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The potential role of antibacterial, antioxidant and antiparasitic activity of *Peltophorum africanum* Sond. (Fabaceae) extracts in ethnoveterinary medicine

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Promoter: Prof Gerald E Swan

Co-promoter: Prof Jacobus N Eloff

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Peltophorum africanum (From Venter & Venter (2002), Making the most of Indigenous Trees)

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Declaration

The experimental material and results described in this thesis is my original work (except where the input of others is acknowledged), conducted in the Phytomedicine Programme , Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, and has not been submitted in any other form to any other University or academic institution. I declare the above statement to be true.

Signed: ……

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Date: ……..

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List of abbreviations

AOX	Antioxidants
TAA	Total antibacterial activity
MIC	Minimum inhibitory concentration
DPPH	1,1-diphenyl-2-picryl hydrazyl
ANOVA	Analysis of variance
WAAVP	World Association for the Advancement of Veterinary Parasitology
TLC	Thin layer chromatography
FAWE	Formic acid: acetic acid: water: ethyl acetate (3:2:30:70)
BEA	Benzene: ethanol: ammonium hydroxide (18:2:0.2)
CEF	Chloroform: ethyl acetate: formic acid (18:8:2)
EMW	Ethyl acetate: methanol : water (10:1.35:1)
INT	p-iodonitrotetrazolium
NCCLS	National Committee for Clinical Laboratory Standards
DMSO	Dimethyl sulfoxide
UPBRC	University of Pretoria Biomedical Research Centre
MTT	3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide
TEAC	Trolox equivalent antioxidant capacity
SEM	Standard error of mean
EPA	Environment Protection Agency
ABTS	2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)

Publications

Full articles prepared from the thesis:

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Summary

There is an increasing interest in ethnomedical and ethnoveterinary practices, especially as it relates to the use of medicinal plants for treating various ailments. As a result, the current trend in government health authorities is to integrate herbal medicine with primary heath care. This arises because nearly 80% of people in the developing world, particularly those from rural communities where modern drugs are unaffordable, inaccessible or, unavailable, depend on phytomedicine for primary healthcare. Despite this, however, most medical and veterinary professionals distrust herbal medicines due to concerns of scientific evidence of efficacy and safety. Hence, there is need for their validation, before herbal medicines gain wider acceptance and use. Traditional healers and rural farmers use extracts of *Peltophorum africanum* (a medicinal plant wide-spread in southern Africa and other tropical regions), to treat diarrhoea, helminths and abdominal parasites, dysentery, HIV-AIDS, acute and chronic pain, anxiety and depression, infertility, and to promote well-being and resistance to diseases.

To evaluate these ethnobotanical leads, dried leaves, bark and root from mature *P. africanum* (Fabaceae) trees were extracted with acetone, ethanol, dichloromethane and hexane. Chromatograms were made on silica gel plates. Thin layer chromatograms (TLC) were sprayed with 0.2% 2, 2-diphenyl-1-picryl hydrazyl (DPPH) for qualitative screening for antioxidants. Quantification of antioxidant activity was done in comparison with L-ascorbic acid and Trolox (6-hydroxy-2, 5, 7, 8-tetranethylchromane-2-carboxylic acid). With regard to the extracts, minimum inhibitory concentrations (MIC) were determined for *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*. The total antibacterial activity (TAA), signifying the volume to which active compounds present in 1 g of plant material can be diluted and still inhibit bacterial growth, was also determined. *In vitro* anthelmintic activity was evaluated by effects of acetone extracts on the egg hatching and larval development of parasitic nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis*. The eggs and larvae of the two parasites were incubated in various concentrations of the leaf, bark and root extracts for two and five days respectively. Furthermore the efficacies of the acetone extracts were tested on lambs artificially induced with *H. contortus* and *T. colubriformis* infections. Toxicity was performed in brine shrimp and MTT assay on Vero monkey kidney cells.

The extracts had substantial activity against both Gram-positive and Gram-negative bacteria, with MIC values of 0.08 mg ml^{-1} for *Staphylococcus aureus* and 0.16 mg ml^{-1} for *Pseudomonas aeruginosa* ; the corresponding TAA values were 1263 and 631 ml g^{-1}. The acetone extracts of

the bark, and root of *P. africanum* had higher antioxidant activity than L-ascorbic acid (Vitamin-C) and Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid), a synthetic vitamin-E analogue, and much higher than *Ginkgo biloba* extract (EGb 761). The standardized extract of *Ginkgo biloba* (EGb 761) is widely employed for its significant benefit in neurological disorders. The respective EC_{50} for the *P. africanum* root , bark and leaf extracts, L-ascorbic acid, and EGb761 were 3.82 µg ml^{-1}, 4.37 µg ml^{-1}, 6.54 µg ml^{-1}, 5.04 µg ml^{-1}, and 40.72 µg ml^{-1}.

The extracts inhibited egg hatchability and larval development (from L_1 to infective stage L_3) of both *H. contortus* and *T. colubriformis* (both parasitic nematodes of ruminants) at concentrations of 0.2-1.0 mg ml^{-1}. The plant extracts, at concentrations of 5-25 mg ml^{-1} completely lysed larval forms (L_1) and eggs of the nematodes. In all assays, the root extracts had higher antibacterial, antioxidant and anthelmintic activity than the bark and leaf. Although the extracts were safe and non-toxic, the reduction in faecal egg and adult worm counts in lambs infected with *H. contortus* and *T. colubriformis* was not statistically significant (P=0.073).

From the acetone extracts of the root, a brownish crystalline compound, bergenin was isolated. Bergenin was also assayed for toxicity with brine shrimp and Vero monkey kidney cells like the extracts, where the compound was found to be not toxic. In a disc diffusion test, the inhibitory activition of bergenin was determined for the bacteria *E. coli*, *P. aeruginosa*, *Mycobacterium vaccae*, and the fungi *Sporobolomyces salmonicolor* and *Penicillium notatum*. Bergenin had reasonable antimicrobial activity against *S. salmonicolor*, moderate activity against *M. vaccae*, *E. coli* and *P. aeruginosa* , but non inhibitory against *P. notatum*.

P. africanum extracts have therefore, potential for treatment of infection-related diseases by either directly inhibiting bacterial growth or by stimulating the immune system of the host. The traditional use of *P. africanum* concoctions against diarrhoea, dysentery and unthriftness, may be also due to anthelmintic activity as these signs are consistent with parasitic gastroenteritis.

Antioxidants are also important in boosting the immunity, critical in the management of helminthosis. There is ample scientific and empirical evidence supporting the use of plant-derived antioxidants in the control of human immunodeficiency virus (HIV) and neurological diseases. Synergistic activity of plant antioxidants has been proposed as a mechanism by which viral replication and immune cell killing in HIV infection can be inhibited. Antioxidants may have neuro-protective (preventing apoptosis), as well as neuro-regenerative roles. Due to the high antioxidant activity of its extracts, *P. africanum* has prospects in the chemotherapy of

HIV and management or control of neurodegenerative diseases. Thus there is great potential of *P. africanum* extracts in medicine.

Further isolation and bioassay characterization of bioactive compounds from *P. africanum* is recommended as well as refinement of *in vivo* tests in target livestock, or clinical trials. Better methods of plant extraction easily adaptable to rural communities for sustainable exploitation of the tree, may have to be devised especially those using the leaves instead of bark or root.

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CHAPTER 1

INTRODUCTION

1.1 Background

McCorkle (1982) defined ethnoveterinary medicine as the folk or traditional beliefs, knowledge, skills and practices relating to health-care of animals. Although health-care of animals has evolved wherever and whenever people and animals co-exist, ethnoveterinary research has a more recent history than other branches of indigenous knowledge systems; only in the mid 1970's did significant systematic scientific investigation begin on this rich body of knowledge (Schwabe and Kuojok 1981; McCorkle, 1982). Today ethnoveterinary medicine, a systemic study of community animal healthcare and its development applications (Schillhorn van Veen, 2001), is a growing field that brings together a synergistic mix of researchers and practitioners.

The World Health Assembly adopted a resolution urging interested governments to give adequate importance to the utilisation of their traditional systems of medicine as suits their national health systems (Akarele, 1983). Resource-poor people in many countries of the developing world can not afford modern pharmaceutically-derived medicines. As increased food, and income from livestock products hold the greatest promise for increased human well-being throughout the developing world, where herd and kraal animals are raised by the poor (WILRTC, 1978), so research results should be useful for hands-on livestock development and extension with a goal of increased human rather than animal well-being. The process to improve plant and animal health-care and management systems ultimately aim at improvement of human health (McCorkle, 1989).

Most of the art, skills and expertise of traditional healers or practitioners have been passed on from generation to generation almost solely by word of mouth (McCorkle and Mathias – Mundy, 1992; Hirt and M'Pia , 1995). Currently such knowledge is maintained within the older members of the community. Due to migrations, regional conflicts, urbanisation and technological transformation in developing countries, such knowledge may disappear (Hirt and M'Pia, 1995). The risk is that the vast knowledge, skills and expertise gathered over centuries may be lost if not documented. Hence it is crucial to identify and make use of the tropical plant *materia medica*, as ethnic groups disappear and their knowledge disappears with them (Goodland, 1981; Farnsworth and Soejarto, 1985).

A great number of plant-derived therapeutic agents have been discovered following leads provided by indigenous knowledge systems (Farnsworth *et al*., 1985; Farnsworth and Soejarto, 1991; Cox, 1994). Often the process begins as folk knowledge of activity of the plant. The traditional healer uses the plant therapeutically. The healer communicates knowledge of the healing potency to a researcher. The researcher in turn collects and identifies the plant. Making crude extracts follows this. The extracts are tested through bioassays. Bioassay-guided fractionation leads to isolation of an active ingredient or compound. The structure of the compound is determined. In a way, the compound is either used in its native form as a source of direct therapeutic agent, serves as raw material for complex synthetic compounds, or as a taxonomic marker in the search for new compounds. The biblical phraseology 'seek and ye shall find' should be the operative principle (Cox, 1994) as we continue to search and study medicinal plants. The ability to isolate compounds depends on ability to screen (Farnsworth, 1994; Houghton and Raman, 1998). Many modern drugs have been derived directly from plants or these plant derivatives have acted as templates for synthesis of modern pharmaceuticals (Balick, 1994; Soejarto, 1996). Tropical rainforests still present a great storehouse of medical genetic resources that may yield important drugs to treat a number of diseases for which cures are not available (Farnsworth and Soejarto, 1991; Akarele *et al*., 1991), or are too expensive to develop. However, as many of these forests and plants are disappearing at a very terrific rate, conservation measures need to be in place (Cunningham, 1991; Eloff, 1998 a).

Use of medicinal plants in treating diseases is an ancient tradition that has existed with human habitations. Many rural communities in the developing world today, especially where modern drugs are unaffordable or inaccessible, use traditional medicines (Cunningham, 1991; Cunningham and Zondi, 1991). Developed countries have become increasingly interested in traditional or 'alternative' medicine of animals and humans alike (Schillhorn van Veen, 2001). Already, at least 25% of all prescriptions dispensed contain substances of plant origin and, there is a renewed interest in the use of crude plant extract materials for medicament (Farnsworth, 1977). As the focus of medicine shifts from treatment of manifest disease to prevention, herbal medicine (with its four pillars of phytochemistry, phytopharmacy, phytopharmacology and phytotherapy) is coming into focus, being a renaissance of age-old human tradition (Weiss and Fintelmann, 2000). The 'Green' movement in Western society has changed attitudes in the general population who conceive naturally derived substances and extracts as being inherently safer and more desirable than synthetic chemical products, with the net effect of increase in sales of herbal preparations (Houghton and Raman, 1998; Capasso *et al*., 2000).

Despite extensive use of plants as medicines, herbal preparations are not always safe (Capasso *et al*., 2000). For their natural defence against pathogens, and possibly against ingestion by man and animals,

plants ordinarily produce many metabolic chemicals such as saponins, tannins, pyrrolizidine alkaloids, cyanogenetic glycosides. These chemicals render the plants poisonous or toxic when consumed, and there are reports of toxicity and poisoning from medicinal plants usage (Stewart *et al*., 1999; Taylor *et al*., 2003; Fennell *et al*., 2004). As ethnomedicine does not follow western paradigms of scientific proof of efficacy, most medical and veterinary professionals distrust the use of herbs, and know little about them (Sofowora, 1982; Thompson, 1997). There is need therefore, for scientific validation of efficacy and safety of herbal medicines before acceptance and worldwide use.

The isolation and characterisation of bioactive compounds from *P. africanum*, therefore is a step in this direction. The present work was in particular prompted by reports of the use of the *P. africanum* by the Setswana-speaking people, who use root and bark concoctions for treating diarrhoea and boosting resistance to disease in cattle (van der Merwe, 2000), and by Manana (2003), who collected the plant from medicinal plant market vendors in Pretoria, and established antibacterial activity in its bark extracts.

1.2 Hypotheses

- Leaf, root and bark extracts of *P. africanum* have bioactive compounds
- *P. africanum* extracts are safe and have potential value in veterinary medicine

1.3 Aim

To evaluate the potential of *Peltophorum africanum* extracts and isolated compounds in ethnoveterinary medicine

1.4 Objectives

To: -

- Determine the best extractant of bioactive compounds from the leaves, bark and root of *Peltophorum africanum*, and screen for antibacterial and antioxidant activity
- Evaluate the extracts for their activity on *Haemonchus contortus* and *Trichostrongylus colubriformis in vitro.*
- Determine the efficacy of the extracts on *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep
- Isolate and characterise pure compound(s) from extracts

- Evaluate the safety of the extracts for possibly clinical application
- Quantify the antioxidant activity of the extracts, in view of potential role in neurodegenerative disorders

CHAPTER 2

LITERATURE REVIEW

2.1 The antibacterial agents

Although Fleming discovered penicillin in 1929, a major step in antibacterial era begun in 1935 with the discovery of prontosil, the first sulphonamide. Penicillin proved a better antibacterial drug in 1940s when it was used successfully against gonorrhoea, scarlet fever, pneumonia and meningitis (Edwards, 1980). The impact of antimicrobial chemotherapy is so great that it is unlikely that anybody will live out his or her life without receiving some form of antimicrobial drug therapy. In the USA, antimicrobial drugs make up to 33% of the pharmacopoeia (USA Pharmacopoeia, 1990). However, there has been widespread misuse of antibiotics, which in turn, has created much resistance. Bizimenyera (1986), while working on chicks and hatcheries in Kenya found some strains of bacteria resistant to as many as six common antibiotics. The development of bacterial resistance against antibiotics is moving ahead of the antibiotics turnover by pharmaceutical industry triggering fears that we may enter a post-antibiotic era (Berkowitz, 1995; Leggiadro, 1995). Efforts to search for new antibacterial compounds from plants have thus been intensified (Vlietinck *et al*., 1995; Eloff, 1998b, c; Martini and Eloff, 1998; Eloff, 1999a; Eloff, 2000; Katerere *et al*., 2003).

2.2 Antioxidants

The increased incidences of nervous and stress-related diseases may be attributed to a pro-oxidative environment caused by among other things, cigarette smoking, alcohol abuse, air pollutants, ionizing radiation, inflammation, or inadequate or inappropriate nutrition. In a meeting point of scientists, industrial researchers, research users, organizations and associations and others working in and/or affected by research and technology, it was estimated that more than two million new cases of degenerative diseases may be detected every year (Nair *et al*., 2003). Due to prevalence, morbidity, and mortality of the neurodegenerative diseases, they present a significant medical, social and financial burden on the society. There is thus an impelling need for scientists in preventive medicine to work on the prevention of diseases, particularly the chronic degenerative forms without a specific cure. Oxidative stress has been referred to as a double edged sword, for whereas transient levels may activate defense mechanisms in the cell, it often induces some enzymes like cyclooxygenase-2 (COX-2), lipoxygenase (LOX) and inducible nitric acid synthase (iNOS) that generate intermediaries that damage cellular macromolecules including DNA .The damage is made on proteins, lipids, and nucleic acids signaling cascades leading to disruption of ion

homeostasis and modification of the genetic apparatus, with consequence of apoptotic cell death (Sun and Chen, 1998; Singh *et al*., 2004). This oxidative stress is implicated inter alia in the cause of carcinogenetic, inflammatory, infectious, cardiovascular and neurological diseases in man and animals (Nair *et al*., 2003). The brain is, in particular, very sensitive to oxidation stress possibly because of its high lipid content, high aerobic metabolic activity and low catalase activity (Halliwell and Gutteridge, 1985; Cao *et al*., 1988; Floyd and Carney J M, 1992; Gilgun-Sherki *et al*., 2001).

Oxidative stress is the result of an imbalance in the pro-oxidant /antioxidant homeostasis leading to the generation of excess reactive oxygen species (ROS), a collective name that includes superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl (HO), peroxyl (ROO), alkoxy (RO), and nitric oxide (NO) in relation to oxygen as it gets reduced to water (Barnham *et al*., 2004; Singh *et al*., 2004). All aerobic organisms are susceptible to oxidative stress because ROS are produced by mitochondria during the respiration processes (Chance *et al*., 1979). Under normal conditions, the body is equipped with defense mechanisms that scavenge ROS and protect the cells from oxidative damage. Oxidative stress occurs when the detoxifying enzyme processes are overwhelmed, saturated, faulty, or are under conditions of low dietary antioxidant intake, inflammation, aging or exposure to environmental factors such as irradiation or tobacco smoke.

Antioxidants (AOX) are substances that inhibit or delay oxidation of a substrate while present in minute amounts (Halliwell and Gutteridge, 1990; Maxwell, 1995). In nature, AOX are grouped as endogenous or exogenous. The endogenous group includes enzymes (and trace elements as part-of) like superoxidase dismutase (zinc, manganese, and copper), glutathione peroxide (selenium) and catalase, and proteins like albumin, transferrin, ceruloplasmin, metalliothionein and haptoglobin. The most important exogenous AOX are dietary phytochemicals (such as polyphenols, quinines, flavonoids, catechins, coumarins, terpenoids) and the smaller molecules like ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E) and beta-carotene vitamin-E, carotenoids, and supplements (Nair *et al*., 2003). The antioxidant processes occur in cytosol, mitochondria or in plasma (Larson 1988; Namiki *et al*, 1993; Berger, 2005). Though their mode of action is not yet completely elucidated and though clinical trials involving them are still relatively scarce, AOX offer a promising approach in the control or slowing down progression of neurodegenerative disorders such as Alzheimer's, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and ischaemic and haemorrhagic stroke (Maxwell, 1995; Floyd, 1999; Mattson, 2000; Moosmann and Behl, 2002; Nair *et al*., 2003; Berger, 2005). Plant derived antioxidants offer prospects in this regard. To write off antioxidants as potentially harmful, is ultimately keeping a powerful weapon out of the therapeutic arsenal.

Strategies aimed at limiting ROS oxidative stress damage, may slow the progression of neurodegenerative diseases (Halliwell, 2001; Singh *et al*., 2004). Since endogenous AOX defences are not always completely effective, and since exposure to damaging environmental factors is increasing, exogenous AOX will find more of a role in diminishing the cumulative effects of oxidative damage (Gilgun-Sherki *et al*., 2001). Many plants nevertheless have been scientifically proved to be effective in control of acute and chronic nervous disorders. As herbal extracts are a complex mixture of compounds, the active molecules, the mode of action, bioavailability and pharmacokinetics, and toxicity issues become difficult to evaluate. Plant-derived AOX are regarded as effective in controlling the effects of oxidative damage, and hence have had influence in what people eat and drink (Viana *et al*., 1996; Sun *et al*., 2002; Pinder and Sandler, 2004).

2.3 Anthelmintic activity

In the tropics and sub-tropics, helminthosis remains one of the most prevalent and economically important parasitoses of livestock (Perry and Randolph, 1999). Gastrointestinal nematodes are a major constraint to economic productivity of livestock as they constitute 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; Perry *et al*., 2002).

Control of gastrointestinal nematode infections of small ruminants is almost exclusively by use of proprietary anthelmintics. 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). Alternatively, misuse and or widespread intensive use of sometimes poor quality pharmaceutically derived anthelmintics has led to development of 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; Jabbar *et al*., 2006). These constraints have made the reliance on pharmaceutically derived anthelmintics difficult in the management of GI parasitic infections in livestock, necessitating novel alternative methods of helminth control. Hence pastoralists and smallholder farmers have continued with the ethnoveterinary practices of using indigenous plant preparations as livestock dewormers (Danø and Bøgh, 1999). The use of traditional remedies may present a cheaper, sustainable alternative if the plant compounds were effectively validated. In addition, there are concerns about the detrimental effects of the chemical anthelmintics on the environment (Cox, 1999) and consumer concerns over potential drug residues in animal products (Knox, 2000).

In recent times, there has been an 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 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). Attempts to dismiss the validity of medical knowledge gained through centuries of practical experience is regarded as unfair, for if a drug has been used for ages, repeatedly asked for by patients and prescribed by doctors, one must assume that it is effective, even without double–blind studies (Weiss and Fintelmann, 2000).

2.4 *Peltophorum africanum*

2.4.1 General aspects

Peltophorum africanum Sond (weeping wattle; African wattle; mosetha; huilboom) is a member of the Fabaceae. It is a deciduous tree that grows up to 15 m high with a wide canopy, and is widespread in sub-Saharan Africa and other tropical regions (Venter and Venter, 2002; Palgrave, 2002). The bark in old trees is grey brown with longitudinal fissures or grooves. The common name 'weeping wattle' is due to sap-sucking insects (*Ptyelus grossus*) that attach to the branches, and some of the sap drips down wetting the ground. The stem and leaves bear no thorns. The leaves are acacia-like, silver grey and covered with fine hairs, and are bi-pinnate up to the tip. The flowers have yellow petals, and the fruits are flat ellipsoidal pods tapering to both ends. Livestock eat young leaves and pods.

2.4.2 Ethnomedical and ethnoveterinary use

P. africanum is a unique plant in that it is traditionally used to treat, *inter alia*, 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). Pastoralists have traditionally used it in combination with other plants in animal healthcare practices (Cunningham and Zondi, 1991). The root is one of the ingredients in the 'Kgatla doctors' mixture to promote fertility and the well-being of cattle, the prepared medicine being known as 'leswalo' (Watt and Breyer-Brandwijk, 1962). An infusion of the root bark is also used as a tonic for general improvement and resistance to disease in cattle (van der Merwe, 2000). A decoction of the mixture of *P. africanum* and *Sclerocarya birrea* is used to treat diarrhoea and dysentery

in cattle (Watt and Breyer-Brandwijk, 1962; van der Merwe, 2000). From the foregoing, the plant may have antimicrobial, antiparasitic and antioxidant compounds.

2.4.3 Phytochemistry

Several authors have investigated the phytohemistry of *P. africanum*. Evans *et al*., 1985 isolated a sulphate ester of trans-4-hydroxypipecolic acid in the seeds. Several condensed flavanoids, a novel cyanomaclurin analogue (Bam *et al*., 1988), profisetinidin-type 4-arylflavan-3-ols and related δ-lactones (Bam *et al*., 1990) were found in the heartwood. Mebe and Makuhunga (1992) isolated new compounds (bergenin, norbergenin, and 11-0 - (E)-p-coumaroylbergenin) from an ethanol extract of the bark. Many flavonoids and coumarins are known antioxidants (Das and Ramanathan, 1992; Paya *et al*., 1992; Haragushi, 2001). Some antioxidants from plants have been shown to have antimicrobial activity (Miski *et al*., 1983; Haragushi *et al*., 1998). However, there are only few reports of detailed studies on the bioactivity of the compounds from roots, leaves and bark of *P. africanum*.

2.4.4 Biological activity

Mlambo and Munjeri (1988) showed that leaf extracts acted on beta-adrenergic receptors in the rabbit jejunum to reduce its contractions (an effect blocked by propranolol) and concluded that the plant was a potential source of pharmacological agents. Mølgaard *et al*., 2001 demonstrated activity for extracts of the leaf and stem against the cestode, *Hymenolepis dimunita*. Leaf extracts have been shown to have antibacterial activity when tested against some nasocomial pathogens (Manana, 2003). *In vitro* antibacterial (Obi *et al*., 2003; Samie *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* have been reported. Nevertheless, much work remains in the study and characterization of bioactive compounds extracted from *P. africanum*, and their clinical evaluation *in vivo*.

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).

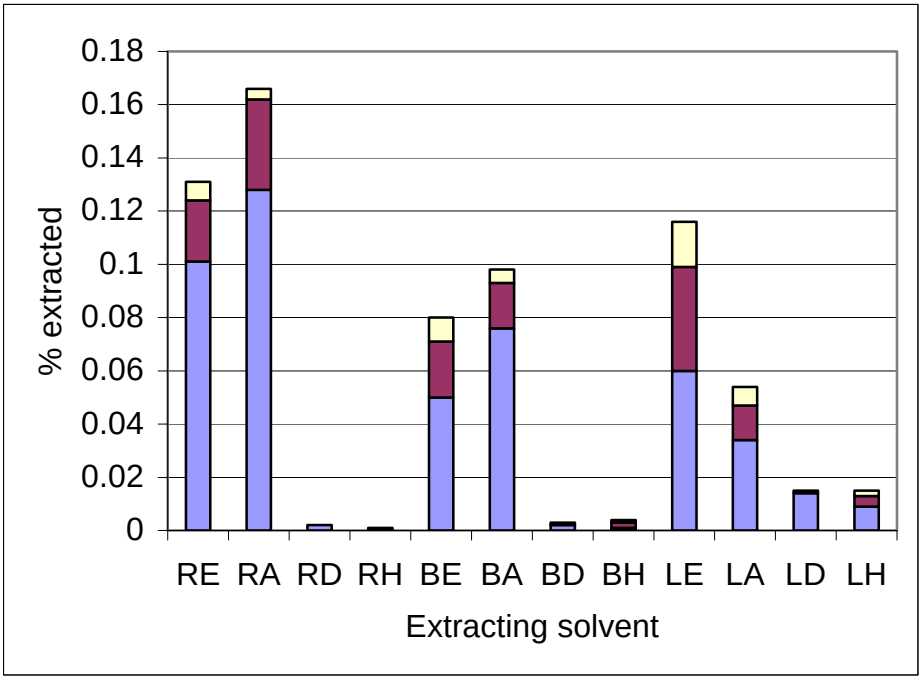

Figure 3.1: Extraction efficiency based on original dry mass of ethanol (E), acetone (A), hexane (H), and dichloromethane (D) from root (R), bark (B), and leaf (L) of P. *africanum*. First extracted at bottom, second in middle, third at top.

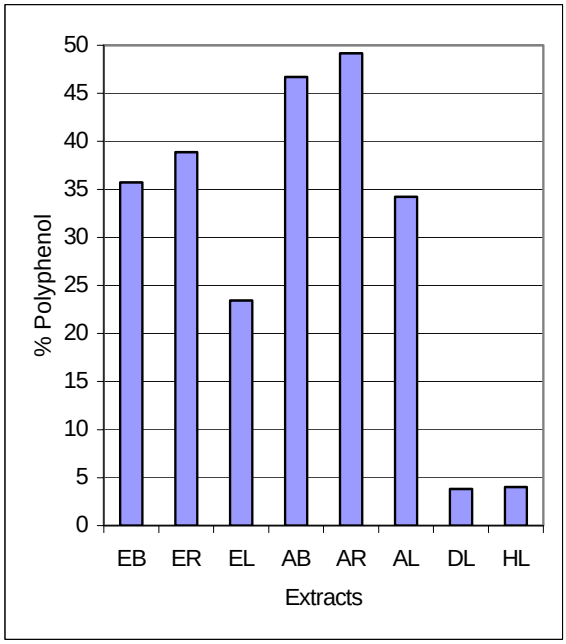

Figure 3.2: Percentage polyphenols in bark (B) , root (R) and leaf (L) extracts of ethanol (E), acetone (A), dichlomethane (D) and hexane (H)

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.

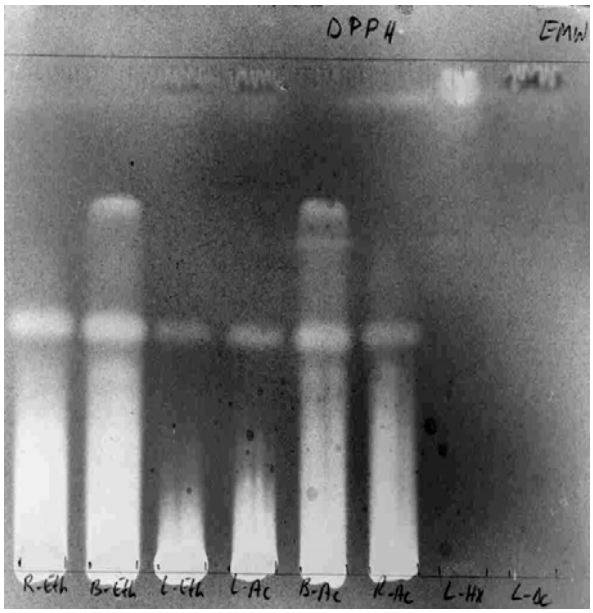

Figure 3.3: Chromatogram of 100 µg of bark (B), root (R) and leaf (L) extracted by Ethanol (Eth), Acetone (Ac), Hexane (Hx) and Dichloromethane (Dc) separated by EMW and sprayed with DPPH reagent. Light areas indicate anti-oxidant activity. Lanes from left to right R-Eth, B-Eth, L-Eth, L-Ac, B-Ac, R-Ac, L-Hx and L-Dc

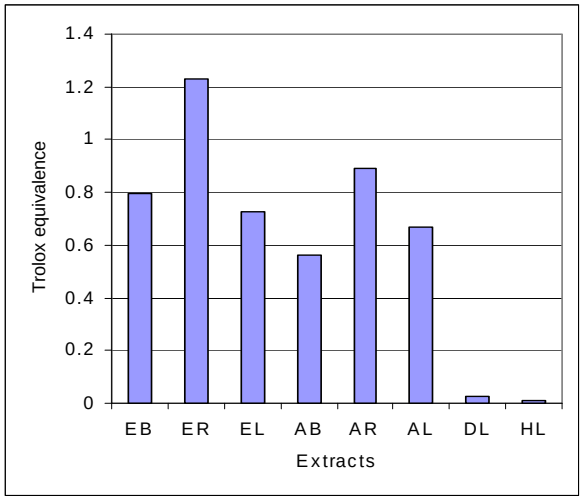

Figure 3.4: Trolox equivalent antioxidant capacity (TEAC) values of bark (B), root (R), and leaf (L) extracted with ethanol (E), acetone (A), dichloromethane (D) and hexane (H)

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

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

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^{o}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}

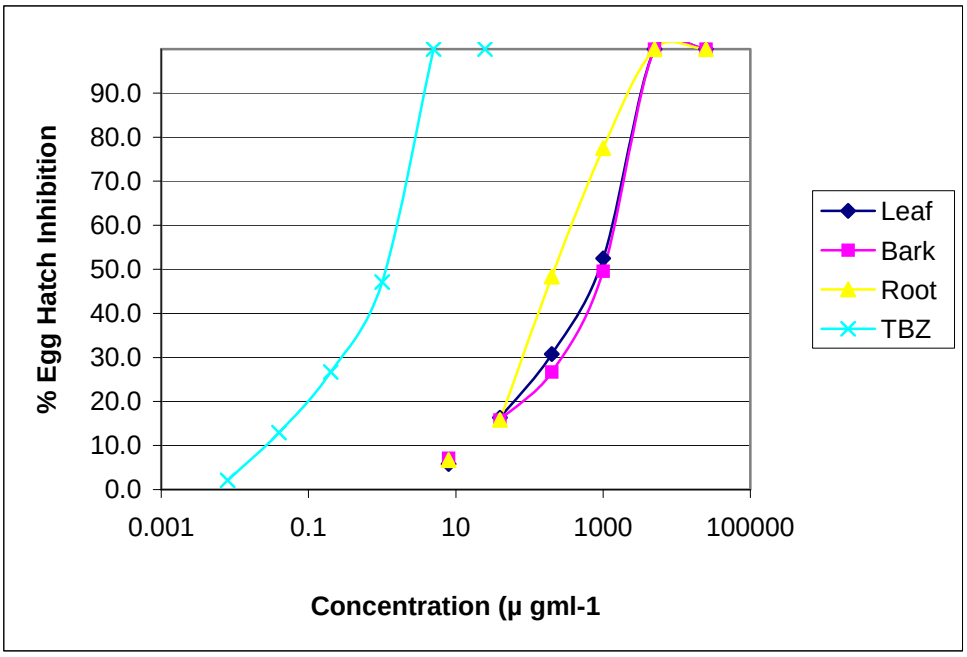

Figure 4.1: Dose-response egg hatch inhibition of *H. contortus* by leaf, bark and root extracts of *P. africanum*

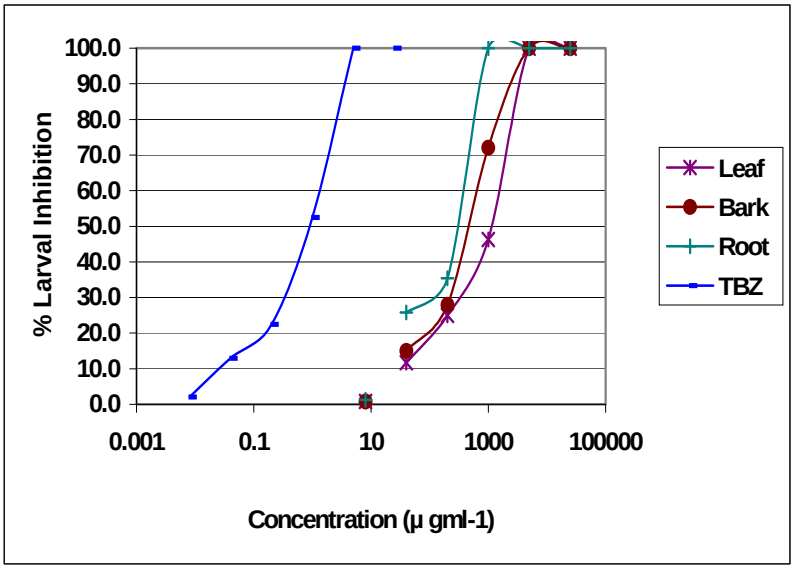

Figure 4.2: Dose-response larval development inhibitions of *H. contortus* by leaf, bark and root extracts of *P. africanum*

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.

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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}

.

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.

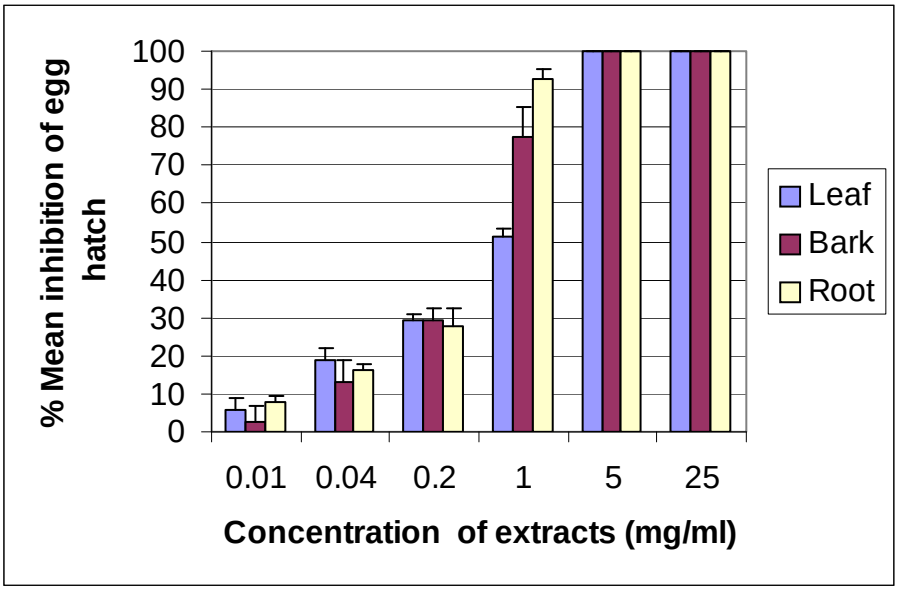

Figure 5.1: Percent mean inhibition of egg hatch of *T. colubriformis* by leaf, bark and root extracts of *P. africanum*

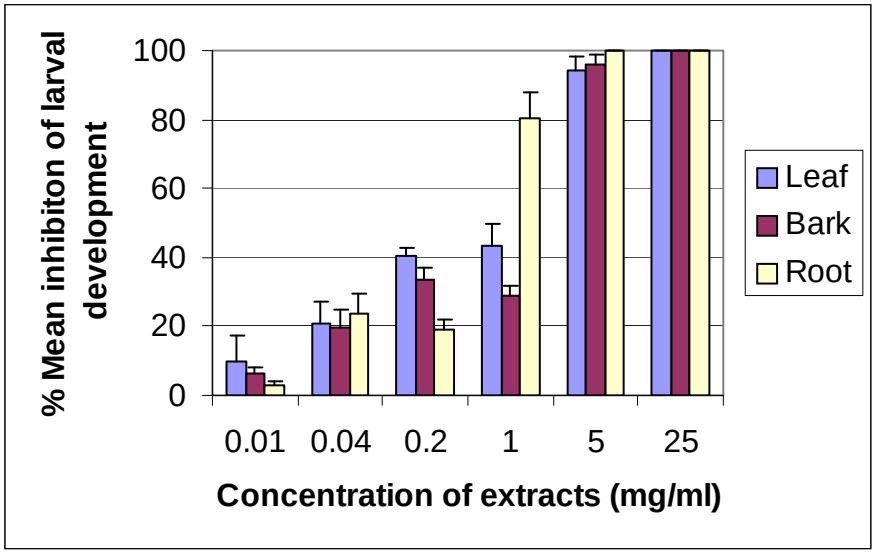

Figure 5.2: Percent mean inhibition of larval development (L_1 to L_3) of *T. colubriformis* by leaf, bark and root extracts of *P.africanum.*

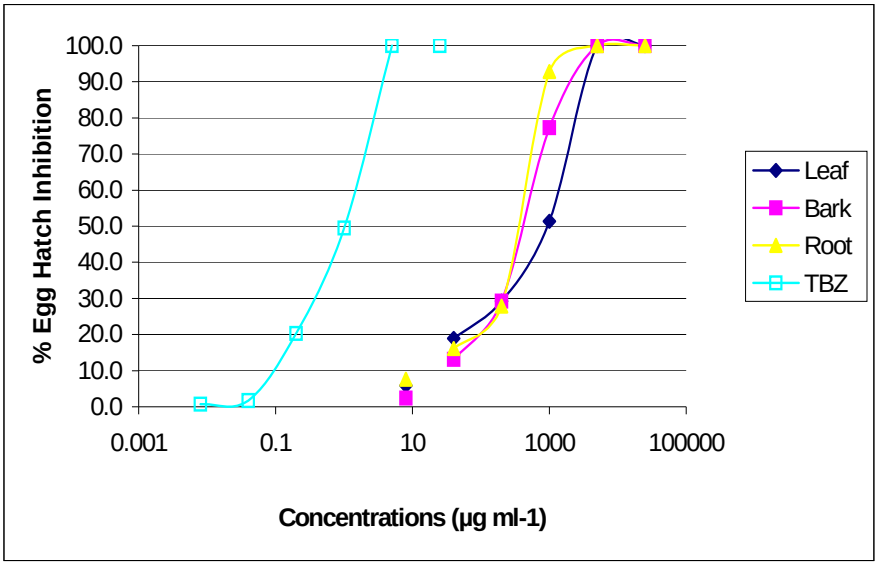

Figure 5.3: Dose-response profile for egg hatch inhibition of *T. colubriformis* by *P. africanum* extracts

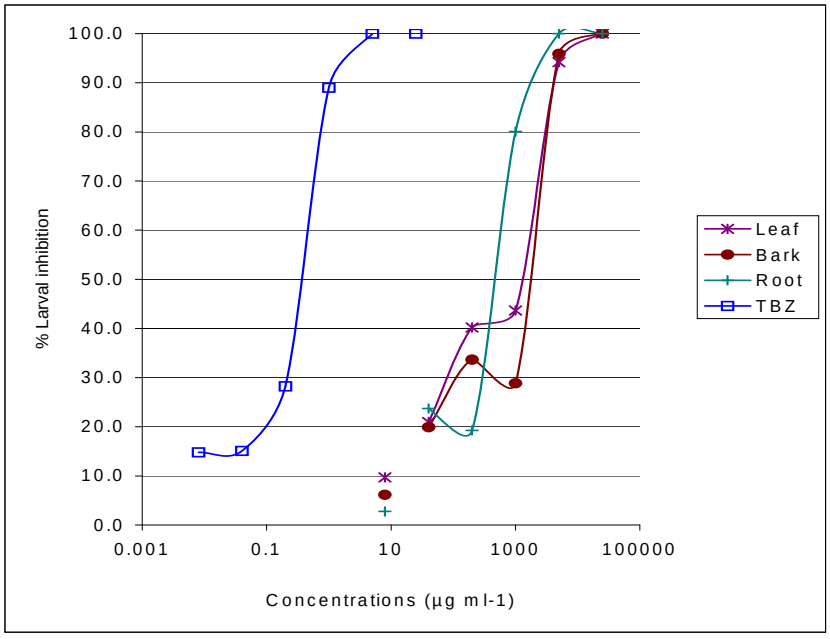

Figure 5.4: Dose-response profile for inhibition of larval development (L_1 to L_3) of *T. colubriformis* by *P. africanum* extracts

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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CHAPTER 6

EFFICACY OF *PELTOPHORUM AFRICANUM* SOND. (FABACEAE) EXTRACTS ON HAEMONCHUS CONTORTUS AND *TRICHOSTRONGYLUS COLUBRIFORMIS* IN SHEEP

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Abstract

The search for sustainable and easily affordable herbal medicines is on worldwide, prompted by emergency of anthelmintic resistance. Following substantially positive *in vitro* work, the efficacy and safety of extracts of *Peltophorum africanum* was determined against the parasitic gastrointestinal nematodes in sheep. Twenty four (24) weaned male Dorper lambs were induced with infections (L_3 infective stages) of *Haemonchus contortus* (2,800 each) and *Trichostrongylus colubriformis* (3,500 each). Four (4) weeks later, the sheep were dosed with acetone extracts of *P. africanum* at doses of 50, 500 and 750 mg kg^{-1}. The control group received no treatment. There was no significant (p=0.073) faecal egg counts reductions at concentrations of up to 750 mg kg^{-1}, or reduction in total worm burden. However, the extracts showed no deleterious effects on the sheep. Feed consumption remained the same for both treated and untreated controls. Further work on appropriate dosage form or on isolation of effective anthelmintic compounds from *P. africanum* is called for.

Key words: Efficacy, safety, sheep, extracts, *Trichostrongylus colubriformis, Haemochus contortus, Peltophorum africanum*

6.1. Introduction

One of the important restraints in livestock production is parasitic diseases. In the tropics and sub-tropics, parasitic gastrointestinal nematodes are prevalent and remain a major constraint to ruminant productivity. Parasitic nematodes rank as number one in causing production losses arising from stock mortality, severe weight loss and poor production, especially in small ruminants of resource–poor farmers in tropical Africa and South Eastern Asia (Perry and Randolph, 1999; Chiejina, 2001; Perry *et al*., 2002). In various surveys, *Haemochus contortus* and *Trichostrongylus colubriformis*, have been listed among the top ten most common nematodes hampering production of goats and sheep in tropical countries (Anon, 1992; Arosemena *et al*., 1999; Horak, 2003; Horak *et al*., 2004). Haemonchosis (caused by *H. contortus*) is characterised by anaemia, haemorrhagic gastroenteritis, hypoproteinemia, sudden death or chronic emaciation, whereas infection with *T. colubriformis* causes protracted diarrhoea with dark stool, weakness,

loss of production and death (Soulsby, 1982; Urquhart et al., 1996). Adult female *H. contortus* have high egg-producing capacity of 5000-15000 eggs per day (Hansen and Perry, 1994), whereas the infective larvae (L_3) of *T. colubriformis* have high capacity to survive even in adverse weather conditions (Urquhart *et al*., 1996). The high fecundity, combined with the high rainfall and temperatures of the tropics, favour permanent larval development in the environment leading to the development of the free-living stages to infective larval forms (L_3) throughout the year.

Control of gastrointestinal nematode infections of small ruminants is almost exclusively by use of proprietary anthelmintics. However, these drugs are expensive and sometimes unavailable or unaffordable by subsistence rural farmers or remote pastoralist communities in developing countries, who as a result end up using adulterated or poor quality products (Monteiro *et al*., 1998). Alternatively the widespread intensive use of anthelmintic drugs by commercial farmers has created multiple drug resistance that has led to a failure to control helminths in ruminants (Prichard, 1990; van Wyk *et al*., 1997; Wolstenholme *et al*., 2004; Jabbar *et al*., 2006). In South Africa, the anthelmintic resistance situation in commercial farms has been described as very serious (van Wyk *et al*., 1999). These constraints have made the reliance on pharmaceutically derived anthelmintics at present difficult, and necessitate alternative strategies of helminth control (Waller, 1997; Danø and Bøgh, 1999; Sanyal, 2001; Jabbar *et al*., 2006). Amongst these strategies is use of plants as an alternative anthelmintic opportunity. Use of effective indigenous plant preparations as livestock dewormers would appear to be a sustainable and affordable method readily adaptable to rural farming communities (Hammond *et al*., 1997; Danø and Bøgh, 1999). However, due to concerns for scientific evidence of efficacy and safety, there is need for scientific validation of herbal medicines before their acceptance and use worldwide.

Peltophorum africanum (weeping wattle), a plant widespread in southern Africa and other tropical regions, is traditionally used to treat, *inter alia,* 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). Condensed flavonoids, a novel cyanomaclurin analogue (Bam *et al*., 1988), profisetinidin-type 4-arylflavan-3-ols and related δ-lactones (Bam *et al*., 1990) were found in the heartwood. Mebe and Makuhunga (1992) isolated bergenin, norbergenin and 11-0-(E)-*p*-coumaroylbergenin from the bark, while Khattab and Nasser (1998) isolated coumarins from the leaves. *P. africanum* extracts have inhibitory effects against human immunodeficiency virus (HIV–1) reverse transcriptase and integrase (Bessong *et al*., 2005, antibacterial (Obi *et al*., 2003; Samie *et al*., 2005) and antioxidant activities (Bizimenyera *et al*., 2005; Bizimenyera *et al*., 2007), and ovicidal and larvicidal activities (Bizimenyera *et al*.,

2006 a, b) against *Haemochus contortus* and *Trichostrongylus colubriformis*. The latter was against both egg hatching and larval development.

As *in vitro* efficacy does not necessarily imply corresponding *in vivo* effect, the aim of the present study was to determine the efficacy and safety of acetone extracts of *P africanum* on the reduction of faecal egg and total worm counts of the gastrointestinal parasites *Haemochus contortus* and *Trichostrongylus colubriformis* in sheep.

6. 2. Materials and Methods

6. 2.1. Collection, storage and preparation of plant material

The plant material (root bark) was collected from mature *Peltophorum africanum* Sond. (Fabaceae) trees growing naturally (and labelled S.A Tree No. 215) at the Onderstepoort campus of University of 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 room temperature before grinding to powder using a Mascalab mill (Eriez®, Bramley). The powdered material , weighing 1.2 kg , was stored in dark tightly closed glass bottles before investigation.

6. 2.2. Plant extraction

The plant material was extracted overnight in acetone (in ratio of 1:10, weight for volume) in glass bottles on a shaker. To circumvent the problem of incomplete solubility in acetone, the extracts of *P. africanum* were not dried (Eloff, 2004). Known volumes of the extract containing 370 mg ml^{-1} were put in bottles, which were sealed and stored in a refrigerator as stock solution before use. Other than use of capsules in the anthelmintic treatment tests (due to the residual acetone) , the stock extract was diluted with distilled water and administered by stomach tube to sheep at three dose concentrations, 50, 500 and 750 mg kg^{-1} body weight for three consecutive days.

6. 2.3. Experimental animals

Twenty four (24) young (6 months) weaned male Dorper lambs were bought from reputed breeders. After weighing on a calibrated scale, the lambs were dosed with albendazole (Valbazen, Pfizer) at the dose of 22 mg kg^{-1} before the start of the study. The lambs were housed indoors in four groups of 6 each at the

University of Pretoria Biomedical Research Centre (UPBRC), Onderstepoort, on concrete floors, under quarantine conditions. The animals were provided with a commercial ration, lucerne and hay and given free access to water. They were under supervision of a veterinarian on a daily basis throughout the study. The health of animals was observed and recorded daily. The cut-off point for salvage treatment for anaemia was a packed cell volume (PCV) value of 15%.

6. 2.4 Experimental design

The experimental design was a slight modification (use of plant extract instead of drug) of the method recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants, Wood *et al*. (1995).

6.2.4.1 Preparation and administration of infective larvae

Mono-specific larval suspensions of *H. contortus* and *T. colubriformis* were obtained from cryopreserved infective larval (L_3) stocks from Onderstepoort Veterinary Institute. After thorough mixing with a magnetic stirrer, twenty aliquots of the larval suspension were counted to establish the number of viable L_3 per ml. The required inocula of L_3 for both *H. contortus* and *T. colubriformis* were mixed in one syringe and orally administered. The L_3 were administered over 5 consecutive days yielding an estimated total dose of 3,500 larvae of *T. colubriformis* and 2,800 of *H. contortus* per animal (Wood *et al*., 1995). Treatment was effected 28 days after infection, after the infection had been confirmed through faecal egg counts using sugar floatation (Hansen and Perry, 1994).

6.2.4.2 Treatment procedures

Each animal was identified by an ear tag and distinctive coat marks. A day before treatment, nine days later, and before necropsy the sheep were weighed along with a full haematology analysis of each. The sheep were randomly allocated according to body weight to 4 treatment groups, following a table of random numbers (Beyer, 1968), and according to the treatment groups: I- Control (no treatment), II- 750 mg kg^{-1}, III- 50 mg kg^{-1} and IV-500 mg kg^{-1} (Table 6.1). Extracts (prepared as in 6.2.2 above) were administered by stomach tube according to body weight on three consecutive days. All animals were subsequently observed daily for any adverse effects of treatment. Feed intake was also noted.

6.2.4.3 Full haematology and liver enzyme analysis

Along with the full haematology, blood samples were analysed for liver enzymes, aspartate aminotransaminase (AST) and gamma-glutamyl transpeptidase (GGT), the enzymes that may reflect liver function (pathology or hepatobiliary dysfunction) in herbivores.

Table 6.1: Treatment groups and individual doses

Group*	No.	Weight (kg)	Dosage (mg/kg)	Total dose (mg)	Volume (ml)
Control (I)	1	32.5	Nil	0	-
	3	30.6	Nil	0	-
	4	23.3	Nil	0	-
	5	28.4	Nil	0	-
	17	25.8	Nil	0	-
	26	33.2	Nil	0	-
II	7	33.3	750	24975	67.5
	8	26.5	750	19875	53.7
	9	24.6	750	18450	50.0
	14	30	750	22500	60.8
	21	26.2	750	19650	53.1
	23	33	750	24750	66.9
III	2	25.7	50	1285	3.57
	6	31.5	50	1575	4.3
	11	29.8	50	1490	4.0
	12	26.2	50	1310	3.5
	19	27.5	50	1375	3.7
	22	34.6	50	1730	4.9
IV	10	25.7	500	12850	34.7
	13	26.1	500	13050	35.3
	15	31.3	500	15650	42.3
	16	21.7	500	10850	29.3
	24	28.1	500	14050	40.0
	25	31.6	500	15800	42.7

* Randomly grouped according to Table of Random Units (Beyer, 1968)

6.2.5 Evaluation

6.2.5.1 Faecal egg counts

From 3 weeks after infection, and on day 1 before treatments, day 1 and 6 after treatment, faecal egg counts were determined on faecal samples collected per rectum with a lubricated gloved hand. The number of eggs per gram (epg) was determined in the standard manner using the McMaster slide. The faecal samples were collected at the same time each day to reduce the number of variables in egg counts.

6.2.5.2 Larval cultures and identification

From faecal eggs collected as 6.2.5.1, an egg suspension (with about 100 eggs per 200 µl water) was incubated in 48-well microtitre plates at room temperature for 48 hours. A drop of Lugol's iodine was added to each well, and the larvae (L_1) were counted, and identified according to established identification keys (Soulsby, 1982; van Wyk *et al*., 2004). This helped to determine the establishment of infection, and evaluation of possible effect of *P. africanum* extracts on the different worm species under investigation.

6.2.5.3 Adult worm counts

At necropsy, the abomasums and small intestines were tied off and freed of excess fat and mesenteric attachments. Each abomasum and small intestine was opened and its contents thoroughly washed into a graduated bucket, and the volume of the wash made up to 2-3 L. After thorough mixing, two 5% aliquots of the contents were fixed in formalin. The nematodes in the 5% aliquots were stained with Lugol's iodine for ease of counting. The contents were poured into flat dishes for counting.

6. 2.6. Calculations and statistical analysis

The efficacy of anthelmintic action was determined by comparing the parasite /egg populations in groups of treated and untreated animals, and dose relationship determined. The following formula (Woods *et al*., 1995) expresses the percent efficacy (%E) of a dose given against a given parasite species (S) in a given treatment group (T) when compared with an untreated control group (C): -

$$\%E = \frac{\text{Mean of S in C - mean of S in T}}{\text{Mean of S in C}} \times 100$$

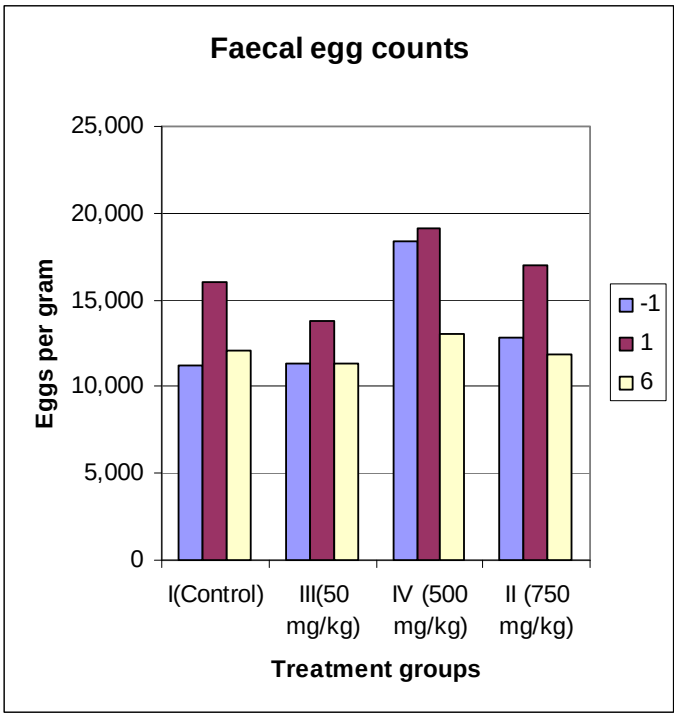
T-Test and ANOVA were used to determine whether there were differences between treated and untreated groups over time schedules.

6.3 Results

Other than transient cough in some animals, no abnormal behavioural changes were observed after treatment of the animals with the extracts. None of the animals required salvage treatment in the four weeks post-inoculation with L_3. There was general decline of the PCV in all the infected lambs. There was no significant difference in faecal egg counts between the treated and control groups (Figure 6.1). By the time the experiment was terminated only a few animals had PCV values below 20%, but none below 15%.

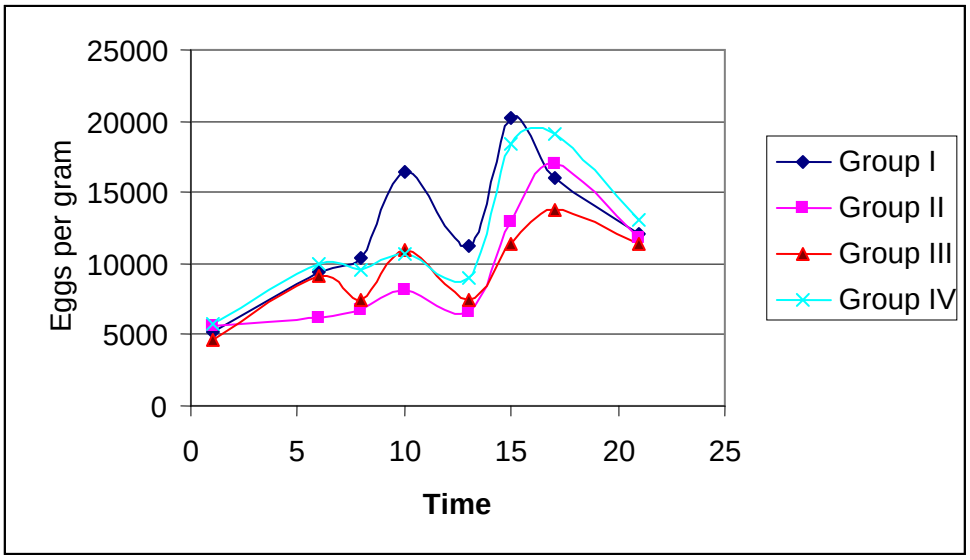
Judging from the analysis of enzymes that reflect liver damage (results not shown), gamma-glutamyl transpeptidase (GGT) and aspartate aminotransaminase (AST), there was no significant pathological or toxic effect on the animals by the extract treatment.

The faecal egg counts fluctuated, with no significant (with p=0.073) difference between the treated and control groups after the various treatments (Figure 6.2). More of the *H. contours* than *T. colubriformis* developed (Figure 6.3, 6. 4), despite the fact that the respective infecting doses with L_3 were 2,800 and 3,500



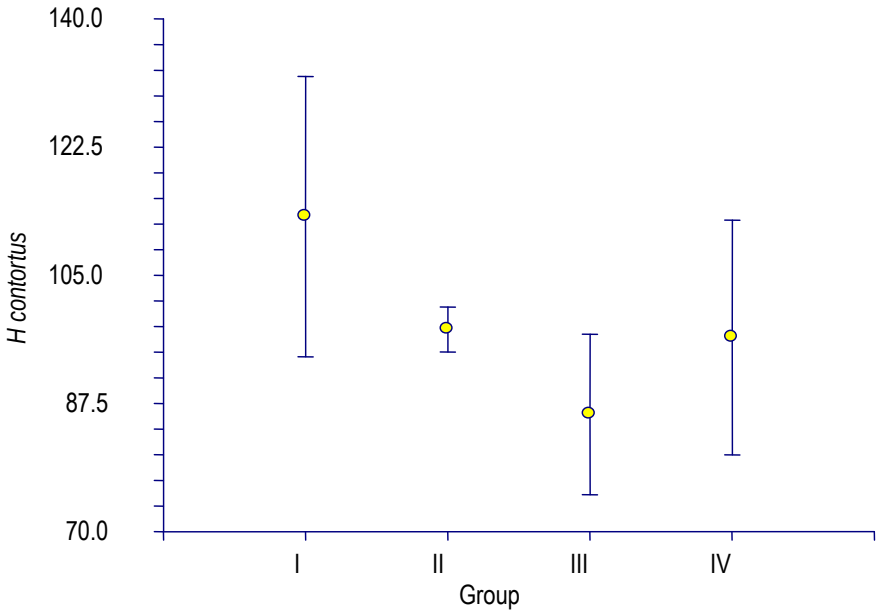
1 = one day before, 1, 6=one and 6 days after treatment

Figure 6.1: Faecal egg counts



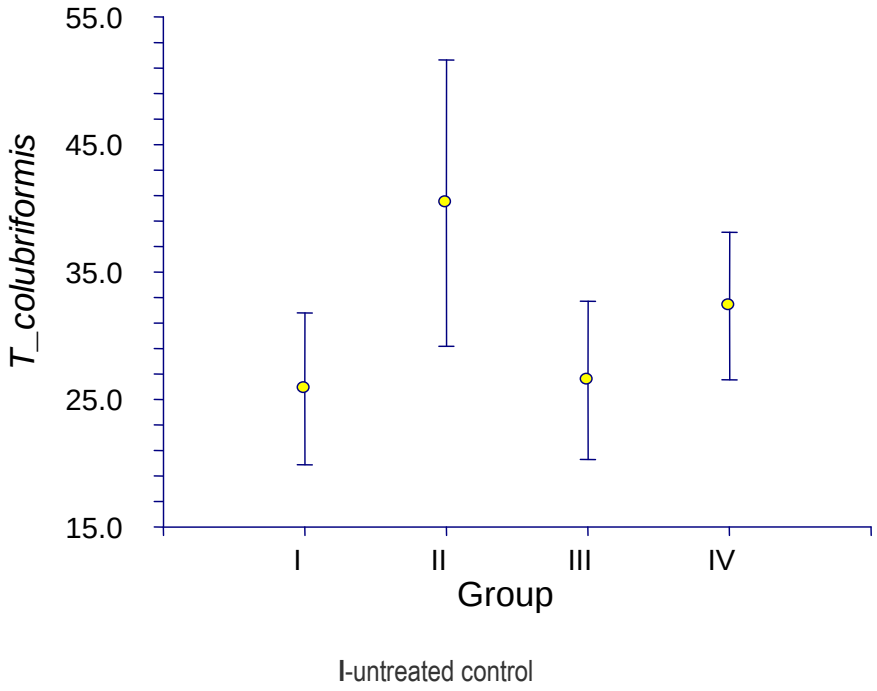
I-untreated control

Figure 6.2: Mean EPG per day of trial



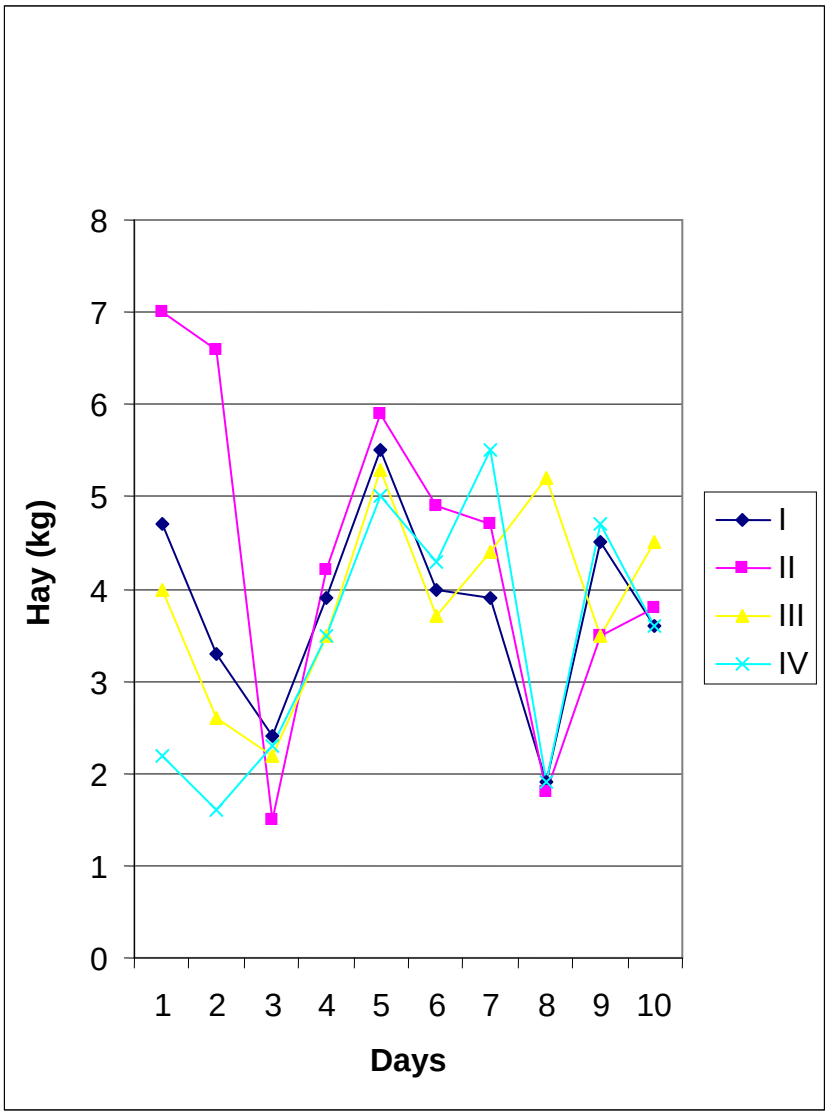
I-untreated control

Figure 6.3: *H. contortus* adult worm counts



I-untreated control

Figure 6.4: *Trichostrongylus colubriformis* adult worm counts



I-untreated control

Figure 6.5: Daily hay consumption (kg) per group, after treatment

The extracts did not effect a significant adult worm reduction in treated compared to control animals (Figure 6.3, 6.4). The administration of extracts did not affect the feed consumption in both treated and control groups significantly, Figure 6.5.

Results of the experiment show that *P. africanum* extracts are safe but not effective against the *H. contortus* and *T. colubriformis* infections in sheep at doses and dosage forms administered in the current work.

6. 4. Discussion

The objective of the study was to establish efficacy and safety of *P africanum* extracts on faecal egg, and adult worm count reduction in sheep artificially infected with *H. contortus* and *T. colubriformis*. This is the first report of such anthelmintic trial in livestock involving *P. africanum*. Other investigators have either used plant powder or plant extracts in analogous work (Githiori *et al*., 2002; Hördegen *et al*., 2003; Hounzangbe-Adote *et al*., 2005; Iqbal *et al*., 2006 a), all modifications of the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (Wood *et al*., 1995). In the *in vivo* model, up to 750 mg kg^{-1} of acetone extracts did not significantly reduce faecal egg counts of *T colubriformis* and *H. contortus*. Githiori *et al*. (2004) found that seven plant species had no significant effect on faecal egg counts on lambs infected with *H. contortus* and fed aqueous extracts of the plants. Some factors could explain why extracts that was effective *in vitro* failed to show positive results *in vivo*. The dosage rate (750 mg kg^{-1}) used in the sheep experiments may not have provided sufficient knock-down effect. Some workers reported good effect when extracts were administered at doses of 1-3 g kg^{-1} body weight (Akhtar *et al*., 2000; Suleiman *et al*., 2005; Iqbal *et al*., 2006b) .The stock extract solution was very concentrated (370 mg ml^{-1}), and some of the extract adhered to the bottle, possibly limiting the effective concentration in the administered extract. There could also be stability factors of anthelmintic components, if plant material had overstayed. However, Eloff (1999) reported that some chemical components did not change and biological activity did not decrease significantly in over 100 year-old herbarium plant specimens. Was three days administration of the extracts too short a period? Ademola *et al*. (2004, 2005) administered ethanolic extracts for two days.

Whereas ovicidal and larvicidal action of *P. africanum* extracts on eggs and larval (L_1) forms of *H. contortus* and *T. colubriformis* has been reported (Bizimenyera *et al*., 2006 a, b) , the extracts did not have a similar action on the adult worm parasites in the current work. 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). The reservoir effect, whereby the extract gets dissolved in the large volume of fluid in the rumen , and an increase in the mean residence time in the rumen due to the extract binding to rumen material, with little passing on to the abomasums may have reduced the bioavailability. What about the potential degradation of the extract by rumen microflora? Possibly different results could have been obtained if the extracts had been administered by direct injection into the abomasums or small intestine; Van Wyk and Gerber (1980) injected *H. contortus* and *T. colubriformis* infective larvae L_3 to establish infections in abomasums and small intestine respectively. Athanasiadouou *et al*., (2001) reported direct anthelmintic astringent effects of condensed tannins on

gastrointestinal nematodes. Alternatively, the extracts could be administered orally after the oesophageal groove is closed by suitable methods, such as pre-administraton of copper sulphate solution (Gibson, 1975). Hence improved methods of extraction to get an anthelmintic rich extract need to be developed.

P. africanum extracts however were found to be safe in the treated animals. Gamma-glutamyl transpeptidase (GGT) and aspartate aminotransaminase (AST), levels used to assess liver pathology, were not statistically different between treated and control lambs. Haematological analysis of these enzymes gives a good indication of liver pathology in herbivores (Reyers, 2005).

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CHAPTER 7

SAFETY PROFILES OF *PELTOPHORUM AFRICANUM* SOND. (FABACEAE) EXTRACTS

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Abstract

Peltophorum africanum Sond (Fabaceae), commonly called 'weeping wattle', is a plant widely used traditionally for medicinal purposes in both man and animals. Traditionally the extracts have been used against diarrhoea, dysentery, helminthosis, acute and chronic pains, resistance to infection and depression. Antibacterial, anthelmintic, and antioxidant activities have been demonstrated in its extracts *in vitro*. The safety and toxicity of the extracts has received little attention. From *in vitro* toxicity tests, the leaf, bark and root extracts were not toxic to brine shrimp and Vero monkey kidney cells. The apparent lack of toxicity of the extracts of *P. africanum* leads to support the promotion of its use in traditional medicine.

Key words: -Herbal extracts; Toxicity; Safety; Bergenin; *Peltophorum africanum*

7.1. Introduction

The use of medicinal plants in treating diseases is an ancient tradition that has co-existed with human habitation. Herbal medicines form a significant part of culture and traditions of rural people in developing countries. As a result there is an increasing trend to integrate traditional medicine with primary health care. This arises because about 80% of people in the developing world today, especially where modern drugs are not affordable, or are inaccessible or unacceptable, depend on traditional herbal remedies [1, 2]. Disease concepts are largely similar in humans and animals. Traditional healers of people are often called to treat animals (and vice versa), often employing the same herbs, compounds or manipulative techniques [3, 4]. In developed Western countries half of all prescriptions dispensed contain substances of natural origin, 50% of which have plant derived active principles [5].The green movement in Western society has changed attitudes in the general population, who now conceive naturally derived substances and plant extracts as being inherently safer and more desirable than synthetic chemical products [6]. Hence there is renewed interest in traditional pharmacopoeias, with researchers determining the scientific rationale of plant usage, discovery of

new compounds, or using plant-derived compounds as models for chemical syntheses of novel pharmaceuticals.

Over 122 drugs from 94 plants, covering a wide range of activity such as antibacterial, anti-inflammatory, antioxidant, anthelmintic, anti-amoebic, antischistosomal, antimalarial, as well as psychotropic and neurotropic activities have been discovered following botanical leads [7]. The trend towards phytotherapy notwithstanding, many medical and veterinary professionals do not trust the use of herbal medicines [3, 8]. Despite extensive use of plants as medicines, herbal remedies are not as safe as frequently claimed. Plants contain substances (such as saponins, viscotoxins, tannins, cyanogenetic glycosides, furanocoumarins, pyrrolizidine alkaloids, sesquiterpenes, etc), possibly naturally produced for defense against pathogens and for discouragement of ingestion by man and animals, which render many herbal medicines poisonous [9]. South Africa has made a world contribution with herbal teas and plant remedies such as Cape aloes, rooibos, buchu, honeybush and devil's claw [10]. Nevertheless surveys have indicated many of the medicinal plants to be toxic or poisonous [11, 12] and many people have died from medicinal plant poisoning [13]. There are similar trends in other parts of the world. Hence the need for their scientific validation (for efficacy and safety) before plant–derived extracts gain wider acceptance and use.

Peltophorum africanum Sond (Fabaceae), is a deciduous tree widely distributed in southern Africa and other tropics. It is a unique plant in that it is traditionally used to treat more or less similar disease conditions in man and animals. The bark and root extracts are traditionally used to treat diarrhea, dysentery, acute and chronic pains, wounds, internal parasites, for boosting resistance to disease, and to treat infertility and depression [14,15, 16]. Traditional healers have used the root extract as a component in the '*Kgatla doctors*' mixture to promote wellbeing, fertility and resistance to disease. Livestock farmers use the root and bark extracts against diarrhoea, dysentery and colic and as a general tonic. In southern Africa, women who lose their spouses take the bark or root decoctions for up to a year, possibly for relief of post-traumatic stress and depression.

The phytochemistry of this species has been investigated by many authors who reported mainly flavonoids, and other phenolic compounds [17, 18, 19, 20, 21]. Reports of testing for biological activity of extracts or isolated compounds are scanty. However, antibacterial [22, 23] , antioxidant and antibacterial [24, 25], antihelmintic [26, 27] and inhibitory properties against HIV- AIDS type 1 reverse transcriptase and integrase [28] have been reported.

P. africanum is one of the dominant plants found in the Pretoria medicinal plant market[16] and very popular among the rural Madikwe community where it is used for livestock treatments [29]. Based on the traditional usage and results of *in vitro* work, the extracts and compounds of *P. africanum* have potential for health of both man and animals. The present study aimed at establishing the safety of the plant extracts.

7. 2. Materials and methods

7. 2.1 Plant material

The leaves, stem bark and root bark collected from mature *Peltophorum africanum* Sond. (Fabaceae) trees naturally growing at Onderstepoort, Pretoria, South Africa (bearing label S.A. Tree No. 215), were dried in the shade at ambient temperature. A voucher specimen (PM 001) is stored in the Medicinal Plant Herbarium, Department of Paraclinical Sciences, University of Pretoria. The dried material was ground to powder in a Macsalab mill (Model 200 LAB), Eriez®, Bramley.

7. 2.2 Extraction

A previous study showed that acetone was the best extractant for *P. africanum* compared to ethanol, hexane and dichloromethane [24]. Therefore, acetone was the solvent selected for the bioassays, in the ratio of plant material to acetone of 1:10 (weight to volume) in an overnight extraction. As the dried extracts extracted by acetone do not fully dissolve back in acetone, the extracts were not concentrated to dryness [30] and made to a stock concentration of 100 mg ml^{-1}.

7.2.3. Toxicity assays

7.2.3.1 Brine shrimp lethality

The brine shrimp lethality test is fully described by Solis *et al*. [31] who used the test for plant extracts in a range of concentrations to obtain an LD_{50} value. The brine shrimp (*Artemia salina*) eggs were obtained from a local pet shop, and hatched in artificial sea water (3.8 g NaCl + 100 ml distilled H_2O), yielding phototrophic nauplii (larvae). The acetone extracts of the leaf, bark and root were tested at concentrations of 0.1, 1, 2, and 5 mg ml^{-1}, in 4 (four) replicates, the test solution made to required volumes with distilled water. The final acetone concentration acted as solvent blank control for the nauplii, whereas Podophyllotoxin (Sigma) was the positive control, and distilled water acted as negative control. Four (4) 96-well microtitre plate replicates, with each well having 100 µl, of plant extract solution and 100 of larvae suspension containing

10-15 nauplii were incubated for 24 h at room temperature (23°C). A stereomicroscope was used in observing and counting the larvae, and any deaths in controls were adjusted for in the treated plates by using Abbott's formula described by Rasoanaivo and Ratsimamanga-Urverg [32].

$$\text{Corrected mortality percent} = \frac{m-M}{S} \times 100$$

m= mean of dead larvae in treated plates %

M= mean of dead larvae in controls %

S= mean of living larvae in controls %

(The reference compound Podophyllotoxin®, LC_{50}= 5 g ml^{-1})

7.2.3.2 MTT assay (cell line cytotoxicity) of extracts

Moosmann [33] fully describes the assay, that essentially is based on the reduction of the yellow coloured 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), by mitochondrial dehydrogenases of metabolically active cells (live cells) to a purple formazan. The viable cell growth after incubation with extracts was determined using MTT (Sigma), for measuring cell proliferation and cytotoxicity. The intensity of colour (measured spectrophotometrically) of the formazan produced by living, metabolically active cells is proportional to the number of live cells present. Formazan is an insoluble purple substance, a result of reduction of the yellow water soluble tetrazolium dye (MTT) by the live and not dead cells.

The growth medium used was Minimum Essential Medium (MEM, Highveld Biological, Johannesburg), supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). Cells of a subconfluent culture of Vero monkey kidney cells , obtained from the Department of Veterinary Tropical Diseases, University of Pretoria, were harvested and centrifuged at 200xg for 5 minutes, and re-suspended in growth medium to 2.4 x 10^3 cells ml^{-1}. A total of 200 µl of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Growth medium (200 µl) was added to wells of column 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37°C in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. Then the MEM was aspirated from the wells using a fine tube attached to a hypodermic needle, and replaced with 200 µl of test extract at different concentrations prepared in growth medium. The cells were disturbed as little as possible during the aspiration of the medium and addition of the test extracts. Each dilution was tested in quadruplicate. The microplates were further incubated for 5 days at 37°C in a 5% CO_2 incubator with the test material. Untreated cells and positive control, Berberine Chloride (Sigma) was included.

After incubation, 30 µl MTT (a stock solution of 5 mg ml^{-1} in PBS) was added to each well and the plates incubated for a further 4 h at 37°C. After incubation with MTT the medium in each well was carefully removed, without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by addition of 50 µl DMSO to each well. The plates were shaken gently until the crystals were dissolved. The amount of MTT reduction was measured immediately by detecting the absorbance in a microplate spectrophotometer reader (Versamax®) at wavelength of 570 nm. The wells in column 1, containing the medium and MTT but no cells, were used to blank the plate reader. The LC_{50} values were calculated as the concentration of test extract resulting in a 50% reduction of absorbance compared to untreated cells.

7.2.4 Safety of extracts in sheep

In a related work reported elsewhere (Chapter 6), acetone extracts were administered by stomach tube to sheep artificially infected with *Haemonchus contortus* and *Trichostrongylus colubriformis*.

7.2.5 Statistical analysis

The Excel package was used in data analysis.

7.3 Results

The leaf, bark and root extracts (at the maximum concentration of 5 mg ml^{-1} employed) did not show toxicity in the brine shrimp or Vero monkey kidney cell line assays, Table 7.1.

Table 7.1: Cytotoxicity of *P. africanum* root, bark and leaf extracts

Item	Brine shrimp[a] LC_{50} (µg ml^{-1})	MTT[b] LC_{50} (µg ml^{-1})
Root*	>1000	>1000
Bark*	>1000	>1000
Leaf*	>1000	>1000
Podophytotoxin	7.01	
Berberine		9.82

Key:
[a]Brine shrimp larval mortality assay
[b]Cytotoxicity assay aginst Vero Monkey kidney cell line
*Extracts at 5 mg ml^{-1} , the highest concentration used

The administration of extracts did not affect the sheep in any manner, whether by way of feed consumption, or any deleterious effect or expression.The haematological parameters and liver enzymes of the sheep that received treatment with *P. africanum* extracts for parasitic nematodes were not affected, Figure 7.1.

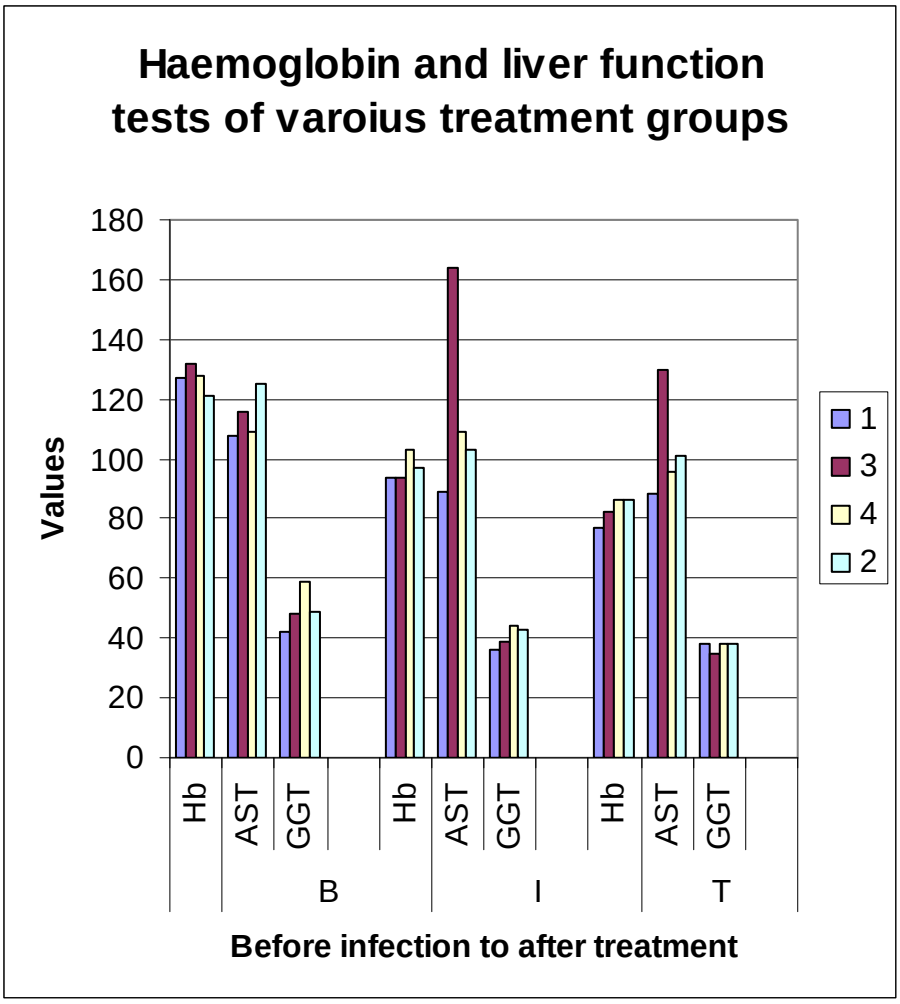

Figure 7.1: Haemoglobin and liver function tests of four treatment groups

Notes: i) Units of values, Hb=g/l and IU/l for AST & GGT

ii) 1-4 are extract treatment groups; 1 being control with no treatment

iii) B=before infection with parasitic nematodes, I= during the course of infection and T= after treatment with extracts.

7. 4 Discussion

P. africanum has been traditionally used in the treatment of a wide range of conditions including diarrheoa, dysentery, acute and chronic pain, wounds, anxiety and depression, and as a tonic for fertility and resistance to diseases[14, 15, 16]. The root and bark from *P. africanum* are important components of the products sold in informal medicinal plant markets in Pretoria [16], cattle markets of Setswana-speaking people

of Madikwe area [29], traditional healers gatherings of Botswana [34], and other regions in southern Africa. This has stimulated scientific research studies on the medicinal plant. In addition to several phytochemists who have isolated various compounds, bioassay characterization studies have shown that extracts of *P. africanum* have antibacterial [22, 23] , antioxidant and anthelmintic [26, 27, 25], as well as anti-HIV/AIDS [28] activities.

P. africanum is not toxic, as the brine shrimp and Vero monkey kidney cell line cytotoxicity assay results have showed. Bessong *et al*. [28] found no toxicity when they tested *P. africanum* extracts in a HeLaP4 cell line. Brine shrimp assay has been used in *in vitro* cytotoxicity screening tests [31] , and the test is also routinely used in the plant extracts [12, 10, 35] in South Africa. However, some plants known to be toxic to livestock have displayed non-toxicity to brine shrimp [35] , casting a doubt whether the brine shrimp assay is capable of detecting toxic effects of plant extracts. Therefore cell-line cytotoxicity was applied alongside the brine shrimp assay for *P. africanum* extracts in the present work, as mammalian cell line gives better correlation.

In another related work pending publishing, *P. africanum* extracts were administered (by stomach tube) to lambs artificially induced with *Haemochus contortus* and *Trichostrongylus colubriformis* infections. There were no abnormal behaviors, toxicity signs or any other abnormality in the lambs attributable to the extracts that were given up to a maximum dosage rate of 750 mg kg^{-1}. Setswana-speaking pastoralists of the Madikwe area of the North West Province, South Africa, who give the extracts to cattle for diarrhea and as a general tonic for resistance to disease, reported no side effects or toxicity in treated animals [29]. To paraphrase Weiss and Fintelmann [36], "if a plant extract has been used for ages, repeatedly asked for by patients and prescribed by doctors, one must assume that it is effective and safe, even without double-blind studies". By the same token, from the traditional use, and from research tests carried out to date, *P. africanum* extracts may be assumed safe. This, nevertheless, gives no room for complacency as many herbal medicines are toxic [9, 11, 12], requiring more defined laboratory and clinical tests. Though Joubert [37] purified a proteinase inhibitor (a potent poison like snake venom) from the seeds of *P. africanum*, animals routinely browse the leaves and young stock [38, 39]. Nevertheless, mature trees are unpalatable and shunned by browsing wild ruminants [40].

In conclusion, the extracts of *P. africanum* Sond. (Fabaceae) appear to be safe. But further laboratory and clinical work is called for. There is a great potential for the ubiquitous plant (*P. africanum*) in the promotion of health, in both man and animals. The traditional use of the bark and root for medication is a practice that may not be sustainable, as in some parts of South Africa the tree has been stripped bare (D. E. N. Mabogo, personal communication). More research is required to innovate better extraction methods that would utilize

the leaves. Furthermore, if the active compounds were isolated, their synthetic varieties could be made available. This would reduce the demand of the plant material, with a view to conserving the environment.

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CHAPTER 8

ISOLATION AND BIOASSAY CHARACTERISATION OF BERGENIN FROM THE ROOT EXTRACT OF *PELTOPHORUM AFRICANUM* SOND. (FABACEAE)

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Abstract

South Africa is home to many medicinal plants. Phytochemists have taken interest in analyzing many of these plants for possible isolation of bioactive compounds. *Peltophorum africanum* Sond (Fabaceae), commonly called 'African wattle', is an outstanding plant traditionally used for treatment of more less similar disease conditions in man and livestock. Concoctions of the plant are used in the treatment of abdominal problems, pain, wounds and depression. Polyphenols, flavonoids, appear to be the most common compounds isolated from the plant by phytochemists, but many of these compounds have yet to undergo bioassay characterizations. Bergenin, the main compound obtained from the root extract, showed antibacterial and antioxidant activities and was not toxic in the cell line assay. This is the first report of bergenin being isolated from the root of *Peltophorum africanum*. Bergenin had antioxidant activity comparable to L-ascorbic acid, with the respective EC_{50} values of 5.86 and 5.03 µg ml-1. The compound exhibited antimicrobial activity, with good action against *Sporobolomyces salmonicolor*, moderate activity against *Mycobacterium vaccae*, *Pseudomonas aeruginosa* and *Escherichia coli*, but no activity against *Penicillium notatum*. Bergenin had no effect on the feline herpesvirus *in vitro*. The apparent lack of toxicity of bergenin, and the extracts of *P. africanum* leads support to the promotion of the use of the plant in traditional medicine. As bergenin has been shown to have neuroprotective effect, *P. africanum* extracts have great potential in treatment of neurodegenerative diseases.

Key words: -Herbal extracts; Antimicrobial; Antioxidant; Bergenin; *Peltophorum africanu*

8.1. Introduction

The green movement in Western society has changed attitudes in the general population, who now conceive naturally derived substances and plant extracts as being inherently safer and more desirable than synthetic chemical products [1] . There is a renaissance of phytotherapy, with phytochemists, pharmacologists and clinicians alike turning to searching for bioactive compounds from plants. South Africa is home to many medicinal plants [2]. One of such medicinal plants is *Peltophorum africanum* Sond (Fabaceae), a deciduous tree widely distributed in southern Africa and other tropical regions.

Despite being a unique plant that is traditionally used to treat more or less similar disease conditions in man and animals, and despite its phytochemistry having been studied by several authors [3, 4, 5, 6, 7, 8], the compounds from *P. africanum* have not received much attention in terms of testing for biological activity. Most of these compounds are flavonoids and other polyphenols. However, antibacterial [9, 10], antioxidant [10, 11], and anthelmintic [12, 13] activities of the extracts have been reported. Bessong *et al*.[14] reported inhibitory activities of the extracts against HIV-AIDS type 1 reverse transcriptase and integrase; the anti-HIV activity was shown both by the extracts and bergenin isolated from the bark.

Since the previous investigation had shown the root had showed most activity (antibacterial, antioxidant and anthelmintic), and acetone appeared to extract more compounds from *P. africanum*, the current work was an attempt to isolate compounds from the root using acetone extracts.

8.2 Methodology

8.2.1 Plant material

The root bark collected from mature *Peltophorum africanum* Sond. (Fabaceae) trees naturally growing at Onderstepoort, Pretoria, South Africa (bearing label S.A. Tree No. 215), was dried in the shade at ambient temperature. A voucher specimen (PM 001) is stored in the Medicinal Plant Herbarium, Department of Paraclinical Sciences, University of Pretoria. The dried material was ground to powder in a Macsalab mill (Model 200 LAB), Eriez®, Bramley.

8.2.2 Isolation of compounds

The acetone extract of the root was used for compound isolation. When an attempt was made to re-dissolve the dry extract back into acetone, a compound crystallized. The extract was further run in a Sephadex LH 20 (Sigma) column eluted with ethylacetate: hexane, in series of 1:1, 5:1, 9:1 from which eight compounds crystallized. Next was elution with chloroform: methanol, in a series of 9:1, 7:1, 5:1, 3:1, 1:1 and 1:3 where a further three compounds crystallized. The crystallized compounds were washed up, and subjected to NMR and mass spectrometer tests, and finally identified.

8.2.3 Toxicity assays with bergenin , the isolated compound

8.2.3.1 Brine shrimp lethality

A modification of the technique of Solis *et al*. [15] was used to test the bergenin in a range of concentrations to obtain an LD_{50} value. Briefly, brine shrimp (*Artemia salina*) eggs, obtained from a local pet shop, were hatched in artificial sea water (3.8 g NaCl + 100 ml distilled H_2O). After 48 h, the phototrophic nauplii (larvae) were collected using a Pasteur pipette and transferred to a fresh beaker.

Bergenin solutions in dimethyl sulphoxide were tested at concentrations of 0.1, 1, 2, and 5 mg ml^{-1}, in 4 (four) replicates, the test solution made to required volumes with distilled water. A solvent blank control was used to test the effect of the final concentration used on the larvae. Podophyllotoxin (Sigma) was the positive control while distilled water acted as negative control.

Bergenin solution (100 µl) was put in each well of a 96-well microtitre plate in 4 replicates per dilution. Nauplii suspension (100 µl, containing 10-15 nauplii) was put in each well. The microtitre plates were covered and incubated in a humidified chamber for 24 h at room temperature (23°C). The numbers of dead and live nauplii were counted using a stereomicroscope. If deaths occurred in the solvent controls at the end of the treatment, corrected percent death values in treated plates were calculated using Abbott's formula described by Rasoanaivo and Ratsimamanga-Urverg [16].

$$\text{Corrected mortality percent} = \frac{m-M}{S} \times 100$$

m= mean of dead larvae in treated plates %
M= mean of dead larvae in controls %
S= mean of living larvae in controls %
(The reference compound Podophyllotoxin®, LC_{50}= 5 g ml^{-1})

8.2.3.2 Determination of cytotoxicity (MTT assay)

The cytotoxicity assay used is based on the reduction of the yellow coloured 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), by mitochondrial dehydrogenases of metabolically active cells (live cells) to a purple formazan, as described by Moosmann [17]. The viable cell growth after incubation with bergenin was determined using MTT (Sigma), for measuring cell proliferation and cytotoxicity. The intensity

of colour (measured spectrophotometrically) of the formazan produced by living, metabolically active cells is proportional to the number of live cells present. MTT is a yellow water-soluble tetrazolium dye that is reduced by live, but not dead cells, to a purple formazan product that is insoluble in aqueous solutions.

Cells of a subconfluent culture of Vero monkey kidney cells , obtained from the Department of Veterinary Tropical Diseases, University of Pretoria, were harvested and centrifuged at 200xg for 5 minutes, and re-suspended in growth medium to 2.4 x 10^3 cells $ml^{-1.}$ The growth medium used was Minimum Essential Medium (MEM, Highveld Biological, Johannesburg), supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). A total of 200 µl of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Growth medium (200 µl) was added to wells of column 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37°C in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. Then the MEM was aspirated from the wells using a fine tube attached to a hypodermic needle, and replaced with 200 µl of test (bergenin) solution at different concentrations prepared in growth medium. The cells were disturbed as little as possible during the aspiration of the medium and addition of the test extracts. Each dilution was tested in quadruplicate. The microplates were further incubated for 5 days at 37°C in a 5% CO_2 incubator with the test material. Untreated cells and positive control, berberine chloride (Sigma) was included.

After incubation, 30 µl MTT (a stock solution of 5 mg ml^{-1} in PBS) was added to each well and the plates incubated for a further 4 h at 37°C. After incubation with MTT the medium in each well was carefully removed, without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by addition of 50 µl DMSO to each well. The plates were shaken gently until the crystals were dissolved. The amount of MTT reduction was measured immediately by detecting the absorbance in a microplate spectrophotometer reader (Versamax®) at wavelength of 570 nm. The wells in column 1, containing the medium and MTT but no cells, were used to blank the plate reader. The LC_{50} values were calculated as the concentration of test solution resulting in a 50% reduction of absorbance compared to untreated cells.

8.2.3.3 Antiviral assay

Crandell feline kidney (CRFK) cells were obtained from the Department of Veterinary Tropical Diseases, University of Pretoria. Cultures were grown in Eagle's essential medium (MEM) containing 10% fetal calf serum (FCS) and 50 µl/ml gentamicin. Confluent cultures were maintained at 37°C in humidified 5% CO_2 atmosphere.

Virus for use in the assay was produced in 75 cm^2 flasks of confluent CRFK cells. Flasks were inoculated with virus stock and then incubated until approximately 90% of the monolayer showed cytopathic effect (CPE). This suspension was centrifuged at 1000 X g for 10 minutes and the supernatant stored at -70^{0}C. The virus used in this case was feline herpesvirus (FHV-1).

Bergenin was diluted in sterile de-ionized water to final dilutions of 1: 50. Virus stock (0.5 ml) was then mixed with 0.5 ml of bergenin (contact time 20 minutes). A serial 10 fold dilution was performed by taking 0.5 ml from the mixture of virus and bergenin and placing in tubes containing 4.5 ml MEM. The above mentioned 10 fold dilutions was performed in 8 different tubes and 200 µ l of each dilution placed in a 96-well flat bottom microtitre plate. Eighty microliter of CRFK cells (480,000 cells/ ml) was added to each well (with 200 µl) in 5 replicates. Each extract test included a virus control and a toxicity test.

The plates were incubated for 5 days at 37^{0}C in a 5% CO_2 atmosphere.

The CPE was observed by the use of an inverted light microscope. One hundred percent cell damage was scored with a 4 while 75% cell damage was scored a 3 and so on. A zero indicated that the cells were viable. The tissue culture infectious dose 50 was calculated using the Karber method [18].

The Karber method was used for calculating the $TCID_{50}$.

Negative log of the ID_{50} end point titer=

[Negative log of the highest virus concentration used] – [{sum of % mortality at each dilution- 0.5} x (log of dilution)]

8.2.4 Bioactive assays with bergenin

8.2.4.1 Antioxidant

The antioxidant assay was done following a previous method [11], comparing the antioxidant activity of bergenin to that of ascorbic acid (Vitamin C).

8.2.4.2 Inhibition of microbial growth

The antimicrobial activity of bergenin was determined by the "hole-plate diffusion method" [19]. The test organisms were *Escherichia coli* (SG 458), *Pseudomonas aeruginosa* (K 799/61), *Mycobacterium vaccae* (IMET 10670), *Sporobolomyces salmonicolor* (SBUG 549), and *Penicillium notatum* (JP 36).The tested microbial suspension was homogeneously seeded onto petri dishes containing 15 ml of the MH agar medium. Holes were aseptically bored into the agar with a hollow punch and 25 µl aliquots of the bergenin solution were placed into wells with a sterile pipette. The plate was kept for 1 h at room temperature for the diffusion of the bergenin into the agar. Subsequently, the plate was incubated at 37°C for 18 h. Chloramphenicol was used as positive control and 70% ethanol was used as negative control. The microorganism control consisted of a seeded petri dish with no plant material, solvent (70% ethanol) or chloramphenicol. Results were recorded as the mean of triplicate experiments.

Microbial growth inhibition was determined as the diameter of the inhibition zones around the holes. The inhibition diameter was the average of four measurements per hole.

8.2.5 Statistical analysis

The Excel package was used in data analysis.

8.3 Results

Bergenin (molecular formula $C_{14}H_{16}O_9.H_2O$),chemically called **3,4,8,10-tetrahydroxymethyl-9-methoxy-3,4,4a, 10b-tetrahydro-2H-pyranol[3,2-c]isochromen-6-one**, molecular weight 328 was found to be a major component of the root extract of *P. africanum*, as more than 5. 641 g were isolated from 36.06 g of the acetone extract of the root material. Out of this amount, 1.554 g of pure bergenin crystallized following attempts to re-dissolve the dry acetone extract residue in acetone. All the nine compounds isolated from the root extract turned to be identical by the nuclear magnetic resonance (NMR) amd mass spectroscopy (MS) analysis. The NMR and MS spectra of bergenin are displayed in Figure 8.1 and Figure 8.2 respectively.

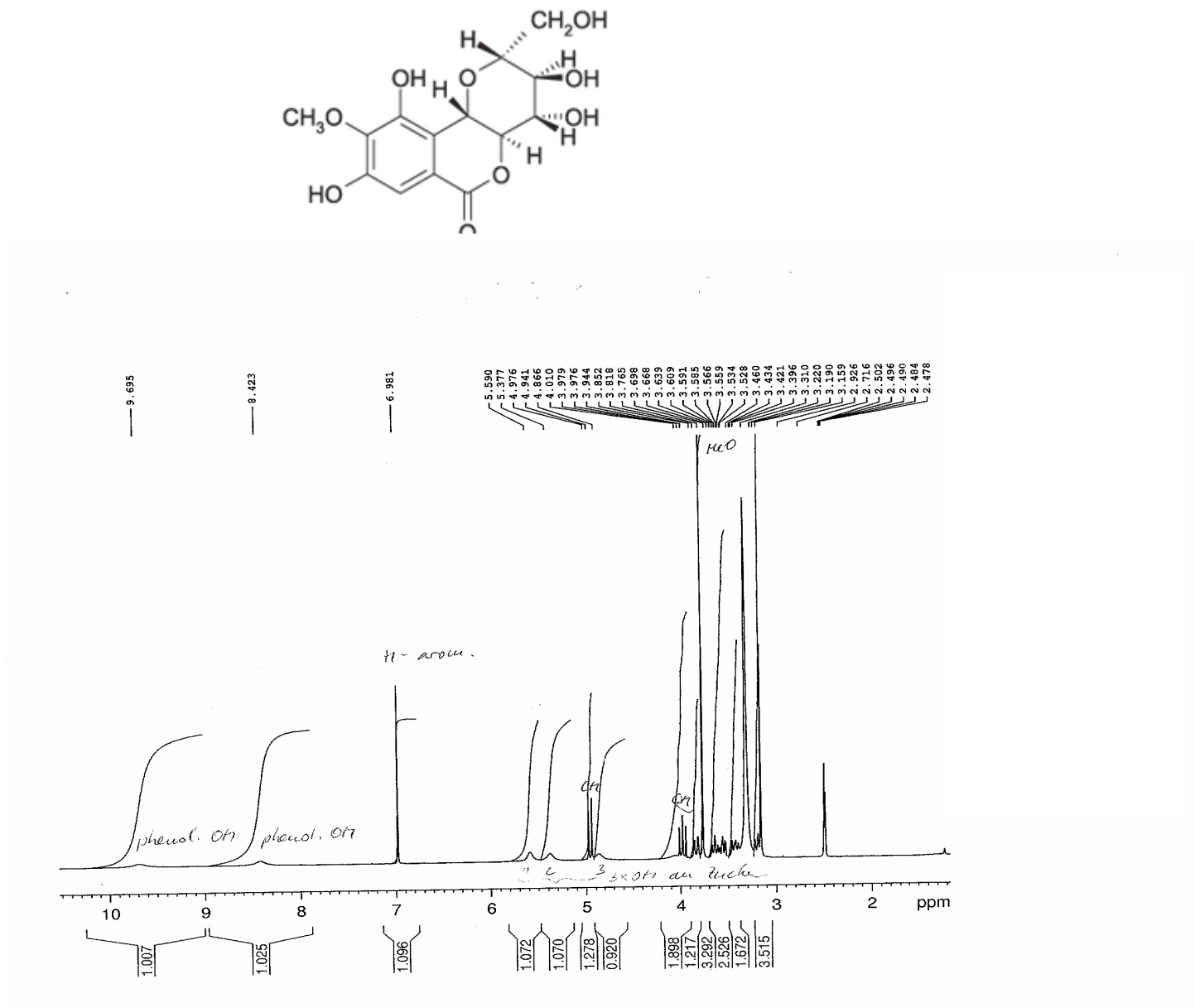

Figure 8.1: Nuclear magnetic resonance (NMR) of bergenin

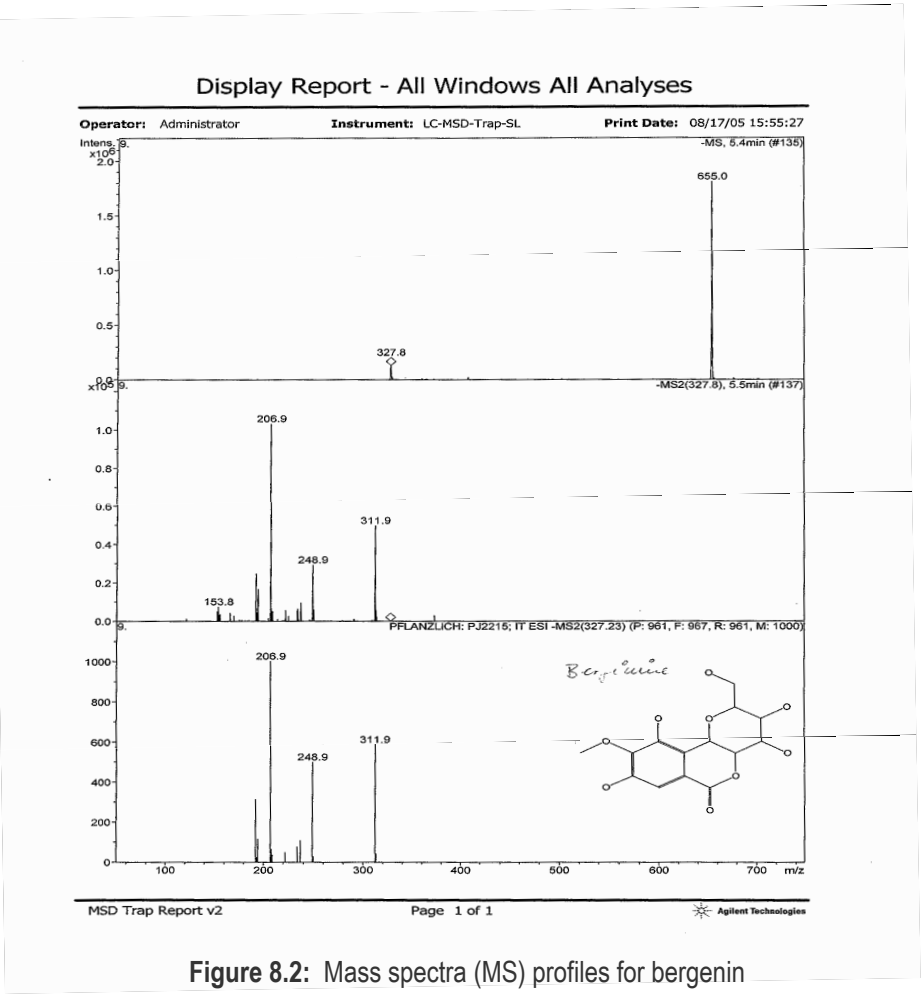

Figure 8.2: Mass spectra (MS) profiles for bergenin

Table 8.1: Cytotoxicity and antioxidant activities of bergenin

Item	Brine shrimp[a] LC_{50} (µg ml^{-1})	MTT[b] LC_{50} (µg ml^{-1})	Antioxidant EC_{50} (µg ml^{-1})
Bergenin	>1000	>1000	5.86± 0.41
Podophytotoxin	7.01		
Berberine		9.82	
Ascorbic acid			5.03± 0.5

Key:
[a]Brine shrimp larval mortality assay
[b]Cytotoxicity assay aginst Vero Monkey kidney cell line
Solutions at 5 mg ml^{-1} , the highest concentration used

Bergenin was obtained as a brown powder with a molecular formula of $C_{14}H_{16}O_9.H_2O$. From ESI-MS, the mass was found to be 328. Proton NMR, ^{13}C NMR and DEPT analyses were performed. The ^{13}C together with DEPT spectrum revealed 14 carbons with carbon signals: δ 118.03 (s), 115.93 (s), 148.04 (s), 140.59 (s), 150.92 (s), 109.50 (d), 163.34 (s), 72.17 (d), 73.73 (d), 79.82 (d), 70.71 (d), 81.77 (d), 61.16 (t), 59.84 (q). After comparing the spectra with literature [20], it deduced as bergenin.

At the maximum concentration used (5 mg ml^{-1}) bergenin did not show toxicity in both brine shrimp and Vero monkey kidney cell line (Table 8.1).

Bergenin had more antioxidant activity than L-Ascorbic acid (Vitamin C), the respective EC_{50} values being 5.86 and 5.03 µg ml^{-1}(Table 8.1).

Bergenin showed good antibacterial activity against *S. salmonicolor*, moderate action against *M. vaccae*, *P. aeruginosa* , and *E.coli*, and no activity against *P. notatum* (Table 8. 2).

Table 8.2: Antimicrobial activity of bergenin against five microbial species

Microorganism	Zone of inhibition	
	(mm)	Colonies
E.coli (SG 458)	15	p
P. aeruginosa (K 799/61)	17	p
Myco. vaccae (IMET 10670)	19	p
S. salmonicolor (SBUG 549)	26	
P. notatum (JP 36)	13	p

Key:
0-15 mm no activity
16-20 mm moderate activity
21-25 mm good activity
> 25 mm strong activity
p= few colonies in the inhibition zone (moderate activity)

There was no activity by bergenin against feline herpesvirus (results not shown). The tissue culture infectious dose 50 ($TCID_{50}$) was used to calculate the antiviral activity of bergenin. Antiviral activity is the difference between the virus titre in the absence and in the presence of the compound (δ $\log_{10}$ $TCID_{50}$/ml). Cytopathic effect (CPE) was determined by microscopic examination of the cells, and the results used to calculate the $TCID_{50}$. In the case of bergenin, there was no difference between the virus titre in the presence of the compound and in its absence.

8. 4 Discussion

Bergenin (molecular formula $C_{14}H_{16}O_9.H_2O$), technically called **3,4,8,10-tetrahydroxymethyl-9-methoxy-3,4,4a, 10b-tetrahydro-2H-pyranol[3,2-c]isochromen-6-one** appears to be a major constituent of the root extract of *P. africanum* since more than 5.641 g of pure bergenin was isolated from 36.06 g of the acetone extract of the plant. It was surprising that all the crystalline compounds that were isolated at different processes turned out to be pure bergenin. Indeed bergenin is a constituent of many medicinal plants, having first been isolated from *Saxifraga stolomifera* by Masotoshi *et al*. [20]. However, it has since then been isolated from other medical plant. Bergenin was first isolated from *P. africanum* ethanolic extract of heartwood by Bam *et al*. [5] and of the bark by Mebe and Makuhunga [7]. This is the first time it is reported isolated from the acetone extract of the root of the plant.

Bergenin was found to have antioxidant activity comparable to L-ascorbic acid (Vitamin C), with their respective EC50 values being 5.86 and 5.03 µg ml^{-1} . Though the antioxidant activity of bergenin was lower than that of the bark and root extract , their respective EC_{50} values being 4.37 and 3.82 µg ml^{-1} (as reported by Bizimenyera *et al*. [11]), bergenin nevertheless might be contributing significantly to the antioxidant activity of *P. africanum*. Antioxidant activity of higher plants is largely due to phenols, however, and various phenolic compounds have been reported in the plant [3, 4, 5, 6, 8]. Various studies of natural and synthetic bergenin, have shown the compound to protect against hepatic toxicity [21, 22], treatment of peptic ulcers [23, 24], anti-HIV/AIDS activity [25, 14] and neuroprotective and antioxidant activity [26]. The present work found bergenin to have strong antioxidant activity, comparable to ascorbic acid (Vitamin C). The abundance of bergenin in *P. africanum*, offers prospects for use of the plant extracts in control of neurodegenerative diseases [11].

The brine shrimp and Vero monkey kidney cell line cytotoxicity assay results have showed that bergenin is not toxic. Brine shrimp assay has been frequently used in *in vitro* cytotoxicity tests [15]. The test is routinely used in the toxicity tests of plant extracts [27, 28, 29] in South Africa. However, some plants known to be toxic to livestock have displayed non-toxicity to brine shrimp [30], prompting skepticism that the brine shrimp assay is capable of detecting toxic effects of plant extracts. That is why cell-line cytotoxicity was applied alongside the brine shrimp assay for the compound in the present work; mammalian cell line gives better correlation.

Antimicrobial (against Gram-positive and Gram-negative bacteria and fungi) activities were displayed by bergenin. Bergenin may be acting in synergy with other compounds, since its activity is less than the crude extract in both cases. This would further appear to validate the traditional use of the plant against infection related diseases [10].

In conclusion, there is a great potential for *P. africanum* Sond. (Fabaceae) in promotion of health in both man and animals. This may in part be because of containing phenolic compounds like bergenin that have antibacterial and antioxidant activity. Synergistic activity of plant-derived antioxidants has been proposed as a mechanism by which HIV replication and immune cell killing (apoptosis) in HIV infected people can be reversed. However suitable laboratory and clinical studies are called for, in addition to further search for effective compounds from plants such as *P. africanum*. Furthermore, if the active compounds were isolated, their synthetic varieties could be made available. This would reduce on the demand on the plant material, with a view to conserving the environment.

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CHAPTER 9

POTENTIAL OF NEUROPROTECTIVE ANTIOXIDANT-BASED THERAPEUTICS FROM *PELTOPHORUM AFRICANUM* SOND. (FABACEAE)

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Annexure 4)

Abstract

There is ample scientific and empirical evidence supporting the use of plant-derived antioxidants for the control of neurodegenerative disorders. Antioxidants may have neuroprotective (preventing apoptosis) and neuroregenerative roles, by reducing or reversing cellular damage and by slowing progression of neuronal cell loss. Although demand for phytotherapeutic agents is growing, there is need for their scientific validation before plant–derived extracts gain wider acceptance and use. We have evaluated antioxidant potential of *Peltophorum africanum* (weeping wattle), a plant widespread in the tropics and traditionally used, *inter alia*, for the relief of acute and chronic pain, anxiety and depression. The dried leaves, bark and root of *P. africanum* were extracted with acetone. Thin layer chromatograms were sprayed with 0.2% 2,2-diphenyl-1-picryl hydrazyl (DPPH) in methanol for screening for antioxidants. Quantification of antioxidant activity was assessed against 6-hydroxy-2, 5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and L-ascorbic acid (both standard antioxidants), using two free radicals, 2,2´-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and DPPH, respectively. Results of our study show that the bark and root extracts had higher antioxidant activity than L-ascorbic acid and Trolox, a synthetic vitamin-E analogue. The respective TEAC (Trolox Equivalent Antioxidant Capacity) values for the bark and root extracts, and Trolox were 1.08, 1.28 and 1.0. L-ascorbic acid (5.04 µg/mL) was more active than the leaf 6.54 (µg/mL), but much less active than the bark (4.37 µg/mL) and root (3.82 µg/mL) extracts. Continued work on *P. africanum*, and other plants rich in antioxidants, may avail neuroscientists with potent neuroprotective antioxidant therapeutics.

Keywords: Antioxidant; Extracts; Neurodegeneration; Neuroprotection; Oxidative stress; *Peltophorum africanum*

9.1 Introduction

Oxidative stress is the result of an imbalance in the pro-oxidant /antioxidant homeostasis leading to the generation of excess reactive oxygen species (ROS), implicated *inter alia* in the cause of carcinogenetic, inflammatory, infectious, cardiovascular and neurological diseases in man and animals (Nair *et al*., 2003). Under normal conditions, the body is equipped with defense mechanisms that scavenge ROS and protect the cells from oxidative damage. However, the detoxifying enzyme processes get overwhelmed, saturated, and faulty under conditions of low dietary antioxidant intake, inflammation, aging or exposure to environmental factors such as irradiation or tobacco smoke, inducing some enzymes like cyclooxygenase-2 (COX-2), lipoxygenase (LOX) and inducible nitric acid synthase (iNOS) that generate intermediaries that damage cellular macromolecules including DNA (Floyd, 1999; Rao and Balachandran, 2000; Nair *et al*., 2003). The damage is made on proteins, lipids, and nucleic acids signaling cascades leading to disruption of ion homeostasis and modification of the genetic apparatus, with consequence of apoptotic cell death (Sun and Chen, 1998; Singh *et al*., 2004). The brain is in particular very sensitive to oxidation stress possibly because of its high lipid content, high aerobic metabolic activity and low catalase activity (Halliwell and Gutteridge, 1985; Cao *et al*., 1988; Floyd and Carney, 1992; Gilgun-Sherki *et al*., 2001.

Antioxidants (AOX) are considered a promising therapeutic approach as they may be playing neuroprotective (preventing apoptosis) and neuroregenerative roles (Moosmann and Behl, 2002). Plant-derived antioxidants offer prospects in this regard. In nature, AOX are grouped as endogenous or exogenous. The endogenous group includes enzymes (and trace elements part-of) like superoxidase dismutase (zinc, manganese, and copper), glutathione peroxide (selenium) and catalase, and proteins like albumin, transferrin, ceruloplasmin, metallothionein and haptoglobin. The most important exogenous AOX are dietary phytochemicals (such as polyphenols, quinones, flavonoids, catechins, coumarins, terpenoids) and the smaller molecules like ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E) and beta-carotene vitamin-E, and supplements. The antioxidant processes occur in cytosol, mitochondria or in plasma (Larson 1988; Namiki *et al*., 1993; Berger, 2005). Though their mode of action is not yet completely elucidated, and clinical trials involving them are still relatively scarce, AOX offer a promising approach in the control or slowing down progression of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis and ischaemic and haemorrhagic stroke (Maxwell, 1995; Floyd, 1999; Mattson, 2000; Moosmann and Behl, 2002; Nair *et al*., 2003; Berger, 2005). To write off antioxidants as potentially harmful, is ultimately keeping a potential weapon out of the therapeutic arsenal.
Strategies aimed at limiting ROS oxidative stress damage, may slow the progression of neurodegenerative diseases (Halliwell, 2001; Singh *et al*., 2004). Since endogenous AOX defences are not always completely

effective, and since exposure to damaging environmental factors is increasing, exogenous AOX will find more roles in diminishing the cumulative effects of oxidative damage (Gilgun-Sherki *et al*., 2001). Plant derived AOX are regarded as effective in controlling the effects of oxidative damage, and hence have had influence in what people eat and drink (Viana *et al*., 1996; Sun *et al*., 2002; Pinder and Sandler, 2004). As the focus of medicine shifts from treatment of manifest disease to prevention, herbal medicine (with its four pillars of phytochemistry, phytopharmacy, phytopharmacology and phytotherapy) is coming into consideration, being a renaissance of age-old human tradition (Weiss and Fintelmann, 2000). The 'Green' movement in Western society has changed attitudes in the general population who now conceive naturally derived substances and extracts as being inherently safer and more desirable than synthetic chemical products, with the net effect of increase in sales of herbal preparations (Houghton and Raman, 1998; Capasso *et al*., 2000). About 80% of people in the developing world rely on phytomedicine for primary healthcare for man and livestock (Plotkin, 1992; McCorkle *et al*., 1996).

However, despite the demand of phytotherapeutic agents growing (Capasso et al., 2000), most medical and veterinary professionals still distrust the use of herbal medicines, due to lack of scientific evidence of efficacy and safety (Sofowora, 1982; Thompson, 1997). Hence the need for their scientific validation before plant-derived extracts gain wider acceptance and use. In this regard, many plants nevertheless have been scientifically proved to be effective in control of acute and chronic nervous disorders (Table 9.1). As herbal extracts are a complex mixture of compounds, the active molecules, mode of action, bioavailability and pharmacokinetics, and toxicity issues become difficult to evaluate.

Peltophorum africanum (weeping wattle), a plant widespread in southern Africa and most tropical areas, is unique in that it is traditionally used to treat more less similar disease conditions in man and domesticated animals. The root and bark decoctions are used to treat wounds, colic (acute pains), joint and back pain (chronic pains), ascites and abdominal disorders, diarrhoea and dysentery, infertility, and depression (Watt and Breyer-Brandwijk, 1962; Venter and Venter, 1996; Van Wyk and Gericke, 2000; Manana, 2003). In southern Africa, women who lose their spouses take the bark/root decoctions for up to a year, possibly for relief of post-traumatic stress. In livestock, the plant is used against diarrhoea, dysentery, colic and as a general tonic. Pastoralists use root as a component in the '*Kgatla doctors'* mixture to promote well-being, resistance to diseases and fertility (Watt and Breyer-Brandwijk, 1962; Cunningham and Zondi, 1991; Van der Merwe, 2000). From the foregoing, the traditional use point to *P. africanum* as having antibacterial, anthelmintic, anti-inflammatory and antioxidant activities. Many of the compounds isolated by phytochemists are polyphenols, typical of antioxidants of higher plants (Larson, 1988; Paya *et al*., 1992; Braca *et al*., 2002).

It is not surprising therefore, that Bizimenyera *et al*. (2005), reported high concentrations (20-50%) of polyphenols in the extracts of *P. africanum* .

Table 9. 1 Commercialised medicinal plants proven effective in control of nervous/chronic conditions (Van Wyk and Wink, 2004)

Plant	Common name	Active ingredients	Activity / action
Harpagophytum procumbens	Devil's claw	Coumarins; phenolic glycosides	Anti-inflammatory; ant-rheumatic
Hypericum perforatum	St. John's wort	Phenolic compounds; hyperforin	Analgesic; psychomotor disturbances; anti-depressant
Withania somnifera	Winter cherry	Steroids; witherferin	Ant-inflammatory; sedative
Ginkgo biloba	Ginkgo	Flavonoids; proanthocyanidins	Enhances memory & learning; dementia; insomnia
Valeriana officinalis	Valerian	Valeranone; sesquiterpenoids	Epilepsy & insomnia; tranquilizer
Rouvolfia serpentium	Indian snake root	Indole alkaloids	Psychomotor disturbances; tranquilizer
Sutherlandia frutescens	Cancer bush	Flavonoids; triterpenoids	Cancer; general tonic
Vitis vinifera	Grape vine	Proanthocyanidins; flavonoids	Circulatory disturbance; antioxidants
Humulus lupulus	Hop plant	Phenolics; proanthoyanidins	Sedative; mood disorders
Papaver somniferum	Opium poppy	Alkaloids	Narcotic; analgesic

Hence, *P. africanum* could have potential for neuroprotective antioxidant –based therapeutics.

In the study described hereafter, the comparative antioxidant activity potential of *P. africanum* extracts was assessed against 6-hydroxy-2, 5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and L-ascorbic acid (both standard antioxidants), using two free radicals, 2,2´-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and 2, 2-diphenyl-1-picryl hydrazyl (DPPH) , respectively.

9.2 Methodology

9.2.1 Collection, storage and preparation of plant material

Leaves, stem bark and root bark were collected from mature *Peltophorum africanum* Sond. (Fabaceae) trees, growing naturally at Onderstepoort, South Africa. A voucher specimen (PM 001) is stored in the Medical Plant Herbarium, Department of Paraclinical Sciences, University of Pretoria, South Africa. The collected plant material was dried in the shade at ambient temperature, and ground to powder before extraction. A known mass of each of the powdered material was then percolated with ten volumes of acetone at room temperature for 24 hours and filtered. Acetone was used as extractant, as it has been found to extract large quantities of bioactive plant material (Eloff, 1998). The extracts obtained were concentrated under vacuum at 40 ^{o}C using a rotary evaporator (Buchi®, Switzerland) to give the crude extracts of each plant material. The dry extracts were stored in sealed vials in the refrigerator prior to further processes.

9.2.2 Chemicals

All chemicals used were of analytical grade. L-ascorbic acid (Merck), potassium persulphate (Sigma), 2,2´-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Sigma), 6-hydroxy-2, 5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®) (Fluka), 2,2-diphenyl-1-picryl hydrazyl (DPPH) (Sigma) and absolute ethanol (Merck).

9.2.3 Evaluation of antioxidant activity

Qualitative screening for antioxidant activity was done using 2, 2-diphenyl-1-picryl hydrazyl (DPPH) according to Takao *et al*. (1994). Thin layer chromatograms (TLC) of extracts developed in EMW (ethyl acetate / methanol / water (10 / 1.35 / 1) solvent system were 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 (Bors *et al*., 1992).

Quantification of AOX activity was determined spectrophotometrically using two radicals, ABTS and DPPH and the Versa-max® microplate reader (Labotec). In one method, use was made of theTrolox equivalent antioxidant capacity (TEAC) assay (Re *et al*., 1999) based on the scavenging of the ABTS radical into a colourless product. The absorbance was read at 734 nm. This method was also used in a previous analysis

(Bizimenyera *et al*., 2005). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid) is a Vitamin-E analogue. If an extract had antioxidant activity equivalent to Trolox, its TEAC value would be 1 and if the extract were more active its TEAC would be greater than 1.

The second method described by Mensor et al. (2001), employed the DPPH free radical assay. Different concentrations of the extracts were prepared between 20.0 and 1.0 µg/ml. Ten µL of 0.4 mM DPPH in ethanol was added to 25 µL of each concentration of extract tested and allowed to react at room temperature in the dark for 30 minutes. Blank solutions were prepared with each test sample solution (25 µL) and 10 µL ethanol only while the negative control was DPPH solution, 10 µL plus 25 µL ethanol. L-ascorbic acid was the positive control. The decrease in absorbance was measured at 518 nm. Values obtained were converted to percentage antioxidant activity (AOXA%) using the formula: -

$$AOXA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}$$

Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control.

L-ascorbic acid (vitamin C) was used as a positive control (antioxidant agent). The antioxidant activity is expressed as effective concentration (EC_{50}) values. The lower the EC_{50} value, the more effective antioxidant activity. The EC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots of concentration of the test extracts (µg/mL) against the mean percentage of the antioxidant activity obtained from three replicate assays. For statistical analysis, the results were expressed as mean ± SEM (standard error of mean) and the EC_{50} values obtained from the regression plots (SigmaPlots^R 2001, SPSS) showed a good coefficient of determination, with most values being $r^2 \geq 0.910$.

9.3 Results

All the extracts (leaf, bark and root), from qualitative screening, contained compounds that exhibited considerable free radical scavenging activity, as shown by the yellow bands (of antioxidant activity) on DPPH chromatograms, Figure 9.1. The root and bark had more antioxidant activity compared to the leaf. The antioxidant activity of the root and bark extracts was higher than both L-ascorbic acid and Trolox (Table 9. 2).

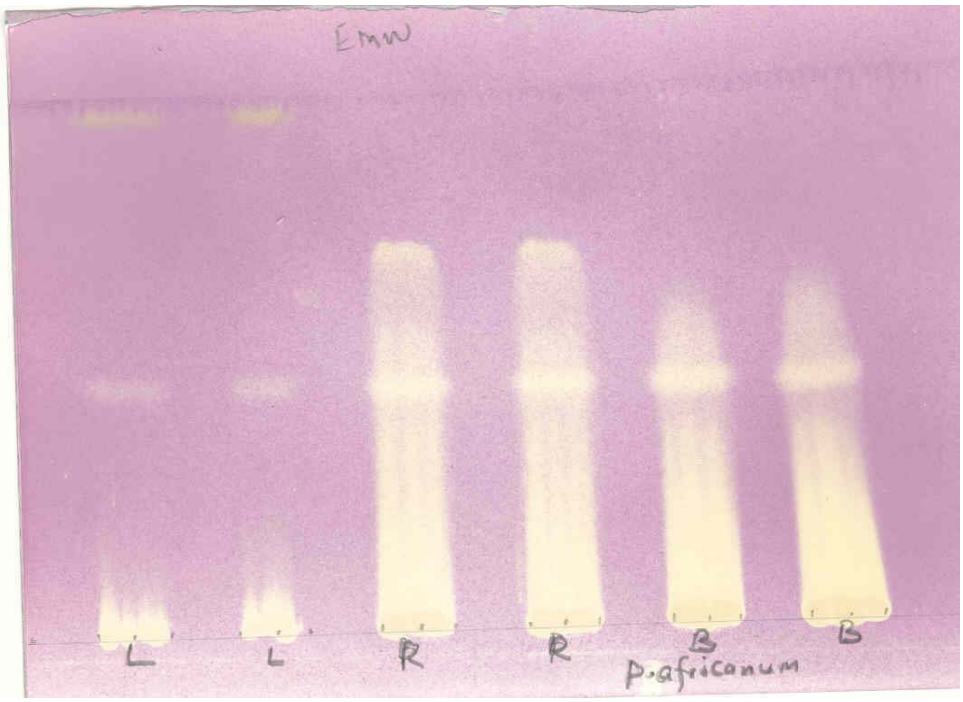

Figure 9. 1 Chromatogram of 200 µg acetone extracts of leaf (L), bark (B) and root (R) of *P. africanum* , separated with EMW and sprayed with DPPH. Note the high antioxidant activity, indicated by yellow areas

Table 9.2 Trolox (TEAC) and Vitamin-C equivalent[#] values of acetone extracts of leaf, bark and root of *P. africanum*

Plant part		**AOX Values***	
		TEAC	**DPPH(EC_{50}±SEM (µg/ml)**
Leaf		0.57	6.54± 0.49
Bark		1.08	4.37± 0.41
Root		1.28	3.82± 0.58
Standards	Trolox	1.0	N/A
	L-ascorbic acid	N/A	5.04± 0.65

{Note: - [#] If the extract had antioxidant activity equivalent to Trolox, its TEAC value would be 1 and if the extract was more active its TEAC value would be greater than. The reverse is true with DPPH.; the values above 5 mean less antioxidant activity and those lower than 5 mean higher activity}

The bark and root extracts had higher TEAC values than Trolox (Vitamin-E analogue), with respective values of 1.08, 1.28 and 1.0. L-ascorbic acid (5.04 µg/mL) was more active than the leaf 6.54 (µg/mL), but much less active than the bark (4.37 µg/mL) and root (3.82 µg/mL) extracts (the higher the µg/mL value, the less AOX activity).

9.4. Discussion and conclusion

Laboratory results showed the extracts had high levels of antioxidant compounds, especially the root and bark extracts. Both Trolox and L-ascorbic acid have been used as standards in quantifications of antioxidant activity (Van den Berg *et al*., 1999; Fukomoto and Mazza, 2000). The EC_{50} of the root (3.82 µg/ml) and bark (4.37 µg/ml) extracts indicated higher antioxidant activity than L-ascorbic acid (5.04 µg/ml), and more effective than *Ginkgo biloba* extract (EGb 761) whose EC_{50} is 40.72 µg/mL (Mensor *et al*., 2001; Bridi *et al*., 2001; Aderogba *et al*., 2004). The standardised extract of *Ginkgo biloba* (EGb 761) has been widely employed for its significant benefit in neurodegenerative disorders (Bridi *et al*., 2001).

The antioxidant activity in plants may largely be due to polyphenols (Thabrew *et al*., 1998), and in particular polyphenols make up nearly 50 % of *P. africanum* root and bark extracts (Bizimenyera *et al*., 2005). The presence of antioxidant compounds also agrees with the phytochemical analyses by other investigators (El Sherbeiny *et al*., 1977; Evans *et al*., 1985; Bam *et al*., 1988 and 1990; Khattab and Nasser, 1998; Mebe and Makuhunga, 1992) who isolated flavonoids, coumarins, gallic acid and other polyphenols from *P. africanum* extracts.

The root and bark appear to have the highest concentrations of the AOX. Coincidentally, traditional healers also use the root and bark concoctions (Watt and Breyer-Brandwijk, 1962; Cunningham and Zondi , 1991; Venter and Venter, 1996; Van der Merwe, 2000; Van Wyk and Gericke, 2000; Manana, 2003). In all the traditional treatments, decoctions and infusions are made from the root or bark. That is why the present authors believe the rationale for traditional healers using *P. africanum* in treating inflammation, pain and depression may be due to high AOX compounds present in the plant.

Various chronic degenerative diseases with different clinical appearances appear to share common biochemical, genetic and cellular alterations that are related to disease pathogenesis (Nair *et al*., 2003; Barnharm *et al*., 2004). Empirical evidence suggests *P. africanum*, due to its high AOX activity, could be influential in the control of these conditions. Mixtures of dietary antioxidants or foods rich in antioxidants have been shown to increase the ability of lymphocytes to withstand DNA oxidation (Duthie *et al*., 1996; Pool-Zobel *et al*., 1997; Porrini and Riso, 2000). Diets rich in flavonoids appear to be protective against ischemic heart disease (Hertog *et al*., 1993; Viana *et al*., 1996; Berger, 2005) and supplementing diets with antioxidants generally appears preventing occurrence and progression of many neurodegenerative diseases (Rice-Evans and Diplock, 1993; Rice-Evans *et al*., 1995; Prasad *et al*., 1999; Singh *et al*., 2004).

The safety of P. *africanum* extracts still has to be confirmed. From inquiries from pastoralists (Van der Merwe, 2000) and Pretoria market vendors of traditional herbs, *P. africanum* extracts were reported safe. Tests in our laboratory involving acetone extracts on monkey kidney cells and brine shrimp (awaiting publication) showed no toxicity effects at the highest dose of 5 mg ml^{-1}used. Bessong *et al*. (2005) reported no toxicity of the extracts. This, however, is no cause for complacency, as toxicity of herbal medicines has been reported (Capasso *et al*., 2000).

This paper reports *P. africanum* leaf, bark and root extracts as showing considerable antioxidant activity. The root and bark had more antioxidants than the leaf. The antioxidant activity of the root and bark extracts, was higher than L-ascorbic acid (Vitamin-C) and Vitamin-E equivalent (Trolox), both standard antioxidants. The results of these *in vitro* tests would appear to justify the traditional use of the plant in treatment of acute and chronic nervous disorders. Antioxidants appear to have neuroprotective and neurodegenerative roles, reducing or slowing down neuronal cell death, and have an important role to play in diminishing the cumulative effects of oxidative damage. Further *in vivo* research and clinical trials are required for generation of conclusive data for use of the plant in treating neurodegenerative diseases. Continued work on plants like *P. africanum*, rich in antioxidants, may avail medicine with antioxidant-based neuroprotective therapeutics.

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CHAPTER 10

GENERAL DISCUSSION AND CONCLUSION

10.1 *Peltophorum africanum* in traditional medicine

This work was stimulated by the reports by van der Merwe (2000) and Manana (2003). The former reported the plant being used by pastoralists of Madikwe area for diarrhoea and dysentery in cattle as well as tonic for well being and general resistance to disease in cattle. The latter found *P. africanum* to be a common medicinal plant in Pretoria medicinal plant market, where the healers claimed the bark had anti-inflammatory activity and was also used for diarrhoea, dysentery, abdominal pains, and helminthosis in people. Earlier, Mabogo (1990) had reported the plant to be used for stomach complaints, intestinal parasites and pain in people. Another dimension of use by traditional health workers has been in relief of anxiety and depression. The bark or root extract is taken by women who lose their spouses for up to a year (Watt and Breyer-Brandwijk, 1962), possibly for relief of post-traumatic stress. From the foregoing, .*P. africanum* was considered to have antimicrobial, anthelmintic and immune boosting activities. There is so much utilisation of *P. africanum* by traditional healers that in some parts of South Africa the plant has been stripped bare through removal of the bark (Mabogo, personal communication).

10.2 Extraction

Extractable compounds from the bark and root were mainly polar, as non polar solvents (hexane and dichloromethane) extracted very little (quantity) material from *P. africanum*, (Chapter 3). Most of the compounds isolated from the plant by many chemists or phytochemists (El sherbeiny *et al*., 1977; Evans *et al*., 1985; Bam *et al*., 1988 and 1990; Khattab and Nasser, 1998; Mebe and Makuhunga, 1992; Bessong *et al*., 2005) have been polyphenols (flavonoids, coumarins, catechins, gallic acid). Acetone was selected as a suitable solvent for the extracts used in bioassays as it extracts compounds of wide polarity range, is miscible with organic and aqueous solvents, and is non-toxic to test organisms. Bergenin in the present work was isolated from the acetone extracts of the root.

10.3 Antibacterial activity

The acetone and ethanol extracts showed considerable antibacterial activity, against both Gram-positive and Gram-negative bacteria (Chapter 3). Using the microplate dilution method of Eloff (1998 a), the minimum inhibitory concentration (MIC) values of 0.08 mg ml^{-1} were obtained for ethanol extracts of the root and dichloromethane extracts of the leaf against *Staphylococcus aureus*. The MIC value for acetone and ethanol extracts of the bark and root against *Pseudomonas aer*uginosa were 0.16 mg ml^{-1}. Bergenin isolated from the acetone extracts of the root had moderate inhibitory action against both *P. aeruginosa* and *E. coli*, by the disc diffusion method of Bennet *et al*. (1996). Bergenin had good antimicrobial activity against *Sporobolomyces salmonicolor* though no activity against *Penicillium notatum* (Chapter 8).

The results of the current work confirmed reports of previous investigators of antimicrobial action of *P. africanum* extracts (Manana, 2003; Obi *et al*., 2003; Samie *et al*., 2003). Manana (2003), using the microdilution technique, found acetone extracts of the bark to have MIC values of 0.08 against *S. aureus*. Samie *et al*. (2003) and Obi *et al*. (2003) employing the disc diffusion method demonstrated good antibacterial activity of the methanol and ethanol extracts of the bark and root. Although to date there are no reports of *in vivo* antibacterial trials with *P. africanum* extracts, the results of *in vitro* work reported would appear to validate the traditional use of the plant extracts for treatment of bacterial diseases of the gastrointestinal tract that cause diarrhoea. The traditional use of *P. africanum* extracts against diarrhoea, dysentery and unthriftness could as well be treating gastrointestinal nematodes, as they share similar clinical symptoms.

10.4 Antioxidant activity

P. africanum is one of the plants rich in antioxidants, plants that have found much use in traditional medicine. The antioxidants appear to be concentrated more in the root and bark than the leaf (Chapters 3 and 9). Antioxidant activity in plants is largely attributable to polyphenols (Thabrew *et al*., 1998). Other investigators have isolated flavonoids, coumarins, gallic acid, catechin, and other polyphenols from *P. africanum* (El Sherbeiny *et al*., 1977; Evans *et al*., 1985, Bam *et al*., 1088, 1990; Mebe and Makuhunga, 1992). The antioxidant activity of the root and bark extracts was higher than L-ascorbic acid (Vitamin C) and Vitamin–E equivalent (Trolox). Traditional healers also use the root and bark concoctions (Watt and Breyer-Brandwijk, 1962; Cunningham and Zondi, 1991; van der Merwe, 2000; Manana, 2003).

Antioxidants are a promising approach in the fight against human immunodeficiency virus (HIV), cardiovascular and neurodegenerative diseases, conditions associated with pro-oxidative conditions in the

body with overabundance of reactive oxygen species (Greenspan *et al*., 1994; Maxwell, 1995; Floyd, 1999; Mattson, 2000; Moosmann and Behl, 2002; Nair *et al*., 2003; Berger, 2005). Compounds of plant origin have been identified that inhibit replication of human immunodeficiency virus (HIV), slowing progression and limiting apoptosis of immune cells (Greenspan *et al*., 1994; Pengsuparp *et al*., 1995; Vlietinck *et al*., 1998; Asres *et al*., 2001). The extracts of *P. africanum* (Bessong *et al*., 2005) and bergenin (Piacente *et al*., 1996) which we have also isolated from the *P. africanum* root extracts has inhibitory effect against HIV. A synergistic action of plant oxidants has been proposed as one of the mechanisms by which replication and cell killing (apoptosis) in HIV infection is inhibited (Greenspan *et al*., 1994). It is not surprising that the extracts of *P. africanum* are used against HIV infections in Botswana (Hargreaves and Mosesane, 2003). The traditional use of the extracts of *P. africanum* by women after losing their spouses (Watt and Breyer-Brandwijk, 1962), could imply therapeutic effects of its compounds on depression and post-traumatic stress, and therefore give the plant a role in neuroscience.

10.5 Anthelmintic activity

The extracts of *P. africanum* had significant inhibitory effect on the egg hatching and larval development of the parasitic nematodes, *Haemonchus contortus* and *Trichostrongylus colubriformis in vitro* (Chapters 4 and 5) at a concentration of 0.2-1 mg ml^{-1}. Though tannins in plant extracts have anthelmintic activity on their own, some anthelmintic activity was retained in tannin-free extract and in the bergenin isolated from the root extract. Traditional healers use extracts of *P. africanum* for gastrointestinal helminths and other abdominal disorders (Watt and Breyer-Brandwijk, 1962; Venter and Venter, 2002). The traditional use of the plant extracts against diarrhoea and dysentery may in fact be due to anthelmintic activity, as these clinical symptoms are consistent with parasitic gastroenteritis. *In vitro* anthelmintic activity of *P. africanum* has been shown against cestodes (Mølgaard *et al*., 2001).

The translation of the *in vitro* anhelmintic activity did not produce significant action in lambs artificially infected with *H. contortus* and *T. colubriformis,* when the extracts were administered at 50, 500 and 750 mg kg^{-1} (Chapter 6). However, this does not nullify traditional usage of the tree, or *in vitro* results reported as higher doses of the extracts may have been more effective. Nevertheless, the extracts did not show toxicity to the lambs even at the highest dose (750 mg kg^{-1}) administered.

Parasitic gastrointestinal nematodes rank as number one cause of production losses in small ruminants (sheep and goat) livestock systems in the tropical and subtropical regions of the world (Perry *et al*., 2002). Due to anthelmintic resistance, and exorbitant cost of pharmaceutically derived drugs (Prichard, 1990; Wolstenholme

et al., 2004; Jabbar *et al*., 2006) there is a trend to seek plant anthelmintics as one of the alternative and cost benefit ways of controlling helminth infections (Hammond *et al*., 1997; Danø and Bøgh, 1999).

10.6 Safety of *Peltophorum africanum*

P. africanum extracts were not found toxic in both brine shrimp and Vero monkey kidney cell line assays (Chapter 7). Bergenin, a major compound of its root was not toxic either (Chapter 8). In a parallel work in lambs infected with nematode parasites, the extracts at 750 mg kg^{-1} did not show any toxic or abnormal effects in the lambs. Toxicity of *P. africanum* has not been reported, by both traditional human and veterinary practitioners. Bessong *et al*. (2005) reported no toxic effect on the HeLaP4 (human epitheloid carcinoma cell line) by the aqueous and methanol extracts of *P. africanum*.

P. africanum is a valuable plant in the veld as its leaves and twigs are eaten by the elephant, rhino, giraffe, kudu, impala and grey duicker, and also browsed by the goats and sheep (Venter and Venter, 2002; van Wyk and Gericke, 2000). It is a tree that grows up to 15 metres tall, with a wide canopy. The common name 'weeping wattle' is due to sap-sucking insects (*Ptyelus grossus*) that attach to the branches, and some of the sap drips to the ground. The larvae of the Van Son's charaxes (*Charaxes vansoni*), Satyr charaxes (*C. ethalion*) and and Braines's charaxes (*C. brainei*) feed on the leaves (Venter and Venter, 2002).

Other than the bitter taste, possibly attributed to the tannin content, *P. africanum* extracts appear to be safe. The apparent lack of toxicity of *P. africanum* leads support to the promotion of its use in traditional medicine. However, more controlled laboratory and clinical tests are required to ascertain the full details its efficacy and safety.

10.7 Conclusions

P. africanum is a widely distributed plant in southern Africa and other tropical regions. In Botswana, because of its ubiquity, and wide availability, *P. africanum* has earned the name '*Lejaja*'. Because of its antibacterial and antioxidant activities, it has good potential in treatment of infection-related diseases either by inhibiting growth of pathogens or indirectly by stimulating the immune system of the host. The use against diarrhoea may be due to its effect on enteric microbial or parasitic agents. *In vitro* activity of *P. africanum* extracts against HIV, and the rich antioxidant content may imply the plant has potential in the management of HIV-AIDS. The rich antioxidant content further puts the plant in line for treatment of neurodegenerative and cardiovascular diseases. There is thus good potential of *P. africanum* in medicine.

However the use of the bark and root by traditional healers, may ultimately lead to exhaustion of the plant resources.

As a result of this study on *P. africanum* a number of recommendations are outlined:-

1. Study of the activity of extracts to determine their stability and seasonal or regional variation of action,
2. Isolation of potent anthelmintic ,antioxidant and antibacterial compounds,
3. Devising better extraction methods and potentiating procedures would yield extracts with a higher activity,
4. More clinical efficacy and safety trials using extracts or isolated compounds, and
5. Preparation of suitable dosages (under low level technology adaptable to rural conditions) for use by resource-poor rural communities.

CHAPTER 11

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APPENDICES/ANNEXURES

Article — Artikel

Rationale for using *Peltophorum africanum* (Fabaceae) extracts in veterinary medicine

S E Bizimenyera[a], G E Swan[a], H Chikoto[a] and J N Eloff[a*]

ABSTRACT

Peltophorum africanum (Fabaceae) is a deciduous tree widespread in southern Africa. The plant has many ethnomedical and ethnoveterinary uses. Root 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 5 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 bacterial infection related diseases.

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

Bizimenyera S E, Swan G E, Chikoto H, Eloff J N **Rationale for using *Peltophorum africanum* (Fabaceae) extracts in veterinary medicine**. *Journal of the South African Veterinary Association* (2005) 76(2): 54–58 (En.). Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, Private Bag X04, Onderstepoort, 0110 South Africa.

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.[11] 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 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 15 m 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].

The phytochemistry of *P. africanum* has been studied by several authors. A sulphate ester of trans-4-hydroxypipecolic acid has been isolated from the seed[10]. 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 Nassar[14] isolated coumarins from the leaves. The chemical structures of the novel compounds isolated were elucidated but the biological activity of isolated compounds has hardly been investigated. 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] in an unpublished M.Sc. thesis of a member of our group. These results further motivated this study.

Use of the bark and root are destructive practices that may lead to destruction of resources and even to 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 not be used. Suitable methods of extraction of bioactive compounds, adapted to resources available in rural communities, could be developed for sustainable use of *P. africanum* extracts in primary health care practices.

MATERIALS AND METHODS

Collection, preparation and storage of plant material

Leaves, stem bark and root bark (referred to as leaf, bark and root in this article) were

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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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In vitro activity of *Peltophorum africanum* Sond. (Fabaceae) 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 °C in the extracts of the leaf, bark and root of *P. africanum* at concentrations of 0.008–25 mg ml^{-1} for 2 and 5 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 0.2–1.0 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.

Keywords: Ovicidal; Larvicidal; Extracts; *Trichostrongylus colubriformis*; *Peltophorum africanum*

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

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POTENTIAL OF NEUROPROTECTIVE ANTIOXIDANT-BASED THERAPEUTICS FROM *PELTOPHORUM AFRICANUM* SOND.(FABACEAE)

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Abstract

There is ample scientific and empirical evidence supporting the use of plant-derived antioxidants for the control of neurodegenerative disorders. Antioxidants may have neuroprotective (preventing apoptosis) and neuroregenerative roles, by reducing or reversing cellular damage and by slowing progression of neuronal cell loss. Although demand for phytotherapeutic agents is growing, there is need for their scientific validation before plant–derived extracts gain wider acceptance and use. We have evaluated antioxidant potential of *Peltophorum africanum* (weeping wattle), a plant widespread in the tropics and traditionally used, *inter alia*, for the relief of acute and chronic pain, anxiety and depression. The dried leaves, bark and root of *P. africanum* were extracted with acetone. Thin layer chromatograms were sprayed with 0.2% 2,2-diphenyl-1-picryl hydrazyl (DPPH) in methanol for screening for antioxidants. Quantification of antioxidant activity was assessed against 6-hydroxy-2, 5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and L-ascorbic acid (both standard antioxidants), using two free radicals, 2,2′-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and DPPH, respectively. Results of our study show that the bark and root extracts had higher antioxidant activity than L-ascorbic acid and Trolox, a synthetic vitamin-E analogue. The respective TEAC (Trolox Equivalent Antioxidant Capacity) values for the bark and root extracts, and Trolox were 1.08, 1.28 and 1.0. EC_{50} values for L-ascorbic acid (5.04 µg/mL) was more active than the leaf 6.54 (µg/mL), but much less active than the bark (4.37 µg/mL) and root (3.82 µg/mL) extracts. Continued work on *P. africanum*, and other plants rich in antioxidants, may avail neuroscientists with potent neuroprotective antioxidant therapeutics.

Key words: Antioxidant; Extracts; Neurodegeneration; Neuroprotection; Oxidative stress; *Peltophorum africanum*

Introduction

Oxidative stress is the result of an imbalance in the pro-oxidant/antioxidant homeostasis leading to the generation of excess reactive oxygen species (ROS), implicated *inter alia* in the cause of carcinogenetic, inflammatory, infectious, cardiovascular and neurological diseases in man and animals (Nair et al., 2003). Under normal conditions, the body is equipped with defense mechanisms that scavenge ROS and protect the cells from oxidative damage. However, the detoxifying enzyme processes get overwhelmed, saturated, and faulty under conditions of low dietary antioxidant intake, inflammation, aging or exposure to environmental factors such as irradiation or tobacco smoke, inducing some enzymes like cyclooxygenase-2 (COX-2), lipoxygenase (LOX) and inducible nitric acid synthase (iNOS) that generate intermediaries that damage cellular macromolecules including DNA (Floyd, 1999; Rao and Balachandran, 2000; Nair et al., 2003). The damage is made on proteins, lipids, and nucleic acids signaling cascades leading to disruption of ion homeostasis and modification of the genetic apparatus, with consequence of apoptotic cell death (Sun and Chen, 1998; Sing et