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In vitro study on the role of the tannins of *Newbouldia laevis and Zanthoxylum zanthoxyloides* on infective larvae of *Trichostrongylus colubriformis*

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***Trichostrongylus colubriformis* is an important cause of parasitic gastroenteritis in small ruminants which causes diarrhea, weakness, loss of production and death. The *in vitro* efficacy of tannins of *Newbouldia laevis* and *Zanthoxylum zanthoxyloides* was determined against this parasitic nematode. Larvae of *T. colubriformis* were incubated at 23°C in the leaf extracts of *N. laevis* and *Z. zanthoxyloides* at concentrations of 150, 300, 600 and 1200 µg ml^{-1} for three hours, respectively. Phosphate buffered saline (PBS) was used as negative controls. Inhibition of larval migration was significantly ($P < 0.05$) observed with increasing concentrations of the extracts. In the range of concentration examined, the results were dose-dependent for *N. laevis* but not for *Z. zanthoxyloides.* The addition of Polyvinyl polypyrrolidone (PVPP) inhibited total or part of the anthelmintic effect. These results suggest that the larval migration is at least in part due to the action of tannins and supports the traditional use of *N. laevis* and *Z. zanthoxyloides* against parasites nematodes. Further research is required to isolate and structurally identify the active anthelmintic compounds, and to improve methods of plant extraction of the effective anthelmintic components that will be readily adaptable for use by rural communities against helminthiasis.**

Key words: Larval migration, tannins, *Trichostrongylus colubriformis*, *Newbouldia laevis*, *Zanthoxylum zanthoxyloides,* Benin.

INTRODUCTION

Gastrointestinal nematodes remain a major constraint to economic productivity of livestock throughout the world,

being the chief parasitoses responsible for disease-related production losses arising from stock mortality, severe weight loss and poor production, especially in small ruminants (Chiejina, 2001; Bizimenyera et al., 2006). *Trichostrongylus colubriformis*, an intestinal nematode, is one of the most important causes of parasitic enteritis causing protracted diarrhoea, weakness, loss of production and death (Bizimenyera et al., 2006).

In the last 30 years, control of gastrointestinal nematode infections of ruminants has been achieved almost exclusively by use of pharmaceutically derived anthelmintic. Indeed, synthetic and semi-synthetically produced anthelmintics have for long been considered the only effective method of controlling helminthoses (Bizimenyera et al., 2006). However, in the extreme situations of subsistence farming where anthelmintic is either unavailable or unaffordable, massive mortalities of young stock are commonplace in tropical Africa and Asia (Bizimenyera et al., 2006). At the other extreme, misuse and or widespread intensive use of sometimes poor quality synthetic or semi-synthetic anthelmintic has led to the development of a high level multiple anthelmintic resistances that may lead to failure of control of worm parasites in ruminants (Wolstenholme et al., 2004; Bizimenyera et al., 2006). These constraints indicate that total reliance on pharmaceutically derived anthelmintic may present difficulties in the management of gastrointestinal parasitic infections in livestock, necessitating alternative methods of helminth control (Sanyal, 2001; Bizimenyera et al., 2006).

In Benin, the breeding of livestock is an economically important activity and is responsible for sources of jobs and income and contributes to 4 to 6% of the gross domestic product. Besides prestige and savings functions, breeding animals contribute to increasing the income of the breeders through on one hand, the sale of animals and their by-products and on the other hand, through the use of the fertilizer for the fertilization of farms. However, this breeding is confronted with numerous constraints: the high cost of medicines, state of financial fragility of the producers, disturbing appearance of crossed resistances in the modern molecules, the lack of sanitary frame of the breeders and the traditional system of the managements of the herds. Among the constraints, animal health is a limitation to animal production. The levying of these constraints requires the implementation of the efficient practices and the use of available endogenous resources. Among these resources are a number of plants which are believed to have bioactive properties and which are readily available at relatively low cost.

In recent times, there has been an increasing interest in ethnomedical and ethnoveterinary practices across the world, especially as it relates to the use of medicinal plants in treating various ailments (Bizimenyera et al., 2006). Use of indigenous plants preparations as livestock dewormers is gaining ground as one of the alternative and sustainable methods readily adaptable to rural farming communities (Hammond et al., 1997). Important opportunities exist through research on the traditional use of herbal medicine, since 80% of people in developing countries rely on phytomedicine for primary healthcare in both humans and animals (Bizimenyera et al., 2006). As ethnomedicine does not follow western paradigms of scientific proof of efficacy and safety, most medical and veterinary professionals distrust the use of herbs, and know little about them (Thompson, 1997). Numerous plant species and extracts have been evaluated and research efforts have mainly focused on condensed tannins (Hoste et al., 2006; Alonzo-Diaz et al., 2008). Due to the quantity of the different types of secondary compounds that exist in plants that could potentially be used as anthelmintics, rapid and cost-effective in vitro screening is necessary (Whitney et al., 2011).

The plants were chosen on the basis of a recent questionnaire survey in Benin which indicated that they were frequently used by small scale farmers against parasitic infections or to treat associated clinical signs (Hounzangbé-Adoté, 2000). *Newbouldia laevis* and *Zanthoxylum zanthoxyloides* have commonly been used in African folk medicine for the treatment of several diseases such as diarrhea, jaundice, hemorrhoids, dysentery, sore throat, gonorrhea and icterus (Arbonnier, 2004). They are also employed against malaria, sexually transmitted disease, dental caries, arthritis pain, gastroenteritis, dysentery and as vermifuge (Eyong et al., 2005). Leaves are used against infertility (Adjanahoun et al., 1991).

The aim of the present in vitro study was to establish the effects of tannins of leaves of *N. laevis* and *Z. zanthoxyloides* on the larvae L_3 migration of the intestinal parasite of sheep and goats (*T. colubriformis*).

MATERIALS AND METHODS

Collection and preparation of plant material

Samples collected in the south of Benin (Atlantic Department) were identified in National Herbarium of Abomey-Calavi University (Herbier National de l'Université d'Abomey-Calavi). Classification of the species was performed by means of the key according to Cronquist (Cronquist, 1988). Voucher specimens are kept at the Herbarium of Abomey-Calavi University for *Z. zanthoxyloides* under N° AA 6301 / HNB and *N. laevis* under N° AA 6302 / HNB. The plants were dried indoors at room temperature before being ground into powder for extraction.

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Plants extracts preparation

Acetone and ethanol used for each plant are recognized to be both solvents which extract more compounds from plants (Eloff, 1998).

Hydro-ethanolic extract

50 g of each plant powder was refluxed in a water bath under magnetic stirring for one hour in 500 mL of ethanol-water (70:30) mixture. The solution is cooled and then filtered. The operation was repeated twice and before the ethanol was removed under pressure at 40°C. The aqueous solution was partitioned successively with dichloromethane (each 3 × 300 ml), and ethyl acetate (each 3 × 300 ml) respectively to defatted and to remove chlorophyll. Thereafter, the remaining aqueous extract were lyophilized and stored at -70°C, and the yields of extraction (mass/mass) were calculated.

Hydro-acetonic extract

50 g of plant powder was refluxed, filtered and washed as described above for hydro-ethanolic extracts with the exception that ethanol-water was replaced with acetone-water (70:30) mixture. The solution is cooled then filtered. The operation is repeated twice and acetone is removed under pressure at 40°C. The aqueous solution was partitioned successively with dichloromethane (each 3 × 300 ml), and ethyl acetate (each 3 × 300 ml) respectively to defatted and to remove chlorophyll. Thereafter, the remaining aqueous extracts were lyophilized, kept at -70°C and the yields of extraction (mass/mass) were calculated.

Determination of condensed tannins (CT) by the method of Butanol-HCl

The determination of CT was performed by the colorimetric method of Butanol-HCl. developed by Porter et al. (1986), based on the depolymerization reaction of CT in acid medium. This reaction leads to the release of anthocyanidins (colored molecules) corresponding to the cleaved monomer (Makkar, 2000; Schofield et al., 2001). It allows a semi-quantitative determination of CT as the released terminal monomers do not produce the corresponding anthocyanidins and therefore they are not dosed (Schofield et al., 2001). In the plant, the CT is present in different forms: free or bound, that is, attached to proteins or plant fibers (Schofield et al., 2001). The existence of these two forms (free or bound) made the determination of CT more difficult. CT content was analyzed using the Butanol-HCl assay according to Makkar (2000).

Larval preparation

Infective larvae of *T. colubriformis* L_3 were obtained by fecal culture of goats previously artificially infected with pure strains of *T. colubriformis*. After egg hatching, L_3 stage was reached after 10 days. The L_3 were then collected by sedimentation using Baermann's devices. These batches of 1-to-2-month-old larvae were used in the assays.

Bioassays: Larval migration inhibition assay

The larval migration inhibition (LMI) assay was used as described by Rabel et al. (1994) adapted for plant extracts (Jackson and Hoste, 2010), in order to measure inhibiting activity against infective larvae. Larvae were incubated for 3 h at 20°C in PBS plus plant extract solutions, at concentrations of 150, 300, 600 or 1200 µg/mL with three repetitions by concentration. The larvae were then washed three times in phosphate buffer (PBS) (pH 7.2, 0.15 M) and centrifuged. After the last washing, 800 µL of larvae at a concentration of 1000 L_3/mL was pipetted onto a 20-µm mesh. The sieve was inserted into a conical tube, so that it just touched the surface of the PBS contained therein. Three replicates were run at room temperature (23°C) for each plant concentration. In addition, a negative control (larvae incubated in PBS) was run in parallel. After 3 h, the L_3 above the sieve were discarded and those which had actively migrated through the mesh into the PBS below, were counted under an optical microscope (at 40x magnification), based on a 10% aliquot technique.

The percentage of LMI was calculated as [(T-M)/T×100)] where T is the total number of L_3 deposited in the sieve and M the number of L_3 having migrated through the mesh into the PBS.

Involvement of the tannins in the anthelmintic activity

Polyvinyl polypyrrolidone (PVPP) forms complexes with tannins and polyphenols and thus blocks their potential biological activity (Makkar, 2003). PVPP was added to the plant extracts at a concentration of 1200 µg/mL for 2 h in a 1:50 ratio (Barrau et al., 2005). These solutions were then centrifuged at 4500 RPM, (5 min, 20°C). After centrifugation, the supernatant and the extracts without adding PVPP were used to incubate sheathed infective larvae of *T. colubriformis*. Thereafter, the LMI assay was performed according to the procedure described previously.

Statistical data analysis

Larval migration bioassay: Excel software was used to calculate averages, standard deviations of larval migration and to generate graphical illustration. A GLM (general linear model) statistical test was performed to determine the difference in the mean percentage of LMI rates between the control and the different dose groups (150, 300, 600 and 1200 µg/mL) procedures using Systat 9 software (SPSS Ltd.). The dose-response effect was determined by considering the level of statistical significance at $p < 0.05$. The same test was applied for the comparison of response in the experiment with the extracts at 1200 µg/mL with or without PVPP.

RESULTS

Yield extractions

From the two selected plants, four extracts were obtained. The Table 1 shows the yields obtained for each plant extracts which is an indication that the hydro-ethanolic extracts gave the best yields.

Condensed tannin content of plants

The method of Butanol-HCl showed that the condensed tannins of *Z. zanthoxyloides* were estimated at 2.5% of the dry matter, while condensed tannins of *N. laevis* were estimated at 1.0%.

Inhibition of larval migration

The extracts of *Z. zanthoxyloides* and *N. laevis* inhibit *in vitro* migration of larvae of *T. colubriformis* (Figure 1). This

Table 1. Yield extractions.

Plants used	Plant part	Type of extraction	Yield (%)
Newbouldia laevis	Leaves	Hydro-ethanolic	13 ± 0.7
		Hydro-acétonic	5.3 ± 0.4
Z. zanthoxyloides	Leaves	Hydro-ethanolic	13 ± 0.5
		Hydro-acetonic	9.8 ± 0.4

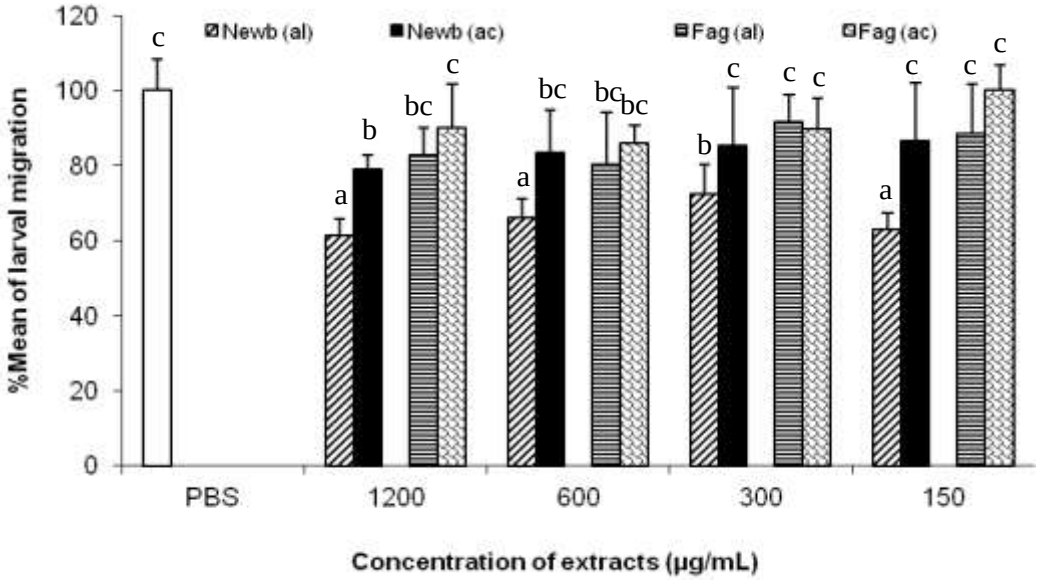

Figure 1. Percent mean of larval migration of *Trichostrongylus colubriformis* incubated with leaf extracts of *Newbouldia laevis* (Newb) and *Z. zanthoxyloïdes* (Fag), ethanolic (al), acetonic (ac).

effect was dependent on plant extracts ($p < 0.001$) but was not concentration dependent ($p > 0.05$) (Figure 1). Ethanol extract of both plants has significantly reduced larval migration ($p < 0.05$). *N. laevis* has showed more effect on larval migration ($p < 0.05$).

Adding polyvinyl polypyrrolidone (PVPP) to the plant extracts completely inhibited the effect of acetone-water extract of *Z. zanthoxyloides* and partially reduced the effect of the ethanol-water extract on parasites (Figure 2). With *N. laevis*, the PVPP only partially reduced the effect of the plant on *T. colubriformis*, regardless of the extraction solvent (acetone-water or ethanol-water).

DISCUSSION

The third stage larvae (L3) are very important on gastrointestinal parasites life cycle such as *T. colubriformis*. It is on this stage that the parasites infest their hosts. Earlier study on West African Dwarf goats showed anthelmintic activity of *Z. zanthoxyloides* and *N. laevis* on the most important gastrointestinal parasites nematodes in small ruminants. The powders of leaves of *Z. zanthoxyloides* and *N. laevis* reduced excretion of strongyles eggs without dose-effect but with a greater efficiency for *N. laevis* (Azando et al., 2011).

The decrease in migration of L_3 larvae of *T. colubriformis* after incubation with plant extracts is due to their immobility or mortality. This inhibition of larval migration depends not on the concentration of plant extracts but on the type of extract.

The aim of the study was to show the involvement of the tannins of *N. laevis* and *Z. zanthoxyloides*, two tropical plants, in the larval migration of *T. colubriformis*, a gastrointestinal hematophagous parasite of small ruminants. Acetone and ethanol were chosen as extraction solvents because they extract polar compounds from plants which are miscible in organic and aqueous solvents and are nontoxic to the organisms used in the tests (Eloff, 1998). The acetone and ethanol extract a number of compounds from plants compared to dichloromethane or hexane (Bizimenyera et al., 2005). Control of gastrointestinal parasites by plants lies among others in the ability of these to affect the viability or fertility of adult

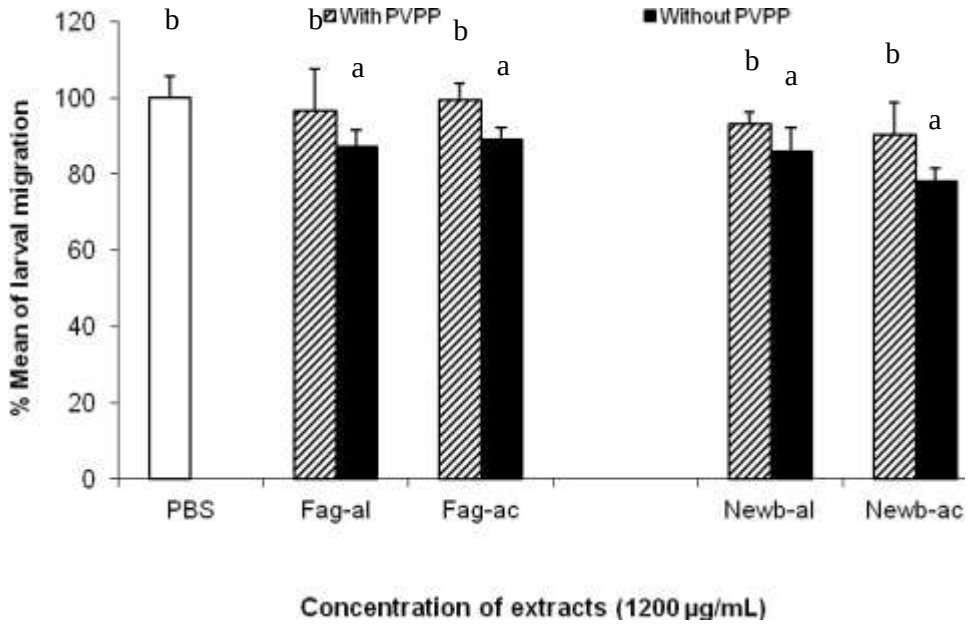

Figure 2. Percent mean of larval migration of *Trichostrongylus colubriformis* incubated with leaf extracts of *Newbouldia laevis* (Newb) and *Z. zanthoxyloïdes* (Fag), ethanolic (al), acetonic (ac) and with or without PVPP.

worms, to reduce egg excretion or to limit the installation of larvae by their immobilization or by inhibition of their exsheathment, thus blocking their life cycle. The reduction in larval migration is due to their immobility or mortality. Aqueous-acetone and aqueous-ethanolic extracts of *N. laevis* and *Z. zanthoxyloides* therefore inhibit *in vitro* migration of infective larvae of *T. colubriformis* and tannins should play an essential role. Brunet et al. (2007) showed that the extract of sainfoin "a plant rich in tannins," affects the kinetics of exsheathing of strongyle L_3 and that this inhibitory effect depends on the extract concentration. Similarly, Bahuaud et al. (2006) showed that some plants more or less rich in tannins can inhibit partially or completely *in vitro* migration of L_3 larvae. But the effectiveness of these tannins depends on their structure and nature of the monomers as shown by the work of Brunet and Hoste (2006). The anthelmintic properties of the plants are related to their phytochemical composition and the results of inhibition tannins suggest the activity of other secondary metabolites. Alkaloids are suspected among others, but according to Bar et al. (2005), flavonoids might also explain the anthelmintic properties of bioactive plants. Some studies have shown that *N. laevis* contains families of compounds such as tannins, flavonoids, alkaloids, anthocyanins, quinone derivatives, saponins, mucilages, steroids, triterpenoids and essential oils (Olounladé, 2005). This plant is also rich in quinones such as newbouldiaquinone (Eyong et al., 2006) or ceramide (Eyong et al., 2005) and alkaloids associated with pigments. Several alkaloids (Dieguez-Hurtado et al., 2003), flavonoids, terpenoids and coumarins (Mara et al., 1992) were isolated from different organs of species of the genus *Zanthoxylum*.

Otherwise, Brunet et al. (2008) found structural damage on L_3 having been in contact with sainfoin extracts. In addition to the inhibition of larval migration, *N. laevis* and *Z. zanthoxyloides* probably induce, on the infective larvae, structural and functional alterations on which it would be interesting to clarify. A dose-dependent inhibition of the migration of L_3 larvae of *T. colubriformis* was highlighted by Hounzangbé-Adoté (2004). The presence of condensed tannins in the two tropical plants, *N. laevis* and *Z. zanthoxyloides* could justify the observed anthelmintic properties. However, the molecules responsible for their anthelmintic effect are still unknown and remain to be identified. Flavonoids and tannins in the polar fraction of *Leuceana leucocephala* (Ademola et al., 2005) and flavonol glycosides in sainfoin (*Onobrychis vicifolia*) (Barrau et al., 2005) showed an effect on the migration of larvae strongle L_3 larvae. The tannins in our plant extracts are partly condensed tannins. Indeed, some condensed tannins (polyphenolic compounds) are known to be active anthelmintic on the different stages of the parasitic cycle of nematodes when tested *in vivo* in sheep and goats (Bahuaud et al., 2006; Hoste et al., 2006; Alonzo-Diaz et al., 2008).

Conclusions

N. laevis and *Z. zanthoxyloides* screened have inhibited larval migration of *H. contortus* with a high rate of reduction

related to *N. laevis* extracts. Findings on both plant extracts support the traditional use of both plants in ruminant helminths controlling by small scale farmers. Research work is ongoing for determining better methods of plants extraction, elucidation of the chemical structure of active compounds, and for *in vivo* tests in suitable target livestock. This work may lead, not only to possible isolation of novel anthelmintic from the plants, but also to the development of better methods of plants extraction which are readily adaptable for use by rural communities against helminthiases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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