Full Length Research Paper

Evaluation of the *In Vitro* Anthelmintic Efficacy of *Anogeissus leiocarpus* and *Daniellia oliveri* Against Haemonchus contortus

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A study was conducted to evaluate *Anogeissus leiocarpus* leaf and *Daniellia oliveri* stem barks as effective remedy for gastrointestinal parasites. The anthelmintic activity of these extracts on eggs, first stage larvae and adults of *Haemonchus contortus* was examined by *in vitro* tests. The extracts were prepared to obtain six increasing concentrations. This was done with Phosphate Buffered Saline (PBS) for egg hatch, embryonated egg assays (75, 150, 300, 600, 1 200 and 2 400 µg/ml) and adult inhibition of motility assay (0.25, 0.5, 1, 2, 4 and 8 mg/ml). PBS and levamisole (at 0.125 µg/ml in PBS) were used as negative and positive control groups, respectively. Both plant extracts induced anthelmintic effects on the three life-cycle stages of *H. contortus* and these effects were significantly different when they were compared to the negative control group (PBS) ($P < 0.05$). The effect was dose-dependent on egg hatching and first stage larvae (L_1) but not on adult worms. Magnitude of effect was proportional to concentration of plant extracts for egg hatching and L_1 but not for adult worms. Besides, the results showed that the *D. oliveri* stem bark extract was more ovicidal and larvicidal than *A. leiocarpus* leaves. It is concluded that these two plants do really possess anthelmintic properties.

Key words: Anthelmintic, leaf, stem bark, extracts, *Anogeissus leiocarpus*, *Daniellia oliveri*, *Haemonchus contortus*.

INTRODUCTION

In Burkina Faso, small ruminant livestock (sheep and goats) improves life conditions of populations in rural farming environment where poverty is a great concern. Indeed, their number is estimated at 17 000 000 animals (MRA, 2004). They contribute actively to improve the social cohesion (weddings and traditional customs) of the farming households, procure some revenue and allow the education of children. Unfortunately, in traditional livestock system, these animals are affected by diseases, among which gastrointestinal nematode parasitism is highly prevalent and creates a huge economic loss. This loss results concretely from the mortality of young animals and the decrease in productions (Fabiyi, 1987; Krecek and Waller, 2006). Some epidemiological investi-gations revealed the potency of gastrointestinal parasi-tism in sheep and goats, animals found in all households in the environment of rural farming of Burkina Faso. In

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the central region of the country, the frequency of the parasitism is high mainly during the rainy season, period favorable to the development of parasites (Belem et al., 2000; Belem et al., 2005a; Belem et al., 2005b).The most dominant parasite is *Haemonchus contortus*, an abomasal bloodsucking nematode which is highly patho-genic and very prolific even in difficult environmental conditions (Jacquiet et al., 1995). Generally nowadays, the control of these parasitic infections is based on mas-sive and strategic use of modern drugs as anthelmintic. Unfortunately, the high cost of these drugs and the shortage of veterinarians in Burkina Faso drove the livestock breeders and herdsmen towards traditional pharmacopoeia using medicinal plants (Kaboré et al., 2007) as done in many developing countries by rural farmers (Akhtar et al., 2000; Githiori et al., 2005; Athanasiadou et al., 2007). In the central region of Burkina Faso, among the medicinal plants commonly used for the treatment of gastrointestinal parasitism in small ruminants, there are *Anogeissus leiocarpus* (DC.) Guill. and Perr. (Combretaceae) and *Daniellia oliveri* (Rolfe) Hucht. and Dalz. (Caesalpiniaceae) (Kaboré et al., 2007). Currently, the availability of the two plants through the country is variable. *A. leiocarpus* is present in all ecological zones, particularly in the surrounding of cool soils whereas *D. oliveri* is generally encountered more in down south Sudanese ecological areas (Arbonnier, 2000).

The aim of this study is to evaluate the *in vitro* effects of *A. leiocarpus* and *D. oliveri* extracts prepared according to traditional way of doing on *H. contortus,* an abomasal nematode in sheep.

MATERIALS AND METHODS

Plant materials and extracts

Samples of *A. leiocarpus* (leaves) and *D. oliveri* (stem bark) were collected from their natural habitat during November 2006 at the experimental station of the Environmental and Agricultural Research Institute, located in Kamboinse, Burkina Faso. They were dried in a well-aerated room protected from sun and dust. The specimens were identified at Botanical Park of the National Center of Scientific and Technological Research of Burkina Faso.

Following the procedures used by veterinary healers in Burkina Faso, an aqueous extraction of each plant was performed by decoction. Briefly, 100 g of each powdered plant materiel was mixed with 1000 ml of distilled water in flask and boiled for 1 h. Then, they were allowed to cool down to 40°C and filtered through muslin gauze and filter paper. The decocted solution of each plant was frozen and then lyophilized. The yields (% w/w) of the extracts were 8.0 and 9.7% respectively for *A. leiocarpus* and *D. oliveri*. The extract yields were stored at 4°C for biological tests.

Parasites' origin

Adult female parasites of *H. contortus* were collected from the abomasa of naturally infected sheep at Ouagadougou International slaughterhouse during August 2007. The abomasums were incised along the greater curvature and washed slowly under tap water several times. Then, adult worms were collected, put in a bottle

containing cool phosphate buffered saline (PBS, pH: 7.2, 4°C), and used immediately for biological test.

Biological assays' procedures

The two extracts's anthelmintic effects were assayed on the three main stages of parasite cycle, i.e. the eggs, first stage larvae (L_1) and the adult worms, through different experimental laboratory procedures (egg hatch assay, embryonated egg assay and adult inhibition of motility).

Egg recovery and tests

The eggs recovery method as described previously by Jabbar et al. (2006) was used in this study.

Egg hatch assay (EHA)

Egg hatch assay was conducted according to procedure described by Coles et al. (2006). Approximately, 50 eggs were collected per tube; each tube contained 1 ml of PBS and 1 ml of increasing concentrations of plant extracts (75, 150, 300, 600, 1 200 and 2 400 µg/ml) prepared with PBS. In addition, positive (levamisole at 0.125 µg/ml) and negative (PBS) controls were considered. The tubes were covered, and the eggs were incubated for 48 h at temperature of 27°C. Thereafter, the number of the first stage larvae (L_1) present per tube was counted using a dissecting microscope. Each concentration was tested on five replicates. An inhibition percent (%) of egg hatching was calculated for each extract concentration using the following modified formula of (Coles et al., 1992):

Inhibition (%) = $100(1-X_1/X_2)$

where X_1 is the number of eggs hatched in test extracts, and X_2 is the respective number in PBS control.

Embryonated egg assay (EEA)

The embryonated eggs of *H. contortus* were used as described by Wabo Poné et al. (2006). Approximately 50 eggs per ml were added in tubes (5 ml) with PBS. The eggs were allowed to stand at laboratory room temperature for about 24 h until they developed to a fully embryonated pre-hatch stage. When the first stage larvae became transparently visible and started moving actively within the egg envelopes (this happened to about 90% of the larvae in the dish), 1 ml of each extract of plant materiel at different concen-trations (75, 150, 300, 600, 1 200 et 2 400 µg per ml) was added to each tube. The tubes were covered and incubated for 6 h to allow for almost complete hatching of all eggs in the control dish. Only hatching which occurred in contact with the test extracts was con-sidered. When the eclodibility in the control dish was higher than 90%, two drops of formaldehyde (10%) were added to each tube to stop the hatching of the eggs. PBS and levamisole prepared with PBS at the same concentration in EHA were used as negative and positive control groups. The test was repeated five times for each concentration of the plant extracts and control groups. A mean percent of eclodibility (%) of eggs was calculated for each extract concentration under dissecting microscope. All the embryonated eggs and first stage larvae (L_1) were counted according to the following formula:

E (%) = (number of L_1 larvae / number of embryonated eggs in culture) x 100
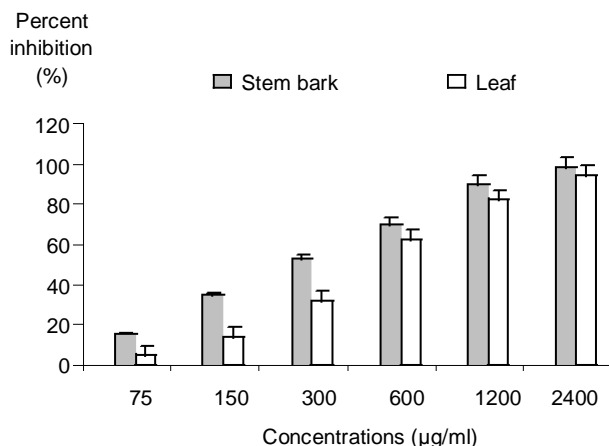
where E is eclodibility.

Figure 1. Dose-dependent profile of the percent hatching egg of *Haemonchus contortus* submitted to the six increasing concentrations of plant extracts (75, 150, 300, 600, 1 200 and 2 400 µg/ml).

Effect on adult worms

The test was performed in plastic Petri dishes (35 x 10 mm). Eight to ten actively moving worms were placed in Petri dishes containing aqueous extracts of plants at concentrations of 0.25, 0.5, 1, 2, 4 and 8 mg/ml in PBS, or PBS alone for the negative control group (final volume of 3 ml). Levamisole diluted in PBS at the same concentration was used in egg test as a positive control. Each concentration of aqueous extracts was tested on three replicates. After 24 h, the aqueous extracts of plant and levamisole were washed away and the worms were suspended in PBS for 30 min to observe the revival of motility. The inhibition of motility of the worms kept in the above treatments was used as criterion for anthelmintic activity. Death of worms was ascertained by absence of motility for an observation period of 5-6 s at the head and tail regions of the body under dissecting microscope. The motility was observed on 24 h intervals. The percent mortality (M) of worms was calculated for each extract concentration using the formula:

Mortality (%) = (number of worm death / number of worms in culture) x 100.

Statistical analysis

In EHA and adult worm test, the mean values were calculated and the effect of increasing the concentration of the plant extracts was determined using a non-parametric test of Kruskall-Wallis. In EEA, the averages of values obtained were compared using the Chi-square test. All analysis was realized with the software CoSTat (version 6.204) at the 5% significance level.

For the comparison of the efficacy of different plant extracts, effective dose$_{50}$ (ED$_{50}$) was determined by the method of Probits using the program SPSS (version 10.0.5) for Windows.

RESULTS

Egg hatching assay (EHA)

The results of *H. contortus* egg hatching inhibition by the leaves of *A. leiocarpus* and the stem barks of *D. oliveri*

are presented in Figure 1 and Table 1. With the levamisole concentration used, all eggs incubated did not hatch and the extracts caused complete lyses of eggs. The mean inhibition values obtained increased significantly (P<0.05) with the increase of the concentrations of the extracts of the leaves of *A. leiocarpus* and the stem barks of *D. oliveri*. For the two higher concentrations of plant extracts (1 200 and 2 400 µg/ml), there were no alive larvae (L$_1$) in the incubated tubes. Both plant extracts showed ovicidal activity in all tested concentrations and the histogram evolution was similar and showed dose-dependency. Statistical differences (P<0.05) were also observed among plant extracts.

The effective dose (ED$_{50}$), calculated using the analysis probit system, for the leaves of *A. leiocarpus* and the stem barks of *D. oliveri* were summarized in Table 1. Comparing the effective dose (ED$_{50}$), the extract of the stem barks of *D. oliveri* (245.9 µg/ml) presented a higher ovicidal activity than the leaves of *A. leiocarpus* (409.5 µg/ml).

Embryonated egg assay (EEA)

The mean percent values of the hatching of embryonated eggs of *H. contortus* submitted to different concentrations of the two plant extracts are presented in Figure 2. The mean eclodibility percentage of the two plant extracts varied from 25 to 68% along with the increase of concentrations. A significant difference (P<0.05) was observed in the effects of the different concentrations tested. The eclodibility of embryonated eggs was dose-dependent for each plant extract studied. The level of eclodibility of the negative control group (PBS) was 94.4%, while reference drug (positive control group) induced 100% eclodibility inhibition. When compared to the concentrations of the two extracts and the levamisole, the mean value of the level of eclodibility obtained of the negative control (PBS) was more significantly elevated (P<0.05). With the two highest concentrations (1200 and 2400 µg/ml) of all the plant extracts, the *H. contortus* L$_1$ which has hatched from embryonated eggs was found dead.

All plant extracts tested presented a linear relationship between the probit of embryonated egg hatching and logarithm of concentrations used. ED$_{50}$ values of *A. leiocarpus* and *D. oliveri* extracts were 411.4 and 362.3 µg/ml, respectively (Table 1).

Effect on adult worms

After 24 h, the percent mortality of *H. contortus* was observed following exposure to each concentration of the tested plant extracts (Figure 3). The percentage of mortality was 100% at 24 h for the levamisole. Compared to the mean percentage of mortality measured in the negative control (PBS: 31%), effects due to the conventional

Table 1. Effective dose50 (ED_{50}) values (μg/ml) of two plants' extracts against *H. contortus*.

Extract plants (family)	ED_{50} (LCL – UCL)[a]	
	EHA	EEA
A. leiocarpus leaf (Combretaceae)	409.5 (376.1 – 446.0)	411.4 (335.2 – 504.1)
D. oliveri stem bark (Caeselpiniaceae)	245.9 (222.3 – 270.7)	362.3 (295.8 – 440.4)

[a]Values at 95% confidence intervals; LCL, lower confidence limit; UCL, upper confidence limit.
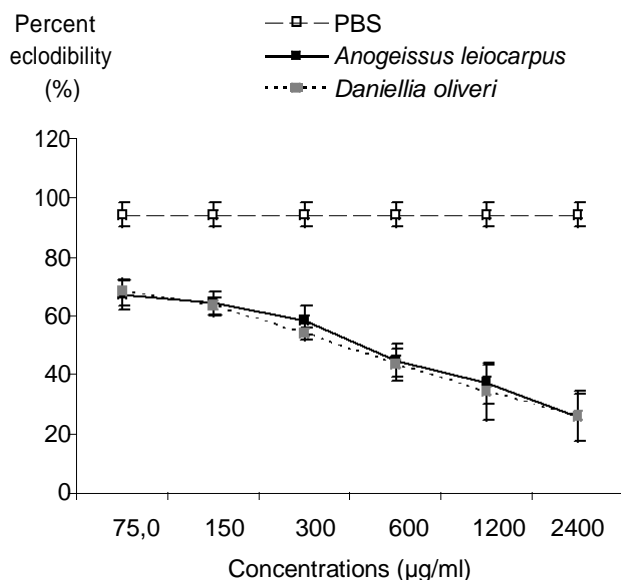

Figure 2. Dose-response profile of the percent embryonated egg hatching of *Haemonchus contortus* of different concentrations of plant extracts (75, 150, 300, 600, 1200 and 2400 μg/ml).

anthelmintic (levamisole) and to the two plant extracts were significantly different (P<0.05). For both plant extracts tested, the effect of mortality was not shown to be dose-dependent during the test.

DISCUSSION

The results of the present study show that the two plant extracts have some anthelmintic properties on the eggs, the first stage larvae and on the adult worms of *H. contortus*. Similar results were found on eggs and adults of *H. contortus* by Hounzangbe-Adote et al. (2005) with alcoholic extracts of four plants in Benin (*Zanthoxylum zanthoxyloides*, *Newbouldia laevis*, *Morinda lucida* and *Carica papaya*). Wabo Poné et al. (2006) have obtained similar results on the eggs of *Ancylostoma caninum* with the stem bark extracts of *Canthium mannii* (Rubiaceae) in Cameroon.

In our study, the anthelmintic effects of the two plant extracts presented nearly an identical evolution in both tests. But the ED_{50} values obtained revealed clearly that *D. oliveri* extract was more efficient than those of *A.*

leiocarpus on inhibition of egg hatching and on first stage larvae (L1). Compared to the ED_{50} values obtained, those in EHA in both plant extracts were low. This could indicate that these extracts were more active on immature eggs than on the paralysis of *H. contortus* L_1 in embryo-nated eggs. Wabo Poné et al. (2006) realized similar observations with *C. mannii* extracts on the eggs of *A. caninum*.

In Ethiopia, Eguale et al. (2006) obtained 0.87, 0.10 and 0.06 mg/ml as ED_{50} of egg hatching of *H. contortus* with the aqueous extracts of *Acacia nilotica*, *Cyperus macrostachyus* and *Elegia capensis* respectively. In Brazil, Maciel et al. (2006) also found 50% inhibition of *H. contortus* egg hatching using *Melia azedarach* ethanol extracts of leaves at 2.2 mg/ml concentration.

In our study, the mortality of the first stage larvae in EHA with higher concentrations (1200 and 2400 μg/ml) indicated that the two aqueous plant extracts possessed larvicidal activity. These results confirmed those obtained during the embryonated egg test.

The mechanism of uptake of anthelmintic drugs in nematodes is well documented in literature. Alvarez et al. (2001) have described two mechanisms: the first is the diffusion of the anthelmintic drugs through the external surfaces such as eggshells and the cuticles of larvae and the second, the diffusion through the intestinal cells. Our results of biological tests suggested that it was possible that secondary metabolites contained in the plant extracts produced similar effects by diffusion through eggshells and transcuticular absorption into adult worms of *H. contortus*. By this mechanism, they could bind to the free protein available for the first stage larvae nutrition and thus, depriving the larvae of food, then provoking their death in embryonated eggs (Athanasiadou et al., 2001) by paralysis as levamisole (Dobson et al., 1986). Indeed, levamisole acts as a selective nicotinic agonist to pro-duce contraction of nematode somatic muscle and drive the paralysis (Sasa et al., 2002). The possible explana-tion for the better activity of both plant extracts on egg compared to the first stage larvae could be their easier diffusion through the eggshells than the cuticles of larvae.

The most important anthelmintic effect of the stem barks of *D. oliveri* than that of the leaves of *A. leiocarpus* was probably related to the concentration of the active compound in the plant extracts than to the variety or specie of plant. According to Kaboré et al. (submitted for publication), the aqueous extract of *D. oliveri* possesses more secondary metabolites than that of *A. leiocarpus*
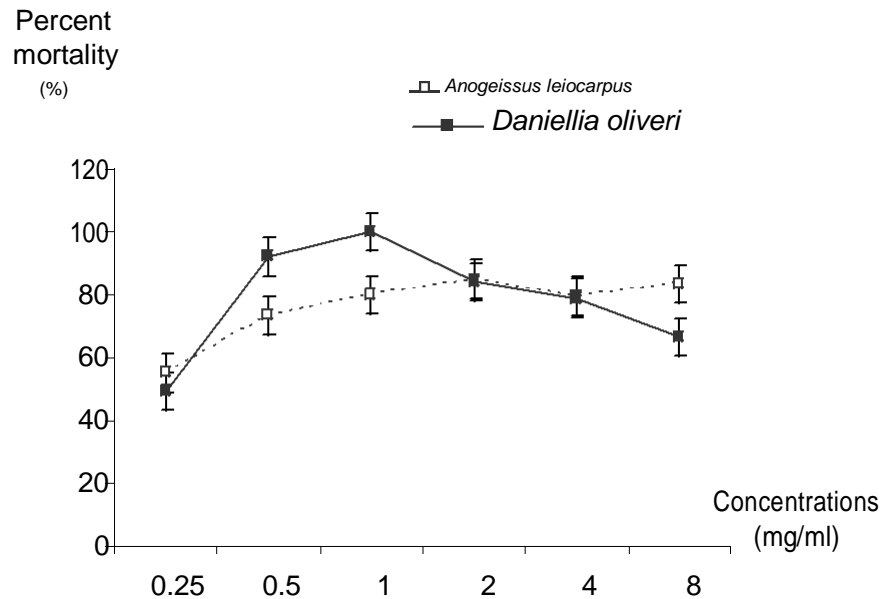
Percent
mortality
(%)

Figure 3. Dose-response profile for mortality (expressed in percentage) of adult worms of *Haemonchus contortus* by the extracts from the leaves of *Anogeisssus leiocarpus* and from stem bark of *Daniellia oliveri*.

resulting from the difference in their solubility in water. According to these authors, the two plant extracts contain flavonoids, saponins and tannins which are known to possess anthelmintic activity. However some studies have evaluated the anthelmintic properties of the other biochemical compounds in the plant extracts. The flavonoids and tannins contained in the polar fraction of *Leuceana leucocepha*la (Ademola et al., 2005) and the flavonol glycosides in sainfoin *(Onobrychis viciifolia)* (Barrau et al., 2005) have been demonstrated to have effects on the third stage larvae (L_3) of *H. contortus*. Tannins contained in our plant extracts are probably condensed tannins which would explain the observed anthelmintic effects on the eggs and the adult worms of *H. contortus.* Indeed, the condensed tannins which are polyphenolic compounds are known to have some anthelmintic properties on the free stages of nematode parasites during *in vivo* tests in sheep and goats (Molan et al., 2000; Bahuaud et al., 2006; Hoste et al., 2006). Some additional studies are necessary to determine the nature of tannins present in the extracts of *A. leiocarpus* and *D. oliveri*.

In conclusion, the results from the present study revealed that extracts from *A. leiocarpus* and *D. oliveri* have shown active *in vitro* anthelmintic effect against eggs, first stage larvae and adult stage of *H. contortus*. Their traditional use by smallholders and pastoralists as anthelmintic seems to be justified. However it would be necessary to achieve some *in vivo* toxicological and parasitological studies to consider the fact of polyparasitism in livestock and the metabolism of the extracts in the animal digestive tract.

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