UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

# A RATIONAL IN VITRO EVALUATION OF 53 MEDICINAL PLANTS USED IN THE TREATMENT OF DIARRHOEA AND THE POTENTIAL USE OF *DEINBOLLIA OBLONGIFOLIA* (SAPINDACEAE) EXTRACTS

**Gabriele Würger**

Mag. Med. Vet. (Veterinärmedizinische Universität Wien)

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Department of Paraclinical Sciences

Faculty of Veterinary Science

University of Pretoria

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Promoter: Prof J.N. Eloff

Co-promoter: Dr L.J. McGaw

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# DECLARATION

This represents an experimental record for the work carried out in the Department of Pharmacology, University of Pretoria, under the supervision of Prof. J.N. Eloff and Dr L.J. McGaw.

I, the undersigned Gabriele Würger, present this document as my authentic material and acknowledge that it has not been submitted in any other form to any other institution. I also acknowledge that I have consulted many publications in compiling this work and the references are all listed.

________________________________

Gabriele Würger

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| | |
|---|---|
| A/G | Albumin/Globulin ratio |
| ALP | Alkaline Phosphatase |
| ALT | Alanine aminotransferase |
| AST | Aspartate aminotransferase |
| BEA | Benzene/Ethanol/Ammonia |
| C | crude extract |
| Ca | Calcium |
| CEF | Chloroform / Ethyl acetate/ Formic acid |
| CF | Chloroform Fraction of *Deinbollia oblongifolia* |
| CNMR | Carbon nuclear magnetic resonance |
| COSY | Correlated spectroscopy |
| Crea | Creatinine |
| CSIR | Council for Scientific and Industrial Research |
| DB | *Deinbollia oblongifolia* n-butanol fraction |
| DC | *Deinbollia oblongifolia* chloroform fraction |
| DEPT | Distortionless enhancement by polarization transfer |
| DH | *Deinbollia oblongifolia* hexane fraction |
| DO | *Deinbollia oblongifolia* |
| DM | *Deinbollia oblongifolia* 35% water in methanol fraction |
| DMSO | Dimethyl sulfoxide |
| DW | *Deinbollia oblongifolia* water fraction |
| EA | Ethyl acetate |
| *E. coli* | *Escherichia coli* |
| EMW | Ethyl acetate/Methanol/Water |
| F | Fraction |
| GGT | Gamma glutamyl transferase |
| GLOB | Globulin |
| H | Hexane |

| | |
|---|---|
| HMBC | Heteronuclear bond correlation |
| HMQC | Heteronuclear multiple quantum correlation |
| HNMR | Proton nuclear magnetic resonance |
| INT | p-Iodonitrotetrazolium violet |
| K | Potassium |
| $LC_{50}$ | Lethal celltoxicity |
| MEM | Minimum essential medium |
| MIC | Minimum Inhibitory Concentration |
| MTT | 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide |
| Na | Sodium |
| NIR | Near-Infrared Reflectance |
| NMR | Nuclear magnetic resonance |
| OECD | Organisation for Economic Cooperation and Development |
| P.a. | *Peltophorum africanum* |
| $R_f$ | Retention factor |
| *S. aureus* | *Staphylococcus aureus* |
| SB | *Spirostachys africana* n-butanol fraction |
| SC | *Spirostachys africana* chloroform fraction |
| SD | Standard Deviation |
| SH | *Spirostachys africana* hexane fraction |
| SI | Selectivity Index |
| SM | *Spirostachys africana* 35% water in methanol fraction |
| SW | *Spirostachys africana* water fraction |
| TA | Total activity |
| T.e. | *Trichilia emetica* |
| TLC | Thin Layer Chromatography |
| TSP | Total serum protein |
| Urea | Urea |

| | |
|---|---|
| UV | Ultraviolet |
| WHO | World Health Organization |
| Z.m. | *Ziziphus mucronata* |

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# ABSTRACT

Antibiotic Feed Additives (AFA) have been used to prevent many bacterial infections during weaning of livestock. The use of these AFA's resulted in the development of multiresistant bacterial strains and was therefore banned by the European Union. The United States also restricted the use of these feed additives considerably. Many scientists have started to search for alternatives in the prophylactic and therapeutic treatment of bacterial infections. Plants have been used traditionally by resource poor people all over the world to treat many infections. Diarrhoea not only causes many problems in the high intensity production of livestock but also leads to many human deaths. A large number of plants have been used to treat diarrhoea in humans and animals. Several authors have selected one or two species based on traditional use to evaluate in depth. In this project several different relevant parameters of 53 plant species used traditionally to treat diarrhoea were investigated in order to develop a model that would identify the species with the highest chance of delivering a useful antibacterial product.

Antibacterial activities against two pathogens important in diarrhoea (*Staphylococcus aureus* and *Escherichia coli)* were positive parameters in selecting species. Because tannins frequently have antibacterial activity, but are not useful as prophylactic agents due to their effect on production, high tannin content was considered to be a negative indication. Cellular toxicity was also used as a negative parameter at a later stage.

In addition to the *in vitro* assays there are also other parameters to be investigated to evaluate the potential use of plants. The influence of season of collection was determined on the antibacterial activity and tannin content of acetone leaf extracts of five plant species traditionally used to treat diarrhoea. They were *Acacia karroo*, *Acacia sieberiana* var. *woodii*, *Peltophorum africanum*, *Trichilia emetica* and *Ziziphus mucronata*.

The antibacterial activity varied depending on the season of collection with the best activity generally in the months of late summer to autumn (January to April). The activity of *Acacia karoo* against *E. coli* was best in the month of April (MIC = 0.11 mg/ml (TA = 332 ml/g)) and against *S. aureus* in the month of March (MIC = 0.06 mg/ml (TA = 334 ml/g)). *Acacia sieberiana* subsp*. woodii* extracts had the best activity against *E. coli* (MIC = 0.10 mg/ml (TA = 303 ml/g)) in March against *S. aureus* in April (MIC = 0.08 mg/ml (TA = 303 ml/g)). *Peltophorum africanum* extracts were most active against *E. coli* in February (MIC = 0.05 mg/ml (TA = 1188 ml/g)) and against *S. aureus* in February and March (MIC = 0.04 mg/ml (TA = 1188 ml/g and 1075 ml/g)). *Trichilia emetica* extracts were generally not very active against the bacterial strains (best activity: MIC = 0.22 mg/ml (TA = 74 ml/g) against *E. coli* in May and MIC = 0.28 mg/ml (TA = 26 ml/g) against *S. aureus* in December). *Ziziphus mucronata* was most active

against both bacterial strains and in May (*E. coli*: MIC = 0.10 mg/ml (TA = 589 ml/g); *S. aureus*: MIC = 0.04 mg/ml (TA = 1099 ml/g)). The tannin content varied in the extracts as well. The antibacterial activity however did not seem to be directly correlated to the tannin content.

Another important parameter in the use of plant species is to determine the interspecies variation of plants based on genetic or environmental influences. Leaves from 42 plants of *Combretum molle* were collected at different locations during the same season. The average MIC against *E. coli* was 0.227 mg/ml. The low standard deviation of 0.07 indicates that there was very little variation in activity. The average value against *S. aureus* was 0.399 mg/ml with a slightly higher standard deviation of 0.16. However due to the fact that the samples from different areas extracted different amounts, the total activity varied. The tannin assays revealed that there was with one exception no correlation between the antibacterial activity and the tannin content. So it can be safe to assume that genetic variation does not influence the activity too much at least in *C. molle* leaves

Fifty three plant species traditionally used to treat diarrhoea in published literature were then ranked using a novel system in order to determine which species had the most potential value. Ranking was based on the lowest MIC value against *E. coli*, lower activity against *S. aureus* (to limit selecting for general metabolic toxins), low tannin concentration and high extract yield.

From this ranking, five plants were chosen to investigate their potential value further: *Acacia sieberiana* var. *woodii* (*E. coli*: MIC = 0.13 mg/ml, TA = 108 ml/g; *S. aureus*: MIC = 0.13 mg/ml, TA = 108 ml/g; Yield = 14 mg), *Albizia adianthifolia* (*E. coli*: MIC = 0.14 mg/ml, TA = 239 ml/g; *S. aureus*: MIC = 0.04 mg/ml, TA = 765 ml/g; Yield = 34 mg), *Deinbollia oblongifolia* (*E. coli*: MIC = 0.17 mg/ml, TA = 158 ml/g; *S. aureus*: MIC = 0.08 mg/ml, TA = 338 ml/g; Yield = 27 mg), *Spirostachys africana* (*E. coli*: MIC = 0.13 mg/ml, TA = 300 ml/g; *S. aureus*: MIC = 0.09 mg/ml, TA = 438 ml/g; Yield = 38 mg) and *Tetradenia riparia* (*E. coli*: MIC = 0.09 mg/ml, TA = 214 ml/g; *S. aureus*: MIC = 0.13 mg/ml, TA = 149 ml/g; Yield = 20 mg). None of the plants contained any tannin.

The next step towards the recommendation of a plant for the development of a commercial product was to evaluate the cytotoxicity of the selected five species. The following values were obtained: *Acacia sieberiana* var. *woodii* $LC_{50}$ = 0.026 mg/ml, *Albizia adianthifolia* $LC_{50}$ = 0.068 mg/ml, *Deinbollia oblongifolia* $LC_{50}$ = 0.078 mg/ml, *Spirostachys africana* $LC_{50}$ = 0.025 mg/ml and *Tetradenia riparia* $LC_{50}$ = 0.028 mg/ml.

*Deinbollia oblongifolia* (for its low $LC_{50}$ value) and *Spirostachys africana* (for its good antibacterial activity and total activity) were potentized by removing inactive compounds through solvent-solvent fractionation. The antibacterial activity against *E. coli* was increased this way (MIC = 0.08 mg/ml for

*Deinbollia oblongifolia* (chloroform fraction) and MIC = 0.08 mg/ml for *Spirostachys africana* (chloroform fraction)) The $LC_{50}$ values for both chloroform fractions were determined ($LC_{50}$ = 0.188 mg/ml for *Deinbollia oblongifolia* and $LC_{50}$ = 0.062 mg/ml for *Spirostachys africana*). The selectivity index (SI) was also determined and proved that the potentization was indeed successful (*Deinbollia oblongifolia* SI = 2.35 compared to a value of 0.45 for the crude extract; *Spirostachys africana* SI = 0.78 compared to a value of 0.19 for the crude extract). Based on these values, the chloroform fraction of *Deinbollia oblongifolia* was chosen as the less toxic one with similar activity and a higher selectivity index to be worked on further. One of the active compounds was isolated and evaluated for its activity against *E. coli* (MIC = 0.74 mg/ml) The $LC_{50}$ value of 0.042 mg/ml indicated that the activity of the extract was a result of synergism rather than being due to a single active compound (the selectivity index (SI) was 0.06 compared to the values of 0.45 for the crude and 2.35 for the potentized extract).

The plant extracts should of course be as effective against pathogenic strains as they were against the ATCC strains and so the extracts and pure compound of *Deinbollia oblongifolia* were tested for their activity against four different pathological *E. coli* strains. The results showed that the crude extract and the fraction were as active as in the preliminary screening results against only one of the four pathological strains. The pure compound on the other hand was more active against all four pathological strains than against the ATCC strain.

The next step was to test the safety of the extracts of *Deinbollia oblongifolia* in mammals. Unfortunately neither the crude extract nor the chloroform fraction of *Deinbollia oblongifolia* could be used safely in a living organism or in an isolated organ study. A part of the problem may have been caused by the vehicle used in the study despite reports in the literature that an acetone water mixture is safe to use.

In general all the species investigated had good antibacterial activity against *E. coli* this supports the traditional use of these species although we used acetone as extractant rather than the water used traditionally. There were major differences in antibacterial activity over a season indicating that mature leaves were more active than young leaves before senescence started. At least in the case of *Combretum molle* there was little difference in the antibacterial activity of many plants collected at different locations during the same season.

The results obtained in this study could be useful in further studies to develop extracts that can be used to control diarrhoea in animals. Possibly more emphasis should be put on the difference in activity towards *E. coli* and *S. aureus* to eliminate the presence of general metabolic toxins. Such an approach would lead to a different priority order for species to examine. A major first step would probably be to test the *in vitro* and *in vivo* toxicity of selected species.

# 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

*karroo*

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).

var.

*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).

*rum*

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).

*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.

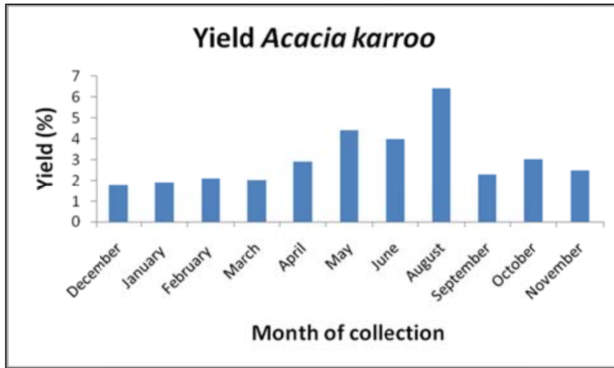



**Fig. 2.6: Average yield of *Acacia karroo* with acetone extraction (no sample in July)**

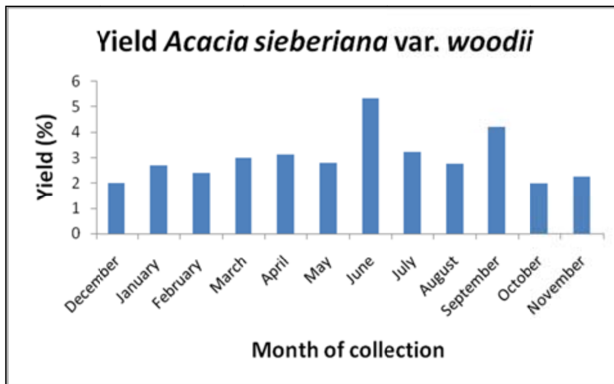



**Fig. 2.7: Average yield of *Acacia sieberiana* var. *woodii* with acetone extraction**

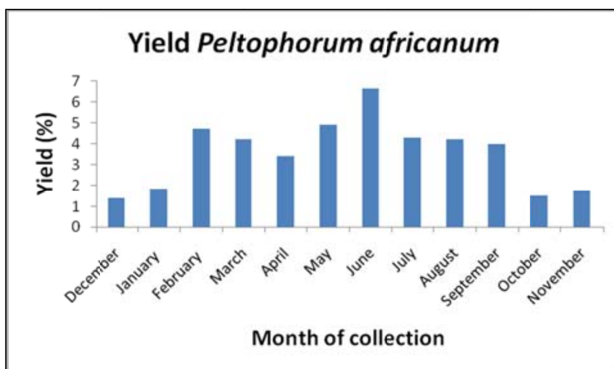



**Fig. 2.8: Average yield of *Peltophorum africanum* with acetone extraction**

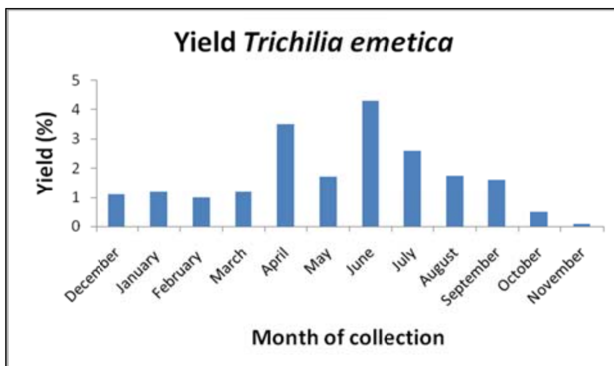



**Fig. 2.9: Average yield of *Trichilia emetica* with acetone extraction**

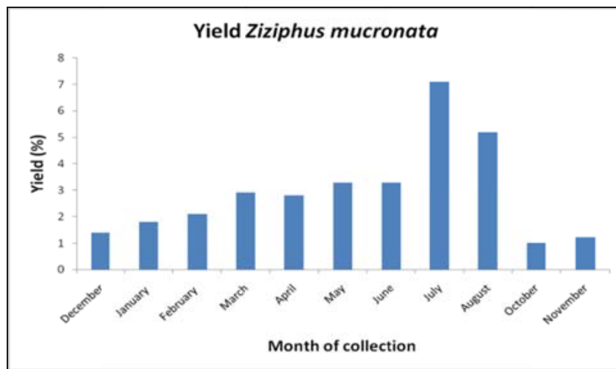



**Fig. 2.10: Average yield of *Ziziphus mucronata* with acetone extraction (no sample in September)**

### 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.

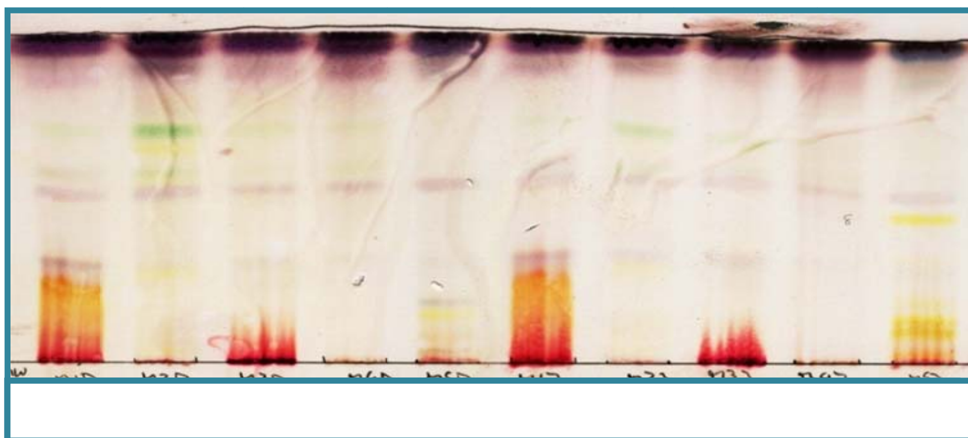


**Fig. 2.11: Chromatograms of monthly samples of five species (M1-M5) collected during December (D) and January (J) separated by using EMW**

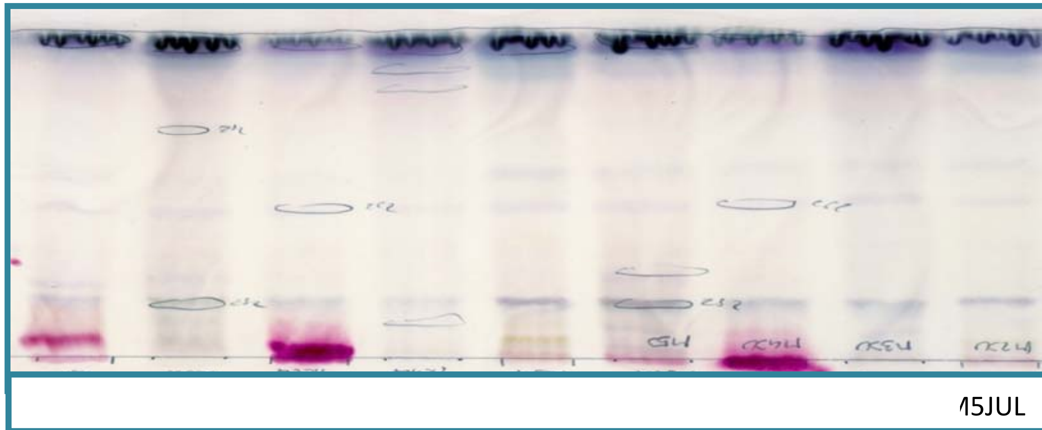



**Fig. 2.12: Chromatograms of monthly samples of five species collected during June and July separated by using EMW**

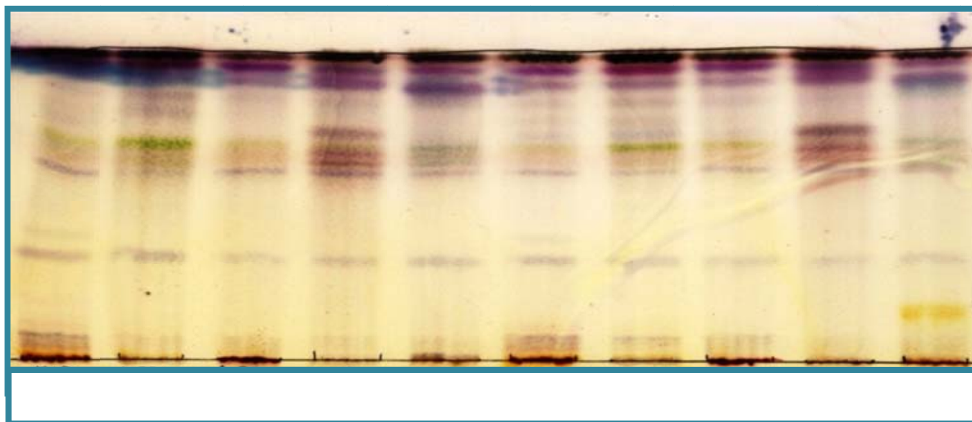


**Fig. 2.13: Chromatograms of monthly samples of five species collected during December and January separated by using CEF**

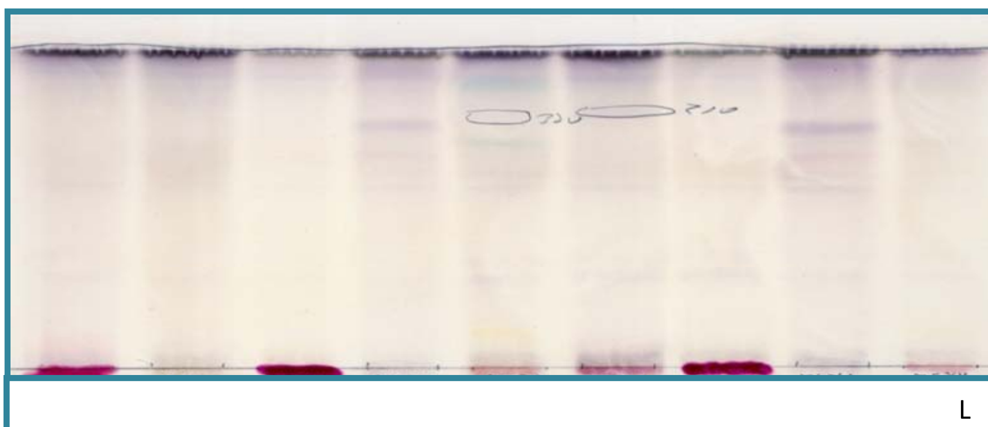


**Fig. 2.14: Chromatograms of monthly samples of five species collected during June and July separated by using CEF**

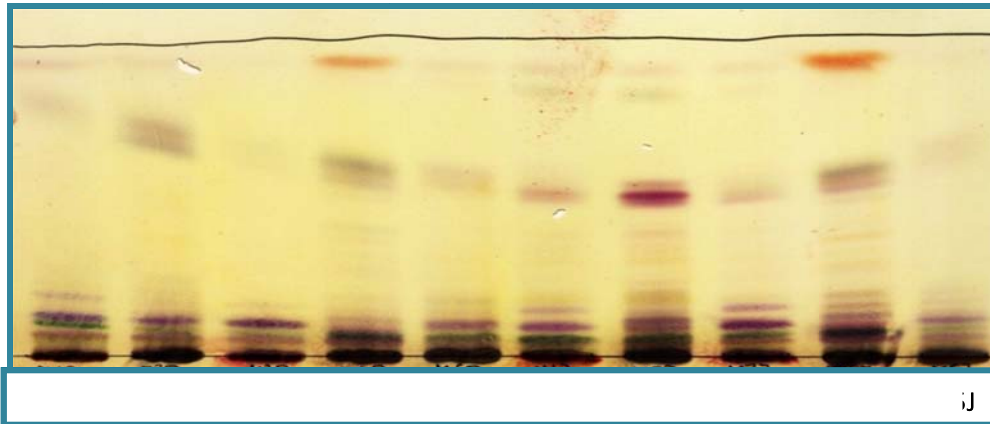


**Fig. 2.15: Chromatograms of monthly samples of five species collected during December and January separated by using BEA**

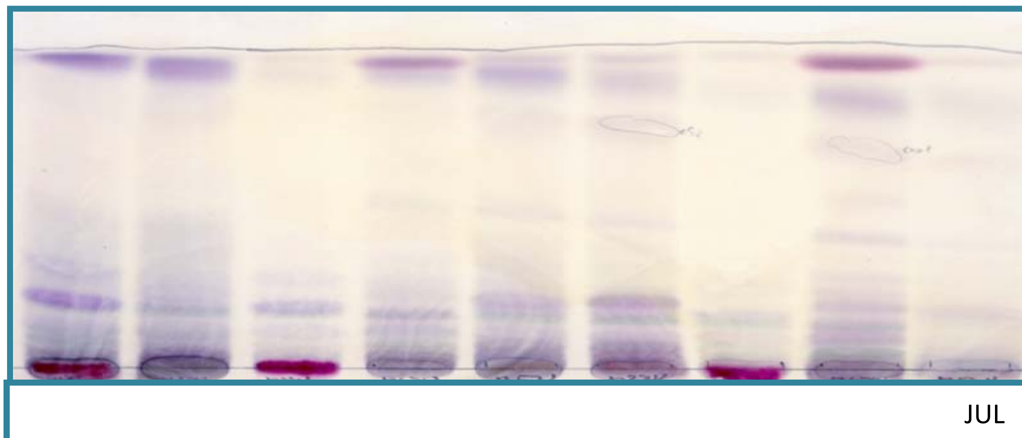



**Fig. 2.16: Chromatograms of monthly samples of five species collected during June and July separated by using BEA**

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.

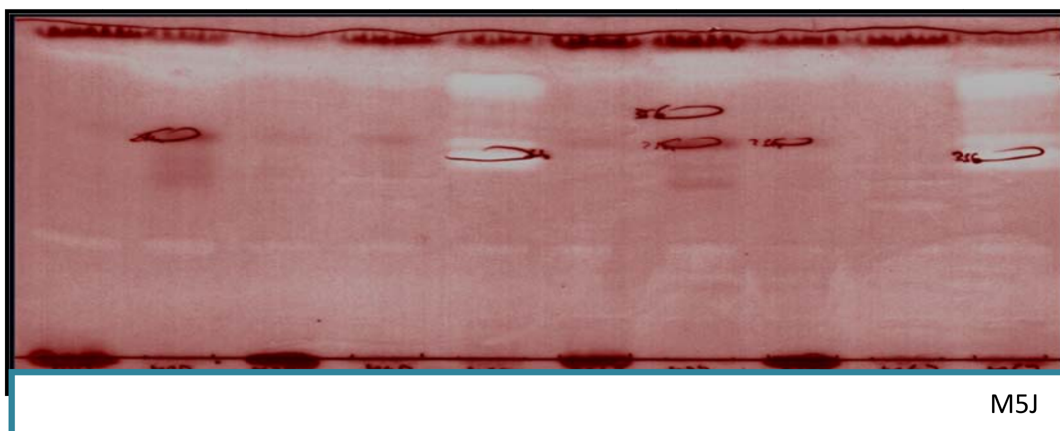



**Fig. 2.17: Bioautography of monthly samples from December and January against *E. coli* (CEF)**

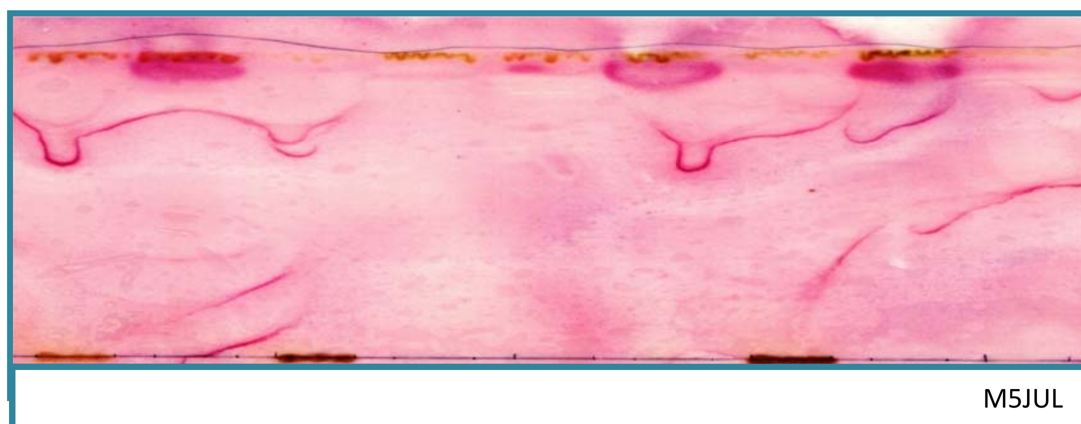



**Fig. 2.18: Bioautography of monthly samples from June and July against *E. coli* (CEF)**

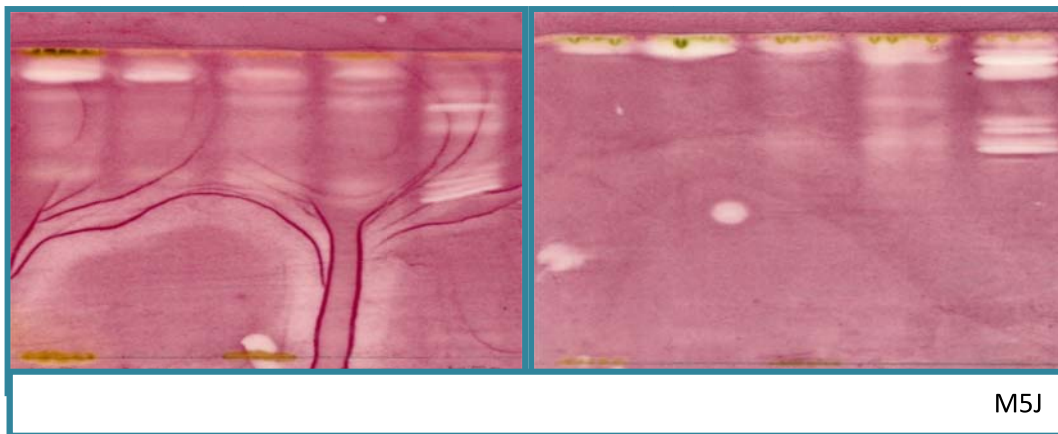



**Fig. 2.19: Bioautography of monthly samples from December and January against *S. aureus* (CEF)**

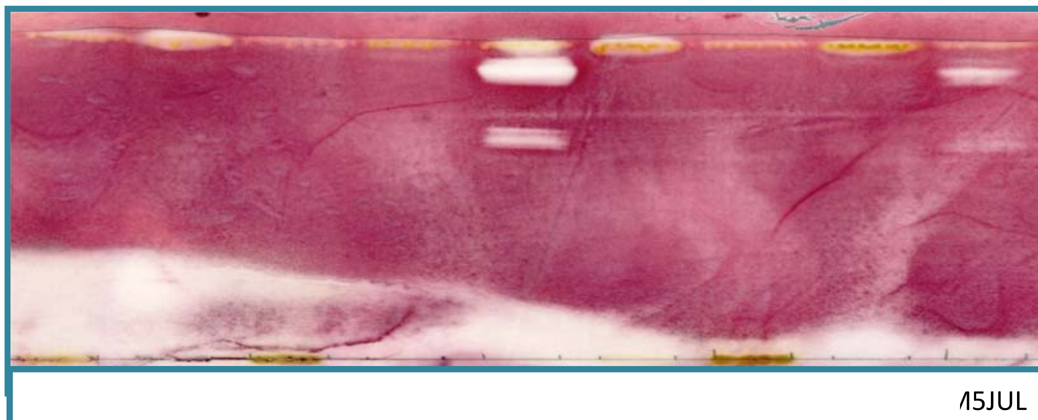



**Fig. 2.20: Bioautography of monthly samples from June and July against *S. aureus* (CEF)**

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).

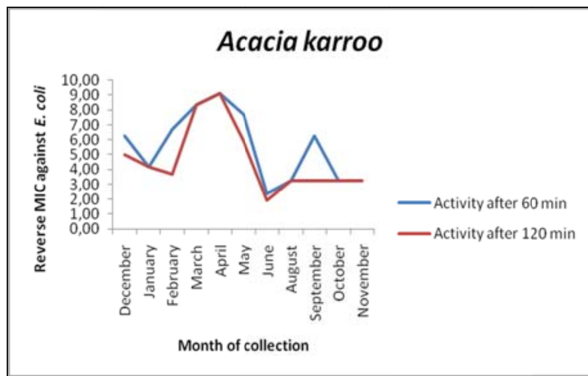
*Peltophorum africanum* had the best activity against *E. coli* in the month of February and against *S. aureus* in the months of February and March with MIC values of 0.05 mg/ml (TA = 1188 ml/g) and 0.04 mg/ml (TA = 1188 ml/g and 1075 ml/g). The worst activity was against *E. coli* in the month of October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



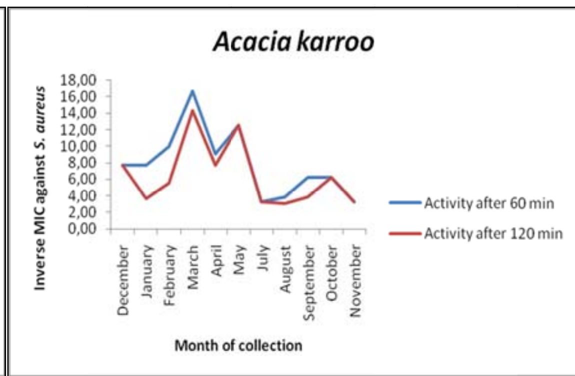
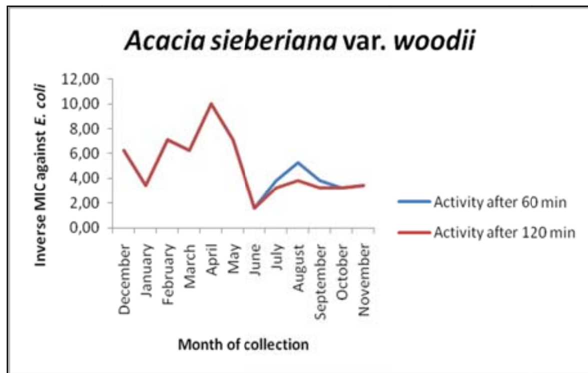




**Fig. 2.21: Activity of *A. karroo* against *E. coli***

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



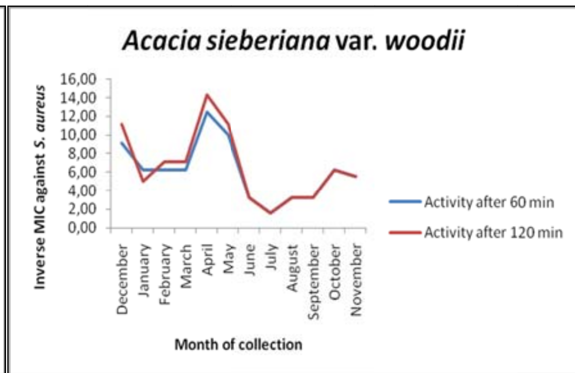
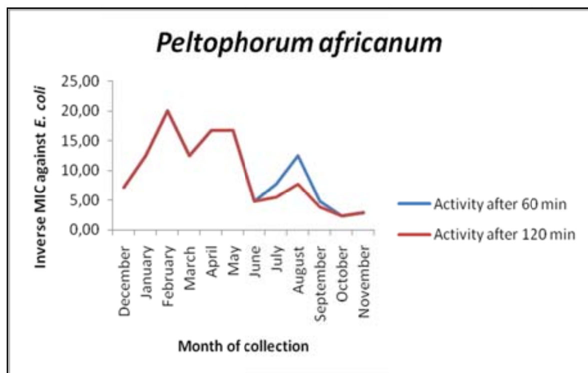




**Fig. 2.23: Activity of *A. sieberiana* against *E. coli***

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



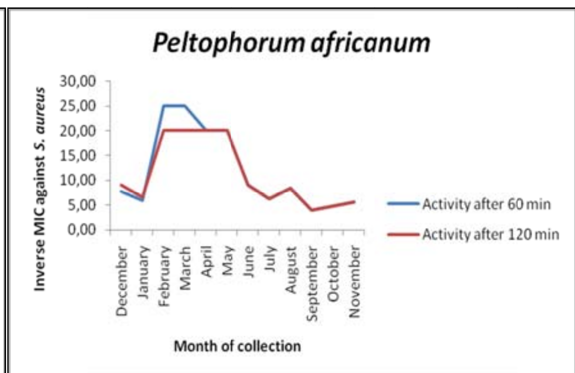
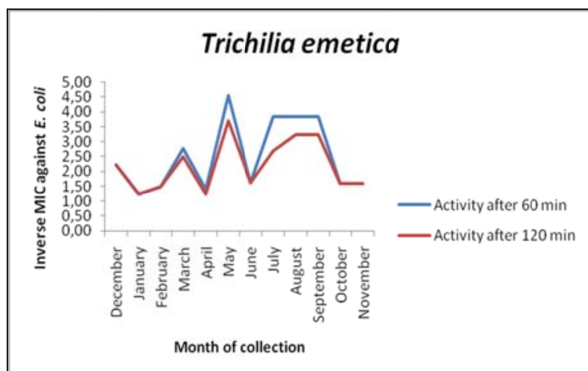




**Fig. 2.25: Activity of *P. africanum* against *E. coli***

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



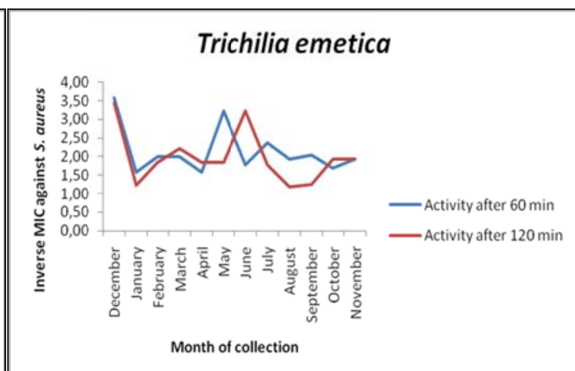



**Fig. 2.27: Activity of** *T. emetica* **against** *E. coli* **Fig. 2.28: Activity of** *T. emetica* **against** *S. aureus*

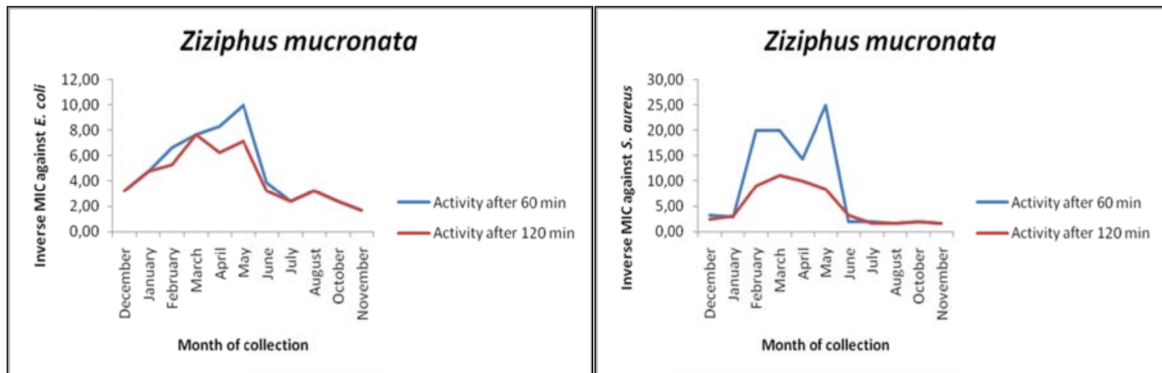



**Fig. 2.29: Activity of** *Z. mucronata* **against** *E. coli* **Fig. 2.30: Activity of** *Z. mucronata* **against** *S. aureus*

**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.

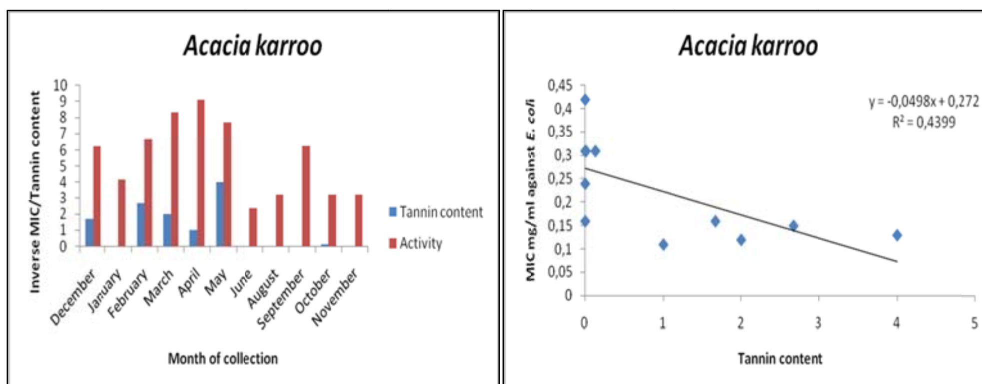
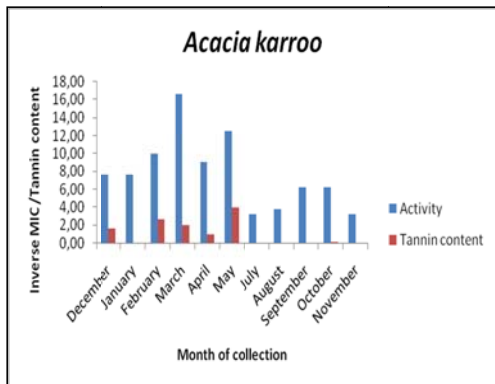



**Fig. 2.31 A** **Fig. 2.31 B**

**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***



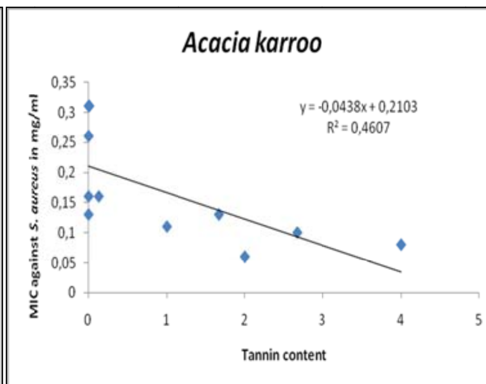
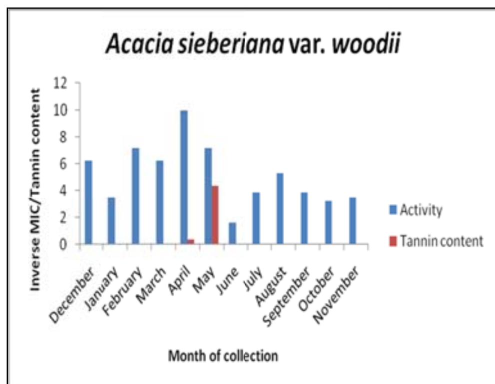




**Fig. 2.32 A** **Fig. 2.32 B**

**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*



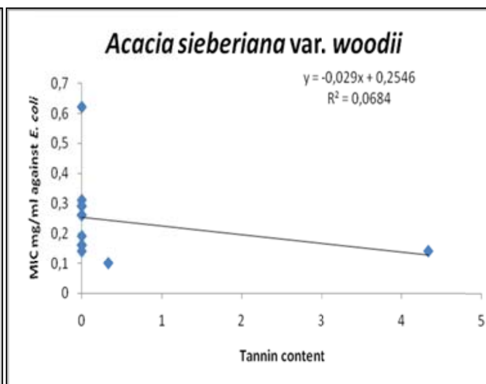
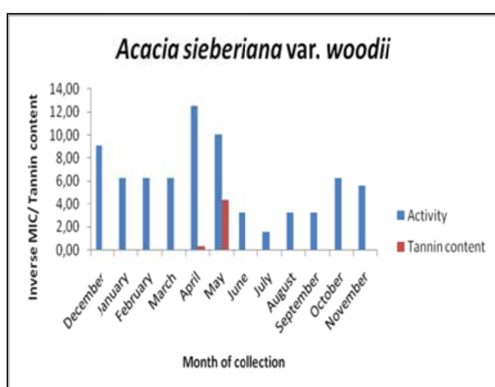




**Fig. 2.33 A** **Fig. 2.33 B**

**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*



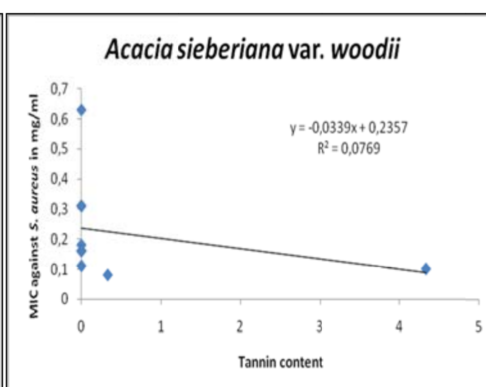
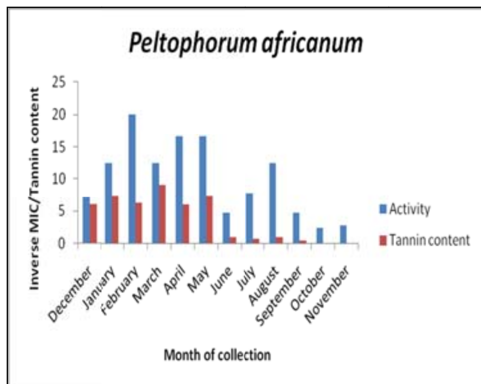




**Fig. 2.34 A** **Fig. 2.34 B**

**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*



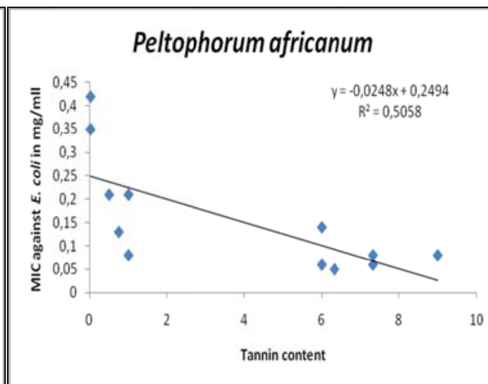
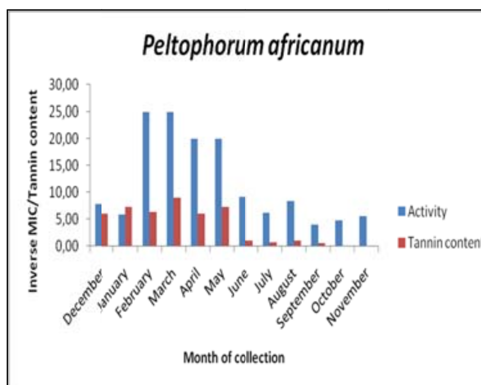




**Fig. 2.35 A** **Fig. 2.35 B**

**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*



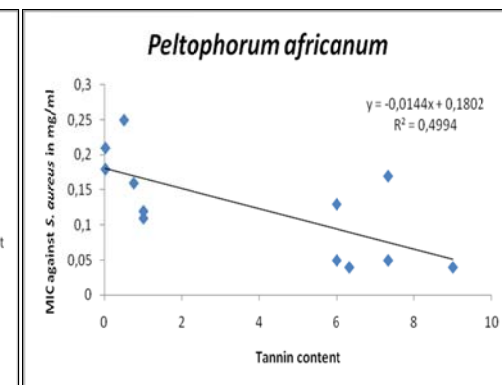
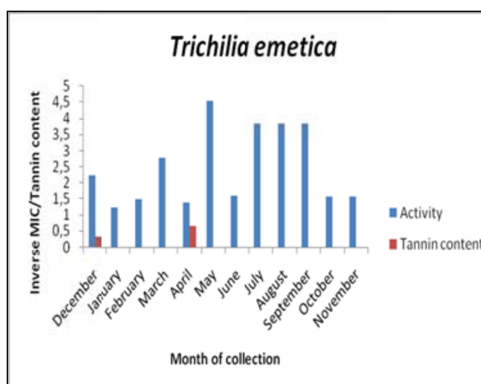




**Fig. 2.36 A** **Fig. 2.36 B**

**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*



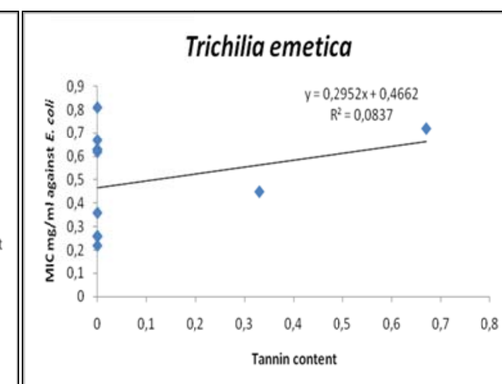
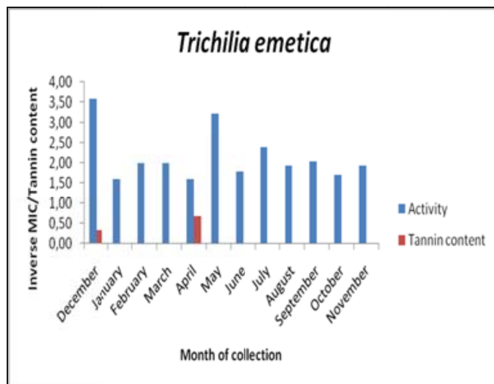




**Fig. 2.37 A** **Fig. 2.37 B**

**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*



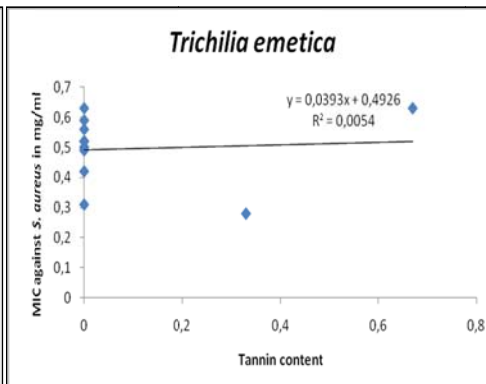
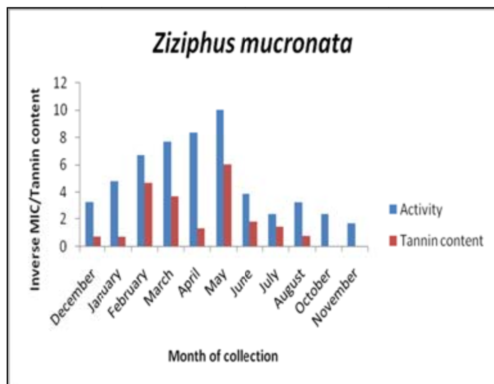




**Fig. 2.38 A** **Fig. 2.38 B**

**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*



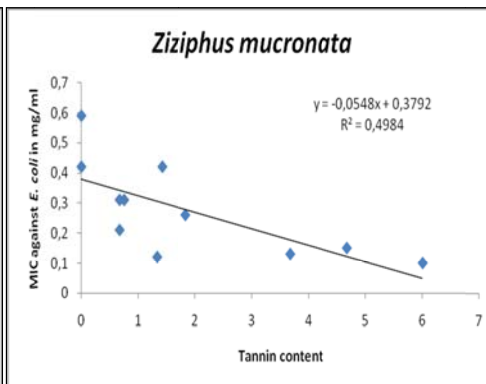
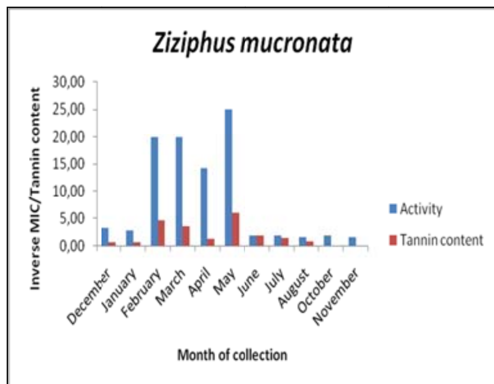




**Fig. 2.39 A** **Fig. 2.39 B**

**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*



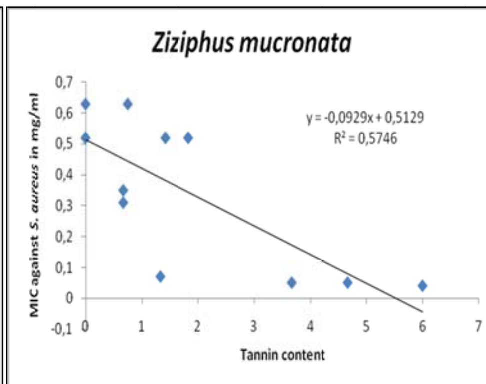




**Fig. 2.40 A** **Fig. 2.40 B**

**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.

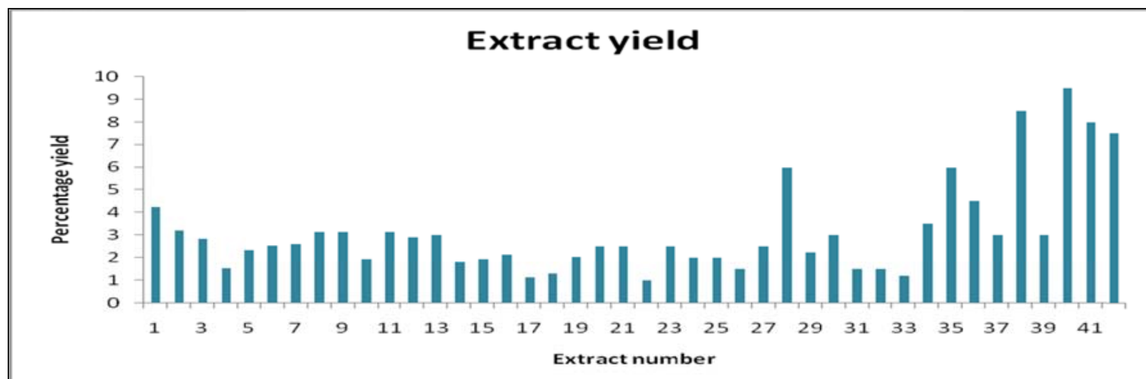



**Figure 3.2: Average yield of acetone extraction of 42 different *Combretum molle* samples**

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.

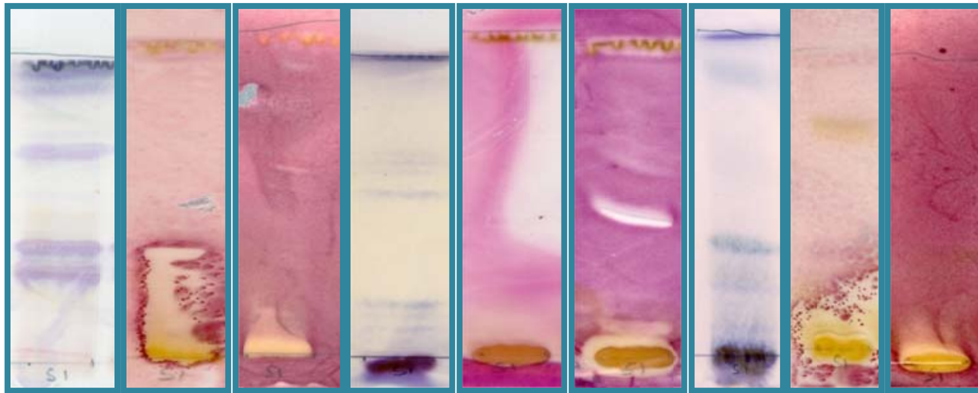


**Fig. 3.3 Fig. 3.4 Fig. 3.5 Fig. 3.6 Fig. 3.7 Fig. 3..8 Fig. 3.9 Fig. 3.10Fig. 3.11**

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*

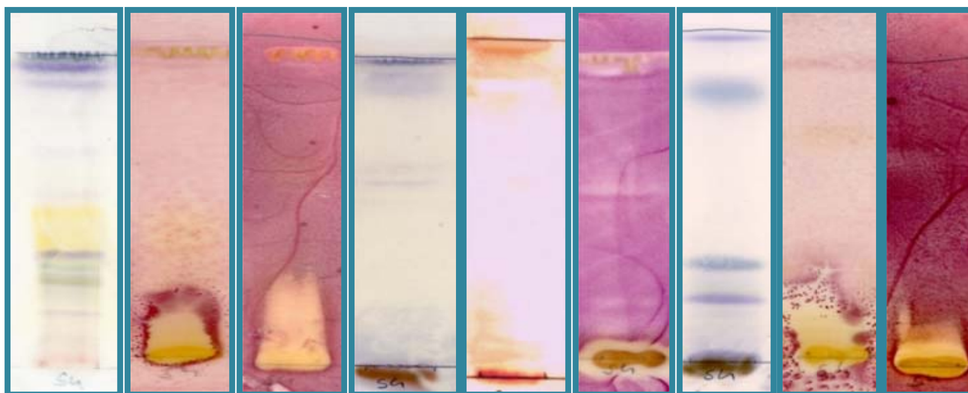


**Fig. 3.12 Fig. 3.13 Fig. 3.14 Fig. 3.15 Fig. 3.16 Fig. 3.17 Fig. 3.18Fig. 3.19 Fig. 3.20**

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*

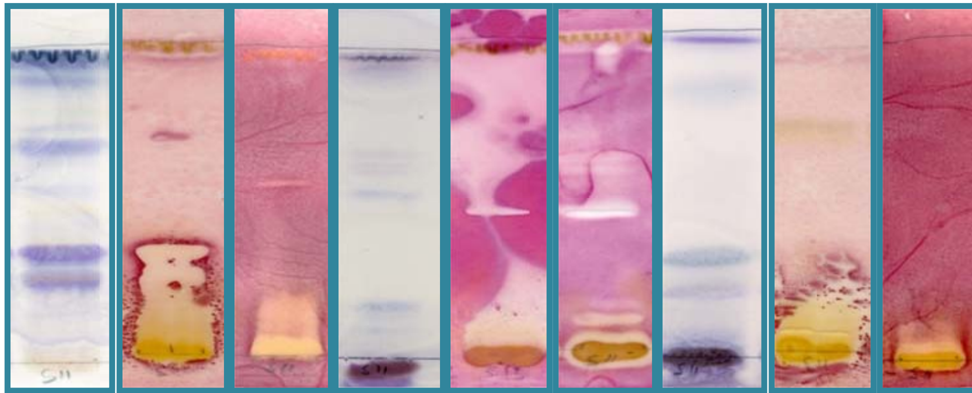


**Fig. 3.21 Fig. 3.22 Fig. 3.23 Fig. 3.24 Fig. 3.25 Fig. 3.26 Fig. 3.27 Fig. 3.28 Fig. 3.29**

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*

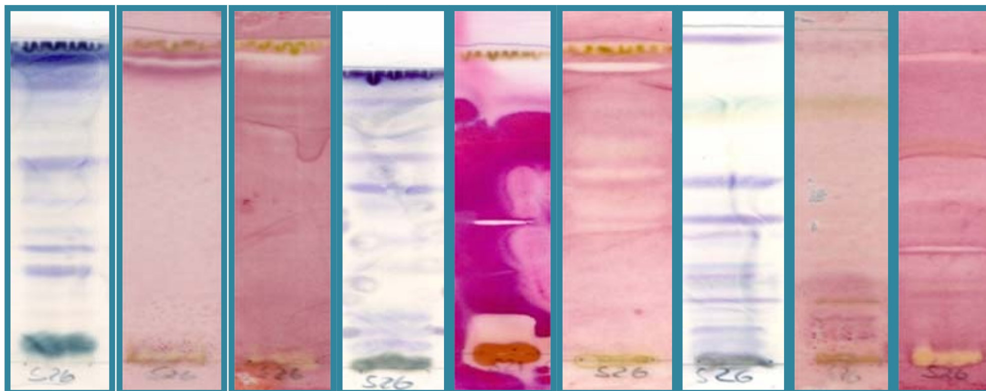


**Fig. 3.30 Fig. 3.31 Fig. 3.32 Fig. 3.33 Fig. 3.34 Fig. 3.35 Fig. 3.36 Fig. 3.37 Fig. 3.38**

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*

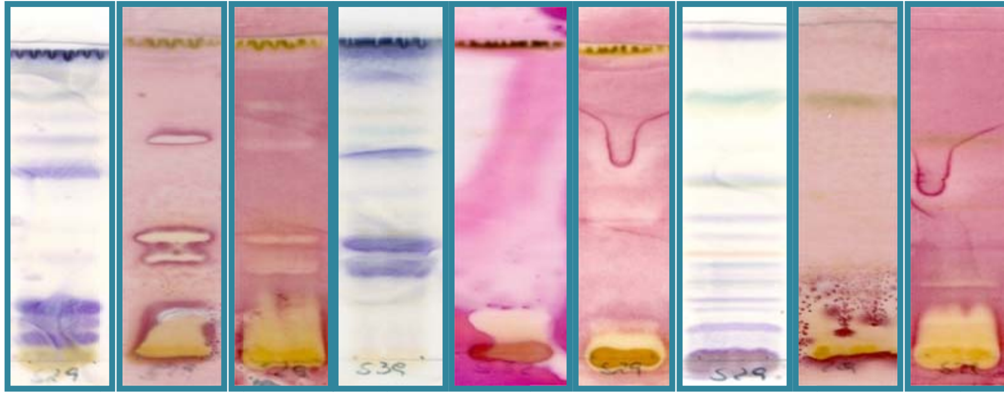


**Fig. 3.39 Fig. 3.40 Fig. 3.41 Fig. 3.42 Fig. 3.43 Fig. 3.44 Fig. 3.45 Fig. 3.46 Fig. 3.47**

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.

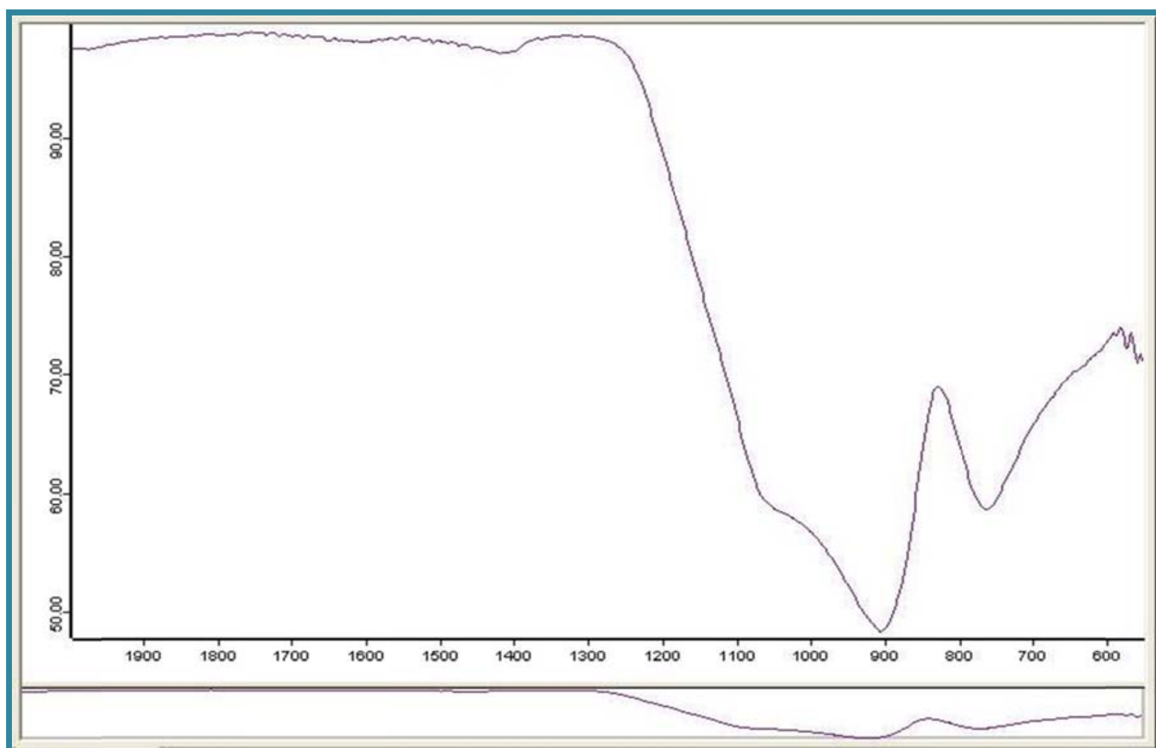



**Fig. 3.48 NIR spectrum of *Combretum molle* collected at SANBI Pretoria Botanical Garden**

### 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.

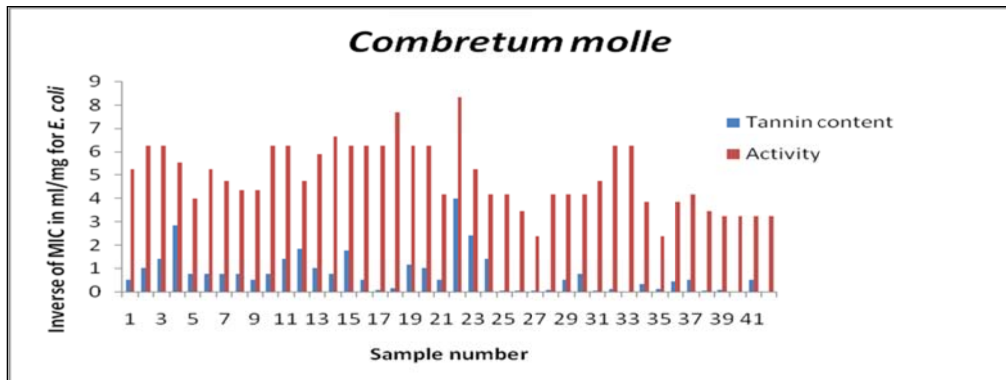



**Fig. 3.49: Comparison of the activity of the different *Combretum molle* samples against *E. coli* and their tannin content**

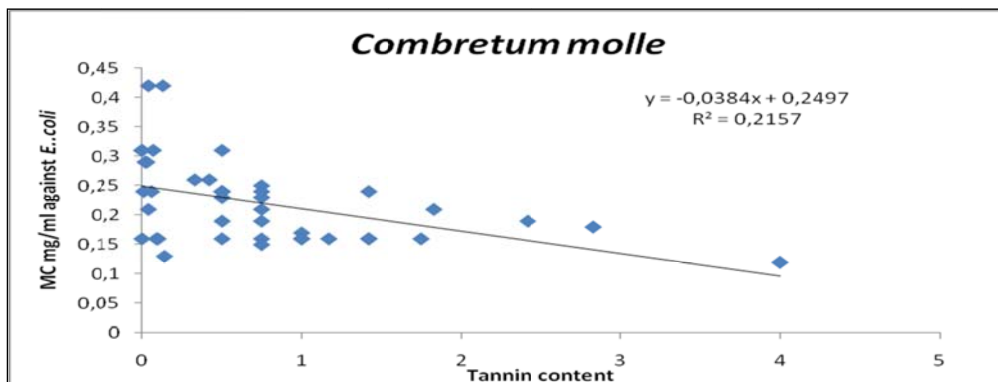



**Fig. 3.50: Correlation of the activity against *E. coli* and the tannin content of the different *Combretum molle* samples**

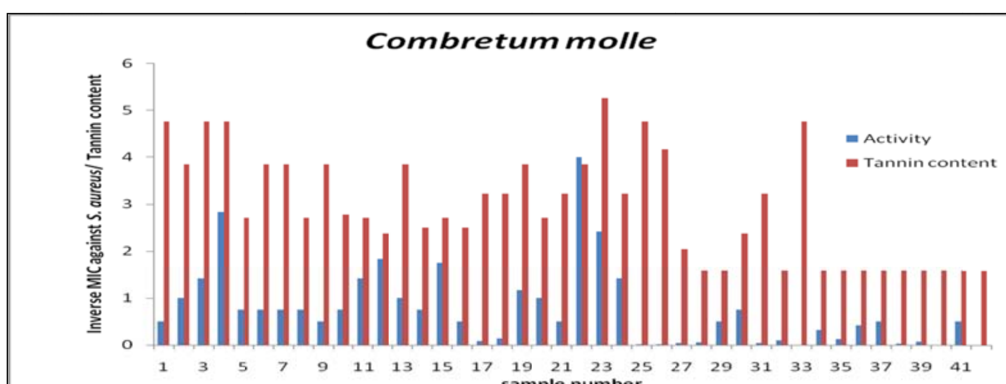



**Fig. 3.51: Comparison of the activity of the different *Combretum molle* samples against *S. aureus* and their tannin content**

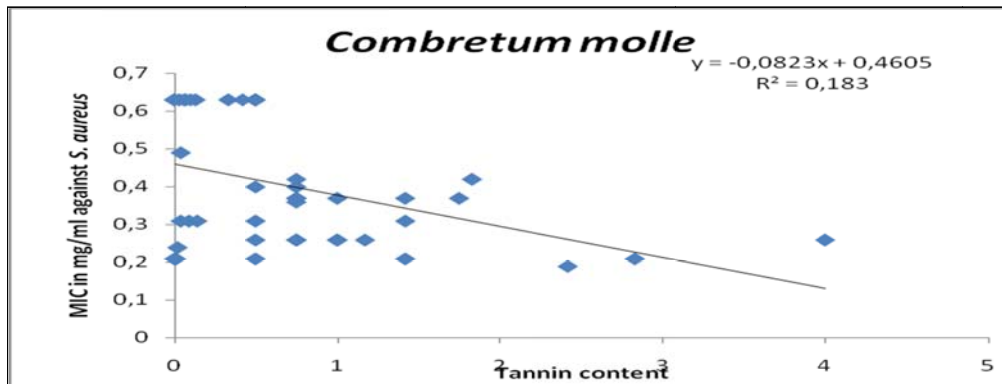



**Fig. 3.52: Correlation of the activity against *S. aureus* and the tannin content of the different *Combretum molle* samples**

## 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.

# Chapter 4

# A proposal for prioritising plant species to be investigated for treating diarrhoea in animals

## 4.1 Introduction

It was previously established in the current research that the extracts exhibited their best activity in the months of late summer and autumn (January until April) and also that there was no detectable intraspecies variation. The next step in the development of a commercially useful product for the treatment of diarrhoea was to investigate which plants had the overall best activity against pathogens and whether their activity was linked to their tannin content or not. A ranking system based on these results was developed to choose the most promising plant species for further in depth studies.

It is well known that infectious diseases account for a high proportion of the health problems in the Third World. Reports of antimicrobial activity of indigenous plants have been published from many regions (Desta, 1993). However, there is no record of an extensive comparison of such plants in southern Africa concerning their MIC values, TLC fingerprints and tannin content as has been performed in this study.

The plants for this study were chosen on their reported use as anti-diarrhoeal agents in ethnomedicine (Hutchings *et al.* 1996; Bossard, 1993; Bryant, 1966).

In the tree screening project conducted at our Phytomedicine Laboratory, the screening of leaves has proven successful to identify species with high antibacterial activity. Although different plant parts are used in ethnomedicine, it was decided to focus on the screening of leaf extracts as leaves are renewable resources. It is also possible that where activity in roots and barks exists, this may be reflected in the activity of leaf material. It has been proven that based on the minimum inhibitory concentration values, even though extracts of the inner bark of *Sclerocarrya birrea* were the most potent, the activity of the leaves and bark extracts did not differ (Eloff, 2001). McGaw *et al.* (2002) also found no statistically significant differences in antibacterial activity for certain bacteria between ethanol extracts of different parts of the species *Schotia brachypetala*.

The results of the preliminary screening will be added to the ever increasing database of the Phytomedicine Programme.

## 4.2 Materials and methods

### 4.2.1 Collection and preparation of plant material

Fifty-three plant species were chosen for the screening process; those species are listed in Table 4.1

**Table 4.1: Selected plants for screening (Hutchings *et al.* 1996; Bossard, 1993; Bryant, 1966)**

| Plant species | Family |
|---|---|
| *Acacia karroo* Hayne | Fabaceae-Mimosoidae |
| *Acacia sieberiana* var. *woodii* DC | Fabaceae-Mimosoidae |
| *Albizia adianthifolia* (Schuhmach.) W.F. Wight | Fabaceae-Mimosoidae |
| *Annona senegalensis* Pers. | Annonaceae |
| *Antidesma venosum* E. Mey. ex Tul. | Euphorbiaceae |
| *Berchemia zeyheri* (Sond.) Grubov | Rhamnaceae |
| *Bridelia micrantha* (Hochst.) Baill. | Euphorbiaceae |
| *Buddleja salviifolia* Lam. | Loganiaceae |
| *Capparis tomentosa* Lam. | Capparaceae |
| *Cassine aethiopica* Thunb. | Celastraceae |
| *Cassine transvaalensis* (Burtt. Davy) Codd | Celastraceae |
| *Cassinopsis ilicifolia* (Hochst.) Kuntze | Icacinaceae |
| *Cassinopsis tinifolia* Harv. | Icacinaceae |
| *Clausena anisata* (Willd.) Hook. F. ex Benth. | Rutaceae |
| *Clerodendrum myricoides* (Hochst.) Vatke | Verbenaceae |
| *Combretum molle* R. Br. ex G. Don | Combretaceae |
| *Combretum zeyheri* Sond. | Combretaceae |
| *Curtisia dentata* (Burm. F.) C.A. Sm. | Cornaceae |
| *Deinbollia oblongifolia* (E. Mey. ex Arn.) Radlk. | Sapindaceae |
| *Dichrostachys cinerea* subsp. *africana* (L.) Wight et Arn. | Fabaceae-Mimosoidae |
| *Dombeya rotundifolia* (Hochst.) Planch. | Sterculiaceae |
| *Ekebergia capensis* Sparrman | Meliaceae |
| *Elephantorrhiza elephantina* (Burch.) Skeels | Fabaceae |
| *Faidherbia albida* (Del.) A. Chev. | Fabaceae-Mimosoidae |
| *Faurea saligna* Harvey | Proteaceae |
| *Ficus sur* Forssk. | Moraceae |
| *Flueggea virosa* (Roxb. Ex. Willd.) Pax & K. Hoffm. | Euphorbiaceae |
| *Hippobromus pauciflorus* (L. F.) Radlk. | Sapindaceae |
| *Jatropha curcas* L. | Euphorbiaceae |
| *Kigelia africana/Kigelia pinnata* (Jacq.) DC | Bignoniaceae |
| *Lannea discolour* (Sond.) Engl. | Anacardiaceae |
| *Lippia javanica* (Burm. f.) Spreng. | Verbenaceae |
| *Lonchocarpus capassa* Rolfe | Fabaceae |
| *Olea eurpoea* subsp. *africana* (Mill.) P.S. Green | Oleaceae |
| *Oncoba spinosa* Forssk. | Flacourtiaceae |
| *Ozoroa obovata* (Oliv.) R. & A. Fernandes | Anacardiaceae |
| *Peltophorum africanum* Sonder | Fabaceae-Caesalpinioideae |
| *Pittosporum viridoflorum* Sims | Pittosporaceae |
| *Ricinus communis* L. | Euphorbiaceae |
| *Schotia brachypetala* Sond. | Fabaceae |
| *Sclerocarya birrea* (A. Rich.) Hochst. | Anacardiaceae |
| *Spirostachys africana* Sond. | Euphorbiaceae |
| *Strychnos spinosa* Lam. | Loganiaceae |
| *Syzigium cordatum* Hochst. Ex Krauss | Myrtaceae |
| *Tecomaria capensis* (Thunb.) Spach | Bignoniaceae |
| *Terminalia phanerophlebia* Engl. & Diels | Combretaceae |
| **Plant species** | **Family** |

| *Tetradenia riparia* (Hochst.) Codd | Lamiaceae |
|---|---|
| *Thespesia acutiloba* (Bak. F.) Exell & Mendonca | Malvaceae |
| *Trema orientalis* (L.) Blume | Ulmaceae |
| *Trichilia emetic* Vahl | Meliaceae |
| *Vangueria infausta* subsp. *infausta* Burchell | Rubiaceae |
| *Ximenia caffra* Sond. | Olacaceae |
| *Zizphus mucronata* Willd. | Rhamnaceae |

The leaf samples of the trees were collected at various sites and identified by qualified personnel (Pretoria Botanical Garden: Khangela Joseph Baloyi, Lowveld National Botanical Garden: Willem Froneman, Manie van der Schyff Botanical Garden University of Pretoria Main Campus: Lorraine Middleton) and by selecting labelled species in the Botanical Garden. The different sites from which plants were collected included: Lowveld National Botanical Garden (Nelspruit), Pretoria National Botanical Garden, the Main Campus of the University of Pretoria and the Campus of the Facultyof Veterinary Science, University of Pretoria (Onderstepoort).

The samples were dried indoors under constant air flow at the Phytomedicine Programme (University of Pretoria). After about a week of drying, the samples were ground to a fine powder with a Macsalab mill (Model 200 LAB). The powdered samples were stored in sealed glass containers in the dark until further use.

### 4.2.2 Extraction

The extracts were prepared as described in section 2.2.2.

### 4.2.3 TLC fingerprinting

The TLC plates were prepared as described in section 2.2.3, and again developed with the three solvent systems developed by the Phytomedicine Laboratory, University of Pretoria, namely BEA, CEF and EMW.

### 4.2.4 Bioautographic assays

The TLC plates were prepared as described in section 2.2.3 and dried for two days under a constant stream of air. Then they were sprayed with *E. coli* and *S. aureus* inoculated liquid cultures and incubated for 18 hours at 37°C and 100% humidity. Afterwards the plates were sprayed with a 2 mg/ml aqueous solution of INT and incubated for another hour for optimal colour development. Clear zones show bacterial growth inhibition due to antibacterial active compounds.

### 4.2.5 Total activity

Total activity was calculated as described in section 2.2.7.

### 4.2.6 Microdilution assay

MIC determinations for each plant extract against *E. coli* and *S. aureus* were conducted as described in section 2.2.6.

### 4.2.7 Tannin assays

Tannin contents for each extract were evaluated as described in section 2.2.6

## 4.3 Results and discussion

### 4.3.1 Quantity extracted

The extraction was performed twice to confirm the results obtained in the first extraction, and both extractions yielded similar results. Amounts in about the same range were extracted from all the species, from 0.68% to 6.2%, except for *Faurea saligna*, where much higher quantities of plant extract resulted. This species was subjected to a third round of extractions to exclude human error as the reason for the difference in extracted quantities. The extracted amount for *Faurea saligna* ranged from 10.5% to 15.1%. What was quite an interesting aspect when comparing the quantity of material extracted from *Combretum molle* (2.9%) and from *Combretum zeyheri* (3.5%) with the quantities of material that Eloff (1999b) extracted (9.8% and 6.6% respectively), was that the plants collected by Eloff in the Lowveld Botanical Garden seemed to yield higher quantities in extraction. This might be due to the different climate and soil conditions in the Mpumalanga area.

The quantities extracted are reported in Table 4.2, and Figure 4.1 supplies a visual comparison of the data.

**Table 4.2: Percentage of plant material extracted with acetone, experiment repeated**

| Extract number | Plant species | 1 | 2 | Average |
|---|---|---|---|---|
| 1 | *Acacia karroo* | 1.5% | 1.3% | 1.4% |
| 2 | *Acacia sieberiana* var. *woodii* | 1.4% | 1.3% | 1.3% |
| 3 | *Albizia adianthifolia* | 3.4% | 2.0% | 2.7% |
| 4 | *Annona senegalensis* | 1.3% | 1.4% | 1.4% |
| 5 | *Antidesma venosum* | 1.9% | 1.6% | 1.8% |
| 6 | *Berchemia zeyheri* | 1.6% | 0.8% | 1.2% |
| 7 | *Bridelia micrantha* | 2.9% | 2.6% | 2.7% |
| 8 | *Buddleja salviifolia* | 3.9% | 1.9% | 2.9% |
| 9 | *Capparis tomentosa* | 2.5% | 1.5% | 2.0% |
| 10 | *Cassine aethiopica* | 3.3% | 2.9% | 3.1% |
| 11 | *Cassine transvaalensis* | 8.1% | 6.1% | 7.1% |
| 12 | *Cassinopsis ilicifolia* | 4.0% | 2.2% | 3.1% |
| 13 | *Cassinopsis tinifolia* | 2.3% | 2.2% | 2.3% |
| 14 | *Clausena anisata* | 4.4% | 4.0% | 4.2% |
| 15 | *Clerodendrum myricoides* | 5.7% | 1.5% | 3.6% |
| 16 | *Combretum molle* | 2.6% | 3.2% | 2.9% |
| 17 | *Combretum zeyheri* | 3.5% | 3.4% | 3.5% |
| 18 | *Curtisia dentate* | 7.2% | 6.0% | 6.6% |
| 19 | *Deinbollia oblongifolia* | 2.7% | 2.3% | 2.5% |
| 20 | *Dichrostachys cinerea* subsp. *africana* | 1.6% | 1.0% | 1.3% |
| 21 | *Dombeya rotundifolia* | 1.7% | 1.1% | 1.4% |
| 22 | *Ekebergia capensis* | 2.3% | 1.8% | 2.1% |
| 23 | *Elephantorrhiza elephantina* | 2.8% | 2.9% | 2.9% |
| 24 | *Faidherbia albida* | 2.8% | 2.1% | 2.4% |
| 25 | *Faurea saligna* | 15% | 12.3% | 13.7% |
| 26 | *Ficus sur* | 2.2% | 1.5% | 1.9% |
| 27 | *Flueggea virosa* | 2.0% | 0.7% | 1.4% |
| 28 | *Hippobromus pauciflorus* | 1.6% | 1.3% | 1.4% |
| 29 | *Jatropha curcas* | 1.9% | 3.6% | 2.7% |
| 30 | *Kigelia africana* | 1.4% | 1.2% | 1.3% |
| 31 | *Lannea discolour* | 2.1% | 2.0% | 2.0% |
| 32 | *Lippia javanica* | 1.9% | 2.0% | 1.9% |
| 33 | *Lonchocarpus capassa* | 1.5% | 1.3% | 1.4% |
| 34 | *Olea europea* subsp. *africana* | 7.0% | 4.4% | 5.7% |
| 35 | *Oncoba spinosa* | 3.7% | 3.6% | 3.7% |
| 36 | *Ozoroa obovata* | 4.3% | 3.6% | 3.9% |
| 37 | *Peltophorum africanum* | 1.0% | 1.2% | 1.1% |
| 38 | *Pittosporum viridoflorum* | 2.2% | 2.4% | 2.3% |
| 39 | *Ricinus communis* | 2.8% | 1.8% | 2.3% |
| 40 | *Schotia brachypetala* | 1.4% | 1.0% | 1.2% |
| 41 | *Sclerocarrya birrea* | 2.3% | 5.0% | 3.7% |
| 42 | *Spirostachys Africana* | 3.8% | 2.9% | 3.3% |
| 43 | *Strychnos spinosa* | 2.6% | 1.8% | 2.2% |
| 44 | *Syzigium cordatum* | 3.5% | 3.7% | 3.6% |
| 45 | *Tecomaria capensis* | 2.7% | 3.2% | 2.9% |
| 46 | *Terminalia phanerophlebia* | 3.0% | 1.4% | 2.2% |
| 47 | *Tetradenia riparia* | 2.0% | 1.1% | 1.6% |
| 48 | *Thespesia acutiloba* | 4.0% | 3.8% | 3.9% |
| 49 | *Trema orientalis* | 3.5% | 4.6% | 4.0% |
| 50 | *Trichilia emetica* | 0.9% | 0.7% | 0.8% |
| 51 | *Vangueria infausta* subsp. *infausta* | 1.1% | 1.2% | 1.2% |
| 52 | *Ximenia caffra* | 3.8% | 4.0% | 3.9% |
| 53 | *Ziziphus mucronata* | 1.0% | 1.5% | 1.2% |

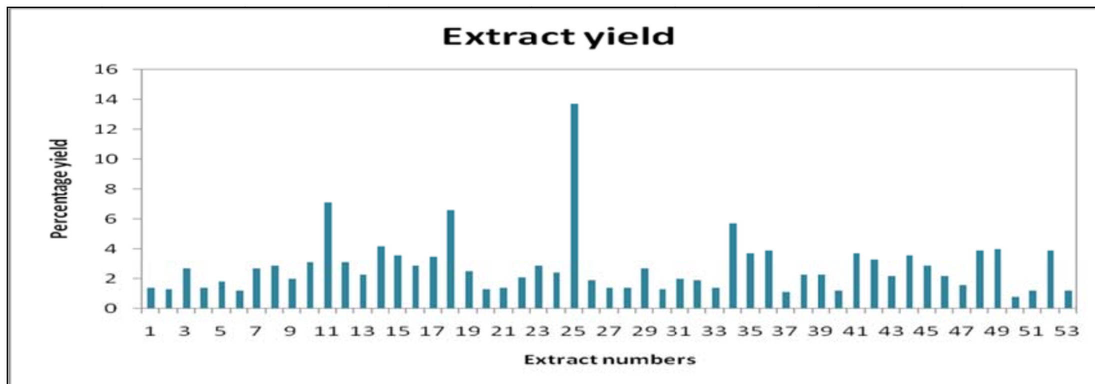



**Figure 4.1: Average percentage extracted from the 53 selected plant species**

### 4.3.2 Microdilution and tannin assays

The microdilution assays gave varying results regarding the antibacterial activity of the different plants. All the determined values are given in Tables 4.3 and 4.4. Some of the plant extracts had bactericidal activity whereas others extracts had bacteriostatic activity. The plants with the bactericidal effects are preferable, since they appear to be directly killing the bacteria, but there is always a risk that this effect means that the extract could be toxic, so it is wise not to disregard the plants with bacteriostatic effects. Furthermore, it will be interesting to investigate plants further that had good antibacterial activity against *E. coli*, but not against *S. aureus*, because if they were equally active against both, they may contain a general metabolic toxin that could also be toxic to mammals. Another important factor in choosing the most promising plant species is how high the extract yield is and of course, plants with a low tannin content (to favour the selection of plants that work with a different mechanism for the antibacterial activity of the extract) should be chosen. The extracts with the best overall average antibacterial activity against the strains of *E. coli* as well as *S. aureus* and also best total activity for both bacterial strains were: *Combretum zeyheri* (*E. coli*: MIC = 0.03 mg/ml, TA = 1077 ml/g, bactericidal; *S. aureus*: MIC = 0.04 mg/ml, TA = 829 ml/g, bactericidal), *Deinbollia oblongifolia* (*E. coli*: MIC = 0.09 mg/ml, TA = 309 ml/g, bacteriostatic; *S. aureus*: MIC = 0.12 mg/ml, TA = 234 ml/g, bactericidal), *Spirostachys africana* (*E. coli*: MIC = 0.12 mg/ml, TA = 317 ml/g, bactericidal; *S. aureus*: MIC = 0.09 mg/ml, TA = 438 ml/g, bactericidal), *Syzigium cordatum* (*E. coli*: MIC = 0.06 mg/ml; TA = 609 ml/g, bactericidal; *S. aureus*: MIC = 0.05 mg/ml, TA = 656 ml/g, bacteriostatic) and *Terminalia phanerophlebia* (*E. coli*: MIC = 0.08 mg/ml, TA = 374 ml/g, bactericidal; *S. aureus*: MIC = 0.03 mg/ml, TA = 900 ml/g, bactericidal). However, since they had such good activity against both the Gram-negative and the Gram-positive bacteria, these extracts could very likely prove to be toxic to animals.

The extract of *Faurea saligna* had a very good total activity (TA = 1022 ml/g) due to the higher extraction percentage in this plant (see 4.3.1). The MIC value against *E. coli* however was only 0.15 mg/ml.

**Table 4.3: MIC values after 60 and 120 min against *E. coli.* Each value represents the average of 9 determinations**

| Plant species | Yield (mg) | Average activity60 (mg/ml) (± SD) | Total activity60 (ml/g) | Average activity120 (mg/ml) (± SD) | Total activity 120 (ml/g) |
|---|---|---|---|---|---|
| *Acacia karroo* | 15 | 0.13 ± 0.04 | 115 | 0.13 ± 0.04 | 115 |
| *Acacia sieberiana* var. *woodii* | 14 | 0.13 ± 0.04 | 108 | 0.13 ± 0.04 | 108 |
| *Albizia adianthifolia* | 34 | 0.13 ± 0.09 | 255 | 0.14 ± 0.04 | 239 |
| *Annona senegalensis* | 13 | 0.12 ± 0.05 | 105 | 0.15 ± 0.08 | 88 |
| *Antidesma venosum* | 19 | 0.09 ± 0.00 | 222 | 0.15 ± 0.11 | 130 |
| *Berchemia zeyheri* | 16 | 0.08 ± 0.04 | 200 | 0.13 ± 0.04 | 120 |
| *Bridelia micrantha* | 29 | 0.13 ± 0.16 | 218 | 0.23 ± 0.12 | 124 |
| *Buddleja salviifolia* | 39 | 0.52 ± 0.10 | 75 | 0.52 ± 0.16 | 75 |
| *Capparis tomentosa* | 25 | 0.17 ± 0.08 | 148 | 0.20 ± 0.07 | 123 |
| *Cassine aethiopica* | 33 | 0.24 ± 0.07 | 136 | 0.35 ± 0.22 | 94 |
| *Cassine transvaalensis* | 81 | 0.19 ± 0.07 | 419 | 0.19 ± 0.07 | 419 |
| *Cassinopsis ilicifolia* | 40 | 0.26 ± 0.28 | 154 | 0.47 ± 0.24 | 85 |
| *Cassinopsis tinifolia* | 23 | 0.31 ± 0.23 | 75 | 0.31 ± 0.28 | 74 |
| *Clausena anisata* | 44 | 0.37 ± 0.05 | 118 | 0.45 ± 0.26 | 97 |
| *Clerodendrum myricoides* | 57 | 0.29 ± 0.06 | 194 | 0.31 ± 0.00 | 184 |
| *Combretum molle* | 16 | 0.18 ± 0.05 | 300 | 0.23 ± 0.08 | 244 |
| *Combretum zeyheri* | 35 | 0.03 ± 0.08 | 1077 | 0.03 ± 0.01 | 1077 |
| *Curtisia dentate* | 72 | 0.24 ± 0.06 | 296 | 0.45 ± 0.28 | 161 |
| *Deinbollia oblongifolia* | 27 | 0.09 ± 0.12 | 309 | 0.17 ± 0.13 | 158 |
| *Dichrostachys cinerea* subsp.*africana* | 16 | 0.19 ± 0.11 | 84 | 0.19 ± 0.12 | 84 |
| *Dombeya rotundifolia* | 17 | 0.20 ± 0.08 | 85 | 0.20 ± 0.11 | 85 |
| *Ekebergia capensis* | 23 | 0.21 ± 0.19 | 110 | 0.21 ± 0.08 | 110 |
| *Elephantorrhiza elephantina* | 28 | 0.26 ± 0.23 | 106 | 0.26 ± 0.19 | 106 |
| *Faidherbia albida* | 28 | 0.30 ± 0.08 | 92 | 0.30 ± 0.23 | 92 |
| *Faurea saligna* | 151 | 0.15 ± 0.26 | 1022 | 0.15 ± 0.08 | 1022 |
| *Ficus sur* | 22 | 0.26 ± 0.04 | 85 | 0.30 ± 0.25 | 73 |
| *Flueggea virosa* | 20 | 0.13 ± 0.12 | 154 | 0.13 ± 0.04 | 154 |
| *Hippobromus pauciflorus* | 16 | 0.11 ± 1.17 | 144 | 0.14 ± 0.13 | 117 |
| *Jatropha curcas* | 19 | 0.95 ± 0.12 | 20 | 0.96 ± 1.16 | 20 |
| *Kigelia africana* | 14 | 0.15 ± 0.06 | 92 | 0.29 ± 0.26 | 49 |
| *Lannea discolour* | 21 | 0.12 ± 0.11 | 183 | 0.12 ± 0.06 | 183 |
| *Lippia javanica* | 19 | 0.12 ± 0.24 | 156 | 0.13 ± 0.10 | 149 |
| *Lonchocarpus capassa* | 15 | 0.34 ± 0.00 | 44 | 0.33 ± 0.26 | 46 |
| *Olea europea* subsp. *africana* | 70 | 0.31 ± 0.16 | 226 | 0.42 ± 0.16 | 168 |
| *Oncoba spinosa* | 37 | 0.15 ± 0.07 | 69 | 0.26 ± 0.08 | 55 |
| *Ozoroa obovata* | 43 | 0.11 ± 0.06 | 403 | 0.11 ± 0.04 | 403 |
| *Peltophorum africanum* | 10 | 0.10 ± 0.13 | 105 | 0.10 ± 0.06 | 105 |
| *Pittosporum viridoflorum* | 22 | 0.22 ± 0.11 | 101 | 0.22 ± 0.13 | 101 |
| *Ricinus communis* | 28 | 0.22 ± 0.13 | 129 | 0.22 ± 0.11 | 129 |
| *Schotia brachypetala* | 14 | 0.20 ± 0.14 | 69 | 0.32 ± 0.25 | 43 |
| *Sclerocarrya birrea* | 23 | 0.22 ± 0.12 | 105 | 0.43 ± 0.30 | 53 |
| *Spirostachys africana* | 38 | 0.12 ± 0.09 | 317 | 0.13 ± 0.11 | 300 |
| *Strychnos spinosa* | 26 | 0.19 ± 0.03 | 135 | 0.19 ± 0.09 | 135 |
| *Syzigium cordatum* | 35 | 0.06 ± 0.26 | 609 | 0.06 ± 0.03 | 609 |
| *Tecomaria capensis* | 27 | 0.34 ± 0.06 | 79 | 0.36 ± 0.23 | 74 |

| Plant species | Yield (mg) | Average activity60 (mg/ml) (± SD) | Total activity60 (ml/g) | Average activity120 (mg/ml) (± SD) | Total activity 120 (ml/g) |
|---|---|---|---|---|---|
| *Terminalia phanerophlebia* | 30 | 0.08 ± 0.05 | 375 | 0.08 ± 0.06 | 375 |
| *Tetradenia riparia* | 20 | 0.09 ± 0.12 | 214 | 0.09 ± 0.05 | 214 |
| *Thespesia acutiloba* | 40 | 0.15 ± 0.21 | 267 | 0.15 ± 0.11 | 262 |
| *Trema orientalis* | 35 | 0.37 ± 0.26 | 95 | 0.37 ± 0.21 | 95 |
| *Trichilia emetica* | 9 | 0.34 ± 0.12 | 26 | 0.37 ± 0.22 | 24 |
| *Vangueria infausta* subsp. *infausta* | 11 | 0.21 ± 0.15 | 52 | 0.21 ± 0.12 | 52 |
| *Ximenia caffra* | 38 | 0.21 ± 0.12 | 178 | 0.22 ± 0.14 | 173 |
| *Ziziphus mucronata* | 10 | 0.15 ± 0.04 | 66 | 0.21 ± 0.10 | 47 |

**Table 4.4: MIC values after 60 and 120 min against *S. aureus*. Each value represents the average of 9 determinations**

| Plant species | Yield (mg) | Average activity60 (mg/ml) (± SD) | Total activity 60 (ml/g) | Average activity 120 (mg/ml) (± SD) | Total activity120 (ml/g) |
|---|---|---|---|---|---|
| *Acacia karroo* | 15 | 0.14 ± 0.04 | 107 | 0.14 ± 0.04 | 107 |
| *Acacia sieberiana* var. *woodii* | 14 | 0.10 ± 0.04 | 140 | 0.13 ± 0.04 | 108 |
| *Albizia adianthifolia* | 34 | 0.04 ± 0.03 | 765 | 0.04 ± 0.03 | 765 |
| *Annona senegalensis* | 13 | 0.07 ± 0.02 | 195 | 0.08 ± 0.00 | 163 |
| *Antidesma venosum* | 19 | 0.08 ± 0.00 | 238 | 0.07 ± 0.02 | 285 |
| *Berchemia zeyheri* | 16 | 0.04 ± 0.03 | 400 | 0.04 ± 0.03 | 400 |
| *Bridelia micrantha* | 29 | 0.05 ± 0.03 | 621 | 0.05 ± 0.03 | 621 |
| *Buddleja salviifolia* | 39 | 0.12 ± 0.15 | 334 | 0.12 ± 0.15 | 334 |
| *Capparis tomentosa* | 25 | 0.26 ± 0.07 | 96 | 0.28 ± 0.07 | 90 |
| *Cassine aethiopica* | 33 | 0.33 ± 0.18 | 100 | 0.47 ± 0.24 | 70 |
| *Cassine transvaalensis* | 81 | 0.18 ± 0.10 | 442 | 0.32 ± 0.24 | 256 |
| *Cassinopsis ilicifolia* | 40 | 0.19 ± 0.09 | 208 | 0.31 ± 0.00 | 129 |
| *Cassinopsis tinifolia* | 23 | 0.23 ± 0.12 | 99 | 0.26 ± 0.07 | 88 |
| *Clausena anisata* | 44 | 0.42 ± 0.16 | 106 | 0.42 ± 0.16 | 106 |
| *Clerodendrum myricoides* | 57 | 0.17 ± 0.11 | 327 | 0.33 ± 0.18 | 172 |
| *Combretum molle* | 16 | 0.21 ± 0.08 | 147 | 0.21 ± 0.07 | 141 |
| *Combretum zeyheri* | 35 | 0.04 ± 0.03 | 829 | 0.05 ± 0.02 | 656 |
| *Curtisia dentate* | 72 | 0.31 ± 0.00 | 232 | 0.31 ± 0.00 | 232 |
| *Deinbollia oblongifolia* | 27 | 0.12 ± 0.07 | 234 | 0.08 ± 0.05 | 338 |
| *Dichrostachys cinerea* subsp. *africana* | 16 | 0.15 ± 0.03 | 106 | 0.16 ± 0.00 | 100 |
| *Dombeya rotundifolia* | 17 | 0.15 ± 0.07 | 113 | 0.15 ± 0.07 | 113 |
| *Ekebergia capensis* | 23 | 0.16 ± 0.00 | 144 | 0.19 ± 0.07 | 119 |
| *Elephantorrhiza elephantina* | 28 | 0.15 ± 0.12 | 193 | 0.11 ± 0.07 | 260 |
| *Faidherbia albida* | 28 | 0.12 ± 0.06 | 233 | 0.13 ± 0.05 | 217 |
| *Faurea saligna* | 151 | 0.23 ± 0.08 | 666 | 0.26 ± 0.07 | 581 |
| *Ficus sur* | 22 | 0.26 ± 0.07 | 85 | 0.26 ± 0.07 | 85 |
| *Flueggea virosa* | 20 | 0.04 ± 0.01 | 450 | 0.05 ± 0.02 | 391 |
| *Hippobromus pauciflorus* | 16 | 0.06 ± 0.03 | 267 | 0.06 ± 0.02 | 248 |
| *Jatropha curcas* | 19 | 0.26 ± 0.07 | 73 | 0.35 ± 0.11 | 55 |
| *Kigelia Africana* | 14 | 0.40 ± 0.16 | 35 | 0.63 ± 0.00 | 22 |
| *Lannea discolour* | 21 | 0.22 ± 0.09 | 96 | 0.31 ± 0.00 | 68 |
| *Lippia javanica* | 19 | 0.18 ± 0.08 | 103 | 0.31 ± 0.00 | 61 |
| *Lonchocarpus capassa* | 15 | 0.23 ± 0.12 | 64 | 0.26 ± 0.07 | 58 |
| *Olea europea* subsp. *africana* | 70 | 0.11 ± 0.04 | 656 | 0.31 ± 0.00 | 226 |
| *Oncoba spinosa* | 37 | 1.67 ± 0.63 | 358 | 1.67 ± 0.63 | 358 |
| *Ozoroa obovata* | 43 | 0.19 ± 0.20 | 224 | 0.23 ± 0.17 | 189 |
| *Peltophorum africanum* | 10 | 0.11 ± 0.04 | 91 | 0.14 ± 0.04 | 71 |
| *Pittosporum viridoflorum* | 22 | 0.26 ± 0.07 | 85 | 0.26 ± 0.07 | 85 |
| *Ricinus communis* | 28 | 0.13 ± 0.04 | 210 | 0.16 ± 0.00 | 175 |
| *Schotia brachypetala* | 14 | 0.07 ± 0.02 | 210 | 0.07 ± 0.02 | 210 |

| Plant species | Yield (mg) | Average activity60 (mg/ml) (± SD) | Total activity60 (ml/g) | Average activity120 (mg/ml) (± SD) | Total activity 120 (ml/g) |
|---|---|---|---|---|---|
| *Spirostachys africana* | 38 | 0.09 ± 0.06 | 438 | 0.09 ± 0.06 | 438 |
| *Strychnos spinosa* | 26 | 0.11 ± 0.07 | 229 | 0.11 ± 0.07 | 229 |
| *Syzigium cordatum* | 35 | 0.05 ± 0.02 | 656 | 0.08 ± 0.00 | 438 |
| *Tecomaria capensis* | 27 | 0.52 ± 0.16 | 52 | 0.52 ± 0.16 | 52 |
| *Terminalia phanerophlebia* | 30 | 0.03 ± 0.01 | 900 | 0.03 ± 0.01 | 964 |
| *Tetradenia riparia* | 20 | 0.08 ± 0.06 | 237 | 0.13 ± 0.13 | 149 |
| *Thespesia acutiloba* | 40 | 0.16 ± 0.00 | 250 | 0.18 ± 0.05 | 226 |
| *Trema orientalis* | 35 | 0.22 ± 0.14 | 159 | 0.22 ± 0.14 | 159 |
| *Trichilia emetica* | 9 | 0.31 ± 0.00 | 29 | 0.43 ± 0.17 | 21 |
| *Vangueria infausta* subsp. *infausta* | 11 | 0.23 ± 0.12 | 47 | 0.23 ± 0.12 | 47 |
| *Ximenia caffra* | 38 | 0.13 ± 0.04 | 285 | 0.13 ± 0.04 | 285 |
| *Ziziphus mucronata* | 10 | 0.31 ± 0.00 | 32 | 0.31 ± 0.00 | 32 |

The tannin assays provided an indication of the tannin content of the different extracts. Returning to the five plant extracts with the best overall activity, only the extracts of *Deinbollia oblongifolia* and *Spirostachys africana* did not contain any detectable tannins.

*Combretum zeyheri*, *Syzigium cordatum* and *Terminalia phanerophlebia* all contained tannins in varying concentrations, with *Terminalia phanerophlebia* exhibiting a small ring of complexed protein, whereas the values for *Combretum zeyheri* and *Syzigium cordatum* were rather large with 4.33 and 4 times gallic acid equivalent.

The values obtained from the tannin assays are shown in Table 4.5.

**Table 4.5: Tannin contents for the 53 selected plant species**

| Plant species | Average equivalent gallic acid | SD |
|---|---|---|
| *Acacia karroo* | 1.67 | 1.00 |
| *Acacia sieberiana* var. *woodii* | 0.00 | 0.00 |
| *Albizia adianthifolia* | 0.00 | 0.00 |
| *Annona senegalensis* | 0.00 | 0.00 |
| *Antidesma venosum* | 0.00 | 0.00 |
| *Berchemia zeyheri* | 0.00 | 0.00 |
| *Bridelia micrantha* | 0.00 | 0.00 |
| *Buddleja salviifolia* | 0.00 | 0.00 |
| *Capparis tomentosa* | 0.67 | 0.58 |
| *Cassine aethiopica* | 0.00 | 0.00 |
| *Cassine transvaalensis* | 7.33 | 0.58 |
| *Cassinopsis ilicifolia* | 3.00 | 1.73 |
| *Cassinopsis tinifolia* | 1.33 | 1.15 |
| *Clausena anisata* | 4.00 | 0.00 |
| *Clerodendrum myricoides* | 0.00 | 0.00 |
| *Combretum molle* | 11.33 | 0.58 |
| *Combretum zeyheri* | 4.33 | 1.53 |
| *Curtisia dentate* | 3.33 | 1.53 |
| *Deinbollia oblongifolia* | 0.00 | 0.00 |
| *Dichrostachys cinerea* subsp. *africana* | 0.33 | 0.58 |
| *Dombeya rotundifolia* | 0.00 | 0.00 |
| *Ekebergia capensis* | 0.00 | 0.00 |
| *Elephantorrhiza elephantina* | 0.00 | 0.00 |
| *Faidherbia albida* | 1.33 | 1.15 |
| *Faurea saligna* | 1.67 | 1.00 |
| *Ficus sur* | 2.67 | 1.15 |
| *Flueggea virosa* | 0.00 | 0.00 |
| *Hippobromus pauciflorus* | 8.00 | 1.15 |
| *Jatropha curcas* | 1.00 | 0.00 |
| *Kigelia Africana* | 0.00 | 0.00 |
| *Lannea discolour* | 4.00 | 0.00 |
| *Lippia javanica* | 0.00 | 0.00 |
| *Lonchocarpus capassa* | 0.00 | 0.00 |
| *Olea europea* subsp. *africana* | 0.00 | 0.00 |
| *Oncoba spinosa* | 1.33 | 1.15 |
| *Ozoroa obovata* | 3.33 | 1.53 |
| *Peltophorum africanum* | 6.00 | 1.73 |
| *Pittosporum viridoflorum* | 0.00 | 0.00 |
| *Ricinus communis* | 3.00 | 1.73 |
| *Schotia brachypetala* | 4.67 | 1.00 |
| *Sclerocarrya birrea* | 11.33 | 0.58 |
| *Spirostachys africana* | 0.00 | 0.00 |
| *Strychnos spinosa* | 0.00 | 0.00 |
| *Syzigium cordatum* | 4.00 | 0.00 |
| *Tecomaria capensis* | 5.67 | 2.08 |
| *Terminalia phanerophlebia* | 0.67 | 0.58 |
| *Tetradenia riparia* | 0.00 | 0.00 |
| *Thespesia acutiloba* | 0.33 | 0.58 |
| *Trema orientalis* | 5.67 | 0.58 |
| *Trichilia emetica* | 0.33 | 0.58 |
| *Vangueria infausta* subsp. *infausta* | 4.08 | 1.04 |
| *Ximenia caffra* | 4.00 | 0.00 |
| *Ziziphus mucronata* | 0.67 | 0.58 |

When the activity against the bacterial pathogens was compared to the tannin content, there was no obvious correlation between those two values. See Figures 4.2 and 4.3 for a visual comparison.

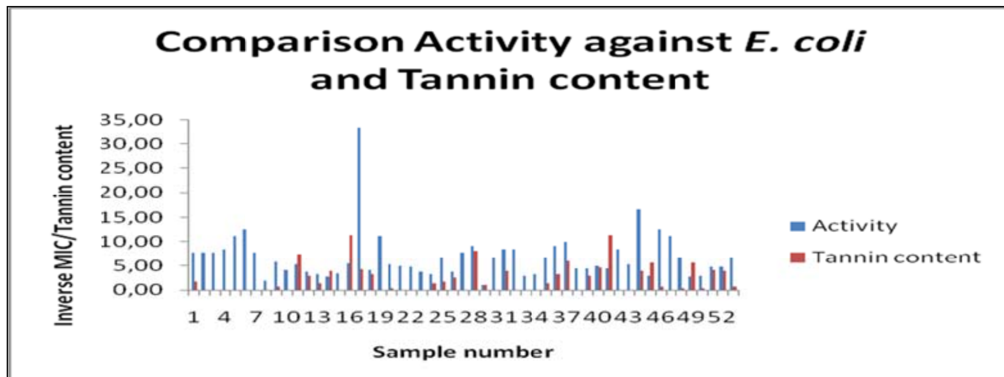



**Fig. 4.2: Comparison of the activity of the 53 different plant samples against *E. coli* and their tannin content**

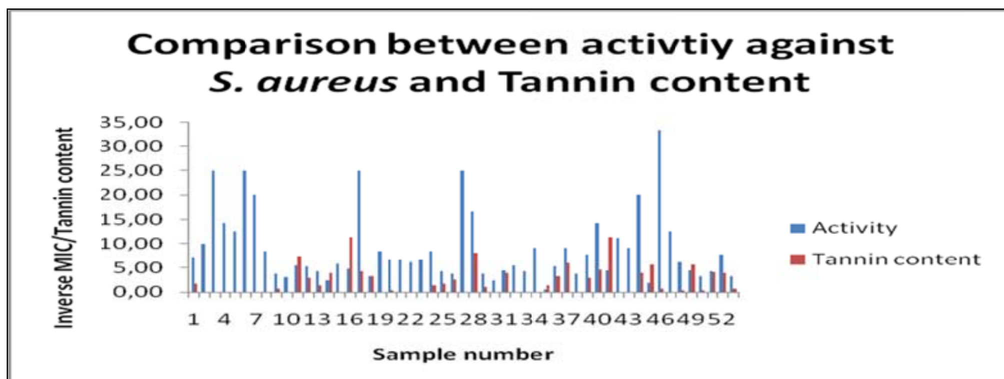



**Fig. 4.3: Comparison of the activity of the 53 different plant samples against *S. aureus* and their tannin content**

#### 4.3.3 Parameters for the selection of the plant species for further investigation

The values obtained in the previous sections helped to select which plant extracts should be used for further investigations. Naturally extracts should have a high activity against *E. coli*, but not necessarily against *S. aureus*. If an extract has strong activity against *E.coli* and *S. aureus* it may contain a general metabolic toxin that could be toxic to other organisms as well. Furthermore the plant extract should have low tannin content since tannins in feed taste bitter and also complex with proteins and would therefore not benefit a prophylactic feed additive. The last point that should be taken into consideration for the selection of the plant species is the extract yield, which should be relatively high.

Therefore those values were tabulated in order of importance based on an assigned percentage value. Low activity against *E. coli*, being the most important point for our study objective, was allocated the

percentage of 60%, the low tannin content 25% (values of 0 were allocated the value of 0.003, resulting from the limit of detection), low activity against *S. aureus* 10% and a high extraction yield was allocated 5%. The sum of these values was calculated by using the following formula:

(0.60/MIC *E. coli*) + (0.25/gallic acid equivalent) + (0.10*MIC *S. aureus*) + (0.05*extract yield) = Rank

(For the plants that did not contain any tannins, the value of the limit of detection in our tannin assay was used, i.e. 0.003).

The values calculated for the different plant species tested (calculated from unrounded values) varied from about 2 to 107 (Table 4.6).

**Table 4.6: Ranking of the extracts based on the formula developed**

| Plant species | MIC *E. coli* (mg/ml) | Tannin content | MIC *S. aureus* (mg/ml) | Yield(mg/g) | Value obtained with formula |
|---|---|---|---|---|---|
| *Tetradenia riparia* | 0.09 | 0.003 | 0.13 | 20.00 | 90.78 |
| *Spirostachys africana* | 0.13 | 0.003 | 0.09 | 38.00 | 89.98 |
| *Albizia adianthifolia* | 0.14 | 0.003 | 0.04 | 34.00 | 89.26 |
| *Lippia javanica* | 0.13 | 0.003 | 0.31 | 19.00 | 89.03 |
| *Flueggea virosa* | 0.13 | 0.003 | 0.05 | 20.00 | 88.95 |
| *Acacia sieberiana* var. *woodii* | 0.13 | 0.003 | 0.13 | 14.00 | 88.66 |
| *Berchemia zeyheri* | 0.13 | 0.003 | 0.04 | 16.00 | 88.64 |
| *Antidesma venosum* | 0.15 | 0.003 | 0.07 | 19.00 | 88.41 |
| *Olea europaea* subs. *africana* | 0.42 | 0.003 | 0.31 | 70.00 | 88.30 |
| *Deinbollia oblongifolia* | 0.17 | 0.003 | 0.08 | 27.00 | 88.19 |
| *Clerodendrum myricoides* | 0.31 | 0.003 | 0.33 | 57.00 | 88.15 |
| *Annona senegalensis* | 0.15 | 0.003 | 0.08 | 13.00 | 88.07 |
| *Strychnos spinosa* | 0.19 | 0.003 | 0.11 | 26.00 | 87.77 |
| *Ekebergia capensis* | 0.21 | 0.003 | 0.19 | 23.00 | 87.36 |
| *Bridelia micrantha* | 0.23 | 0.003 | 0.05 | 29.00 | 87.36 |
| *Pittosporum viridiflorum* | 0.22 | 0.003 | 0.26 | 22.00 | 87.20 |
| *Dombeya rotundifolia* | 0.20 | 0.003 | 0.15 | 17.00 | 87.20 |
| *Elephanthorrhiza elephantina* | 0.26 | 0.003 | 0.11 | 28.00 | 87.02 |
| *Cassine aethiopica* | 0.35 | 0.003 | 0.47 | 33.00 | 86.74 |
| *Buddleja salviifolia* | 0.52 | 0.003 | 0.12 | 39.00 | 86.44 |
| *Kigelia africana* | 0.29 | 0.003 | 0.63 | 14.00 | 86.20 |
| *Lonchocarpus capassa* | 0.33 | 0.003 | 0.26 | 15.00 | 85.95 |
| *Combretum zeyheri* | 0.03 | 4.333 | 0.05 | 35.00 | 20.27 |
| *Syzigium cordatum* | 0.06 | 4.000 | 0.08 | 35.00 | 12.26 |
| *Faurea saligna* | 0.15 | 1.667 | 0.26 | 151.00 | 11.79 |
| *Terminalia phanerophlebia* | 0.08 | 0.667 | 0.03 | 30.00 | 9.38 |
| *Ozoroa obovata* | 0.11 | 3.333 | 0.23 | 43.00 | 7.87 |
| *Cassine transvaalensis* | 0.19 | 7.333 | 0.32 | 81.00 | 7.22 |
| *Combretum molle* | 0.11 | 11.333 | 0.18 | 26.00 | 6.97 |
| *Peltophorum africanum* | 0.10 | 6.000 | 0.14 | 10.00 | 6.87 |
| *Thespesia acutiloba* | 0.15 | 0.333 | 0.18 | 40.00 | 6.70 |
| *Lannea discolour* | 0.12 | 4.000 | 0.31 | 21.00 | 6.36 |
| *Acacia karroo* | 0.13 | 1.667 | 0.14 | 15.00 | 5.53 |
| *Hippobromus pauciflorus* | 0.14 | 8.000 | 0.06 | 16.00 | 5.23 |
| *Curtisia dentate* | 0.45 | 3.333 | 0.31 | 72.00 | 5.05 |
| *Dichrostachys cinerea* subsp. *africana* | 0.19 | 0.333 | 0.16 | 16.00 | 4.71 |
| *Ximenia caffra* | 0.22 | 4.000 | 0.13 | 38.00 | 4.70 |
| *Capparis tomentosa* | 0.20 | 0.667 | 0.28 | 25.00 | 4.61 |
| *Ricinus communis* | 0.22 | 3.000 | 0.16 | 28.00 | 4.27 |
| *Ziziphus mucronata* | 0.21 | 0.667 | 0.31 | 10.00 | 3.72 |
| *Clausena anisata* | 0.45 | 4.000 | 0.42 | 44.00 | 3.63 |
| *Faidherbia albida* | 0.30 | 1.333 | 0.13 | 28.00 | 3.58 |
| *Vangueria infausta* subsp. *infausta* | 0.21 | 4.083 | 0.23 | 11.00 | 3.47 |
| *Trema orientalis* | 0.37 | 5.667 | 0.22 | 35.00 | 3.45 |
| *Cassinopsis ilicifolia* | 0.47 | 3.000 | 0.31 | 40.00 | 3.38 |
| *Cassinopsis tinifolia* | 0.31 | 1.333 | 0.26 | 23.00 | 3.30 |
| *Ficus sur* | 0.30 | 2.667 | 0.26 | 22.00 | 3.20 |
| *Tecomaria capensis* | 0.36 | 5.667 | 0.52 | 27.00 | 3.10 |
| *Oncoba spinosa* | 0.66 | 1.333 | 0.42 | 36.30 | 2.95 |
| *Trichilia emetica* | 0.37 | 0.333 | 0.43 | 9.00 | 2.85 |
| *Schotia brachypetala* | 0.32 | 4.667 | 0.07 | 14.00 | 2.62 |
| *Sclerocarya birrea* | 0.43 | 11.333 | 0.33 | 23.00 | 2.59 |
| *Jatropha curcas* | 0.96 | 1.000 | 0.35 | 19.00 | 1.86 |

The effect of the tannin content within the calculation can be clearly seen in the rather big difference in the values of *Combretum zeyheri* (with a value of 20.27) and *Lonchocarpus capassa* (with a value of 85.95). The values of all the species containing tannins are much lower than those of the species without detectable tannins in this research.

Based on this list, 5 plants were selected based on their ranking values, their availability and on the extent of research that has been done on them already (plants with little research done on them being preferable). Those five plants were: *Acacia sieberiana* var. *woodii* (MIC *E. coli* = 0.13 mg/ml, MIC *S. aureus* = 0.13 mg/ml, Yield = 14 mg, Tannin content = 0 Gallic acid equivalent), *Albizia adianthifolia* (MIC *E. coli* = 0.14 mg/ml, MIC *S. aureus* = 0.04 mg/ml, Yield = 34 mg, Tannin content = 0 Gallic acid equivalent), *Deinbollia oblongifolia* (MIC *E. coli* = 0.17 mg/ml, MIC *S. aureus* = 0.08 mg/ml, Yield = 27 mg, Tannin content = 0 Gallic acid equivalent), *Spirostachys africana* (MIC *E. coli* = 0.13 mg/ml, MIC *S. aureus* = 0.09 mg/ml, Yield = 38 mg, Tannin content = 0 Gallic acid equivalent ) and *Tetradenia riparia* (MIC *E. coli* = 0.09 mg/ml, MIC *S. aureus* = 0.13 mg/ml, Yield = 20 mg, Tannin content = 0 Gallic acid equivalent). Even though the activity of *Deinbollia oblongifolia* against *E. coli* was not that high after two hours of incubation with INT, we chose to further investigate it, because the activity had much better values after one hour of incubation with INT against *E. coli* (MIC = 0.09 mg/ml) and might therefore prove interesting if tested *in vivo.* A literature search also revealed that not much work had been done on this species so far. This might also provide the possibility to isolate novel compounds from this plant.

#### 4.3.4 Bioautographic assays and TLC fingerprinting

Bioautographic assays on all of the 53 selected plant species were done, but only results of the 5 chosen plants (*Acacia sieberiana* var.woodii, *Albizia adianthifolia*, *Deinbollia oblongifolia*, *Spirostachys africana* and *Tetradenia riparia*) are discussed at this point. The number of compounds active against *E. coli* and *S. aureus* as representatives for a Gram-negative and a Gram-positive species were determined. White bands on the assays represent the compound(s) in the plant extracts responsible for inhibiting bacterial growth. *S. aureus* was included to help find selectivity of the different extracts toward *E. coli*, since we wanted to exclude general metabolic toxins. With the three different solvent systems used, some information on the character, such as polarity, of the antibacterial compounds could also be obtained.

No compounds present in any extracts had significant inhibition bands when separated by the non-polar BEA solvent system. Only *Acacia sieberiana* var. *woodii*, *Deinbollia oblongifolia* and *Tetradenia riparia* had active compounds against both bacterial strains, but those inhibition bands were positioned at the bottom of the plate. The compounds responsible for the activity in the extracts appeared to be relatively

polar, since they did not show any significant inhibition bands or only bands right on the bottom of the plate when developed with this BEA solvent, as this solvent system separates non-polar compounds well.

The bioautograms obtained with the CEF solvent system had significant bands in all the samples. All the bioautographies obtained when run with the EMW solvent system had active bands near the top of the plate. One faint inhibition band against *E. coli* developed when an *Acacia sieberiana* var. *woodii* extract was developed with the CEF solvent. Against *S. aureus* one faint and one pronounced inhibition zone developed after this plant extract was developed with the CEF solvent.

Three faint inhibition bands against *E. coli* could be seen when *Albizia adianthifolia* was run with the CEF solvent. It also had two faint inhibition bands against *S. aureus* when run with this solvent.

*Deinbollia oblongifolia* had various active bands against *S. aureus* and *E. coli* when developed with the CEF solvent, but there were also some other inhibition bands present that were only selectively active against *E. coli*.

*Spirostachys africana*, developed in the CEF solvent, had one inhibition band that was selectively active against *E. coli*.

*Tetradenia riparia* developed several inhibition bands against both bacteria after being run with the CEF solvent. It seems however that this plant extract possesses more active compounds against *E. coli*.

Even though TLCs of all the plant samples were done, only the results of the five chosen species (See section 4.3.2) are shown here to be able to visualize and compare the active compounds.

EMW and CEF were the best solvents for separating the compounds with high and intermediate polarity in the extracts. The BEA solvent system did not separate many plant compounds as they were usually situated on the bottom of the plates, except for the case of *Albizia adianthifolia* and *Tetradenia riparia*. This suggested that the polarity of the compounds in the other extracts was too high to be moved along the TLC plate by the non-polar solvent, showing that the most of the compounds possessed intermediate or high polarity.

The compounds present in the extract of *Deinbollia oblongifolia* were separated well with both the EMW and the CEF solvent systems indicating that the most of the plant's compounds possess intermediate as well as high polarity.

The compounds present in the extract of *Albizia adianthifolia* and *Tetradenia riparia* were separated well with each of the three solvent systems, suggesting that the plant possesses compounds of high, intermediate and low polarity.

The EMW and CEF solvent systems separated the compounds of the *Acacia sieberiana* var. *woodii* and *Spirostachys africana* extract very well indicating that all these plants possess compounds of intermediate and high polarity.

Scans of the chromatograms and bioautographic assay plates are presented in Figures 4.4 to 4.48.

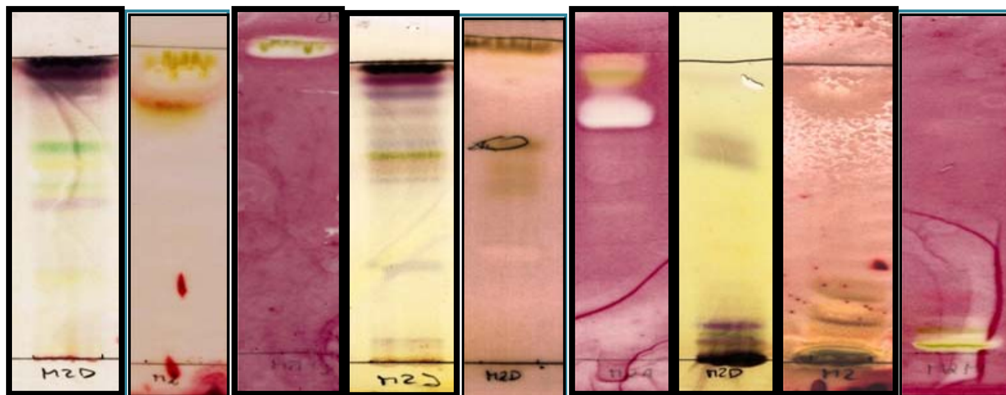


**Fig. 4.4 Fig. 4.5 Fig. 4.6 Fig. 4.7 Fig. 4.8 Fig. 4.9 Fig. 4.10 Fig. 4.11 Fig. 4.12**

Fig. 4.4: *Acacia sieberiana* var. *woodii* EMW; Fig. 4.5: *Acacia sieberiana* var. *woodii* EMW against *E. coli*; Fig. 4.6: *Acacia sieberiana* var. *woodii* EMW against *S. aureus*; Fig. 4.7: *Acacia sieberiana* var. *woodii* CEF; Fig. 4.8: *Acacia sieberiana* var. *woodii* CEF against *E. coli*; Fig. 4.9: *Acacia sieberiana* var. *woodii* CEF against *S. aureus*; Fig. 4.10: *Acacia sieberiana* var. *woodii* BEA; Fig. 4.11: *Acacia sieberiana* var. *woodii* BEA against *E. coli*; Fig. 4.12: *Acacia sieberiana* var. *woodii* BEA against *S. aureus*

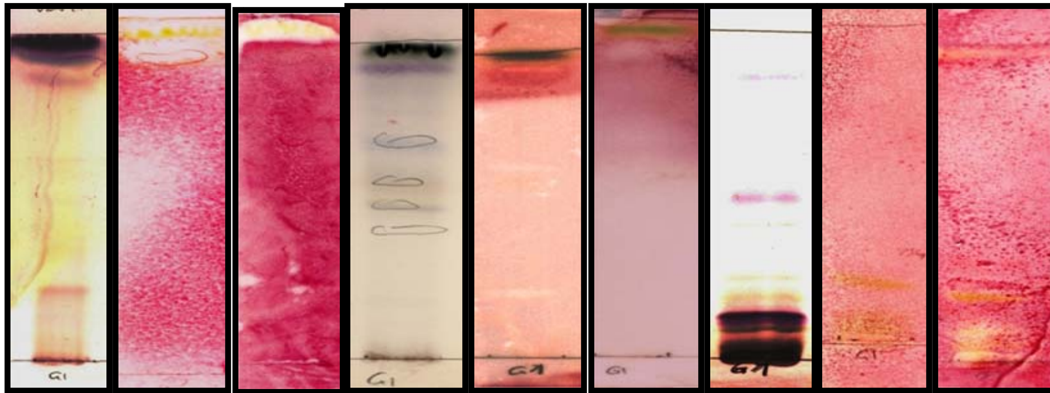


**Fig. 4.13 Fig. 4.14 Fig. 4.15 Fig. 4.16 Fig. 4.17 Fig. 4.18 Fig. 4.19 Fig. 4.20 Fig. 4.21**

Fig. 4.13: *Albizia adianthifolia* EMW; Fig. 4.14: *Albizia adianthifolia* EMW against *E. coli*; Fig. 4.15: *Albizia adianthifolia* against EMW *S. aureus*; Fig. 4.16: *Albizia adianthifolia* CEF; Fig. 4.17: *Albizia adianthifolia* CEF against *E. coli*; Fig. 4.18: *Albizia adianthifolia* CEF against *S. aureus*; Fig. 4.19: *Albizia adianthifolia* BEA; Fig. 4.20: *Albizia adianthifolia* BEA against *E. coli*; Fig. 4.21: *Albizia adianthifolia* BEA against *S. aureus*

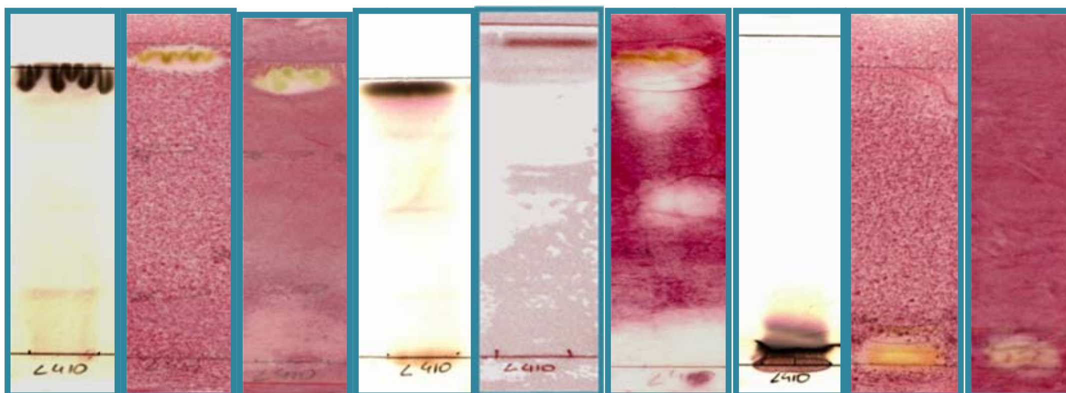


**Fig. 4.22 Fig. 4.23 Fig. 4.24 Fig. 4.25 Fig. 4.26 Fig. 4.27 Fig. 4.28 Fig. 4.29 Fig. 4.30**

Fig. 4.22: *Deinbollia oblongifolia* EMW; Fig. 4.23: *Deinbollia oblongifolia* EMW against *E. coli*; Fig. 4.24: *Deinbollia oblongifolia* EMW against *S. aureus*; Fig. 4.25: *Deinbollia oblongifolia* CEF; Fig. 4.26:*Deinbollia oblongifolia* CEF against *E. coli*; Fig. 4.27: *Deinbollia oblongifolia* CEF against *S. aureus*; Fig. 4.28: *Deinbollia oblongifolia* BEA; Fig. 4.29: *Deinbollia oblongifolia* BEA against *E. coli*; Fig. 4.30: *Deinbollia oblongifolia* BEA against *S. aureus*

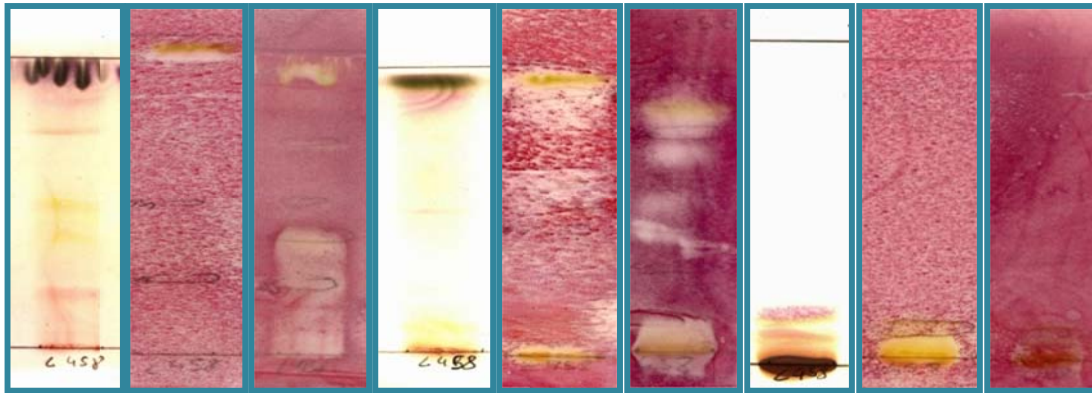


**Fig. 4.31 Fig. 4.32 Fig. 4.33 Fig. 4.34 Fig. 4.35 Fig. 4.36 Fig. 4.37 Fig. 4.38 Fig. 4.39**

Fig. 4.31: *Spirostachys africana* EMW; Fig. 4.32: *Spirostachys africana* EMW against *E. coli*; Fig. 4.33: *Spirostachys africana* EMW against *S. aureus*; Fig. 4.34: *Spirostachys africana* CEF; Fig. 4.35: *Spirostachys africana* CEF against *E. coli*; Fig. 4.36: *Spirostachys africana* CEF against *S. aureus*; Fig. 4.37: *Spirostachys africana* BEA; Fig. 4.38: *Spirostachys africana* BEA against *E. coli*; Fig. 4.39: *Spirostachys africana* BEA against *S. aureus*

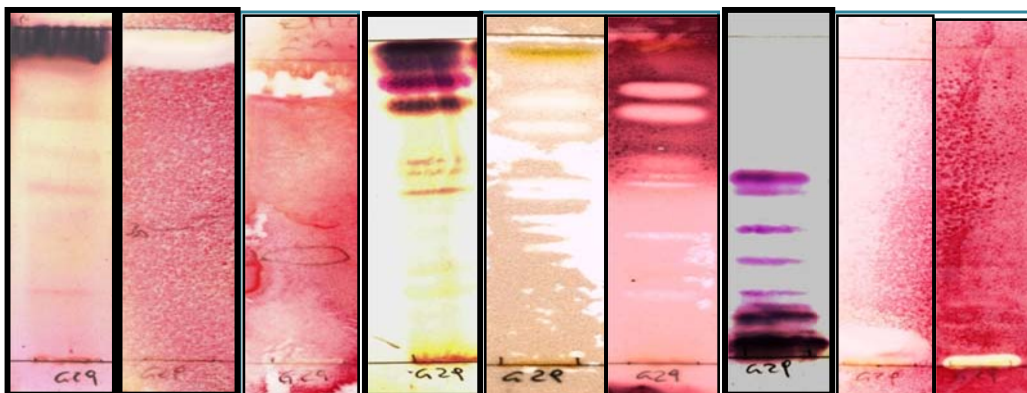


**Fig. 4.40 Fig. 4.41 Fig. 4.42 Fig. 4.43 Fig. 4.44 Fig. 4.45 Fig. 4.46 Fig. 4.47 Fig. 4.48**

Fig. 4.40: *Tetradenia riparia* EMW; Fig. 4.41: *Tetradenia riparia* EMW against *E. coli*; Fig. 4.42: *Tetradenia riparia* EMW against *S. aureus*; Fig. 4.43: *Tetradenia riparia* CEF; Fig. 4.44: *Tetradenia riparia* CEF against *E. coli*; Fig. 4.45: Tetradenia ripariaCEF against *S. aureus*; Fig. 4.46: *Tetradenia riparia* BEA; Fig. 4.47: *Tetradenia riparia* BEA against *E. coli*; Fig. 4.48: *Tetradenia riparia* BEA against *S. aureus*

## 4.4 Conclusions

This study compared 53 different plants, all extracted with acetone, in terms of the antibacterial activity of these plants with claimed medicinal value. The tannin determination assists in determining whether the antibacterial activity of the plant extracts is directly related to high tannin content or not. These results will help students of the Phytomedicine Programme in the future to choose plants with good activity but low tannin content for *in vivo* -and other further studies, which is important since tannins are

known to hinder the absorption of proteins and alkaloids, which might accelerate drug metabolism and result in blood levels of activities too low for a therapeutic effect (Williamson, 2001).

The five plants showing the overall best results regarding selectivity and activity in the antibacterial assays as well as a low tannin content and a high extract yield were *Acacia sieberiana* var. *woodii*, *Albizia adianthifolia*, *Deinbollia oblongifolia*, *Spirostachys africana* and *Tetradenia riparia*.

These five plants were selected based on the aspects discussed as well as on availability, on their bioautographic results and on the amount of work that has been done already on the plants as it is preferable to investigate plant species, on which little research has been done. The next step will be to determine the cytotoxicity *in vitro* of extracts of the five plant species. Based on these results, one of the five plants will be chosen for further analysis (i.e. animal experiments, isolation of active compounds and structure elucidation).

# Chapter 5

# Cytotoxicity of the five chosen extracts

## 5.1 Introduction

After selecting the five plant species *Acacia sieberiana* var. *woodii*, *Albizia adianthifolia*, *Deinbollia oblongifolia*, *Spirostachys africana* and *Tetradenia riparia* with the help of the ranking system established in chapter 4, the next step was to determine their *in vitro* cytotoxicity in order to make the decision regarding extracts to work on further.

*In vitro* cytotoxicity assays are useful for screening purposes to define cellular toxicity. This is considered primarily as the potential of an extract to induce cell death, in different cell types (Eisenbrand *et al.*, 2002). In order to produce a commercially useable product, the first step following bioactivity testing is to evaluate the toxicity of the plant extracts. Cell toxicity may be evaluated using the MTT (3 (4, 5-dimethyl thiazol-2yl)-2, 5-diphenyltetrazolium bromide) assay in which mitochondrial enzymes in viable but not dead cells cleave the yellow tetrazolium salt (MTT) to a blue formazan product (Mosmann, 1983). This assay is widely used as a preliminary screen to quantify cell proliferation, cytotoxicity and sensitivity (Mosmann, 1983; Fokkema *et al.*, 2002; Itamochi *et al.*, 2002). The MTT reduction is determined by the use of a microplate reader at a wavelength of 570 nm. The results are presented as a percentage of the value of the control cells. The concentration leading to a 50% death ($LC_{50}$) is calculated from concentration-response curves after linear regression analysis. Other cytotoxicity assays are available, for example the sulphorhodamine B assay and the neutral red assay which measure other aspects of cellular toxicity. The principle of the sulphorhodamine assay is based on the ability of the protein dye sulforhodamine B to bind electrostatically and pH dependently on protein basic amino acid residues of trichloroacetic acid-fixed cells (Houghton *et al.*, 2007). Under mild acidic conditions it binds to cells, and under mild basic conditions it can be extracted from cells and solubilised for measurement. Its sensitivity is comparable with that of several fluorescence assays (Voigt, 2005). The neutral red assay is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes (Mensah *et al.*, 2001; Repetto *et al.*, 2008). Since our laboratory at the Phytomedicine Programme has established a standard procedure for the MTT assay, this assay was used for the determination of the cell toxicity of the five extracts.

## 5.2 Materials and Methods

To determine the toxicity of the extracts, the MTT assay developed by Mosmann (1983) was used on African Green monkey kidney (Vero) cells (obtained from the Department of Veterinary Tropical Diseases of the Veterinary Faculty of the University of Pretoria) in a slightly modified version.

### 5.2.1 Cell culture

Minimum Essential Medium (MEM, Sigma) supplemented with 5% foetal calf serum (Highveld Biological), glutamine (Sigma) and gentamicin (Virbac) was used to culture the Vero cells. Cells were subcultured in 75 $cm^2$ culture flasks (Greiner) every week using trypsin-versene (Sigma) to remove cells from the culture flask.

### 5.2.2 Counting of cells and preparation of plates

The cells were counted using a Neubauer haemocytometer. Trypan blue (0.4%) in an equal volume was added to the cell aliquot to determine the number of viable cells. Those cells able to actively exclude the trypan blue appeared clear, while dead cells or those with a damaged cell membrane appeared blue.

In preparing cell suspensions for plating, cells were constituted to 10 000 cells/ml and 200 µl (2 500 cells per well) were pipetted into each well of a 96-well microtitre plate, except for columns 1 and 12 which contained medium alone to minimize evaporation from other wells. The plates were incubated overnight at 37°C in a 5% $CO_2$ incubator to allow the cells to attach.

### 5.2.3 MTT assay working method

Stock solutions of the plant extracts were prepared by reconstitution to a concentration of 100 mg/ml in acetone. Appropriate dilutions of each extract were prepared in growth medium and pipetted onto the cell monolayers after removing the original MEM from the wells. The viable cell growth after 120 h incubation (5 days in a 5% $CO_2$ incubator) with plant extracts was determined using the tetrazolium-based colorimetric assay (MTT assay) described by Mosmann (1983) with slight modifications. In this assay, the extract-containing MEM was removed and the cells were washed with 150 µl PBS. The PBS was removed and 200 µl fresh MEM was added to the wells. An aliquot of 30 µl MTT (stock solution of 5 mg/ml in PBS) was added to each well and the plates were further incubated for 4 h. After incubation, the MEM was removed from each well carefully, without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 µl DMSO to each well. The plate was shaken gently until the solution was dissolved (or shaken in the microplate reader). The amount of MTT reduction was recorded by immediately measuring the absorbance at 570 nm (reference wavelength of

630 nm) in a Versamax microplate reader (Molecular Devices). The wells in column 1, which previously contained medium and MTT but no cells were used to blank the plate reader. The results were described as a percentage of the control cells. Berberine chloride (Sigma) was used as a positive control, and acetone served as the negative control. Tests were carried out in quadruplicate and each experiment was repeated three times. The assay was repeated two to three times to ensure reproducible results.

## 5.3 Results and Discussion

### 5.3.1Determination of the $LC_{50}$ values of the five different plant extracts

After determining in which range the cytotoxicity of the extracts lay, a second round of MTT assays was performed to determine a more accurate $LC_{50}$ by testing 6 different concentrations within the range. The assay was repeated two to three times. The diagrams depicting these results can be seen in Figures 5.2 to 5.7.

*Albizia adianthifolia* was tested in the concentrations of 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml 0.01 mg/ml and 0.0075 mg/ml. From these results a response curve was drawn and the trend line was determined. With the trend line it was possible to determine the values for the equation $y = kx + d$. With this formula we determined that the $LC_{50}$ was 0.068 mg/ml (standard deviation: 0.004).

*Acacia sieberiana* var. *woodii* was tested in the concentrations of 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml and 0.0075 mg/ml. The response curve was drawn and the trend line determined. The resulting $LC_{50}$ was 0.026 mg/ml (standard deviation: 0.005).

When doing the preliminary screening it was noted that the $LC_{50}$ of *Deinbollia oblongifolia* lay somewhere between 1.0 mg/ml and 0.1 mg/ml, but when the second round of screening was done in the concentrations of 1.0 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.1 mg/ml and 0.075 mg/ml it became obvious that the $LC_{50}$ was closer to 0.075 mg/ml and so the assay was redone at six lower concentrations (0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml and 0.0075 mg/ml). The value of the $LC_{50}$ was 0.078 mg/ml (standard deviation: 0.00).

*Spirostachys africana* was tested in the concentrations of 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml and 0.0075 mg/ml. The response curve was drawn and the trend line determined. The resulting $LC_{50}$ was 0.025 mg/ml (standard deviation: 0.005 mg/ml).

*Tetradenia riparia* was tested in the concentrations of 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml and 0.0075 mg/ml. The response curve was drawn and the trend line determined. The resulting $LC_{50}$ was 0.028 mg/ml (standard deviation: 0.005 mg/ml).

Figure 5.1 gives an example of how the linear regression curve was plotted to determine the $LC_{50}$ in case of one of the *Tetradenia riparia* samples.

The selectivity index (SI) of all five species was determined using the following formula:

SI = $LC_{50}$ (mg/ml) / MIC (mg/ml)

Table 5.1 lists all the $LC_{50}$ values and the values for the SI obtained.

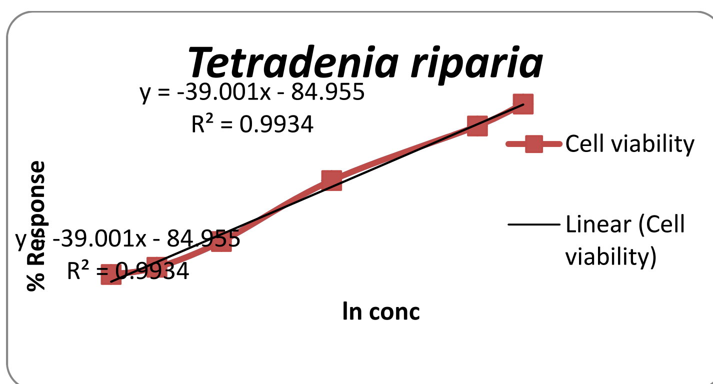



**Fig. 5.1: Linear regression curve and equation for one of the *Tetradenia riparia samples***

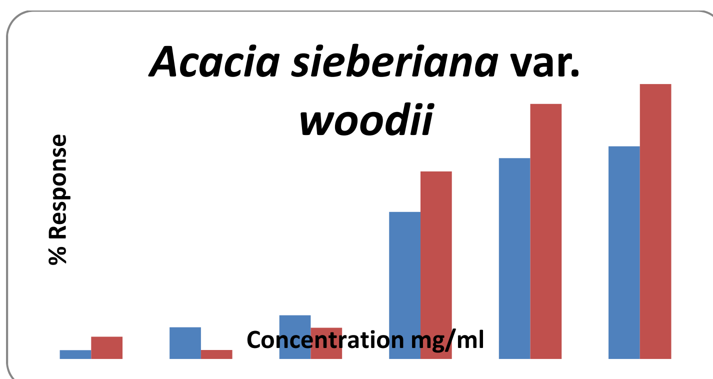



**Fig. 5.2: Toxicity of *Acacia sieberiana* var. *woodii* extract**

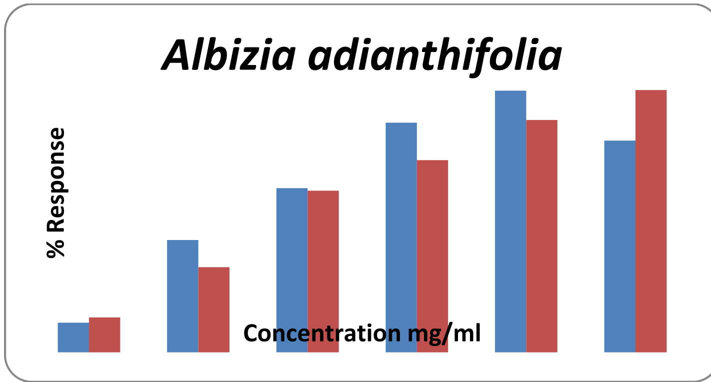



Fig. 5.3: Toxicity of *Albizia adianthifolia* acetone extract

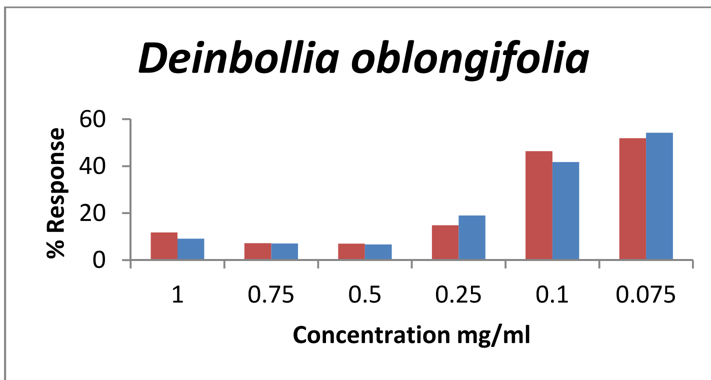



Fig. 5.4: Toxicity of *Deinbollia oblongifolia* extract1st experiment

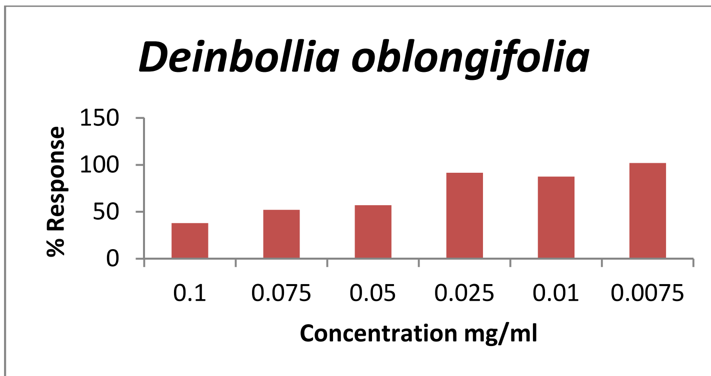



Fig. 5.5: Toxicity of *Deinbollia oblongifolia*extract

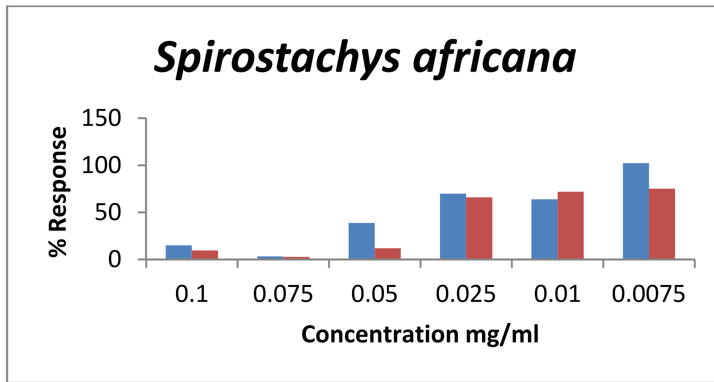



**Fig. 5.6: Toxicity of *Spirostachys africana* extract**

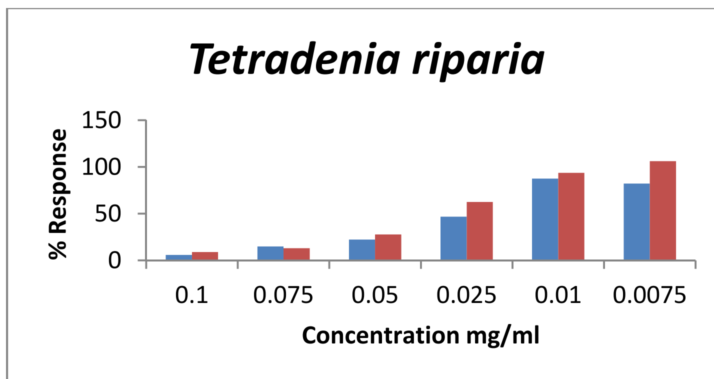



**Fig. 5.7: Toxicity of *Tetradenia riparia* extract**

**Table 5.1: $LC_{50}$ and SI values of the different species**

| Species | $LC_{50}$ (mg/ml) (± SD) | SI |
|---|---|---|
| *Acacia sieberiana* var. *woodii* | 0.026 ± 0.005 | 0.20 |
| *Albizia adianthifolia* | 0.068 ± 0.004 | 0.49 |
| *Deinbollia oblongifolia* | 0.078 ± 0.00 | 0.46 |
| *Spirostachys africana* | 0.025 ± 0.005 | 0.19 |
| *Tetradenia riparia* | 0.028 ± 0.005 | 0.31 |

## 5.4 Conclusion

Results from the MTT assays demonstrated that the least toxic extract was derived from *Deinbollia oblongifolia*. As a second plant on which to conduct further studies, *Spirostachys africana* was selected even though the extract was relatively toxic with an $LC_{50}$ value of 0.025 mg/ml. The next step was to potentize these two extracts by increasing their activity while attempting to minimize toxicity.

# Chapter 6

# Potentization of *Deinbollia oblongifolia* and *Spirostachys africana* extracts

## 6.1 Introduction

After the previous study regarding cell toxicity, *Deinbollia oblongifolia* and *Spirostachys africana* were chosen to be potentized by solvent-solvent fractionation. These two plants were relatively active against *E. coli* (*Deinbollia oblongifolia* MIC = 0.17 mg/ml and *Spirostachys africana* MIC = 0.13 mg/ml) and based on the tannin assays performed earlier had a low concentration of tannins. The evaluation of their cytotoxicity resulted in relatively low cytotoxicity values ($LC_{50}$). The method of potentization allows us to enhance the activity as well as possibly reduce the toxicity of the plant extracts by splitting the crude extract into fractions which may separate active and toxic compounds. During this fractionation process inactive compounds might be concentrated in some of the fractions, leaving other fractions with the more active compounds and therefore enhancing their activity by reducing the dilution on a mass basis of the inactive compounds. The five resulting fractions in the method used (Suffness and Douros, 1979) each have different chemical properties due to the solvent-solvent fractionation process where solvents of increasing in polarity are used, i.e. hexane, chloroform, 35% water in methanol, n-butanol and water.

This process might allow for some fractions to be less toxic than others due to the fact that the toxic compounds might be present in a fraction with a different polarity from that of the active non-toxic compounds. It has already been proven that through solvent-solvent fractionation, the toxicity of certain extracts can be lowered due to the removal of toxic compounds (Liu et al, 2008). Unfortunately it is often the case that the compound(s) responsible for antibacterial activity are the same ones causing cytotoxicity.

## 6.2 Materials and Methods

For the potentization of the extracts the solvent-solvent fractionation method described by Suffness and Douros (1979) and modified by Eloff (1998) was used. With this method five different fractions were obtained, which were then evaluated for their antibacterial properties.

### 6.2.1 Mass extraction

Leaves of the two tree species, *Deinbollia oblongifolia* and *Spirostachys africana*,were collected at the SANBI Pretoria National Botanical Garden in the months of November and December 2009. The trees were identified by Joseph Khangela Baloyi, an employee of the Botanical Garden (Voucher specimens are being lodged at the herbarium). The leaves were dried in a dark room for one week and then ground to a fine powder using a Macsalab mill (Model 200 LAB). The resulting powder was stored in sealed glass containers.

Five hundred g of the ground leaves of *Spirostachys africana* and 300 g of the ground leaves of *Deinbollia oblongifolia* were exhaustively extracted with acetone in a 1:10 ratio (i.e. 5 l and 3 l of acetone respectively) in a 10 l glass container and shaken for one hour on a Labotec shaker model 20.2. This process was repeated three times with fresh aliquots of acetone on the same plant material. The extract was then left to settle and was filtered through cotton wool and then through Whatman No1 filter paper using a Buchner funnel. The extract was concentrated to a minimum volume using a Büchi rotavapor R-114 (Labotec) at 45°C. The remaining extracts were transferred to pre-weighed glass jars and left to completely dry under a constant flow of air.

### 6.2.2 Solvent-solvent fractionation

The *Spirostachys africana* and the *Deinbollia oblongifolia* extracts were fractionated using the solvent-solvent fractionation method developed by Suffness and Douros (1979) in a slightly modified version. The technique and schematic procedure of the solvent-solvent fractionation procedure is provided in Figure 6.1.

The method developed by Suffness and Douros (1979) was modified by leaving out one of the fractionation steps, namely the carbon tetrachloride step due to potential health problems with using large volumes of this solvent. There were therefore four fractionation steps in the solvent-solvent fractionation process. Each step of the fractionation was carried out once, and the steps are described below.

**Step 1:** Chloroform-water fractionation step: The dried extract was mixed with equal volumes of chloroform and water (100 ml each) and was transferred to a separating funnel. The liquid was mixed well by careful rotation of the funnel. After partition was achieved, the bottom phase which contained the chloroform was placed into a pre-weighed büchi (round-bottomed) flask.

**Step 2:** Water and n-butanol fractions: To the 100 ml of the water fraction 100 ml of n-butanol was added to yield a n-butanol and a water fraction after separation in a separating funnel. These fractions were placed in pre-weighed glass jars and then left to dry under a constant air flow.

**Step 3:** Hexane fraction: The chloroform fraction was reduced to a minimum volume in a rotavapor and left to dry under a constant flow of air. The dried fraction was dissolved in equal volumes of hexane and 10% water in methanol (each 100 ml) and placed into a separating funnel. Since no partition was achieved that way, a further 600 ml of the 10% water in methanol solvent was added to achieve partition into a hexane and a 10 % water in methanol fraction. The hexane fraction was collected into pre-weighed glass jars and left to dry under a constant flow of air.

**Step 4:** Chloroform and 35% water in methanol fraction: The 10% water in methanol fraction resulting from the hexane extraction was diluted to 35% water in methanol. An equal volume of chloroform was added and the mixture was extracted in the separating funnel and the resulting phases collected in pre-weighed glass jars. They were then dried under a constant flow of air.

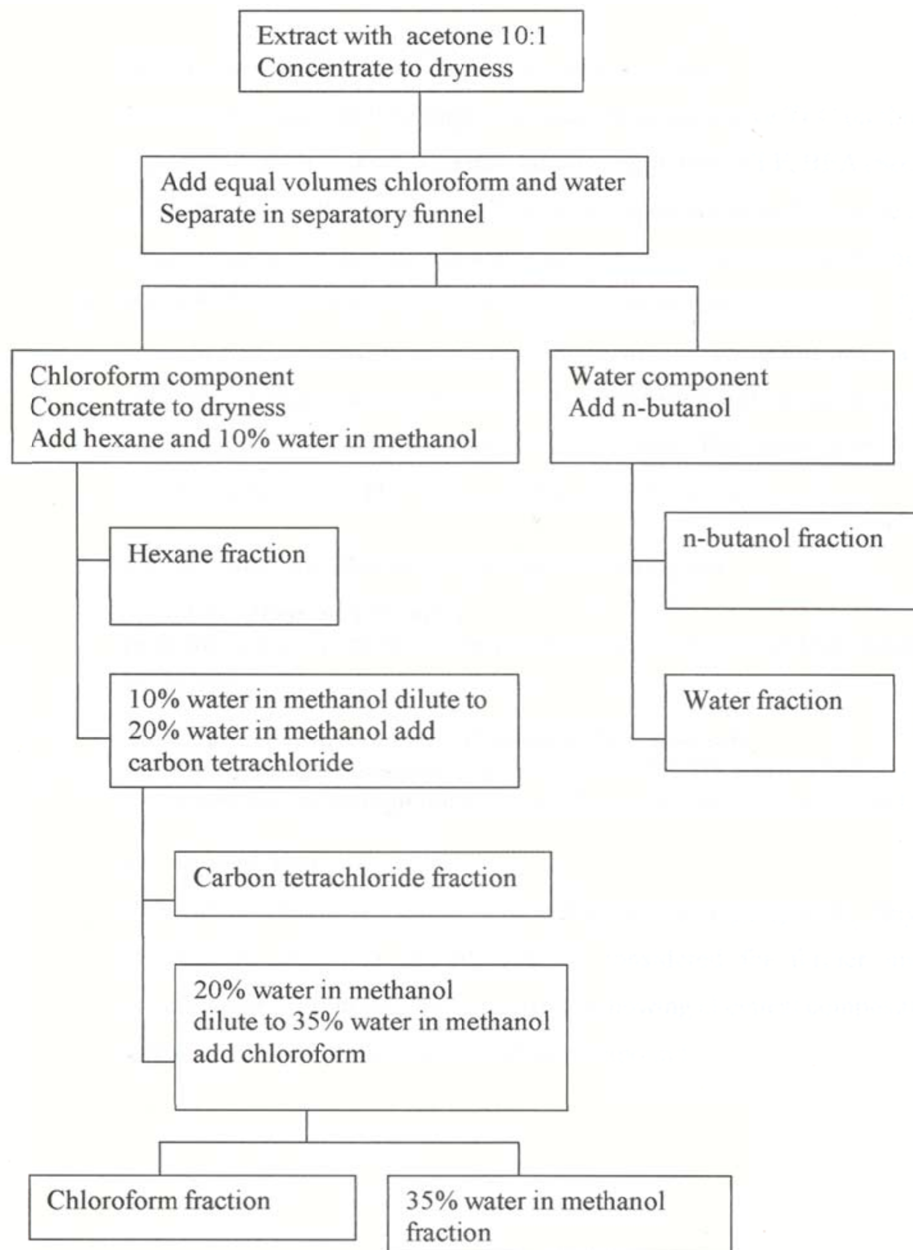



**Fig 6.1: Representation of solvent-solvent fractionation procedure (Suffness and Douros, 1979)**

### 6.2.3 TLC fingerprinting and bioautographic assays

The dried fractions were dissolved in acetone to a concentration of 10 mg/ml and then loaded (100 µg) onto two sets of three TLC plates and run in each of the three solvent systems developed in our lab (EMW, CEF, BEA). One set was sprayed with vanillin-sulphuric acid reagent to serve as a reference chromatogram.

The second set of TLC plates was used for bioautography using *E. coli* as test organism as described in section 2.2.5.

### 6.2.4 Minimum inhibitory concentration determination

The minimum inhibitory concentrations of the resulting fractions were determined by employing the 2-fold serial dilution assay described in section 2.2.6. This assay was done three times in triplicate to ensure reproducibility.

### 6.2.5 Cytotoxicity assays

#### 6.2.5.1 Cell culture

The same method as described in section 5.2.1 was used to prepare the cell culture.

#### 6.2.5.2 Counting of cells and preparation of plates

The same method as described in section 5.2.2 was used to count the cells and prepare the plates.

#### 6.2.5.3 MTT-assay working method

The same method as described in section 5.2.3 was used.

## 6.3 Results and Discussion

### 6.3.1 Mass extraction

The resulting dried extract from 300 g *Deinbollia oblongifolia* weighed 11.18 g and the resulting dried extract from 500 g *Spirostachys africana* weighed 22.51 g. This means the extract yield for *Deinbollia oblongifolia* was 3.7% and for *Spirostachys africana* with acetone was 4.5%.

### 6.3.2 Solvent-solvent fractionation

The chloroform fraction resulting from the first step by mixing the dried extract with chloroform and water and separating the two phases had a dry weight of 16.2 g for *Spirostachys africana* and 10.38 g for *Deinbollia oblongifolia*.

The resulting fraction masses and their percentage in relation to the total extraction yield for *Deinbollia oblongifolia* are shown in Table 6.1.

**Table 6.1: Fraction masses and percentages of *Deinbollia oblongifolia***

| Fraction | Mass (g) | Percentage of total extraction yield |
|---|---|---|
| **Water** | 1.32 g | 0.44 % |
| **n-Butanol** | 0.27 g | 0.09 % |
| **Hexane** | 8.02 g | 2.67 % |
| **Chloroform** | 1.11 g | 0.37 % |
| **35% water in methanol** | 0.53 g | 0.18 % |

These resulting masses total 11.25 g, which means only 0.05 g were lost during the separation process.

The resulting fraction masses and their percentage in relation to the total extraction yield for *Spirostachys africana* are shown in Table 6.2.

**Table 6.2: Fraction masses and percentages of *Spirostachys africana***

| Fraction | Mass (g) | Percentage of total extraction yield |
|---|---|---|
| **Water** | 3.49 g | 0.70% |
| **n-Butanol** | 3.59 g | 0.72% |
| **Hexane** | 8.00 g | 1.6% |
| **Chloroform** | 3.69 g | 0.74% |
| **35% water in methanol** | 0.55 g | 0.11% |

These resulting masses total 19.32 g, which means that 3.19 g were lost during the separation process.

### 6.3.3 Minimum inhibitory concentration

The minimum inhibitory concentrations against *E. coli* of the two potentized extracts were determined by the MIC assay described in section 2.2.6.

The resulting MIC values for the fractions of each plant are shown in Tables 6.3 and 6.4.

**Table 6.3: MIC values of the fractions of *Deinbollia oblongifolia* against *E. coli***

| Fraction | Average MIC against *E. coli* after 60 min (mg/ml) (± SD) | Average MIC against *E. coli* after 120 min (mg/ml) (± SD) |
|---|---|---|
| **Water** | 1.38 ± 0.92 | 1.38 ± 0.92 |
| **n-Butanol** | 0.75 ± 0.39 | 0.75 ± 0.39 |
| **Hexane** | 0.18 ± 0.10 | 0.30 ± 0.22 |
| **Chloroform** | 0.07 ± 0.02 | 0.08 ± 0.00 |
| **35% water in methanol** | 0.12 ± 0.04 | 0.20 ± 0.11 |

**Table 6.4: MIC values of the fractions of *Spirostachys africana* against *E. coli***

| Fraction | Average MIC against *E. coli* after 60 min (mg/ml) (± SD) | Average MIC against *E. coli* after 120 min (mg/ml) (± SD) |
|---|---|---|
| **Water** | 0.50 ± 0.15 | 0.82 ± 0.33 |
| **n-Butanol** | 0.18 ± 0.10 | 0.25 ± 0.07 |
| **Hexane** | 0.20 ± 0.07 | 0.25 ± 0.07 |
| **Chloroform** | 0.05 ± 0.02 | 0.08 ± 0.06 |
| **35% water in methanol** | 0.18 ± 0.10 | 0.40 ± 0.15 |

The chloroform fractions of both extracts exhibited the best activity against *E. coli.* In the case of *Deinbollia oblongifolia* the chloroform fraction had a much better activity (MIC = 0.08 mg/m) than the crude extract (MIC = 0.17 mg/ml). With *Spirostachys africana* the chloroform fraction also had a better activity (MIC = 0.08 mg/ml) than the crude extract (MIC = 0.13 mg/ml).

### 6.3.4 TLC fingerprinting and bioautographic assays

The TLC plates gave a reference to the compounds in the plants and helped to identify the active compounds when the fractions were subjected to bioautography. The most active antibacterial compounds were detected in the chloroform fractions of both extracts. The compounds that were active in *Deinbollia oblongifolia* seemed to inhibit the bacteria to a stronger degree than the ones from *Spirostachys africana*.

The chromatograms and bioautographic assays can be seen in Figures 6.2 to 6.7

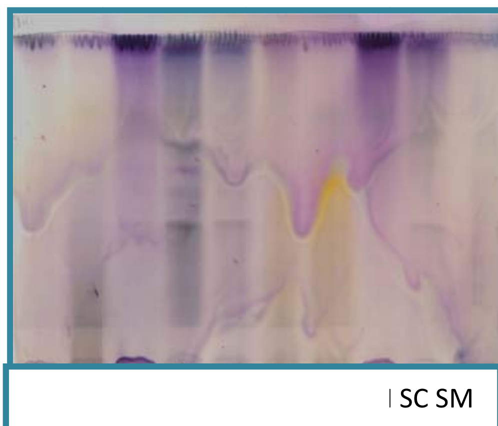



**Fig. 6.2: Chromatograms EMW**

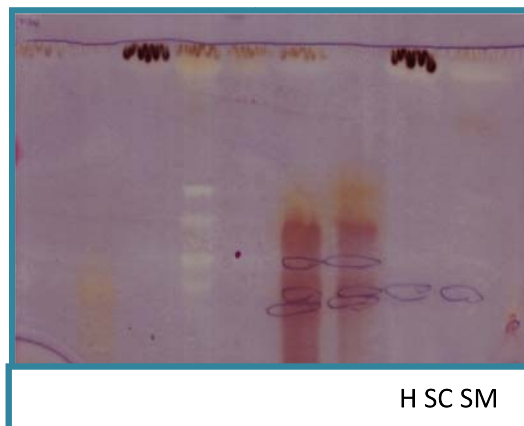



**Fig. 6.3: Bioautography against *E. coli* EMW**

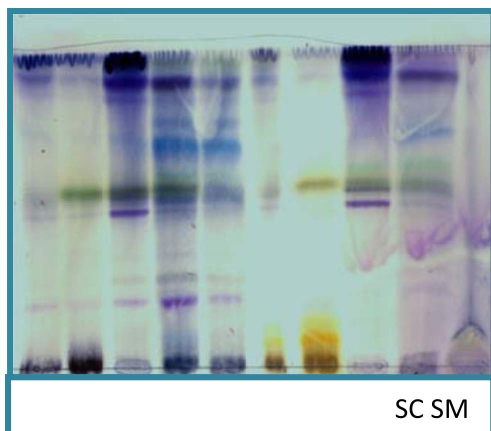



**Fig. 6.4: Chromatograms CEF**

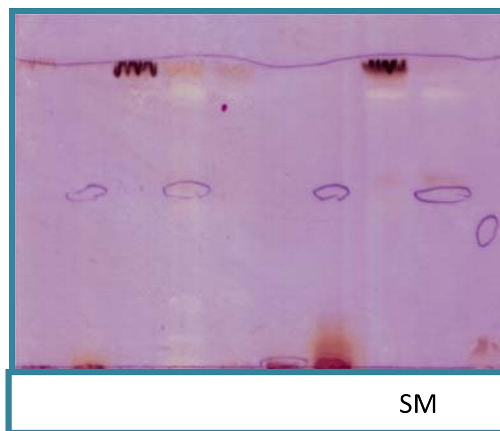



**Fig. 6.5: Bioautography against *E. coli* CEF**

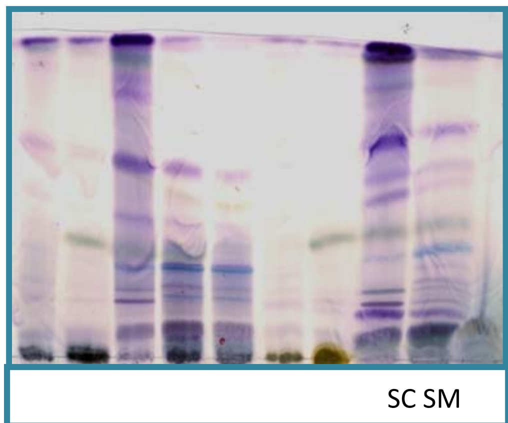



**Fig. 6.6: Chromatograms BEA**

**Fig. 6.7: Bioautography against *E. coli* BEA**

### 6.3.5 Cytotoxicity assays

The chloroform fraction of *Deinbollia oblongifolia* was tested in the range of 1 mg/ml to 0.1 mg/ml in the concentrations of 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.1 mg/ml and 0.075 mg/ml. The resulting $LC_{50}$ value was 0.188 mg/ml (standard deviation: 0.017).

The chloroform fraction of *Spirostachys africana* was tested in the range of 0.1 mg/ml and 0.01 mg/ ml in the concentrations of 0.1 mg/ml, 0.075 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.01 mg/ml and 0.0075 mg/ml. The resulting $LC_{50}$ value was.0.062 mg/ml (standard deviation: 0.02).

The assay was repeated twice to ensure reproducibility. The results are also shown in Figures 6.8 and 6.9.

The selectivity index (SI) was again determined and it was 2.35 for the chloroform fraction of *Deinbollia oblongifolia* and 0.78 for the chloroform fraction of *Spirostachys africana*.

The results for the values for the $LC_{50}$ and the SI are shown in table 6.5.

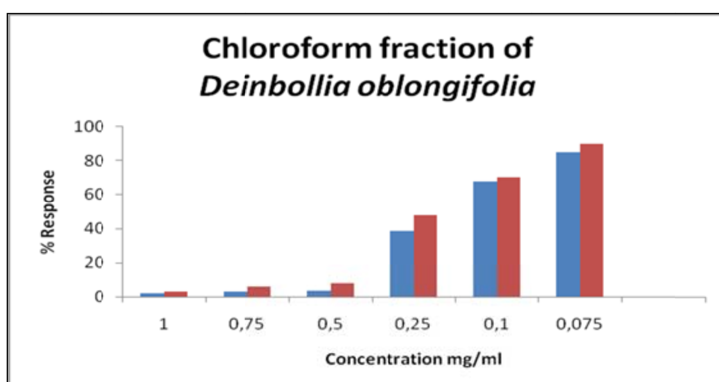



**Fig. 6.8: Toxicity of the chloroform fraction of *Deinbollia oblongifolia***

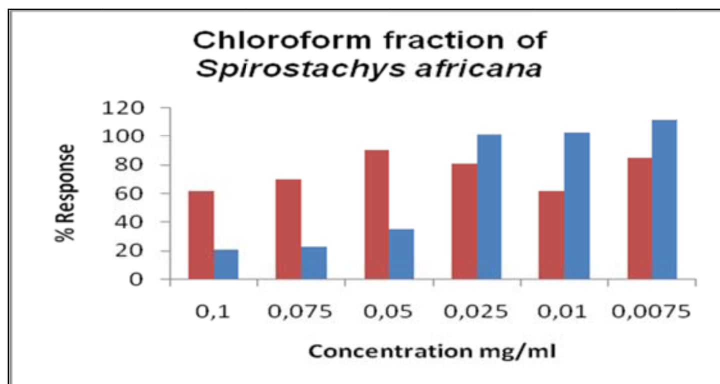



**Fig. 6.9: Toxicity of the chloroform fraction of *Spirostachys africana***

**Table 6.5: $LC_{50}$ and SI of the chloroform fractions**

| **Extract** | **$LC_{50}$ (mg/ml) (± SD)** | **SI** |
|---|---|---|
| Chloroform fraction of *Deinbollia oblongifolia* | 0.188 mg/ml ± 0.017 | 2.35 |
| Chloroform fraction of *Spirostachys Africana* | 0.062 ± 0.02 | 0.78 |

## 6.4 Conclusion

The potentization of the two crude extracts of *Deinbollia oblongifolia* and *Spirostachys africana* showed that the intermediate polarity compounds in the chloroform fraction of both extracts had the highest activity. The next step was to determine the cytotoxicity of these chloroform fractions to determine which one of them is least toxic before testing the *in vivo* efficacy.

The chloroform fraction of *Deinbollia oblongifolia* was less toxic than that prepared from *Spirostachys africana*. Therefore further work was done on the chloroform fraction of *D. oblongifolia*. Even though the $LC_{50}$ value of 0.188 mg/ml represented a rather high cellular toxicity, it is possible that the toxicity could be lower in a biological system since it may be metabolised and changed *in vivo* before it affects the animal. Since a lot of medications are excreted through the kidneys and therefore affect them, and most laboratories have available Vero kidney cells for cytotoxicity assays, the kidney cells were chosen for the cytotoxicity evaluation.

The values for the selectivity Index (2.35 for the potentized extract of *Deinbollia oblongifolia* compared to a value of 0.45 for the crude extract and 0.78 for the potentized extract of *Spirostachys*

*africana*compared to the lower value of 0.19 for the crude extract) show that a potentization of both extracts was indeed achieved.

The next step in this project was to isolate the active compounds from the extract and then to investigate the acute and subacute toxicity of the chloroform fraction as well as of the crude extract in a live rat model. The crude extract was not as active *in vitro* against *E. coli* as the chloroform fractions, but many substances are metabolically modified in a biological system and it is possible that the crude extract could exhibit a higher activity in an animal than in the laboratory tests, which would be ideal since it is economically more feasible to work with the crude extract.

# Chapter 7

# Isolation of active compounds from *Deinbollia oblongifolia*

## 7.1 Introduction

To discover new bioactive compounds from plants, bioassay-guided fractionation is often employed. Novel isolated bioactive compounds could lead to the development of new drugs if they are sufficiently active or non-toxic, and possibly the chemical structure of promising targets can be modified to achieve this objective. There is one major drawback to this process in that it frequently happens that known metabolites are isolated. However, new activities for known compounds are sometimes discovered, so useful information is still obtained.

The compounds in plant extracts are commonly isolated using column chromatography. In principle, plant constituents are distributed between the solid phase (for example silica gel or Sephadex) and the mobile phase, which comprises an eluting solvent. In silica gel the separation of compounds in an extract from each other is based on a number of factors including the polarity of compounds, hence compounds are eluted separately from the column with solvent systems of differing polarity. Silica gels constitute polar ends which interact strongly with polar compounds and they are eluted later from the column. In Sephadex gel filtration the separation of constituents in an extract depends on the size of the molecules. Constituents with a small molecular weight interact strongly with the matrix of the gel and tend to move slowly through the gel and are eluted later while the large molecular weight constituents are eluted early because they move fast through the column.

*Deinbollia oblongifolia* (Sapindaceae) or Dune soap-berry tree is a small tree or shrub up to 3.5 m in height, occurring in coastal open woodland, dune bush and forest, along riverine fringes and in bush clumps. Its distribution is mainly in KwaZulu Natal. To the knowledge of the author, no active compounds have been isolated from this species so far.

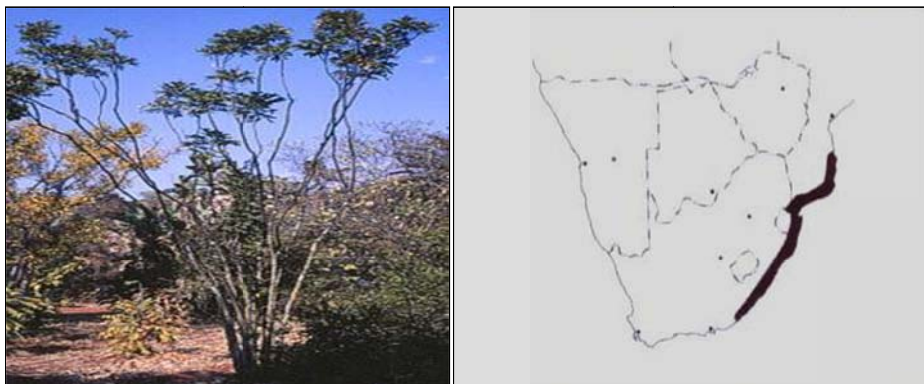


**Fig. 7.1:** *Deinbollia oblongifolia* **Fig. 7.2: Distribution of** *Deinbollia oblongifolia*

## Materials and methods

### 7.2.1 Preparation of the bulk extract

Leaves of the tree species, *Deinbollia oblongifolia* were collected at the SANBI Pretoria National Botanical Garden in the months of November and December 2009. The trees were identified by Joseph Khangela Baloyi, an employee of the Botanical Garden. The leaves were dried in a dark room for one week and then ground to a fine powder using a Macsalab mill (Model 200 LAB). The resulting powder was stored in sealed glass containers.

The ground leaves (300 g) of *Deinbollia oblongifolia* were exhaustively extracted with the extracting solvent acetone in a 1:10 ratio (e.g. 3 l of acetone respectively) in a 10 l glass container and shaken for one hour on a Labotec shaker model 20.2. This process was repeated three times with fresh aliquots of acetone on the same plant material. The extract was then left to settle and was filtered through cotton wool and then through Whatman No1 filter paper using a Buchner funnel. The extract was concentrated to a minimum volume using a Büchi rotavapor R-114 (Labotec) at 45°C. The remaining extract was transferred to pre-weighed glass jars and left to completely dry under a constant flow of air. The mass of the resulting extract was 11.18 g.

### 7.2.2 Solvent fractionation

Since the chloroform fraction was the most active, the isolation of the active compounds was done using the chloroform fraction obtained by the solvent fractionation that was done as described in section 7.3.2. The weight of the resulting chloroform fraction was 3.69 g.

### 7.2.3 Column chromatography

A glass column of diameter 5 cm was used to separate the constituents of 3.69 g of the chloroform fraction of the *D. oblongifolia* extract. Silica gel 60 (400 g) (Merck) was mixed with hexane to form a slurry and this was then packed into the glass column up to a height of 37 cm. The chloroform fraction of the acetone extract (4 g) was dissolved in a small volume of acetone, mixed with about 25 g of silica gel 60 (Merck) and left to completely dry under a constant flow of air. The dried extract and silica powder combination was then delicately placed on top of the packed column. Initially the column was eluted with 200ml of 100% hexane and subsequently the polarity of the eluting solvent was sequentially increased by replacing hexane with ethyl acetate in an increasing percentage (80ml of 5%, 320ml of 10%, 120ml of 15%, 120 ml of 20% and 60 ml of 50%). The rest of the column was eluted with 100% methanol (200ml). The fractions were labelled 1 to 38. Fractions of approximately 20 ml each were collected in test tubes. In Table 7.1 the fractions are listed with their corresponding eluting solvent.

**Table 7.1: Fractions with their corresponding solvent systems**

| **Fraction** | **Solvent-system** |
|---|---|
| 1-2 | 100% hexane |
| 3-6 | 95% hexane, 5% ethyl acetate |
| 7-22 | 90% hexane, 10% ethyl acetate |
| 23-28 | 85% hexane, 15% ethyl acetate |
| 29-34 | 80% hexane, 20% ethyl acetate |
| 35-37 | 50% hexane, 50% ethyl acetate |
| 38 (200ml collected in a 250 ml flask) | 100% methanol |

### 7.2.4 Thin layer chromatography

The fractions were left to dry in front of a fan until the volume was decreased to concentrate the compounds. Then an aliquot of every third fraction was loaded onto 10 x 20 cm TLC plates. Two plates each were developed in a 10% ethyl acetate and 90% hexane solvent system. One set of TLC plates was sprayed with vanillin-sulphuric acid spray reagent and served as reference chromatograms. The second set of plates was sprayed with *E. coli* and incubated for 18 hours and then sprayed with INT to visualize the compounds that are active against *E. coli*. Fractions with similar $R_f$ values and antibacterial activity were combined and placed in pre-weighed glass vials. They were then left to dry under a constant flow of air.

### 7.2.5 Purification of column fractions

From the TLC plates in section 8.2.4 any fraction(s) containing impurities were washed by adding 2 ml of hexane and shaking the glass vial to try and dissolve any impurities soluble in hexane. The dissolved fraction was transferred into a preweighed glass vial and both fractions were dried under a constant flow of air. This was done to obtain pure compounds, but the active compound also dissolved in the hexane and therefore this washing step did not prove effective.

### 7.2.6 Preparative thin layer chromatography

The active fraction was further separated by preparative TLC on silica gel plates (Merck Silica gel 60 $F_{254}$, 0.25 mm thick) using EMW. The active fraction was scraped off the TLC plates and eluted from the silica with ethanol. The active compound in ethanol was filtered through Millipore filters (0.45 µm and 0.22 µm) to remove the silica. To ensure complete removal of the silica particles, the filtrate was passed through a Silica 60 (Merck) column prepared in a Pasteur pipette. The Silica 60 (Merck) was loaded to a height of 30 mm, and the pipette had an internal diameter of 5 mm. The purity of the isolated compound was confirmed by TLC using various solvent systems.

### 7.2.7 Minimum inhibitory concentration

The minimum inhibitory concentration of the pure compound against *E. coli* was determined using the method described in section 2.2.6.This assay was carried out three times in triplicate.

### 7.2.8 Cytotoxicity of the isolated compound

The toxicity of the pure compound was tested against Vero kidney cells following the method described in section 5.2 with the only difference being the range of concentrations in which the compound was tested. The compound was dissolved in extracting solvent, i.e. acetone, to produce a 20 mg/ml solution which was used for testing in the assays. The compound was screened at a range of concentrations to determine the $LC_{50}$.

### 7.2.9 Identification of purified active compound

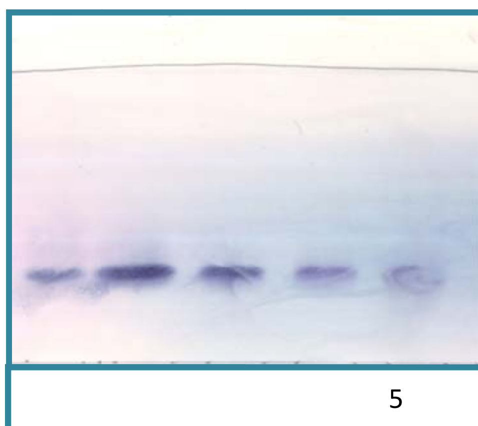
#### *7.2.9.1 Nuclear Magnetic Resonance Spectroscopy*

Nuclear Magnetic Resonance (NMR) Spectroscopy ($^{1}$H NMR and $^{13}$C NMR) was carried out to confirm the structure of the compound using a Varian Inova 500 MHz spectrometer, based at the CSIR, Pretoria.

## 7.3 Results and Discussion

### 7.3.1 Thin layer chromatography and bioautography of the fractions resulting from column chromatography:

Every fraction was loaded onto two sets of TLC plates that were run in the 10% ethyl acetate and 90% hexane solvent system. One plate was sprayed with the vanillin-sulphuric acid spray reagent and the other plate was sprayed and incubated with *E. coli* for 18 hours and then sprayed with INT to visualize the bacterial inhibition bands. The fractions that showed the relevant compound are shown below in Figures 7.1 and 7.2.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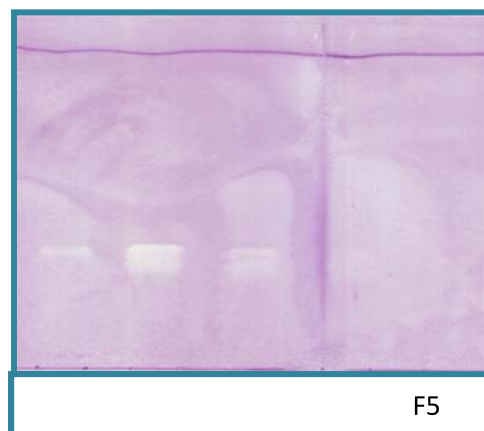

F5

**Fig. 7.3: Chromatograms 10% EA / 10%H**

**Fig. 7.4: Bioautography 10% EA / 90% H**

The fractions 1 to 4 were combined and dried under a constant flow of air. They were spotted onto three sets of TLC plates together with the chloroform fraction for reference and run in the three solvent systems (EMW, CEF and BEA) and in the 10% ethyl acetate and 90% hexane solvent system. They were then sprayed with vanillin sulphuric acid spray reagent to show possible impurities. The combined fractions were also spotted onto another TLC plate and after being developed in the EMW solvent system, the plate was incubated with *E. coli* to show the activity. Figure 7.3 shows the respective plates.

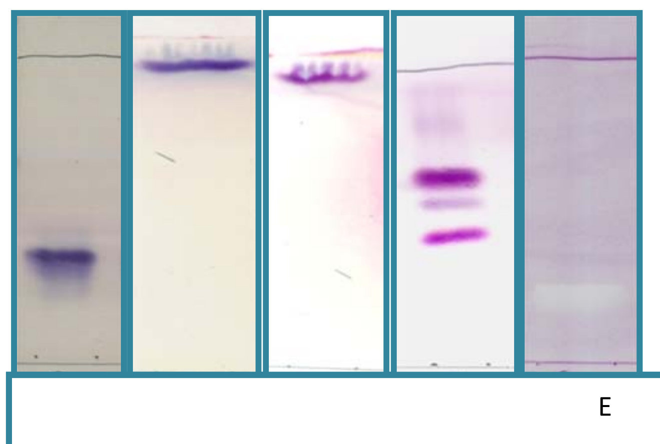



**Fig. 7.5: A: Chromatogram combined fractions 10% EA / 90% H; B: Chromatogram combined fractions EMW; C: Chromatogram combined fractions CEF; D: Chromatogram combined fractions BEA; E: Bioautography combined fractions 10% EA / 90% H**

The fractions proved to still contain impurities. These became especially obvious in the plate run with the BEA solvent system. The next step was to try and isolate the combined fractions further with preparative thin layer chromatography.

### 7.3.2 Preparative thin layer chromatography

The combined fractions were diluted with acetone to a concentration of 20 mg/ml and then loaded onto four TLC plates and run in the EMW solvent system. One small spot of the combined fractions was run alone at the edge of the plate to serve as a reference for the preparative TLC and was sprayed with vanillin-sulphuric acid reagent after development of the plate.

After preparative TLC was performed we were left with a pure compound. The compound was loaded onto three sets of two TLC plates that were developed in the EMW, CEF and BEA solvent systems. One plate developed in each solvent system was sprayed with the vanillin-sulphuric acid spray reagent to show the purity of the compound. The second plate developed in each of the solvent system was incubated with *E. coli* to show the compound's activity. The respective TLC and bioautographic plates can be seen in the Figures 7.4 to 7.5 below.

After the pure compound was dried, the total yield was between 10 and 20 mg.

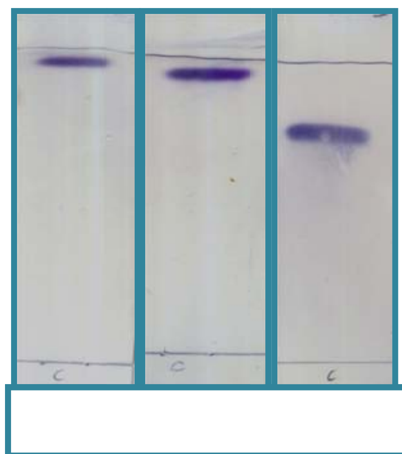


Fig. 7.6: Chromatograms of compound

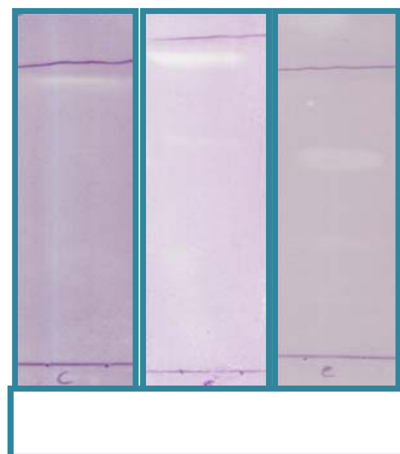


Fig. 7.7: Bioautography of compound against *E. coli*

**7.3.3 Minimum inhibitory concentration**

When determining the minimum inhibitory concentration of the purified compound against *E. coli* (ATCC 25922), it became apparent from the MIC value of 0.74 mg/ml after 60 and 120 minutes, that the antibacterial activity decreased by quite a lot in comparison with the potentized and even the crude extract. The total activity value was 0.07 ml/g, so it is also from the economic point of view clear that it would not be sensible to produce the pure compound as a treatment for diarrhoea.

**7.3.4 Cytotoxicity of the isolated compound:**

The cytotoxicity of the pure compound was tested using the MTT assay on Vero kidney cells. The range of concentrations tested lay between 10 and 200 µg/ml.

The $LC_{50}$ for the pure compound, determined from the linear regression curve, was 42 µg/ml. The diagram depicting the cytotoxicity results is shown in Figure 7.6.

Based on the $LC_{50}$ the selectivity index (SI) was determined to be 0.06.

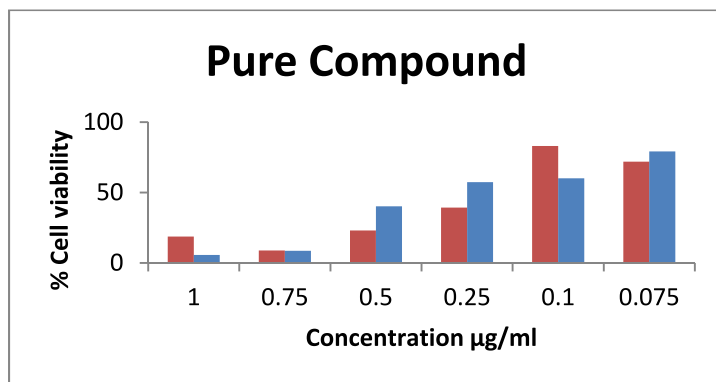



**Fig. 7.8: Cytotoxicity of the isolated compound**

### 8.3.5 Identification of the purified active compound

The NMR spectra were run at the CSIR by Ms Teresa Faleschini. Dr Ahmed Aroke interpreted the chemical structure.

Twenty mg of a white powder were isolated. To determine the structure, 2-dimensional NMR (HMBC-NMR, HSQC-NMR, $^{1}$H-NMR, $^{13}$C-NMR and COSY-NMR) spectra were interpreted and compared with available literature data (Mahato and Kundu, 1994). The correlation of the results can be seen in Tables 7.2 and 7.3.

The structure was determined to be a mixture of the two compounds 3β-OH-α-amyrin and a 3β-OH-β - amyrin, two triterpenoids. The structures can be seen in Figure 7.9.

**Table 7.2: NMR-Spectra 1 of the isolated bioactive compound**

| C | H | HSQC | HMBC (C->H) |
|---|---|---|---|
| **1** | 1.64 ($CH_2$) | 38.76 | H 28, H 16 |
| **2** | 1.60 ($Ch_2$) | 27.22 | H 15 |
| **3** | 3.2 (CH) | 29.00 | H 28, H 19, H 16 |
| **4** | - (C) | 42.07 | H 18, H 16, H 29 |
| **5** | 0.715 (CH) | 55.00 | H 18, H 16, H 15, H19 |
| **6** | 1.54 ($CH_3$) | 18.00 | H 15 |
| **7** | - (C) | - | - |
| **8** | - (C) | 40 | H 27, H 24 |
| **9** | 1.53 (CH) | 48 | H 18, H 16, H 15 |
| **10** | - (C) | 36.9 | H 16 |
| **11** | 1.07 ($CH_2$) | 25.35 | - |
| **12** | 5.10 (CH) | 124 | - |
| **13** | - (C) | 139 | H 23.3 |
| **14** | - (C) | 42 | H 27, H 24 |
| **15** | 1.25 ($CH_2$) | 29 | - |
| **16** | 0.87 ($CH_2$) | 24 | H 33 |
| **17** | - (C) | 33 | H 24, H 29 |
| **18** | 1.31 (CH) | 59 | H 29 ($CH_3$) |
| **19** | - (CH) | 39.6 | H 16 |

| | | | |
|---|---|---|---|
| **20** | - (CH) | 39.6 | H 33 |
| **21** | - ($CH_2$) | | - |
| **22** | - ($CH_2$) | 41.57 | - |
| **23** | 0.81 ($CH_3$) | 29 | H 16 |
| **24** | 0.748 ($CH_2$) | 15.48 | H 55 |
| **25** | 0.96 ($CH_3$) | 15.37 | H 18 |
| **26** | - $CH_3$ | 12 | - |
| **27** | - $CH_3$ | 24 | - |
| **28** | - $CH_3$ | - | - |
| **29** | - $CH_3$ | - | - |
| **30** | - CH3 | - | - |

**Table 7.3: NMR-Spectra 2 of the isolated bioactive compound**

| C | H | HSQC | HMBC (C->H) |
|---|---|---|---|
| **1** | 1.64 ($CH_2$) | 38.76 | H 28, H 16 |
| **2** | 1.60 ($Ch_2$) | 27.22 | H 15 |
| **3** | 3.2 (CH) | 29.00 | H 28, H 19, H 16 |
| **4** | - (C) | 42.07 | H 18, H 16, H 29 |
| **5** | 0.715 (CH) | 55.00 | H 18, H 16, H 15, H19 |
| **6** | 1.54 ($CH_3$) | 18.00 | H 15 |
| **7** | - (C) | - | - |
| **8** | - (C) | 40 | H 27, H 24 |
| **9** | 1.53 (CH) | 48 | H 18, H 16, H 15 |
| **10** | - (C) | 36.9 | H 16 |
| **11** | 1.07 ($CH_2$) | 25.35 | - |
| **12** | 5.10 (CH) | 122 | - |
| **13** | - (C) | 145 | H 23.3 |
| **14** | - (C) | 42 | H 27, H 24 |
| **15** | 1.25 ($CH_2$) | 29 | - |
| **16** | 0.87 ($CH_2$) | 24 | H 33 |
| **17** | - (C) | 33 | H 24, H 29 |
| **18** | 1.31 (CH) | 45 | H 29 ($CH_3$) |
| **19** | - (CH) | 39.6 | H 16 |
| **20** | - (CH) | 39.6 | H 33 |
| **21** | - ($CH_2$) | | - |
| **22** | - ($CH_2$) | 41.57 | - |
| **23** | 0.81 ($CH_3$) | 29 | H 16 |
| **24** | 0.748 ($CH_2$) | 15.48 | H 55 |
| **25** | 0.96 ($CH_3$) | 15.37 | H 18 |
| **26** | - $CH_3$ | 12 | - |
| **27** | - $CH_3$ | 24 | - |
| **28** | - $CH_3$ | - | - |
| **29** | - $CH_3$ | - | - |
| **30** | - CH3 | - | - |

The literature survey showed that this compound has not been isolated from the genus *Deinbollia* before.

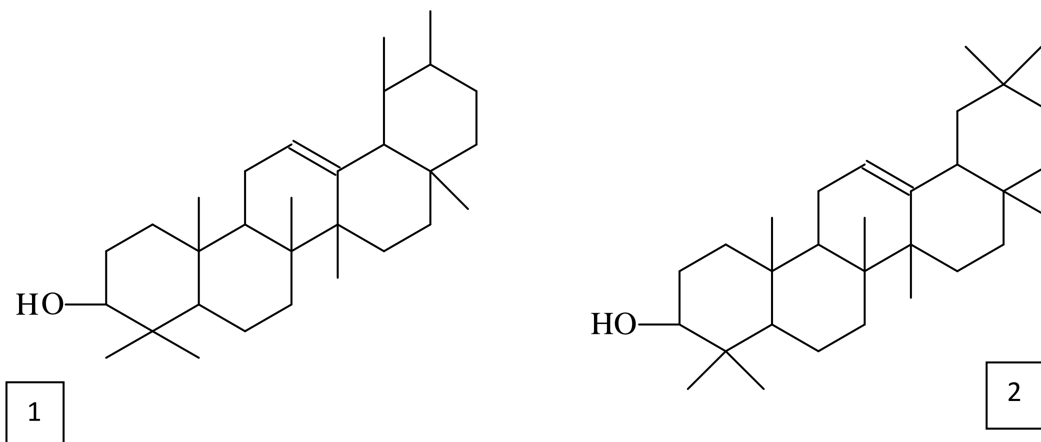



**Fig. 7.9: Structures of 3β-OH-α-amyrin (1) and a 3β-OH-β-amyrin (2)**

The $^{13}C$ chemical shift analysis made possible the identification of the structural features, namely oxygen-bearing methane carbons at δc=79.055 and 79.024 (C-3), and twoolefinic carbons atδc=124.411 and 121.713 (C-12) respectively. In the HMBC spectra, the signal at δc=124.411 correlates with 138.02, which is a characteristic feature of ursene derivatives, while δc= 121.713 correlates with 144, acharacteristic feature of oleanene derivatives. The carbon resonating at δc=59.056 (C-18) confirmed the presence of an ursene derivative with no carbonyl functional group at C-17. Also, a carbon resonating at δc=47.6 (C-18) confirmed the presence of an oleanene derivative with no carbonyl functional group at C-17. These observations indicate the presence of two closely related isomers as all signals were duplicated with minor differences. Detailed analysis of the$^{13}C$ NMR spectra, HMBC, HSQC and COSY and comparison of the data reported in the literature (Mahato and Kundu, 1994) confirmed the mixture to be 3β-OH-α-amyrin (1)and 3β-OH-β-amyrin (2).

## 7.4 Conclusions

One compound that was active against the ATCC strain of *E. coli* and that also showed a distinct band in the bioautographic plates was isolated. Its toxic effect on Vero cells was also investigated and it has a $LC_{50}$ value of 0.042 mg/ml which is a more toxic value than that obtained from the potentized fraction. The compound's activity against *E. coli* was also much lower than the activity of the potentized extract, 0.74 mg/ml opposed to 0.08 mg/ml. This proves that the activity of the plant is more owing to a synergistic effect of a number of compounds than being caused by the presence of a single compound. The structure of the compound was elucidated to be mixture of the two compounds 3β-OH-α-amyrin and a 3β-OH-β-amyrin,two triterpenoids.

# Chapter 8

# Efficacy of the potentized extract of *Deinbollia oblongifolia* against different *E. coli* strains

## 8.1 Introduction

All the tests performed to evaluate the antibacterial activities of the plant extracts thus far were done using only the ATCC 25922 strain of *E. coli*. To see whether the extracts are also active when tested against pathological *E. coli* strains, four of these strains were obtained and the extracts were tested against them. Bioautographic assays were also done to see whether the active compounds in the extract and the potentized version changed. The pure compound was also tested against the four pathological strains.

## 8.2 Materials and Methods

### 8.2.1 Bacterial cultures

Four *E. coli* strains that were isolated from different organs in different animals by the Department of Veterinary Tropical Diseases (Section Bacteriology, University of Pretoria) were used in the screening process. These included one strain from the sinus of poultry (B 1295), one from the large intestine of a pig (B1297), one isolated from a bovine liver and spleen (B 1314) and the last one was a bursal swab from poultry (B 1279).

The bacteria were cultured on blood agar plates. For the assay, liquid cultures were used. The preparation of the liquid medium and cultures was done as described in section 2.2.4.

### 8.2.2 Minimum inhibitory concentration determination

The activity of the crude extract, the potentized extract and the isolated compound against the different pathological *E. coli* strains was measured by the use of the microdilution assay described in section 2.2.6.The assay was repeated three times to ensure reproducibility.

### 8.2.3 Bioautographic assays

The method described in section 2.2.5 was used to show the activity of the crude extract, the potentized extract and the isolated compound against the different pathological *E. coli* strains.

## 8.3 Results and Discussion

### 8.3.1 Minimum inhibitory concentration

The minimum inhibitory concentration of the crude extract, the potentized extract and the compound was determined against all four pathogenic bacterial strains. The results show that there were noticeable differences in the minimum inhibitory concentrations of the different extracts against the four different pathogenic bacteria.

The crude extract had the same activity as previously determined with the ATCC 25922 strain against the pathological strains isolated from the poultry sinus B 1295 and the poultry bursal swab B1279 (both 0.16 mg/ml). The activity against the *E. coli* strains isolated from the large intestine from the pig B 1297 and the liver and spleen of a bovine B 1314 was not as good as previously determined (0.31 mg/ml and 0.28 mg/ml respectively).

The chloroform fraction had the same activity against the *E. coli* strain isolated from the poultry sinus (B 1295) as it had against the ATCC 25922 strain (0.08 mg/ml). The activity against the other three pathological strains B 1279, B 1297 and B 1314 was not as good as it was against the ATCC 25922 strain (all 0.16 mg/ml). This difference in activity however only correlated to a difference in one well and was therefore not statistically significant.

The pure compound had a better activity against all four pathogenic strains than against the ATCC 25922 strain (B1279: 0.31 mg/ml; B1295: 0.31 mg/ml; B1297: 0.42 mg/ml and B1314: 0.36 mg/ml compared to ATCC 25922: 0.74 mg/ml value).

The values for all the minimum inhibitory concentrations against all four pathological bacterial strains are given in Table 8.1.

**Table 8.1: MIC values in mg/ml (± SD) of the crude extract (C), the chloroform fraction (CF) and the pure compound (P) isolated from *Deinbollia oblongifolia* after 60 or 120 minutes incubation against four strains of pathogenic bacteria**

| Bacterial strain | MIC 60 C | MIC 120 C | MIC 60 CF | MIC 120 CF | MIC 60 P | MIC 120 P |
|---|---|---|---|---|---|---|
| **B 1279** | 0.16 ± 0.00 | 0.16 ± 0.00 | 0.08 ± 0.00 | 0.16 ±0.00 | 0.31 ± 0.00 | 0.31 ± 0.00 |
| **B 1295** | 0.08 ± 0.00 | 0.16 ± 0.00 | 0.04 ± 0.00 | 0.08 ± 0.00 | 0.29 ± 0.01 | 0.31 ± 0.00 |
| **B1297** | 0.15 ± 0.03 | 0.31 ± 0.00 | 0.16 ± 0.00 | 0.16 ± 0.00 | 0.31 ± 0.00 | 0.42 ± 0.17 |
| **B1314** | 0.28 ± 0.08 | 0.28 ± 0.08 | 0.16 ± 0.00 | 0.16 ± 0.00 | 0.31 ± 0.00 | 0.36 ± 0.13 |

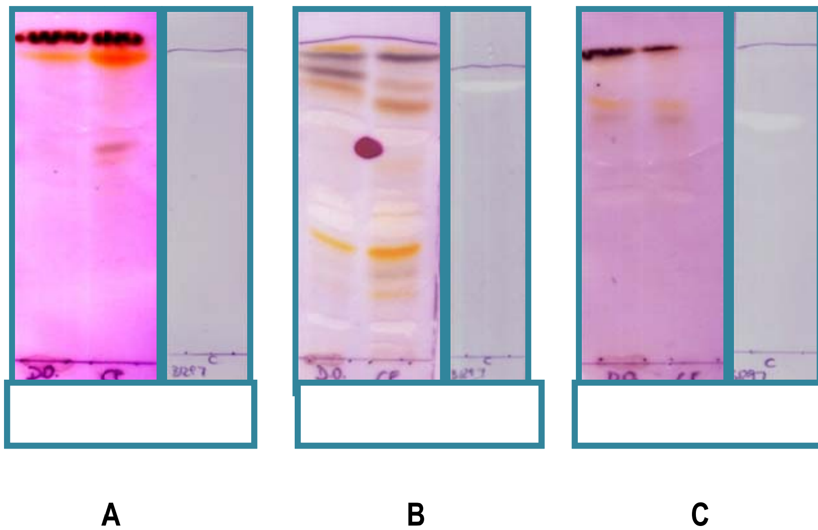
### 8.3.2 Bioautographic assays

The crude extract (C) was active in all the bioautographic plates sprayed with the four bacterial strains. However some of the assays resulted in more visible inhibition bands than others. Against the bacterial strain isolated from the poultry bursal swab, four different compounds in the chloroform fraction were active in the CEF bioautogram. Against the bacterial strain isolated from the poultry sinus, two compounds in the chloroform fraction (CF) were active when run with the CEF solvent system and 3 compounds of the chloroform fraction were active when run with the BEA solvent system. Against the bacteria isolated from the porcine large intestine and the one isolated from the bovine spleen and liver, two compounds of the fraction were active when run with the EMW solvent system.

The chloroform fraction of the extract was active in all the bioautographic plates sprayed with the four bacterial strains. However some of the assays had more visible inhibition bands than others. Against the bacterial strain isolated from the poultry bursal swab, four different compounds in the chloroform fraction were active when the plate was run with the CEF system. Against the bacterial strain isolated from the poultry sinus, two compounds of the chloroform fraction were active when run with the CEF solvent system and three compounds of the chloroform fraction were active when run with the BEA solvent system. Against the bacteria isolated from the porcine large intestine and the one isolated from the bovine spleen and liver, two compounds of the fraction were active when run with the EMW solvent system.

The pure compound (P) exhibited its activity in all the bioautographic assays for the bacterial strain isolated from the poultry bursal swab and against the strain isolated from the large porcine intestine. The activity was also visible in the plates sprayed with the bacterial strains isolated from the bovine liver and spleen and the poultry sinus that had been developed with CEF and EMW. However, the active band was not visible in the plates eluted with BEA and sprayed with these two bacterial strains.

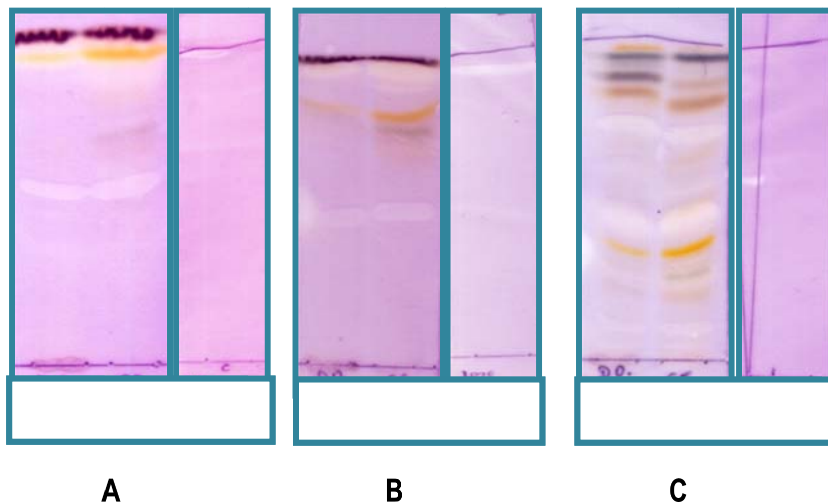
Allthe bioautographic assays are shown in Figures 8.1 to 8.4.



A B C

Fig. 8.1: Bioautography against B 1279

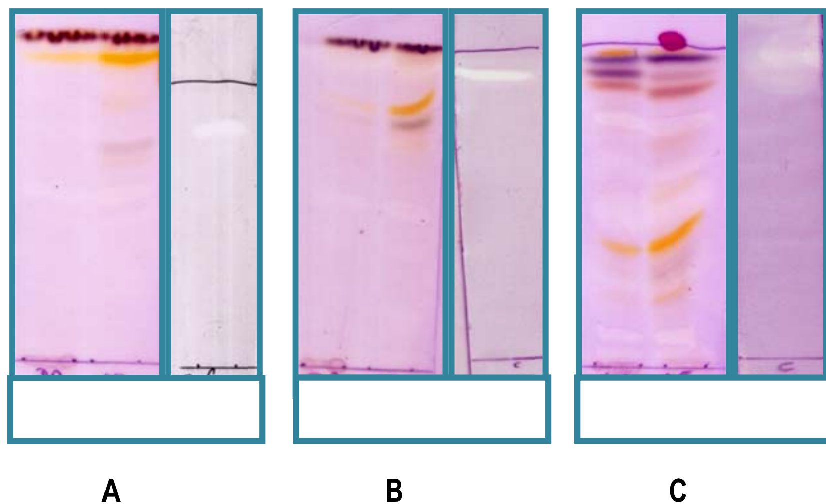
Fig. 8.1 A: Bioautography against B 1279 for C, CF and P EMW; Fig. 8.1 B: Bioautography against B 1279 for C, CF and P CEF; Fig. 8.1 C: Bioautography against B 1279 for C, CF and P BEA



A B C

Fig. 8.2 Bioautography against B 1295

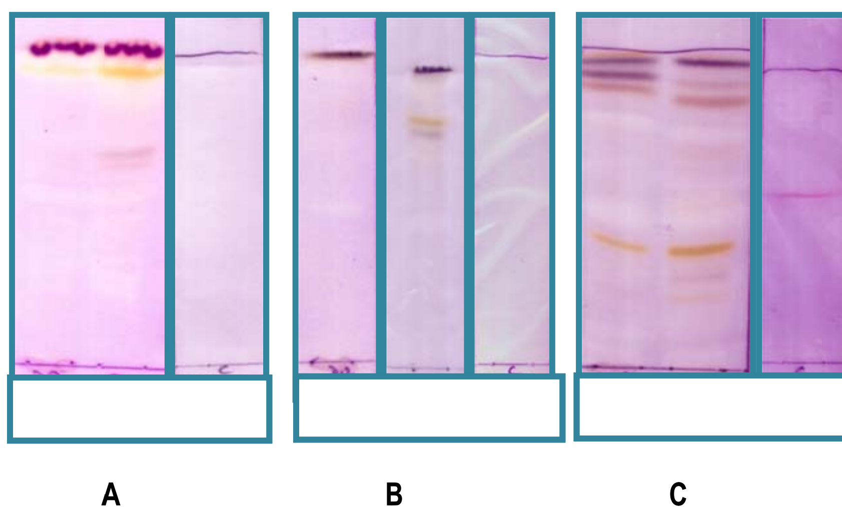
Fig. 8.2 A: Bioautography against B 1295 for C, CF and P EMW; Fig. 8.2 B: Bioautography against B 1295 for C, CF and P CEF; Fig. 8.2 C: Bioautography against B 1295 for C, CF and P BEA



A B C

**Fig. 8.3: Bioautography against B 1297**

**Fig. 8.3 A: Bioautography against B 1297 for C, CF and P EMW; Fig. 8.3 B: Bioautography against B 1297 for C, CF and P CEF; Fig. 8.3 C: Bioautography against B 1297 for C, CF and P BEA**



A B C

**Fig. 8.4 Bioautography against B 1314**

**Fig. 8.4 A: Bioautography against B 1314 for C, CF and P EMW; Fig. 8.4 B: Bioautography against B 1314 for C, CF and P CEF; Fig. 8.4 C: Bioautography against B 1314 for C, CF and P BEA**

## 8.4 Conclusion

The crude extract and the chloroform fraction were as active as in the preliminary screening results against at least one of the four pathological *E. coli* strains. The pure compound was even more active against all the four pathological strains than it was against the ATCC strain.

These results prove that the crude extract and the chloroform fraction could be used to combat at least some pathogenic bacteria *in vitro*, and possibly in stock animals. Now the next step was to evaluate the safety of this extract in a living organism.

# Chapter 9

# Acute, subacute and larval toxicity of the extract of *Deinbollia oblongifolia* and its chloroform fraction

## 9.1 Introduction

The next step in order to develop a safe product is to evaluate the safety of the extract and its chloroform fraction in a living organism. The OECD method for determining acute and subacute toxicity was used (OECD/OCDE, adopted 17th of December 2001) in trial rats.

It is the principle of the test that, based on a stepwise procedure with the use of a minimum number of animals per step, sufficient information is obtained on the acute and subacute toxicity of the test substance to enable its classification. In the acute and subacute toxicity tests, animals were randomly allocated to 2 groups, one group for the crude extract and one group for the chloroform fraction. For the acute toxicity test, animals were dosed in a step-wise fashion to determine the $LD_{50}$. For the subacute toxicity test, the two groups of rats were each divided into four groups, which received varying dosages of the crude plant extract or chloroform fraction before being evaluated.

## 9.2 Materials and Methods

### 9.2.1 General overview of the study design

#### 9.2.1.1 STUDY DESIGN

The experiment was conducted at the University of Pretoria Biomedical Research Centre (UPBRC), at the Faculty of Veterinary Science, University of Pretoria, in a conventional experimental animal room. Permission to perform the trial was obtained from the Animal Use and Care Committee (AUCC) of the University of Pretoria.

#### 9.2.1.2 TRIAL ANIMALS

Animals were housed in pairs and individually identified by ear notching. The animals were randomly selected and kept in their cages for at least 5 days prior to dosing to allow for acclimatisation to the laboratory conditions. A total of 6 animals per step were used.

**Housing and management**

Upon arrival at the research site, the rats were examined and only healthy rats were put into the prepared housing. Conventional rat cages (Tekniplast Type III) with 2 rats per cage were used. Enrichment for these cages was provided (tissue, toilet rolls, apples and carrots). The temperature in the experimental room was 22°C (± 3°C) and the relative humidity 50-60%. Artificial lightning was provided with a sequence of 12 hours light, 12 hours dark. A conventional laboratory diet was used for feeding (EPOL rat pellets) as well as an unlimited supply of potable water.

#### 9.2.1.3 STUDY TERMINATION

For the acute toxicity the rats were weighed and humanely sacrificed a minimum of 14 days after each dosage step.

Forthe subacute toxicity the rats were intended to be killed on day 14.

All test animals were subjected to gross necropsy. All gross pathological changes were recorded for each animal and a microscopic examination of the organs showing evidence of gross pathology in animals surviving 24 hours or more were deducted. Terminal cardiac exsanguinations were done for serum chemistry for the subacute test whilst the animals were under isoflurane anaesthesia.

#### 9.2.1.4 COLLECTION AND STORAGE OF SAMPLES

On all animals, gross pathological and histological changes were recorded. Pathology was done by Vetpath, a contracted private company located on the Onderstepoort Campus, that provides veterinary pathological services for, among others, projects done at the Faculty of Veterinary Science, University of Pretoria. Terminal cardiac exsanguinations were done for haematology and serum chemistry in the subacute study (performed by the Department of Companion Animals Clinical Studies, Section of Clinical Pathology), whilst the animals were under isoflurane anaesthesia.

#### 9.2.1.5 MEASUREMENTS AND OBSERVATIONS

**1.** ***Rats:*** Origin and disease status were obtained from the supplier. Rats were weighed daily on an individual basis. Mass was also determined after euthanasia.

**2.** ***Mortality:*** Cages were checked twice daily for mortalities. All dead rats were weighed and submitted for *post mortem* examination.

**3.** ***General observations:*** Records were kept in terms of daily procedures.

### 9.2.2 Acute toxicity

A total of 24 female rats were used. The animals were randomly allocated to 6 animals per group. Healthy young adult animals of commonly used laboratory strains were employed (nulliparous and non-pregnant). Each animal was approximately 8 weeks old and weighed about 200 g at the start of the study. The dose level used as the starting dose was 50 mg/kg body weight. Depending on the toxic effects on 6 rats, the dose was changed to either 300 mg/kg (for the chloroform fraction) or 5 (for the crude extract) mg/kg body weight. The treatment groups were as follows:

**Group 1:** The plant material was ground with the Macsalab mill to a fine powder and mixed with acetone in a ratio of 1:10. Then the mixture was shaken on a Labotec Model 20.2 for 3 hours and left to settle overnight. The extract was filtered into preweighed glass vials and dried under constant airflow. The dried extract was then dissolved in acetone to the final concentration that was needed for the particular step of the trial (e.g. 50 mg/ml).

Rats were treated with 50 mg/kg of the plant extract of *Deinbollia oblongifolia* and observed for 3 days. Depending on the toxic effects, the dose was changed to 5 mg/kg.

**Group 2:** The chloroform fraction was prepared by first making the plant extract of *Deinbollia oblongifolia* (as mentioned in the previous paragraph in **Group 1**). The dried extract was then potentized by solvent-solvent fractionation. Hereby the dried extract was mixed well with equal volumes of chloroform and water and separated in a separatory funnel. The chloroform fraction was then removed from the funnel and concentrated to dryness. Then hexane and 10% water in methanol were added in equal volumes to the dried chloroform component. The 10% water in methanol fraction was diluted to 35% water in methanol and the equal volume of chloroform was added. This resulting chloroform fraction was dried and dissolved in acetone to the final concentration that was needed for the trial (e.g. 50 mg/ml).

Rats were treated with 50 mg/kg of the chloroform fraction of *Deinbollia oblongifolia* and observed for 3 days. Depending on the toxic effects, the dose was changed to 300 mg/kg.

The animals were dosed by oral gavage with the extract and observed individually after dosing (at least once during the first 30 minutes, and periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter) for a total of 14 days, except when they needed to be removed from the study and humanely killed for animal welfare reasons or were found dead. If toxic signs were observed, the treatment was repeated with a lower dose under the same conditions.

Depending on the observation of toxic signs, the dosage was adjusted to either 5 mg/kg or 300 mg/kg (limit test). An approximate $LD_{50}$ was assigned according to the OECD method.

The test substances were administered in a constant volume of 1 ml over the range of doses to be tested by varying the concentration of the dosing preparation. The test substance was administered in a single dose by gavage using an intubation canula. The animals were fasted beforehand by withholding food, but not water, overnight for about 8 hours. After fasting, the animals were weighed and the test substance administered. After the administration, food was withheld for a further 3-4 hours. The time interval between treatment groups was determined by the onset, duration, and severity of the toxic signs. The treatment of the animals at the next dose was delayed until the survival of the previously dosed animals was ensured.

At the end of the study (after 14 days) or after the animal died, necropsies and micropathological examinations were performed on all the animals.

Since the acute toxicity trial is designed to assign a $LD_{50}$ to a previously untested chemical substance, no control group is included in this trial.

### 9.2.3 Subacute toxicity

A total of 21 female rats were used for each group. The animals were purchased following conclusion of the acute toxicity trial, and randomly allocated to 6 animals per dosage group in a total of 3 dosage groups for both the plant extract and the chloroform fraction. There was also one extra group with 6 animals that was treated with just the solvent to serve as a control group. Each animal was between 8 and 12 weeks old and weighed about 200 g. The dose levels used were determined by the acute toxicity study

For the subacute study, there were seven groups. Each group was treated daily with the same dose of the extracts for a time period of 2 days (the initially planned treatment for 2 weeks could not be carried out due to observed toxic signs in the animals).

**Group 1:** rats were treated with 5 mg/kg of the plant extract of *Deinbollia oblongifolia*

**Group 2:** rats were treated with 20 mg/kg of the plant extract of *Deinbollia oblongifolia*

**Group 3:** rats were treated with 50 mg/kg of the plant extract of *Deinbollia oblongifolia*

**Group 4:** rats were treated with 50 mg/kg of the chloroform fraction of *Deinbollia oblongifolia*

**Group 5:** rats were treated with 125 mg/kg of the chloroform fraction of *Deinbollia oblongifolia*

**Group 6:** rats were treated with 300 mg/kg of the chloroform fraction of *Deinbollia oblongifolia*

**Group 7:** rats were treated with 0 mg/kg of the plant extract of *Deinbollia oblongifolia*

The animals were dosed once daily by oral gavage with the extract (in different concentrations in the different groups) and observed individually after dosing (at least once during the first 30 minutes, and periodically thereafter, with special attention given during the first 4 hours) for a total of 3 days, except when they needed to be removed from the study and humanely killed for animal welfare reasons, or were found dead.

The test substances were administered in a constant volume of 1 ml over the range of doses to be tested by varying the concentration of the dosing preparation. The test substance were administered once daily in a single dose by gavage using an intubation canula. Before dosing, the animal was weighed and the test substance administered. After 14 days the experiment should have been terminated, but it had to be prematurely terminated 3 days into the study.

### 9.2.4 Larval assay

For the larval assay a modified version of the method developed by Khater and Khater in 2009 was used. The larvae were collected from different vulture restaurants that are run by the Vulture Programme, Rhino and Lion, a Non-profit Organization, South Africa. The emerging adult flies were identified as *Chrysomya marginalis* and *Lucilia cuprina*. Larvae were hatched by placing the flies in an enclosed space and supplying them with matured tenderised steak to lay their eggs in. The eggs were then incubated together with the meat and after hatching early stages of the larvae were harvested for conduction of the assay.

One millilitre of the diluted extracts (in acetone) at a concentration of 50 mg/ml was added to a piece of tenderised steak and left outside until the solvent had evaporated.

Then 15 to 20 of the obtained larvae were counted and transferred onto this piece of meat, which was then placed in a plastic container and covered with tidy roll (paper towel) secured with an elastic band. In the first eight hours checks were done every half an hour on the larvae. Another check was carried out after a total of 12 hours and after 24 hours a final check was performed and the larvae were placed in glass Petri dishes and filmed using a video camera to compare the movement of the larvae of the different dosage groups. A piece of steak where the pure solvent had been added served as negative control and a piece of steak where 1 ml of Virbamec (Virbac, 1% m/v ivermectin) had been added served as positive control. The assay was done in triplicate to increase reproducibility.

## 9.3 Results and Discussion

### 9.3.1 Acute toxicity study

#### Crude extract

The first treatment started with 50 mg/kg dissolved in 1 ml of acetone. Six rats were dosed with this treatment. The taste was apparently not very pleasant since all the rats fought against the gavaging. All the rats showed acute toxic signs immediately after dosing, including dizziness, ataxia and troubled breathing. All the rats seemed very subdued after the dosing. The first animal was euthanized after 30 minutes. After an hour a further toxic sign was observed. The animals started to scratch themselves excessively. After forty minutes the breathing of two of the rats became laboured and they made vocal sounds while trying to breath. One hour and a half after dosing, mucosal discharge out of the nose and eyes was observed as well as warfarin stains (reddish coloured stains on the fur resulting from stress). The second animal was put down two hours after dosing.

Four hours after dosing, food was supplied, but not one of the animals seemed to show any appetite. The animals started eating after a total of eight hours. Ten hours after dosing the fur of the animals was piloerect. Twelve hours after dosing the animals seemed to improve, but after leaving the animals to rest overnight, the symptoms became more severe and all the animals had to be euthanized. They had all lost weight during the trial period, ranging from 5.7%-18.1% reduction of the body mass they had at the starting dose.

In the pathological report all the rats showed congestion of the liver and gas accumulation in the stomach. Two of the rats had a splenomegaly and four had renal congestion. Three rats showed signs of congested ovaries and one had pathological lung symptoms (atelectasis and emphysema). The pathological report stated that these six rats revealed signs of acute shock without aspirated material present in the trachea or aspiration pneumonia. These pathological findings and the clinical signs of disorientation, laboured breathing and a mild degree of nervous signs suggested a possible toxic effect of the dosed extract. A summary of the clinical signs, pathological findings and the percentage of body weight gained or lost is shown in Table 9.1.

After 50 mg/ml showed severe toxic effects, the next group of three animals was treated with a dosage of 5 mg/kg. Since the carrier solvent seemed to not settle well with the animals (they were fighting the gavaging and seemed to aspirate rather than swallow the administered extracts) the carrier solvent was changed to a 5% acetone in water mixture. This was the lowest concentration of acetone the extract was soluble in. Again there were difficulties with getting the animal to swallow the substance and it was

assumed that the extract might cause pharyngeal spasms. Therefore the application needle was changed to a longer needle to try and place the solvent a bit further down into the oesophagus of the animal, so that the pharynx did not get into contact with the extract. The animals were again scratching after dosing, but no other signs were observed and the animals appeared alert and curious and the scratching had subsided by the next morning. The animals were further observed for 14 days and then euthanized and sent for pathological examination.

A second group of three animals was treated with the same dosage (5 mg/kg). Again, scratching during the first day was observed, but otherwise the animals were alert and active and eventually euthanized after 14 days and sent for pathological examination. Upon weighing on the day of euthanasia it was seen that none of the rats had experienced a weight loss, indicating that this dosage had at least a less toxic effect than the previously used dose.

The pathological report of the six animals dosed with 5 mg/kg crude extract stated that all the animals were in a good body condition and that the only consistent morphological finding was the liver congestion and the accentuated lobulation of the liver. This was stated to not be a specific finding and that the correlation to administration of the crude extract was uncertain.

A summary of the clinical signs, pathological findings and the percentage of body weight gained or lost is shown in Table 9.1.

**Table 9.1: Dosing regimen, clinical signs and macroscopic lesions and percentage of body weight gained or lost during the trial in animals dosed with *Deinbollia oblongifolia***

| Rat | Dosing quantity | Clinical signs | Macroscopic lesions | % (+, -) |
|---|---|---|---|---|
| 1 | 50 mg/kg | Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 28 hours | Moderate gas accumulation in stomach; moderate splenomegaly, moderate liver and renal congestion; mild ovarian congestion; blood around mouth nose and on front feet; dry content in large intestine | -13.0% |
| 2 | 50 mg/kg | Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 2 hours | Mild bilateral emphysema; moderate gas accumulation in stomach; mild splenomegaly; severe liver congestion; bilateral renal congestion | -5.7% |
| 3 | 50 mg/kg | Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 28 hours | Prominent gas accumulation in stomach; moderate ovarian congestion; mild hepatomegaly and liver congestion; bilateral renal congestion; blood on skin and around nose, mouth and one front foot; | -14.2% |
| 4 | 50 mg/kg | Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 28 hours | Gas accumulation in stomach with petechial haemorrhages in mucosa; mild gas accumulation in the anterior intestine; mild swollen and congested liver; congestion of ovaries; blood around mouth and nose | -18.1% |
| 5 | 50 mg/kg | Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 1 hour | Moderate gas accumulation in stomach; Severe liver congestion and mild hepatomegaly; bilateral renal congestion; moderate bilateral congestion of ovaries; focal atelectasis and multifocal areas of emphysema in both lungs | -10.2% |
| 6 | 50 mg/kg | Depression, piloerection, unsteady gait, pulmonary distress; euthanized after 27 hours | Gas accumulation in stomach; moderate liver congestion; small amount of gas in anterior intestine; congested ovaries bilateral; blood on skin, around mouth, nose and front feet; dry contents in the large intestine | -9.8% |
| 8 | 5 mg/kg | None except for warfarin spot on the day of euthanasing; searched for food after dosing; euthanized on day 14 | Liver congestion with paler peripheral lobulation and congested centrilobular areas; | +13.7% |
| 10 | 5 mg/kg | While dosing spasms; else no clinical signs; euthanized on day 14 | Moderate liver congestion with accentuated lobulation, which appears as a pale reticular pattern surrounding sunken dark red central areas of less than1 mm disseminating throughout the parenchyma; bilateral moderate renal congestion; mild splenic white pulp prominence | +15.6% |

| Rat | Dosing quantity | Clinical signs | Macroscopic lesions | % (+, -) |
|---|---|---|---|---|
| 11 | 5 mg/kg | None except for some scratching and a light 1 hour head tilt 2 hours after dosing; euthanized day 14 | Moderate liver congestion with accentuated lobulation, which appears as a pale reticular pattern surrounding sunken dark red central areas of less than 1 mm disseminated throughout the parenchyma; bilateral moderate renal congestion; mild splenic white pulp prominence | +13.6% |
| 12 | 5 mg/kg | None; euthanized day 14 | Mild liver congestion and mild accentuated lobulation in liver | +0.6% |
| 13 | 5 mg/kg | Scratching; euthanized day 14 | Moderate liver congestion and accentuated lobulation, which appears as a pale reticular pattern surrounding sunken dark red central areas of less than 1 mm disseminate throughout the parenchyma; mild bilateral kidney congestion; spleen slightly enlarged with white pulp prominence; mild bilateral lung congestion; mild extensive haemoragae subcutaneous in the neck area (from handling?) | +15% |
| 14 | 5 mg/kg | None; euthanized day 14 | Moderate liver congestion with severe accentuated lobulation in the liver parenchyma | +11.2% |

### Chloroform fraction of *Deinbollia oblongifolia*

The first treatment dose was 50 mg/kg in 10% acetone in water (the amount of acetone was determined by the solubility of the extracts). The first group of three rats was dosed. The only obvious sign of possible toxicity was again the occurrence of scratching on the first day in all the dosed animals. The animals were observed for 14 days and then sacrificed and sent for pathological evaluation. According to OECD protocol a second group of 3 animals was dosed with the same treatment and observed for 14 days. They again scratched more than the undosed animals during the first 24 hours. After 14 days the animals were sacrificed and sent for pathological evaluation. Both groups of rats seemed more subdued during the first day after dosing compared to the untreated animals.

The next step was to dose the animals with 300 mg/kg of the chloroform fraction. The extract was dissolved in a mixture of 50% acetone and 50% water (the lowest concentration of acetone that the extracts were soluble in). Right after dosing the animals started to excessively shred the paper in the cage and scratch. The animals also showed intermittently symptoms of laboured breathing, piloerect hair, warfarin stains and a lack of coordination during the first 24 hours. The animals returned back to their normal behaviour after 24 hours and were further observed for 14 days and then sacrificed and sent for pathological evaluation. Upon euthanasia the animals were weighed and it was discovered that there was no weight loss in any of the trial animals. The pathological results showed that all the animals

were in a good body condition and that the only consistent macroscopical finding was the hepatic congestion and the accentuated lobulation of the liver, which was observed in many of the rats. The pathological report stated that this was not a specific finding and that the correlation to the dosed extract was uncertain. Table 9.2 shows a summary of all the clinical signs, pathological findings and the percentage of weight gained or lost during the trial.

A second group of three rats was dosed with 300 mg/kg in a 50% acetone and 50% water mixture. The rats showed again toxic signs during the first 24 hours (shredding of paper, piloerect hair, warfarin stains, laboured breathing and lack of coordination) which again subsided after the first day. The rats were sacrificed after 14 days and sent for pathological evaluation. Upon euthanasia the animals were weighed and it was discovered that there was no weight loss in any of the trial animals. The pathological results showed that all the animals were in a good body condition and that the only consistent macroscopical finding was the hepatic congestion and the accentuated lobulation of the liver, which was observed in many of the rats. The pathological report stated that this was not a specific finding and that the correlation to the dosed extract was uncertain. Table 9.2 records a summary of all the clinical signs, pathological findings and the percentage of weight gained or lost during the trial.

**Table 9.2: Dosing regimen, clinical signs and macroscopic lesions and percentage weight gain or loss in animals dosed with the chloroform fraction of *Deinbollia oblongifolia***

| Rat | Dosing quantity | Clinical signs | Macroscopic lesions | % (+,-) |
|---|---|---|---|---|
| 7 | 50 mg/kg | Scratching; warfarin spot on day 14; euthanized day 14 | Hepatic liver congestion with prominent accentuated lobulation and congestion | +14.3% |
| 9 | 50 mg/kg | None; euthanized day 14 | Moderate liver congestion and moderate accentuated lobulation in the liver; moderate oedema of the uterus; mild cranial ventral bilateral pulmonary atelectasis | +8.4% |
| 16 | 50 mg/kg | Scratching; euthanized day 14 | Mild accentuated lobulation and moderate congestion of the liver; mild oedema of the uterus and congestion of the ovaries; moderate lung congestion | +13.9% |
| 17 | 50 mg/kg | Scratches; euthanized day 14 | Mild liver congestion with accentuated lobulation of moderate degree | +16.1% |
| 18 | 50 mg/kg | Scratches; euthanized day 14 | Mild hepatocellular congestion with accentuated lobulation on the periphery of the lobules; mild uterine oedema and congestion of the ovaries | +13.4% |

| Rat | Dosing quantity | Clinical signs | Macroscopic lesions | % (+,-) |
|---|---|---|---|---|
| 19 | 300 mg/kg | Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for three hours; piloerection; euthanized day 14 | Mild liver congestion with moderate accentuated lobulation of the liver parenchyma | +15.0% |
| 21 | 300 mg/kg | Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for four hours; piloerection; euthanized day 14 | Hepatic congestion with moderate accentuated lobular pattern; mild cranial ventral congestion of the lung | +12.1% |
| 25 | 300 mg/kg | Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for four hours; piloerection; euthanized day 14 | Liver congestion with mild accentuated lobulation; mild oedema of the uterus; mild pulmonary congestion | +14.3% |
| 26 | 50 mg/kg | Scratching; euthanized day 14 | Hepatic congestion centrilobular with accentuated pale lobulation of the liver parenchyma; mild bilateral renal congestion | +13.2% |
| 31 | 300 mg/kg | Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for three hours; piloerection; euthanized day 14 | Mild liver congestion with moderate accentuated lobulation of the liver parenchyma | +14.0% |
| 33 | 300 mg/kg | Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for four hours; piloerection; euthanized day 14 | Moderate liver congestion with a pale accentuated lobular pattern of the liver parenchyma; mild lung congestion | 15.0% |
| 34 | 300 mg/kg | Extensive scratching; frantically trying to eat paper; slight pulmonary distress and disorientation right after dosing for four hours; piloerection; euthanized day 14 | Moderate liver congestion with moderate accentuated lobulation | 15.1% |

From these results the dosage groups for the subacute toxicity study were calculated to be the following for the crude extract: 5 mg/kg; 20 mg/kg and 50 mg/kg. The chloroform fraction was less toxic with

dosages for the chloroform fraction calculated as 50 mg/kg, 125 mg/kg and 300 mg/kg for the subacute toxicity study.

### 9.3.2 Subacute toxicity study

From the results of the acute toxicity study, the dosage groups for the subacute toxicity study were determined to be 5 mg/kg, 20 mg/kg and 50 mg/kg for the crude extract and 0, 50 mg/kg, 125 mg/kg and 300 mg/kg for the chloroform fraction. One group was just dosed with the solvent (50% acetone in water) to serve as negative control. Six rats were allocated to each group. All the animals were dosed once in the mornings of every day.

*Group 1 (5 mg/kg crude extract):*The rats showed clinical signs of pulmonary distress (except for one of them). Some of them also showed signs of neurological irritation (scratching, piloerection, pica), which can be associated with a toxic effect. The pica in rats has been shown to indicate a toxic effect (Mitchell et al, 1976). All the rats lost weight during the trial period (only rats terminated on day 3 were weighed).The pathological findings ranged from gas accumulation in the stomach, adrenal congestion, liver congestion, lung congestion and atelectasis and splenomegaly, but none of these findings was found in all of the rats of the group and so these findings could not be directly associated with a toxic shock. However the animal that was euthanized the day following the first dosing did show consistent signs of toxic shock (gas accumulation in stomach, signs of pulmonary oedema and hepatic blood pooling due to heart failure) that can be associated either to the plant material or the dosing solvent. See Appendix Table B 1 for a summary of all the clinical signs, pathological findings and the percentage of weight gain/loss the animals experienced during the trial. The blood chemistry results indicated that the liver in the rats was affected (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect (see Appendix Table B 2 for blood chemistry results).

*Group 2 (20 mg/kg crude extract):* The rats showed clinical signs of pulmonary distress. Some of them also showed signs of neurological impairment (unsteady gait, depression, scratching, piloerection, and pica). All these symptoms can again be linked to a toxic effect. In two of the rats it was noted that the abdomen was swollen, which was most likely due to the splenomegaly found in the pathological examination. The rats did not lose weight during the trial period (only rats that were terminated on day 3 were weighed). All of the rats showed upon pathological examination a spleen with extramedullary haemopoiesis in the red pulp. Other pathological findings ranged from gas accumulation in the stomach, adrenal congestion, liver congestion, lung congestion and atelectasis and splenomegaly, but none of these findings was found in all of the rats of the group and so these findings could not be directly associated with a toxic shock that was directly linked to the dosing of the animals with the plant extract.

However the animals that were euthanized the day following the first dosing did show consistent signs of toxic shock (gas accumulation in stomach, signs of pulmonary oedema and hepatic blood pooling due to heart failure) that can be associated either to the plant material or the dosing solvent. See Appendix Table B 1 for a summary of all the clinical signs, pathological findings and the percentage of weight gain/loss the animals experienced during the trial. The blood chemistry results indicated that the liver in the rats was affected again (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect. The urea and creatinine values were also elevated suggesting an involvement of the kidneys into the picture of a general toxic shock (see Appendix Table B 3 for blood chemistry).

*Group 3 (50 mg/kg crude extract):* The rats showed clinical signs of pulmonary distress. Some of them also were neurologically impaired judging from their unsteady gait, their obvious depression, the pathological scratching and the piloerection of the hair as well as the exhibition of pica. This suggests again a toxic effect. Two of the rats again exhibited a swollen abdomen. The rats did not lose weight during the trial period. Five of the rats showed upon pathological examination a spleen with extramedullary haemopoiesis in the red pulp. Other pathological findings ranged from gas accumulation in the stomach, adrenal congestion, liver congestion, lung congestion and atelectasis and splenomegaly, but none of these findings was found in all of the rats of the group and so these findings could not be directly associated with a general toxic shock due to the dosing of the rats with the crude extract. See Appendix Table B 1 for a summary of all the clinical signs, pathological findings and the percentage of weight gain/loss the animals experienced during the trial.
The blood chemistry results again indicated that the liver in the rats was affected (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect (see Appendix Table B4 for blood chemistry).

*Group 4 (50 mg/kg chloroform fraction):* The rats showed signs of pulmonary distress and neurological impairment (scratching, depression, unsteady gait, piloerection, warfarin spots and pica). One of the rats had an apparent swollen abdomen. Two of the rats were euthanized the same day after they were first dosed due to the signs of acute shock they showed. Upon euthanasia three of the animals had gained weight during the trial whereas one animal lost weight (only animals terminated on day 3 were weighed). Upon pathological examination it was concluded that the animals that were euthanized the day following the first dosing showed prominent gas accumulation in the stomach as well as signs of a pulmonary oedema and hepatic blood pooling due to heart failure which suggests a shock and is most likely associated with the dosing of the plant material or the dosing solvent. Pathological findings in the other rats included adrenal congestion, kidney congestion, liver congestion, lung congestion and atelectasis as well as extramedullary haemopoiesis in the spleen. See Appendix Table B 5 for a full summary of clinical signs, pathological findings and the percentage of the weight gain or loss the animals

experienced during the trial. The blood chemistry results indicated that the liver in the rats was affected (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect (see Appendix Table B 6 for blood chemistry).

*Group 5 (125 mg/kg chloroform fraction):* All the rats showed signs of pulmonary distress and neurological impairment (scratching, depression, unsteady gait, piloerection, warfarin spots and pica). Upon euthanasia it was discovered that 4 of the animals had gained weight during the trial whereas two animals lost weight. Pathological findings in the rats included adrenal congestion, kidney congestion, lung congestion and atelectasis as well as extramedullary haemopoiesis in the spleen, but none of the findings was seen consistently in all the rats and could therefore not be directly associated with the dosing See Appendix Table B 5 for a full summary of clinical signs, pathological findings and the percentage of the weight gain or loss the animals experienced during the trial. The blood chemistry results indicated that the liver in the rats was affected (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect (see Appendix Table B 7 for blood chemistry).

*Group 6 (300 mg/kg chloroform fraction):* The clinical signs included pulmonary distress and neurological impairment (scratching, depression, unsteady gait, piloerection, warfarin spots and pica). One of the rats had an apparent swollen abdomen. Upon euthanasia it was discovered that all of the animals except for one had lost weight during the trial. Pathological findings in the rats included mild gas accumulation in the stomach, adrenal congestion, kidney congestion, lung congestion and atelectasis as well as extramedullary haemopoiesis in the spleen, but none of the findings was seen consistently in all the rats and could therefore not be directly associated with the dosing. See Appendix Table B 5 for a full summary of clinical signs, pathological findings and the percentage of the weight gain or loss the animals experienced during the trial. The blood chemistry results indicated that the liver in the rats was affected (especially the ALT, AST, GGT, ALP and ALB values) suggesting a toxic effect (see Appendix Table B 8 for blood chemistry).

*Group 7 (Solvent acetone):* Three of the animals of the control group showed severe clinical signs of toxic shock after the first day of dosing (pulmonary distress, piloerection, depression, one even died during the night) and had to be euthanized the same or the next day. It was concluded that aspiration of the solvent could be responsible for these severe effects and therefore the rats were dosed intraperitoneally on the second day. All of them showed severe depression after dosing, but got gradually better during the day, however they still showed signs of toxic shock (piloerection, depression, scratching, pica, unsteady gait) and so it was decided to euthanize the rest of the control group along with the other groups and terminate the trial. Two of the animals that were terminated on the third day showed weight loss whereas one gained weight slightly. Upon pathological examination it was

discovered that the animals that were euthanized on the second day showed signs of shock (gas accumulation in stomach, signs of pulmonary oedema and hepatic blood pooling due to heart failure). The other animals showed several symptoms (pulmonary congestion, inflammation in the peritoneum, extramedullary haemopoiesis, and adrenal congestion). See Table B 9 for a full summary of the clinical signs, pathological findings and the percentage of weight gain or loss the rats experienced during the trial. The blood serum chemistry indicated that the extract had had a toxic effect on the liver (especially the ALT, AST, GGT, ALB and ALP values). See Appendix Table B 10 for the full serum chemistry.

It was also discovered that most of the animals had a haemothorax, but this was interpreted as being directly associated with the blood collection upon euthanasia by cardiac puncture.

The original outline of the study also required haematology to be done (full blood count, haematocrit, etc.), but since the rats were euthanized on the weekend and the laboratory was not available, the blood had to be frozen and the haematology work was therefore not possible.

These results showed that an unambiguous conclusion about the toxicity of the extracts cannot be drawn since the solvent also seemed to have had an effect on the rats. Acetone was chosen as solvent based on the solubility of the extract, the $LD_{50}$ value of acetone in rats based on literature reports and because acetone is generally considered relatively safe in rats due to its low level of toxicity. This has been established in a 13-week drinking water study (NTP, 1991; Dietz *et al.*, 1991) where rats were exposed to different levels of acetone in the drinking water where no mortalities or overt clinical signs were observed within the experimental period. It could however be possible that acetone could cause toxic effects since experimental animal data characterizing the effect of long-term oral exposure to acetone are not available, due probably to its low toxicity (EHC 207, 1998). In a study on the mammalian acetone metabolism it was found that acetone is toxic at high doses and that its primary target sites are liver, kidney and bone marrow (Kalapos, 2003).

One of the PhD students Ms Lilian Mukandiwa in the Phytomedicine Programme had developed an assay to test the efficacy of different plant extracts on larval motility and development. Therefore it was decided to perform a larval toxicity assay to investigate the toxic effect of the extracts and acetone on another life form.

### 9.3.3 Larval assay

The crude extract of *Deinbollia oblongifolia* and the potentized extract of this plant were tested for their larvicidal activity in this assay. The extracts were added in a concentration of 50 mg/ml to pieces of tenderised steaks. Acetone served as a negative control and ivermectin as positive control. Checks

were done every half an hour during the first 8 hours and then once after a total of 12 hours and after 24 hours.

The larvae that were dosed with the crude extract showed the first visible toxic signs (paralysis, partial paralysis) after six hours. These effects lasted until the last check after 24 hours. It was also discovered that the process of the further development was noticeably compromised (slower development, some did not develop further at all and just died).

The larvae that were dosed with the potentized extract showed toxic signs even earlier. The first paralytic effects in the larvae were observed 1.5 hours after dosing. The effects lasted until the final observation 24 hours after dosing. The further development was also seriously impaired. Mortalities and slowed developmental processes occurred in the larvae.

The positive control group that was treated with ivermectin showed toxic effects straight away and all the larvae died.

The negative control group that was treated with acetone did not show any toxic effects, neither right after dosing nor in the further development.

## 9.4 Conclusion

The results of the acute toxicity study and the larval assay proved that the extracts were of a toxic nature. The results of the subacute toxicity trial did indicate that the solvent control also seemed to have a toxic effect on the rats. In older literature it has been postulated that acetone enhances the hepatotoxic effects of a number of compounds (Plaa and Traiger, 1972; Moldeus and Gergely, 1980; Liu *et al.*, 1991; EHC 207, 1998). It could therefore be a possibility that toxic nature of the extracts was further intensified by the use of acetone as a solvent.

The results of this experiment unfortunately showed that the extract cannot be used safely in a living organism in this form as an antidiarrhoeal agent. The idea of the further study of the efficacy of the extracts in a living organism challenged with *E. coli* therefore had to be abandoned.

# Chapter 10

# General Conclusions

## 10.1 Introduction

Since prehistoric times, people have used natural resources for medicinal purposes (Anesini and Perez, 1993) including combating infections. Therefore, it is only logical that in times of growing resistance of pathogenic microorganisms against presently used drugs, research should again turn to these resources.

The existence of diarrhoea in humans and livestock has always been a source of tremendous problems. In livestock, diarrhoea causes huge economic losses for farmers due to the higher mortality rate especially during the weaning of the young animals. Weaning in the young is related to a higher susceptibility to diarrhoea due to their age-related increased susceptibility to bacterial infections. In many cases, this diarrhoea is caused by bacterial agents such as *E. coli*. To deal with this problem, farmers have been using antibiotic feed additives for years. But, as recent research and medical cases show, this usage has led to the creation of drug-resistant bacterial strains, e.g. multiresistant *Enterococcus faecalis*. Because of this, the European Union banned antibiotic feed additives and now the farming community, as well as the veterinary community, are forced to find alternatives to prevent economic losses.

Since plants have always been used, especially in rural areas, for medical purposes and many plant species have proven antibacterial activity – plant extracts provide a potential solution to the problem especially since natural products are the source of many useful pharmaceuticals. It is important to develop a useful system to recommend which plant species would be the most likely to yield a commercial product. To verify the efficacy of selected plant extracts used traditionally different aspects were investigated: good activity against *E. coli* was seen as a positive parameter since this pathogen plays an important role in the occurrence of diarrhoea, while high tannin content was viewed negatively. Species with low tannin content are recommended because even though tannins may have antibacterial activity and have been used traditionally for the treatment of diarrhoea, they do have reported unwanted interactions for prophylactic use. For example, the presence of tannins in a herbal drug may hinder the absorption of proteins and lead to lower productivity. Tannins may affect the induction of enzymes such as cytochrome p450, which may accelerate drug metabolism to result in blood levels of actives too low for a therapeutic effect (Williamson, 2001). Furthermore, tannins produce a bitter taste in the feed. The presence of tannins in a prophylactic drug would therefore be impractical.

Therefore the aim of this project was to develop a formula based on *in vitro* assays to predict the potential value of traditionally used plants. In the introduction a number of objectives were identified to attain this aim.

## 10.2 Evaluate the magnitude of seasonal variation in the antibacterial activity

Since some scientists are sceptical about the use of plant extracts due to the possible variation of activity during the season. The magnitude of seasonal variation in the antibacterial activity of 5 selected plant species (collected monthly) against *E. coli* and *S. aureus* and the tannin content was determined. A monthly screening of acetone leaf extracts of five chosen plant species (*Acacia karroo*, *Acacia sieberiana* var. *woodii*, *Peltophorum africanum*, *Trichilia emetica*, and *Ziziphus mucronata*) was done over a period of one year. All the samples for the monthly evaluation were collected in the Gauteng area due to the easier accessibility and the quicker transport to the drying room to ensure optimum quality of the dried product. Leaves had the highest activity between the months of January to April (summer to autumn) after they were fully developed and before senescence started. There was little to no correlation between the activity against the bacteria and the tannin content of the plant even though tannins are commonly associated antibacterial activity. Therefore it can be assumed that tannin did not influence the antibacterial activity in the plant, but that other compounds were responsible.

## 10.3 Evaluate the genetic and geographic variation in antibacterial activity

The next objective was to determine the magnitude of interspecies variations. This is an important step in the rational use of plant material and predicting activity for a species. Leaves from 42 different trees of the same species (*Combretum molle*) were collected in a range of areas (Waterberg, Onderstepoort, SANBI National Botanical Garden Pretoria, Magaliesberg and Lowveld Botanical Garden Nelspruit) and screened for their antibacterial properties and tannin content. There was limited variation in antibacterial activity between different plants of the same species tested in this study. The average MIC against *E. coli* was 0.227 mg/ml. The low standard deviation of 0.07 indicated that there was very little variation in activity. The average value against *S. aureus* was 0.399 mg/ml with a slightly higher standard deviation of 0.16. Although, only plants from Gauteng and Mpumalanga provinces were investigated the climatic and geological conditions were sufficiently different to conclude that at least for *Combretum molle* the possible variation in activity between different plants of the same species is not a major factor to take into consideration. Differences in the tannin content were however detected. The number of antibacterial compounds based on bioautography varied without influencing the overall antibacterial activity. Further investigation in this matter by isolating the different active compounds and comparing their structures might be warranted. Again there was no correlation between the activity of the plant and its tannin

content. Some authors have decided that because many members of the Combretaceae contain large quantities of tannins in roots and bark, it is not a viable option to investigate members of the family for antimicrobial compounds. These results show that at least *Combretum molle* leaves have a low tannin content and that tannin content is not associated with antibacterial activity.

## 10.4 Verify the efficacy of selected plant extracts used traditionally to treat diarrhoea and develop a ranking system

After it was determined that the season had an effect on antibacterial activity of the plant extracts and different plants of the same species growing in different areas have a similar antibacterial activity, the next objective was to evaluate the efficacy and to develop a ranking system for plants that have been used to treat diarrhoea in ethnomedicine, in order to recommend the most promising species for further investigation. To do that, 53 tree species with reported medicinal use in Zulu medicine as antidiarrhoeal agents, or to treat dysentery that were available were chosen. The antibacterial activity of acetone leaf extracts of these species against the nosocomial agents *S. aureus* and *E. coli* was evaluated. *E. coli* was chosen due to its role in the occurrence of diarrhoea and *S. aureus* was included in the assays to detect possible selective antibacterial activity of the extracts. The tannin content was again also determined. All the investigated species were active to differing degrees against the bacterial pathogens. A formula was designed that would incorporate high activity against *E.coli,* low activity against *S. aureus*, low tannin content and high extractability with acetone to determine the species with the highest potential value: (0.60/MIC *E. coli*) + (0.25/gallic acid equivalent) + (0.10*MIC *S. aureus*) + (0.05*extract yield) = Rank.

## 10.5 Select species to be used for further analysis

Five plants were chosen for further in depth studies based on the ranking system that was obtained from application of the formula. The most important aspect for choosing these species for further research was that they had a high activity against *E. coli*, but other aspects were taken into account as well, i.e. low tannin content, a lower activity against *S. aureus* and a high extract yield. The five selected species based on these criteria were *Acacia sieberiana* var. *woodii*, *Albizia adianthifolia, Deinbollia oblongifolia*, *Spirostachys africana* and *Tetradenia riparia*.

## 10.6 Determine the *in vitro* cytotoxicity

Even though it is a very attractive idea to use plant medicines to treat diseases in animals, it is dangerous to assume that because they are natural they should be safe. Many natural products are extremely toxic. Also, because nearly all herbal remedies contain multiple biologically active constituents, interaction with conventional drugs is a matter of concern (Wynn et al, 2007). Therefore it is important to investigate the toxicity of herbal drugs and of their constituent compounds. The determination of the $LC_{50}$ value was also necessary in order to determine the selectivity index (SI) for the plants (SI = $LC_{50}$ / MIC): *Acacia sieberiana* var. *woodii* SI = 0.20; *Albizia adianthifolia* SI = 0.49; *Deinbollia oblongifolia* SI = 0.46; *Spirostachys africana* SI = 0.19; *Tetradenia riparia* SI = 0.31.

## 10.7 Potentize (enhance the activity of) two extracts

The next objective was to potentize two extracts and to determine their antibacterial activity against *E. coli* as well as their cytotoxicity to recommend which one should be investigated in further detail. The crude extract of *Deinbollia oblongifolia* had the lowest toxicity on the cells and was therefore selected for further study. *Spirostachys africana* was also chosen for further study due to its good activity and total activity against *E. coli*. It may be possible to increase the activity and/or decrease the toxicity of an extract by simple techniques. The two extracts were subjected to solvent-solvent fractionation and in both cases, the chloroform fraction was the most active against *E. coli*. Cytotoxicity of the two fractions was determined and the chloroform fraction of *Deinbollia oblongifolia* was the least toxic. The $LC_{50}$ of 0.188 mg/ml may appear to be quite toxic, but it has to be kept in mind that the kidney cells were directly exposed to the extract in the cytotoxicity test, whereas in a biological system the extract may undergo metabolic changes in the gut or while travelling through the body. Also, relative to other plant extracts tested in the same assay system, this $LC_{50}$ value is among the highest of a large number of extracts tested. The Selectivity Index (2.35 for the potentized extract of *Deinbollia oblongifolia* compared to a value of 0.45 for the crude extract and 0.78 for the potentized extract of *Spirostachys africana* compared to the lower value of 0.19 for the crude extract) showed that a potentization of both extracts was indeed achieved.

## 10.8 Isolate and characterise the active compound

Another objective of the current research was to isolate and characterise the active compound(s) of the chosen potentized plant extract, so the chloroform fraction of *Deinbollia oblongifolia* was fractionated by chromatographic isolation procedures and an antibacterial compound was successfully isolated. It was determined to be a mixture of the two compounds, 3β-OH-α-amyrin and 3β-OH-β-amyrin, two

triterpenoids. The efficacy of the crude extract, the chloroform fraction and the pure compound was tested against four pathological strains of *E. coli* isolated from different organs of different animals. The activity varied compared to the original activity that was determined against the ATCC 25922 strain. The extracts proved to be less effective against three of these bacterial strains, but showed the same efficacy against one of the strains. The isolated compound was even more active against all the pathological strains than it was against the ATCC strain.

## 10.9 Determine the acute and subacute toxicity in *in vivo* tests in rats

To determine whether either the crude or the potentized extract could be used safely in a biological system, acute and subacute toxicity studies in rats were done using the protocol recommended by the OECD. Unfortunately it became clear that the crude extract and the chloroform fraction were toxic to the rats. There might also have been an interaction between the carrier solvent and the drug that caused the toxicity, even though the acetone-water carrier mixture has been reported to be relatively safe in rats. Even though the extracts were toxic, they could still be used for medicinal purposes. For example some chemotherapeutic drugs were developed from toxic plant products (e.g. taxol), since many oncology drugs induce cellular toxicity and death through free radical generation (Wynn et al, 2007). In terms of antidiarrhoeal drugs, as with others such as anthelmintics, the balance needs to be achieved between toxicity to the pathogen and toxicity to the host.

It would be very interesting to determine genotoxicity and mutagenicity in these extracts to determine whether they could be used for developing such a chemotherapeutic agent. This however will have to be done in the scope of a different project since it surpasses the scope for this dissertation.

## 10.10 Test the efficacy of the potentized extract in *in vivo* feeding experiments

Due to the toxicity of the extracts in rats this aspect could not be investigated any further.

## 10.11 Recommend further steps in the development of a model

The results obtained in this project highlight the general difficulties in the development of a new drug or preparation against diarrhoea and other bacterial-related diseases because of toxicity concerns. Many promising plant species with excellent antibacterial activity have to be rejected owing to unacceptable toxicity, so toxicity is an aspect that needs to be tested early. Another point is that it is absolutely imperative to test the toxicity of plant extracts *in vivo* as well as *in vitro* since the effects can vary following metabolic activity, and an extract that was deemed relatively safe in an *in vitro* cytotoxicity screening can prove to be highly toxic in an animal. Many traditionally used medicines have never been

evaluated for their toxic potential and are being used based on their history of ethnomedicinal use, and are generally regarded as safe. Toxicity issues are probably the major stumbling block to the development of new therapeutic preparations. One may ask why if *Deinbollia* has been traditionally used apparently without ill effects, such toxicity is now found. The answer is probably in the extractant used to extract compounds from the plant material. Rural people would have mainly water as an extractant and water extracts a totally different group of compounds from plants (Kotze and Eloff, 2002).

This work was the first step in the development of a ranking system for the selection of medicinal plants to be used to treat diarrhoea on a rational basis. It resulted in a large quantity of new data that is now available for other scientists to build upon. To improve the ranking system, more weight should possibly be placed on the low activity against *S. aureus* to prevent the selection of general metabolic toxins. Determining the activity of the extracts on other microorganisms such as beneficial microorganisms such as *Lactobacillus spp*. would be very useful. It would be very interesting to perform *in vitro* and *in vivo* toxicity studies on all the plants and include these results in the ranking system. This should definitely increase the potential of developing a useful therapeutic product to combat diarrhoea in stock production.

Much research still has to be done to perfect the system, but this work represents the first step and may help scientists in the future to select the most promising plant species for in depth screening as opposed to the current random selection, or selection based on only one or two appropriate parameters.

# Chapter 11

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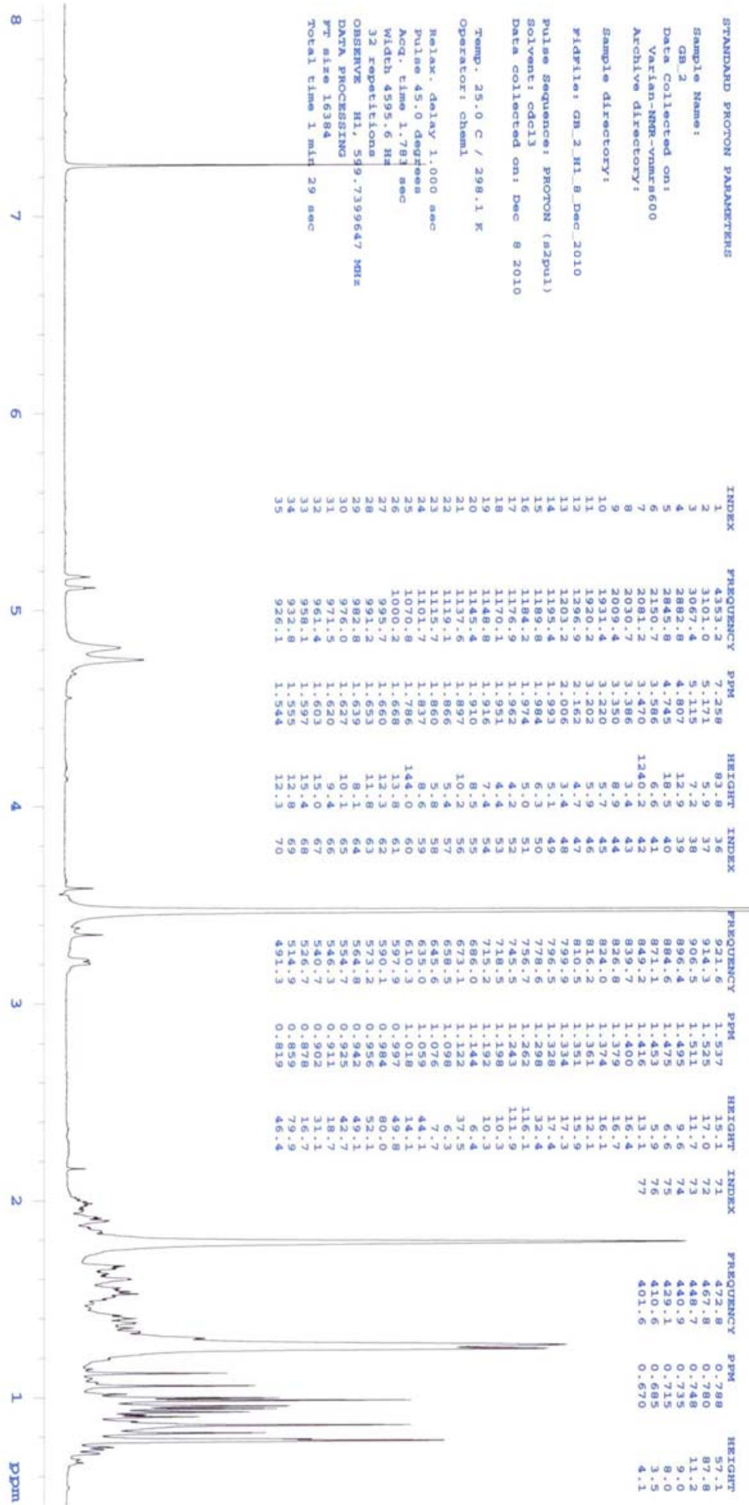
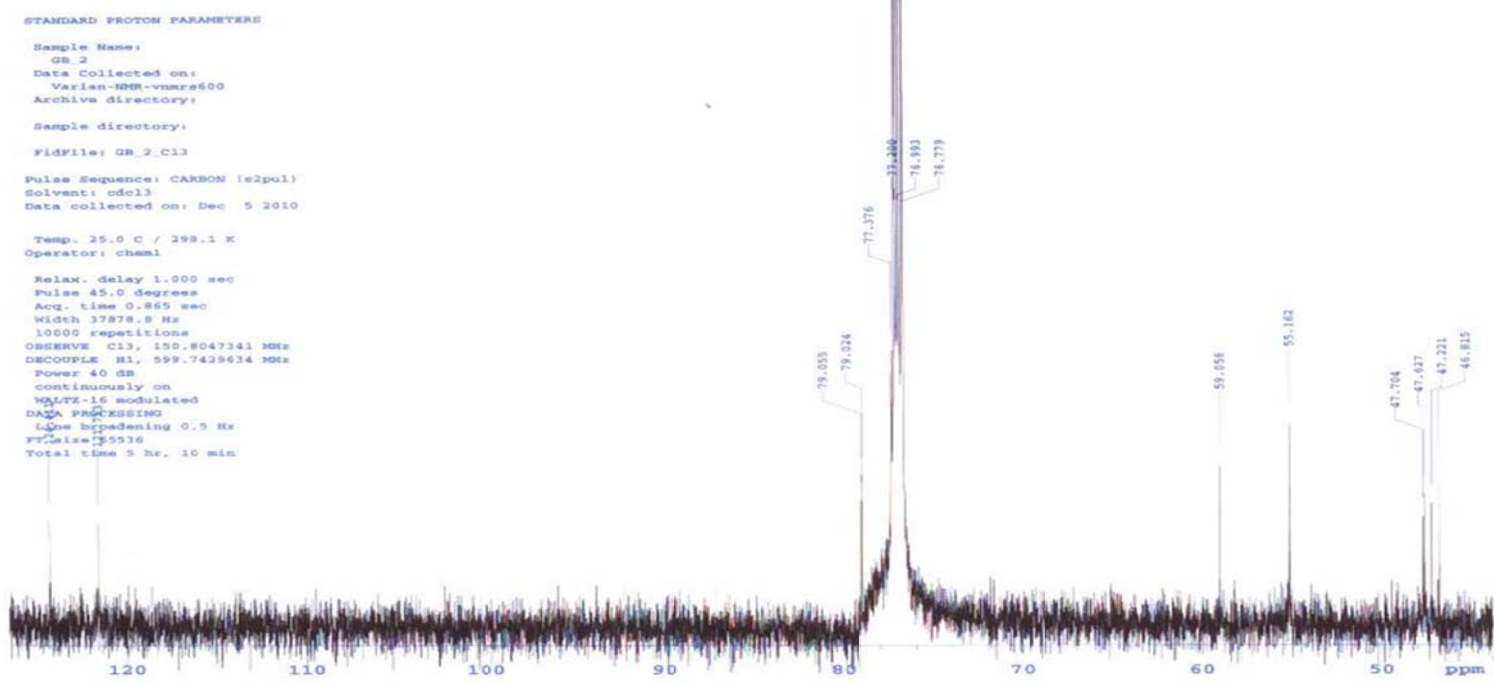
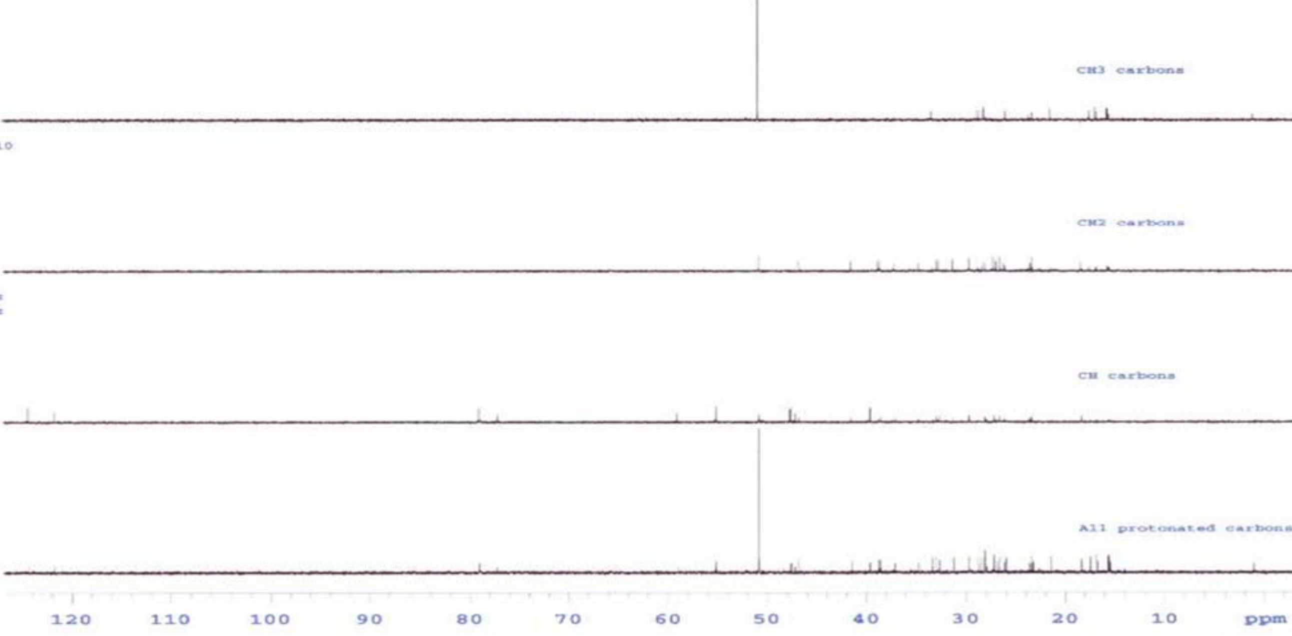
# Appendix A



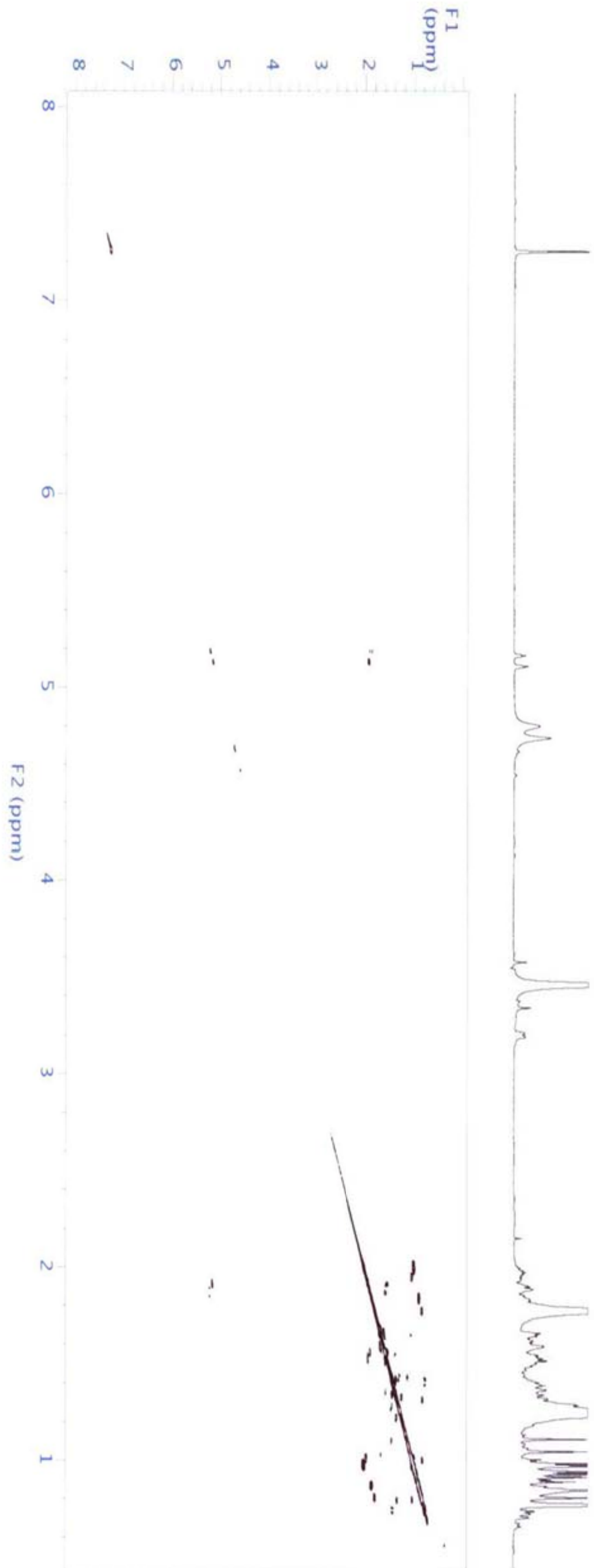
A 1: $^{1}$H-NMR Spectroscopy of C1



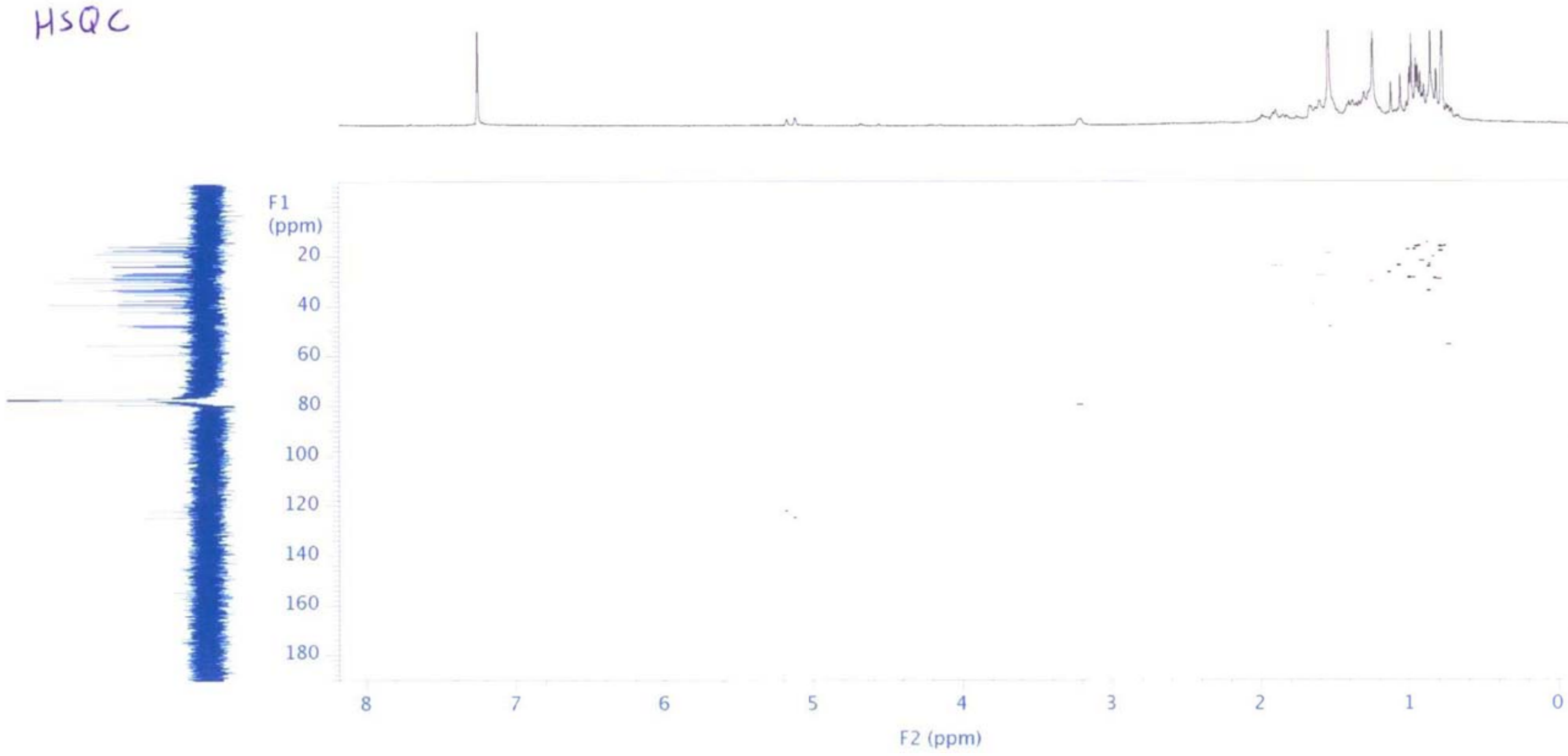

A 2: $^{13}$C-NMR Spectroscopy of C1



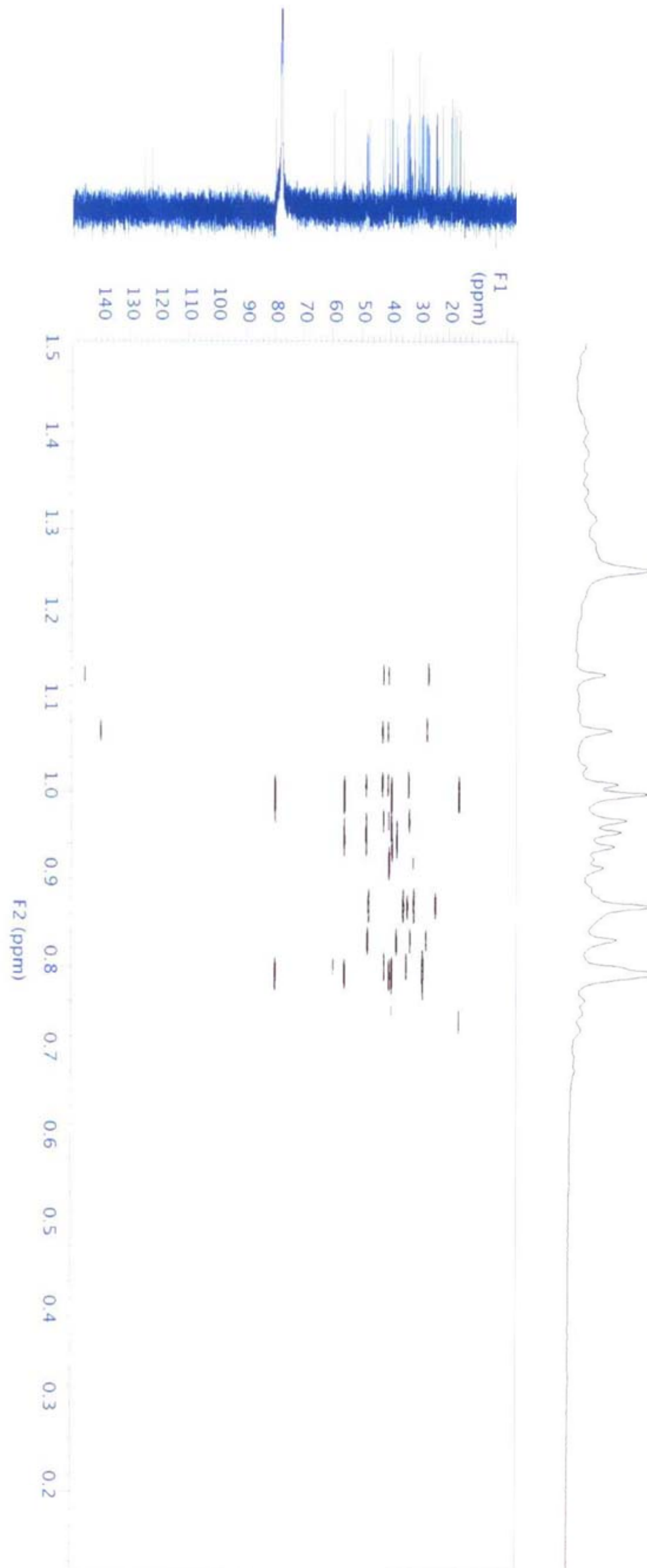

A 3: $^{13}C$-DEPT-NMR Spectroscopy of C1




A 4: COSY-NMR Spectroscopy of C1




**A.5: HSQC-NMR Spectroscopy of C1**


F1 (ppm)
20
30
40
50
60
70
80
90
100
110
120
130
140
F2 (ppm)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2

# Appendix B

**Table B 1: Dosing regimen, clinical signs and macroscopic lesions and percentage of weight gain or loss in animals dosed with *Deinbollia oblongifolia***

| Rat | Dosing quantity | Clinical signs | Macroscopic lesions | % (+, -) |
|---|---|---|---|---|
| 1 | 5 mg/kg | Scratching; eye discharge after dosing; pulmonary distress from 2 hours after dosing; gasping blood on day 2; warfarin spots; euthanized day2 | Moderate gas accumulation in the stomach; Moderate hepatic centrolobular congestion and blood pooling with dilated centrilobular sinousoids; spleen with extramedullary haemopoiesis in moderately congested red pulp; mild lung congestion with mild protein rich lung oedema and focal areas of atelectasis and emphysema; mild haemothorax | NA |
| 24 | 5 mg/kg | Frantically eating paper; depression; unsteady gait; scratching right after dosing; piloerection; pulmonary distress after dosing on day 2; euthanized day 3 | Mild adrenal medullary congestion; mild autolysis in the large and small intestine; pulmonary congestion of the alveolar walls; active white pulp in spleen | -1.5% |
| 28 | 5 mg/kg | Depression; after dosing on day 2 slight pulmonary distress; euthanized day 3 | Mild bilateral medullary congestion and sinusoidal dilatation of the adrenal; mild liver congestion; mild pulmonary congestion; mild splenomegaly with moderate congestion in splenic red pulp | |
| 29 | 5 mg/kg | Scratching; after dosing on day 2 pulmonary distress; piloerection, unsteady gait from day 2 on; euthanized day 3 | Mild autolytic changes in uterine horn; mild pulmonary congestion; active normal white pulp and mild extramedullary haemopoiesis; mild haemothorax | -4.2% |
| 33 | 5 mg/kg | None; euthanized day 3 | Minimal autolysis in small intestine; mild pulmonary congestion and oedema; splenic white pulp is active hyperplastic while mild extramedullary haemopoiesis within the splenic red pulp; mild haemothorax | -0.2% |
| 36 | 5 mg/kg | Piloerection; after dose on day 2 pulmonary distress; euthanized day 3 | Mucometra; pulmonary congestion and minimal oedema; mild haemothorax | -12.8% |
| 3 | 20 mg/kg | Pica; after dose day 2 swollen abdomen; euthanized day 3 | Minimal autolytic changes in intestinal mucosa; mild lung atelectasis; prominent splenic white pulp and | +0.4% |

| | | | | |
|---|---|---|---|---|
| | | | moderate extramedullary haemopoiesis in splenic red pulp; severe haemothorax | |
| 9 | 20 mg/kg | Scratching; depression; piloerection; nasal discharge; pulmonary distress; terminated after four hours | Moderate gas accumulation in stomach and small intestine; mild congestion in adrenal medulla; mild multifocal tubular dilatation of collecting ducts in both kidney sections; pale lung; mild lung oedema and foci of atelectasis and emphysema; mild focal autolysis in pancreas; mild extramedullary haemopoiesis in the spleen with normal splenic red pulp and with pulp and mild haemosiderosis; mild congestion of the thymic medulla; small amount of blood in pericardium and in the thorax | NA |
| 18 | 20 mg/kg | Scratching; after dose on day 2 swollen abdomen detected and pulmonary distress; euthanized day 3 | Pulmonary congestion; moderate extramedullary haemopoiesis in splenic red pulp; moderate haemothorax | +4-5% |
| 23 | 20 mg/kg | Depression; scratching; after dose on day 2 pica; piloerection on day 2; euthanized day 3 | Mild autolytic changes in the small intestine; mild pulmonary congestion; mild extramedullary haemopoiesis in spleen; moderate haemothorax | +3.0% |
| 25 | 20 mg/kg | None; euthanized day 3 | Moderated congested adrenal medulla; moderate autolysis in intestinal mucosa of large intestine; moderate autolysis in the intestinal mucosa in the small intestine; ; moderate autolytic foci in pancreas; mild extramedullary haemopoiesis in splenic red pulp; moderate haemothorax | +2.6% |
| 35 | 20 mg/kg | Pulmonary distress; unsteady gait; depression; piloerection; euthanized after 5 hours | Minimal gas accumulation in stomach; adrenal congestion; moderate congestion of the leptomeninges and neuroparenchyma; mild autolysis in small intestine; congestion in liver cortex; moderated accentuated lobulation in liver with moderated centrilobular blood pooling; moderate lung congestion with lung oedema and multifocal areas of atelectasis; mild foci of autolysis in pancreas; moderate extramedullary haemopoiesis in the splenic red pulp; mild haemothorax | NA |
| 2 | 50 mg/kg | Scratching; piloerection day 2; euthanized day 3 | Gas accumulation in the stomach; mild congestion in adrenal medulla; minimal autolytic changes in small | +3.5% |

| | | | | |
|---|---|---|---|---|
| | | | intestine; mild cortical congestion in kidney; mild hepatic congestion in the central part of the hepatic nodules and mild accentuated lobulation; mild pulmonary congestion; moderate extramedullary haemopoiesis in spleen; unilateral anophtalmia from birth | |
| 5 | 50 mg/kg | Scratching; piloerection; depression; warfarin spots on day 2 as well as swollen abdomen; after dosing on day 2 pulmonary distress and unsteady gait; euthanized day 3 | Mild renal tubular dilatation; moderate hepatic congestion and blood pooling; focal autolytic changes in pancreas; moderate hyperplasia of the lymphoid tissue in the splenic white pulp and extramedullary haemopoiesis is moderate in the red pulp; mild congestion of the thymic tissue; internal congestion and splenomegaly; severe haemopericardium | -1.7% |
| 11 | 50 mg/kg | Scratching; depression; piloerection; unsteady gait; pulmonary distress; euthanized day 3 | Mild pulmonary oedema and congestion; minimal pancreatic autolysis; active white pulp and moderate congestion in red pulp | -1.5% |
| 12 | 50 mg/kg | Depression; unsteady gait; piloerection; scratching; pulmonary distress; after dose on day 2 severe depression; euthanized day 3 | Mild hepatic congestion in the portal areas; mild lung oedema and areas of atelectasis; moderate extramedullary haemopoiesis in the splenic red pulp; mild splenic congestion | -2.6% |
| 26 | 50 mg/kg | Depression; scratching; euthanized day 3 | Mild sinusoidal dilatation and congestion of the adrenal medulla; mild portal and parenchymal foci of lymphocytic infiltrations in liver; mild lung oedema; moderate extramedullary haemopoiesis in spleen; mild autolysis in stomach; mild haemopericardium and haemothorax | +1.3% |
| 38 | 50 mg/kg | Scratching; dose on day 2 struggle to dose; bloated abdomen on day 2; euthanized day 3 | Minimal mucosal autolysis in small intestine; mild sinusoidal blood pooling in liver; foci of autolysis in pancreas; mild extramedullary haemopoiesis in the red pulp of the spleen | -1.2% |

**Table B 2: Clinical chemistry parameters of rats dosed with 5 mg/kg of the crude extract of** ***Deinbollia oblongifolia***

| Analytes | Reference values | Rat 1 | Rat 24 | Rat 28 | Rat 29 | Rat33 | Rat 36 |
|---|---|---|---|---|---|---|---|
| **TSP (g/l)** | 58.5 (± 2.3) | 55 | 55.3 | 53 | 53.1 | 53.4 | 59 |
| **ALB (g/l)** | 30.8 (± 1.1) | 39.6 | 35.7 | 37.4 | 35.7 | 31.3 | 41.9 |
| **GLOB (g/l)** | 31 - 33[b] | 15.4 | 19.6 | 15.6 | 17.4 | 22.1 | 17.1 |
| **A/G** | 0.95 – 0.96[c] | 2.59 | 1.82 | 2.4 | 2.06 | 1.42 | 2.44 |
| **ALT (U/l)** | 57.5 (±22.5) | 31 | 75 | 71 | 45 | 55 | 30 |
| **ALP (U/l)** | 290 (± 63) | 94 | 132 | 114 | 93 | 146 | 108 |
| **AST (U/l)** | 78.1 (± 13.0) | 92 | 191 | 139 | 88 | 118 | 109 |
| **GGT (U/l)** | 5 – 6[b] | 5 | 2 | 0 | 1 | 0 | 0 |
| **Urea (mmol/l)** | 9.46 (± 0.84) | 4.2 | 6.7 | 7.7 | 9.3 | 7.7 | 7.9 |
| **Crea (µmol/l)** | 47.6 (± 7.4) | 33 | 30 | 31 | 35 | 23 | 37 |
| **Na** | 145 (± 5) | 136 | 139.6 | 141.5 | 140.5 | 139.2 | 137.6 |
| **K** | 4.95 (± 0.65) | 3.59 | 4.57 | 4.01 | 4.31 | 4.1 | 3.99 |
| **Ca** | 2.4 (± 0.2) | 2.47 | 2.67 | 2.75 | 2.52 | 2.57 | 2.52 |

**Table B 3: Clinical chemistry parameters of rats dosed with 20 mg/kg of the crude extract of** ***Deinbollia oblongifolia***

| Analytes | Reference values | Rat 3 | Rat 9 | Rat 18 | Rat 23 | Rat25 | Rat 35 |
|---|---|---|---|---|---|---|---|
| **TSP (g/l)** | 58.5 (± 2.3) | 46.9 | ND | 53.8 | 56.2 | 55.4 | ND |
| **ALB (g/l)** | 30.8 (± 1.1) | 10.8 | ND | 40 | 40.4 | 40.3 | ND |
| **GLOB (g/l)** | 31 - 33[b] | 36.1 | ND | 13.8 | 15.8 | 15.1 | ND |
| **A/G** | 0.95 – 0.96[c] | 0.3 | ND | 2.89 | 2.56 | 2.66 | ND |
| **ALT (U/l)** | 57.5 (±22.5) | 52 | ND | 65 | 86 | 68 | ND |
| **ALP (U/l)** | 290 (± 63) | 104 | ND | 127 | 175 | 151 | ND |
| **AST (U/l)** | 78.1 (± 13.0) | 218 | ND | 168 | 166 | 157 | ND |
| **GGT (U/l)** | 5 – 6[b] | 7 | ND | 1 | 1 | 1 | ND |
| **Urea (mmol/l)** | 9.46 (± 0.84) | 5 | ND | 8.5 | 8.3 | 8.1 | ND |
| **Crea (µmol/l)** | 47.6 (± 7.4) | 22 | ND | 35 | 31 | 28 | ND |
| **Na** | 145 (± 5) | 130.7 | ND | 139.4 | 142.7 | 141.1 | ND |
| **K** | 4.95 (± 0.65) | 5.44 | ND | 5.27 | 4.66 | 4.99 | ND |
| **Ca** | 2.4 (± 0.2) | 2.11 | ND | 2.93 | 2.72 | 2.86 | ND |

**Table B 4: Clinical chemistry parameters of rats dosed with 50 mg/kg of the crude extract of *Deinbollia oblongifolia***

| Analytes | Reference values | Rat 2 | Rat 5 | Rat 11 | Rat 12 | Rat26 | Rat 38 |
|---|---|---|---|---|---|---|---|
| **TSP (g/l)** | 58.5 (± 2.3) | 56.7 | 52.8 | 56.8 | 57.1 | 56.1 | 53.9 |
| **ALB (g/l)** | 30.8 (± 1.1) | 42.1 | 35.3 | 42.1 | 43.5 | 41.7 | 40 |
| **GLOB (g/l)** | 31 - 33[b] | 14.6 | 17.5 | 14.7 | 13.6 | 14.4 | 13.9 |
| **A/G** | 0.95 – 0.96[c] | 2.88 | 2.02 | 2.85 | 3.18 | 2.9 | 2.87 |
| **ALT (U/l)** | 57.5 (±22.5) | 67 | 53 | 57 | 65 | 71 | 65 |
| **ALP (U/l)** | 290 (± 63) | 170 | 106 | 177 | 158 | 165 | 137 |
| **AST (U/l)** | 78.1 (± 13.0) | 115 | 179 | 214 | 212 | 136 | 123 |
| **GGT (U/l)** | 5 – 6[b] | 2 | 2 | 3 | 3 | 1 | 0 |
| **Urea (mmol/l)** | 9.46 (± 0.84) | 9 | 10 | 6.1 | 10.9 | 6.3 | 7.3 |
| **Crea (µmol/l)** | 47.6 (± 7.4) | 28 | 36 | 36 | 34 | 27 | 29 |
| **Na** | 145 (± 5) | 140.5 | 140.9 | 142.4 | 141.1 | 141.2 | 141.7 |
| **K** | 4.95 (± 0.65) | 4.17 | 5.09 | 4.92 | 5.07 | 4.21 | 3.9 |
| **Ca** | 2.4 (± 0.2) | 2.68 | 2.66 | 3.08 | 2.71 | 2.63 | 2.62 |

**Table B 5: Dosing regimen, clinical signs and macroscopic lesions and percentage of weight gain or loss in animals dosed with the chloroform fraction of *Deinbollia oblongifolia***

| Rat | Dosing quantity | Clinical signs | Macroscopic lesions | % (+, -) |
|---|---|---|---|---|
| 4 | 50 mg/kg | Scratching; euthanized day 3 | Moderate cortical congestion bilateral in adrenal glands; mild autolytic changes in the intestinal mucosa of small intestine; mild cortical congestion in kidney; mild hepatic congestion; focal lung congestion; moderated extramedullary haemopoiesis in the red pulp of the spleen with moderate haemosiderosis; moderate haemorrhages in the heart and mild haemopericardium | -1.5% |
| 13 | 50 mg/kg | Scratching; depression; pulmonary distress; unsteady gait; piloerection; warfarin spots on day 2 and gasping blood; euthanized after 21 hours | Severe gas accumulation in the stomach; mild autolytic changes in the intestinal mucosa; mild congestion in both Kidneys; moderate centrilobular congestion with sinusoidal dilatation in liver; protein-rich lung oedema and focal congestion; mild extramedullary haemopoiesis and haemosiderosis of the red splenic pulp and active lymphoid tissue in the splenic white pulp | NA |
| 16 | 50 mg/kg | Pulmonary distress; euthanized | Small amount of gas in small intestine; mild medullary | NA |

| | | | | |
|---|---|---|---|---|
| | | after four hours | congestion in adrenal; mild congestion in leptomeninges and neuroparenchyma; moderate dilatation of uterus and few lymphocytic cells in the endometrial lamina propria; moderate mucometra; mild congestion of mucosa of the small intestine; centrilobular blood pooling in liver with mild to moderate accentuated liver lobulation; focal atelectasis in the lung; congestion of red splenic pulp | |
| 19 | 50 mg/kg | Pica; swollen abdomen on day 2; euthanized day 3 | Minimal congestion in the adrenal medulla; mild autolysis in large and small intestine; in the renal crest, small foci of lymphocytic infiltrations in one of the kidney sections; mild atelectasis and lung congestion; mild autolytic changes in pancreas; congestion of red splenic pulp | +2.1% |
| 31 | 50 mg/kg | Pulmonary distress after dosing on day 2; warfarin spots day 2; piloerection day 3; euthanized day 3 | Mild mucosal autolysis in small intestine; mild congestion of the cortico –medullary junction; mild atelectasis in one lung section; moderate extramedullary haemopoiesis and mild splenic congestion | +3.3% |
| 39 | 50 mg/kg | Unsteady gait; piloerection, depression, unsteady gait and pulmonary distress day 2; euthanized day3 | Mild autolytic changes in small intestine; mild pulmonary congestion; mild extramedullary haemopoiesis in spleen; mild haemopericardium and haemothorax | +4.4% |
| 7 | 125 mg/kg | Pulmonary distress; depression; nasal discharge; piloerection; pica; depression; euthanized day 3 | Mild autolysis in the small intestine; cortical congestion in both kidneys; bilateral pulmonary atelectasis and lung congestion; moderate extramedullary haemopoiesis and active lymphoid tissue; moderate haemothorax | -11.4% |
| 8 | 125 mg/kg | Depression; blood on nose after dose on day 2; piloerection; pulmonary distress day 2; unsteady gait day 2; euthanized day 3 | Small amount of gas in stomach; mild autolytic changes in small intestine; mild renal cortical congestion in both kidneys; mild atelectasis in the lung; mild autolytic changes in spleen; moderate congestive splenomegaly and active lymphoid tissue in the splenic white pulp | -9.0% |

| 10 | 125 mg/kg | Depression; scratching; pulmonary distress after dose on day2; euthanized day 3 | Congested adrenal cortices; mild autolysis in large and small intestine; mild pulmonary atelectasis; mild autolysis in pancreas; active splenic white pulp and mild extramedullary haemopoitic cells in red pulp; mild haemopericardium | +3.7% |
|---|---|---|---|---|
| 14 | 125 mg/kg | Scratching on day2; depression; piloerection, unsteady gait; pulmonary distress day2; euthanized day 3 | Mild cortical congestion in adrenal; mild autolytic changes in small intestine; mild hepatic congestion and sinusoidal blood pooling; moderate pulmonary congestion; mild autolytic changes in pancreas; moderated extramedullary haemopoiesis in the splenic red pulp | +4.5% |
| 17 | 125 mg/kg | Depression; blood after dosing on day2; pulmonary distress on day2; piloerection; euthanized day 3 | Mild adrenal cortical congestion; mild leptomeningeal congestion; mild autolysis in large intestine and small intestinal mucosa; mild pulmonary congestion; active white pulp and moderate extramedullary haemopoiesis in the splenic red pulp; haemothorax and haemopericardium | +1.7% |
| 20 | 125 mg/kg | Pica; scratching; pulmonary distress on day2; euthanized day 3 | Mild adrenal congestion; mild atelectasis and congestion of the anterior lobe of the lung; mild extramedullary haemopoiesis and mild haemosiderosis; mild haemothorax | +1.7% |
| 22 | 300 mg/kg | Scratching; depression; piloerection; warfarin spots on eyes on day 2; piloerection; pulmonary distress; euthanized day3 | Moderate autolytic changes in the small intestine; pulmonary congested and show atelectasis; mild autolysis in pancreas; red splenic pulp with moderate extrtamedullary haemopoiesis; moderate haemothorax | -2.1% |
| 30 | 300 mg/kg | Unsteady gait; scratching; depression; piloerection; pulmonary distress after dosing on day2; euthanized day3 | Mild pulmonary congestion; mild congestion of the red splenic pulp | -1.4% |

| | | | | |
|---|---|---|---|---|
| 37 | 300 mg/kg | Depression; piloerection; euthanized day 3 | Adrenal cortex and medulla congested; mild pulmonary congestion with foci atelectasis; prominent and active splenic lymphoid tissue; mild extramedullary haemopoiesis in the red splenic pulp; mild haemothorax | -0.1% |
| 40 | 300 mg/kg | Depression, piloerection; warfarin spots; pulmonary distress day 2; euthanized day 3 | Mild gas accumulation in stomach; mild congestion of the deeper cortical layer and adrenal medulla; mild hepatic centrilobular congestion; moderated atelectasis and pulmonary congestion; mild autolytic changes in pancreas; active splenic white pulp | -0.3% |
| 41 | 300 mg/kg | Depression; pulmonary distress; piloerection; unsteady gait after dose on day2; euthanized day 3 | Mild autolysis in large and small intestine; minimal pulmonary congestion; mild autolytic changes in the pancreas; active splenic white pulp | +1.0% |
| 42 | 300 mg/kg | Unsteady gait; depression; drinking a lot; piloerection; unsteady gait and warfarin spots on eyes as well as swollen abdomen on day 2; pulmonary distress day 2; euthanized day 3 | Mild gas accumulation in stomach; mild blood pooling and congestion in liver; mild pulmonary congestion and atelectasis; moderated extramedullary haemopoiesis in the splenic red pulp; well populated lymphocytes in thymus; moderate haemothorax | -3.2% |

**Table B 6: Clinical chemistry parameters of rats dosed with 50 mg/kg of the chloroform fraction of *Deinbollia oblongifolia***

| Analytes | Reference values | Rat 4 | Rat 13 | Rat 16 | Rat 19 | Rat31 | Rat 39 |
|---|---|---|---|---|---|---|---|
| **TSP (g/l)** | 58.5 (± 2.3) | 53.2 | 51.7 | ND | 54.9 | 56.1 | 55.6 |
| **ALB (g/l)** | 30.8 (± 1.1) | 36.3 | 26.9 | ND | 42.1 | 41.6 | 39.2 |
| **GLOB (g/l)** | 31 - 33[b] | 16.9 | 24.8 | ND | 12.8 | 14.5 | 16.4 |
| **A/G** | 0.95 – 0.96[c] | 2.15 | 1.09 | ND | 3.3 | 2.86 | 2.38 |
| **ALT (U/l)** | 57.5 (±22.5) | 58 | 93 | ND | 67 | 67 | 60 |
| **ALP (U/l)** | 290 (± 63) | 124 | 64 | ND | 137 | 157 | 163 |
| **AST (U/l)** | 78.1 (± 13.0) | 159 | 420 | ND | 150 | 88 | 90 |
| **GGT (U/l)** | 5 – 6[b] | 4 | 19 | ND | 2 | < 0 | 0 |
| **Urea (mmol/l)** | 9.46 (± 0.84) | 7.1 | 3.8 | ND | 6.6 | 7.8 | 8.3 |
| **Crea (µmol/l)** | 47.6 (± 7.4) | 35 | < 18 | ND | 32 | 29 | 29 |
| **Na** | 145 (± 5) | 140.1 | 113.1 | ND | 141.7 | 141.8 | 139.7 |
| **K** | 4.95 (± 0.65) | 3.93 | ND | ND | 4.38 | 3.85 | 4.8 |
| **Ca** | 2.4 (± 0.2) | 2.58 | 1.85 | ND | 2.61 | 2.7 | 2.68 |

**Table B 7: Clinical chemistry parameters of rats dosed with 125 mg/kg of the chloroform fraction of *Deinbollia oblongifolia***

| Analytes | Reference values | Rat 7 | Rat 8 | Rat 10 | Rat 14 | Rat17 | Rat 20 |
|---|---|---|---|---|---|---|---|
| **TSP (g/l)** | 58.5 (± 2.3) | 49.4 | 48.7 | 52.4 | 55.9 | 52.4 | 50.7 |
| **ALB (g/l)** | 30.8 (± 1.1) | 23.1 | 36.1 | 35.9 | 39.7 | 40.3 | 37.3 |
| **GLOB (g/l)** | 31 - 33[b] | 26.3 | 12.6 | 16.5 | 16.2 | 12.1 | 13.4 |
| **A/G** | 0.95 – 0.96[c] | 0.88 | 2.86 | 2.19 | 2.45 | 3.34 | 2.77 |
| **ALT (U/l)** | 57.5 (±22.5) | 69 | 44 | 78 | 63 | 55 | 46 |
| **ALP (U/l)** | 290 (± 63) | 62 | 111 | 142 | 139 | 138 | 138 |
| **AST (U/l)** | 78.1 (± 13.0) | 250 | 155 | 100 | 156 | 97 | 132 |
| **GGT (U/l)** | 5 – 6[b] | 7 | 2 | 3 | 1 | 0 | 1 |
| **Urea (mmol/l)** | 9.46 (± 0.84) | 10.8 | 10.3 | 6.9 | 9.1 | 7.4 | 9.4 |
| **Crea (µmol/l)** | 47.6 (± 7.4) | 40 | 35 | 28 | 25 | 31 | 30 |
| **Na** | 145 (± 5) | 135.1 | 139.4 | 140.5 | 140.9 | 141.9 | 143.2 |
| **K** | 4.95 (± 0.65) | 5.73 | 4.64 | 4.16 | 2.16 | 4.52 | 4.08 |
| **Ca** | 2.4 (± 0.2) | 2.55 | 2.49 | 2.55 | 2.6 | 2.68 | 2.6 |

**Table B 8: Clinical chemistry parameters of rats dosed with 300 mg/kg of the chloroform fraction of *Deinbollia oblongifolia***

| Analytes | Reference values | Rat 22 | Rat 30 | Rat 37 | Rat 40 | Rat41 | Rat 42 |
|---|---|---|---|---|---|---|---|
| **TSP (g/l)** | 58.5 (± 2.3) | 56.8 | 54.7 | 54.4 | 54.5 | 53.1 | 49 |
| **ALB (g/l)** | 30.8 (± 1.1) | 41.7 | 40.8 | 41.6 | 34.6 | 38 | 23 |
| **GLOB (g/l)** | 31 - 33[b] | 15.1 | 13.9 | 12.8 | 19.9 | 15.1 | 26 |
| **A/G** | 0.95 – 0.96[c] | 2.77 | 2.93 | 3.25 | 1.74 | 2.53 | 0.89 |
| **ALT (U/l)** | 57.5 (±22.5) | 69 | 66 | 73 | 80 | 67 | 55 |
| **ALP (U/l)** | 290 (± 63) | 134 | 135 | 148 | 130 | 142 | 127 |
| **AST (U/l)** | 78.1 (± 13.0) | 108 | 147 | 98 | 252 | 134 | 86 |
| **GGT (U/l)** | 5 – 6[b] | 1 | 1 | < 0 | 3 | < 0 | < 0 |
| **Urea (mmol/l)** | 9.46 (± 0.84) | 7.7 | 9.5 | 7.5 | 6.1 | 7 | 8.1 |
| **Crea (µmol/l)** | 47.6 (± 7.4) | 33 | 35 | 31 | 30 | 26 | 31 |
| **Na** | 145 (± 5) | 140.8 | 137.6 | 139.9 | 138.6 | 139.5 | 136.9 |
| **K** | 4.95 (± 0.65) | 4.37 | 4.75 | 4.5 | 5.2 | 3.89 | 4.01 |
| **Ca** | 2.4 (± 0.2) | 2.82 | 2.68 | 2.67 | 2.62 | 2.54 | 2.32 |

**Table B 9: Dosing regimen, clinical signs and macroscopic lesions and percentage of weight gain or loss in animals dosed with the solvent control acetone**

| Rat | Dosing quantity | Clinical signs | Macroscopic lesions | % (+, -) |
|---|---|---|---|---|
| 6 | 0 mg/kg | Pulmonary distress; piloerection; depression; euthanized after 9 hours | Moderated gas accumulation in stomach and small intestine; mild leptomeningeal congestion in brain; prominent apoptosis and moderated centrilobular blood pooling  and accentuated lobulationin the liver; moderate diffuse protein-rich lung oedema and atelectasis and haemorrhage in the lung; mild lung congestion; moderate haemosiderosis and extramedullary haemopoiesis; severe haemothorax | NA |
| 15 | 0 mg/kg | Depression; intraperitoneal dosing day 2 then unsteady gait, piloerection, heart palpitations; swollen abdomen; pulmonary distress; euthanized day 3 | Mild medullary congestion in kidney; mild pulmonary congestion; moderate extramedullary haemopoiesis and normal splenic white pulp; mild haemothorax and haemopericardium | +0.1% |

| | | | | |
|---|---|---|---|---|
| 21 | 0 mg/kg | Dose on day 2 intraperitoneally; depression; pica; unsteady gait; piloerection; scratching; day 3 slight tendency to the left; euthanized day 3 | Mild acute peritoneal inflammation in the mesentery of the small intestine; focal atelectasis and mild congestion in lung; active lymphoid tissue in the splenic white pulp with mild extramedullary haemopoiesis of the red pulp; mild thymus congestion, but thymic lymphoid tissue normal; mild haemothorax | -7.8% |
| 27 | 0 mg/kg | Slight depression; died during the night | Stomach severely dilated with gas; segmental gas accumulation in small intestine; moderated congestion of the leptomeninges in brain; moderate autolysis in small and large intestine; moderated congestion of renal cortex; moderate to severe centrilobular blood pooling in liver; moderated pulmonary congestion and moderate lung oedema with protein-rich fluid in the alveoli; foci of atelectasis and emphysema; foci of autolytic changes in pancreas; moderate haemosiderosis in splenic red pulp; mild thymus congestion | NA |
| 32 | 0 mg/kg | Depression; intraabdominal dosing day 2; depression; unsteady gait; piloerection; euthanized day 3 | Mild congestion of the medullary adrenal; mild inflammation in the peri-adrenal fat; mild peritoneal mesenteric macrophage-rich reaction; moderate autolysis in the mucosa and neutrophils and macrophages in the mesentery; foci of autolysis and few macrophages on the peritoneal surface of pancreas; mild extramedullary haemopoiesis and moderate hamosiderosis in spleen | -7.5% |
| 34 | 0 mg/kg | Warfarin spots; nasal discharge; depression; pulmonary distress; unsteady gait; oedema at eye; euthanized after 4 hours | Mild gas accumulation in stomach; mild dilatation of uterus associated with mucometra; mild kidney congestion; blood pooling and accentuated lobulation in liver; mild atelectasis and congestion in lung; active white pulp in spleen; mild splenic red pulp haemosiderosis; severe haemothorax | NA |

**Table B 10: Clinical chemistry parameters of rats dosed with the pure solvent (Acetone)**

| Analytes | Reference values | Rat 6 | Rat 15 | Rat 21 | Rat 27 | Rat32 | Rat 34 |
|---|---|---|---|---|---|---|---|
| **TSP (g/l)** | 58.5 (± 2.3) | ND | 45.7 | 50.4 | ND | 50 | ND |
| **ALB (g/l)** | 30.8 (± 1.1) | ND | 26.2 | 32 | ND | 24.7 | ND |
| **GLOB (g/l)** | 31 - 33[b] | ND | 19.5 | 18.4 | ND | 25.3 | ND |
| **A/G** | 0.95 – 0.96[c] | ND | 1.35 | 1.74 | ND | 0.98 | ND |
| **ALT (U/l)** | 57.5 (±22.5) | ND | 151 | 163 | ND | 157 | ND |
| **ALP (U/l)** | 290 (± 63) | ND | 103 | 104 | ND | 113 | ND |
| **AST (U/l)** | 78.1 (± 13.0) | ND | 478 | 410 | ND | 444 | ND |
| **GGT (U/l)** | 5 – 6[b] | ND | 3 | 1 | ND | 5 | ND |
| **Urea (mmol/l)** | 9.46 (± 0.84) | ND | 20.3 | 9.4 | ND | 6.1 | ND |
| **Crea (µmol/l)** | 47.6 (± 7.4) | ND | 32 | 33 | ND | 26 | ND |
| **Na** | 145 (± 5) | ND | 130.8 | 134.3 | ND | 132 | ND |
| **K** | 4.95 (± 0.65) | ND | 4.52 | 5.57 | ND | 5.49 | ND |
| **Ca** | 2.4 (± 0.2) | ND | 2.3 | 2.41 | ND | 2.34 | ND |