In Vitro Effect of Degradation in *Sacco* in the Rumen on the Anthelmintic Properties of *Parkia Biglobosa* and *Pterocarpus Erinaceus* on *Haemonchus Contortus*

Vidjinnangni Fifamè Grâce Nadège Dèdéhou (PhD)
Laboratoire d'Ethnopharmacologie et de Santé Animale, Faculté des Sciences Agronomiques, Université d'Abomey Calavi, Cotonou, Bénin.
Laboratoire d'Ecologie, de Santé et de Production Animale, Faculté d'Agronomie, Université de Parakou, Parakou, Bénin
Sévérin Babatoundé (PhD, Full Professor)
Laboratoire de Zootechnie, Université d'Abomey Calavi, Cotonou, Bénin.
Mahulé Sylvie Hounzangbé-Adoté (PhD, Full Professor)
Laboratoire d'Ethnopharmacologie et de Santé Animale, Faculté des Sciences Agronomiques, Université d'Abomey Calavi, Cotonou, Bénin.

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Abstract

Parkia biglobosa and *Pterocarpus erinaceus* are traditionally used to treat various ailments including helminth infections. This study was undertaken to evaluate the effect of the rumen degradability on anthelmintic properties of the two plants and to examine the possible role of tannins and/or polyphenols on these properties. Hydro-acetone extracts of *in sacco* degradability residues of *P. biglobosa* pods and *P. erinaceus* leaves were screened *in vitro* to determine the possible anti-parasitic effects against eggs and infective larvae of *Haemonchus contortus*. In addition, the possible involvement of tannins and/or polyphenols was examined by comparing the levels of inhibition of larval migration obtained with the same extracts, after of not addition of PVPP. Extracts of both plants induced significant egg hatch inhibition ($p < 0.001$). The effects were dose and incubation period dependent. Extracts of both plants have shown again remarkable larval migration inhibition compared to the PBS ($p < 0.05$). The effect of incubation period was not significant ($p > 0.05$). These results suggest that the plants did not lose their anthelmintic properties after rumen degradation. The use of the PVPP indicated for almost all of the extracts that tannins and/or polyphenols are largely involved in the effect. Complementary investigations are necessary to understand the metabolism of these plants in the digestive tract of the animals.

Keywords: *Parkia biglobosa, Pterocarpus erinaceus,* rumen degradation, anthelmintic, *Haemonchus contortus*.

Introduction

Gastrointestinal nematode parasitism, especially *Haemonchus contortus* constitutes one of the most serious constraints affecting ruminant production in developing countries such as Benin (Attindehou *et al.*, 2012). It has an impact on production which results in economic losses. Parasitic nematodes cause mortality, severe weight losses, low milk output and reproductive failure in livestock (Alawa *et al.*, 2002; Bizimenyera *et al.*, 2008). The control of gastrointestinal nematodes predominantly depends upon chemotherapy. However, development of drug resistance in the parasites (Kaplan, 2004), effect of drug residues on human and high costs of the synthetic drugs have led in search for the treatment and/or control of parasites (Brunet, 2008; Olounladé *et al.*, 2011).

Although alternatives such as grazing management, selective breeding for resistant hosts, worm vaccines and the use of biological agents have been proposed, their adoption and success under conditions that exist in most developing countries has not been satisfactory, because of factors such as finance, technical know-how (Krecek and Waller, 2006). In recent years, there has been a resurgence of interest in traditional health care practices and the use of plants with anthelmintic properties which can reduce or control parasitic nematode infestations is one of the alternative sustainable approaches.

Parkia biglobosa and *Pterocarpus erinaceus* are important multipurpose trees and are well known in many African countries. They provide building materials, wood, food, fodder and other commodities and are especially important as traditional medicines (Zerbo *et al.*, 2011; Tchacondo *et al.*, 2011). They showed in previous study anti-parasitic activity against *H. contortus* (Dèdéhou *et al.*, 2014).

The aim of this study is to evaluate the effect of the rumen degradability on anthelmintic activity of *P. biglobosa* and *P. erinaceus* against eggs and infective larvae of *H. contortus*.

Material And Methods

Plants Collection

The plant materials were the leaves of *P. erinaceus* and the pods of *P. biglobosa*. The plants were collected from their natural habitats in the center part of Benin. Samples of *P. erinaceus* and *P. biglobosa* were identified by plant taxonomist at the National Herbarium of Benin under numbers

AA6368/HNB and AA6385/HNB respectively. Plant parts were air dried at room temperature, ground and kept in bottle until processed.

Animals and diets

Five African Dwarf sheep (average weight: 30 kg) were used for the *in sacco* experiments. Each sheep was fitted with a rumen cannula (40 mm) and penned individually. Each sheep received a daily ration containing 35 g/kg of body weight (BW) of *Panicum maximum* var. C1, 7.5 g/kg BW of cotton seeds and 7.5 g/kg BW of peelings of cassava (*Manihot esculenta*). The diet was given in two equal portions at 8 hour intervals. Water and a mineral lick were always available.

In *sacco* study and plants residues extraction

The nylon bag method was used for plant powders degradability in sheep rumens. The heat-sealed bags, measuring 10 x 15 cm, were made of nylon cloth having a calibrated pore size of either 40 µm. Approximately 5 g of powder of *P. erinaceus* leaves or *P. biglobosa* pods, ground through a 2 mm sieve, were put into each bag. Three bags were fixed on a plastic pipe 30 cm long connected to the plug of the rumen cannula. The bags were introduced into the rumen just before the morning feeding and incubated for 24, 48, and 72 hours. Three repetitions were carried out on each sheep. Thus 45 nylon bags were incubated for each powder.

At the end of incubation, the bags were removed from the rumen and manually washed at least three times in cold water under a water jet of tap. They were then squeezed gently by hand and dried at 40 °C for 48 hours. The dried bags were weighed to determine the amount of residual dry matter. The residue was ground and preserved for the extraction.

After *in sacco* degradation, hydro-acetone extraction was performed by soaking 10g of dried and ground residues in 100 ml of 70% acetone for 1 h at 40 °C. After filtration, the solvent was evaporated using a Rota vapor and dried at 40 °C. The extract was kept in a sample at 4 °C until used for the assays.

Egg hatch assay

Eggs used in the assay were freshly collected from feaces of a donor sheep experimentally infected with *Haemonchus contortus*. The egg hatch assay was conducted according to World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles *et al.,* 1992). The eggs were extracted, repeatedly washed and distributed in 96-multi-well plates at a density of 100 eggs per well. Increasing concentrations of plant extracts (75, 150, 300,600, 1200 and 2400 µg dry matter (DM) per ml were obtained from dry ground extracts dissolved in phosphate buffered saline

(PBS 0.15 M, pH = 7) and then added to each well. Each concentration was tested on six replicates. In addition, a positive (thiabendazole at 125, 250 and 500 µg/ml and negative 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.

Larval migration inhibition assay

This test is aimed at evaluating the anthelmintic effect of the plant extracts on the migration capacity of the L_3s larvae. This test was performed according to Rabel *et al.* (1994). *H. contortus* L_3s were obtained by fecal culture. Eggs reached the L_3s stage after 10 days. The L_3s were then collected using Baermann's devices. The anthelmintic effect of each extracts was tested using 150, 300, 600, 1200 µg/ml concentrations. Negative (PBS) and positive controls (levamisole at 125, 250 and 500 µg/ml) were also prepared in PBS and incorporated to the assay. Three replicates were run for each extract and for the controls. After 3 h of incubation at room temperature (25 °C), the larval suspensions were rinsed out 3 times with PBS buffer. After the final washing, 1 ml of larvae at the concentration of 1000 L_3s/ml 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. After 3 h of incubation, the inserts were removed and the number of larvae which had actively migrated though the mesh was determined.

The percentage of LMI was calculated as

$$LMI = \frac{T - M}{T} x100$$

where T is the total number of L_3s deposited in the sieve and M the number of L_3s present in the PBS.

Polyvinyl polypyrrolidone (PVPP) is used to show the involvement of tannins on larval migration. PVPP forms complexes with tannins and polyphenols and thus blocks their potential biological activity (Makkar, 2003). PVPP was added to the 2 plant extracts at a concentration of 1200 µg/ml for 2 hours 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 infective larvae of *H. contortus*. Thereafter, the larval migration inhibition assay was performed according to the procedure described previously.

Statistical analysis of results

The mean values were calculated and the increasing effect of the concentration of plant extracts was performed using GLM process through the package MASS of free software (Venables and Ripley, 2002). The hierarchical organization of mean was made using Multiple Comparison Test of Tukey through HSD test procedure of the package agricolae (De Mendiburu, 2013) of free software R (R Core Team, 2013).

Results

Dry matter disappearance in the rumen

P. biglobosa and *P. erinaceus* dry matter disappearance percentages according to the incubation period are presented in Figure 1. The matter loss observed for *P. biglobosa* was greater than of *P. erinaceus*. After 72 hours of incubation the matter loss was 33.3% for *P. biglobosa* and 16.7% for *P. erinaceus*. The disappearance of the dry matter was almost linear for the two plants. Two kinetic times namely 24 and 72 hours were appointed for the biological tests on the parasites and their effects were compared with 0 hour of incubation.

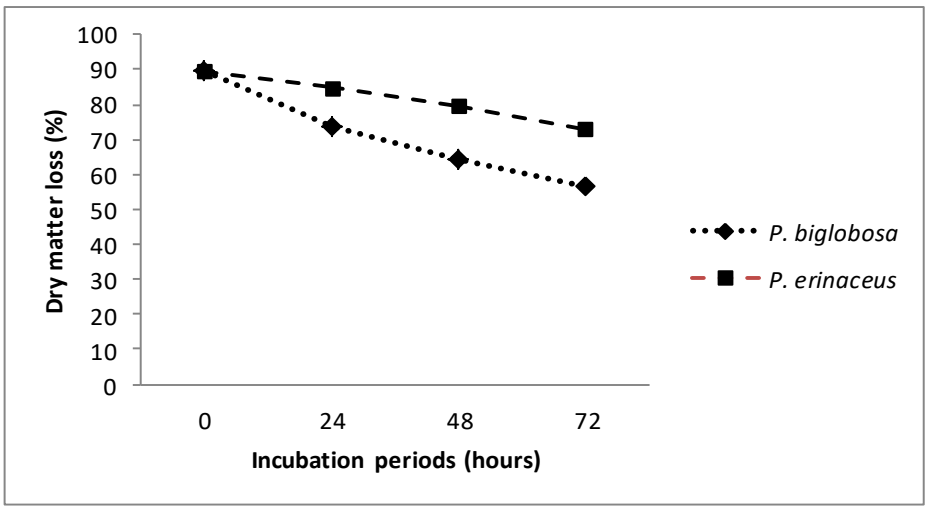

Figure 1: *Parkia biglobosa* and *Pterocarpus erinaceus* dry matter degradation in the rumen.

Effect on egg hatching

The extracts of residues of *P. biglobosa* pods and *P. erinaceus* leaves showed significant effect on egg hatching compared to the negative control ($p < 0.001$). The mean hatching rate measured in the negative control was 88%. With the positive control (Thiabendazole) concentrations used, more than 99% of eggs incubated did not hatch. The effect of highest concentration of extract (2400 µg/ml) was similar to positive control (Thiabendazole) ($p < 0.05$). The

mean eggs hatching values obtained increased significantly ($P < 0.05$) with the decrease of the concentrations of the extracts (Figure 2). The ovicidal activity observed is dose and incubation period-dependent ($p < 0.001$). Both plants showed the same activity on eggs ($p < 0.05$).

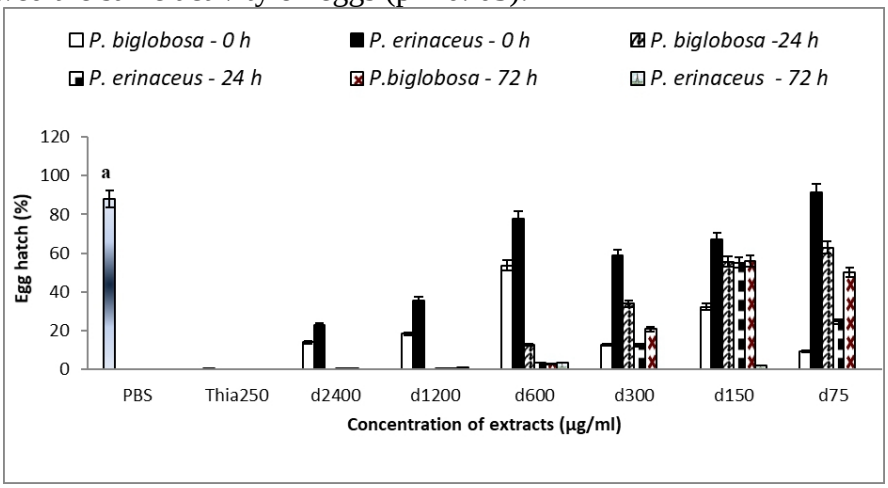

Figure 2: Dose-dependent profile of the percent hatching egg of *H. contortus* at concentrations (75 – 2400 µg/ml) of plant residues extracts.

Effects on larval migration

The mean percent values of the migration of *H. contortus* infective larvae exposed to different concentrations of *P. biglobosa* and *P. erinaceus* extracts are presented in Figure 3. The mean migration rate observed for the larvae of negative control was 97%. The extracts exhibited larval migration inhibition at all concentrations tested compared to negative control (PBS) ($p < 0.05$) but less than positive control (levamisole) ($p < 0.05$). The mean migration rate observed for the larvae of extracts was less than 47%. The effect of incubation period was not significant ($p > 0.05$). Addition of PVPP to the extracts decreased very partially the inhibiting activity of *P. biglobosa* at 0 hour of incubation. For the other extracts, the addition of PVPP induced a larval migration restoration. The percentage of migration was doubled or tripled compared to the extracts untreated with PVPP (Figure 4).

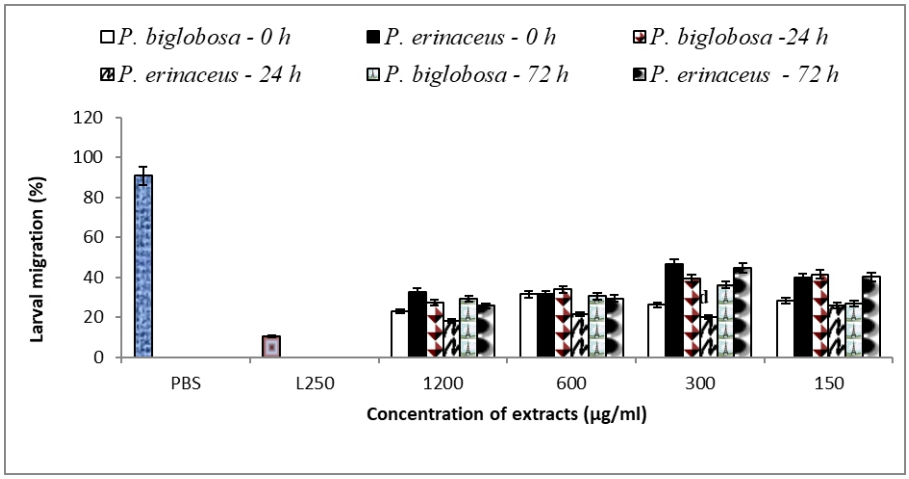

Figure 3: Variation of the rate of larval migration of *H. contortus* with PBS, levamisole and plant residues extracts at concentrations (150 – 1200 µg/ml).

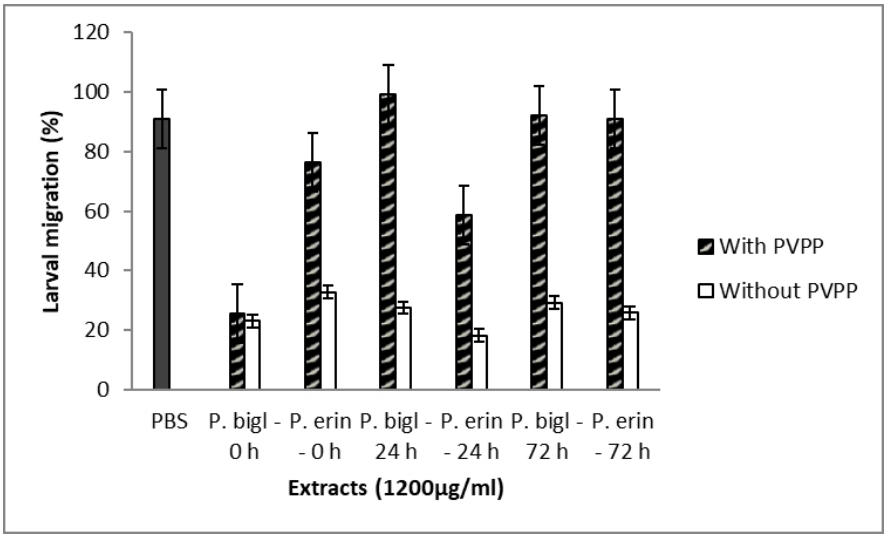

Figure 4: Variation of the effect of the extracts of *Parkia biglobosa* (P. bigl) and *Pterocarpus erinaceus* (P. erin) in presence (With) or not (Without) of the PVPP on the larval migration of *Haemonchus contortus*.

Discussion

Acetone was the solvent of extraction used in this study. This solvent was selected because of the greatest effectiveness of the hydro-acetone extracts of *Parkia biglobosa* and *Pterocarpus erinaceus* brought back by previous studies (Dèdéhou *et al.*, 2014). These extracts gave *in vitro* the best results on the larvae and the adult worms of *H. contortus* compared with the hydro-methanol extracts (Dèdéhou *et al.*, 2014).

The extracts of *P. biglobosa* and *P. erinaceus* inhibited dose-dependent egg hatching and larval migration of *H. contortus*. The results obtained for egg hatching are comparable with those reported by other authors with *Senna occidentalis, Leonotis ocymifolia, Leucas martinicensis, Rumex abyssinicus, Albizia schimperiana, Maesa lanceolata and Plectranthus punctatus* (Tadesse *et al.,* 2009; Eguale *et al.,* 2011). These results are better than those reported by Nwosu *et al.* (2006) with *Azadirachta indica*. For larval migration inhibition, the results obtained confirm those previously announced by Dèdéhou *et al.* (2014). Also, Soetan *et al.* (2011) reported the anthelmintic effect of extracts of seeds and leaves of *P. biglobosa* on bovine nematode eggs. Phytochimical screening of the hydro-acetone extracts of *P. biglobosa* and *P. erinaceus* revealed for two plants the presence of several bioactive molecules like tannins, triterpenes, flavonoids, saponins and alkaloids (Dèdéhou *et al.,* 2014). The role of some of these molecules was suspected or shown in the anthelmintic effects of both plants (Ayers *et al.,* 2008; Brunet, 2008; Olounladé *et al.,* 2011). Molan *et al.* (2003) and Brunet (2008) showed that flavonoids induced structural deteriorations on infective larvae thus preventing their migration. According to Maciel *et al.* (2006) and Marie-Magdeleine *et al.* (2009), the development of the larvae could be inhibited by terpenoids. Eguale *et al.* (2007) and Marie-Magdeleine *et al.* (2009) suspected the role of saponins in egg hatch inhibition.

The two plants did not lose their anthelmintic effects after 24 to 72 hours' incubation in the rumen. After 72 hours' incubation, the larval migration inhibition activity of the two plants was preserved and their egg hatch inhibition activity was increased. This could be related to a greater concentration in bioactive substances of the residues resulting from the matter loss of the powders incubated during digestion in the rumen. These results are similar to those found by Alowanou *et al.* (2015) with *Mitragyna inermis, Combretum glutinosum* et *Bridelia ferruginea* and those of Minaflinou Sacca Sidi *et al.* (2017) with *Newbouldia laevis,* where plants kept their anthelmintic properties after incubation in the rumen of sheep. On the over hand, with *Zanthoxylum zanthoxyloides* a reduction in anthelmintic activity was observed after incubation in the rumen (Minaflinou Sacca Sidi *et al.,* 2017).

Many hypotheses were emitted as for the implication of certain bioactive molecules in the anthelmintic effects of the plants. However, few studies were interested in becoming of these molecules in the digestive tract of the animals. Hydrolysable tannins are hydrolyzed by bacteria in the rumen causing the release of the gallic acids and can be further metabolized to other compounds such as pyrogallol (Singh *et al.,* 2001; Min *et al.,* 2003). The breakdown products are then absorbed and circulate in blood. This can reduce the availability of the secondary metabolites of the plants and consequently their therapeutic effectiveness. Condensed tannins have the properties to form

complexes with macromolecules, including proteins, especially prolinerich proteins (Waterman, 1999). The degree of complexation of tannins with proteins depends on the molecular mass, structure and the configuration of both substances (Mueller-Harvey, 2006; Poncet-Legrand *et al.* 2006). These interactions are usually due to hydrogen bonds and/or hydrophobic interactions (Poncet-Legrand *et al.* 2006). In the acidic conditions of the abomasum, condensed tannins are released and are thus available in portions of digestive tract which follows upon the rumen. This would justify the aptitude of the plants which contain these tannins to disturb the viability of the worms or the fertility of the females (Azando *et al.*, 2011). The effect of degradation in the rumen on terpenes would vary according to the nature of terpene. Malecky *et al.* (2009) after using *in vitro* incubation of seventeen terpenes with mixed rumen bacteria from dairy goats obtained recovery rates different markedly among terpenes, partly in relation to the presence of oxygen and rings in the molecules. Mathison *et al.* (1999) indicated that alfalfa (*Medicago sativa*) saponins were rapidly released into rumen fluid and extensively degraded in the digestive tract of sheep but no attempt was made to determine how closely the products of degradation were related to the original molecules.

Our second objective was to obtain indications on the nature of the active compounds responsible for the anthelmintic activity of *P. biglobosa* and *P. erinaceus*. In particular, we tested the hypothesis that tannins and/or polyphenols were involved in this activity by comparing the effects of the same extracts with or without addition of PVPP which is known to create complexes with polyphenols in particular tannins (Makkar, 2003). The use of the PVPP induced for almost all of the extracts an increase in the larval migration as in the PBS. This indicates that the tannins and/or polyphenols are mainly responsible for the inhibiting activity observed. However, the similar effects exerted by the extracts of *P. biglobosa* at 0 hour treated or not with PVPP indicates the presence of nonphenolic substances which are implied in the action of this plant. The results obtained with 24 and 72 hours would indicate a degradation of these substances in the rumen.

Conclusion

The present study has shown that extracts of *P. biglobosa* and *P. erinaceus* have some anthelmintic activities against egg and larvae of *Haemonchus contortus*. The two plants preserved their anthelmintic properties after incubation *in sacco* in the rumen. Incubation during 72 hours in the rumen increased the action of the two plants on eggs.

Taking into consideration these results, it would be interesting in later studies to proportion the main bioactive molecules of these plants in different parts of digestive tract of sheep to know which molecules act on the parasites.

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