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Research Article

In Vitro Combined Effects of *Zanthoxylum zanthoxyloides* and *Newbouldia laevis* Methanolic Extracts on Three Life-Cycle Stages of the Parasitic Nematode, *Haemonchus contortus*

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Abstract | Methanolic extracts of *Zanthoxylum zanthoxyloides* (Fagara), *Newbouldia laevis* and three of their combinations (50% *N. laevis* -50% *Z. zanthoxyloides*, 25% *Z. zanthoxyloides* -75% *N. laevis* and 75% *Z. zanthoxyloides* - 25% *N. laevis*) were screened *in vitro* for potential anti-parasitic effects against eggs, infective larvae and adults of *Haemonchus contortus*. Significant effects were obtained but differences were observed depending on the parasitic stage. The effects of the two plant extracts and their combinations were no dose dependent on egg hatching and infective larvae although more potent effects were usually observed with low concentrations. In contrast, dose–response relationship was found for adult worms. On eggs hatching, the combined extracts 50% *N. laevis* - 50% *Z. zanthoxyloides* and 25% *Z. zanthoxyloides* - 75% *N. laevis* have showed the same effectiveness like *Fagara* and *N. laevis* extracts. Combined plant extracts seemed to be more effective than individual plant extracts on larval migration at less concentrations. Extracts of *Fagara* and two plant associations (i.e., 50% *Z. zanthoxyloides* - 50% *N. laevis* and 75% *Z. zanthoxyloides* - 25% *N. laevis*) reduced adult worm motility. Overall, the combination of 50% *Z. zanthoxyloides* -50% *N. laevis* was most effective on all three stages of *H. contortus*. These results showed that *Z. zanthoxyloides* (*Fagara*) and *N. laevis* used in combined form (50%:50%) were more effective on *H. contortus* thus both plants association could be used as potential alternative of synthetic drugs to control parasitic infections of *H. contortus* in small ruminants.

Keywords | *Zanthoxylum zanthoxyloides*, *Newbouldia laevis, Haemonchus contortus,* Plants combination, Benin

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INTRODUCTION

In tropical developing countries, gastro-intestinal nematode infection remains one of the main causes of impaired production in small ruminants and can lead to fatalities. *Haemonchus contortus* is one of the most important species because of its high prevalence and pathogenicity (Hounzangbé-Adoté et al., 2005a). This kind of parasite causes adverse effects on the host like haematological and biochemical disturbances (Hoste et al., 2006), loss of body weight (Perry et al., 2002) and huge economic losses (Jabbar et al., 2006). Control of these parasitic infections is generally based on the strategic use of anthelmintic. Unfortunately, these substances are not only less available in the countries of the South but also their cost in local markets is relatively high. In addition, the misuse of these drugs for

many years had led to the development of resistant worm strains. Some side effects are noted and the use of disinfectants to control free stage of parasites is harmful to the environment (Wabo-Poné et al., 2011). For these reasons, alternative strategies for nematode control are needed.

In developing nations, traditional methods of controlling nematodes, used by small farmers, remain largely dependent on medicinal plants (Hounzangbé-Adoté et al., 2005a). In this regard, some ethnobotanical investigations have been undertaken everywhere in Africa and particularly in Benin on plants used by breeders to helminthes controlling. Among these plants, *Zanthoxylum zanthoxyloides* (Fagara), *Newbouldia laevis* were identified on the basis of a recent questionnaire survey in south of Benin which indicated that they were frequently used by small scale farmers against parasitic infections or to treat associated clinical signs (Hounzangbé-Adoté, 2001). Also sometime, according to Hounzangbé- Adoté (2001) leaves of both plants are used in association to helminthes controlling in this zone of Benin. In earlier studies, Hounzangbé-Adoté et al. (2005a) and (2005b) were assessed the *in vitro* effects of extracts from these two tropical plants on three life-cycle stages of *H. contortus* and *Trichostrongylus colubriformis*. Leaves extract of each plant was significantly reduced from each parasite, eggs hatching, larvae migration and adult worm's motility. As well, the *in vivo* effects of fresh and powder leaves of two plants were assessed on sheep and goat infested naturally and artificially with gastrointestinal nematodes parasites (Hounzangbe-Adoté et al., 2005c; Azando et al., 2011a; Minaflinou et al., 2015). Results obtained from these studies showed that these two plants possess anthelmintic properties and confirm their traditional uses by small farmers. However, it is important to verify if both plants used in association will be effective to helminthes controlling according to the investigations from small farmers. The current study was therefore conducted to explore the potential anti-parasitic properties of different combinations of both plants (*Z. zanthoxyloides* and *N. laevis*) from Western Africa against *H. contortus*. *In vitro* methods were employed targeting three life-cycle stages of *H. contortus*, *i.e.* the eggs, the infective larvae and the adult worms.

MATERIALS AND METHODS

Preparation of Plant Leaves Extracts

Materials screened were leaves of *Z. zanthoxyloides* (Fag) and leaves of *N. laevis* (New). Samples of each plant were collected from their natural habitat and dried indoors at room temperature. *Z. zanthoxyloides* (Lam.) Zepernick and Timler to Rutaceae family and *N. laevis* (P. Beauv.) Seemann ex Bureau to Bignoniaceae family were identified at national herbarium of Benin respectively under the numbers: AA 6301 /HNB and AA 6302 / HNB. Thereafter, for each plant, 50g of leaves powder were sampled and for both plants combination, respectively 37.5g/12,5g, 25g/25g and 12.5g/37,5g of powdered leaves from each plant was extracted in 500 mL of solvent for two hours at 40°C. A mixture methanol/water was used in proportion to 70:30. Following this, the solution was filtered, and the resulting filtrate was then evaporated in an oven at 47°C. Once the solvent had evaporated, the extract was obtained and dried in a drier for 5 days.

Biological Assays' Procedures

Extracts effects on the three main stages of the parasite cycle, *i.e.* the egg, the third-stage larvae (L_3s) and the adult worms were measured using different laboratory procedures that is eggs hatching assay, inhibition of larval migration and adult motility inhibition.

Effects on *H. contortus* Eggs

The method was based on a modification of the egg hatch assay performed to assess anthelmintic resistance by Coles et al. (1992). Briefly, parasite eggs were freshly obtained from donor sheep experimentally infected with *H. contortus*. The eggs were extracted, washed repeatedly with saline and distributed in 96 multiwell plates at a concentration of 100 eggs /well in 100 µl. Following this, 100 µL of plant association extracts prepared with PBS at different concentrations (75, 150, 300, 600, 1200, and 2400 µg/mL) were added into each well containing eggs solution. A negative control (PBS) and a positive control (Thiabendazole 500, 250 and 125 µg/mL in PBS) were also included. The whole culture plate were then incubated at 27°C for 48 hours. Hatched eggs were counted under a microscope. The test was repeated five times for each plant dose. The percentage inhibition of hatching for each concentration was evaluated microscopically using the modified formula of Coles et al. (1992).

Effects on Infective Larvae

The larval migration inhibition (LMI) bioassay was used as described by Rabel et al. (1994) in order to measure inhibiting activity against infective larvae which were obtained from stool of sheep previously infected artificially with strains of *H. contortus*. Droppings were collected and left to culture at room temperature for 10 days. The larvae were then extracted from the fecal mass using a Baermann apparatus, which utilizes the water tropism of larvae. After, larvae were incubated for 3 h at 20°C in PBS solutions of plant association extracts, at concentrations of 75, 150, 300 600 or 1200 µg/mL. 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 L3s/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. Four replicates were run at room temperature for each plant concentration. In addition, negative (larvae incubat-

ed in PBS) and positive (larvae incubated in Levamisole at concentrations of 250 µg/mL) controls were run in parallel. After 3 h, the L3s above the sieve were discarded and those which had actively migrated through the mesh into the PBS below, were counted under a microscope at magnification 20X. The percentage of LMI was calculated as:

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

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.

Effects on Adult Worms

The purpose of this assay was to test the anthelmintic effect of the three plant extracts on adult worm motility. This test was performed according to Hounzangbé-Adoté et al. (2005a) and (2005b). Adult worms were obtained from sheep which were experimentally infected with a pure strain of *H. contortus*. Four weeks after infection, the sheep were euthanized. Immediately after death, the abomasum was collected, opened, briefly washed with saline and placed in a Baermann apparatus with saline at 37°C. After 2 h, the worms that had migrated into the saline were collected and quickly placed in a 48-multiwall plate at a concentration of three worms/ well. The worms were first washed for one hour in PBS with penicillin and streptomycin at a concentration of 4%. Thereafter, 1 mL of the different concentrations of plant association extracts, from 75 to 2400 µg/mL, diluted in PBS were added to the wells. Positive (Levamisole) and negative (PBS plus antibiotic) controls were included on each plate. For each treatment, the measurements were performed on six replicates per dose and per plate. The supernatant was changed every 24 h. The mobility of the adult worms was noted by careful observation under a microscope at magnification 40X after each 6h. At each observation, a motility index was calculated as the ratio between the total numbers of immobile worms/total number of worms, referring in each case to the overall number of worms present in the six replicates.

Statistical Analysis

For assays on egg hatch and larval migration, significant differences in means for the proportion of unhatched eggs and inhibition migration rates between treatments were assessed by the general linear model (GLM) procedures using package MASS (Venables and Ripley, 2002) of R software (R Core Team, 2013). Adult worm assay: for each treatment, the number of immobile worms was recorded with time and survival analyses were assessed using t test of Student. The comparison of the two plants and their combination for each dose was done using Student Newman and Keuls test (SNK) of R software.

RESULTS

Egg Hatch Assay

Z. zanthoxyloides (Fagara), *N. laevis* extracts and their associations (50%New-50% *Z. zanthoxyloides*, 25% *Z. zanthoxyloides* -75% *N. laevis* and 75% *Z. zanthoxyloides* -25% *N. laevis*) have significantly ($p < 0.001$) reduced *in vitro* *H. contortus* eggs hatching. The reduction of eggs hatching rate have varied from 18.84 to 65% following eggs exposure to increasing concentrations (Figure 1). This inhibition was no dose-dependent ($p > 0.05$) but was function of the plant species tested ($p < 0.001$). Two plants association extracts 50% *N. laevis* -50% *Z. zanthoxyloides* and 25% *Z. zanthoxyloides* -75% *N. laevis* have showed the same effectiveness as two plants extracts (Fagara and *N. laevis*). The association of 75% of Fagara and 25% of *N. laevis* was the lowest effective ($p > 0.05$).

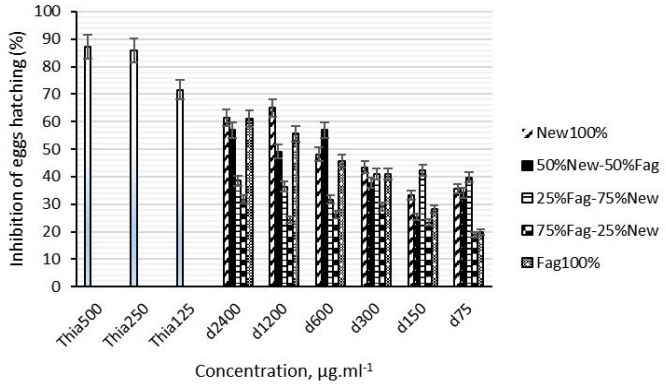

Figure 1: Effects of *N. laevis*, *Z. zanthoxyloides* extracts and their different associations extracts (50%New-50%Fag, 25%Fag-75%New and 75%Fag-25%New) on *H. contortus* eggs hatching. **New)** *Newbouldia laevis*; **Fag)** *Zanthoxylum zanthoxyloides*; **Thia)** Thiabendazole

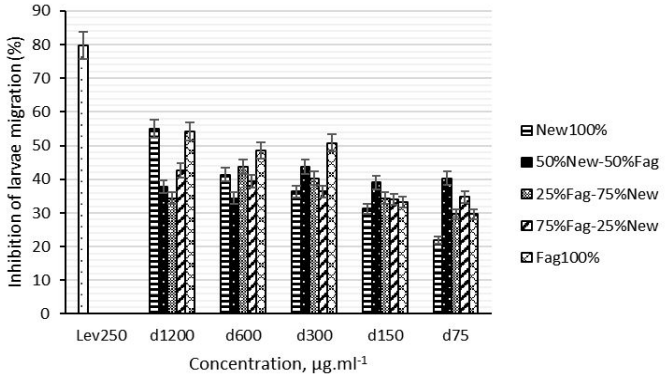

Figure 2: Effects of *N. laevis*, *Z. zanthoxyloides* methanolic extracts and their combinations extracts (50%New-50%Fag, 25%Fag-75%New and 75%Fag-25%New) on *H. contortus* larvae migration. **New)** *Newbouldia laevis*; **Fag)** *Zanthoxylum zanthoxyloides*; **Lev)** Levamisole

Larval Migration Inhibition (LMI)

The inhibition of larval migration induced by *Z. zanthoxyloides* (Fagara), *N. laevis* and their associations (50%New-

50%Fag, 25%Fag-75%New and 75%Fag-25%New) extracts was neither no dose-depend ($p > 0.05$) nor the plant species used ($p > 0.05$) (Figure 2). A significant effect on larval migration was observed for higher (1200 and 600µg/mL) and middle (300µg/mL) concentrations extracts of each plant and each two plant associations. At weak doses, plant association extracts seemed to be more effective than two plants extracts (Figure 2).

Table 1: Effects of various concentrations of methanolic extracts of two plants and their combinations (50%New-50%Fag, 25%Fag-75%New and 75%Fag-25%New) on adult worms' motility

		Mobile worms (%)			
Treatment*	**Doses (µg/ml)**	**Time (hour)**			
		6h	**24h**	**36h**	**42h**
PBS	00	100	50	25	0
Lev125	125	100	12.5	0	0
Lev250	250	87.5	0	0	0
Lev500	500	50	0	0	0
New 100%	2400	100	12.5	0	0
	1200	100	25	12.5	0
	600	100	12.5	0	0
	300	100	25	0	0
	150	100	37.5	0	0
Fagara 100%	2400	87.5	0	0	0
	1200	100	0	0	0
	600	100	12.5	0	0
	300	100	25	0	0
	150	100	50	0	0
75%Fag-25%New	2400	100	0	0	0
	1200	100	0	0	0
	600	100	0	0	0
	300	100	25	0	0
	150	100	37.5	0	0
50%Fag-50%New	2400	100	0	0	0
	1200	100	0	0	0
	600	100	0	0	0
	300	100	25	0	0
	150	100	37.5	0	0
25%Fag-75%New	2400	100	0	0	0
	1200	100	37.5	0	0
	600	100	0	0	0
	300	100	25	0	0
	150	100	12.5	0	0

* **PBS:** Phosphate Buffered Saline; **New:** *Newbouldia laevis*; **Fag:** *Zanthoxylum zanthoxyloides*; **Lev:** Levamisole

Adult Worm Motility

Adult worms' motility inhibition induced by plant extracts and their combination extracts (50%New-50%Fag, 25%Fag-75%New and 75%Fag-25%New) was dose-dependent ($p<0.001$) and function of incubation time ($p<0.001$).

50 percent of worms were immobilized at high concentration of positive control (500 µg/mL) and less of 25 percent at middle dose (250 µg/mL) and high dose (2400 µg/mL) respectively for positive control (250 µg/ml) and Fagara extract, 6 hours after exposure. After 24 hours of incubation, total inhibition was observed in wells of positive control (500 and 250 µg/ml) and those of highest concentrations, 2400 and 1200 µg/mL for Fagara, 2400, 1200 and 600 µg/ml for 75%Fag-25%New and 50%Fag-50%New and 2400 µg/mL for 25%Fag-75%New (Table 1). A strong inhibition of *H. contortus* adult worms' motility was observed following exposure to each concentration of plants extracts and their combination extracts after 36 h (Table 1). The motility reduction was total for all extract doses excepted dose 1200 µg/ml of *N. laevis* extract (Table 1). Nevertheless, after 48 hours of exposure to different concentrations of plant extracts and their association extracts, the reduction of *H. contortus* motility was total. Extracts of Fagara and two plant associations 50%Fag-50%New and 75%Fag-25%New have in this assay a significant effect on the survival of adult worms of *H. contortus* (Table 1).

DISCUSSION

Zanthoxylum zanthoxyloides (*Fagara*) and *Newbouldia laevis* are present throughout tropical areas of Africa (Bekalo et al., 1996). It has been identified as plants most commonly used by farmers in Benin, as shown by a previous survey (Hounzangbé-Adoté, 2001). According to them the farmers usually used both plants in association for small ruminant gastro-intestinal parasites controlling. Moreover, screening based on *in vitro* studies using *Fagara* and *N. laevis* extracts have provided evidence indicating that different concentrations of extracts modified the viability of both adult worms and third-stage larvae of *Haemonchus contortus* and *Trichostrongylus colubriformis* and also affected egg hatching (Hounzangbe-Adote et al., 2005a, b). Recent *in vivo* studies of Fagara and *N. laevis* powder leaves realized by Azando et al. (2011a) on same parasites have confirmed both plants anthelmintic properties. However, in this time, no data was not obtained about both plants association effectiveness against nematode infections as indicated by farmers.

The *in vitro* methods provide a means to screen rapidly for potential anthelmintic activities of the different plant extracts and to analyze the possible mechanisms involved in the interactions between active compounds and parasites. Results obtained from eggs hatching assay indicate

that extracts of both plants combination possessed *in vitro* ovicidal activity. This inhibition was function of the plant species tested and no dose-dependent. Similar observation was done on same plants and parasite in earlier study by Hounzangbé-Adoté et al. (2005a) and recent study by Olounladé et al. (2011). Besides, according to Hounzangbé-Adoté et al.(2005a), the reduction in egg excretion observed in the present study suggest that plants combination extracts could affect the biology of parasitic eggs when sprayed on pastures. By itself, a reduction in egg hatching can also help to modulate the risk of parasitism by limiting the infectivity of pastures grazed by ruminants. The *in vitro* migration assay is a rapid and effective tool for the determination of drug effects which paralyze nematodes (D'Assonville et al., 1996). The principle of the LMI test depends on an active migration process through the sieve, the reduction in migration rate could be due either to larval mortality or to larval paralysis. These observations were remarked in the present study especially on *H. contortus* larvae migration. Because a significant reduction in larvae migration rate was observed especially at higher and middle concentration extracts of plant associations. The reduction of adult worms of *H. contortus* mobility induced by both plants combination extracts was dose dependent and function of time incubation. In this study, a high rates of immobility was recorded after 24 hours of exposure to plant combination extracts, especially plants association 50%Fag-50%New and 75%Fag-25%New. Similar results were reported on adult worms of *H. contortus* by Hounzangbé-Adoté et al. (2005a) with alcoholic extracts of *N. laevis* and *Z. zanthoxyloïdes*. In that study, they recorded high rates of immobility after only six hours of exposure to *N. laevis* and after 24 hours of exposure to *Z. zanthoxyloïdes*. As well in others specie, same observations were noticed by Alowanou et al. (submitted) with *M. inermis* and *C. glutinosum* extracts on adult worms of same parasite with 100% no mobile worms after 24 hours to highest dose of 2400 µg/mL.

The ovicidal, larvidal and deworming activities of plants combination extracts observed in this study may be attributed to the presence of secondary metabolites such as saponins, alkaloids, tannins and flavonoids (Brunet et al., 2008; Azando et al., 2011b). Bogning et al. (2016) reported that saponins are known to destabilize cell membranes, hence increase cell permeability by combining with membranes associated sterols, which could have significant effect on parasites eggs hatching.

CONCLUSION

The association extracts 50% *N. laevis* -50% *Z. zanthoxyloides*, 25% *Z. zanthoxyloides* -75% *N. laevis* and 75% *Z. zanthoxyloides* -25% *N. laevis* were effective against eggs, larvae, and adult worms of *H. contortus*. The combination extract of 50% *N. laevis* -50% *Z. zanthoxyloides* was most effective as compared to other combinations and individual plants on at least two stages of the parasite. These results suggested that these plants could be used as alternative to synthetic drugs and accordingly as a great asset for the control of small ruminant's gastro-intestinal nematodes, particularly *H. contortus*.

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CONFLICT OF INTEREST

There was no conflict of interest from any of Co-authors concerning this work. It was unanimously packaged.

AUTHORS' CONTRIBUTION

Irvine Yèïnou Minaflinou Sacca Sidi conducted the Biological assays in accordance with the protocol and wrote the manuscript. Géorcelin Goué Alowanou, monitored the execution of the protocol and participated in the writing of the manuscript. Pascal Abiodoun Olounladé, followed the biochemical part of the protocol, statistical analyses and participated in the correction of the manuscript. Vidjinnangni Fifamè Nadège Dedehou, monitored the execution of the protocol and participated in the writing of the manuscript. Sylvie Mawulé Hounzangbé-Adoté designed the protocol and participated in the correction of the manuscript.

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