In vitro Ovicidal and Larvicidal Activity of the Leaf, Bark and Root Extracts of *Peltophorum africanum* Sond. (Fabaceae) on *Haemonchus contortus*

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Abstract: The *In vitro* efficacy of the extracts of *Peltophorum africanum* Sond. (Fabaceae), was determined against *Haemonchus contortus*. Acetone extracts of the leaf, bark and root, at concentrations of 0.008 to 25 mg mL^{-1} were incubated at 23°C with the eggs and larval stage (L$_1$) of the parasite for two and five days, respectively. Thiabendazole and water were positive and negative controls, respectively. Increasing the concentration of extracts caused a significant (p<0.05) increase in inhibition of egg hatching, and larval development. At concentrations of 0.2 and 1.0 mg mL^{-1} the extracts inhibited egg hatching and development of L$_1$ to the infective stage (L$_3$). No eggs and larvae (L$_1$) of *H. contortus* were detected at concentrations of 5 and 25 mg mL^{-1}. The *in vitro* model may provide support of the traditional use of *P. africanum* extracts against helminthosis. Suitable methods of plant extraction, adaptable to rural use may help rural communities control helminthosis.

Key words: Ovicidal, larvicidal, extracts, *Haemonchus contortus*, *Peltophorum africanum*

INTRODUCTION

In the tropics and sub-tropics, helminthosis remains one of the most prevalent and economically important parasitoses of domesticated animals[1,2]. Gastrointestinal nematodes are the chief parasitoses responsible for disease-related production losses arising from stock mortality, severe weight loss and poor production, especially in small ruminants[3]. Haemonchosis (caused by *Haemonchus contortus*) has been listed among the top 10 most important conditions hampering production of sheep and goats in tropical countries[1,4]. The disease is characterised by anaemia, haemorrhagic gastroenteritis, hypoproteinaemia (manifested by oedema or 'bottle jaw'), sudden death or chronic emaciation[5,6]. Adult *H. contortus* females have high egg-producing capacity, of 5000-15000 eggs per day[7]. The high fecundity combined with the high rainfall and temperatures, favour permanent larval development in the environment leading to heavy contamination of pastures with the infective larval (L$_3$) forms.

Use of synthetic and semi-synthetically produced anthelmintic drugs has for long been considered the only effective method of control of gastrointestinal nematode infections of small ruminants. However, most of the proprietary drugs are expensive and unavailable to rural subsistence livestock keepers, who are tempted to use substandard doses. Conversely, in more developed farming systems, the massive use of the drugs has created multiple anthelmintic resistance against all of the major families of broad spectrum anthelmintics[8-10], that may lead to failure of control of worm parasites in ruminants. Surveys in South Africa, indicate anthelmintic resistance to be serious on sheep and goat farms[11]. The foregoing has created delicate situations, where at one extreme there are heavy mortalities of young stock, while at the other the economic control of helminth parasites is difficult. These constraints indicate that entire reliance on synthetic anthelmintics may present difficulties in the management of gastrointestinal parasitic infections in livestock, necessitating novel alternative methods of helminth control[12-14].

Use of indigenous plant preparations as livestock dewormers is gaining ground as one of the alternative and sustainable methods readily adaptable to rural farming communities[15,13]. About 80% of people in the developing world rely on phytomedicine for primary healthcare[16,17]. Ethnomedicine often does not follow the western paradigms of scientific proof of efficacy; hence the medical and veterinary professionals distrust herbal remedies[18,19]. There is need therefore, for scientific validation of efficacy of herbal medicines before their acceptance and use.

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Peltophorum africanum (weeping wattle) is a unique plant in that it is traditionally used to treat almost similar disease conditions in both man and domesticated animals. Traditional healers use the plant to treat among other conditions, diarrhoea, dysentery, helminthosis, and wounds and for promotion of well-being and resistance to diseases in man and animals[20-22]. Phytochemists have found several condensed flavonoids, a novel cyanomaclurin analogue[23], profisetinidin-type-4-aryflavan-3-ols and related δ-lactones[24] in the heartwood. New compounds (bergenin, norbergenin and 11-0(E)-p-courmaroylbergenin) were isolated from ethanol extracts of the bark (Mebe and Makuhunga,[25]), and leaves yielded coumarins (Khattab and Nasser,[26]). *In vitro* antibacterial (Obi *et al.*,[27]), antioxidant and antibacterial (Bizimenyera *et al.*,[28]) activities, and inhibitory properties against the Human Immunodeficiency Virus (HIV) type 1 reverse transcriptase and integrase (Bessong *et al.*,[29]) of the leaf, bark and root extracts of *P. africanum* extracts have been reported. The compounds responsible for the biological activities of *P. africanum* extracts have not yet been sufficiently characterise, and many are not yet determined.

The aim of the present study was to evaluate the *in vitro* effects of *P. africanum* acetone extracts on the egg hatching and larval development (L_1 to infective stage L_3) of *Haemonchus contortus,* the abomasal nematode of sheep and goats. This study is part of the ongoing work on the isolation and characterisation of bioactive compounds from *P. africanum.*

MATERIALS AND METHODS

Collection, storage and preparation of plant material: Leaves (L), stem Bark (B), and Root bark (R) were collected in spring from mature *Peltophorum africanum* Sond. (Fabaceae) trees growing naturally (and labelled No. S.A Tree No. 215) at the Onderstepoort Faculty of Veterinary Science, University of Pretoria in South Africa. A voucher specimen (PM 001) was stored in the medicinal plant herbarium, Department of Paraclinical Sciences, University of Pretoria. The collected plant material was dried in the shade, at ambient temperature. Dried material was ground to powder in a Mascalab mill, (Model 200 LAB), Eriez®, Bramley. The powdered material was separately stored in dark tightly closed glass bottles prior to extraction with acetone.

Preparation of plant extracts: In a preliminary work, the composition of the extracts was determined by Thin Layer Chromatography (TLC) using four solvents of varying polarity, that is acetone, ethanol, dichloromethane and hexane. For the present study, three gram of each plant part (L, B, and R) was extracted in triplicate with 30 mL of technical grade acetone in glass bottles on a shaking machine for one h. After the solvent was dried off in a stream of air at room temperature, the dried extract was reconstituted in acetone to make a 100 mg mL^{-1} of a stock extract that was stored at 5°C in sealed vials before use. For the ovicidal and larvicidal tests, the stock extract was diluted with distilled water in vials to concentrations of 25, 5, 1, 0.2, 0.04 and 0.008 mg mL^{-1}. A preliminary test run had shown that at the 25 mg mL^{-1} dilution, the acetone in the extract had no effect on the eggs and larvae of *H. contortus.*

Egg recovery and preparatio: The egg preparation, egg hatch and larval development inhibition assays is based on the recommendations of the World Association for the Advancement of Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance (Coles *et al.*,[30]). An analogous method has been used for ovicidal tests of plant extracts[31,32]. Faecal pellets were collected using harnesses and collecting bags, from lambs with monospecific infections of *H. contortus.* The lambs, under strict veterinary care and supervision, were housed indoors on concrete floor, fed hay and pellets, and given free access to water. Water was slowly added to the faeces and pellets mashed in a food blender to make a relatively liquid suspension. This suspension was filtered through a sieve of 400 μm mesh to remove coarse plant debris. The suspension was serially filtered through sieves of pore sizes from 250 μm, 150, 90, 63, and finally eggs collected from the 38 μm mesh. The material on the 38 μm mesh was washed into 50 mL centrifuge tubes filled with distilled water. The tubes were centrifuged at 3000 rpm for 5 min. The supernatant was discarded and the sediment re-suspended in saturated sodium chloride in water in another set of centrifuge tubes to separate the eggs with a lower density from other debris. The tubes were again centrifuged at 3000 rpm for 5 min. The supernatant was washed with water on a 38 μm mesh that trapped the eggs. The eggs were carefully washed off from the 38 μm pore mesh into a 1 litre conical cylinder with distilled water where they were allowed to settle for one h. The eggs were siphoned from the bottom of the conical flask into a beaker. After magnetic stirring the egg suspension in the beaker, the concentration of eggs were estimated by counting the number of eggs in 10 aliquots of 50 μL of the suspension on a microscope slide. A final concentration of 100 eggs per well (for both egg hatch and larval development assays) was selected, and such egg suspension was used within 1 h of preparation.

Egg Hatch inhibition (EH) assay: About 100 eggs in every 200 µL of the egg suspension was pipetted into each well of the 48 well microplates. In the test wells 200 µL of the appropriate plant extract (25, 5, 1, 0.2, 0.04 and 0.008 mg mL^{-1}) was added. Positive control plates contained 200 µL of 25, 5, 1, 0.2, 0.04 and 0.008 µg mL^{-1} of thiabendazole. Negative control wells contained 200 µL of distilled water. Three replicates were analysed for each treatment. The microplates were incubated under 100% relative humidity at room temperature (.23°C) for 48 h. A drop of Lugol's iodine solution was then added to each well to stop further hatching, and all the unhatched eggs and L$_1$ larvae in each well were counted. The percentage inhibition of hatching was calculated.

Larval Development (LD) inhibition assay: The same egg suspension and 48 well microplates as in 2.4 above were used. Into each well 170 µL of the egg suspension was placed. Each well also contained 50 µL of a suspension of lyophilised *Escherichia coli* (ATCC 9637), essential for the development of nematodes by Hubert and Kerboeuf,[33]. Then 10 µL of Amphotericin B$^{®}$ (Sigma) was added to each well to control fungal growth followed by 20 µL of nutritive media (comprising of 1gm yeast extract in 90 mL of normal saline and 10 mL of Earle's balanced salt solution) was added. The well contents were well mixed. The plates were incubated under 100% relative humidity at room temperature for 48 h. Once the larvae had hatched, 250 µL of the test extracts at the same concentrations mentioned in 2.4 above and thiabendazole control concentrations was added to each plate (negative control plates had 250 µL of distilled water added). As in 2.4 above, there were three replicates for each treatment. The plates were further incubated under 100% relative humidity at room temperature for 5 days. All the plates were checked to determine at which concentration in the wells all the larvae had died. Then, further development was stopped by addition of one drop of Lugol's iodine solution. All the L$_1$ and L$_3$ larvae in each well were counted. The inhibition of development to L$_3$ was calculated.

Calculations and statistical analysis: The percentage inhibition of egg hatching and larval development (L$_1$ to L$_3$) was calculated using the formula modified after Coles *et al.*,[30]: -

$$(\%)\text{Inhibition} = 100(1-P_{test}/P_{control}),$$

where P_{test} = the number of eggs hatched (or larval forms (L$_1$), in case of EH assay), or the number of hatched larvae that developed into infective larvae (L$_3$) (in study of LD assay) in test extracts, and $P_{control}$ = the respective numbers in water control. The mean values, as well as the dose-response curves were determined using the Excel statistical package. The non-parametric Kruskal-Wallis test by Hammer *et al.*,[34]), instead of ANOVA variance calculations, was used in the statistical analysis; the numbers of eggs were only estimates of 100 per well (see 2.3). The Environment Protection Agency (EPA) Probit Analysis Programme was used to calculate the dose that was effective against 50% of the cells (ED$_{50}$) in the different treatments.

RESULTS

The leaf, bark and root extracts of *P. africanum* inhibited the egg hatching and larval development (from L$_1$ to L$_3$) of *H. contortus* at concentrations of 0.2-1
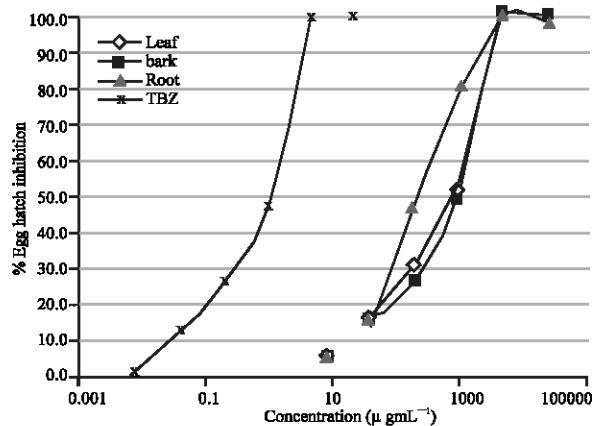

Fig. 1: Dose-response egg hatch inhibition of *H. contortus* by leaf, bark and root extracts of *P. africanum*
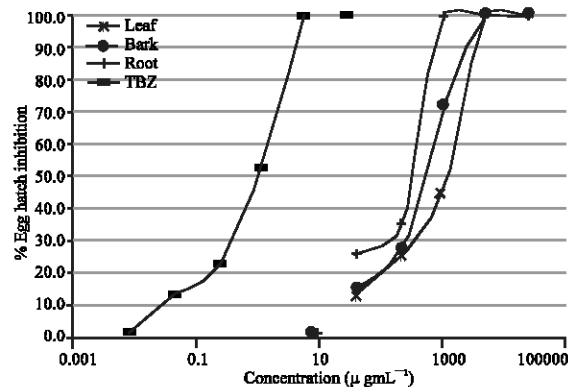

Fig. 2: Dose-response larval development inhibitions of *H. contortus* by leaf, bark and root extracts of *P. africanum*

Table 1: % Mean inhibition of egg hatch (EH) and larval development (LD) of *H contortus* by *P. africanum* leaf, bark and root extracts

	Concentration of extracts (mg mL^{-1})	25	5	1	0.2	0.04	0.008
% Mean inhibition	EH Leaf	100	100	50.67 (2.52)	28.67 (3.51)	15.67 (3.06)	1 00 (1.73)
	Bark	100	100	47.67 (5.03)	21.33 (6.66)	13.67 (2.31)	1.67 (1.15)
	Root	100	100	77.00 (4.58)	46.33 (4.04)	10.00 (4.58)	1.33 (2.31)
	LD Leaf	100	100	43.00 (5.57)	19.00 (2.00)	9.67 (1.53)	2.00 (1.00)
	Bark	100	100	71.33 (7.57)	28.67 (3.06)	14.00 (3.61)	1.00 (1.73)
	Root	100	100	100	32.67 (4.51)	19.33 (4.04)	1.00 (1.00)

Note: - Standard deviation (in brackets)

Table 2: Kruskal-wallis and effective dose$_{50}$ (ED$_{50}$) values of extracts of *P. africanum* against *H. contortus*

	Egg hatch (EH)*		Larval development (LD) *	
Test material	P-value (Kruskal-Wallis)	ED$_{50}$ (mg mL^{-1})	P-value (Kruskal-Wallis)	ED$_{50}$ (mg mL^{-1})
Leaf	0.00773	0.515	0.00655	0.717
Bark	0.00894	0.383	0.00655	0.475
Root	0.00683	0.259	0.0131	0.261
Thiabendazole**	0.00796	0.810	0.00655	0.625

Note: * Values at 95% confidence intervals ** Thiabendazole values are in μmL^{-1}

Table 3: Larvicidal activities of acetone extracts of *P. africanum* against *H. contortus*

Conc of extracts (mg mL^{-1})	Leaf	Bark	Root	Thiabendazole*
25	X	X	X	X
5	X	X	X	X
1	X	X	X	X
0.2	√	√	√	X
0.04	√	√	√	√
0.008	√	√	√	√

Key: - X= all larvae₁ dead √= larvae₁ alive Note: The larvae in concentrations 5 and 25 mg mL^{-1} were completely lysed * Thiabendazole values are in μ mL^{-1}

mg mL^{-1}, Table 1. Increasing the concentration of the extracts caused a dose dependent significant (p<0.05) increase in inhibition of egg hatching and larval development (Fig. 1, 2 and Table 2). The root extracts were more effective than the bark and leaf (Table 1 and 2). The eggs and larvae (L₁) were lysed at concentrations of 5 and 25 mg mL^{-1}, and could not be observed in the respective wells. There was no single larva alive in the wells at concentrations of 1 mg mL^{-1} and higher with any of the extracts, or at a thiabendazole concentration of 0.2 μg mL^{-1} and higher than (Table 1 and 3). It is interesting that the plant extracts had a similar dose response curve as thiabendazole at a c. thousand-fold higher concentration (Fig. 1 and 2).

DISCUSSION

We used acetone as extractant because it extracts compounds with a wide polarity range from plants, is non-toxic to test organisms, is miscible with organic and aqueous solvents and is easy to remove to recover extracted compounds[35]. Experience with hundreds of plant species in our laboratory has confirmed the value of acetone as an extractant for many diverse compounds from plants. Acetone also extracted the largest quantity of compounds from *P. africanum* compared to ethanol, dichloromethane, and hexane (Bizimenyera *et al.*,[28]).

The egg hatch assay as recommended for determining the anthelmintic resistance[30], has been modified to test ovicidal effects of plant extracts[36,32]. The *in vitro* model reported in this study demonstrated ovicidal and larvicidal effects of acetone extracts of *P. africanum* against *H. contortus*. The extracts inhibited egg hatching and larval development (L₁ to L₃). The inhibition of larval development was most probably due to larval (L₁) mortality. This is the first report of the *P. africanum* extracts on *H. contortus*. Earlier work (Mølgaard *et al.*,[37]) had shown that 0.5 mg mL^{-1} of leaf and bark and 0.8 mg mL^{-1} of root extracts of *P africanum* were effective against newly excysted cestodes of the worm *Hymenolepis dimunita* after a 24 h incubation. Their work was not extended to cover other classes of helminths. Furthermore, only water extracts were tested, whereas organic solvents extract more material from plants than water (Kotze and Eloff,[38]).

There was a problem of counting every egg or larva as the extract at concentrations above 5 mg mL^{-1} completely lyses eggs and L₁ larvae. Therefore, the count is still an estimation based on numbers of eggs put in the plates. The ovicidal and larvicidal effects were not timed. Whereas it has been shown that tannins in plant extracts exert anthelmintic action on their own (Athanasiadou *et al.*,[39]), the removal of tannins from *P. africanum* extracts only slightly reduced their

anthelmintic activity (results not shown). Compounds isolated from the root extracts to date had less anthelmintic activity than the extracts (results not shown). Given that the polyphenol content of the root extract of *P. africanum* is higher than the bark and leaf (Bizimenyera *et al.,*[28]), and could account for the higher anthelmintic activity, there appears to be other compounds in the extracts acting singly or in synergy.

Extracts from a number of plants have been tested against *H. contortus*. Seven plant species (Githiori *et al.,*[40]) had no effect on faecal egg counts in lambs infected with *H. contortus* and fed water extracts of the plants. Assis *et al.,*[32] reported that a 50 mg mL^{-1} ethyl acetate extract of *Spigelia anthelmia* inhibited 100% egg hatching and 81% larval development of *H. contortus*. Water extracts of *Vernonia amygdalina* did not show any activity at concentrations of 11.2 mg mL^{-1} while *Annona senegalensis* at 7.1 mg mL^{-1} showed significant egg hatch of *H. contortus* (Alawa *et al.,*[31]). The activity of the *P. africanum* extracts at concentrations of 0.2-1 mg mL^{-1} is comparable to the range of 0.5-1.0 mg mL^{-1} reported elsewhere (Akhtar *et al.,*[41]; Hördegen *et al.,*[42]). In the *in vitro* model, 1.0 mg mL^{-1} of acetone extract of the root inhibits 77% of hatching and 100% larval development. Efficacy at 1.0 mg mL^{-1} whereas significant for extracts is still low compared to the same effect with thiabendazole at 1 μg mL^{-1}.

The *in vitro* activity of *P. africanum* extracts may not necessarily be transferable wholesale to in vivo efficacy, as the latter is influenced by physiology and bioavailability factors in body (Githiori *et al.,*[43]). Furthermore activity against eggs and larval forms (L$_1$) of *H. contortus* may not automatically imply action on adult worm parasites. Direct effects of plant forages on anthelmintic load in grazing animals have been reported[44,41,42]; Athanasiadou *et al.,* . [4]If our next experiments testing the effect of plant extracts in vivo give positive results, administration *P. africanum* leaves to infected animals may lead to a reduction in faecal counts and therefore to lowered environment or pasture contamination.

If all goes we, we intend to isolate and characterise the anthelmintic compounds and also investigate whether extracts made in a low technology environment in rural areas are effective.

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