CHAPTER 5

IN VITRO ACTIVITY OF *PELTOPHORUM AFRICANUM* SOND. (FABACAE) EXTRACTS ON THE EGG HATCHING AND LARVAL DEVELOPMENT OF THE PARASITIC NEMATODE *TRICHOSTRONGYLUS COLUBRIFORMIS*

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Abstract

Trichostrongylus colubriformis is an important cause of parasitic gastroenteritis in ruminants, where it causes protracted diarrhoea, rapid loss of weight, loss of production and death. The *in vitro* efficacy of extracts of *Peltophorum africanum* was determined against this parasitic nematode. Eggs and larvae of *T. colubriformis* were incubated at 23^{o}C in the extracts of the leaf, bark and root of *P. africanum* at concentrations of 0.008 – 25 mg ml^{-1} for two and five days respectively. Thiabendazole and water were used as positive and negative controls, respectively. Inhibition of egg hatching and larval development increased significantly ($P<0.05$) with increasing concentrations of the extracts. Concentrations of 0.2 to 1.0 mg ml^{-1} of the extracts of leaf, stem bark, and root bark of *P. africanum* completely inhibited the hatching of eggs and development of larvae. No eggs and larvae of T. colubriformis could be observed in wells incubated with all the three extracts at concentrations of 5 and 25 mg ml^{-1}. The in vitro model results support the traditional use of *P. africanum* against nematode parasites. 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 helminthosis.

Key words: - Ovicidal; larvicidal; extracts; *Trichostrongylus colubriformis*; *Peltophorum africanum*

5.1 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 (Perry and Randolph, 1999; Chiejina, 2001). *Trichostrongylus colubriformis*, an intestinal nematode, is one of the most important causes of parasitic enteritis causing protracted diarrhoea, weakness, loss of production and death. Infestation of sheep with *T. colubriformis* causes a severely infected animal to pass dark diarrhoea that has earned the parasite the name of "black scours worm" (Soulsby, 1982). The parasite is frequently identified in large numbers in infected sheep and cattle in South Africa (Horak, 2003; Horak *et al*., 2004). The infective larvae (L_3) of *T. colubriformis* have a high capacity to survive even in adverse weather conditions (Urquhart *et al*,

1996). In tropical areas, the high temperatures and rainfall favour the development of the free-living stages to infective stage throughout the year.

In the last 30 years, control of gastrointestinal nematode infections of ruminants has been achieved almost exclusively by use of pharmaceutically derived anthelmintics. Indeed, synthetic and semi-synthetically produced anthelmintics have for long been considered the only effective method of controlling helminthosis. However, in the extreme situations of subsistence farming where anthelmintics are either unavailable or unaffordable, massive mortalities of young stock are tragically commonplace in tropical Africa and Asia (Anon, 1992; Griggs, 1996). At the other extreme, misuse and or widespread intensive use of sometimes poor quality synthetic or semi-synthetic anthelmintics has led to development of a high level multiple anthelmintic resistance that may lead to failure of control of worm parasites in ruminants (Prichard, 1990; Maciel *et al*, 1996; Monteiro *et al*, 1998; Wolstenholme *et al*, 2004). These constraints indicate that entire reliance on pharmaceutically derived anthelmintics may present difficulties in the management of gastrointestinal parasitic infections in livestock, necessitating alternative methods of helminth control (Waller, 1997; Sanyal, 2001).

In recent times, there has been increasing interest in ethnomedical and ethnoveterinary practices across the world, especially as it relates to the use of medicinal plants in treating various ailments. 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 (Hammond *et al*, 1997; Danø and Bøgh, 1999). 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 (Plotkin, 1992; McCorkle *et al*, 1996). 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 (Sofowora, 1982; Thompson, 1997).

Peltophorum africanum (weeping wattle) is a plant that is traditionally used to treat among other conditions, diarrhoea, dysentery, helminthosis and promotion of well-being and resistance to diseases in man and animals (Watt and Breyer-Brandwijk, 1962; Van der Merwe, 2000; Van Wyk and Gericke, 2000). Several authors have investigated the phytochemistry of this species without testing the activity of extracts or isolated components (Bam *et al*, 1988; Bam *et al*, 1990; Mebe and Makuhunga, 1992; Khattab and Nasser, 1998). The extracts of *P. africanum*, however, have inhibitory effects against human immunodeficiency virus (HIV–1) reverse transcriptase and integrase (Bessong *et al*, 2005), and also antibacterial and antioxidant

activities (Bizimenyera *et al*, 2005). Nevertheless, much work remains in the study and characterization of bioactive compounds extracted from *P. africanum*.

The aim of the present in vitro study was to establish the effects of acetone extracts of leaf, stem bark and root bark of *P. africanum* on the egg hatchability, larval viability and larval development (to infective stage, $L_{3,}$) of the intestinal parasite of sheep and goats, *Trichostrongylus colubriformis*. This study is part of the ongoing work on isolation and characterisation of bioactive compounds from *P. africanum*.

5. 2.Materials and methods

5. 2.1. Collection, storage and preparation of plant material

Leaves (L), stem bark (B), and root bark (R) (referred to further as leaf, bark and root) were collected in spring from mature *Peltophorum africanum* Sond. (Fabaceae) trees growing naturally (and labelled S.A tree number 215) at Onderstepoort, Pretoria, 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 plant material was ground to powder using a Mascalab mill, (Model 200 LAB), Eriez®, Bramley. The powdered material was separately stored in dark tightly closed glass bottles before investigation.

5. 2. 2. Plant extracts preparation

Acetone has been shown to be a good extractant of compounds from plants (Eloff, 1998). A previous study on extraction of bioactive compounds from *P. africanum* (Bizimenyera *et al*, 2005) showed that acetone extracted the largest amount (quantity) of extracts from the plant material when compared to ethanol, dichloromethane, and hexane. Three grams (3g) of each plant part (L, B, and R) were extracted in triplicate with 30 ml of technical grade acetone in glass bottles on a shaking apparatus for one hour. Typically, extracts have to be dried and subsequently re-dissolved in suitable solvents to make up known concentrations for bioassays. As preliminary work had shown that there is incomplete solubility in acetone of the acetone extracts of *P. africanum*, the extracts were not dried to circumvent this problem. A small aliquot of the acetone extract was dried, and the values obtained were used to calculate the concentration of the original extract. Known volumes of the extract containing 100 mg ml^{-1} were put in sealed vials and stored in an ordinary refrigerator as a stock solution before use. For the ovicidal and larvicidal tests, the stock solution 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 evaluation had shown that at the 25 mg ml^{-1} dilution, the acetone in the extract had no effect on the eggs and larvae of *T. colubriformis*.

5. 2.3. Recovery and preparation of eggs

The recommendations by the World Association for the Advancement of Veterinary Parasitology (WAAVP) for the detection of anthelmintic resistance in nematodes of veterinary importance (Coles *et al* 1992), were modified for the egg preparation, and for the egg hatch and larval development inhibition assays. Faeces were collected from lambs that had monospecific infections of *T. colubriformis* .The lambs were housed on concrete floor indoor, fed hay, commercial concentrate pellets and had free access to water. Faecal pellets were collected using sterilised harnesses and collecting bags. Water was added to the faeces and pellets mashed in an ordinary fruit blender to make a relatively liquid suspension (slurry). The slurry was filtered through a household sieve of 400 µm mesh to remove coarse plant debris. Thereafter, the suspension was serially filtered through sieves of pore sizes from 250, 150, 90, 63 µm, and finally eggs were trapped on the 38 µm pore mesh. The material on the 38 µm mesh was washed into 50 ml centrifuge tubes that were filled with distilled water. The tubes were centrifuged at 3000 rpm for 5 minutes and the supernatant discarded and the sediment re-suspended in saturated salt solution. The suspension was centrifuged in another set of centrifuge tubes again at 3000 rpm for 5 minutes and the supernatant was washed through a 38 µm mesh sieve where the eggs were collected.

The eggs collected were carefully washed off from the 38 µm sieve into a 1 litre conical cylinder with distilled water where they were allowed to sediment for two hours. The eggs were siphoned from the bottom of the conical flask into a beaker that was then subjected to electromagnetic stirring of the egg suspension. The concentration of eggs was estimated by counting the number of eggs in 10 aliquots of 50 µl of the suspension on a microscope slide. By either concentrating the egg suspension by centrifugation, or by diluting, a final concentration of 100 eggs per the required volume of the suspension was selected for use in the assays and such egg suspension was used within 1 hour after estimation.

5. 2.4. Egg hatch inhibition assay (EH)

Approximately 100 eggs in 200 µl of the egg suspension were pipetted into each well of a 48-well microtitre plate. In the test wells, 200 µl of the appropriate plant extract in concentrations of 25, 5, 1, 0.2, 0.04 and 0.008 mg ml^{-1} was added. Thiabendazole (Sigma®) at concentrations of 25, 5, 1, 0.2, 0.04 and 0.008 µg ml^{-1} was used as a positive control while distilled water was utilised as a negative control; each control well

content was 200 µl. Three replicates for each concentration, extract and control were used. The plates were incubated under humidified conditions at ambient temperature (23 ^{0}C) for 48 hours. A drop of Lugol's iodine solution was added to each well to stop further hatching, and all the unhatched eggs and L_1 larvae in each well were counted. The percent inhibition of egg hatching was calculated.

5. 2.5. Larval development assay (LD)

Eggs were separated and estimated as described in 5.2.4 above, and approximately 100 eggs in 170 µl of the egg suspension was put into each well in a 48-well microtitre plate. To each well in addition, 50 µl of a suspension of lyophilised *Escherichia coli* (ATCC 9637) was added, as bacteria are necessary for the development of nematode larvae (Hubert and Kerboeuf, 1992), as well as 10 µl of amphotericin B (Sigma) to control fungal growth. A further, 20 µl of nutritive media (comprising of 1g yeast extract in 90 ml of normal saline and 10 ml of Earle's balanced salt solution) was added into each well. The well contents were well mixed. The plates were then incubated under humidified conditions at ambient temperature for 48 hours. After this period, 250 µl of the test extracts at the same concentrations mentioned in 5.2.4 above and thiabendazole control concentrations were added to respective plates (control plates had 250 µl of distilled water added). There were three replicates for each extract concentration and controls. The plates were further incubated for 5 days. All the plates were inspected to determine at which concentration all the larvae had died in the wells. 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. A percentage inhibition of development to L_3 was calculated.

5. 2.6. Calculations and statistical analysis

The experiment was to determine efficacy of the extracts, based on their inhibition of egg hatching and larval development (L_1 to L_3) of T. colubriformis.

The percent (%) inhibition of egg hatching and larval development was calculated using the formula (modified after Coles *et al*, 1992): -

$$(\%)\text{Inhibition} = 100(1-P_{test} / P_{control}),$$; where P_{test} is 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 case of LD assay) in test extracts, and $P_{control}$ is the respective numbers in water control.

The mean values were calculated using the Excel statistical package.

As the number of eggs or larvae varied per well (the number of eggs put per well was an estimation, (see 5.2.3), the effect or significance of increasing the concentration of the extract on ovicidal and larvicidal effect was determined by the Kruskall-Wallis test (Hammer *et al*, 2001).

For the comparison of the efficacy of different plant parts (leaf, bark, and root) the ED_{50} (Effective $Dose_{50}$) was determined using the Environmental Protection Agency (EPA) Probit Analysis Programme.

5. 3. Results

The percent inhibition (of egg hatch and larval development) increased significantly ($P< 0.05$) with increase of concentration of the extracts of the leaf, bark and root, Table 5.1 and Figure 5.1, 5. 2.

Table 5.1: p-values (Kruskal-Wallis) and effective dose $_{50}$ (ED_{50}) values of extracts of *P. africanum* against *T. colubriformis*

Test material	**Egg hatch (EH)***		**Larval development (LD) ***	
	P-value (Kruskal-Wallis)	ED_{50} (mg ml^{-1})	P-value (Kruskal-Wallis)	ED_{50} (mg ml^{-1})
Leaf	0.00655	0.619	0.00735	0.724
Bark	0.00655	0.383	0.00581	0.365
Root	0.00655	0.280	0.00767	0.284
Thiabendazole**	0.00796	0.715	0.00796	0.686

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

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Concentrations of 0.2-1.0 mg ml^{-1} of the leaf, bark and root extracts of *P. africanum* were found to be completely ovicidal and larvicidal. There were no live larvae (L_1) in wells incubated at extract concentrations of 0.2 mg ml^{-1} and above, for the leaf and bark, and at 0.04 mg ml^{-1} and above for the root extracts. No eggs or larvae (L_1) of *T. colubriformis* could be observed in wells incubated with all the plant extracts at concentrations of 5 and 25 mg ml^{-1}; at these concentrations, the extracts caused complete lyses of the eggs and larval forms (L_1) of *T. colubriformis* (Figure 5.1, 5. 2). The root extracts were more effective than the bark and leaf extracts (Table 5.1 and Figure 5.1, 5. 2, 5. 3, 5.4). The ED_{50} for egg hatch inhibition for the root extracts was 0.28 mg ml^{-1} compared to 0.619 mg ml^{-1} for the leaf, and respectively 0.284 mg ml^{-1} and 0.724 mg ml^{-1} for the larval development. Thiabendazole (TBZ) was more effective than the extracts, but the

compounds in the extracts have more less similar dose-response profiles, Figures 5. 3 and 5. 4. The *P. africanum* extracts appeared to inhibit egg hatching more than larval development, Figure 5.1, 5. 2.

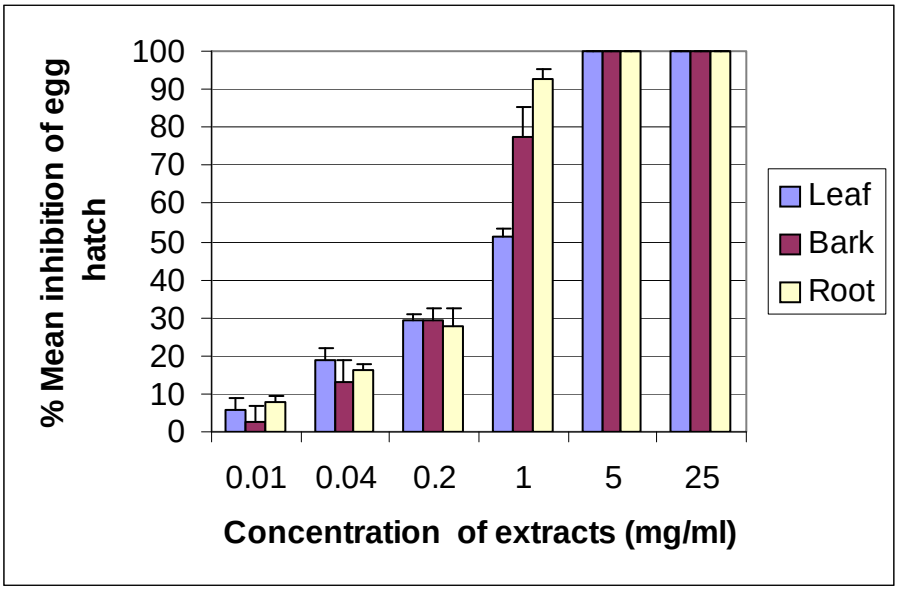

Figure 5.1: Percent mean inhibition of egg hatch of *T. colubriformis* by leaf, bark and root extracts of *P. africanum*

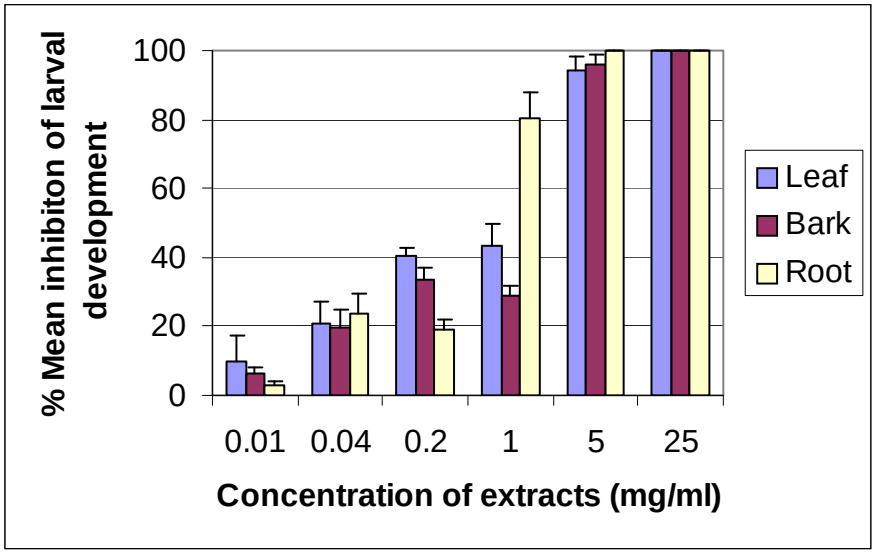

Figure 5.2: Percent mean inhibition of larval development (L_1 to L_3) of *T. colubriformis* by leaf, bark and root extracts of *P.africanum.*

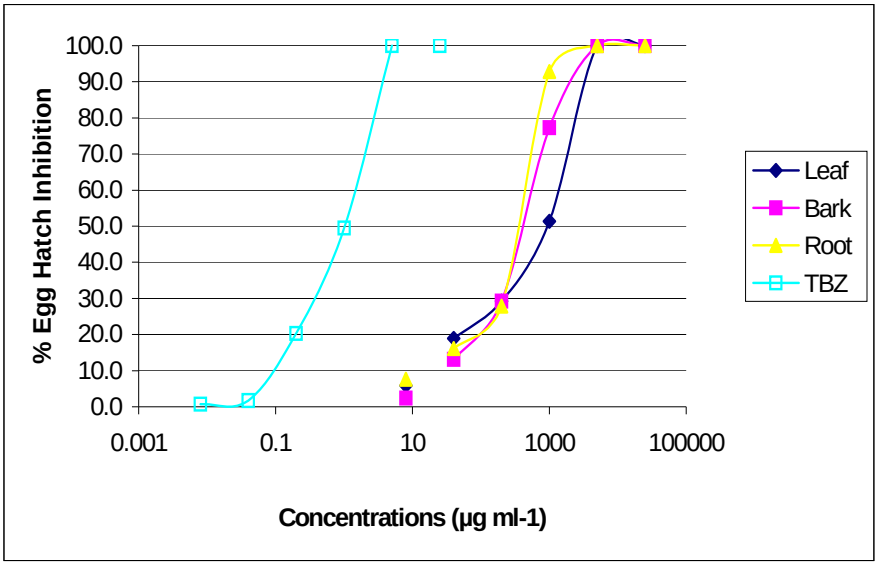

Figure 5.3: Dose-response profile for egg hatch inhibition of *T. colubriformis* by *P. africanum* extracts

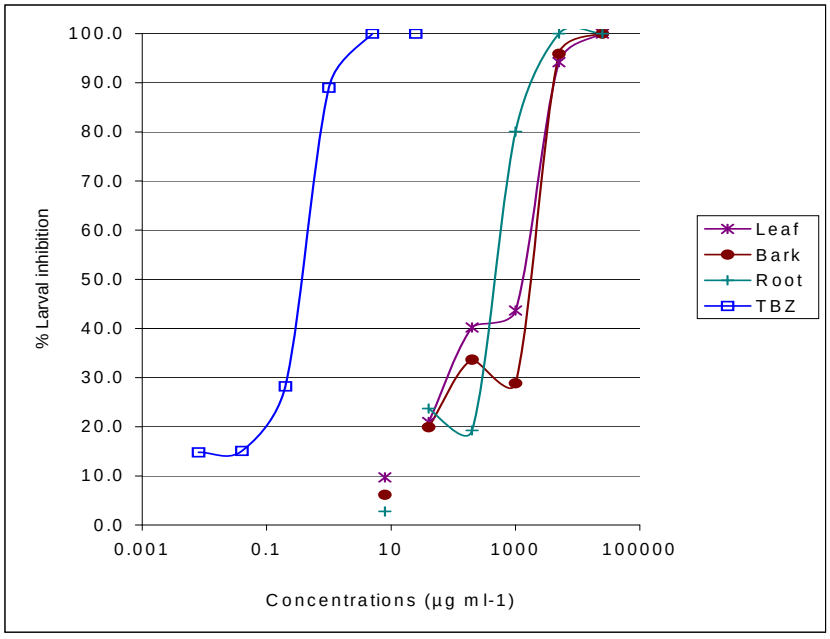

Figure 5.4: Dose-response profile for inhibition of larval development (L_1 to L_3) of *T. colubriformis* by *P. africanum* extracts

5. 4. Discussion and conclusion

The objective of the study was to establish the effects of *P. africanum* extracts on egg hatching, larval viability and development (L_1 to L_3) of *T. colubriformis*. An analogous method has been used for the test of ovicidal and larvicidal effects of plant extracts (Lorimer, *et al* 1996; Molan *et al*, 2003) all, in effect, modifications of the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P) methods for the detection of anthelmintic resistance in nematodes of veterinary importance (Coles *et al*, 1992). Acetone was selected as a suitable extractant as it extracts compounds of a wide polarity range, is miscible with organic and aqueous solvents, and is non-toxic to test organisms (Eloff, 1998), and indeed organic solvents extract more plant material than water. Besides, acetone extracts the largest amounts of compounds from *P. africanum* compared to ethanol, dichloromethane and hexane (Bizimenyera *et al*, 2005).

The *in vitro* model reported in this study demonstrated ovicidal and larvicidal effects of acetone extracts of *P. africanum* against *T. colubriformis*. No work to date on effects of *P. africanum* extracts on parasitic nematodes has been reported. Analogous previous work (Mølgaard *et al*, 2001) using aqueous extracts 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 24-hour incubation. Their work, however, was not extended to cover other classes of helminths.

No eggs and larvae (L_1) of *T. colubriformis* could be observed in wells incubated with all the three extracts at concentrations of 5 and 25 mg ml^{-1}, as they were completely lysed. The ovicidal and larvicidal effects were not timed as some worms died within 3 minutes (not included in results) of adding the extract, whereas in other cases the worms were weak or moribund after 5 days incubation. The problem of counting each and every egg or larva, compounded with the likely uneven numbers of eggs put in each well, made ANOVA calculations for variance difficult. Hence the non-parametric Kruskal-Wallis test (Hammer *et al*, 2001) was used in the statistical analysis in the present study.

The mechanism of the anthelmintic action is yet to be determined. Tannins in plant extracts have anthelmintic activity on their own attributed to physical astringent action on helminths (Athanasiadou *et al*, 2001; Molan *et al*, 2000). The present study showed the ovicidal and larvicidal action of the leaf, bark and root extracts were nearly similar, yet the polyphenol (most polyphenols are tannins) content of the leaves of *P. africanum* is much lower than the root and bark (Bizimenyera *et al*, 2005). The extracts with tannins removed exhibited slightly less activity than the crude extracts (results not shown). Likewise compounds isolated from the extracts had less activity than the crude extract (results not shown). Although the tannin

effect of the extracts at the 5 and 25 mg ml^{-1} concentrations cannot be ruled out, the compounds in the extracts may be acting singly or in synergy for anthelmintic action. The mode of action of the anthelmintic compounds could be similar to that of thiabendazole, judging from the dose-response curves, but more work needs to done for rational conclusions.

The root of *P. africanum* was shown to be more effective than the bark and leaf. Most of the recorded ethnomedical use of the plant employ root and bark extracts (Watt and Breyer-Brandwijk, 1962; van Wyk and Gericke, 2000; van der Merwe, 2000). The antibacterial and antioxidant activity is higher in the root compared to the leaf and bark (Bizimenyera *et al*, 2005). The presence of bioactive compounds in the root and bark would render easy extractability by polar solvents available to rural users. However, extensive use of the bark and root would not be sustainable due to possible plant overexploitation.

In the *in vitro* model, 1.0 mg ml^{-1} of acetone extract of the root inhibits egg hatching by 92.8 % and larval development by 80.1 %. The activity of *P. africanum* at concentrations of 0.2-1 mg ml^{-1} is comparable to the range of 0.2-1.0 mg ml^{-1} reported by other investigators (Akhtar *et al*, 2000; Hördegen *et al*, 2003), in regard to anthelmintic action of plant extracts. Efficacy at 1.0 mg ml^{-1}, whereas significant for extracts is still low compared to the same effect with thiabendazole at 1µg ml^{-1}. This would imply that the activity of the extract is a hundred times plus lower than the activity of thiabendazole. The activity of thiabendazole used in this work at 1µg ml^{-1} suggest the *T. colubriformis* strain used was not resistant as this is the normal plasma concentration of the drug (Prichard, 1978) normally administered at 44 mg kg^{-1} body weight. Hence, if there were no pharmacokinetic differences with thiabendazole, about 44g of the crude extract of *P. africanum* would probably treat an adult sheep infected with *T. colubriformis*. Hence improved methods of extraction to get an anthelmintic rich extract need to be developed.

The ovicidal and larvicidal action of *P. africanum* extracts on eggs and larval (L_1) forms of *T. colubriformis* may not automatically imply that the extracts have similar action on the adult parasites. The relevance of *in vitro* studies to *in vivo* efficacy, in regard to anthelmintic activity, is greatly influenced by the differences in the physiology and the bioavailability of plant preparations within animal hosts (Githiori *et al*, 2005). Hence a conclusive answer can only be made following an appropriate in vivo study. However, if the effects shown *in vitro* could apply *in vivo*, administration of extracts to animals infested with the adult worms would be followed by reduction in faecal egg counts and therefore lowered environment or pasture contamination. This may be beneficial in the control of the parasite, as its L_3 forms are known to have high capacity to survive in adverse weather conditions (Urquhart *et al*, 1996). The anthelmintic activity demonstrated by *P.*

africanum extracts against *T. colubriformis* could as well be applicable to other helminth types; for instance the extracts have already been shown to have effects on cestodes (Mølgaard *et al*, 2001).

The traditional use of the *P. africanum* extracts against diarrhoea, dysentery and unthriftness, may also be due to anthelmintic activity, as these signs are consistent with parasitic gastroenteritis. The anthelmintic activity of *P. africanum* extracts, in addition to the antibacterial, antioxidant and anti-HIV activities, further support the traditional use of the plant. Research work is ongoing for determining better methods of plant extraction, elucidation of the chemical structure of the compounds isolated, and for *in vivo* tests in suitable target livestock. This work may lead, not only to possible isolation of novel anthelmintics from the plant, but also to identification of better methods of plant extraction which are readily adaptable for use by rural communities against helminthosis.

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