CHAPTER 3

RATIONALE FOR USING PELTOPHORUM AFRICANUM (FABACEAE) EXTRACTS IN VETERINARY MEDICINE

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Abstract

Peltophorum africanum (Fabaceae) is a deciduous tree widespread in southern Africa. The plant has many ethnomedical and ethnoveterinary uses. The roots and bark decoctions are used to treat diarrhoea, dysentery, sore throat, wounds, back and joint pains, HIV-AIDS, venereal diseases and infertility. Pastoralists and rural farmers use the root and bark extracts to treat diarrhoea, dysentery, infertility, and to promote well-being and resistance to diseases in cattle. To evaluate these ethnobotanical leads, dried leaves, stem bark and root bark were extracted with ethanol, acetone, dichloromethane and hexane. Polyphenols in the extract were determined by the Folin-Ciocalteu method with gallic acid as standard. Qualitative antioxidant activity was screened by spraying thin layer chromatograms (TLC) of the extracts with 0.2% 1, 1-diphenyl-2-picryl hydrazyl (DPPH), and quantified with Trolox equivalent antioxidant capacity (TEAC) assay. Minimum inhibitory concentration (MIC) and total antibacterial activity (TAA) were determined by serial microplate dilution for *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*, with gentamicin as standard and tetrazolium violet as growth indicator. Acetone and ethanol extracted the largest quantity of material. Polyphenols concentration was 49.2% in acetone extract of the root and 3.8% in dichloromethane extract of the leaf. Antioxidant activity of at least five antioxidant compounds as measured by TEAC ranged from 1.34 (ethanol extract of the root) to 0.01 (hexane extract of the leaf). The total antibacterial activity (volume to which active compounds present in 1 g plant material can be diluted and still inhibit bacterial growth) was 1263 ml/g for ethanol extract of the root against *S. aureus* , and 800 ml/g for acetone extract of the root against *P. aeruginosa*. There was substantial activity against both Gram-positive and Gram-negative bacteria, with MIC values of 0.08 mg/ml for *S. aureus* and 0.16 mg/ml for *P. aeruginosa*. There is therefore a rationale for the traditional use of root and bark of *P. africanum* in treating infection related diseases.

Key words: antioxidant, antibacterial, ethnoveterinary, extracts, and *Peltophorum africanum*

3.1 Introduction

Phytotherapy, the treatment and prevention of disease using medicinal plants, is an ancient tradition that has existed with human habitations. About 80% of the world's people still depend on the traditional healthcare practices using herbs. This is so mainly in rural communities in the developing world where

modern drugs may be unaffordable or inaccessible. Disease concepts are largely similar in humans and animals; in many traditional systems, healers of people are often called upon to treat animals and vice versa [28]. Healers frequently use the same herbs, compounds or manipulative techniques. Many pharmacologically active compounds have been discovered following ethnobotanical leads [4, 11]. As tropical forests still present a great storehouse of medicinal genetic resources, the search for compounds with novel bioactivity from plants continues [32].

Peltophorum africanum (Sond), commonly called 'weeping wattle' or 'huilboom', is a member of the Fabaceae. It is a deciduous tree growing up to 15m high with a wide canopy that occurs widely in medium to low altitudes in wooded grassland areas of southern Africa [24]. Whereas the genus is found throughout the tropics, *P. africanum* is the only member of the genus in southern Africa. The plant has many traditional medicinal uses in humans and animals. The roots and bark are used to treat sore throat, wounds, diarrhoea, dysentery, helminthosis, abdominal pains, ascites, back and joint pains, HIV-AIDS, venereal diseases, infertility, colic and eye infections[12, 31, 34]. Pastoralists and rural farmers use the root and bark to treat diarrhoea, dysentery, and infertility in cattle and to promote well-being and resistance to disease [30, 34]. Bark from *P. africanum* was identified as one of the most important products sold in informal medicinal plant markets in Pretoria [17].

However, bioactive compounds from *P. africanum* have not been widely studied. Evans *et al*[10] isolated a sulphate ester of trans-4-hydroxypipecolic acid from the seed. Several condensed flavonoids, a novel cyanomaclurin analogue [1], profisetinidin-type 4-arylflavan-3-ols and related δ-lactones[2] were found in the heartwood. Mebe and Makuhunga[19] isolated new compounds (bergenin, norbergenin and 11-0(E)-p-coumaroylbergenin from ethanol extracts of the bark. Khattab and Nasser [14] isolated coumarins from the leaves.The chemical structures of the novel compounds isolated were elucidated but their biological activities require more study. Leaf extracts have beta-adrenergic activity on the rabbit jejunum, an effect that was blocked by propranolol [20], and anticestodal activity [21]. Bark acetone extracts of *P. africanum* had MIC values of 0.02 to 0.08 mg/ml towards *Staphylococcus aureus* [17].

Utilisation of the bark and root are destructive practices that may lead to the plant extinction. Qualitative and quantitative investigation of the bioactive compounds present in the leaves, bark and root merits further study, to determine if there is a rationale in the traditional use of the plant by rural farmers and whether leaves may be used. Suitable methods of extraction of bioactive compounds, easily adapted to rural communities, could be developed for sustainable use of *P africanum* extracts in primary health care practices.

3.2 Materials and Methods

3.2 .1 Collection, preparation and storage of plant material

Leaves, stem bark and root bark (referred to as leaf, bark and root in this presentation) were collected in May 2003 from a mature tree growing naturally at the campus of the Faculty of Veterinary Sciences at Onderstepoort. A voucher specimen (PM 001) is stored in the Medicinal Plant Herbarium, Department of Paraclinical Sciences, University of Pretoria. The plant material was dried in the shade at ambient temperature and ground to fine powder in a Macsalab mill (Model 200 LAB), Eriez®, Bramley. The powdered material was separately stored in the dark in tightly closed glass bottles before analyses.

3.2.2 Extraction

Four solvents with varying polarities [13] were selected to extract a diversity of compounds from the plant material i.e. technical grade ethanol, acetone, dichloromethane and hexane (Merck®). One gram of the leaf, bark and root was extracted in 10 ml of the respective solvent in a centrifuge tube by vigorously shaking on an orbital shaker (Labotec®, model 202, South Africa) for 10 minutes. After centrifugation for 10 minutes, the extract was decanted into pre-weighed glass vials. The process was repeated three times. The solvent was evaporated in a stream of air at room temperature overnight to determine the mass extracted [5].

3.2.3 Chromatography

Dried extracts were re-dissolved in the corresponding extraction solvent to yield a 10-mg/ml solution. An aliquot of 10 µl of each solution (i.e. 100 µg) was applied on thin layer chromatography (TLC) plates (Merck®, silica gel 60). Four solvent systems were used as eluents: benzene/ethanol/ammonium hydroxide (18/2/0.2) (BEA); chloroform/ethyl acetate/formic acid (10/8/2) (CEF); formic acid/acetic acid/water/ethyl acetate (3/2/30/70) (FAWE); and ethyl acetate/methanol/water (10/1.35/1) (EMW).
Separated compounds were inspected and marked under visible and ultraviolet light (255 and 360 nm Camac Universal UV lamp). The TLC plates were subsequently sprayed with vanillin spray reagent [0.1 g vanillin (Sigma) in 28 ml methanol to which 1 ml of concentrated sulphuric acid was carefully added]. Corresponding plates were sprayed with p-anisaldehyde spraying agent [1 ml p-anisaldehyde (Sigma) in 18 ml ethanol to which 1 ml concentrated sulphuric acid was carefully added]. Thereafter, the plates were heated at 100˚C for a few minutes to optimal colour development.

3.2.4 Polyphenols

Polyphenols were quantified by the Folin-Ciocalteu method, with gallic acid as a standard [27].

3.2.5 Antioxidant screening

1, 1-diphenyl-2-picryl hydrazyl (DPPH) is a purple stable free radical. Reaction of DPPH with an antioxidant results in discoloration of the free radical. The TLC plates were uniformly sprayed with 0.2% DPPH in methanol. Antioxidant activity is detected on the chromatogram when the initially purple DPPH background turns yellow in bands where an antioxidant is present [29].

Quantification of antioxidant activity was by the Trolox equivalent antioxidant capacity (TEAC) assay. The decolourising assay method [26] was used with minor modifications. Free radical was preformed by reacting potassium persulphate (Sigma) with 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Sigma) and was stored at 4^0C before use. The free radical was diluted with absolute ethanol (Merck) to an absorbance of 0.7 ± 0.02 at 734 nm (Beckman). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid) (Fluka), was prepared fresh in ethanol solution and used as the antioxidant standard. Initially, four different concentrations of the Trolox and extracts were made. One ml of ABTS was added to 10 μl (in quadruplicate) of each concentration of Trolox and extracts. Only the solutions that gave absorbency of between 20% and 80% of the initial absorbency were considered. The TEAC values were measured after 6 minutes. If an extract had equivalent antioxidant activity to Trolox (a synthetic water soluble vitamin E analogue), its TEAC value would be 1 and if the extract was more active its TEAC value would be greater than 1. Re *et al.*[26] used equivalent mM concentrations of different standards to obtain their results. With unknown compounds in a plant extract this is not possible and we used equivalent mg/ml concentrations. By bringing the molecular mass into consideration, we can convert the published data [26] to data comparable to our results.

3.2.6 Antibacterial screening

We used acetone to dissolve dried extracts to 10 mg/ml due to the low toxicity of acetone for the test organisms[5]. A modification of the bioautography procedure described by Begue and Kline [3] was used. TLC plates were sprayed with concentrated broth culture suspensions of actively growing cells of *S. aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Enterococcus faecalis* (ATCC 29219). These are the strains recommended by the National Committee for Clinical Laboratory Standards [22]. Cultures grown overnight were centrifuged and the pellet was resuspended with fresh broth in about 50% of the original volume to yield a high concentration of the bacteria. The numbers of bacterial cells in the overgrown Muller-Hinton broth cultures as quantified by a modified serial dilution technique[16] ranged from 28-50 x 10^{16} colony forming units per millilitre (cfu/ml). The plates were incubated at 38˚C and 100% relative humidity overnight. The plates were then sprayed with 2 mg/ml p-iodonitrotetrazolium (INT). Clear zones on the chromatogram indicated inhibition of bacterial growth after further incubation at 37^0C for 30-120 minutes.

A microplate serial dilution method [6] was used to determine the minimum inhibitory concentrations (MIC) for the test bacteria, i.e. the lowest concentrations of the extract that inhibit bacterial growth. INT was used as growth indicator dye and gentamicin (50 µg/ml) was the positive control.

Total antibacterial activity (TAA) equivalent (ml/g) of the plant parts was determined by dividing the quantity of material (mg) extracted from 1 g of dried plant material by the MIC value (mg/ml) [8]. The TAA value is the volume in ml to which the extract obtained from 1 g of plant material can be diluted and still inhibits bacterial growth [8].

3.3 Results

Whereas the non-polar extractants extracted a small quantity of compounds from root and bark, ethanol and acetone extracted the largest quantity in total from all plant parts (Figure 3. 1).
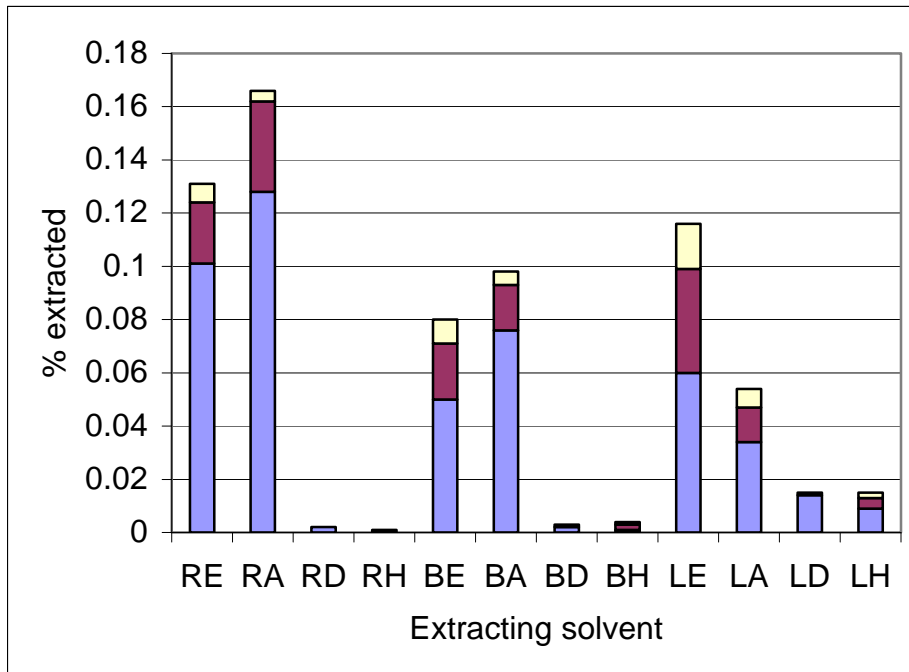
Figure 3.1: Extraction efficiency based on original dry mass of ethanol (E), acetone (A), hexane (H), and dichloromethane (D) from root (R), bark (B), and leaf (L) of P. *africanum*. First extracted at bottom, second in middle, third at top.
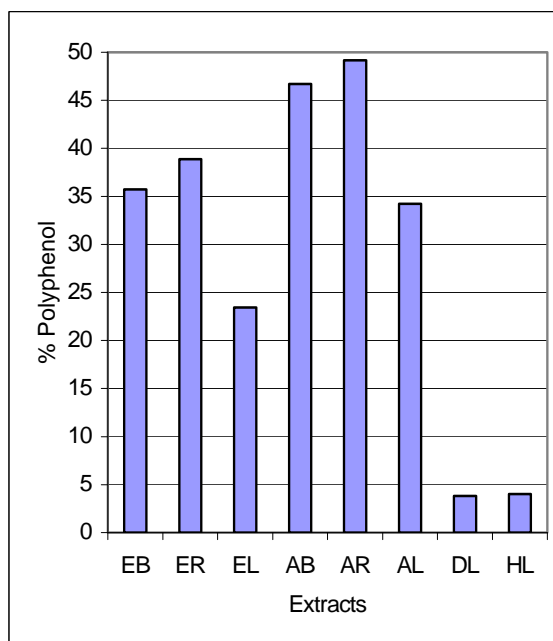

Figure 3.2: Percentage polyphenols in bark (B) , root (R) and leaf (L) extracts of ethanol (E), acetone (A), dichlomethane (D) and hexane (H)

There was hardly any extract obtained at the third extraction with hexane and dichloromethane, and only a small quantity with acetone and ethanol, indicating that extraction was exhaustive.

Polyphenol concentrations were high (more than 20%) in the acetone and ethanol extracts of the root, bark and leaves (Figure 3. 2).

There were 5-6 antioxidant compounds present in acetone and ethanol extracts of especially root and bark, and the highest antioxidant activity was in the ethanol and acetone extracts of the root and the ethanol extract of the bark (Figure 3. 3, 3. 4). In all the acetone and ethanol extracts, a compound with an R_f value of c. 0.48 (similar to that of catechin) in the EMW TLC system was present.
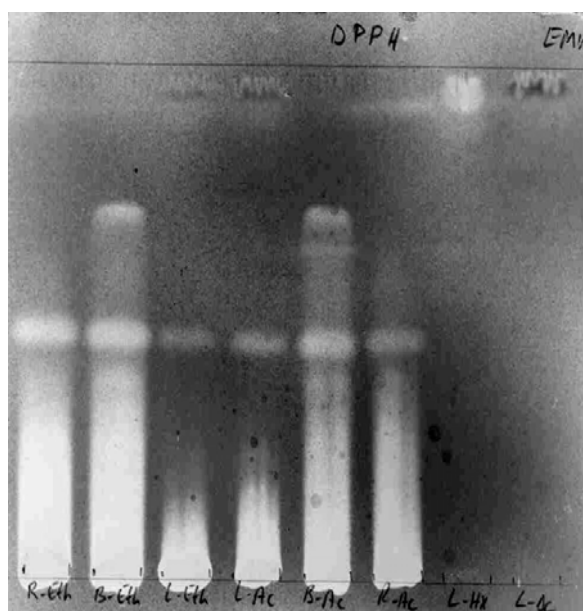

Figure 3.3: Chromatogram of 100 µg of bark (B), root (R) and leaf (L) extracted by
Ethanol (Eth), Acetone (Ac), Hexane (Hx) and Dichloromethane (Dc) separated
by EMW and sprayed with DPPH reagent. Light areas indicate anti-oxidant activity.
Lanes from left to right R-Eth, B-Eth, L-Eth, L-Ac, B-Ac, R-Ac, L-Hx and L-Dc
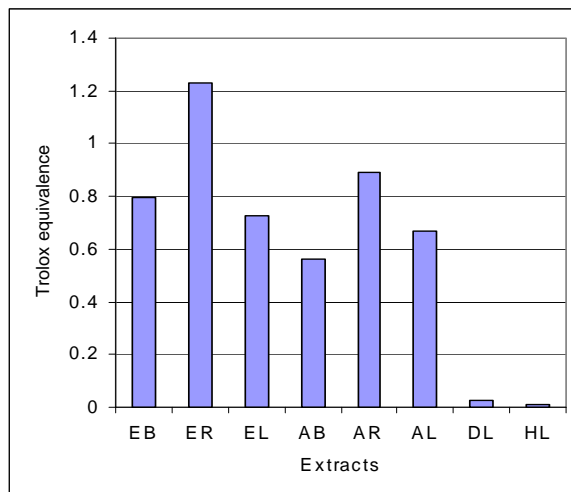
Figure 3.4: Trolox equivalent antioxidant capacity (TEAC) values of bark (B), root (R), and leaf (L) extracted with ethanol (E), acetone (A), dichloromethane (D) and hexane (H)

Table 3. 1: Minimum inhibitory concentration (MIC) values of the bark (B), root (R) and leaf (L) extracts of ethanol (E), acetone (A), dichloromethane (D) and hexane (H) against four bacteria

	S. aureus	*E. faecalis*	*P. aeruginosa*	*E. coli*
EB	0.32	0.32	0.63	0.63
AB	0.16	0.16	0.16	0.63
ER	0.08	0.32	0.16	0.63
AR	0.16	0.16	0.16	0.63
EL	0.32	0.32	0.16	0.63
AL	0.16	0.16	0.16	1.25
DL	0.08	2.5	2.5	2.5
HL	2.5	2.5	2.5	2.5

Table 3.2: Total antibacterial activity (volume to which active compounds present in 1 g of plant material can be diluted and still inhibit growth) values (ml/g) of ethanol (E), acetone (A), dichloromethane (D), and hexane (H) extracts of the bark (B), root (R) and leaf (L) tested on four bacteria

	Mass (mg) extracted from 1 g	*S. aureus*	*E. faecalis*	*P. aeruginosa*	*E. coli*
EB	50	156	156	79	79
AB	76	475	475	475	121
ER	101	1263	316	631	160
AR	128	800	800	800	203
EL	60	188	188	375	95
AL	34	213	213	213	27
DL	15	188	6	6	6
HL	15	6	6	6	6

The antibacterial compounds in root and bark were highly polar and hardly moved from the origin when EMW was used with *S. aureus* as test organism (results not shown). In all leaf extracts there were two or three non-polar antibacterial compounds present with R_f values of 0.74, 0.85 and 0.92 (EMW system). These values corresponded to some of the R_f values of antioxidant compounds present in root, bark and leaves (Figure 3.3).

The minimum inhibitory concentration (MIC) values of the extracts varied from 0.08 to 2.5 mg/ml (Table 3. 1). There was substantial activity against both Gram-positive and Gram-negative bacteria, with an MIC value 0.08 mg/ml for *S. aureus* and 0.16 mg/ml for *P. aeruginosa*. The ethanol extract of the root had the same MIC value (0.08 mg/ml) as the dichloromethane extract of the leaf against *S. aureus*. *E. coli* was the most resistant against the extracts.

The total antibacterial activity values obtained for the ethanol and acetone extracts of the root and barks were high, varying from 79 to 1263 ml/g (Table 3.2).

3.4 Discussion

Pharmacologically active plant derived compounds have been discovered by following the ethnobotanical use of plants [11]. The ability to isolate compounds depends on the ability to screen using the 'seek and ye shall find' as the operative principle[4]. Use of organic solvents is the most popular method for getting plant extracts [13]. There was hardly any extract obtained at the third extraction with hexane and dichloromethane, and only a small quantity with acetone and ethanol. This would imply that the ground particles were small and that the shaking and separation of phases by centrifugation was efficient. Acetone and ethanol extracted the largest quantity in total, from all plant parts. Acetone has already been shown to be a good extractant for a diversity of plant compounds [5]. Extractable compounds from the root and bark were mainly polar, as few compounds were extracted from the root and bark by non-polar solvents. This indicates that *P. africanum* does not have a high concentration of non-polar compounds in the bark and root. This conclusion is supported by the TLC and bioautography results where root and bark extracts had low R_f values even in polar solvent systems.

Our results show that *P. africanum* had several antioxidant compounds especially in the root and bark (Figure 3. 3). Antioxidants in plants prevent damage caused by free radicals to DNA and other molecules, reduce inflammation, and promote good health and resistance to disease [15, 18, 25]. In the qualitative antioxidant analysis most of the compounds had a high polarity as would be expected of polyphenols or tannins. The antioxidant activity in *P. africanum* may be due to polyphenols or tannins as these compounds have been found in its heartwood [1, 2, 14, 19]. The Follin-Ciocalteu method does not differentiate between tannins and many other phenolics that are not tannins[27]. The TEAC values of 1.32, 0.95 and 0.83 for the ethanol and acetone extracts of the root and acetone extracts of the bark respectively, compare favourably with the value of 1.49 for pure Vitamin C found by Re *et al*[26].

Presence of antibacterial compounds in the root, bark and leaf was established confirming earlier work[17, 23]. In the current work, the best MIC values of 0.08 mg/ml were obtained for ethanol extracts of the root and dichloromethane extracts of the leaf against *S. aureus*. Manana[17] found the acetone extracts of the bark to have MIC values of 0.02-0.08 mg/ml against *S. aureus*. Obi *et al*[23] using disc diffusion methods demonstrated antibacterial action of *P. africanum* extracts against some Gram-negative bacteria (not done for Gram-positive). In the present work, the best values for Gram-negative bacteria were 0.16 mg/ml for acetone and ethanol extracts of the bark and root against *P. aeruginosa*. Though the MIC values show that the extracts have considerable activity against both Gram-positive and Gram-negative bacteria, it is important to calculate the total antibacterial activity (TAA) present in the plant to determine the potential

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value for isolating antibacterial compounds or for using the plant extracts in primary health care [8]. The TAA values of 1263 and 800 ml/g for ethanol and acetone extracts of the root respectively, are much higher than those obtained to date from the members of Combretaceae and Celastraceae [7, 9]. For plant extracts, the TAA value has more applications. As Eloff [8] pointed out, when selecting plants for investigation in rural areas, not only MIC values but also the quantity extracted should be incorporated to calculate the total antibacterial activity. Whereas the MIC values for ethanol extract of the root and dichloromethane extract of the leaf against *S. aureus* were same (0.08 mg/ml) extracts from one gram of plant material could have been diluted to 1263 ml in the former and 188 ml in the latter and still inhibit growth of *S. aureus*. This in effect would imply that much less plant material would have to be extracted by acetone compared to the amount of plant material that would be required with extraction by dichloromethane to achieve same antibacterial activity. This has a bearing on the sustainable use of the plant in rural areas.

The high level of antibacterial and antioxidant activity in acetone and ethanol extracts validates some of the traditional uses of *P. africanum* in people and animals [12,17,30,34], and in particular may be contributing to the successful use of the plant in treating infection-related diseases in cattle by rural farmers in Madikwa area of South Africa[30]. The antibacterial activity in the roots and bark are probably largely due to the tannins[33], due to the high polyphenol content of these extracts and the high polarity of the active compounds based on bioautography results. The compounds in *P. africanum* may be contributing to the use of the plant in treating infection related diseases by rural people, either directly by inhibiting growth of pathogens or indirectly by stimulating the immune system of the host. The high polarity of the bioactive compounds in the root and bark also means that these compounds may be extractable by the polar solvents available to rural users.

This preliminary screening has shown the highest antioxidant and total antibacterial activities were found in the root and bark extracts, factors that may explain some of the uses of the plant in traditional healthcare practices. The antibacterial activity was from polar compounds in the root, and non-polar compounds in the leaf extracts. We intend to isolate and characterize the antioxidant and antibacterial compounds from roots and leaves of plants growing in a variety of regions and climatic conditions in South Africa.

Although *P. africanum* is widely distributed in rural areas, the use of roots and bark is more destructive than use of leaves in the long run. Leaves may have potential for the antibacterial activity in their extracts, but not as a source of antioxidant compounds. For sustainable use of the plant in veterinary medicine and primary health care practices in rural areas, suitable means of leaf extractions may have to be developed and adopted.

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CHAPTER 4

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*

4.1. Introduction

In the tropics and sub-tropics, helminthosis remains one of the most prevalent and economically important parasitoses of domesticated animals (Anon, 1992; Perry and Randolph, 1999). 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 (Chiejina, 2001). Haemonchosis (caused by *Haemonchus contortus*) has been listed among the top 10 most important conditions hampering production of sheep and goats in tropical countries (Anon, 1992; Arosemena *et al*, 1999). The disease is characterised by anaemia, haemorrhagic gastroenteritis, hypoproteinaemia (manifested by oedema or 'bottle jaw'), sudden death or chronic emaciation (Soulsby, 1982; Urquhart *et al*, 1996). Adult *H. contortus* females have high egg-producing capacity, of 5000-15000 eggs per day (Hansen and Perry, 1994). The

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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 (Waller, 1987; Maciel *et al*, 1996; Wolstenholme *et al*, 2004), 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 (van Wyk *et al*, 1999). 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 (Waller, 1997; Danø and Bøgh, 1999; Sanyal, 2001).

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). About 80% of people in the developing world rely on phytomedicine for primary healthcare (Plotkin, 1992; McCorkle *et al*, 1996). Ethnomedicine often does not follow the western paradigms of scientific proof of efficacy; hence the medical and veterinary professionals distrust herbal remedies (Sofowora, 1982; Thompson, 1997). There is need therefore, for scientific validation of efficacy of herbal medicines before their acceptance and use.

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 (Watt and Breyer-Brandwijk, 1962; Van de Merwe, 2000; Van Wyk and Gericke, 2000). Phytochemists have found several condensed flavonoids, a novel cyanomaclurin analogue (Bam *et al*, 1988), profisetinidin-type-4-aryflavan-3-ols and related δ-lactones (Bam *et al*, 1990) in the heartwood. New compounds (bergenin, norbergenin and 11-0(E)-p-courmaroylbergenin) were isolated from ethanol extracts of the bark (Mebe and Makuhunga, 1992), and leaves yielded coumarins (Khattab and Nasser, 1998). In vitro antibacterial (Obi *et al*, 2003), antioxidant and antibacterial (Bizimenyera *et al*, 2005) activities, and inhibitory properties against the human immunodeficiency virus (HIV) type 1 reverse

transcriptase and integrase (Bessong *et al*, 2005) 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*.

4.2. Materials and methods

4.2.1 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.

4.2.2 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 work, three grams 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 machine for one hour. 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*.

4.2.3 Egg recovery and preparation

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*, 1992). An analogous method has been used for ovicidal tests of plant extracts (Alawa *et al* 2003; Assis *et al*, 2003). 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 minutes. 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 minutes. 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 hour. 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 was 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 hour of preparation.

4.2.4 Egg hatch inhibition (EH) assay

About 100 eggs in every 200µl of the egg suspension were 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 ^{0}C) for 48 hours. 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.

4.2.5 Larval development (LD) inhibition assay

The same egg suspension and 48 well microplates as in 4.2.4 above were used. Into each well 170µl 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 (Hubert and Kerboeuf, 1992). Then 10µl 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 hours. Once the larvae had hatched, 250 µl of the test extracts at the same concentrations mentioned in 4.2.4 above and thiabendazole control concentrations was added to each plate (negative control plates had 250µl of distilled water added). As in 4.2.4 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.

4.2.6 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*, 1992): -

(%) 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 case 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 (as cited by Hammer *et al*, 2001), instead of ANOVA variance calculations, was used in the statistical analysis; the numbers of eggs were only estimates of 100 per well (see 4.2.3).

The Environment Protection Agency (EPA) Probit Analysis Programme was used to calculate the dose that was effective against 50% of the eggs or larvae (ED_{50}) in the different treatments.

4.3. 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 mg ml^{-1}, Table 4.1.

Table 4.1: Percent 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 4.2: Kruskal-Wallis and effective dose$_{50}$ (ED$_{50}$) values of extracts of *P. africanum* against *H. contortus*

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	0.00773	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

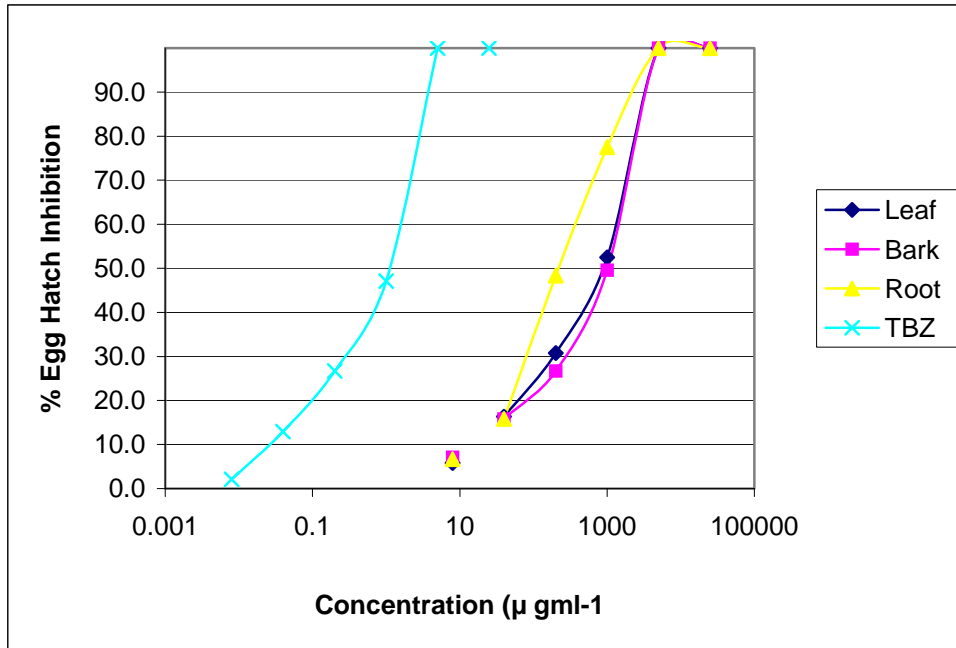** Thiabendazole values are in µg ml^{-1}

Figure 4.1: Dose-response egg hatch inhibition of *H. contortus* by leaf, bark and root extracts of *P. africanum*
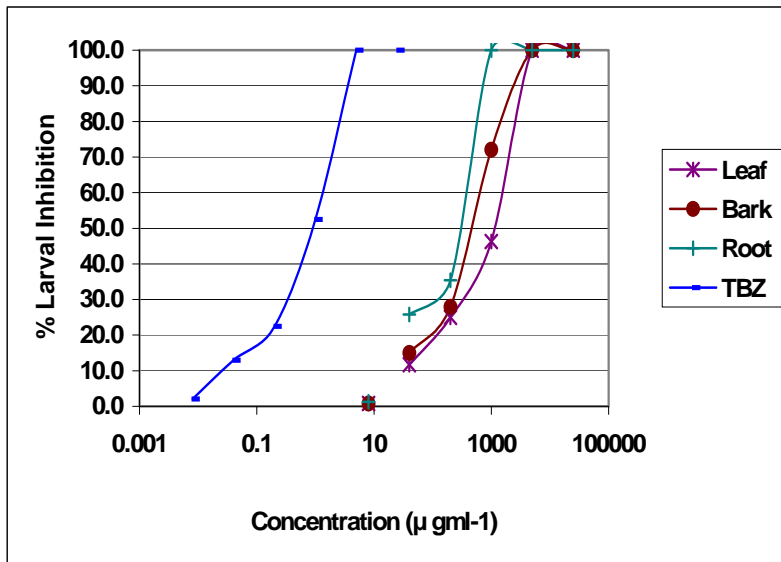

Figure 4.2: Dose-response larval development inhibitions of *H. contortus* by leaf, bark and root extracts of *P. africanum*

Increasing the concentration of the extracts caused a dose dependent significant (p<0.05) increase in inhibition of egg hatching and larval development (Figures 4.1, 4. 2 and Table 4. 2). The root extracts were more effective than the bark and leaf (Table 4.1, 4. 2).

Table 4.3: 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 larvae$_1$ dead

√= larvae$_1$ alive

Note: The larvae in concentrations 5 and 25 mg/ml were completely lysed

* Thiabendazole values are in µg ml^{-1}

The eggs and larvae (L_1) 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 (Table 4.1, 4. 3). It is interesting that the plant extracts had a similar dose response curve as thiabendazole at a thousand-fold higher concentration (Figure 4.1, 4. 2).

4.4 Discusion and conclusion

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 (Eloff, 1998). 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.* 2005). The egg hatch assay as recommended for determining the anthelmintic

resistance (Coles *et al*, 1992), has been modified to test ovicidal effects of plant extracts (Lorimer *et al*, 1996; Assis *et al*, 2003).

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 (L_1 to L_3). The inhibition of larval development was most probably due to larval (L_1) mortality. This is the first report of the *P. africanum* extracts on *H. contortus*. Earlier work (Mølgaard *et al*, 2001) 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 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, 2002).

There was a problem of counting every egg or larva as the extract at concentrations above 5 mg ml^{-1} completely lyses eggs and L_1 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*, 2001), 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*, 2005), 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*, 2004) had no effect on faecal egg counts in lambs infected with *H. contortus* and fed water extracts of the plants. Assis *et al* (2003) 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*, 2003). 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*, 2000; Hördegen *et al*, 2003). 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*, 2005). 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 (Niezen *et al*, 1998; Akhtar *et al*, 2000; Hördegen *et al*, 2003; Athanasiadou *et al*, 2005). 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 well, 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.

Acknowledgements

4.5 References

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