 4%. Thereafter, 1 ml of each of the four concentrations of plant extracts, i.e. 300, 600, 1200 and 2500 $\mu g\,ml^{-1}$ diluted in PBS, were added to the wells. Positive (levamisole at 125, 250, 500 and 1000 $\mu g\,ml^{-1}$) and negative (PBS and antibiotics) controls were included on each plate. For each treatment, measurements were made on four replicates per plate. The supernatant was changed every 24 h. The mobility of adult worms was noted by careful observation under a stereomicroscope at magnification 40 × after 24 and 48 h. At each time, a motility index was calculated as the ratio between the number of immobile worms/total number of worms.

Statistical analyses

For assays on egg hatch and LMI, significant differences in means for the proportion of unhatched eggs and LMI rates between treatments were assessed by the general linear model (GLM) procedures (Systat 9 software, SPSS Ltd). In the adult worm assay, for each treatment (plant extract and concentration), the number of immobile worms was recorded with time and survival analyses were assessed using a non-parametric, stratified Cox regression test (Systat 9 software SPSS Ltd).

Results

Egg hatch assay

Compared to the mean hatching rate measured in the control (79.3%), the effects of the chemical anthelmintic and of the four tropical plant extracts on the hatching rate were significant ($P < 0.01$) (fig. 1). In contrast, no statistical difference was detected between the control and the values measured on *Trichostrongylus* eggs exposed to increasing concentrations of rye grass hay. For the four tropical plants and oxfendazole, the inhibition of egg hatching was dose-dependent and appeared similar between the five treatments.

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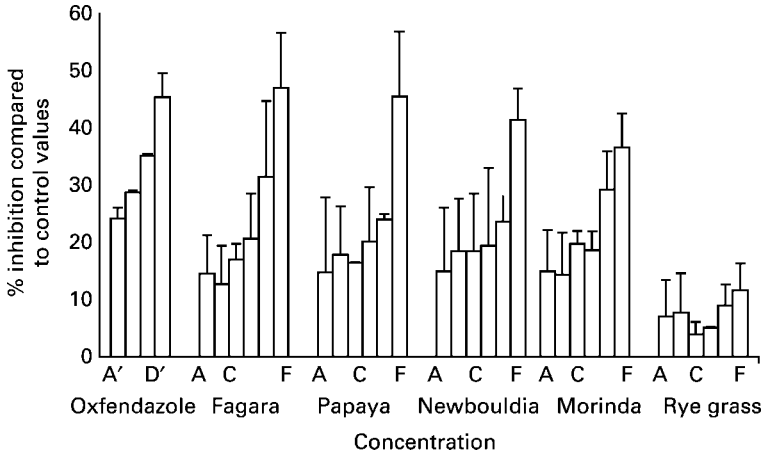

Fig. 1. Inhibition of egg hatching with six increasing concentrations (75 (A); 150; 300 (C); 600; 1200 and 2500 (F) $\mu g\,ml^{-1}$) of extracts of four tropical plants and with four concentrations of oxfendazole (0.5 (A′); 1.0; 5.0 and 10.0 (D′) $\mu g\,ml^{-1}$). Six increasing concentrations of rye grass were used as negative control for the extraction procedure. Compared to PBS control, the inhibitory effects were dose-dependent and significant ($P < 0.01$) for oxfendazole and the four tropical plant extracts. They were non-significant for the rye grass extracts.

Larval migration inhibition

The mean migration rate of the larvae in the negative control group was 61.6% (fig. 2). With levamisole, the inhibition of migration rates was significant and ranged between 76.3% and 94.3% depending of the applied concentration.

A significant effect on larval migration was observed for extracts of each of the four plants ($P < 0.05$). However, no clear dose response was observed for any of the plant extract in the LMI.

Adult worm motility

After 24 h, reductions in the motility of *T. colubriformis* were observed following exposure to each concentration of *N. laevis* (table 1). After 48 h, changes in the motility were also recorded following exposure to extracts of *C. papaya* and *Z. zanthoxyloides*, but not to *M. lucida* (table 1). However, taking into account the different times and concentrations of the plants, significant effects on worm motility were shown only for *C. papaya* and *N. laevis*, but not for *Z. zanthoxyloides*. For *C. papaya* and *N. laevis*, the effects were also shown to be statistically dose-dependent.

Discussion

The four plants examined in the present study were chosen because they had been identified as part of the traditional pharmacopoeia used by local farmers in the Republic of Benin to control digestive parasitic diseases (Hounzangbe-Adote, 2000). These four plants are also distributed widely throughout Africa and an assessment of their possible efficacies was considered to be of interest

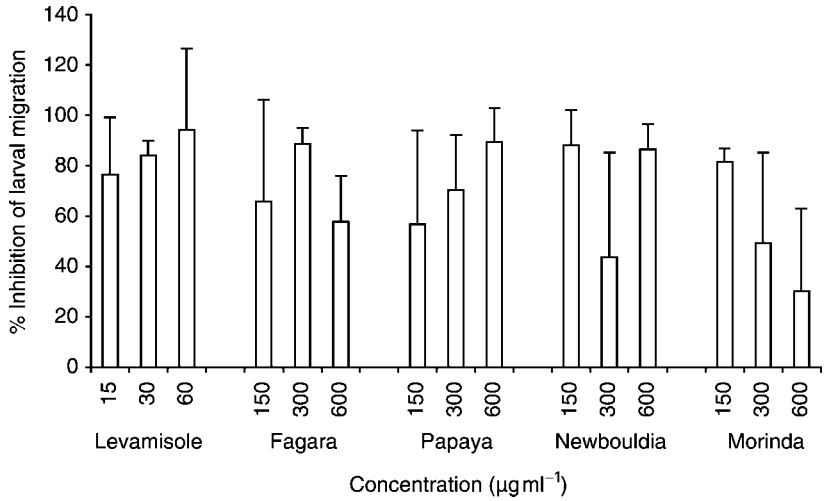

Fig. 2. Inhibition of larval migration with three concentrations of levamisole (15 to 60 $\mu g\,ml^{-1}$) and of extracts of the four tropical plants (150 to 600 $\mu g\,ml^{-1}$). Significant effects were observed for levamisole ($P < 0.01$) and the four plant extracts ($P < 0.05$) but the latter were not dose-dependent.

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Table 1. The effects of various concentrations of extracts from four tropical plants on adult worms of *Trichostrongylus colubriformis*, expressed as a percentage of immobile worms compared to the total number in the wells.

	Concentration ($\mu g\,ml^{-1}$)	% of immobile worms	
		24 h	48 h
PBS	0	2	8
Levamisole	125	33	65
	250	41	64
	500	44	66
	1000	18	20
Zanthoxylum zanthoxyloides	300	7.1	7.1
	600	10	30
	1200	7.7	92
	2500	26.7	87
Carica papaya	300	0	0
	600	23	31
	1200	11.8	41.2
	2500	38.1	90.5
Newbouldia laevis	300	70.6	82.4
	600	69.2	84.6
	1200	82.3	100
	2500	100	100
Morinda lucida	300	0	0
	600	5	5
	1200	5	5
	2500	15	18

Four replicates per plant or levamisole concentrations and 20 replicates for controls were used.

not only in western Africa but also in other parts of the continent.

Haemonchus contortus is usually described as the main gastrointestinal parasitic nematode of ruminants occurring in tropical areas, due to its high prevalence and the severity of the signs associated with its presence in the abomasum. However, other nematode species, parasites of the intestine, are also abundant and pathogenic (Kagira & Kanyari, 2001), with *Trichostrongylus colubriformis* being one of the most common and ubiquitous species. For example, the prevalence of *H. contortus* was described to be 92.5% in Benin while the prevalence for *T. colubriformis* was 88% (Salifou, 1996). Indeed, in another epidemiological survey in western Africa (Gambia), *T. colubriformis* was shown to be the most common nematode, with regard to both the prevalence and intensity of infection (Fristche *et al.*, 1993). In eastern Africa (Kenya), *Trichostrongylus* and *Haemonchus* were also identified as being the two most common genera occurring in farmed small ruminants (Rugutt, 1999).

Results on the efficacies of the same four plant extracts against the three parasitic stages of *H. contortus* have been reported previously (Hounzangbe-Adote *et al.*, 2004). Comparing those data with the present results is useful to determine whether their potential antiparasitic effects are specific to one particular nematode species. To summarize, the overall results for the four plant extracts were similar for the two worm species. The main difference between the two studies was observed on adult worms. All four plant extracts were found to be active against *H. contortus*, whereas only *N. laevis* and *C. papaya* extracts showed significant activity against *T. colubriformis* adult worms. Such variability in activity has previously been reported for tanniferous plants or condensed tannins both from *in vitro* and *in vivo* studies. In *in vitro* assays on third-stage larvae of *Haemonchus* and *Trichostrongylus*, Molan *et al.* (2000) showed the divergent effects of extracts of sulla (*Hedysarum coronarium*), a legume forage. On the other hand, recent *in vivo* studies in goats suggested that the distribution of quebracho (a source of condensed tannins) could have differing effects depending upon the developmental stage (infective larvae or adult worm) and the species of nematode (Paolini *et al.*, 2003a,b).

Little information is available on the possible antiparasitic activities of the four tropical plants tested. In fact, for two of these plants (*N. laevis* and *M. lucida*), data from the present study and from a previous one on *H. contortus* (Hounzangbe-Adote *et al.*, 2004) represent the first experimental evidence of anthelmintic activity associated with these extracts. For *C. papaya* and *Z. zanthoxyloides*, an indication of potential anthelmintic effects has been acquired previously but mostly on non-ruminant, host–parasite models (Satrija *et al.*, 1994, 1995; Navarette & Hong, 1996). With *C. papaya*, one assay which was conducted in goats concerned *H. contortus* infection and did not indicate a high *in vivo* efficacy, both in terms of egg production and worm numbers (Vieira *et al.*, 1999). *In vivo* experiments are therefore needed in sheep to verify these *in vitro* results. In particular, it would be interesting to determine the effects of *C. papaya* and *N. laevis* on parasitic stages under *in vivo* conditions.

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