Chapter 1

Introduction and objectives

All over the world people who keep livestock have developed their own ideas and techniques for meeting the health and husbandry needs of their food, farm and work animals. Their knowledge and skills may be hundreds or even thousands of years old (Wynn and Fougére, 2007). Plants were previously the primary source of all medicines in the world and continue to provide mankind with new remedies (Van Wyk *et al.* 1997). In South Africa, several species of medicinal plants are used by many ethnic groups for the treatment of various ailments in both humans and domestic animals (Masika and Afolayan, 2002). The treatment of livestock diseases using traditional remedies is widely practiced in rural communities and the practice dates back some centuries (Smith, 1895).

Another aspect that contributes to the importance of plant medicines is that alternative healing methods have increased in significance, especially in Europe and the United States. For a long time antibiotic food additives in livestock have been used regularly to improve animal growth and production. Recently those antibiotic food additives have been banned by the European Union in its member countries (Witte, 2000). Therefore the search for alternatives has become urgent.

The World Health Organisation (WHO) has observed that up to 80% of the rural population in developing countries greatly depend on herbal or alternative medicine. Therefore it has asked its member countries to research safe indigenous medicines for their national health care. Although the efficacy of herbal remedies in developing countries is rated very highly, information is lacking on the appropriate dosage and safety of the plant materials that are used. Such information is necessary for the scientific validation of the materials, as well as to the methods applied, in order to properly evaluate the actual efficacy of these remedies (Masika and Afolayan, 2002).

1.1 Literature review

The need for new products to treat diseases in stock animals has been increasing tremendously over the past few years. Therefore the possibility of using plant extracts as an alternative has gained more and more importance.

However there are limitations to using plant products.

1. The provision of plant material is often a problem, because many plant species are threatened. We have found a way to overcome this problem by only using leaves from trees, which are a renewable source.

2. The second problem is the possible seasonal variation that would limit the collection period of the plant material.

3. Another problem is the genetic and geographic variation that occurs within plants of the same species.

4. The selection of which plants to use is often difficult since many plants with reported traditional ethnic use have shown no or very low activity as antimicrobial agents by using in vitro assays. Furthermore traditional healers usually use water extracts of plants but our results in the Phytomedicine Programme have shown that water extracts are frequently not active in *in vitro* assays.

In this thesis I will focus on exploring these limitations in order to select and investigate some plants as possible future antidiarrhoeal agents.

1.1.1 Importance of diarrhoea in animal production

Diarrhoea is especially important in animal production. Due to weak immune systems, especially in young stock, the occurrence of diarrhoea poses a big threat to animal health and productivity. Titus said in 1865 “This disease is better known than the method of cure. It is more difficult to cure this disease in horned cattle than in man, or any other animal.” (Wynn and Fougére, 2007). This disease condition has several different causes.

One of the most important causes of diarrhoea is infection with bacterial strains (e.g. *Escherichia coli*), but fungal and parasitic infections also play an important role. Certain *E. coli* strains can cause diarrhoea. The enteric or diarrhoea strains cause a fever, depression and diarrhoea in calves, followed by rapid dehydration and refusal to drink (Oberem *et al.* 2006).

Newborn calves and piglets which are deficient in immunoglobulin are much more susceptible to diarrhoea than animals with adequate levels. The stress of weaning in pigs is considered an important contributory cause of weanling diarrhoea (Blood *et al.* 1979).

Withdrawal of colostrum too early and a wrong diet, as well as giving the stock medication with laxative side effects, psychological influences, malabsorption, maldigestion, tumours or hormone–related maladies are further causes for diarrhoea (Schilcher et al, 2007). The use of antibacterial agents orally in all species may alter the intestinal microflora and permit the development of a superinfection by organisms which would not normally cause disease (Blood *et al.* 1979). Dietary diarrhoea occurs in all species and all ages, but is most common in the newborn which ingests too much milk or a diet which is indigestible (Blood *et al.* 1979).

Diarrhoea in ruminants can have many causes. If feeding of dry hay does not alleviate diarrhoea in a cow that continues to eat, phytotherapy with astringent products may be warranted. Most astringents derive their action from tannins (Wynn and Fougére, 2007).

The occurrence of diarrhoea often leads to weaning in the young, high treatment costs and the death of animals. All of this results in economical loss for the farmer. Ameliorating economic hardship was one of the reasons for the development of AFA (antibiotic feed additives). However selective pressure exerted by the use of antibiotics as growth promoters in food animals appears to have created large reservoirs of transferable antibiotic resistance in these ecosystems (Witte, 2000). This has led to the ban of antibacterial growth promoters that might interfere with human chemotherapy that has been introduced in European Union countries (Witte, 2000). The European Parliament and Council Regulation (EC) No 1831/2003 lays down provisions phasing out the authorisations of antibiotic feed additives as from 1 January 2006. Thiscreates a significant opportunity for alternative feed additives. Moreover, following the ban, countries outside the EU that import animal products such as chicken and pork meat will find it difficult to continue doing so. This opens up new opportunities for those within the European animal feed industry to supply such countries with alternative feed additives. There is also important scope for South Africa to develop plant-based alternatives to antibiotic feed additives. Further research needs to be done on alternative products to prove their effectiveness (Frost and Sullivan, 2005).

1.1.2 Medicinal plants

Herbal medicine is one of the oldest forms of treatment known and used by all traditional healers. The WHO estimates that botanical medicines are used by 70% of the world's population, and it is no surprise that people have used similar plant medicines for the animals in their care as long as animals have been associated with human life (Wynn and Fougère, 2007).

According to the WHO, a medicinal plant is defined as any plant which contains substances that can be used for therapeutic purposes or which contain precursors of chemo-pharmaceutical semi-synthesis (World Health Organization, 1979). Traditionally used medicinal plants produce a variety of compounds, some of which have known therapeutic properties (Chopra *et al.* 1992,Harborne and Baxter, 1995, Ahmad and Beg, 2001). In the case of plants with antimicrobial activity, substances that can either inhibit the growth of pathogens or kill them, and also have no or low toxicity to host cells are considered candidates for the development of new antimicrobial drugs. In recent years, antimicrobial properties of medicinal plants have been increasingly reported from different parts of the world (Nimri*et al.* 1999, Saxena *et al.* 1999).

Another important aspect of medicinal efficacy is the tannin content of the plant extracts used. It has been postulated and partly proven that this tannin content contributes a large part to the efficacy of herbal medicine in the treatment of diarrhoea (Galvez *et al.* 1991).

A major problem in the use of tannins is that they bind to proteins and therefore may be useful to treat acute diarrhoea but because the protein binding has an effect on the growth it may not be useful in treating chronic diarrhoea in animal production systems.

1.1.3 Sources of antimicrobial activity in plants

Due to their direct exposure to the environment and inability to move away from their surroundings, plants are highly susceptible to attack by pathogenic organisms. Especially important in this defence mechanism is the production of secondary metabolites, which protect plants from pathogens and herbivores. People have recognized the therapeutic properties of many of these secondary metabolites, and plants have been used for this reason for a long time in the treatment of humans and animals against infections. If we can isolate and identify the biologically active compounds in the plants, it might help us understand and prove the efficacy of plant medicine used traditionally.

1.1.4 Plant extracts and their effects

Because some plant extracts have been used in human medicine for centuries they are frequently considered safe. The reason for focusing on plant extracts rather than on isolated compounds is that scientists have focused on looking for compounds that can be used as single substance pharmaceuticals. The Phytomedicine Programme however has found substantial evidence for synergistic antimicrobial effects in plant extracts. In many cases the activity of isolated compounds was several folds lower than could be expected. Extracts, especially if they were potentized, frequently had the same level of activity as isolated compounds. (Eloff *et al.* 2006). Therefore we believe that there is a reasonable chance to develop anti-infective extracts rather than isolate single compounds that can be patented.

Over the past several years, the Phytomedicine Programme at the University of Pretoria has developed methods to determine the antimicrobial activity of plant extracts (Eloff, 1998c, Masoko *et al.* 2007). Methods have also been developed elsewhere to determine the tannin content of plant extracts (Hagerman, 1986). Some aspects of the results of the Phytomedicine Programme and those of other medicinal plant researchers that are relevant to the current project are given below:

(1) Synergy occurs when the effect of two or more compounds occurring together in an extract is greater than the sum of the effects when identical amounts of each constituent are used. Because plant extracts

contain more than one biologically active compound, synergy may explain why the activity of an extract is frequently greater than that of pure isolated compounds from the plant (Williamson, 2001).

(2) Each plant is a unique chemical factory capable of synthesizing large numbers of highly complex and new chemical substances (Farnsworth, 1984). For this reason the wide selection of plant species provides the possibility to find many natural and novel chemical substances.

(3) About 70-80% of the world population, particularly in developing countries, rely on non-conventional medicine in their primary healthcare as reported by the World Health Organisation (Akerele, 1993). The growth in popularity of over-the counter health foods (nutraceuticals) and medicinal products from plants or other natural sources has translated to a very large share of the healthcare market (Johnson, 1997).

(4) Less time and resources are used in the development of an effective extract than in the purification of a single active chemical from a plant. It is therefore more economical to produce biologically active extracts than to produce pure compounds. This concept, that a whole or partially purified extract of a plant offers advantages over a single isolated ingredient, also underpins the philosophy of herbal medicine (Eloff *et al.* 2005).

(5) The main sources of biological activity in plants are secondary metabolites (Farnsworth, 1984). Plant extracts may contain more than one antimicrobial compound, and if resistance should develop against one compound, the other compounds present in the extract could still remain active. There may also be increased stability of compounds present in an extract since some constituents may prevent the deterioration of others, e.g. antioxidant compounds like flavonoids may preserve molecules susceptible to oxidation.

(6) Eloff (2004) could show that not only the MIC values of the extracts, but also the quantity of the extracted plant material should be taken into account to determine the activity of different fractions during bioassay guided fractionation. Total activity (TA) can also be used to compare the activity between different plants by dividing the quantity in mg extracted from one gram of dried plant material by the MIC in mg/ml. The total activity indicates the degree to which the active compounds in one gram of plant material can be diluted and still inhibit bacterial growth. This takes the quantity of the extracted plant material into account. The higher the total activity of a plant extract, the more effective the original plant (Eloff, 2000).

1.1.5 Aim

It is time consuming and expensive to conduct animal experiments under controlled conditions to test the efficacy of plant species used traditionally to control diarrhoea in humans and animals. It is possible

that wider *in vitro* assays on several relevant aspects could lead to a rational selection of plant species with a good potential of delivering a useful product in combating diarrhoea in production animals. The aim of this project was to develop a formula based on *in vitro* assays to predict the potential value of traditionally used plants.

1.1.6 Objectives

The objectives of this research project are to:

1. Evaluate the magnitude of seasonal variation in the antibacterial activity of 5 selected plant species collected monthly by determining their antibacterial activity against *E. coli* and *S. aureus* and by measuring their tannin content.
2. Evaluate the genetic and geographic variation in antibacterial activity against *E. coli* and *S. aureus* and tannin content between plants of the same species growing in different areas. .
3. Verify the efficacy of selected plant extracts used traditionally by determining the *in vitro* antibacterial activity against *E. coli* and tannin content and develop a ranking system for further selection based on these values
4. Select species to be used for further analysis.
5. Determine the *in vitro* cytotoxicity of the recommended extracts selected for further study.
6. Potentize (enhance the activity of) two extracts and determine the antibacterial activity and cytotoxicity of the potentized extracts to recommend which one of the two should be investigated in further detail.
7. Isolate and characterise the active compound(s) to in order to determine the nature of the compound.
8. Determine the acute and subacute toxicity in *in vivo* tests in rats.
9. Test the efficacy of the potentized extract in *in vivo* feeding experiments if the extract proves to be non-toxic.
10. Recommend further steps in the development of a model that would facilitate the selection of plant species likely to yield a therapeutically useful product.

Chapter 2

Seasonal variation in antibacterial activity of five selected plant species

2.1 Introduction

Due to the exposure to different weather and soil conditions the activity of leaf extracts may vary. In order to develop an antidiarrhoeal drug, it is important to be able to achieve the same level of activity in the source material. Therefore in this study the first step was to investigate, how the different seasons affect the activity, as well as the tannin content of leaf samples that were picked over a period of one year.

Many studies have been conducted on the seasonal variation of plant compounds (Booth *et al.* 2006; Riipi *et al.* 2002) and of leaf extracts (McGaw *et al.* 2002). Several plant-herbivore hypotheses are based on the assumption that plants cannot simultaneously allocate resources to growth and defence. Co-occurring changes in physical leaf traits and concentrations of several compounds indicated a seasonal decline in foliage suitability for herbivores (Riipi *et al.* 2002). If the seasonal variation of the activity has been established, another interesting aspect would be to determine to what degree the variation of activity is related to the tannin content that has been already found to vary during the season (Riipi *et al.* 2002).

In this project we investigate leaf material collected from tree species, because this is easy to collect and does not damage botanical garden plants. Furthermore it is easy to establish the identity, and if needed, additional material can be collected from the same plant at a later stage, especially since leaves are a renewable source. Random screening of leaf extracts of tree species against bacteria, fungi and parasites of medical importance has been one of the main focuses of the Phytomedicine Programme at the University of Pretoria [www.up.ac.za/phyto] for several years. A database of close to 600 tree species with records of activities of extracts prepared from leaves of these trees against eight species of fungi and bacteria has been developed.

Five tree species were selected to study the seasonal variability of the antibacterial activity of plant extracts. These five plant species were selected based upon their reported use as anti-diarrhoeal agents in ethnomedicine as well as on their accessibility. Since representatives of all of the five species are located on the campus of the Faculty of Veterinary Sciences, Onderstepoort, where the Phytomedicine Programme is also located, they were easily accessible and leaf material could be collected at the same time each month without major difficulties.

Acacia karroo Hayne (Fabaceae)is a shrub to medium sized tree with a variable shape, which manifests typically in a somewhat rounded crown. It occurs in bushveld, grassland and is associated with coastal dune forest (van Wyk and van Wyk, 1997). Bark and fruit contain tannin (Watt and Breyer-Brandwijk, 1962). Gum exudates, bark and leaves are used in various parts of southern Africa as emollients and astringents and for colds, ophthalmia, diarrhoea, dysentery and haemorrhage (Watt and Breyer-Brandwijk, 1962; Mabogo, 1990).

karroo

Acacia sieberiana var. *woodii* DC (Fabaceae) is a medium to large sized tree with spreading branches and an umbrella-shaped or flattened crown, that occurs in the bushveld and grassland in deep soil and along rivers (van Wyk and van Wyk, 1997). In the Democratic Republic of Congo, the bark exudate is used as an astringent and haemostatic and for diarrhoea and ophthalmia. In Nigeria, the leaves are used as a vermifuge, for inflammation of the urethra and of the genitals while the stems and twigs are used in unspecified parts of West Africa to treat teeth caries (Hutchings *et al.* 1996).

var.

The third selected species was *Peltophorum africanum* Sond. (Fabaceae), a small to medium sized tree with a dense crown that occurs in bushveld and often on sandy soil (van Wyk and van Wyk, 1997). Root and bark contain tannins (Watt and Breyer-Brandwijk, 1962). Traditional healers in Gauteng use aqueous extracts of the root and bark as an antidote to gastric irritation caused by overdoses of *Jatropha curcas* L. (Mampane *et al.* 1987). In Zimbabwe, roots are used for abdominal pain, dropsy, diarrhoea, infertility, venereal diseases, sore throats, as diuretics and diaphoretics and to prevent abortion (Gelfand *et al.* 1985). Bark is also used for abdominal pain and diarrhoea (Hutchings *et al.* 1996).

rum

emetica

Trichilia emetica Vahl (Meliaceae)is a medium to large sized evergreen tree with a dense, spreading crown. The sexes are separate on different plants. It occurs in riverine forest and bushveld (van Wyk and van Wyk, 1997). The bark and leaves contain tannin and the bark is resinous (Watt and Breyer-Brandwijk, 1962). Infusions of the bark and leaf are used in Zulu medicine for lumbago, rectal ulceration in children, and dysentery (Watt and

Breyer-Brandwijk, 1962). Leaves are used for dysentery in several other parts of Africa (Hutchings et al., 1996).

Ziziphus mucronata Willd (Rhamnaceae) is a shrub or small to medium sized tree, occurring in a wide variety of habitats(van Wyk and van Wyk, 1997). Peptide alkaloids have been isolated from the bark and leaves (Tscheche *et al.* 1974). Plant extracts show antifungal activity against *Candida albicans* (Gundidza, 1986a, b). Leaves, fruit and stems are reported to be used variously for colds, diarrhoea and as carminatives in West Africa (Dalziel, 1937). Unspecified parts are used for bloody diarrhoea, dysmenorrhoea and urogenital complaints in Angola (Bossard, 1993).

2.2 Materials and methods

2.2.1 Plant collection

The five species, *Acacia karroo*, *Acacia sieberiana* subsp. *africana*, *Peltophorum africanum*, *Trichilia emetica* and *Ziziphus mucronata* were collected on a monthly basis from the same labelled trees from December 2007 to November 2008. These trees are located on the Onderstepoort Veterinary Campus, University of Pretoria.

The plant material was dried in a dark room in the Phytomedicine Programme, University of Pretoria, under a constant stream of air. After a week of drying, the plant material was ground to a fine powder with a Macsalab mill (Model 200 LAB) and then stored in sealed glass containers in the dark at room temperature.

2.2.2 Extraction

One gram (1 g) of the ground plant material of each of the species listed in section 2.2.1 was extracted with 10 ml acetone (technical grade-MERCK) in 30 ml glass tubes. Eloff has rationalized in 1998 that acetone is the most suitable extractant for the screening of antimicrobial components in plants (Eloff, 1998b). The mixture was shaken for 20 minutes on a Labotec Model 20.2 shaking machine at high speed and left to settle. Then the extracts were filtered through Whatman No 1 filter paper into preweighed glass vials. This extraction process was repeated three times on the same plant material. The resulting extracts were combined and then dried under constant air flow.

2.2.3. TLC fingerprinting

The dried samples were dissolved in acetone to a concentration of 10 mg/ml. An aliquot of 10 µl (representing 100 µg of the extract dry mass) of each sample was loaded onto three separate aluminium backed thin layer chromatography (TLC) plates (Silica gel 60 F_{254}, Merck). The TLC plates were developed in a closed tank with a saturated atmosphere in the three mobile phase systems of different polarity, developed at the Phytomedicine Programme, University of Pretoria (Kótze and Eloff, 2002). Those three systems were the following:

BEA: benzene/ethanol/ammonia (18:2:0,2), non-polar, alkaline

CEF: chloroform/ethyl acetate/formic acid (10:9:2), intermediate polarity, acidic

EMW: ethyl acetate/methanol/water (10:1,35:1), polar, neutral

The developed plates were examined under UV light (at 254 and 365 nm wavelength) to detect fluorescing compounds.

A spray reagent of 0.1 g vanillin dissolved in 28 ml methanol with 1 ml sulphuric acid was prepared (Stahl, 1969). The developed plates were sprayed with the vanillin-sulphuric acid spray reagent and then heated at 110°C to optimal colour development.

2.2.4 Bacterial cultures

Bacterial strains of *Escherichia coli* (Gram-negative; ATCC 25922) and *Staphylococcus aureus* (Gram-positive; ATCC 29213) were obtained from the Microbiology Laboratory (Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria). These specific strains of nosocomial pathogens are recommended as test strains for comparing the activity of antibiotics (National Committee for Clinical Laboratory Standards, 1992). The cultures were maintained on Mueller Hinton (MH) agar at 4°C and were inoculated in MH broth and incubated overnight at 37°C to produce working cultures. The density of bacterial cultures used for the screening procedures were approximately: 2.6 x 10^{12} cfu/ml (*S. aureus)* and= 3.0 x 10^{11} cfu/ml *(E. coli)*.

2.2.5 Bioautographic assays

Bioautography is a useful tool in the qualititative determination of how many antibacterial compounds are present in different extracts and also to determine whether there exists a qualitative difference in different treatments with the same species. The method described by Begue and Kline (1972) was used.

Chromatograms were prepared as described in section 2.2.3 and then left under a constant stream of air for two days to completely evaporate the solvent.

Ten ml of the overnight bacterial cultures of *E. coli* and *S. aureus* were measured into two test tubes and centrifuged at a very high speed (5300 x g) for 20 minutes to concentrate the bacteria. After discarding the supernatant, the pellets were visible at the bottom of the tubes. The sedimented bacterial pellets were resuspended in fresh Mueller-Hinton broth. Separate chromatograms were sprayed with these bacterial suspensions and incubated at 37°C in 100% humidity for 18 hours. After the incubation period the plates were sprayed with a 2 mg/ml aqueous solution of *p*-iodonotrotetrazolium violet (INT, Sigma) and left to develop for 1 hour at 37°C in 100% humidity. The inhibition of bacterial growth by individual compounds was indicated by white zones visible on the plates against a reddish-purple background. A set of TLC plates previously sprayed with vanillin-sulphuric acid was used as a reference to detect the corresponding antibacterial compounds in the extract.

2.2.6 Microdilution assays

The two-fold serial dilution microplate method of Eloff (1998c) was used to determine the minimum inhibitory concentration of the plant extracts against the two bacterial strains, *E. coli* and *S. aureus.*

The dried extracts were dissolved in acetone to a concentration of 10 mg/ml. Aliquots (100 µl) of the plant extracts were added to the first row of wells of a 96 well microtitre plate in triplicate, and were serially diluted two-fold with water. As a positive control, 100 µl of 0.1 mg/ml gentamicin (Virbac) was used, and acetone was used as a solvent control. The negative control was distilled water. Overnight incubated bacterial cultures (100 µl) of *E. coli* and *S. aureus* in MH broth were then added to each well. The plates were covered and incubated for 16 hours at 37°C and then 40 µl of a 0.2 mg/ml aqueous solution of INT were added to each well and again incubated at 37°C.

The MIC was recorded as the lowest concentration of the extract to inhibit bacterial growth. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a formazan product by biologically active organisms (Eloff, 1998c). The MIC values were read after 1 and 2 hours of incubation with INT. Where the bacterial growth was inhibited, the intensity of the purple colour was reduced from the wells at a lower concentration with actively growing bacteria. In each experiment there were three replicates and the experiment was repeated three times to determine the variability of the results. The average MIC values for the 9 determinations and standard deviations were calculated.

2.2.7 Total activity

To compare the antibacterial activity of extracts of different species and the same plant over different periods, not only the MIC, but also the quantity extracted must be taken into consideration by calculating the total activity (Eloff, 2000). The total activity was determined as follows:

Total activity = quantity in mg extracted from 1 g/ MIC value (mg/ml).

The units for total activity are ml/g, reflecting the number of ml to which an extract prepared from 1 g of plant material can be diluted and still retain antibacterial activity.

2.2.8 Tannin assays

The tannin content in the plant extracts was determined using the radial diffusion method, a simple protein precipitation method (Hagerman, 1998). There exist many methods to test the tannin content of a plant, but since it was intended in this project to extract the plant material with acetone, and there were numerous samples, the radial diffusion method was the best and most time-effective choice.

The tannin was allowed to react with a protein and then the precipitated complex was quantified. In the method, a tannin-containing solution was placed in a well in a protein-containing agar slab in a Petri dish. The protein used was bovine serum albumin. The agar slab consisted of 1 g of prepared agarose type 1, in which 0.1 g of bovine serum albumin was diluted. There were four wells per Petri dish, into each of which 8 µl of plant extract resuspended in acetone at a concentration of 10 mg/ml were transferred. Afterwards the plates were sealed with parafilm and placed in a level incubator at 30°C for 96 hours. As the tannin diffused into the gel and formed a complex with the protein, a visible ring of precipitation developed. The area of the ring is proportional to the amount of tannin in the extract (Hagerman, 1998). The detection limit of the method is 0.025 mg tannic acid or condensed tannin, and the precision is 6% (relative standard deviation). With a plastic ruler the diameter of the ring was measured. The accuracy of the values was 0.5 mm. The plates can be stored after development at 4°C for several weeks. They should be covered and sealed with parafilm.

The square of the diameter is proportional to the tannin content in the sample. There was also a solvent (acetone) control included in the tannin determination assay, as well as gallic acid (10 mg/ml) as a positive control to quantify the amount of tannin in the plant samples.

The method has several advantages over other methods for determining tannin: it is very simple and requires neither complex reagents nor instruments. Components of the plant extract such as non-tannin phenolics or water-insoluble compounds do not interfere with the method. The assay is not subject to

interference from the organic and aqueous solutions which are commonly used to extract tannin from plants (Hagerman, 1998).

Gallic acid was included as a reference since this method does not supply us with a determination of the amount of tannin in a sample but rather just determines their presence. Gallic acid was included in the assays at a concentration of 10 mg/ml and the diameter for the zone of the complexed tannin obtained was 1 cm. The assays were performed in triplicate to confirm results.

2.3 Results and discussion

2.3.1 Quantity extracted

Interestingly, the extracted quantity was directly related to the time of collection of the plant material, as can be seen in Figures 6 to10. *Acacia karroo* collected in December yielded an extraction percentage of 1.8% and reached its peak percentage in August with 6.4% and then declined to 2.2% in November. In the month of July the leaf quality of *Acacia karroo* was unsatisfactory and therefore it was not sampled for this month. *Acacia siebereriana* var. *woodii* reached its peak extraction percentage in the month of June (5.4%) and then declined to the same amount in November as was extracted in December (2.0%). *Peltophorum africanum* in December yielded an extraction percentage of 1.6%, in June the percentage was the highest amount of 6.6% and then it declined to 1.7% in November. *Trichilia emetica* started off with an extraction percentage of 1.1% in December and had a maximum percentage in June with 4.2%. The percentage then declined to under 1.0% in the months of October and November. *Ziziphus mucronata* had an extraction percentage of 1.4% in December, which then rose to 7.1% in July, and then it decreased to 1.2% in November. However the leaf quality of *Ziziphus mucronata* degenerated rapidly in the months of July, August and September with the result that the leaves could not be sampled in September.

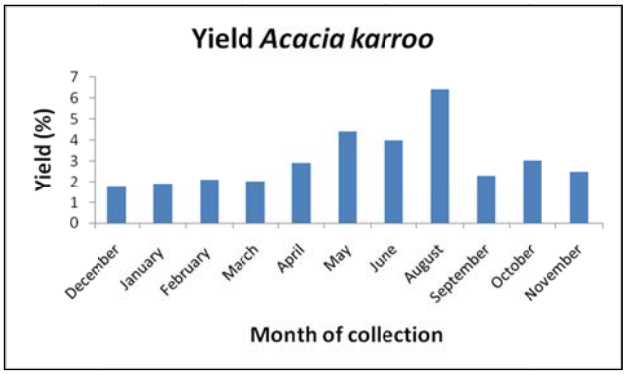


Fig. 2.6: Average yield of *Acacia karroo* with acetone extraction (no sample in July)

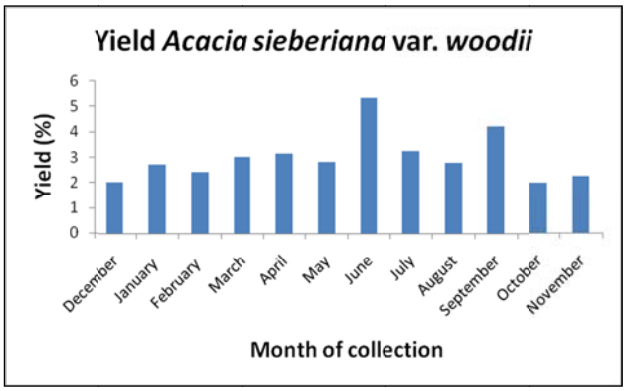


Fig. 2.7: Average yield of *Acacia sieberiana* var. *woodii* with acetone extraction

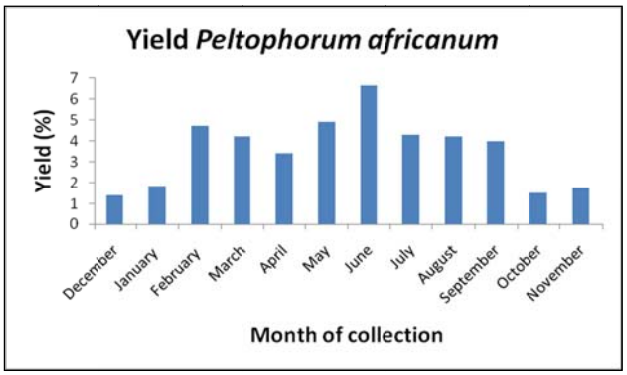


Fig. 2.8: Average yield of *Peltophorum africanum* with acetone extraction

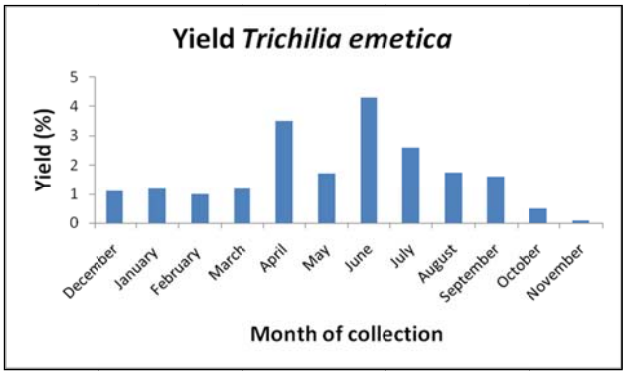


Fig. 2.9: Average yield of *Trichilia emetica* with acetone extraction

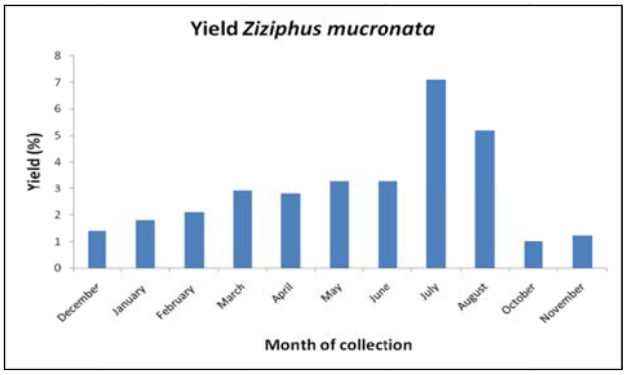


Fig. 2.10: Average yield of *Ziziphus mucronata* with acetone extraction (no sample in September)

2.3.2 TLC fingerprinting

The TLC chromatograms showed that similar compounds were present in each of the five plants every month. One difference was that in the winter months the compounds did not seem as pronounced as they were in the summer months. This can be seen most prominently in the samples developed using CEF.

Figures 2.11 to 2.16 show the chromatograms of the five plants in the summer months of December and January and of the winter months June and July. Due to the bad leaf quality of *Acacia karroo* in July, this sample is not displayed in the TLC fingerprints.

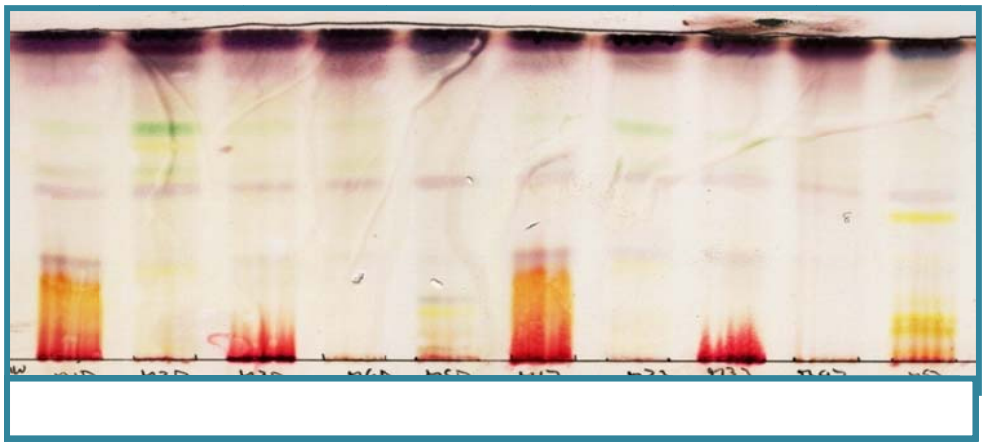


Fig. 2.11: Chromatograms of monthly samples of five species (M1-M5) collected during December (D) and January (J) separated by using EMW

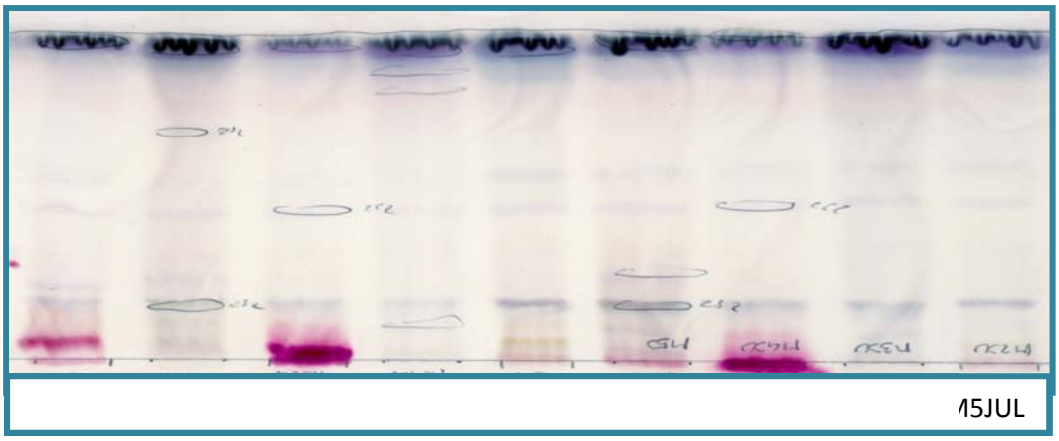


Fig. 2.12: Chromatograms of monthly samples of five species collected during June and July separated by using EMW

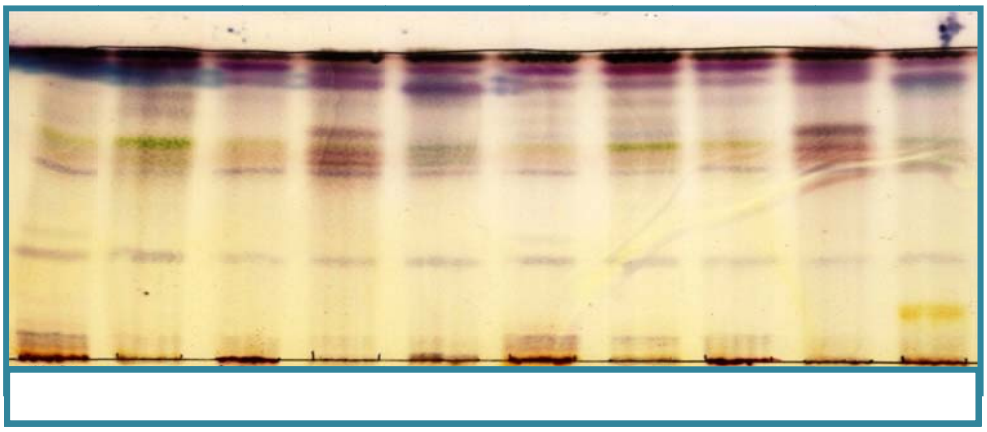


Fig. 2.13: Chromatograms of monthly samples of five species collected during December and January separated by using CEF

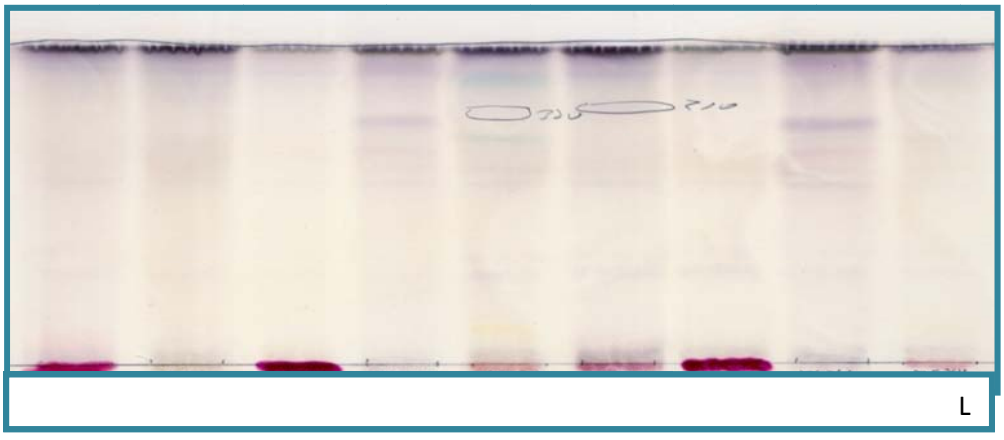


Fig. 2.14: Chromatograms of monthly samples of five species collected during June and July separated by using CEF

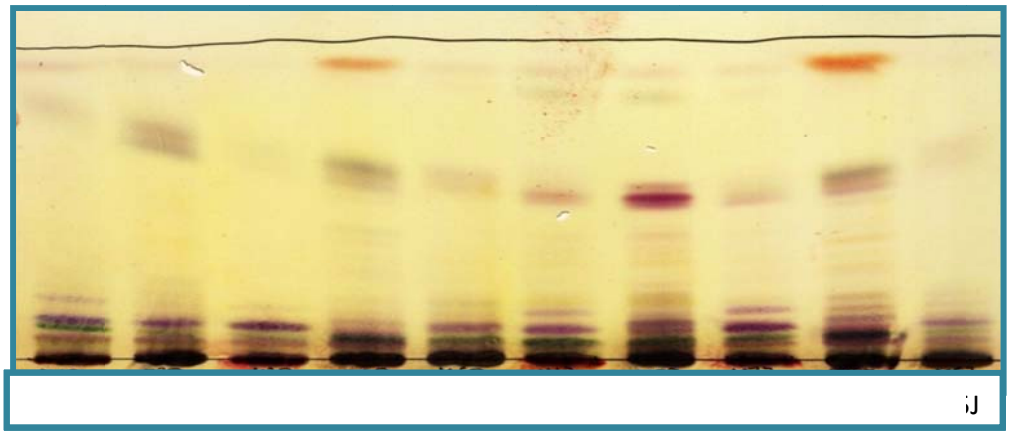


Fig. 2.15: Chromatograms of monthly samples of five species collected during December and January separated by using BEA

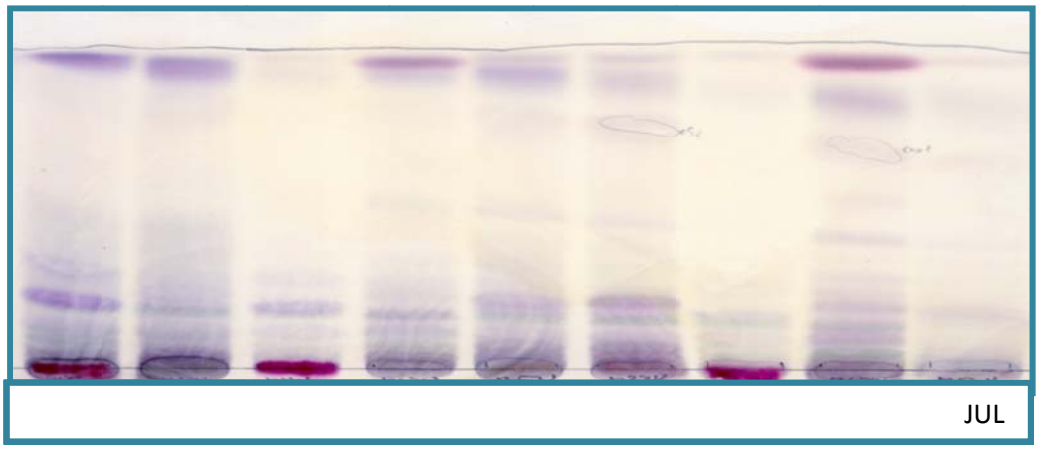


Fig. 2.16: Chromatograms of monthly samples of five species collected during June and July separated by using BEA

M1D: *Acacia karroo* December; M2D: *Acacia sieberiana* var. *woodii* December; M3D: *Peltophorum africanum* December; M4D: *Trichilia emetica* December; M5D: *Ziziphus mucronata* December; M1J: *Acacia karroo* January; M2J: *Acacia sieberiana* var. *woodii* January; M3J: *Peltophorum africanum* January; M4J: *Trichilia emetica* January; M5J: *Ziziphus mucronata* January; M1JU: *Acacia karroo* June; M2JU: *Acacia sieberiana* var. *woodii* June; M3JU: *Peltophorum africanum* June; M4JU: *Trichilia emetica* June; M5JU: *Ziziphus mucronata* June; M2JUL: *Acacia sieberiana* var. *woodii* July; M3JUL: *Peltophorum africanum* July; M4JUL: *Trichilia emetica* July; M5JUL: *Ziziphus mucronata* July

2.3.3 Bioautographic assays

The bioautographic assays showed clear inhibition bands in the extracts prepared during the summer months. The zones in the extracts prepared during the winter months were either less pronounced or not present at all. This section shows the bioautography images of the five plants in the summer months

December and January and of them in the winter months June and July. Due to the unsatisfactory leaf quality of *Acacia karroo* in June, this extract is not displayed in the bioautographic assays.

The inhibitory bands for *E. coli* were more distinct for extracts from the summer months than from those in the winter months, suggesting that compounds responsible for activity against *E. coli* are present in higher concentrations during the warm period. However, the inhibitory bands against *S. aureus* were equally pronounced in the winter and in the summer months, and in the case of separation with the BEA solvent were slightly more distinct in the winter months suggesting that the time of collection does not really matter in this case. The compound separation was done with the CEF, BEA and EMW solvent systems. BEA and EMW, however, did not separate the compounds very well and so only the bioautography representations using the CEF solvent system are displayed in the following figures. The bioautograms are displayed in Figures 2.17 to 2.20.

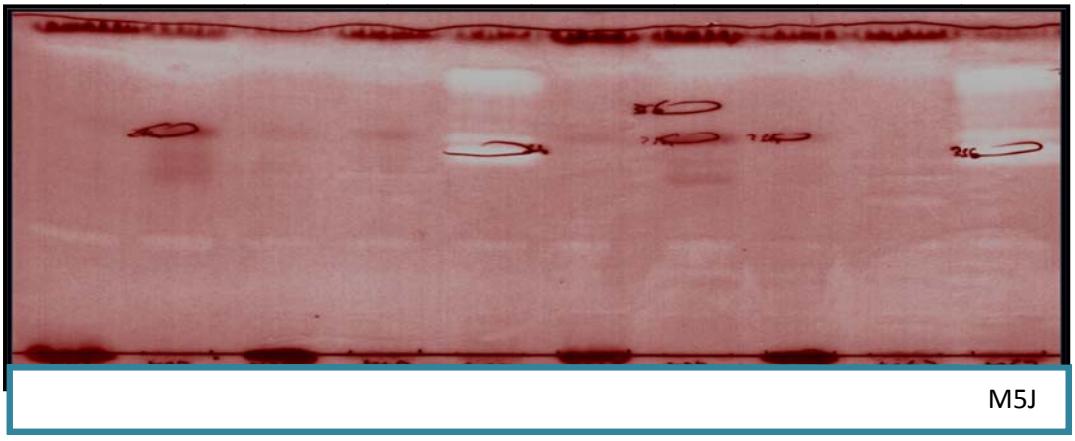


Fig. 2.17: Bioautography of monthly samples from December and January against *E. coli* (CEF)

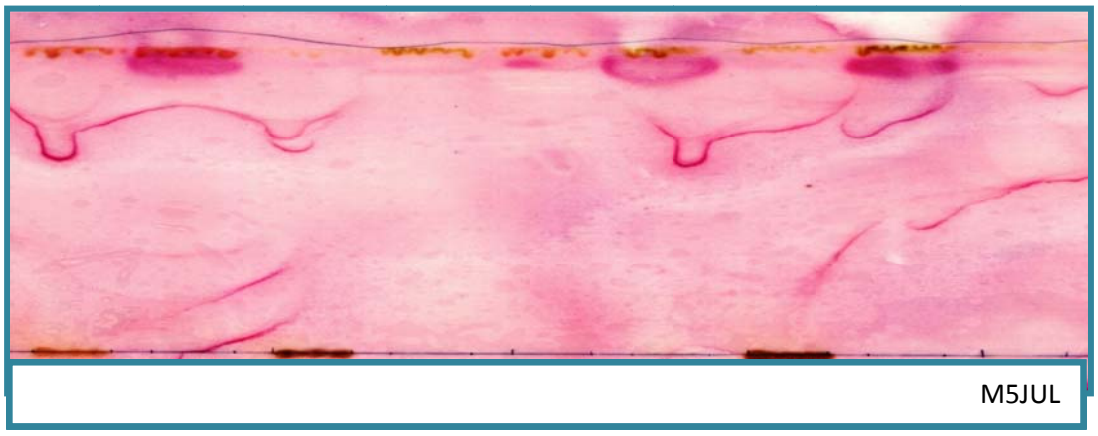


Fig. 2.18: Bioautography of monthly samples from June and July against *E. coli* (CEF)

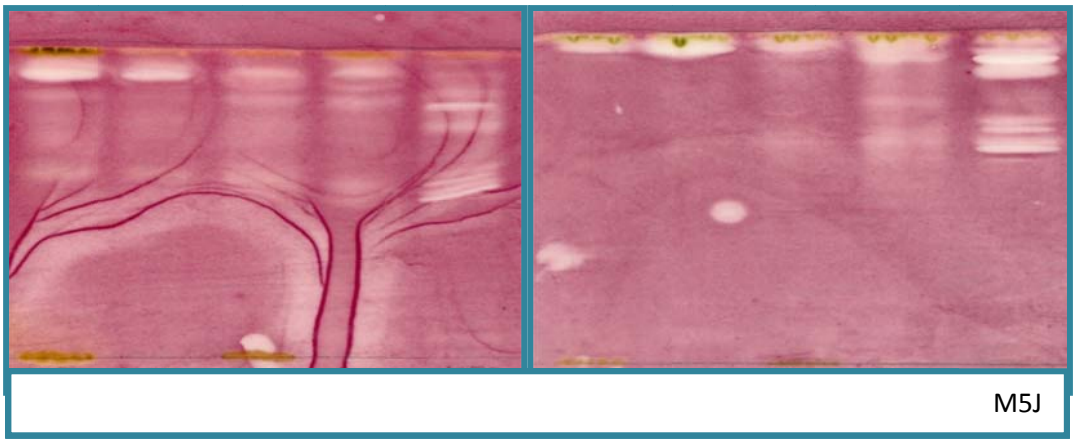


Fig. 2.19: Bioautography of monthly samples from December and January against *S. aureus* (CEF)

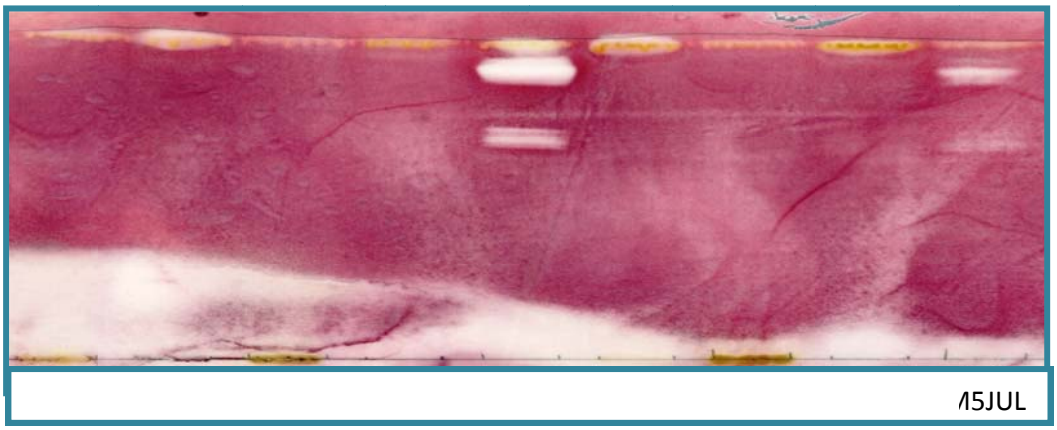


Fig. 2.20: Bioautography of monthly samples from June and July against *S. aureus* (CEF)

M1D: *Acacia karroo* December; M2D: *Acacia sieberiana* var. *woodii* December; M3D: *Peltophorum africanum* December; M4D: *Trichilia emetica* December; M5D: *Ziziphus mucronata* December; M1J: *Acacia karroo* January; M2J: *Acacia sieberiana* var. *woodii* January; M3J: *Peltophorum africanum* January; M4J: *Trichilia emetica* January; M5J: *Zizphus mucronata* January; M1JU: *Acacia karroo* June; M2JU: *Acacia sieberiana* var. *woodii* June; M3JU: *Peltophorum africanum* June; M4JU: *Trichilia emetica* June; M5JU: *Ziziphus mucronata* June; M2JUL: *Acacia karroo* July; M3JUL: *Peltphorum africanum* July; M4JUL: *Trichilia emetica* July; M5JUL: *Ziziphus mucronata* July

2.3.4 Microdilution assays

The microdilution assay showed variations in the antibacterial activity within species on a monthly basis (Tables 2.1 and 2.2). *Acacia karroo* had the lowest MIC with regard to its activity against *E. coli* in the month of April and against *S. aureus* in the month of March, with MIC values of 0.11 mg/ml (TA = 332 ml/g) and 0.06 mg/ml (TA = 334 ml/g) respectively. The MIC values reflecting the worst activity were recorded against *E. coli* in the month of June with 0.42 mg/ml (TA = 96 ml/g) and for *S. aureus* in the

months of June and November with MIC = 0.31 mg/ml (TA = 129 ml/g and 80 ml/g respectively). The quality of the leaves was so bad in July that the plants could not be sampled.

Acacia sieberiana var. *woodii* recorded the best activity against *E. coli* with MIC = 0.10 mg/ml (TA = 182 ml/g) in the month of March and the worst in the month of June with MIC = 0.62 mg/ml (TA = 86 ml/g). The best activity against *S. aureus* was found in the extract prepared during April with a value of 0.08 mg/ml (TA = 303 ml/g), and the worst activity was present in the July extract with a value of 0.63 mg/ml (TA = 51 ml/g).

Peltophorum africanum had the best activity against *E. coli* in the month of February and against *S. aureus* in the months of February and March with MIC values of 0.05 mg/ml (TA = 1188 ml/g) and 0.04 mg/ml (TA = 1188 ml/g and 1075 ml/g). The worst activity was against *E. coli* in the month of October and for *S. aureus* in the month of September, with values of 0.42 mg/ml (TA = 36 ml/g) and 0.25 mg/ml (TA = 163 ml/g) respectively.

Trichilia emetica extracts had high MIC values throughout the year against both bacterial strains, showing the highest value at 0.81 mg/ml against *E. coli* in the month of January and at 0.63 mg/ml in the months of January and April against *S. aureus*. The lowest MIC values were reached for *E. coli* in the month of May with a value of 0.22 mg/ml (TA = 74 ml/g) and for *S. aureus* in the month of December with a value of 0.28 mg/ml (TA = 26 ml/g)

Extracts of *Ziziphus mucronata* had the best MIC values against *E. coli* in the month of May and against *S. aureus* in the month of May with MIC values of 0.10 mg/ml (TA = 589 ml/g) and of 0.04 mg/ml (TA = 1099 ml/g) respectively. Extracts had the highest MIC value in the month of November against *E. coli* and in the months of August and November against *S. aureus* with values of 0.58 mg/ml (TA = 21 ml/g) and of 0.63 mg/ml (TA = 83 ml/g, 19 ml/g) respectively.

The antibacterial activities of the extracts of all five plants seemed to be bactericidal since the values for the longer incubation did not significantly differ (with differences in only one dilution factor, or well in the assay).

The results are shown in Figures 2.21 to 2.30 as well as in Tables 2.1 and 2.2.

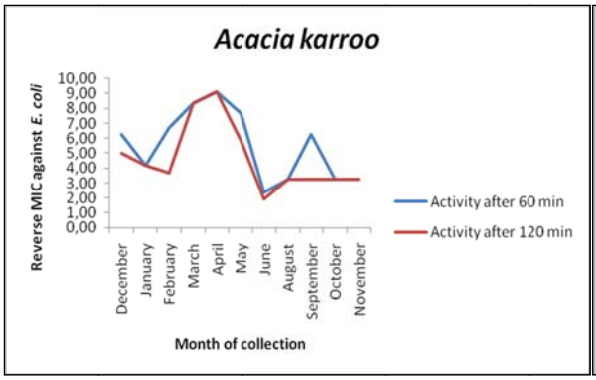


Fig. 2.21: Activity of *A. karroo* against *E. coli*

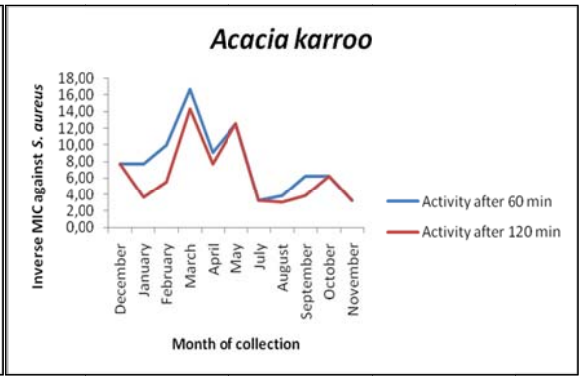


Fig. 2.22: Activity of *A. karroo* against*S. aureus*

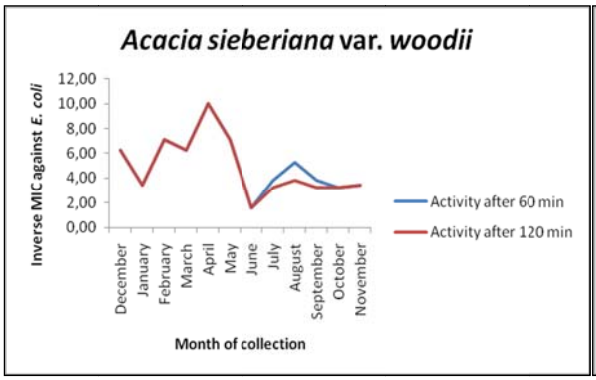


Fig. 2.23: Activity of *A. sieberiana* against *E. coli*

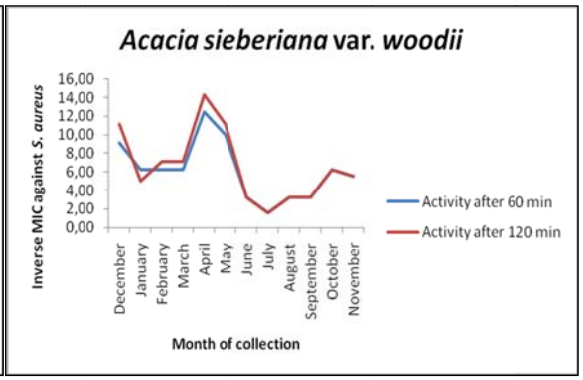


Fig. 2.24: Activity of *A. sieberiana* against *S. aureus*

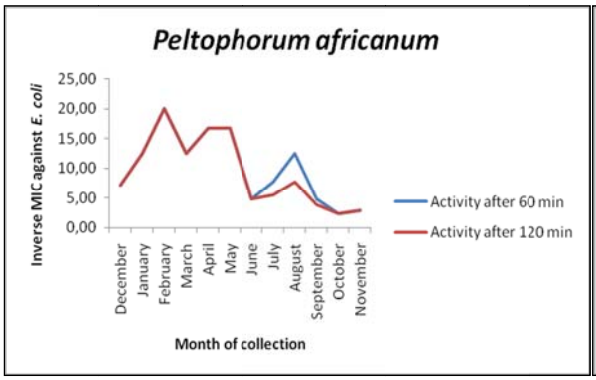


Fig. 2.25: Activity of *P. africanum* against *E. coli*

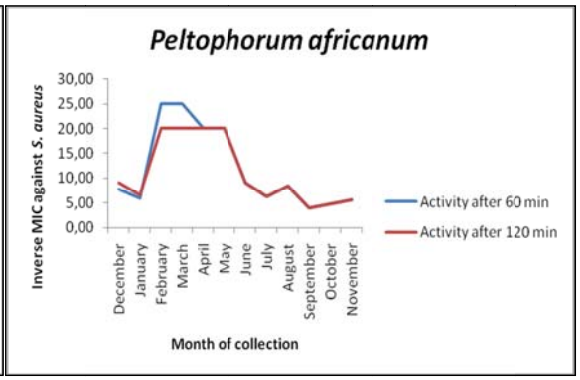
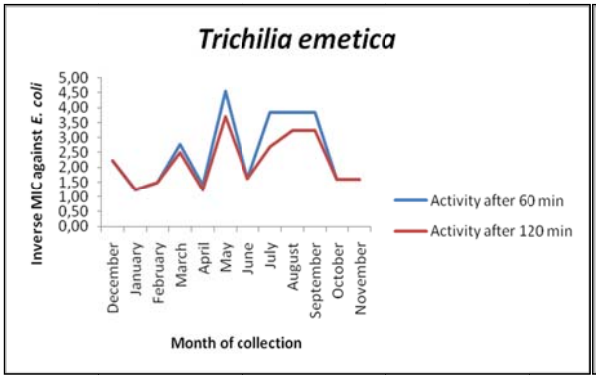


Fig. 2.26: Activity of *P. africanum* against *S. aureus*



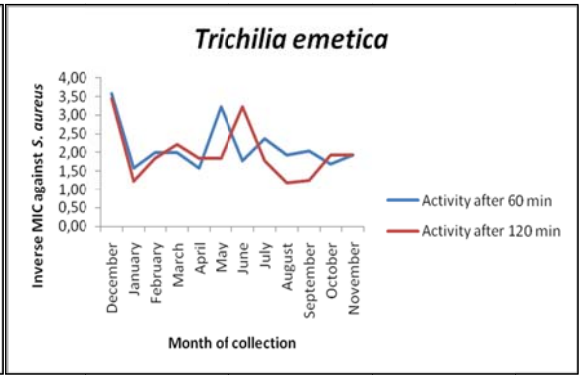


Fig. 2.27: Activity of *T. emetica* **against** *E. coli*

Fig. 2.28: Activity of *T. emetica* **against** *S. aureus*

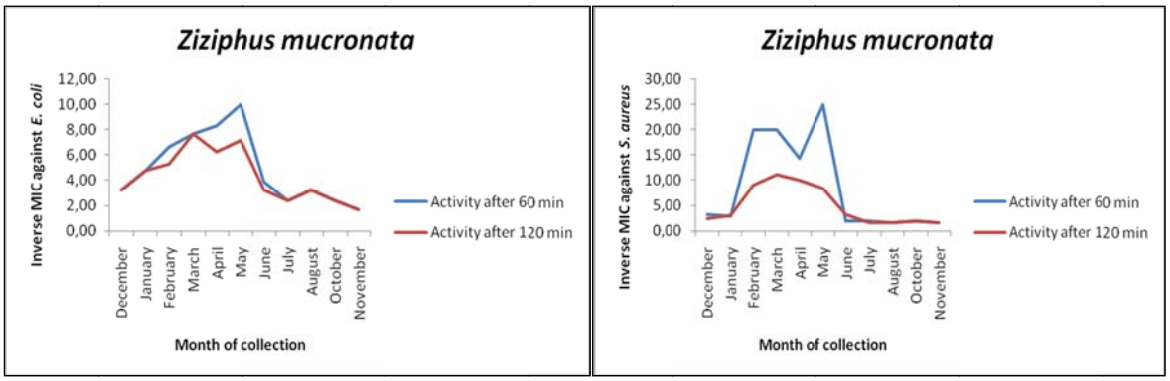


Fig. 2.29: Activity of *Z. mucronata* **against** *E. coli* **Fig. 2.30: Activity of** *Z. mucronata* **against** *S. aureus*

Table 2.1: MIC values of monthly samples against *E. coli*after 60 min and 120 min

Plant extract	Month of collection	Extract yield (mg)	Average activity *E. coli* 60 (mg/ml) (± SD)	Total activity *E. coli* 60 (ml/g)	Average activity *E. coli* 120 (mg/ml) (± SD)	Total activity *E. coli* 120 (ml/g)
Acacia karroo	December	18	0.16 ± 0.00	65	0.20 ± 0.07	81
Acacia karroo	January	19	0.24 ± 0.27	158	0.24 ± 0.27	158
Acacia karroo	February	21	0.15 ± 0.11	235	0.27 ± 0.25	165
Acacia karroo	March	20	0.12 ± 0.09	209	0.12 ± 0.09	209
Acacia karroo	April	29	0.11 ± 0.04	332	0.11 ± 0.04	332
Acacia karroo	May	44	0.13 ± 0.09	450	0.17 ± 0.13	450
Acacia karroo	June	40	0.42 ± 0.16	96	0.52 ± 0.16	76
Acacia karroo	July	ND	ND	ND	ND	ND
Acacia karroo	August	64	0.31 ± 0.00	207	0.31 ± 0.00	207
Acacia karroo	September	23	0.16 ± 0.00	144	0.31 ± 0.00	74
Acacia karroo	October	30	0.31 ± 0.00	97	0.31 ± 0.00	97
Acacia karroo	November	25	0.31 ± 0.00	80	0.31 ± 0.00	80
Acacia sieberiana var. *woodii*	December	20	0.16 ± 0.00	80	0.16 ± 0.00	80
Acacia sieberiana var. *woodii*	January	27	0.29 ± 0.23	135	0.29 ± 0.23	135
Acacia sieberiana var. *woodii*	February	24	0.14 ± 0.04	191	0.14 ± 0.04	191
Acacia sieberiana var. *woodii*	March	30	0.16 ± 0.00	182	0.16 ± 0.00	182
Acacia sieberiana var. *woodii*	April	31	0.10 ± 0.04	303	0.10 ± 0.04	303
Acacia sieberiana var. *woodii*	May	28	0.14 ± 0.04	226	0.14 ± 0.04	226
Acacia sieberiana var. *woodii*	June	54	0.62 ± 0.47	86	0.62 ± 0.47	86
Acacia sieberiana var. *woodii*	July	32	0.26 ± 0.07	123	0.31 ± 0.00	103
Acacia sieberiana var. *woodii*	August	27,5	0.19 ± 0.07	142	0.26 ± 0.08	106
Acacia sieberiana var. *woodii*	September	42	0.26 ± 0.07	162	0.31 ± 0.00	136
Acacia sieberiana var. *woodii*	October	20	0.31 ± 0.00	65	0.31 ± 0.00	65
Acacia sieberiana var. *woodii*	November	23	0.29 ± 0.05	77	0.29 ± 0.05	77
Peltophorum africanum	December	14	0.14 ± 0.04	106	0.14 ± 0.04	106
Peltophorum africanum	January	18	0.08 ± 0.00	175	0.08 ± 0.00	175
Peltophorum africanum	February	47	0.05 ± 0.02	1188	0.05 ± 0.02	1188
Peltophorum africanum	March	42	0.08 ± 0.06	968	0.08 ± 0.06	968
Peltophorum africanum	April	34	0.06 ± 0.02	572	0.06 ± 0.02	572
Peltophorum africanum	May	49	0.06 ± 0.02	926	0.06 ± 0.02	926
Peltophorum africanum	June	67	0.21 ± 0.08	317	0.21 ± 0.08	317
Peltophorum africanum	July	43	0.13 ± 0.04	323	0.18 ± 0.10	235
Peltophorum africanum	August	42	0.08 ± 0.00	525	0.13 ± 0.04	315
Peltophorum africanum	September	40	0.21 ± 0.08	191	0.26 ± 0.07	154
Peltophorum africanum	October	15	0.42 ± 0.16	36	0.42 ± 0.16	36
Peltophorum africanum	November	17	0.35 ± 0.11	50	0.35 ± 0.11	50
Trichilia emetica	December	11	0.45 ± 0.17	15	0.45 ± 0.17	15
Trichilia emetica	January	12	0.81 ± 0.30	17	0.81 ± 0.30	17
Trichilia emetica	February	10	0.67 ± 0.42	37	0.67 ± 0.42	37
Trichilia emetica	March	12	0.36 ± 0.20	34	0.40 ± 0.16	34
Trichilia emetica	April	35	0.72 ± 0.39	42	0.81 ± 0.30	42
Trichilia emetica	May	17	0.22 ± 0.08	74	0.27 ± 0.07	74
Trichilia emetica	June	42	0.62 ± 0.47	67	0.62 ± 0.47	67
Trichilia emetica	July	26	0.26 ± 0.07	100	0.37 ± 0.21	71
Trichilia emetica	August	18	0.26 ± 0.07	67	0.31 ± 0.00	57

Plant extract	Month of collection	Extract yield (mg)	Average activity *E. coli* 60 (mg/ml) (± SD)	Total activity *E. coli* 60 (ml/g)	Average activity *E. coli* 120 (mg/ml) (± SD)	Total activity *E. coli* 120 (ml/g)
Trichilia emetica	September	16	0.26 ± 0.07	62	0.31 ± 0.00	52
Trichilia emetica	October	5	0.63 ± 0.00	8	0.63 ± 0.00	8
Trichilia emetica	November	9	0.63 ± 0.00	14	0.63 ± 0.00	14
Ziziphus mucronata	December	14	0.31 ± 0.00	48	0.31 ± 0.00	48
Ziziphus mucronata	January	18	0.21 ± 0.08	100	0.21 ± 0.08	100
Ziziphus mucronata	February	21	0.15 ± 0.03	181	0.19 ± 0.09	181
Ziziphus mucronata	March	29	0.13 ± 0.12	709	0.13 ± 0.12	709
Ziziphus mucronata	April	28	0.12 ± 0.06	425	0.16 ± 0.11	425
Ziziphus mucronata	May	33	0.10 ± 0.06	589	0.14 ± 0.12	589
Ziziphus mucronata	June	33	0.26 ± 0.07	127	0.31 ± 0.00	107
Ziziphus mucronata	July	71	0.42 ± 0.16	170	0.42 ± 0.16	170
Ziziphus mucronata	August	52	0.31 ± 0.00	168	0.31 ± 0.00	168
Ziziphus mucronata	September	ND	ND	ND	ND	ND
Ziziphus mucronata	October	10	0.42 ± 0.16	24	0.42 ± 0.16	24
Ziziphus mucronata	November	12	0.59 ± 0.11	21	0.59 ± 0.11	21

Table 2.2: MIC values of monthly samples against *S. aureus* after 60 min and 120 min

Plant extract	Month of collection	Yield (mg)	Average activity *S. aureus* 60 (mg/ml) (± SD)	Total activity *S. aureus* 60 (ml/g)	Average activity *S. aureus* 120 (mg/ml) (± SD)	Total activity *S. aureus* 120 (ml/g)
Acacia karroo	December	18	0.13 ± 0.04	113	0.13 ± 0.04	113
Acacia karroo	January	19	0.13 ± 0.04	110	0.27 ± 0.25	110
Acacia karroo	February	21	0.10 ± 0.04	212	0.18 ± 0.10	165
Acacia karroo	March	20	0.06 ± 0.02	334	0.07 ± 0.04	334
Acacia karroo	April	29	0.11 ± 0.04	295	0.13 ± 0.04	295
Acacia karroo	May	44	0.08 ± 0.00	428	0.08 ± 0.00	428
Acacia karroo	June	40	0.31 ± 0.00	129	0.31 ± 0.00	129
Acacia karroo	July	ND	ND	ND	ND	ND
Acacia karroo	August	64	0.26 ± 0.08	246	0.33 ± 0.12	195
Acacia karroo	September	23	0.16 ± 0.00	144	0.26 ± 0.08	89
Acacia karroo	October	30	0.16 ± 0.00	188	0.16 ± 0.00	188
Acacia karroo	November	25	0.31 ± 0.00	80	0.31 ± 0.00	80
Acacia sieberiana var. *woodii*	December	20	0.11 ± 0.04	128	0.09 ± 0.05	128
Acacia sieberiana var. *woodii*	January	27	0.16 ± 0.00	135	0.20 ± 0.07	135
Acacia sieberiana var. *woodii*	February	24	0.16 ± 0.00	136	0.14 ± 0.04	136
Acacia sieberiana var. *woodii*	March	30	0.16 ± 0.00	182	0.14 ± 0.04	182
Acacia sieberiana var. *woodii*	April	31	0.08 ± 0.00	303	0.07 ± 0.02	303
Acacia sieberiana var. *woodii*	May	28	0.10 ± 0.04	267	0.09 ± 0.03	267
Acacia sieberiana var. *woodii*	June	54	0.31 ± 0.00	173	0.31 ± 0.00	173
Acacia sieberiana var. *woodii*	July	32	0.63 ± 0.00	51	0.63 ± 0.00	51
Acacia sieberiana var. *woodii*	August	28	0.31 ± 0.00	89	0.31 ± 0.00	89
Acacia sieberiana var. *woodii*	September	42	0.31 ± 0.00	136	0.31 ± 0.00	136
Acacia sieberiana var. *woodii*	October	20	0.16 ± 0.00	125	0.16 ± 0.00	125
Acacia sieberiana var. *woodii*	November	23	0.18 ± 0.05	127	0.18 ± 0.05	127
Peltophorum africanum	December	14	0.13 ± 0.04	106	0.11 ± 0.04	106
Peltophorum africanum	January	18	0.17 ± 0.08	97	0.15 ± 0.08	97
Peltophorum africanum	February	47	0.04 ± 0.00	1188	0.05 ± 0.02	1188
Peltophorum africanum	March	42	0.04 ± 0.00	1075	0.05 ± 0.02	1075

Plant extract	Month of collection	Yield (mg)	Average activity *S. aureus* 60 (mg/ml) (± SD)	Total activity *S. aureus* 60 (ml/g)	Average activity *S. aureus* 120 (mg/ml) (± SD)	Total activity *S. aureus* 120 (ml/g)
Peltophorum africanum	April	34	0.05 ± 0.02	572	0.05 ± 0.02	572
Peltophorum africanum	May	49	0.05 ± 0.03	1158	0.05 ± 0.02	1158
Peltophorum africanum	June	67	0.11 ± 0.04	623	0.11 ± 0.04	623
Peltophorum africanum	July	43	0.16 ± 0.00	269	0.16 ± 0.00	269
Peltophorum africanum	August	42	0.12 ± 0.04	364	0.12 ± 0.04	364
Peltophorum africanum	September	40	0.25 ± 0.16	163	0.25 ± 0.16	163
Peltophorum africanum	October	15	0.21 ± 0.08	71	0.21 ± 0.08	71
Peltophorum africanum	November	17	0.18 ± 0.05	99	0.18 ± 0.05	99
Trichilia emetica	December	11	0.28 ± 0.07	26	0.29 ± 0.06	26
Trichilia emetica	January	12	0.63 ± 0.00	17	0.81 ± 0.30	17
Trichilia emetica	February	10	0.50 ± 0.18	37	0.54 ± 0.16	37
Trichilia emetica	March	12	0.50 ± 0.18	29	0.45 ± 0.17	29
Trichilia emetica	April	35	0.63 ± 0.00	37	0.54 ± 0.16	37
Trichilia emetica	May	17	0.31 ± 0.00	52	0.54 ± 0.16	52
Trichilia emetica	June	42	0.56 ± 0.14	54	0.31 ± 0.00	54
Trichilia emetica	July	26	0.42 ± 0.16	75	0.56 ± 0.14	75
Trichilia emetica	August	18	0.52 ± 0.16	33	0.84 ± 0.31	21
Trichilia emetica	September	16	0.49 ± 0.17	33	0.80 ± 0.35	20
Trichilia emetica	October	5	0.59 ± 0.11	19	0.52 ± 0.16	19
Trichilia emetica	November	9	0.52 ± 0.16	17	0.52 ± 0.16	17
Ziziphus mucronata	December	14	0.31 ± 0.00	48	0.40 ± 0.16	48
Ziziphus mucronata	January	18	0.35 ± 0.33	163	0.33 ± 0.26	163
Ziziphus mucronata	February	21	0.05 ± 0.03	603	0.11 ± 0.06	422
Ziziphus mucronata	March	29	0.05 ± 0.03	788	0.09 ± 0.05	630
Ziziphus mucronata	April	28	0.07 ± 0.02	365	0.10 ± 0.05	365
Ziziphus mucronata	May	33	0.04 ± 0.03	1099	0.12 ± 0.13	942
Ziziphus mucronata	June	33	0.31 ± 0.00	107	0.31 ± 0.00	107
Ziziphus mucronata	July	71	0.52 ± 0.16	136	0.63 ± 0.00	113
Ziziphus mucronata	August	52	0.63 ± 0.00	83	0.63 ± 0.00	83
Ziziphus mucronata	September	ND	ND	ND	ND	ND
Ziziphus mucronata	October	10	0.52 ± 0.16	19	0.52 ± 0.16	19
Ziziphus mucronata	November	12	0.63 ± 0.00	19	0.63 ± 0.00	19

2.3.5 Tannin assays

The results of the tannin assays are presented in Table 2.3. Extracts of *Acacia sieberiana* var. *woodii* did not have any tannin content except for the months of April and May. Neither did extracts of *Trichilia emetica* except for the months of December and April. *Acacia karroo* had no tannin content in the months of January, June, August and September. Furthermore it could not be sampled due to poor leaf quality in July. These results could account for the difference in results obtained in the antibacterial assays throughout the year, since tannins may have antibacterial activity and could contribute to the antibacterial activity of the extract. *Peltophorum africanum* constantly had tannin in the assays in varying amounts. So did *Ziziphus mucronata* with the exceptions of the months of October and November. However the lack of tannin in those samples could be explained by the quality of the leaves from *Ziziphus mucronata* in those two months, which was much worse than in the other months of the year

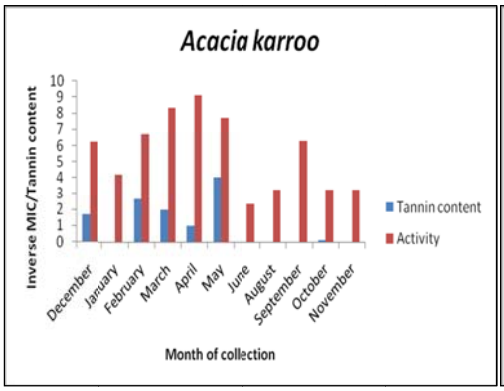
except for the month of September, where the quality and quantity of the leaves was so bad that they could not even be sampled.

Table 2.3: Results of tannin assays for extracts prepared from leaf material collected monthly (results for average square diameter of zone are not given since the average equivalent gallic acid translated 1:1 to this value)

Plant extract	Month of collection	Average equivalent gallic acid	SD
Acacia karroo	December	1.67	0
Acacia karroo	January	0	0
Acacia karroo	February	2.67	1.15
Acacia karroo	March	2	0.58
Acacia karroo	April	1	0
Acacia karroo	May	4	0
Acacia karroo	June	0	0
Acacia karroo	July	ND	
Acacia karroo	August	0	0
Acacia karroo	September	0	0
Acacia karroo	October	0.13	0.15
Acacia karroo	November	0.01	0.06
Acacia sieberiana var. *woodii*	December	0	0
Acacia sieberiana var. *woodii*	January	0	0
Acacia sieberiana var. *woodii*	February	0	0
Acacia sieberiana var. *woodii*	March	0	0
Acacia sieberiana var. *woodii*	April	0.33	0.58
Acacia sieberiana var. *woodii*	May	4.33	1.53
Acacia sieberiana var. *woodii*	June	0	0
Acacia sieberiana var. *woodii*	July	0	0
Acacia sieberiana var. *woodii*	August	0	0
Acacia sieberiana var. *woodii*	September	0	0
Acacia sieberiana var. *woodii*	October	0	0
Acacia sieberiana var. *woodii*	November	0	0
Peltophorum africanum	December	6	1.15
Peltophorum africanum	January	7.33	0.58
Peltophorum africanum	February	6.33	1.15
Peltophorum africanum	March	9	0
Peltophorum africanum	April	6	1.73
Peltophorum africanum	May	7.33	0.58
Peltophorum africanum	June	1	0
Peltophorum africanum	July	0.75	0.29
Peltophorum africanum	August	1	0
Peltophorum africanum	September	0.5	0.29
Peltophorum africanum	October	0.02	0.06
Peltophorum africanum	November	0.02	0.06
Trichilia emetica	December	0.33	0.58
Trichilia emetica	January	0	0
Trichilia emetica	February	0	0
Trichilia emetica	March	0	0
Trichilia emetica	April	0.67	0.58
Trichilia emetica	May	0	0
Trichilia emetica	June	0	0
Trichilia emetica	July	0	0
Trichilia emetica	August	0	0
Trichilia emetica	September	0	0
Plant extract	**Month of collection**	**Average equivalent gallic acid**	**SD**

Trichilia emetica	October	0	0
Trichilia emetica	November	0	0
Ziziphus mucronata	December	0.67	0.58
Ziziphus mucronata	January	0.67	0.58
Ziziphus mucronata	February	4.67	1
Ziziphus mucronata	March	3.67	1.15
Ziziphus mucronata	April	1.33	1.15
Ziziphus mucronata	May	6	1.73
Ziziphus mucronata	June	1.83	0.29
Ziziphus mucronata	July	1.42	0.29
Ziziphus mucronata	August	0.75	0.29
Ziziphus mucronata	September	ND	ND
Ziziphus mucronata	October	0	0
Ziziphus mucronata	November	0	0

The correlation between the tannin content and the activity of the plants was compared to see whether there was any correlation between the two. In figures it can be seen that there is no to little correlation between these values. Therefore the activity seems not to be affected by the tannin content of the plants. See Figures 2.31 to 2.40 for a visual comparison.



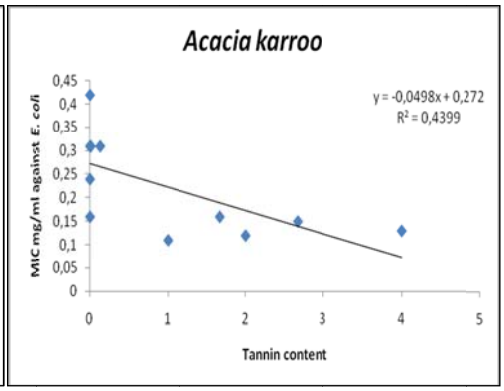
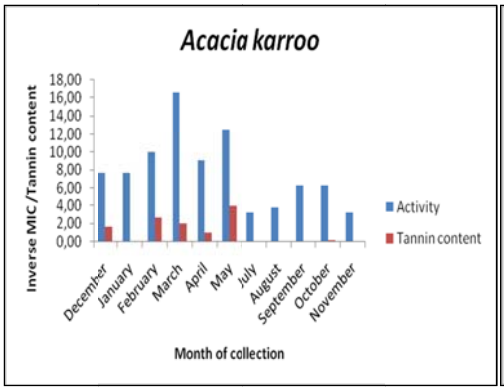


Fig. 2.31 A **Fig. 2.31 B**

Fig. 2.31 A: Comparison between tannin content and activity against *E. coli* **for** *Acacia karroo*
Fig. 2.31 B: Correlation between tannin content and activityagainst *E. coli* **in** *Acacia karroo*



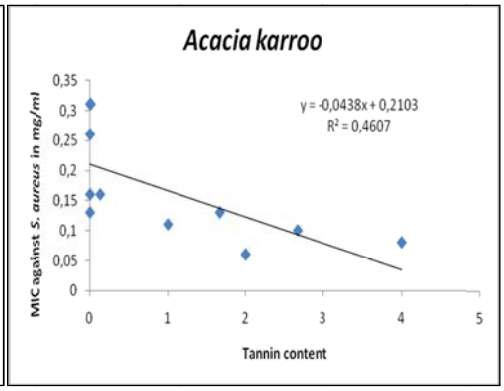
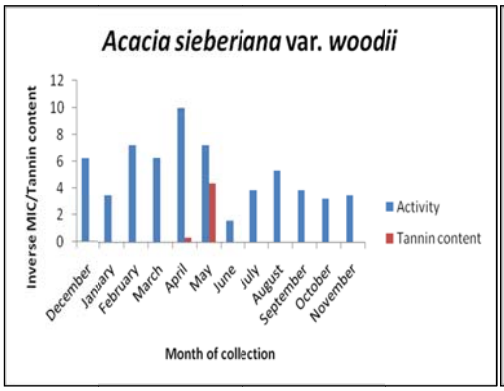


Fig. 2.32 A **Fig. 2.32 B**

Fig. 2.32 A: Comparison between tannin content and activity against *S. aureus* **for** *Acacia karroo* **Fig. 2.32 B: Correlation between tannin content and activity against** *S. aureus* **in** *Acacia karroo*



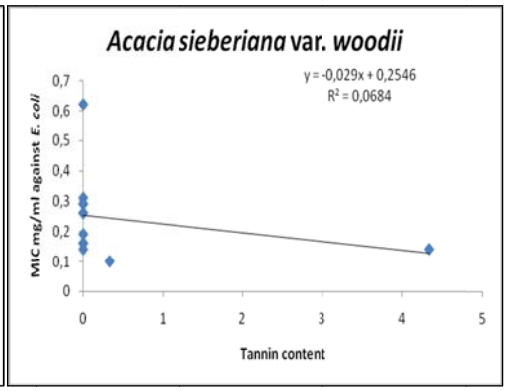
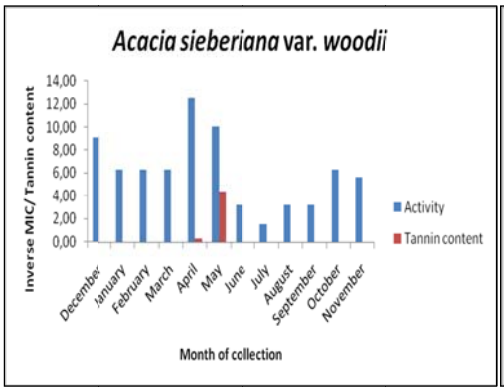


Fig. 2.33 A **Fig. 2.33 B**

Fig. 2.33 A: Comparison between tannin content and activity against *E. coli***for** *Acacia sieberiana* **var.** *woodii* **Fig. 2.33 B: Correlation between tannin content and activity against** *E. coli* **in** *Acacia sieberiana* **var.** *woodii*



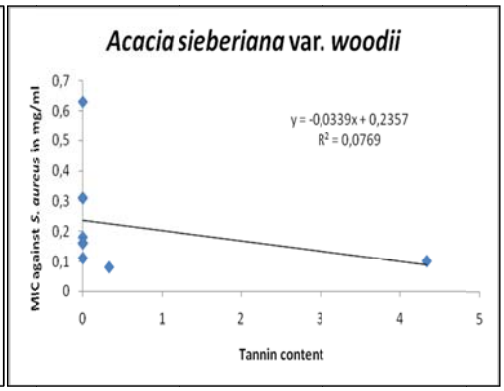
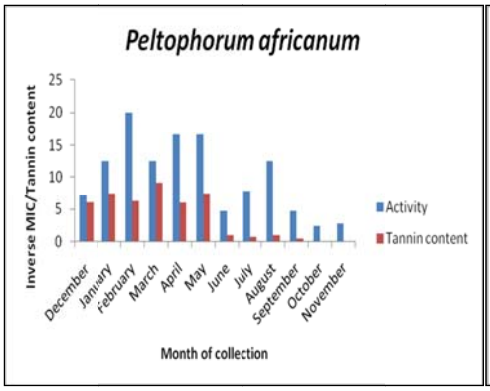


Fig. 2.34 A **Fig. 2.34 B**

Fig. 2.34 A: Comparison between tannin content and activity against *S. aureus* **for** *Acacia sieberiana* **var.** *woodii* **Fig. 2.34 B: Correlation between tannin content and activity against** *S. aureus* **in** *Acacia sieberiana* **var.** *woodii*



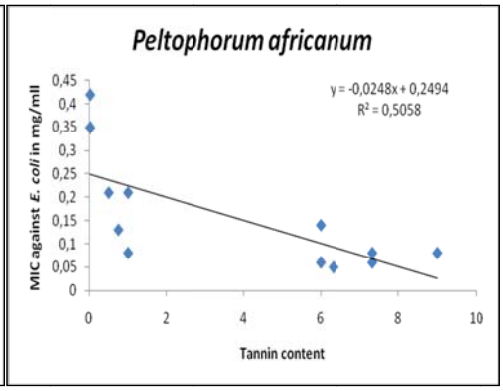
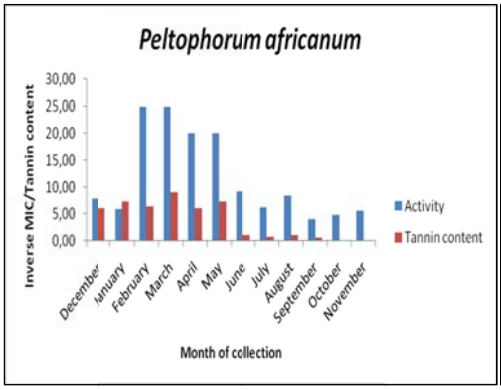


Fig. 2.35 A **Fig. 2.35 B**

Fig. 2.35 A: Comparison between tannin content and activity against *E. coli* **for** *Peltophorum africanum* **Fig. 2.35 B: Correlation between tannin content and activity against** *E. coli* **in** *Peltophorum africanum*



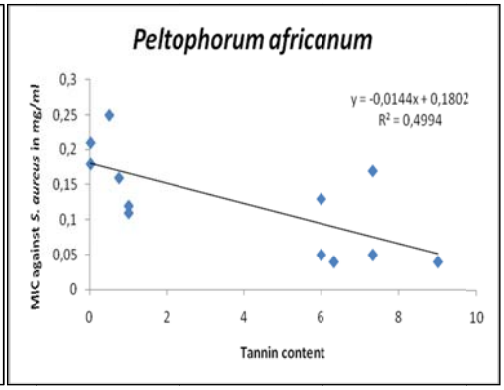
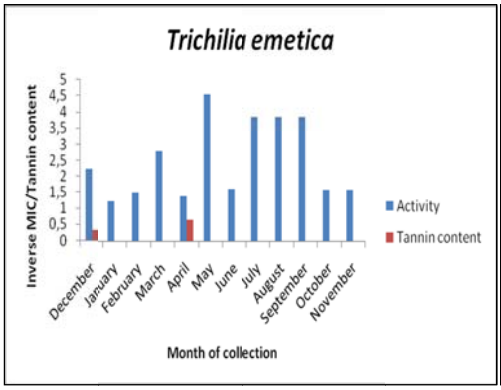


Fig. 2.36 A **Fig. 2.36 B**

Fig. 2.36 A: Comparison between tannin content and activity against *S. aureus* **for** *Peltophorum africanum* **Fig. 2.36 B: Correlation between tannin content and activity against** *S. aureus* **in** *Peltophorum africanum*



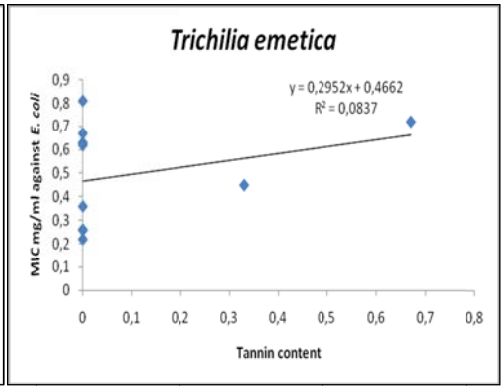
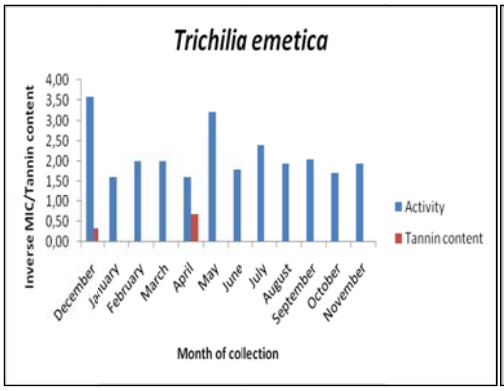


Fig. 2.37 A **Fig. 2.37 B**

Fig. 2.37 A: Comparison between tannin content and activity against *E. coli* **for** *Trichilia emetica*
Fig. 2.37 B: Correlation between tannin content and activity against *E. coli* **in** *Trichilia emetica*



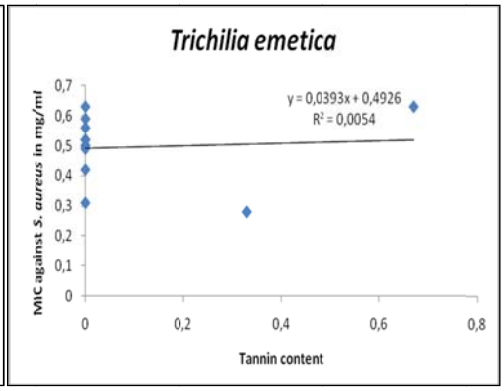
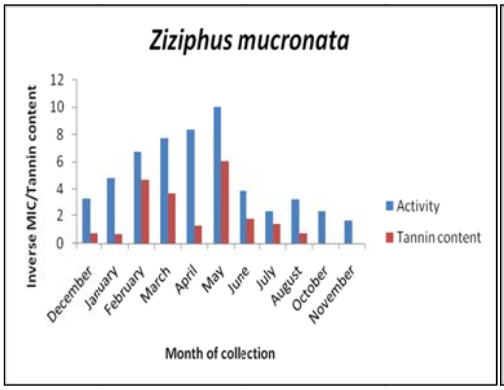


Fig. 2.38 A **Fig. 2.38 B**

Fig. 2.38 A: Comparison between tannin content and activity against *S. aureus* **for** *Trichilia emetica* **Fig. 2.31 B: Correlation between tannin content and activity against** *S. aureus* **in** *Trichilia emetica*



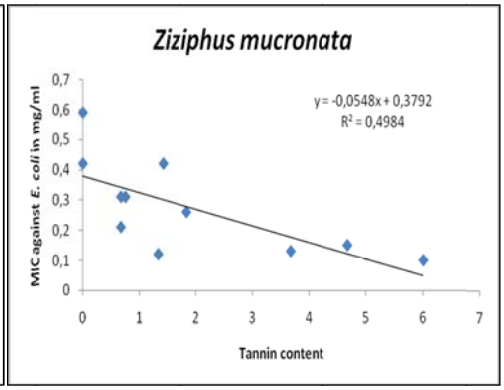


Fig. 2.39 A **Fig. 2.39 B**

Fig. 2.39 A: Comparison between tannin content and activity against *E. coli* **for** *Ziziphus mucronata* **Fig. 2.39 B: Correlation between tannin content and activity against** *E. coli* **in** *Ziziphus mucronata*

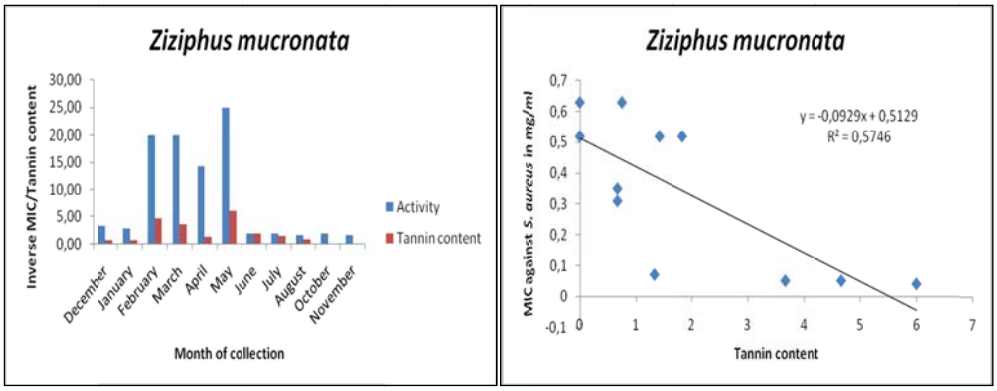


Fig. 2.40 A **Fig. 2.40 B**

Fig. 2.40 A: Comparison between tannin content and activity against *S. aureus* **for** *Ziziphus mucronata* **Fig. 2.40 B: Correlation between tannin content and activity against** *S. aureus* **in** *Ziziphus mucronata*

2.4 Conclusions

These results show that the season is an important factor to consider when plant samples are collected for antibacterial activity screening. The antibacterial activity of plant material of the species tested in this study varied throughout the year. From results obtained in the TLC fingerprinting, the strength of the inhibition bands in bioautography as well as the better MIC results, it was shown that the summer months and especially February to May are preferable for collection. The tannin assays also show that the tannin content in the plants varies throughout the year with more tannins being present in the winter months.

The variation in the tannin content is most probably linked with the patterns of antibacterial activity in the samples of *Peltophorum africanum* and *Ziziphus mucronata*. This can be assumed because the best antibacterial activity was achieved in the months where the highest tannin content was detected and the activity was worse in the months where the plants contained less tannin. However, when comparing the activity with the tannin content in a graph, no constant general correlation can be shown. Therefore the higher activity in the months with higher tannin content seems to be coincidental. Other mechanisms seem to be responsible for the antibacterial activity in all five plants.

In the bioautography assay of *Ziziphus mucronata* extracts, where TLC plates were developed in the EMW and BEA eluents, it can be seen from the R_f-values of the antibacterial compounds that polar compounds are responsible for the inhibition bands in winter as well as in summer against both bacteria. These compounds could be tannins, which tend to be more polar, but to confirm this, the tannins would have to be removed and the bioautography repeated to see if the inhibition bands are still present or

not. However since the correlation between the activity and the tannin content could not be shown, it is unlikely that tannins are the only active compounds.

The *Peltophorum africanum* extract bioautogram showed some faint inhibition bands surrounding polar compounds, especially in the summer months, but not significantly in the winter months.

Given these results, it became clear that the best period to sample leaves for extracts would be the months from January until April for most plants, since there seems to be a correlation between the activity and the season. This will be an important aspect in the production of a commercially produced plant product. The next step was to see whether there also occurred a genetic variability in plants collected in different areas.

Chapter 3

Antibacterial screening of *Combretum molle* leaf extracts to detect possible intraspecies variation

3.1 Introduction

In the previous chapter, it was determined that the activity of plant extracts is dependent on the season in which the leaves are picked, with autumn (January until April) being the best time to collect leaves. The next step in order to ascertain a constant level of activity was to detect whether any changes in activity occurred due to genetic variability. Therefore samples from one plant collected at different areas were investigated in relation to their activity and tannin content.

The reason for selecting *Combretum molle* R.Br.ex G.Don (Combretaceae) for the screening to detect potential variation in intraspecies antibacterial activity was its relatively easy identification and its abundance in the research area, as well as its reported use as an anti-diarrhoeal medication in ethnomedicine (Hutchings *et al.* 1996) and known antibacterial activity against *E. coli* and *S. aureus* (Eloff, 1999).

Combretum molle is a small to medium sized semi-deciduous to deciduous tree (van Wyk and van Wyk, 1997) and is a member of the family Combretaceae. Triterpenoids, mollic acid 1-α-hydroxycycloartenoid and mollic acid 3-β-D-xyloside and their glucosides, xyloside and arabinoside have been isolated from the leaves (Pegel and Rogers, 1976; Pegel and Rogers, 1985; Rogers and Thevan, 1986; Lawton and Rogers, 1993). Mollic acid glucosides from the leaves could be used as a molluscicide (Lawton *et al.* 1991). Extracts from various parts of the plant have antitumor activity against sarcoma 180 (Kerharo and Adam, 1974). The Vhavenda use the leaves for stomach complaints (Mabogo, 1990) and the roots for abdominal pain, diarrhoea, infertility, bleeding after childbirth, convulsions, as an aphrodisiac, for fattening infants and for weakness and backache in Zimbabwe (Gelfand *et al.* 1985). Root infusions are taken for diarrhoea in Tanzania (Hedberg *et al.* 1982) and root decoctions are used for dysentery in East Africa (Hutchings *et al.* 1996).

3.2 Materials and methods

3.2.1 Plant collection

Samples of *Combretum molle* were collected from 42 different trees located in different areas of the northern parts of South Africa to test for possible intraspecies variation and location-dependent variation in antibacterial activity and chemical composition as determined by TLC fingerprints.

The collection areas were the Lowveld Botanical Garden (Nelspruit), the Pretoria Botanical Garden (SANBI), a private game farm in the Waterberg, the Magaliesberg Mountains and the Onderstepoort Campus of the Faculty of Veterinary Science, University of Pretoria (the location of the trees was marked).

After collection the plants were handled as described in section 2.2.1.

3.2.2 TLC fingerprinting

The plates were prepared as described in section 2.2.3.

3.2.3 Bioautographic assays

The bioautographic assays were performed as described in section 2.2.5.

3.2.4 Total Activity

As described in section 2.2.7.

3.2.5 Microdilution assays

As described in section 2.2.6.

3.2.6 Tannin assays

As described in section 2.2.8.

3.3 Results and discussion

3.3.1 Quantity extracted

The plant samples differed clearly in their extraction quantity depending on where they were collected. Plant samples from the Lovweld Botanical Garden in Nelspruit (samples 28-42) extracted in some samples much higher quantities (ranging between 1.5% and 9.5%) than those from the Gauteng area (ranging between 1% and 4.25%). Eloff (1999b) determined that the extracted quantity of a sample of

Combretum molle collected at the Lowveld Botanical Garden in Nelspruit was about 9.8% which correlates well with the results obtained in this study.

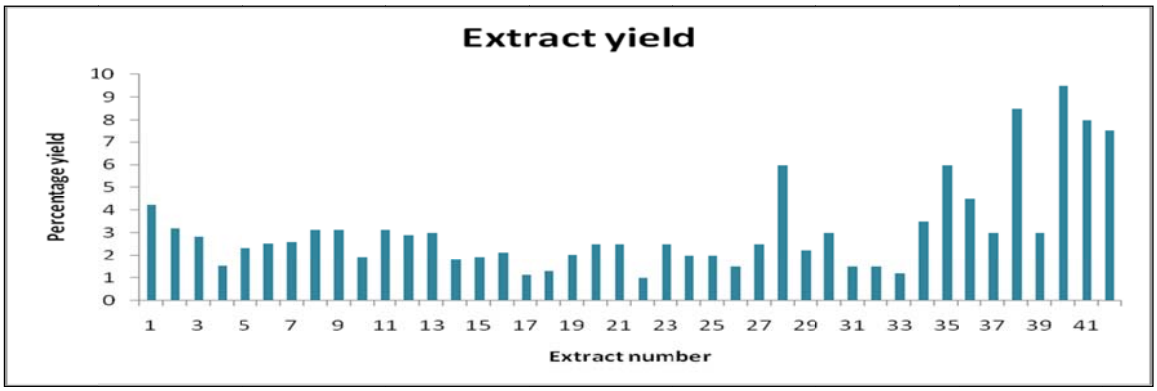


Figure 3.2: Average yield of acetone extraction of 42 different *Combretum molle* samples

Sample 1 - 3 collected at Waterberg; Sample 4 collected at Onderstepoort; Sample 5 – 16 collected at Pretoria Botanical Garden; Sample 17 - 27 collected at Magaliesberg; Sample 28 – 42 collected at Lowveld Botanical Garden

3.3.2 TLC fingerprinting and Bioautographic assays

The TLC fingerprinting showed that all the plants, regardless of their location, had basically the same compound constitution. The only noticeable variation was that some of the compounds were more pronounced in some samples than in others. In the CEF and BEA solvent systems, the Magaliesberg and the Lowveld samples showed some of the compounds more prominently, whereas with the EMW solution system more or less all the compounds were visible with the same intensity. This suggests that the intermediate polarity and non-polar compounds are present in a higher concentration in the Magaliesberg and Lowveld area.

The bioautograms showed that even though the different samples had similar compounds that reacted with vanillin-sulphuric acid in the TLC fingerprinting, they did not all show the same inhibition bands. Some bands were present in all the samples, but others were missing in the different bioautograms. Also noticeable was that the inhibitory bands were variously strongly pronounced among the samples of the different areas. In the samples from the Magaliesberg areas the inhibitory bands against *E. coli* as well as against *S. aureus* were the least pronounced in all three solvent systems. The samples from Waterberg, Onderstepoort and SANBI had more or less the same inhibitory bands in all three solvent systems whereas the sample from Lowveld had extra inhibitory bands against *E. coli* and against *S. aureus* in the EMW eluent, suggesting that the polar antibacterial compounds are present in a higher concentration in the plants of that area.

The chromatograms and bioautographies are displayed in Figures 3.3 to 3.47 below.

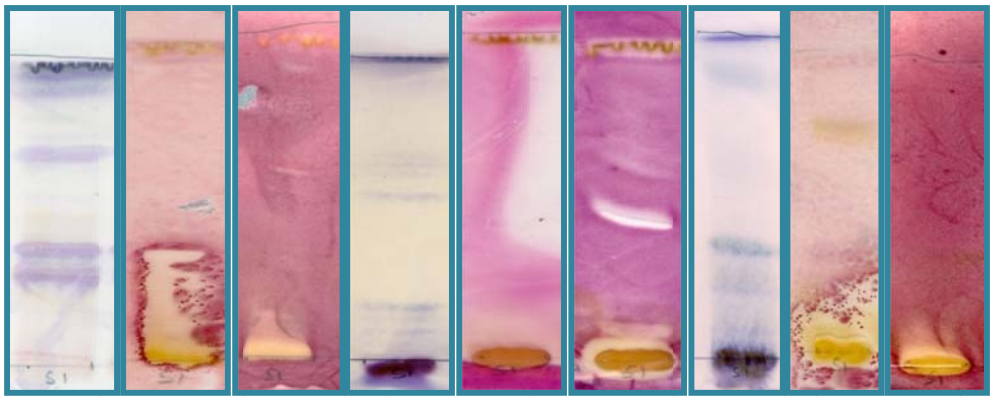


Fig. 3.3 Fig. 3.4 Fig. 3.5 Fig. 3.6 Fig. 3.7 Fig. 3..8 Fig. 3.9 Fig. 3.10Fig. 3.11

Fig. 3.3: *Combretum molle* Waterberg EMW; Fig. 3.4: *Combretum molle* Waterberg EMW against *E.coli;* Fig. 3.5: *Combretum molle* Waterberg EMW against *S. aureus*; Fig. 3.6: *Combretum molle* Waterberg CEF; Fig. 3.7: *Combretum molle* Waterberg CEF against *E. coli*; Fig. 3.8: *Combretum molle* Waterberg CEF against *S.* aureus; Fig. 3.9: *Combretum molle* Waterberg BEA; Fig. 3.10: *Combretum molle* Waterberg BEA against *E.coli*; Fig. 3.11: *Combretum molle* Waterberg BEA against *S. aureus*

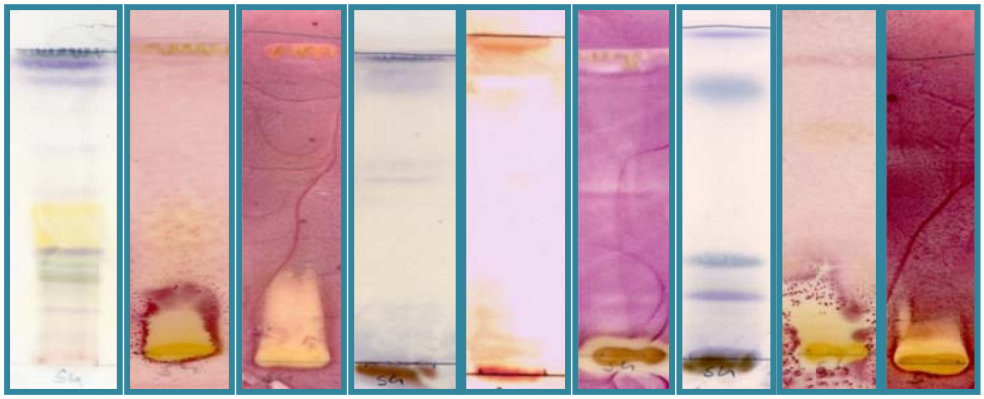


Fig. 3.12 Fig. 3.13 Fig. 3.14 Fig. 3.15 Fig. 3.16 Fig. 3.17 Fig. 3.18Fig. 3.19 Fig. 3.20

Fig. 3.12: *Combretum molle* Onderstepoort EMW; Fig. 3.13: *Combretum molle* Onderstepoort EMW against *E. coli*; Fig. 3.14: *Combretum molle* Onderstepoort EMW against *S. aureus*; Fig. 3.15: *Combretum molle* Onderstepoort CEF; Fig. 3.16: *Combretum molle* Onderstepoort CEF against *E. coli*; Fig. 3.17: *Combretum molle* Onderstepoort CEF against *S. aureus*; Fig. 3.18: *Combretum molle* Onderstepoort BEA; Fig. 3.19: *Combretum mollle* Onderstepoort BEA against E. *coli*; Fig. 3.20: *Combretum molle* Onderstepoort BEA against *S. aureus*

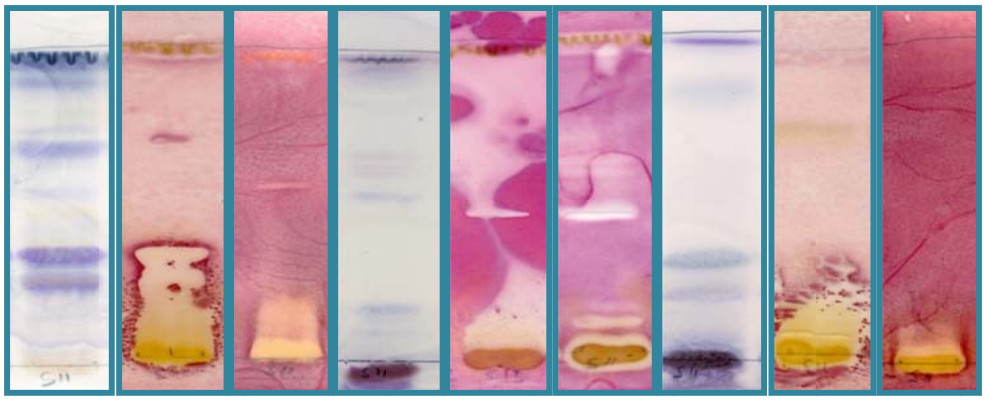


Fig. 3.21 Fig. 3.22 Fig. 3.23 Fig. 3.24 Fig. 3.25 Fig. 3.26 Fig. 3.27 Fig. 3.28 Fig. 3.29

Fig. 3.21: *Combretum molle* SANBI Pretoria Botanical Garden EMW; Fig. 3.22: *Combretum molle* SANBI Pretoria Botanical Garden EMW against *E. coli*; Fig. 3.23: *Combretum molle* SANBI Pretoria Botanical Garden EMW against *S. aureus*; Fig. 3.24: *Combretum molle* SANBI Pretoria Botanical Garden CEF; Fig. 3.25: *Combretum molle* SANBI Pretoria Botanical Garden CEF against *E. coli*; Fig. 3.26: *Combretum molle* SANBI Pretoria Botanical Garden CEF against *S. aureus*; Fig. 3.27: *Combretum molle* SANBI Pretoria Botanical Garden BEA; Fig: 3.28: *Combretum molle* SANBI Pretoria Botanical Garden BEA against *E. coli*; Fig. 3.29: *Combretum molle* SANBI Pretoria Botanical Garden BEA against *S. aureus*

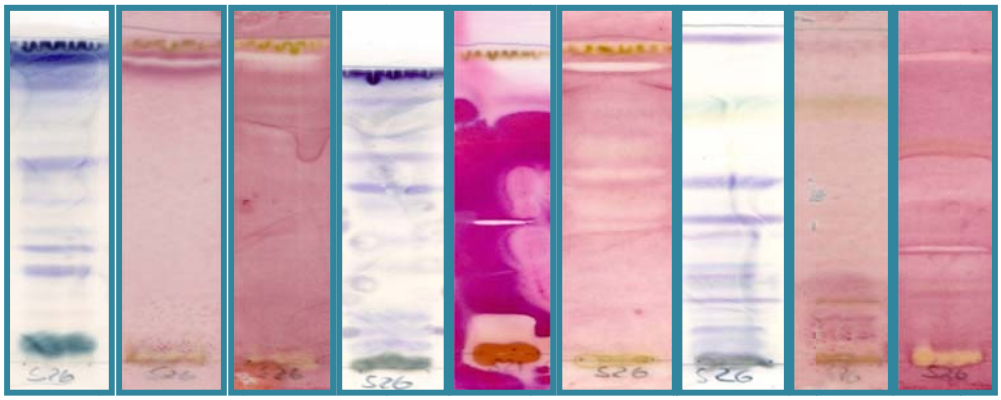


Fig. 3.30 Fig. 3.31 Fig. 3.32 Fig. 3.33 Fig. 3.34 Fig. 3.35 Fig. 3.36 Fig. 3.37 Fig. 3.38

Fig. 3.30: *Combretum molle* Magaliesberg EMW; Fig. 3.31: *Combretum molle* Magaliesberg EMW against *E. coli*¸Fig. 3.32: *Combretum molle* Magaliesberg EMW against *S. aureus*; Fig. 3.33: *Combretum molle* Magaliesberg CEF; Fig. 3.34: *Combretum molle* Magaliesberg CEF against *E. coli*; Fig. 3.35: *Combretum molle* Magaliesberg CEF against *S. aureus*; Fig. 3.36: *Combretum molle* Magaliesberg BEA; Fig. 3.37: *Combretum molle* Magaliesberg BEA against *E. coli*; Fig. 3.38: *Combretum molle* Magaliesberg BEA against *S. aureus*

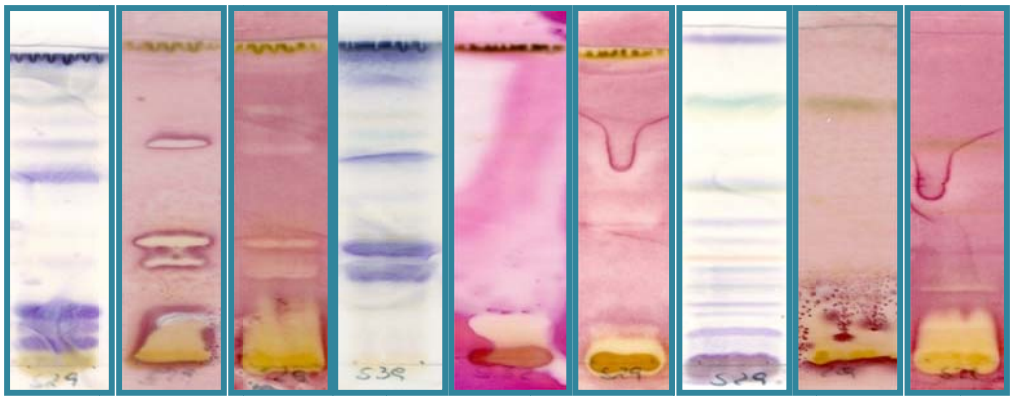


Fig. 3.39 Fig. 3.40 Fig. 3.41 Fig. 3.42 Fig. 3.43 Fig. 3.44 Fig. 3.45 Fig. 3.46 Fig. 3.47

Fig. 3.39: *Combretum molle* Lowveld Botanical Garden Nelspruit EMW; Fig. 3.40: *Combretum molle* Lowveld Botanical Garden Nelspruit EMW against *E. coli*; Fig. 3.41: *Combretum molle* Lowveld Botanical Garden Nelspruit EMW against *S. aureus*; Fig. 3.42: *Combretum molle* Lowveld Botanical Garden Nelspruit CEF; Fig. 3.43: *Combretum molle* Lowveld Botanical Garden Nelspruit CEF against *E. coli*; Fig. 3.44: *Combretum molle* Lowveld Botanical Garden Nelspruit CEF against *S. aureus*; Fig. 3.45: *Combretum molle* Lowveld Botanical Garden Nelspruit BEA; Fig. 3.46: *Combretum molle* Lowveld Botanical Garden Nelspruit BEA against *E. coli*; Fig. 3.43.4: *Combretum molle* Lowveld Botanical Garden Nelspruit BEA against *S. aureus*

3.3.3 Microdilution assays

The microdilution assays (Tables 3.1 and 3.2) showed that all the extracts had about the same antibacterial activity against the test strains of *E. coli* and *S. aureus*, differing in only up to two wells (or dilution factors) between each sample. However due to the fact that the samples from different areas extracted different amounts, the total activity varied much more.

The values obtained for the minimum inhibitory concentration against *E. coli* ranged from 0.15 to 0.42 mg/ml and for *S. aureus* from 0.19 to 0.63 mg/ml. The average value against *E. coli* was 0.227 mg/ml and the average value against *S. aureus* was 0.399mg/ml. Interestingly, the ranges of the values for total activity were from 57 to 307 ml/g and from 24 to 202 ml/g respectively. What was also noticeable was that the effect was mostly bactericidal for both the bacterial strains as activity was not overcome by the bacteria after a longer period of incubation.

Table 3.1: MIC values of 42 different *Combretum molle* samples against *E. coli* after 60 min and 120 min

Extract number	Place of collection	Extract yield (mg)	Average activity *E. coli* 60 (mg/ml) (± SD)	Total activity *E. coli* 60 (ml/g)	Average activity *E. coli* 120 (mg/ml) (± SD)	Total activity *E. coli* 120 (ml/g)
1	Waterberg	42.50	0.19 ± 0.07	220	0.21 ± 0.08	202
2	Waterberg	32.00	0.16 ± 0.00	200	0.20 ± 0.07	162
3	Waterberg	28.50	0.16 ± 0.00	178	0.21 ± 0.08	136
4	Onderstepoort	15.50	0.18 ± 0.05	88	0.23 ± 0.08	68
5	SANBI	23.50	0.25 ± 0.16	96	0.25 ± 0.16	96
6	SANBI	25.50	0.19 ± 0.07	132	0.21 ± 0.08	121
7	SANBI	26.00	0.21 ± 0.08	124	0.21 ± 0.08	124
8	SANBI	31.50	0.23 ± 0.08	139	0.23 ± 0.08	139
9	SANBI	31.50	0.23 ± 0.08	139	0.26 ± 0.07	121
10	SANBI	19.50	0.16 ± 0.00	122	0.21 ± 0.08	93
11	SANBI	31.50	0.16 ± 0.00	197	0.21 ± 0.08	150
12	SANBI	29.00	0.21 ± 0.08	138	0.26 ± 0.07	112
13	SANBI	30.00	0.17 ± 0.06	179	0.17 ± 0.06	179
14	SANBI	18.00	0.15 ± 0.03	119	0.16 ± 0.00	113
15	SANBI	19.50	0.16 ± 0.00	122	0.16 ± 0.00	122
16	SANBI	21.50	0.16 ± 0.00	134	0.16 ± 0.00	134
17	Magaliesberg	11.50	0.16 ± 0.00	72	0.16 ± 0.00	72
18	Magaliesberg	13.00	0.13 ± 0.04	98	0.16 ± 0.00	81
19	Magaliesberg	20.50	0.16 ± 0.00	128	0.21 ± 0.08	98
20	Magaliesberg	25.00	0.16 ± 0.00	156	0.16 ± 0.00	156
21	Magailiesberg	25.00	0.24 ± 0.08	106	0.31 ± 0.00	81
22	Magaliesberg	10.00	0.12 ± 0.04	83	0.16 ± 0.00	63
23	Magaliesberg	25.00	0.19 ± 0.06	135	0.16 ± 0.16	156
24	Magaliesberg	20.00	0.24 ± 0.08	85	0.21 ± 0.08	95
25	Magaliesberg	20.00	0.24 ± 0.08	85	0.21 ± 0.08	95
26	Magaliesberg	15.00	0.29 ± 0.06	53	0.24 ± 0.08	62
27	Magaliesberg	25.00	0.42 ± 0.17	60	0.38 ± 0.14	66
28	Nelspruit	60.00	0.24 ± 0.08	255	0.26 ± 0.07	231
29	Nelspruit	22.25	0.24 ± 0.08	95	0.26 ± 0.07	86
30	Nelspruit	30.00	0.24 ± 0.08	128	0.26 ± 0.07	115
31	Nelspruit	15.00	0.21 ± 0.08	71	0.24 ± 0.08	62
32	Nelspruit	15.00	0.16 ± 0.00	94	0.21 ± 0.08	71
33	Nelspruit	12.00	0.16 ± 0.00	75	0.21 ± 0.08	57
34	Nelspruit	35.00	0.26 ± 0.08	135	0.23 ± 0.08	154
35	Nelspruit	60.00	0.42 ± 0.17	144	0.49 ± 0.17	123
36	Nelspruit	45.00	0.26 ± 0.08	173	0.28 ± 0.07	163
37	Nelspruit	30.00	0.24 ± 0.08	128	0.26 ± 0.07	115
38	Nelspruit	85.00	0.29 ± 0.06	298	0.29 ± 0.05	290
39	Nelspruit	30.00	0.31 ± 0.00	97	0.31 ± 0.00	97
40	Nelspruit	95.00	0.31 ± 0.00	307	0.31 ± 0.00	307
41	Nelspruit	80.00	0.31 ± 0.00	258	0.31 ± 0.00	258
42	Nelspruit	75.00	0.31 ± 0.00	242	0.31 ± 0.00	242

Table 3.2: MIC values of 42 different *Combretum molle* samples against *S. aureus* after 60 min and 120 min

Extract number	Place of collection	Extract yield (mg)	Average activity*S. aureus* 60 (mg/ml) (± SD)	Total activity*S. aureus* 60(ml/g)	Average activity*S. aureus* 120 (mg/ml) (± SD)	Total activity *S. aureus* 120 (ml/g)
1	Waterberg	42.50	0.21 ± 0.08	202	0.37 ± 0.20	116
2	Waterberg	32.00	0.26 ± 0.07	123	0.37 ± 0.20	87
3	Waterberg	28.50	0.21 ± 0.10	135	0.37 ± 0.20	78
4	Onderstepoort	15.50	0.21 ± 0.08	74	0.21 ± 0.07	74
5	SANBI	23.50	0.37 ± 0.21	64	0.37 ± 0.20	64
6	SANBI	25.50	0.26 ± 0.07	98	0.26 ± 0.07	98
7	SANBI	26.00	0.26 ± 0.07	100	0.26 ± 0.07	100
8	SANBI	31.50	0.37 ± 0.21	86	0.37 ± 0.20	86
9	SANBI	31.50	0.26 ± 0.07	121	0.37 ± 0.20	86
10	SANBI	19.50	0.36 ± 0.20	54	0.37 ± 0.20	53
11	SANBI	31.50	0.37 ± 0.21	86	0.37 ± 0.20	86
12	SANBI	29.00	0.42 ± 0.16	70	0.47 ± 0.15	62
13	SANBI	30.00	0.26 ± 0.07	115	0.26 ± 0.07	115
14	SANBI	18.00	0.40 ± 0.18	45	0.40 ± 0.17	45
15	SANBI	19.50	0.37 ± 0.21	53	0.37 ± 0.20	53
16	SANBI	21.50	0.40 ± 0.22	54	0.40 ± 0.21	54
17	Magaliesberg	11.50	0.31 ± 0.00	37	0.31 ± 0.00	37
18	Magaliesberg	13.00	0.31 ± 0.00	42	0.31 ± 0.00	42
19	Magaliesberg	20.50	0.26 ± 0.07	79	0.26 ± 0.07	79
20	Magaliesberg	25.00	0.37 ± 0.21	68	0.37 ± 0.20	68
21	Magaliesberg	25.00	0.31 ± 0.00	81	0.31 ± 0.00	81
22	Magaliesberg	10.00	0.26 ± 0.08	39	0.40 ± 0.20	25
23	Magaliesberg	25.00	0.19 ±0.07	129	0.19 ± 0.06	135
24	Magaliesberg	20.00	0.31 ± 0.00	65	0.31 ± 0.00	65
25	Magaliesberg	20.00	0.21 ± 0.08	95	0.16 ± 0.07	125
26	Magaliesberg	15.00	0.24 ± 0.08	62	0.21 ± 0.08	71
27	Magaliesberg	25.00	0.49 ± 0.17	51	0.42 ± 0.16	60
28	Nelspruit	60.00	0.63 ± 0.00	95	0.63 ± 0.00	95
29	Nelspruit	22.25	0.63 ± 0.00	35	0.63 ± 0.00	35
30	Nelspruit	30.00	0.42 ± 0.16	72	0.47 ± 0.15	64
31	Nelspruit	15.00	0.31 ± 0.00	48	0.31 ± 0.00	48
32	Nelspruit	15.00	0.63 ± 0.00	24	0.63 ± 0.00	24
33	Nelspruit	12.00	0.21 ± 0.08	57	0.24 ± 0.07	51
34	Nelspruit	35.00	0.63 ± 0.00	56	0.63 ± 0.00	56
35	Nelspruit	60.00	0.63 ± 0.00	95	0.63 ±0.00	95
36	Nelspruit	45.00	0.63 ± 0.00	71	0.63 ± 0.00	71
37	Nelspruit	30.00	0.63 ± 0.00	48	0.63 ± 0.00	48
38	Nelspruit	85.00	0.63 ± 0.00	135	0.63 ± 0.00	135
39	Nelspruit	30.00	0.63 ± 0.00	48	0.63 ± 0.00	48
40	Nelspruit	95.00	0.63 ± 0.00	151	0.63 ± 0.00	151
41	Nelspruit	80.00	0.63 ± 0.00	127	0.63 ± 0.00	127
42	Nelspruit	75.00	0.63 ± 0.00	119	0.63 ± 0.00	119

Another aspect noticeable from the MIC results was that trees from the Lowveld Botanical Garden in Nelspruit varied in their activity. Samples 34 to 42 were all growing in the same place in the garden where they have been planted whereas samples 28 to 33 were collected at different locations spread out all over the garden where they grew naturally. The variation in activity could result from the different

soil quality in the different parts of the garden as well as from the natural occurrence opposed to the human influenced one.

After the determination of the antibacterial activity, Near Infrared Reflectance (NIR) Spectra of all the samples were evaluated. Near Infrared Spectroscopy is an analytical technique used in phytomics and is mainly used for structure elucidation of unknown samples, but it also plays an important role in phytoanalysis (Stecher *et al.* 2003). One ml of extract of each sample was dripped onto a microscope slide and dried. Those microscope slides were then read by a Near Infrared Reflectance (NIR) Bruker Tensor 27 apparatus situated at Biomox Pharmaceuticals (Pty) Ltd in Pretoria. All the samples had the same basic spectrum even though the concentration varied significantly due to the measuring method (during measurement one ml of the extract in a concentration of 10 mg/ml was dropped onto an objective slide and the slide was then inserted into the machine and measured; since the flow pattern of the extract was different on every slide, the concentration varied significantly). An example of the spectra is shown in Figure 3.48 below.

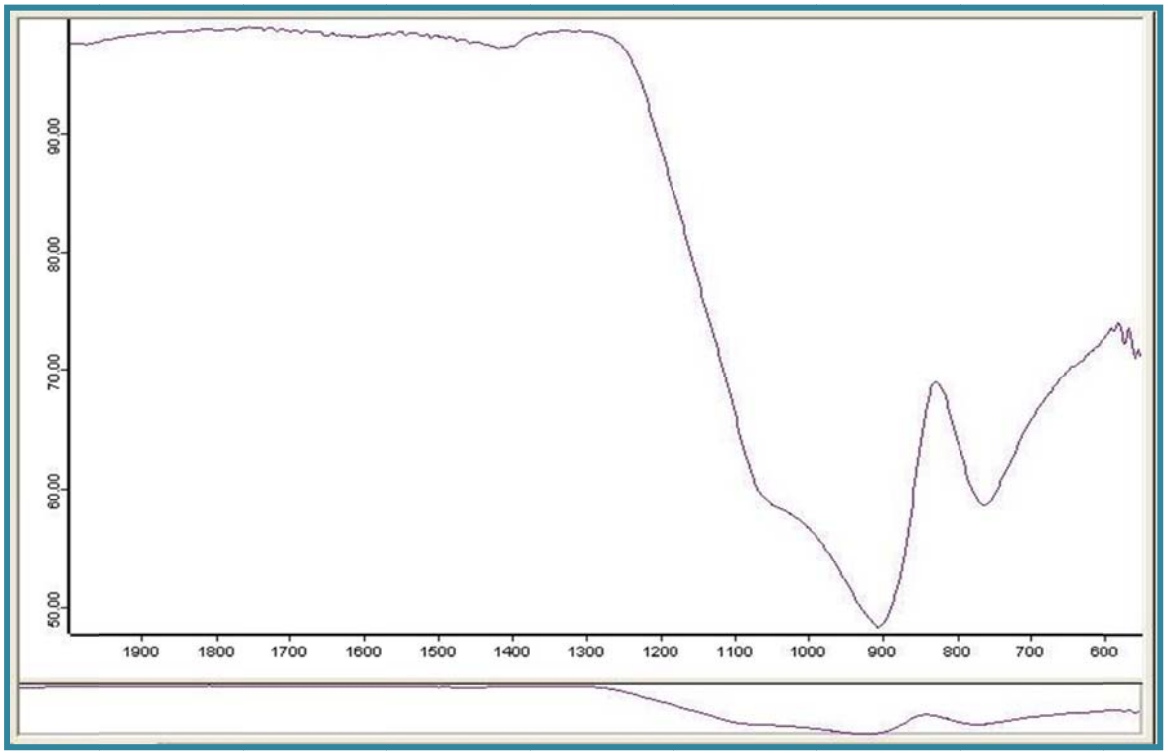


Fig. 3.48 NIR spectrum of *Combretum molle* collected at SANBI Pretoria Botanical Garden

3.3.4 Tannin assays

The tannin assays, performed in triplicate, showed that most of the samples contained tannins (Table 3.3). This was to be expected since they are all from the same plant species, belonging to a family where it is known that several of its members are often tanniferous and produce ellagic and gallic acids (Hutchings *et al.* 1996).

However, two of the samples collected in the Lowveld did not show any tannin in the assay. This could be due to a human error in the extraction process or in the performance of the actual assay despite repeating these processes, but it could be also due to the fact that the concentrations of tannins present in the extract were too low to be detected by this method.

It is observable by these results that the tannin content in different plants from the same species varies. It is conceivable that tannin content may remain relatively constant in certain species, but this study provides evidence that variations occur as the results in this study show. The average square diameter of the zone of the precipitated protein in one of the samples from the Magaliesberg area was 4.00, the highest value, and no zone of precipitation at all in a sample from the Lowveld Botanical Garden was the lowest value.

Table 3.3: Results of tannin assays of 42 different *Combretum molle* samples (results for average square diameter of zone are not given since the average equivalent gallic acid translated 1:1 to this value)

Extract number	Place of collection	Average equivalent gallic acid	SD
1	Waterberg	0.50	0.29
2	Waterberg	1.00	0.00
3	Waterberg	1.42	0.29
4	Onderstepoort	2.83	0.29
5	SANBI	0.75	0.29
6	SANBI	0.75	0.29
7	SANBI	0.75	0.29
8	SANBI	0.75	0.29
9	SANBI	0.50	0.29
10	SANBI	0.75	0.29
11	SANBI	1.42	0.29
12	SANBI	1.83	0.29
13	SANBI	1.00	0.00
14	SANBI	0.75	0.29
15	SANBI	1.75	0.76
16	SANBI	0.50	0.29
17	Magaliesberg	0.09	0.00
18	Magaliesberg	0.14	0.12
19	Magaliesberg	1.17	0.50
20	Magaliesberg	1.00	0.00
21	Magaliesberg	0.50	0.29
22	Magaliesberg	4.00	0.00
23	Magaliesberg	2.42	0.50
24	Magaliesberg	1.42	0.29
25	Magaliesberg	0.01	0.12
26	Magaliesberg	0.02	0.10
27	Magaliesberg	0.04	0.15
28	Nelspruit	0.06	0.17
29	Nelspruit	0.50	0.29
30	Nelspruit	0.75	0.29
31	Nelspruit	0.04	0.00
32	Nelspruit	0.10	0.21
33	Nelspruit	0.00	0.06
34	Nelspruit	0.33	0.58
35	Nelspruit	0.13	0.15
36	Nelspruit	0.42	0.50
37	Nelspruit	0.50	0.29
38	Nelspruit	0.03	0.06
39	Nelspruit	0.07	0.06
40	Nelspruit	0.00	0.00
41	Nelspruit	0.50	0.29
42	Nelspruit	0.00	0.00

When comparing the activity against *E. coli* of all the different samples with their tannin content it became clear that, as seen already in the previous chapter, there was little to no correlation between the two values. The only exception was sample 23 (when tested against *E. coli*, but not when tested against *S. aureus*). It also could be seen that samples collected from Nelspruit (samples 28-42) generally had a

lower activity volume than plants from other areas. See Figures 3.48 to 3.51 for a visual comparison of the data.

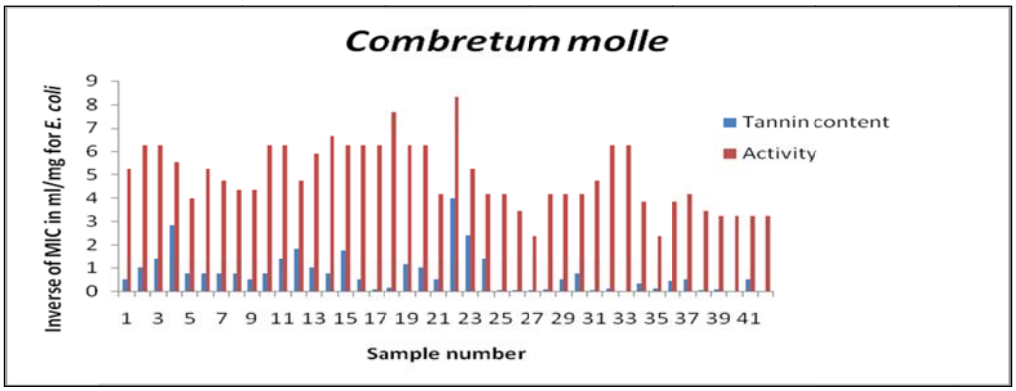


Fig. 3.49: Comparison of the activity of the different *Combretum molle* samples against *E. coli* and their tannin content

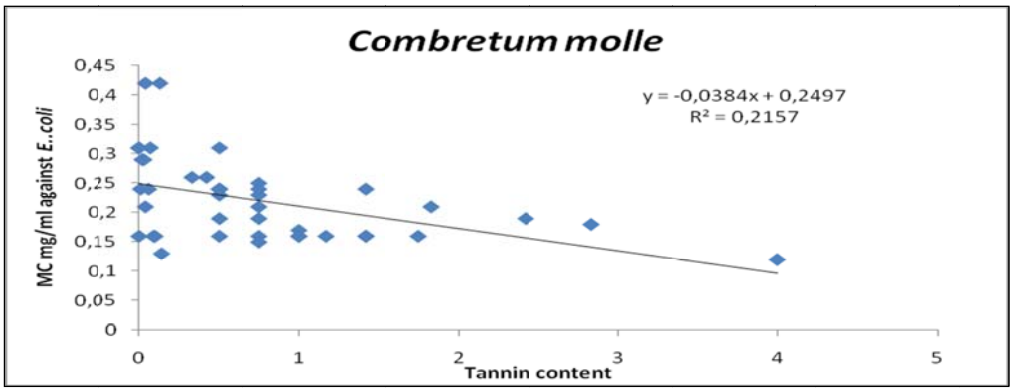


Fig. 3.50: Correlation of the activity against *E. coli* and the tannin content of the different *Combretum molle* samples

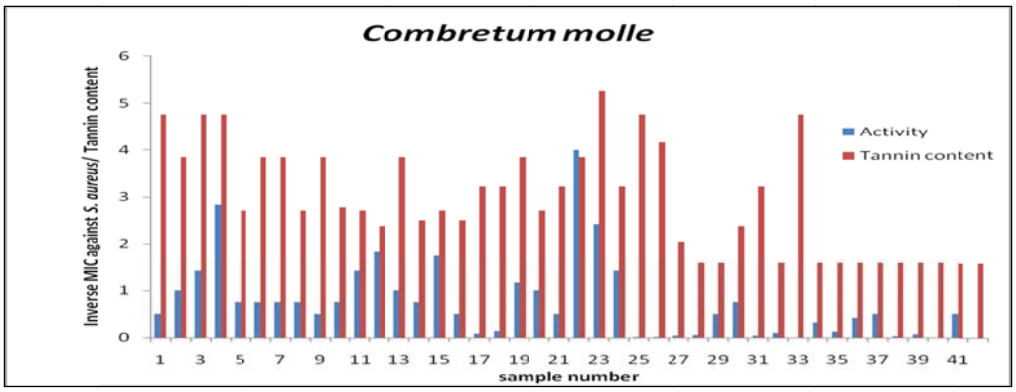


Fig. 3.51: Comparison of the activity of the different *Combretum molle* samples against *S. aureus* and their tannin content

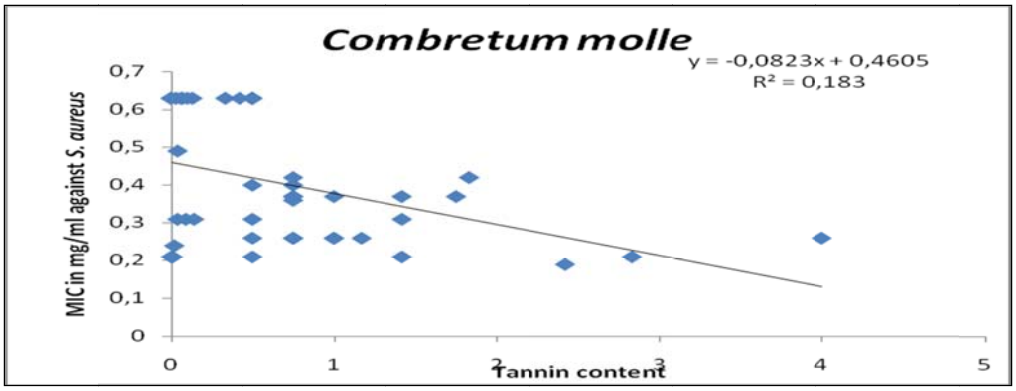


Fig. 3.52: Correlation of the activity against *S. aureus* **and the tannin content of the different** *Combretum molle* **samples**

3.4 Conclusions

These results clearly show that there is no obvious intraspecies variation in antibacterial activity, reflected by the MIC values. Some samples however demonstrated inhibition bands at different R_f values in the bioautography assays. This could mean that even though the MIC values stayed constant, different compounds could be responsible for the activity. The sample from the Onderstepoort campus had the lowest number of inhibition bands, which might also be due to the fact that the leaves were still very young at the time of collection. This occurrence might also explain the difference in the quantities extracted from the different plants, since a lower quantity was extracted from plants growing in the Gauteng area than from the Mpumalanga area. Even though all the samples were collected around the same time, they differed, with the samples from Mpumalanga having already further developed leaves during collection than those in the Gauteng area. This is most probably due to the different climate. Gauteng was experiencing a dry period whereas there was more rainfall in the Mpumalanga area at the time of collection.After comparing the activity of the different samples with their tannin content, it became clear that with the exception of one sample against *E. coli*, there was little to no correlation between the tannin content and the activity volume of the plants. Therefore the contribution of tannin to the activity seems to be negligible.

It is very good news that there was not much difference in MIC values between different communities, at least when looking at *Combretum molle* since this means that the location of collection is not important in the production of extracts with the same antibacterial properties.

The next step after seeing that the location of the trees does not, but the season does indeed affect the activity of the extracts, was to develop a system to select the most promising plant species for further indepth studies.