Isolation and characterization of antimicrobial compounds from *Funtumia africana* (Apocynaceae) leaf extracts

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DECLARATION

I declare that the thesis hereby submitted to the University of Pretoria has not previously been submitted by me or any other person for a degree at this or any other university, that it is my own work in design and in execution, and that all material contained herein has been duly acknowledged.

Mr. T.E. Ramadwa

Prof. J.N. Eloff (Promoter)

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LIST OF ABBREVIATIONS

Aj	*Androstachy johnsonii*
As	*Acalypha sonderiana*
BEA	Benzene:ethanol:ammonium hydroxide (90:10:1)
But	Butanol fraction
C	Carbon
CEF	Chloroform:ethyl acetate:formic acid (5:4:1)
Chlo	Chloroform fraction
COX	Cyclooxygenase
CSIR	Council for Scientific and Industrial Research
Dm	*Dracaena mannii*
DMSO	Dimethyl sulfoxide
Dn	*Drypetes natalensis*
DPM	Disintegrations per minute
EtAC	Ethyl acetate
Ec	*Escherichia coli*
Ef	*Enterococcus faecalis*
EMW	Ethylacetate:methanol:water (10:1.35:1)
F	Fraction
Fa	*Funtumia africana*
GC	Gas chromatography
H	Hexane
Hex	Hexane fraction
Hlc	Hydrochloric acid
HPLC	High performance liquid chromatography
INT	Iodonitrotetrazolium violet
M	Molarity
MIC	Minimal inhibitory concentration
MLC	Minimum lethal concentration
MU	Methyl ursolate
MS	Mass spectrometry
Na	Sodium

Nc	*Necepsia castaneifolia*
NMR	Nuclear Magnetic Resonance
NSAIDs	Non-steroidal anti-inflammatory drugs
O	Oxygen
Ot	*Oncinotus tenuiloba*
Pa	*Pseudomonas aeruginosa*
R_f	Retention factor
RTA	Relative total activity
Sa	*Staphylococcus aureus*
SEM	Standard deviation of mean
TA	Total activity
Tf	*Turraea floribunda*
TLC	Thin layer chromatography
UV	Ultraviolet
WHO	World Health Organization
Xt	*Xylia torreana*

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PRESENTATIONS OF RESULTS

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Oral: TE Ramadwa, EE Elgorashi, JN Eloff. Preliminary screening of leaf extracts of nine plant species for antibacterial activity. **Phytomedicine lectures, Faculty of Veterinary Sciences, University of Pretoria, South Africa.**

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Oral: TE Ramadwa, EE Elgorashi, JN Eloff. Isolation of antibacterial compounds from leaf extracts of *F. africana* (Benth) stapf. **Phytomedicine lectures, Faculty of Veterinary Sciences, University of Pretoria, South Africa.**

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ABSTRACT

Medicinal plants have played an important role in drug discovery, with many pharmaceutical products originating from plants. Isolation and characterization of antibacterial compounds is still relevant today because of continuing development of resistance of bacteria to antibiotics.

The aim of the study was to evaluate the antibacterial activity of leaf extracts of nine tree species (*Acalypha sonderiana*, *Androstachys johnsonii*, *Dracaena mannii*, *Drypetes natalensis*, *Funtumia africana*, *Necepsia casteneifolia*, *Oncinotus tenuiloba*, *Turraea floribunda*, and *Xylia torreana*) selected from the Phytomedicine Programme Database based on good antimicrobial activities. The next step was to select the most active plant species and to isolate and characterize the antibacterial compounds. A serial microplate dilution method was used to determine the minimal inhibitory concentration and bioautography was used to determine the number of antibacterial compounds in the extract and their R_f values. Four nosocomial infection pathogens (*Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa,* and *Staphylococcus aureus*) were used as test organisms.

Extracts of all the plant species were active with average MIC values ranging from 0.13 to 2.0 mg/ml against the four bacteria. MIC values as low as 0.08 mg/ml was obtained with *F. africana* and *O. tenuiloba* extracts against *S. aureus*. In bioautography seven of the nine leaf extracts had activity with clear zones of inhibition on bioautograms against the red background. *F. africana* was active against all four bacteria while *O. tenuiloba* had selective activity against *P. aeruginosa* with clear bands on the bioautogram. *F. africana* was chosen for further investigation because (a) it had good antibacterial activity against the four tested bacteria with MIC value as low as 0.08 mg/ml, (b) there were several active compounds against all the tested bacteria based on bioautography, (c) it is common in nature, and (d) as far as our literature survey could ascertain there was no published information on the antimicrobial activity of this plant species.

The bulk powdered leaves of *F. africana* were extracted with acetone. The acetone extract was fractionated into five fractions (hexane, chloroform, butanol, H_2O and 30% H_2O in methanol) using solvent-solvent fractionation, to group the phytochemicals based on their polarity. Hexane and chloroform fractions were the most active with MIC values as low as 0.02 mg/ml for the chloroform fraction.

One of the traditional uses of *F. africana* is to treat burns. As a result, the crude extract and its five fractions were also tested for anti-inflammatory activity using both the COX-1 and COX-2 assays. The crude extract and the hexane and chloroform fraction had moderate activity against both cyclooxygenase 1 and 2. The chloroform fraction was more active than the crude extract (59.7 ± 1.4%) with an inhibition of 68.2 ± 6.6%. Because there was no activity in the aqueous extracts and traditional healers usually use water as extractant, the pain relief experiences traditionally must be due to another anti-inflammatory mechanism.

One antibacterial compound was isolated from the hexane fraction using column chromatography with silica gel as the stationary phase and a hexane ethyl acetate gradient as the mobile phase from low to high polarity. The isolated compound was identified as methyl ursolate using nuclear magnetic resonance (NMR) and mass spectrometry. Methyl ursolate has been isolated from a number of plant species. However, this is the first report on the isolation from *Funtumia* genus and the first report of its antimicrobial activity. Previous phytochemical investigation from the stem bark of *F. africana* led to the isolation of steroidal alkaloids of the conanine group.

Methyl ursolate had a low activity with MIC values of >250 µg/ml against the four tested bacteria, but had better activity against five fungal (*Candida albicans, Cryptococcus neomeforms, Fusarium oxysporum, Penicillium janthinellium,* and *Rhizoctonia solani)* species with an MIC value of 63 µg/ml against *F. oxysporum*. The chloroform fraction had excellent activity with an MIC of 20 µg/ml and may be developed to become a useful complex drug. The more than one hundred fold lower activity of the isolated methyl ursolate compared to the activity of the chloroform fraction from which it was isolated, provides strong evidence of synergism. This may be a good model system for studying synergism in antimicrobial preparations.

CHAPTER 1

INTRODUCTION

Throughout the ages humans have relied on nature for basic needs for the production of food, shelter, clothing, means of transportation, fertilizers, flavours, fragrances, and medicines (Newman *et al*., 2000). The great ancient Chinese, Indian, and North African civilizations provided written evidence of man's utilization of plants for the treatment of a wide range of diseases (Phillipson, 2001). In South Africa, western and traditional systems of medicine exist together, the first dating back only 300 years with the influx of European settlers and the latter possibly to palaeolithic times (Van Wyk *et al*., 1997). Evidence accruing from observation of animals demonstrated that even chimpanzees use a number of plant species for their medicinal value (Huffman and Wrangham, 1993).

A medicinal plant can be defined as any plant used in order to prevent, relieve or cure a disease or to alter physiological and pathological process or any plant employed as a source of drugs or their precursors (Arias, 1999). Plant derived medicines have made a large contribution to human health and well-being (Iwu *et al*., 1999). Even today, plant materials remain an important source for combating illness, including infectious diseases (Konig, 1992). Dependence on plants as the source of medicine is more prevalent in developing countries where traditional medicine plays a major role in health care (Farnsworth, 1994; Srivastava *et al*., 1996).

Levels of sanitation, hygiene and living conditions for the majority of African people are not comparable to those of industrialized countries. This exposes African people to a wider array of microbial pathogens, which increase their susceptibility to bacterial infections (Taylor *et al*., 2001). The problem is also exacerbated by lack of proper health care facilities and where these exist, the majority of the population cannot afford to pay for conventional medicines (Matu and Van Staden, 2003). The World Health Organization reported that 80% of the world's population rely chiefly on traditional medicine and a major part of the traditional therapies involve the use of plant extracts or their active constituents (WHO, 1993). Indigenous medicines are relatively inexpensive and locally available and readily accepted by the local population (Gurib-Fakim, 2006).

The treatment of infectious diseases with antimicrobial agents continues to present problems in modern-day-medicine with many studies showing a significant increase in the incidence of bacterial resistance to several antibiotics (Finch, 1998; Kunin, 1993). Medicinal plants contain many phytochemicals, with some having antibacterial activity. There are many published reports on the effectiveness of traditional herbs against Gram-positive and Gram-negative microorganisms, and as a result, plants are still recognized as the bedrock for modern medicine to treat infectious diseases by some (Evans *et al*., 2002).

With an estimated 27 million South Africans depending on traditional medicine for their primary health care needs (Mander, 1998), and the increasing acceptance of herbal medicine as an alternative form of health care, the screening of plants extracts and isolation of antibacterial compounds is still essential and relevant (Rabe and Van Staden, 1997).

From research in the Phytomedicine Programme it became clear that the antimicrobial compounds in more than 50 plant species examined to date are relatively non-polar compounds. These compounds would not be available with the extractants normally used by traditional healers in South Africa. Acetone leaf extracts of more than 600 southern African species have been evaluated for activity against eight important bacterial and fungal pathogens. Nine trees species with promising activities were selected for examination in this thesis.

Frequently extracts of plants used traditionally have low antimicrobial activity, but the therapeutic effect may be due to anti-inflammatory activity. The search into plants with alleged folkloric used as pain relievers, anti-inflammatory agents, should therefore be viewed as beneficial and logical strategy in the search for anti-inflammatory drugs (Farnsworth, 1989; Eisner, 1990). Plant secondary metabolites such as flavonoids, triterpenoids, alkaloids etc, are capable of modifying the activities of inflammatory cells (Iwalewa *et al.,* 2007).

1.1 AIM OF THE STUDY

The aim of the study was to evaluate the antibacterial activity and anti-inflammatory activity of leaf extracts of nine tree species selected from the Phytomedicine Programme database and then to isolate and characterize the bioactive compounds from the most active plant species.

1.2 OBJECTIVES

The objectives of the study were to:

- Screen leaf extracts of nine different plant species for antibacterial activity and to identify the one with the highest activity.
- Isolate the antibacterial compounds from the selected plant species.
- Determine the anti-inflammatory activity of different fractions of the selected species to evaluate the traditional use of the plant.
- Elucidate the structure of the isolated compounds.
- Determine the antibacterial activity and antifungal activity of the isolated compounds.

CHAPTER 2

LITERATURE REVIEW

2.1 Traditional medicines and their challenges in Africa

Plants have been used for medicinal purposes in Africa for centuries, partly as a result of the high cost of western pharmaceuticals and health care, and partly because the traditional medicines are more acceptable from cultural and spiritual perspectives (Cunningham, 1988).

A problem with traditional medicine is that it does not keep pace with scientific and technological advancement (WHO, 1978). The methods, techniques, and training are often kept secret. The diagnosis of the diseases by the traditional medicine practitioner and the dosage of the medicaments prescribed are often imprecise (Sofowora, 1996). This warrants scientific validation of their safety, efficacy, quality and the dosage of the plant material used (Masika and Afolayan, 2002).

Southern Africa has a rich diversity of plants with about 10% of the world's total number of higher plants species (Van Wyk, 2008). Despite this diversity and the range of medicinal plants used in South Africa, only a relatively small number of plants species have been scientifically validated (Springfield *et al*., 2005). Some of the South African indigenous plant species may become extinct before their potential as sources of pharmaceutical drugs is investigated and applied (Shai *et al*., 2008).

Some of the medicinal plants that are used traditionally are becoming endangered, rare, or threatened due to unsustainable harvesting methods (Gates, 2000). Other factors such as exposure to modern culture and urbanization also play a role in the loss of the traditional use of plants (Shrestha and Dhillion, 2003; Tabuti, 2007). Some of these plant species may have certain traits that enable them to recover quickly and are able to adapt to continuous harvesting (Siebert, 2004), others exhibit traits that make them very sensitive to uncontrolled harvesting and these species may not recover for a long time (Pfab and Scholes, 2004). If plant leaf extracts are as effective as the bark, bulbs or roots, these endangered species can be utilized in a sustainable way (Eloff, 2001; Shai *et al*., 2008).

2.2 Medicinal plants as an alternative to antibiotics

Since the discovery of the first antibiotic, penicillin, by Alexander Fleming in 1928, antimicrobial drugs have proved remarkably effective for the control of bacterial infections. However, the emergence of resistance to antibiotics in several pathogenic microorganisms in the past two decades has gradually rendered conventional treatment less effective (Sritharan and Sritharan, 2004).

Bacteria can be resistant to the action of antimicrobial drugs because of the inherent structure or physiology of the bacteria (constitutive resistance), or they can develop a mechanism to circumvent the action of the drugs through genetic mutation or through acquisition of the genetic elements (acquired resistance) (Morley *et al*., 2005). Although resistance genes are believed to transfer most commonly among bacteria of the same species, evidence is increasing that genetic elements can transfer among bacteria of different species, or even from different genera (Sritharan and Sritharan, 2004; Morley *et al.,* 2005). Deoxyribonucleic acid (DNA) fragments like transposons can be transferred from one bacterium to another (Sheldon, 2005). These small mobile genetic elements contained in the plasmids have the ability to move from the plasmid to chromosome and vice versa. Transposons can also be introduced into bacterial cells by bacteriophages that can act as a transport vectors, by uptake of naked DNA, or by transfer from other plasmids (Morley *et al.,* 2005). Consequently, this might create bacterial populations with one or more of the following properties; an increased ability to degrade antibacterial compounds, decreased cell permeability to drugs, decreased affinity for the antibiotic or increased efflux of many different antibiotics (Sritharan and Sritharan, 2004). Mechanisms associated with acquired antimicrobial resistance includes: production of competitive metabolites, target mutation, target substitution and failure to metabolize a drug to its active form (Morley *et al.,* 2005). Examples of microorganisms that gained resistance to antimicrobials over the years includes *Escherichia coli, Proteus sp, Pseudomonas aeruginosa, Shigella dysenteriae, Salmonella enteritidis, Salmonella typhi, Staphylococcus aureus, Streptococcus faecalis and Candida albicans* (Barbour *et al*., 2004). The four most important nosocomial bacteria i.e. bacteria causing infections of hospital patients are *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Enterococcus faecalis (*Sacho and Schoub, 1993).

Some antibiotics are also associated with adverse effects on the host which include hypersensitivity, depletion of beneficial gut and mucosal microorganisms, immunosuppression and allergic reactions (Idose et al., 1968). Side effects may also be a result of abusive and/or incorrect use of synthetic drugs (Rates, 2001).

Plants have the major advantage of still being the cheapest and most effective alternative source of drugs (Van der Watt and Pretorius, 2001). Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Cos *et al*., 2006). Since plants produce a variety of compounds with antimicrobial properties, screening plant leaf extracts and isolation of antibacterial compounds may become the base for the development of a medicine, a natural blueprint for the development of new drugs, or a phytomedicine to be used for the treatment of diseases (Iwu *et al*., 1999). The presence of antibacterial, antifungal and other biological activities have been demonstrated in extracts of different plant species used in traditional medicine practices (Shai *et al*., 2008, 2009).

2.3 Commercialization of medicinal plants in South Africa.

The global demand for herbal medicines is not only large, but growing (Srivastava, 2000). Factors contributing to the growth in demand for traditional medicine include the increasing human population and the frequently inadequate provision of western (allopathic) medicine in developing countries (Marshall, 1998).

Over 20 000 tons of plant material harvested, and processed were sold annually as traditional medicine in South Africa (Mander, 1997). In KwaZulu Natal, more than 700 plant species with a value of about $10-30 million are traded annually (Crouch and Arnold, 1997; Mander, 1998). About 500 plant species are traded as medicinal plants in Witwatersrand (Gauteng province). Parts of the plants sold more frequently are barks and roots which makes endangered plant species very vulnerable. The stems, leaves, whole plants and bulbs are also sold (Van Wyk *et al.,* 1997).

Around 38 South African indigenous species have been commercialized to some extent (i.e. they are available as processed material in modern packaging and in various dosage forms as tea, tinctures, tablets, capsules or ointments), out of an estimated 3000 medicinal plants species that are regularly used in traditional medicine in South Africa (Cunningham, 1988; Mander, 1998; Williams *et al*., 2000). The process has been rapidly gaining momentum during last decade. Before 1995, only nine plants (*Agothosma betulina, Aloe ferox, Aspalathus linearis, Cyclopia* species, *Harpagophytum procumbens, Hypoxis hemerocallidea, Lippia javanica, Pelargonium sidoides and Xysmalobium undulatum)* were developed to some extent. Six newcomers to the pharmaceutical market are *Artemisia afra, Hoodia gordonii, Mesembryanthemum tortuosum, Siphonochilus aethiopica, Sutherlandia frutescens* and *Warburgia salutaris* (Van Wyk, 2008).

2.4 The role of medicinal plants in drug discovery.

Plants contain different classes of compounds such as phenolics and polyphenols, flavonoids, alkaloids and many others. Some of these substances serve as plant defence mechanisms against predation by microorganisms, insects, and herbivores. Some, such as terpenoids, give plants their odours; others such as quinones and tannins are responsible for plant pigmentation (Cowan, 1999).

Several methods have been used to acquire compounds for drug discovery including isolation from plants and other natural sources, synthetic chemistry, combinatorial chemistry, and molecular modelling (Ley and Baxendale, 2002; Geysen *et al*., 2003; Lombardino and Lowe, 2004). However, natural products, and particularly medicinal plants, remain an important source of new drugs, new drugs leads, and new chemical entities (Newman *et al*., 2000, 2003; Butler, 2004). The compounds have provided the basic scaffold for medicinal chemistry modifications to expand the spectrum and/or potency of improved analogues in subsequent

years (Walsh, 2003). Isolation and characterization of pharmacologically active compounds from medicinal plants continue even today.

Plants have given western pharmacopoeia about 7000 different pharmaceutical important compounds and a number of top selling drugs of modern time, e.g. quinine, artemisinin, taxol, camptothecin among others. (Tshibangu *et al*., 2002). A study of the 25 best-selling pharmaceutical drugs in 1997 found that 11 of them (42%) were either natural products or entities derived from natural products, with a total value of $17,5 billion (Laird and Kate, 2002). Natural plant products and the organic sector are considered the fastest growing, with sales of nutrition products totalled about US$128.5 billion worldwide in 1999. This trend was reflected by a growth of sales in the United States of America from US$15 billion in 1999 to US$23 million in 2002 (Kelly *et al*., 2005). The US leads the market with 35%, closely followed by Europe (33%), and Japan (Asia) (18%) while Africa contributes less than 1% of the market (US$520 million) (De Kock, 2004). Seventy-four percent of the 121 biologically active plant-derived compounds presently in use worldwide were discovered through follow-up research work to verify their ethnomedicinal use (Sofowora, 1996). This illustrates the potential value and relevance of plant derived secondary metabolites as viable compounds for modern drug development (Balandrin *et al*., 1993).

2.5 Which method to use for selecting plant species and antimicrobial investigation?

2.5.1 Selection of plant species

The selection of suitable plants for investigation is a very important and decisive step, to avoid wasting unnecessary time (Houghton, 1995). There are several ways in which this could be done, including traditional use, chemical content, toxicity, randomised selection or a combination of several criteria (Ferry and Baltassat-Millet, 1977; Soejarto, 1996; Williamson *et al*., 1996).

The most common method used is ethnopharmacology or ethnobotany. The method involves a careful observation of the use of natural resources in folk medicine in different cultures. The preparation procedure serves as an indication of the best extraction method, and formulation used will provide information about pharmacological activity, oral versus non-oral intake and the doses to be tested. Another good and specific approach is to screen samples from existing collection such as a botanical garden. The advantage of this approach is the availability of species from different parts of the world, plant material is already identified and accessibility to greater amount of plant species showing promising results (Houghton, 1995). The search for anti-tumour drugs is an example where the investigator decides on well-defined pharmacological activity and performs a randomised search. Chemotaxonomic approach involves selection of plants mainly on genera or

families, based on chemical classes with known pharmacological activity (Gottlied and Kaplan, 1993; Souza Brito, 1996). It is possible and often desirable and inevitable, to use a combination of several criteria.

2.5.2 Selection of an antimicrobial assay method

There are a number of antibacterial bioassays that are used: agar diffusion, micro-dilution and bioautography. Initially, disc diffusion was used possibly due to its simplicity, capacity to analyze a large number of test samples and its ability to work well with defined inhibitors (Rasoanaivo and Ratsimamanga-Urverg, 1993). However, this method has some limitations; it is not appropriate for non-polar samples. They do not diffuse well through agar, does not distinguish between bactericidal and bacteriostatic effects, agar type and salt concentration influence results, and minimal inhibitory concentration cannot be determined (Gould, 2000). To determine minimum inhibitory concentrations (MIC) or minimum lethal concentrations (MLC) of active plant extracts, a sensitive 96-well microtitre plate (microdilution) method can be used (Eloff, 1998a). This method can determine static and cidal activity at a particular concentration. Bioautography combines thin-layer chromatography (TLC) with a bioassay in situ and therefore allows the researcher to localize the active compounds within a sample (Hamburger and Cordell, 1987; Gibbons and Gray, 19

CHAPTER 3

PRELIMINARY SCREENING OF LEAF EXTRACTS OF NINE PLANT SPECIES FOR ANTIBACTERIAL ACTIVITY

3.1 INTRODUCTION

The majority of studies dedicated to antimicrobial activity of South African plants focus on extracts (Van Vuuren, 2008). This might be due to the fact that when traditional medicinal plants are prepared, efficacious compounds are not extracted individually from the plants (Street *et al*., 2008). Instead the whole plant, parts thereof or crude extracts are used (Drewes *et al*., 2006). Many traditional health practitioners believe that the whole plant extract is more active than isolated compounds (Rodriguez-Fragoso *et al*., 2008).

Initially, five plant species (*Xylia torreana, Necepsia castaneifolia, Turraea floribunda, Dracaena mannii, Acalypha sonderiana*) were selected for preliminary screening; however none of the species exhibited high antibacterial activity. Four plant species (*Androstachys johnsonii, Funtumia africana, Drypetes natalensis and Oncinotus tenuiloba*) were then added to the initial list of the selected plant species. All nine plant species were part of the Tree Screening Project in the Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria which focuses on randomly screening plant species for antibacterial and antifungal activity. For conservation and sustainability purposes, only leaves were used. The plant species used in the study are summarized in Table 3.1 with their traditional uses and distribution in Southern Africa.

The Phytomedicine Programme [www.up.ac.za/phyto] is a multidisciplinary and collaborative research programme that investigates the use of medicinal plants growing in Africa for the benefits of its people and animals. More than 600 plant species have been screened against eight important bacterial and fungal pathogens using serial microplate dilution and bioautography. Other important parameters which were used when selecting plant species for preliminary screening includes availability of species, number of antibacterial compounds on bioautography and literature reports on antimicrobial activity. Extracts have been considered to possess some antibacterial activity when the MIC values are below 8 mg/ml (Fably *et al*., 1998) but in the Phytomedicine Programme activities higher than 0.1 mg/ml are not considered to be interesting.

Table 3.1: Summary of selected plant species for this study, their medicinal uses and distribution.

Family & plant species	Medicinal uses	Distribution	Reference
Apocynaceae			
Funtumia africana (Benth.) Stapf	Burns, incontinence	Zimbabwe, Mozambique	Wagner *et al* (1987)
Oncinotus tenuiloba Stapf	No use reported in literature. Species from the family are used to treat fevers and diarrhoea	South Africa (KwaZulu-Natal & Eastern Cape Province). Swaziland	Palgrave (2002)
Dracaenaceae			
Dracaena mannii Baker	Swellings, dropsy; gout generally healing	South Africa (KwaZulu-Natal), Zimbabwe, Mozambique	Burkill (1985); Palgrave (2002)
Euphorbiaceae			
Acalypha sonderiana Müll. Arg	No use reported in literature	South Africa (KwaZulu-Natal)	Palgrave (2002)
Androstachys johnsonii Prain	Stomach problems	South Africa (Limpopo & Mpumalanga Province)	Palgrave (2002); Samie *et al* (2005)
Drypetes natalensis (Harv.) Hutch	No use reported in literature	South Africa (KwaZulu- Natal), Zimbabwe, Mozambique	Palgrave (2002)
Necepsia castaneifolia (Baill) Bouchart & J. Leonard	No use reported in literature	Zimbabwe	Palgrave (2002)
Meliaceae			
Turraea floribunda (Honchst)	Rheumatism, dropsy & painful joint	South Africa (KwaZulu-Natal & Eastern Cape), Zimbabwe, Mozambique, Swaziland	Palgrave (2002)
Mimosoideae			
Xylia torreana (Brenan)	No use reported in literature	South Africa (Limpopo), Mozambique, Zimbabwe, Zambia	Palgrave (2002)

3.2 MATERIALS AND METHODS

3.2.1 Plant collection and extraction

Leaves of the selected plant species were collected from Lowveld National Botanical Gardens, Nelspruit, Mpumalanga and the Natal National Botanical Gardens, KwaZulu-Natal, during November 2006. The leaves were collected in open woven orange bags, dried at room temperature in the shade and powdered using Macsalab mill (Model 200 Lab). The powdered materials were then stored in closed honey jars at room temperature in the shade until needed.

Exactly 1 g of powdered leaves material from each species was extracted with 10 ml of technical graded acetone (Merck) in centrifuge tubes on a shaking machine for about an hour. Centrifugation was carried out on the extract at 3500 × rpm for 10 minutes. The supernatant was filtered using Whatman No. 1 filter paper and transferred into pre-weighed vials. The extraction was repeated three times on pellet to exhaustively extract the plant material before the solvent was removed under a stream of cold air.

3.2.2 Phytochemical analysis

All the extracts were reconstituted in acetone to a concentration of 10 mg/ml before analyzed with aluminium-backed thin layer chromatography (TLC) (Merck, silica gel 60 F_{254}). Ten microlitre of each extract was loaded on 10 cm × 20 cm TLC plates in a line of 1 cm long. The TLC plates were developed in saturated chambers using three eluent systems of different polarities, namely, benzene:ethanol:ammonium hydroxide (90:10:1) (BEA, non-polar/basic), ethyl acetate:methanol:water (40: 5.4:4) (EMW, polar/neutral) and chloroform:ethyl acetate:formic acid (5:4:1) (CEF, intermediate polarity/ acidic) (Kotze and Eloff, 2002).

The developed TLC plates were visualized under the ultraviolet light (254 and 360 nm, Camac universal UV lamp TL-600) and then sprayed with vanillin-sulphuric acid (0.1 g vanillin (sigma), 28 ml methanol, 1 ml sulphuric acid) (Stahl, 1969) and heated in an oven at 110ºC to detect the phytochemicals.

3.2.3 Bacterial species

Bacterial species selected for the study were the four nosocomial pathogens, namely, two Gram-positive *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212) and two Gram-negative *Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853) (Sacho and Schoub 1993). The selection of the specific bacterial strains is based on the recommendation of the national committee for clinical laboratory standards (NCCL, 1990). All the cultures were maintained on Mueller-Hilton agar at 4ºC. The cells were inoculated and incubated at 37ºC in Mueller-Hilton broth for 12 hours prior to the screening procedure.

The densities of bacterial cultures for use in the screening procedures were as follows; *S. aureus,* (2.6×10^{12} *cfu*/ml); *E. faecalis,* (1.5×10^{10}); *P. aeruginosa,* (5.2×10^{9}); *E. coli* (3.0×10^{11}).

3.2.4 Bioautographic antibacterial method

Qualitative analyses of the number of antibacterial compounds were determined by the bioautography method (Begue and Kline, 1972). Exactly 100 µg of each plant extract was loaded in a line of 1 cm wide on the TLC plates and developed in the three eluent systems mentioned in section 3.2.2. The TLC plates were dried under a stream of air to evaporate the solvents. Overnight bacterial cultures which were grown in Mueller-Hilton in an incubator at 37ºC were centrifuged at 3000 × g for 10 minutes. The pellets were resuspended in 10 ml of fresh MH broth. The developed plates were sprayed with the fresh bacterial cultures until completely moist using a spraying gun. The moist plates were incubated at 37ºC in a humidified atmosphere for about 18 hours. The plates were sprayed with 2 mg/ml aqueous solution of *p*- iodonitrotetrazolium violet (INT) (Sigma) and incubated for a further 2-6 hours depending on the organism. Bacterial growth led to the emergence of purple–red colour resulting from the reduction of INT into the formazan. Clear zones indicated the inhibition of the bacteria used by the compound present at that R_f on the chromatogram.

3.2.5 Minimal inhibitory concentration (MIC)

The serial microplate dilution method developed by Eloff (1998a) was used to determine the minimal inhibitory concentration (MIC) for plant extracts using tetrazolium violet reduction as an indicator of growth. Residues of the different extracts were redissolved in acetone to a concentration of 10 mg/ml. For each of the four bacteria used, 100 µl of each plant extract tested were two-fold serially diluted with 100 µl sterile distilled water in a sterile 96-well microtitre plates. A similar two-fold serial dilution of gentamicin (sigma) (0.1 mg/ml) was used as a positive control against each bacterium. One hundred microlitres of each bacterial culture were added to each well. The plates were covered and incubated overnight at 37ºC. To indicate bacterial growth 40 µl of 0.2 mg/ml *p*- iodonitrotetrazolium violet (INT) were added to each well and the plates incubated at 37ºC for 30 minutes. Bacterial growth in the wells was indicated by a red colour, whereas clear wells indicated inhibition of the bacterial growth by the plant extracts.

3.2.6 Total activity (TA)

Total activity indicates the degree to which the active compound in one gram of plant material can be diluted and still inhibit the growth of the tested bacterial microorganisms (Eloff, 2004). This was calculated as follows:

Total activity = quantity of material extracted from 1 g of plant material in mg divided by MIC in mg/ml

3.3 RESULTS AND DISCUSSION

3.3.1 Percentage yield of the different plant leaf extracts

Acetone was the only extractant used, because of its ability to extract both non-polar and polar constituents and its low toxicity to microorganisms in bioassays (Eloff, 1998b). The highest percentage yield of the nine plant leaf extracts screened was obtained from *Acalypha sonderiana* (8%), with the lowest from both *Xylia torreana* and *Dracaena mannii* (4%) as shown in Figure 3.1. These results obtained are comparable with the data reported by Eloff (1999), where acetone extracted 2.6 to 22.6% with average value of 10.7%, in a study involving biological activity of 27 different members of the Combretaceae family.

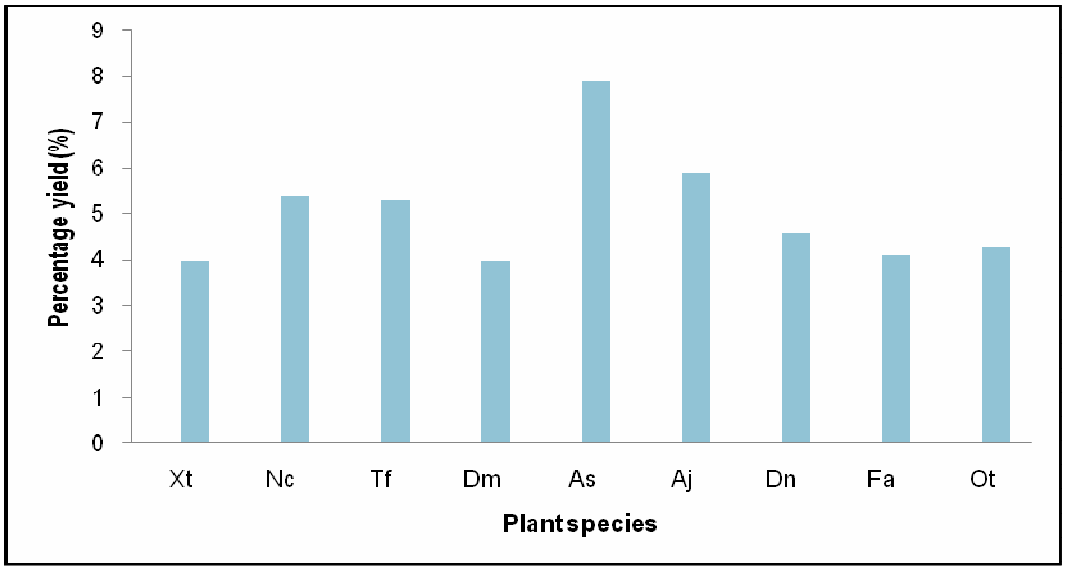


Figure 3.1: Percentage yield of the nine plant leaf extracts (*Xt, Xylia torreana; Nc, Necepsia castaneifolia; Tf, Turraea floribunda; Dn, Dracaena mannii; As, Acalypha sonderiana; Aj, Androstachys johnsonii; Dn, Drypetes natalensis; Fn, Funtumia africana; Ot, Oncinotus tenuiloba*) extracted with acetone.

3.3.2 Phytochemical analysis

The BEA solvent system separated more phytochemicals for all of the extracts after spraying with vanillin-sulphuric acid compared to CEF and EMW as shown in Figure 3.2. This means that all the species selected contain a high percentage of non-polar compounds visualized with the vanillin spray reagent. Only *Androstachys johnsonii* and *Xylia torreana* contained reasonable quantities of polar compounds that were extracted with acetone and reacted with the vanillin spray reagent.

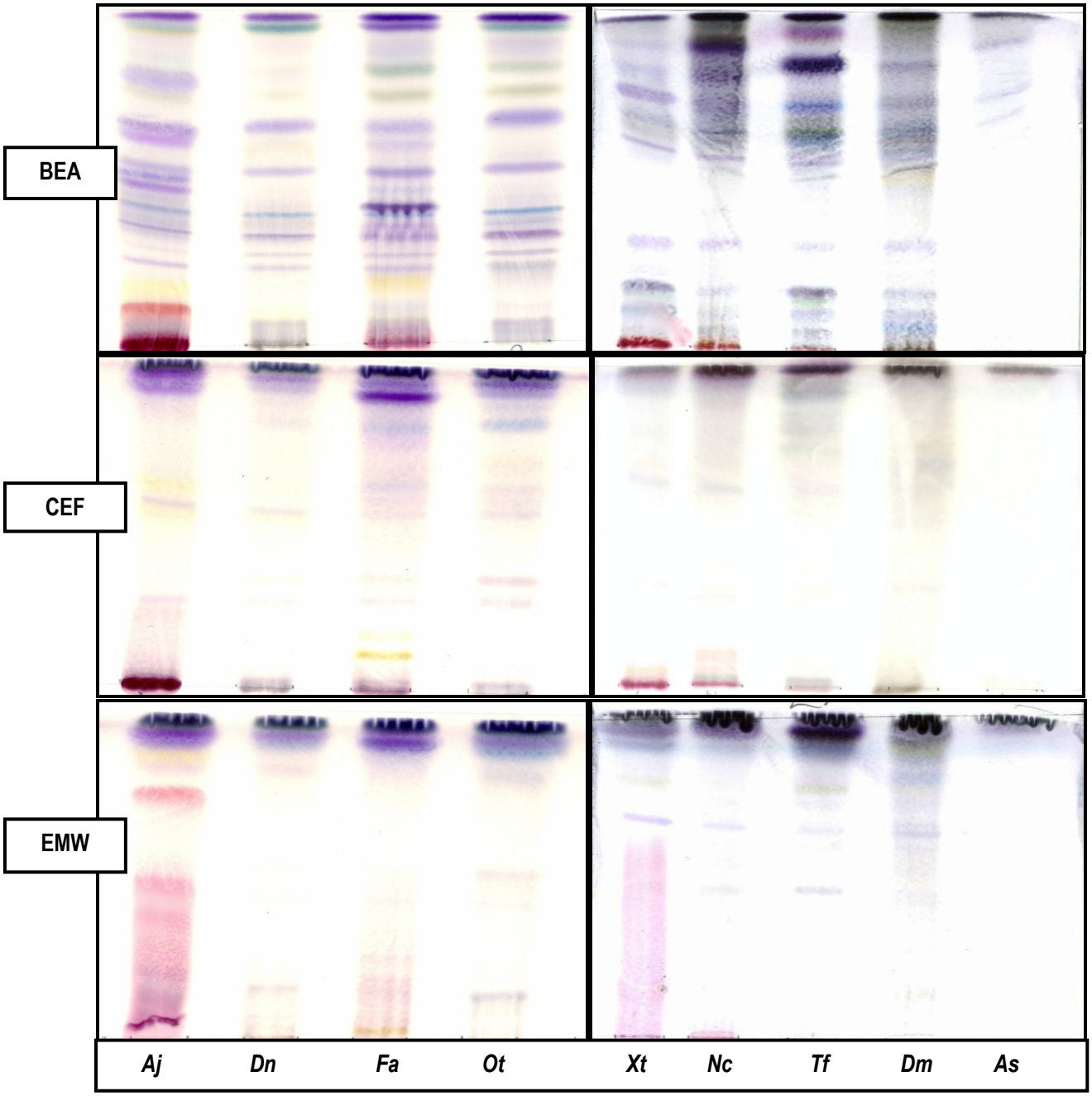


Figure 3.2: TLC bioautograms of nine plant leaf extracts developed in BEA, CEF and EMW solvent systems (top to bottom) sprayed with vanillin-sulphuric acid. Lanes from left to right**,** *Androstachys johnsonii (Aj), Drypetes natalensis (Dn), Funtumia africana (Fa), Oncinotus tenuiloba (Ot), Xylia torreana (Xt), Necepsia castaneifolia (Nc), Turraea floribunda (Tf), Dracaena mannii (Dm), Acalypha sonderiana (As).*

3.3.3 Bioautography of the plant extracts

All the nine leaf extracts were tested for antibacterial activity using the antibacterial bioautography method as shown in Figure 3.3. With the exception of *X. torreana*, all other extracts showed activity with clear zones of inhibition against the red background. Four plant leaf extracts (*A. johnsonii, D. natalensis, F. africana, O*

tenuiloba) were tested against all the four bacterial species (*S aureus, P aeruginosa, E coli, E faecalis)* as shown in figure 3.4. The activity of *F. africana* was consistent against all four bacterial species. *A, johnsonii* was active against *S. aureus* and *P aeruginosa*. *O. tenuiloba* had selective activity against only *P. aeruginosa* with four bioactive compounds.

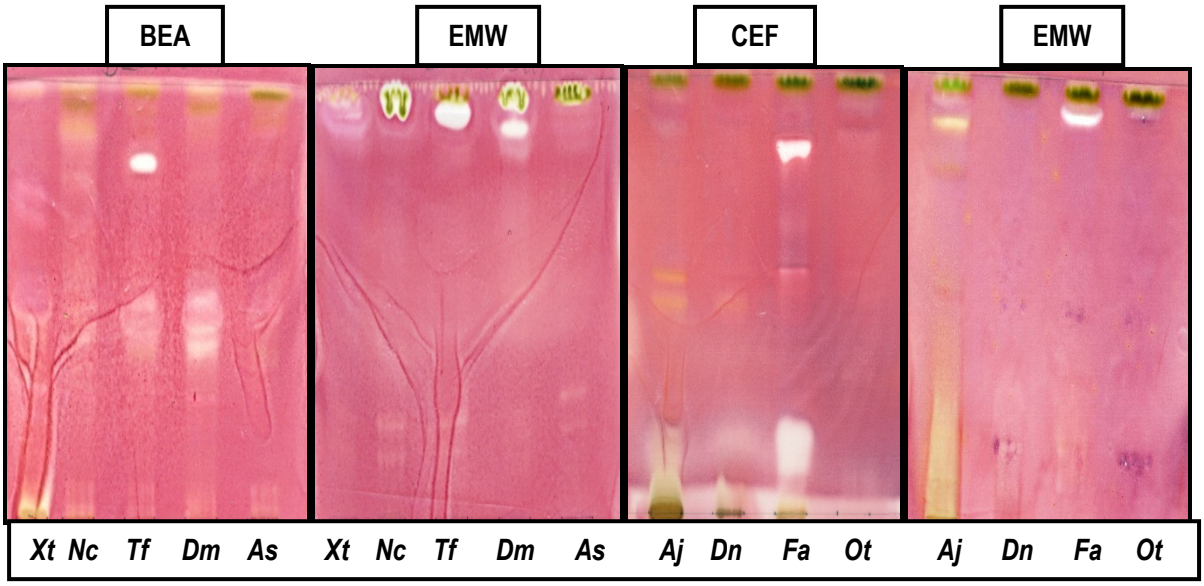


Figure 3.3: TLC bioautograms of nine plant leaf extracts (*Xt, Xylia torreana; Nc, Necepsia castaneifolia; Tf, Turraea floribunda; Dm, Dracaena mannii; As, Acalypha sonderiana; Aj, Androstachys johnsonii; Dn, Drypetes natalensis; Fa, Funtumia africana; Ot, Oncinotus tenuiloba*) developed in BEA, EMW, CEF and EMW and then Sprayed with *Staphylococcus aureus*.

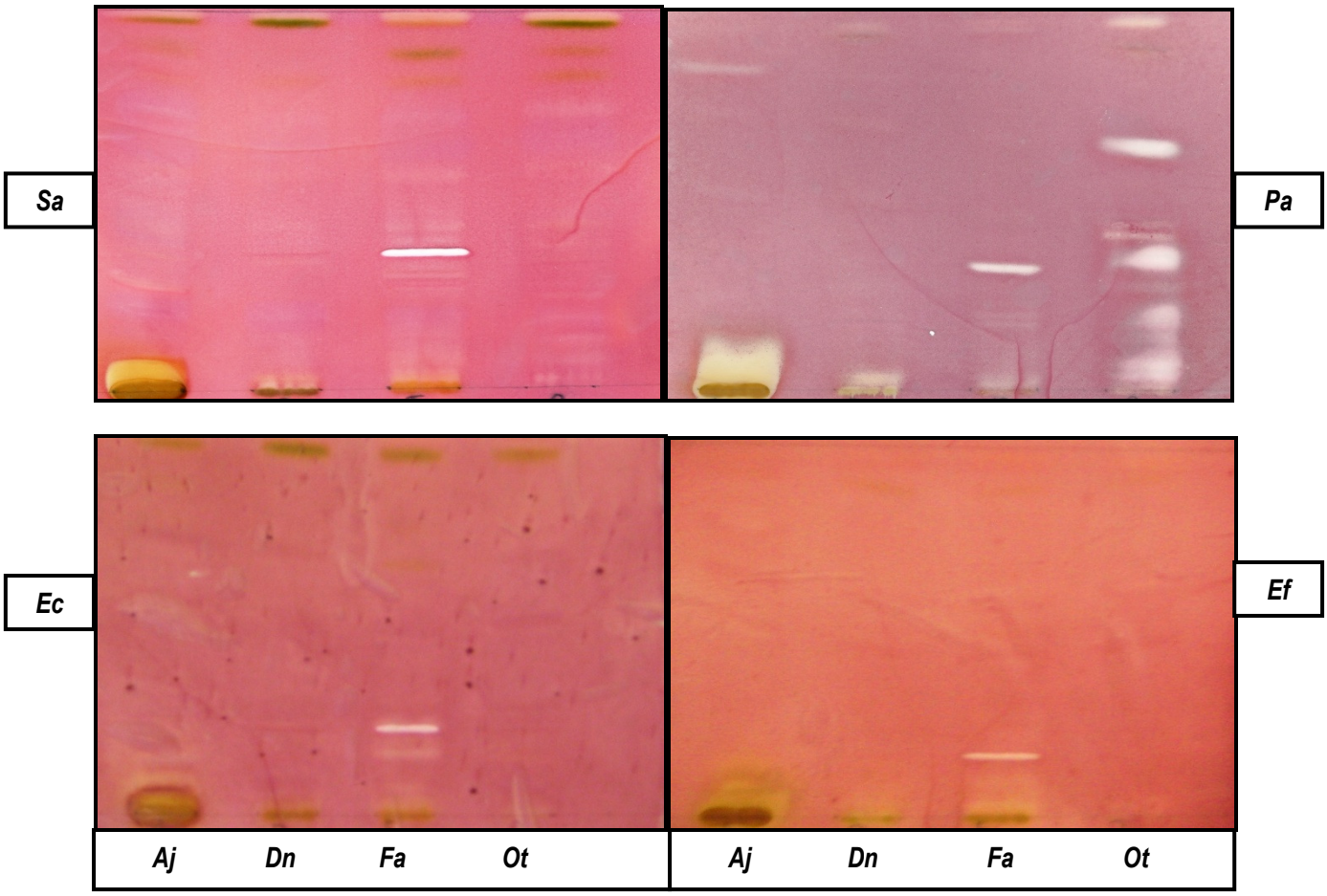


Figure 3.4: Comparison of TLC bioautograms of plant leaf extracts (*Aj*, *Androstachys johnsonii; Dn, Drypetes natalensis; Fa, Funtumia africana; On, Oncinotus tenuiloba*) developed in BEA solvent system against four test bacterial suspensions (*Sa, Staphylococcus aureus; Pa, Pseudomonas aeruginosa; Ec, Escherichia coli; Ef, Enterococcus faecalis*).The red colour indicates the growth of the bacteria, while clear white zones indicate inhibition of bacterial growth.

3.3.4 Microdilution method

The MIC values and total activity of the nine acetone extracts against all the tested bacteria are presented in Table 3.2 and 3.3 respectively. *F. africana* and *O. tenuiloba* extracts had better activity against all the tested organisms compared to other extracts. MIC value as low as 0.08 mg/ml was obtained from *F. africana* extracts against both *P. aeruginosa* and *S. aureus. Oncinotus tenuiloba* had MIC value of 0.08 mg/ml against *S. aureus* only. *Drypetes natalensis* extracts also had noteworthy MIC values of 0.16 mg/ml and 0.24 mg/ml against *S. aureus* and *E. faecalis* respectively. *X. torreana* and *N. castaneifolia* were the only two plant species with average MIC values of above 1 mg/ml.

Previous work on the acetone extract of *A. johnsonii* using micro-dilution method in Venda (Limpopo Province) had low activity against *S. aureus, E. coli* and *P. aeruginosa* with MIC values of 3 mg/ml, 6 mg/ml and 3 mg/ml respectively (Samie *et al.,* 2005). In this study, *A. johnsonii* had better activity against *S. aureus* (0.31 mg/ml), *E. coli* (0.31 mg/ml) and *P. aeruginosa* (0.63 mg/ml). These substantial differences might be due to seasonal and geographical variations. The leaves of this study were collected from Lowveld National Botanical Gardens in Nelspruit, Mpumalanga Province whereas Samie *et al* (2005) collected the same species in Venda. Other conditions that might influence variation in biological activity includes genetic variability, plant age and growth climatic conditions. Plants harvested from the wild generally vary in quality and consistency of active compounds (Bopana and Saxena, 2007). In cases where mature trees or plants cannot be found, the younger ones suffice, which results in availability of inconsistent plant material of the same species (Von Ahlefeldat *et al*., 2003). Previous investigation on *A. johnsonii* led to the isolation of atisane diterpene: ent-16α-Hydroxyatis-13-en-3-one (Piacenza *et al*., 1985). Six limonoids have been isolated from *T. floribunda (*Mcfarland *et al*., 2004). Limonoids are known to have antimicrobial activity (Roy and Saraf, 2006). Therefore the possibility that limonoids might be responsible for antibacterial activity of *T. floribunda* should not be ruled out. No previous work has been reported on the antibacterial activity of *F. africana* as far as our literature survey could ascertain.

Table 3.2: MIC values (mg/ml) of the nine plant leaf extracts of this study: *Androstachys johnsonii, Acalypha sonderiana, Dracaena mannii, Drypetes natalensis, Funtumia africana, Necepsia castaneifolia, Turraea floribunda, Oncinotus tenuiloba, Xylia torreana* against four bacteria: *Escherichia coli* (*Ec), Enterococcus faecalis* (*Ef)*, *Pseudomonas aeruginosa (Pa)* and *Staphylococcus aureus (Sa).* Gentamicin was used as a positive control.

Plant species	*Ec*	*Ef*	*Pa*	*Sa*
A. johnsonii	0.31	0.47	0.63	0.31
A. sonderiana	1.25	0.63	0.63	0.63
D. mannii	0.47	0.31	0.63	0.63
D. natalensis	0.31	0.24	0.78	0.16
F. africana	0.24	0.12	0.08	0.08
N. castaneifolia	0.63	0.63	1.25	2.5
T. floribunda	0.31	0.63	1.25	0.63
O. tenuiloba	0.24	0.16	0.12	0.08
X. torreana	0.24	0.16	0.12	0.08
Gentamicin (µg/ml)	8.0	1.6	0.2	0.3

The highest total activity was obtained on *O. tenuiloba* (538 ml/g) and *F. africana* (513 ml/g) as shown on Table 3.3. *F. africana* and *O. tenuiloba* had consistent total activity across all the bacteria. Other plant species like *D. natalensis*, *A, johnsonii* and *A. sonderiana* had a moderate total activity against more than two bacteria. The lowest total activities were obtained with *X. torreana*, *N. castaneifolia* and *T. floribunda* extracts respectively.

Table 3.3: Total activity (ml/g) *of Androstachys johnsonii, Acalypha sonderiana, Dracaena mannii, Drypetes natalensis, Funtumia africana, Necepsia castaneifolia, Turraea floribunda, Oncinotus tenuiloba, Xylia torreana* of against the four bacterial strains; *Escherichia coli (Ec); Enterococcus faecalis* (*Ec) Pseudomonas aeruginosa (Pa);* and *Staphylococcus aureus (Sa)*.

Plant species	*Ec*	*Ef*	*Pa*	*Sa*
A. johnsonii	190	126	95	190
A. sonderiana	63	125	125	125
D. mannii	85	129	64	64
D. natalensis	148	192	59	148
F. africana	171	342	513	513
N. castaneifolia	86	86	43	22
T. floribunda	171	84	42	84
O tenuiloba	179	263	358	538
X. torreana	64	16	16	16

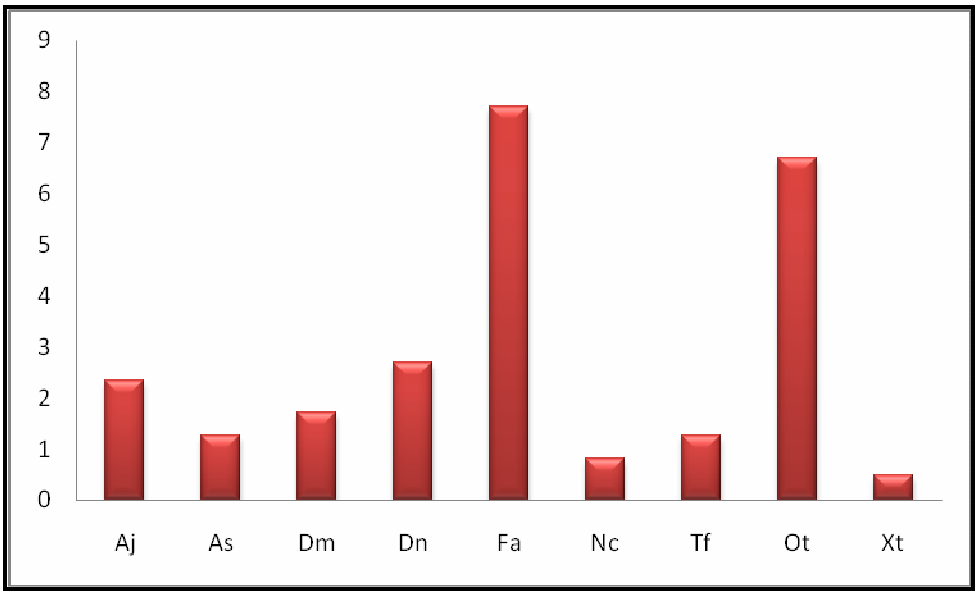


Figure 3.5: Average antibacterial activity in ml/mg of the different plant extracts (*Aj, Androstachys johnsonii; As, Acalypha sonderiana; Dm, Dracaena mannii; Dn, Drypetes natalensis; Fa, Funtumia africana; Nc, Necepsia castaneifolia; Tf, Turraea floribunda; Ot, Oncinotus tenuiloba Xt, Xylia torreana;)* against all the four bacterial species (*Sa, Staphylococcus aureus; Pa, Pseudomonas aeruginosa; Ec, Escherichia coli; Ef, Enterococcus faecalis*) by calculating inverse of MIC in mg/ml. Values indicates the volume to which 1 mg of extract can be diluted and still kill bacteria.

3.3 CONCLUSION

With only the exception of *X. torreana* and *N. castaneifolia*, all other plant species which were screened had reasonable activity against all the tested bacterial species. *F. africana* and *O. tenuiloba* had the most consistent MIC values with an overall average of 0.13 mg/ml and 0.15 mg/ml respectively (Figure 3.5). The highest total activity was obtained with *O. tenuiloba* and *F. africana* extracts. Though eight of the nine tested plant species had clear zones of inhibition on bioautograms as shown in Figure 3.3, *F. africana* had compounds with antibacterial activity against all the bacteria (Figure 3.4). It was against this background that *Funtumia africana* was chosen for further investigation.

CHAPTER 4

ISOLATION OF ANTIBACTERIAL COMPOUND FROM THE LEAF EXTRACT OF *F. Africana*

4.1 INTRODUCTION

Plants contain a wide range of many secondary metabolites. Their initial site of synthesis is often restricted to a single organ such as roots, fruits or leaves. The storage site often differs based on the polarity of the compounds. Hydrophilic compounds such as alkaloids, glucosinolates and tannins are stored mainly in the vacuoles or idioblasts and lipophilic compounds such as terpene-based essential oils are stored in trichomes, glandular hairs, resin ducts, thylakoid membranes or on the cuticle (Wiermann, 1981). Although the primary use of many plant secondary metabolites is probably for defence, secondary metabolites also act as volatile attractants to promote pollination by birds and insects, and they may also have a possible nutritional role (Wink, 2004).

To obtain antibacterial compounds, the plant extracts are first qualitatively analyzed by thin layer chromatography (TLC) and other chromatographic methods to determine the biological activity. For purification and isolation, the plant extracts are sequentially fractionated (Verpoorte, 1989). This strategy is called bioassay-guided fractionation. The chromatographic techniques that are used to isolate compounds include column chromatography, high performance liquid chromatography (HPLC) and gas chromatography (GC).

Funtumia africana (Benth.) stapf belongs to the family Apocynaceae, which generally contains alkaloids (Gurib-Fakim, 2006). The genus *Funtumia* consists of two species namely; *F. africana* and *F. elastic*. The leaves of both species are very similar, glabrous, leathery, elongated, elliptic more or less acuminate, cuneate at the base with short stalks (Keay *et al.,* 1964). *F. africana* is distinguished through the flowers and fruits which are both longer than those of *F. elastic* (Burkill, 1997).

Funtumia africana which is known as Silk-rubber in English, is a medium-sized tree up to 8-27 m in height; frequently common in the understory of moist ever-green forest. Bark: grey, smooth; bole sometimes slightly buttressed; milky latex rapidly becoming sticky on exposure to air. Leaves: simple, opposite, oblong-elliptic, 5-32 × 2-17 cm, usually about 14-17 × 4-6.5 cm, leathery, dark green above, paler green below, midrib and 6-13 pairs of more or less parallel lateral veins particularly prominent on the under surface, hair-tuft domatia often in the axils; apex abruptly attenuate; base broadly tapering; margin entire, wavy, slightly rolled under; petiole squat, 0.3-1.5 cm long; axillary glands small and numerous; stipules absent. Flowers: creamy yellow, sweetly scented, about 1.5 cm in diameter, corolla tube about 5 mm long, very thick and fleshy in the upper half, the mouth very narrow, lobes narrow and hairless, overlapping to the right, in dense, shortly stalked axillary clusters, or cymes; bisexual; floral parts in 5s; calyx small, with flattened glands at the base; stamens enclosed

within the corolla tube; ovary comprising 2 carpels, free, joined by the base of the style (October-December, but may continue on to April). Fruit: paired follicles, each narrowly elliptic, flattened, 9-30 × 0.5-3.5 cm, at the right angels to the stalk and resembling a 2-bladed propeller, pale brown, splitting along the upper surface to release the many seeds (November-April). Seeds: small, wind-distributed, each with a tuft of silvery silky hairs at 1 end (Palgrave, 2002).

Figure 4.1: Leaves of *F. africana* photographed at the Lowveld National Botanical Gardens in Nelspruit, Mpumalanga Province on the 22nd of October 2008.

The latex has been used as bird-lime. The wood is white and even-textured, and cheap furniture has been made from it; it burns well and might be suitable for matches (Palgrave, 2002). It is used traditionally to treat incontinence and burns in West Africa (Wagner *et al.,* 1987).

Previous phytochemical investigation from the stem bark of *Funtumia africana* led to the isolation of steroidal alkaloids of the conanine groups named 12α-hydroxy norcona-*N* (18).1.4-trienin-3-one, 11α, 12α-dihydroxy norcona-*N* (18).1.4-trienin-3-one and 11α-hydroxy norcona-*N*(18).1.4-trienin-3-one (Wagner *et al*., 1987). The leaves have not been investigated before for their phytochemicals.

4.2 MATERIALS AND METHODS

4.2.1 Extraction and preliminary fractionation procedure

The powdered plant leaf material (400 g) of *F. africana* were extracted with 4 L of acetone and shaken vigorously for eight hours on a Labotec shaking machine. The supernatant was filtered through Whatman No.1 filter paper using a Buchner funnel and evaporated using a Büchi rotavaporator R-114 (Labotec). The concentrated extract was then poured into a pre-weighed beaker. The same procedure was repeated two times on the pellet. The extract was then left to dry under a stream of cold air. The quantity extracted was 31.34 g. Solvent-solvent fractionation was used to fractionate the acetone extract based on polarity of the compounds as shown in Figure 4.2 (Suffness and Douros 1979).

The acetone extract was reconstituted in 300 ml of chloroform and mixed with an equal volume of distilled water in a separatory funnel to give a chloroform fraction. The water fraction was then mixed with an equal volume of n-butanol to yield the water and butanol fractions. The chloroform fraction was dried in a vacuum rotary evaporator and extracted with an equal volume of hexane and 10% water in methanol mixture, which yielded hexane fraction. The 10% water: methanol was then further diluted to 30% water in methanol and mixed with chloroform to yield 30% water in methanol fraction and chloroform fraction. A total of five fractions were collected, namely; water, butanol, hexane, 30% water in methanol and chloroform fraction.

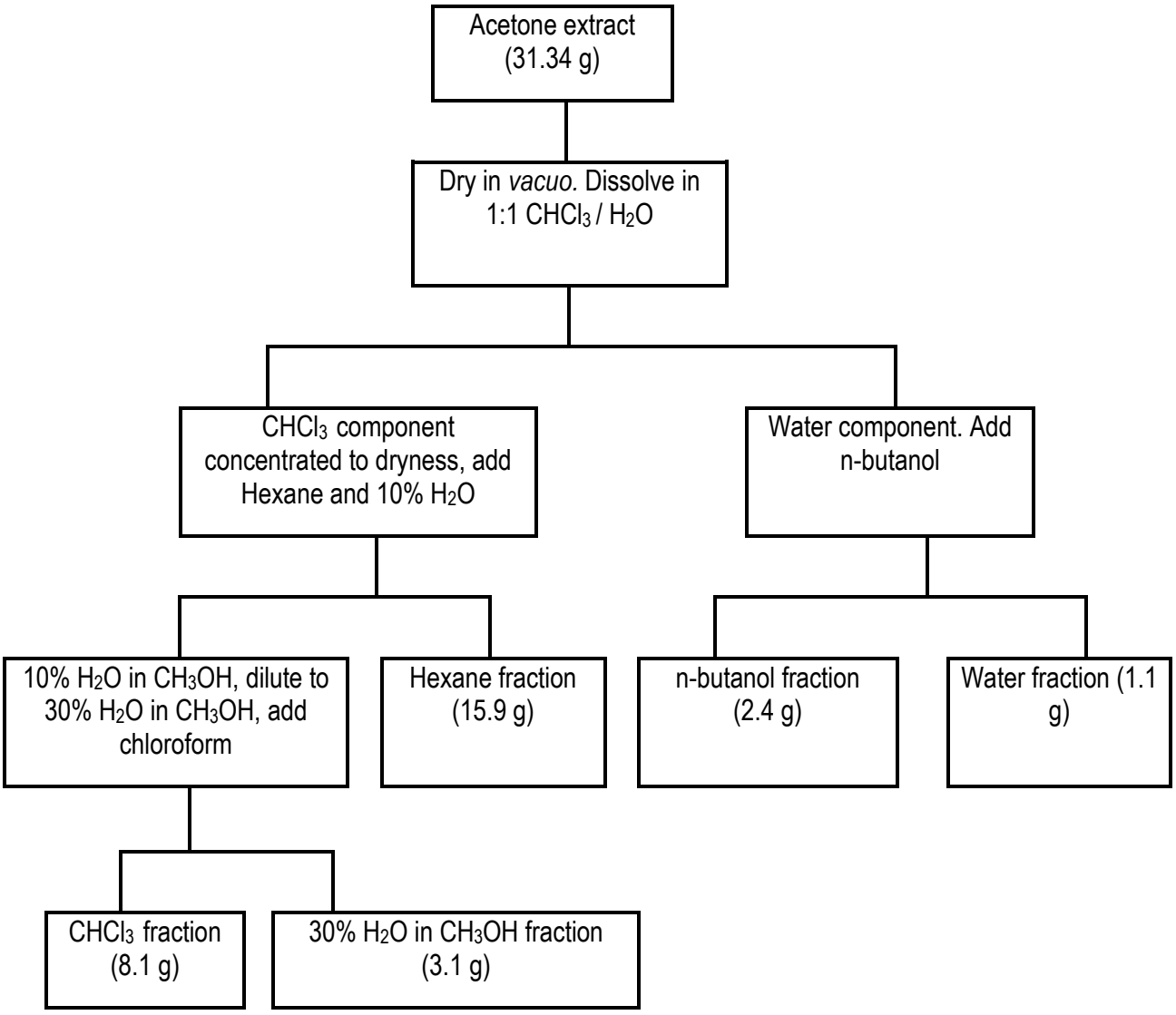


Figure 4.2: Flow chart of solvent-solvent fractionation used to categorize different compounds of *F. africana* based on polarity as described by Suffness and Douros (1979).

4.2.2. Isolation of bioactive compounds from hexane fraction.

The procedure used to isolate the bioactive compounds from the leaves of *Funtumia africana* is schematically shown in Figure 4.3.

Column chromatography was chosen for separation of compounds using silica gel as a stationary phase. About 200 g of Silica gel 60 (Merck) was mixed with hexane to form a slurry and stirred using a stirring rod. Twenty millilitre of hexane was added into a column of 40 cm length and 2.5 cm diameter whose vent was plugged with cotton to avoid bubbles when the slurry was packed into the column. The solvent was allowed to flow out the column to allow the gel to settle. Hexane extracts (16 g) was dissolved in 50 ml acetone mixed with 15 g silica gel and the mixture was allowed to dry under stream of cold air for about 5 hours, before it was layered on the column bed. Initially, hexane (600 ml) was gradually added into the column to remove fats, waxes and some chlorophyll. The polarity was increased by addition of ethyl acetate (EtAC) at an interval of 5% until 100% EtAC.

Hundred and seven fractions collected in test tubes were allowed to concentrate under a stream of cold air. Fractions containing similar constituents were combined (monitored by TLC fingerprinting and bioautography). The fractions were labelled fraction 1 to 7 as shown in Figure 4.3.

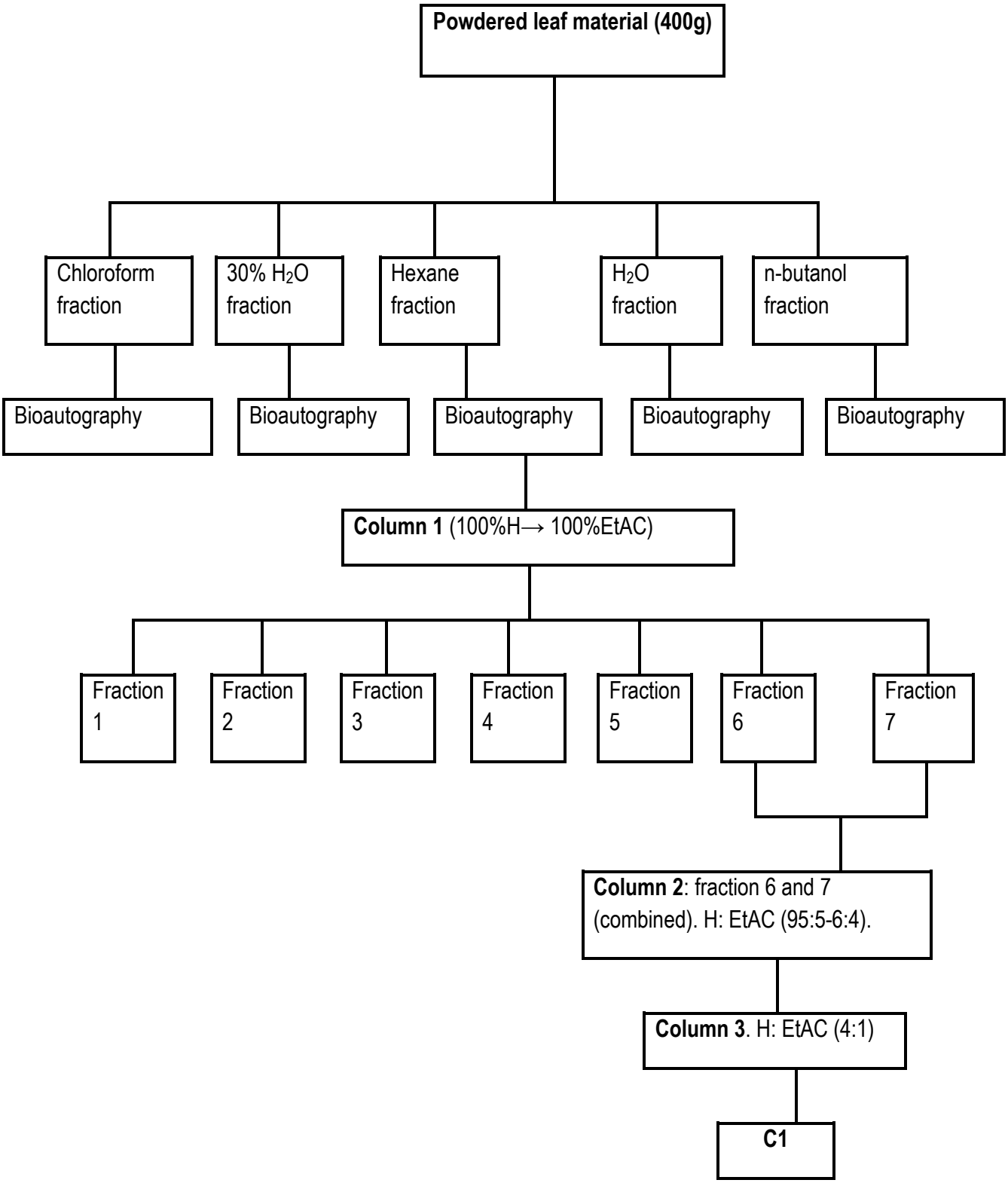


Figure 4.3: Schematic representation of the isolation of bioactive compounds from *F. africana* (monitored by TLC finger printing and bioautography) using column chromatography with silica gel 60 as the stationary phase.

4.2.2.1 Isolation of compounds in Fraction 6 and 7

Fraction 6 and 7 from the first column were combined to yield 1.12 g, because the separation of the phytochemicals was the same especially antibacterial compound. This was mixed with 10 g of silica gel 60 in hexane. A glass column of 40 cm length and 2 cm diameter was used. The column was packed as in Section 4.2.2. The 1.12 g yield-silica gel mixture was added on top of the column packed with 100 g silica gel 60 slurry. The column was eluted with 95% H in EtAC to 40% EtAC with an increment of 5%. An equal volume (500 ml) was added for each increment. Fractions were collected and developed on TLC using H:EtAC (7:3) as an eluent, sprayed with vanillin: sulphuric acid in methanol. Fractions containing same compounds were combined.

4.2.2.2 Isolation of compound C1 from column 3.

A glass column (40 cm × 1.5 cm) packed with 70 g silica slurry prepared as in Section 4.2.2.1 was used. The column was loaded with the dried mixture of the resultant yield (320 mg) containing the bioactive compound of interest from Section 4.2.2.1 and 5 g silica gel in H. The column was eluted with 100 ml of 5%, 10%, 15% and 20% EtAC in H. The fractions were monitored by TLC fingerprinting.

4.3 RESULTS AND DISCUSSION

4.3.1 Mass of the fractions of *F. africana* leaves extracted using solvent-solvent fractionation.

The acetone extracts (31.34 g) of *F. africana* was fractionated using solvent-solvent fractionation into five fractions. The highest masses were obtained on the two least polar fractions, namely; hexane and chloroform with 15.9 g and 8.1 g respectively compared to other more polar fractions (Figure 4.4). The overall total mass of the five fractions after solvent-solvent fractionation was 30.6 g compared to the initial amount of 31.34 g after bulk extraction. This represents a good recovery of 97.6% indicating a minimal loss through the formation of a pellicle during solvent-solvent fractionation.

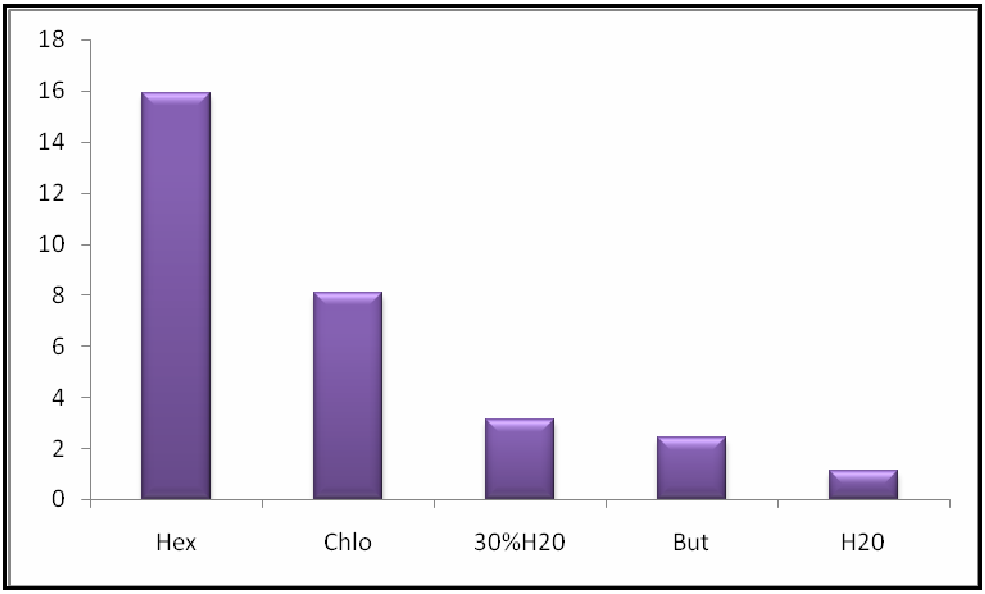


Figure 4.4: Mass of five fractions (Hex, hexane; Chlo, chloroform; 30%H_2O in methanol; But, butanol; H_2O, Water) of *F. africana* after solvent-solvent fractionation (See Fig. 4.2 for exact values) indicating the preponderance of non polar compounds in the original acetone extract.

4.3.2 TLC fingerprinting and bioautography of the five fractions.

TLC fingerprinting of the five fractions of crude extracts of *F. africana* were developed in BEA and CEF eluent system are shown in Figure 4.5. The hexane and chloroform fractions separated well on the two eluent systems used as expected while the other fractions which were more polar (30%H_20, H_2O and butanol) did not move from the base on the chromatograms. Both hexane and chloroform are less polar and the eluent systems used were also less polar. It is possible that the phytochemicals visible on TLC plates after spraying with vanillin on the more polar fractions are phenolic glycosides, sugars and amino acids which are also very polar. It would appear that hexane and chloroform contain similar compounds, but with slight difference in their concentration as shown in Figure 4.7. Because of this hexane fraction was chosen for further investigation. The hexane fraction was also chosen since it gave the highest yields of extraction of all the fractions (Figure 4.4)

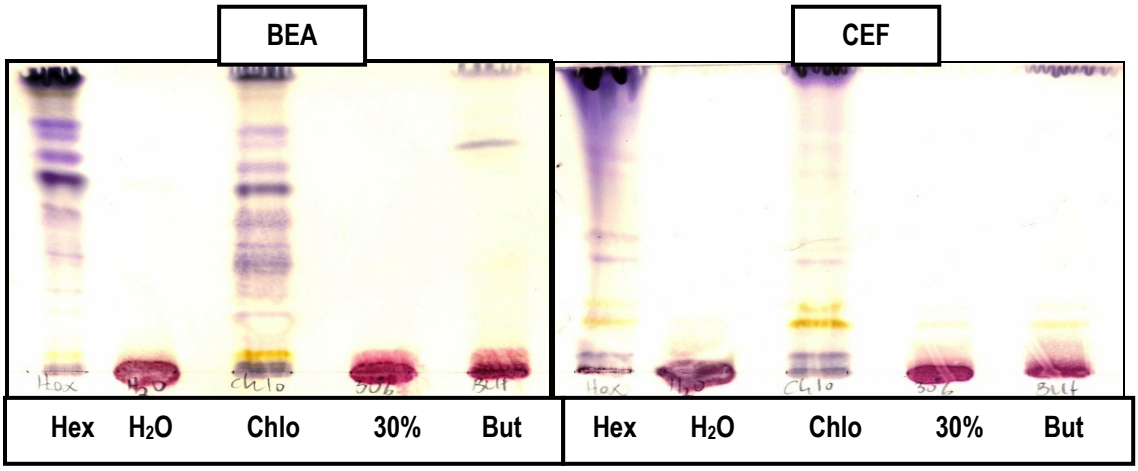


Figure 4.5: TLC fingerprinting of five fractions of *F. africana* developed in BEA and CEF solvent systems sprayed with vanillin-sulphuric acid. Lanes from left to right: Hex, hexane; H_2O in methanol; Chlo, chloroform; 30%H_2O; But, butanol.

Bioautograms of five fractions of crude extracts of *F. africana* developed in BEA and CEF eluent systems and sprayed with *S. aureus* are shown in Figure 4.6. The hexane and chloroform fractions showed activity with clear zones of inhibition on both BEA and CEF solvent system. The active compounds of the two fractions are non-polar, because they moved to the top of the TLC plate in the moderate polarity CEF mobile system. This was confirmed by the fact that the same active compounds only moved slightly in the BEA solvent system which is non-polar. The other three fractions (30%H_20, H_2O and butanol) did not show any activity. The comparison of hexane and chloroform fraction as shown in Figure 4.7 demonstrated that the active compounds had similar R_f values and might be the same.

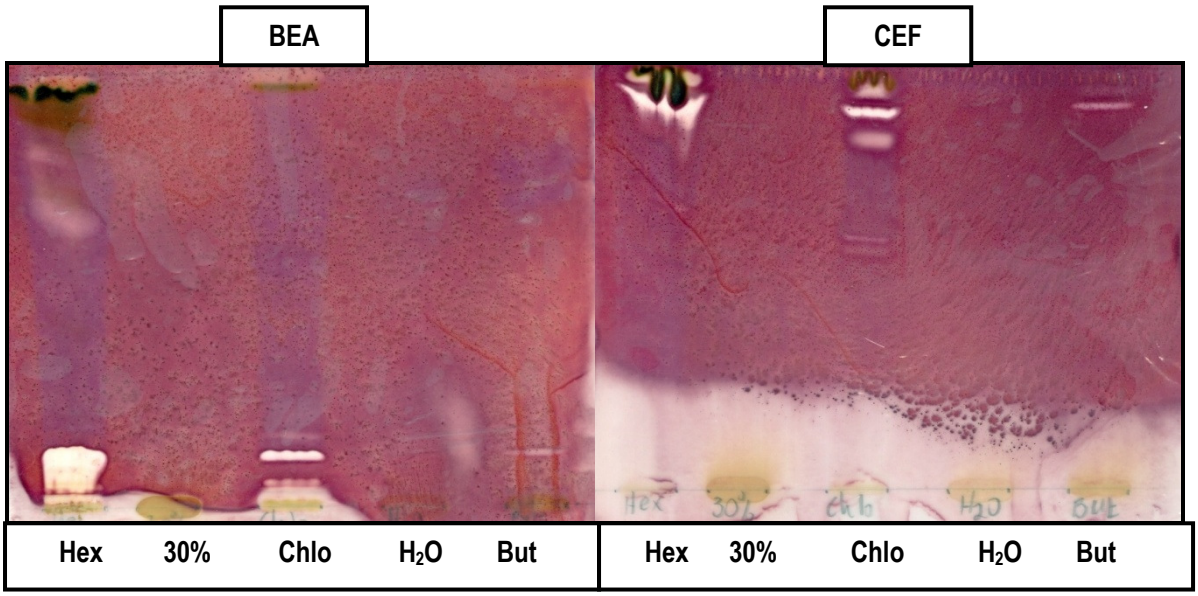


Figure 4.6: TLC bioautograms of five fractions of a crude extract of *F. africana* developed in BEA and CEF solvent systems sprayed with *S. aureus*, with the red colour indicating the growth of the bacterium and clear white zones indicating bacterial inhibition. Lanes from left to right: Hex, hexane; 30%H_2O in methanol; Chlo, chloroform; H_2O; But, butanol.

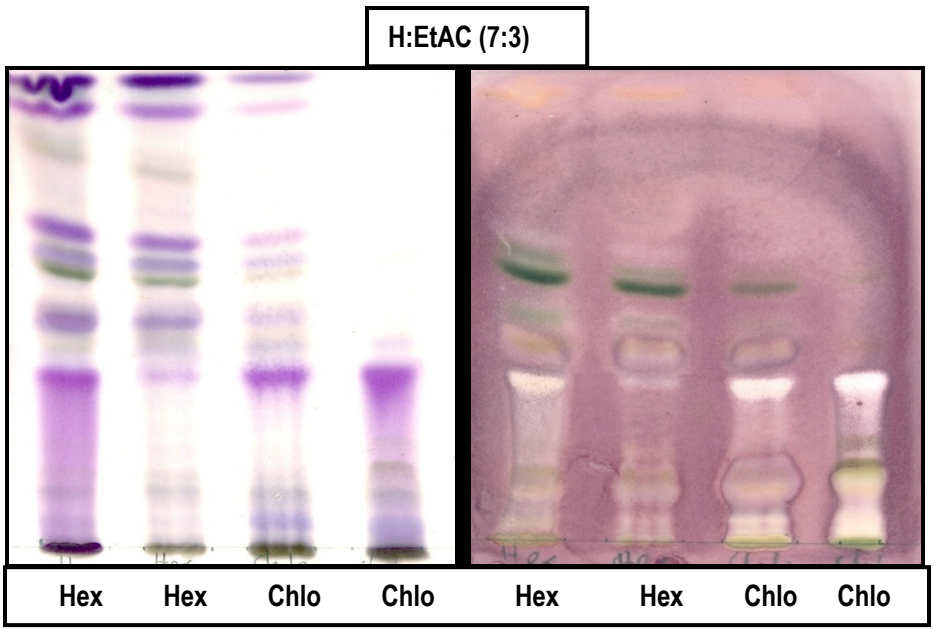


Figure 4.7: Comparison of the hexane (Hex) and chloroform (Chlo) fractions developed in Hexane (H): Ethyl acetate (EtAC) [7:3] and sprayed with vanillin-sulphuric acid (left) and *S. aureus* (right) from duplicate experiments.

4.3.3 MIC values of the five fractions against the tested bacteria.

The MIC values (mg/ml) and total activity (ml/g) of the five fractions of the crude extracts of *F. africana* against *S. aureus, P. aeruginosa, E. coli,* and *E. faecalis* are presented in Table 4.1. The hexane and chloroform fractions had low MIC values and high total activities compared to the other fractions. MIC value as low as 0.02 mg/ml were obtained with the chloroform fraction against *P. aeruginosa* followed by hexane fraction with an MIC value of 0.08 mg/ml against *S. aureus*. The MIC values of the chloroform fraction were higher than that of the crude extract except in the case of *S. aureus,* where the values were the same. As indicated in paragraph 4.3.1 there was excellent quantitative recovery of the crude after solvent-solvent fractionation. The differences may be due to synergistic activities of compounds present together in the crude but separated from each other in the fractions.

Eloff (2004) suggested that by determining the total activity of fractions the loss or gain of activity during fractionation may be detected. Total activity takes the MIC and the quantity present in the crude and fractions into consideration and is calculated by dividing the mass in mg of the crude extract and each fraction with the relevant MIC in mg/ml. The highest total activity was obtained with the chloroform fraction against *P. aeruginosa* (405000 ml) compared to other fractions of *F. africana* as shown in Table 4.2. The total activity in bioassay-guided fractionation helps to determine if there is a loss of biological activity during each step of fractionation and to ascertain the presence of synergistic effects (Eloff, 2004). If one keeps in mind the inaccuracy of the two-fold serial dilution technique to give accurate results by comparing the total activity of the crude with the sum of the total activities of the fractions there was no substantial loss or increase of activity. It is interesting that in the case of the two Gram negative organisms there was an increase of 22 and 25% and when comparing the two Gram positive organisms there was a decrease of 20% in both cases. The main point however is that there appeared to be no evidence of synergism between the different solvent-solvent fractions.

Table 4.1: MIC values in mg/ml of the five fractions: hexane (Hex); chloroform (Chlo); 30%H_2O in methanol; butanol (But); H_2O of *F. africana* against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and, *Enterococcus faecalis.*

Bacterial species	Crude	Hex	Chlo	30% H_2O	But	H_2O
E. coli	0.24	0.16	0.16	0.63	0.63	2.5
E. faecalis	0.12	0.16	0.08	0.63	1.25	1.25
P. aeruginosa	0.08	0.24	0.02	0.32	0.32	2.5
S. aureus	0.08	0.08	0.08	0.32	0.32	1.25

Table 4.2: Total activity in ml of the five fractions: hexane (Hex); chloroform (Chlo); 30%H_2O in methanol; butanol (But); H_2O of *F. africana* against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli,* and, *Enterococcus faecalis*.

Bacterial species	Crude	Hex	Chlo	30% H_2O	But	H_2O	Total of all fractions	% of Crude
E. coli	130583	99375	50625	4920	3809	440	159169	122
E. faecalis	261166	99375	101250	4920	1920	880	208345	80
P. aeruginosa	391750	66250	405000	9688	7500	440	488878	125
S. aureus	391750	198750	101250	9688	7500	880	318068	81

TLC plates of the combined fractions from an open column developed using H:EtAC (7:3) solvent system, sprayed with vanillin-sulphuric acid reagent (right) and sprayed with *S. aureus* (left) are shown in Figure 4.8. Four fractions (F4-7) had activity with a number of bioactive compounds on the bioautogram. Fraction 6 and 7 were combined as they contained similar compounds and investigated further. Fraction 4 and 5 were also combined (800 mg), however the attempts to isolate the antibacterial compounds were unsuccessful. Certain amounts of compounds are generally lost after each step of isolation. Consequently, there were still a number of compounds present in small concentration.

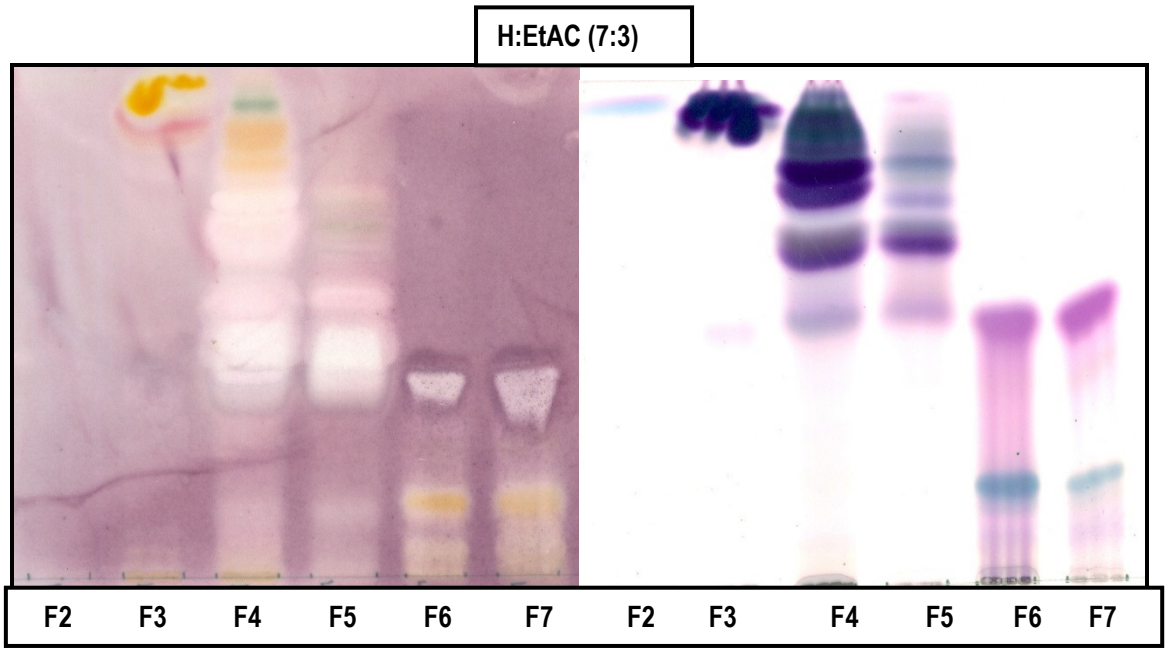


Figure 4.8: Chromatograms of combined fractions after the first open column chromatography on hexane fraction using hexane (H) and ethyl acetate (EtAC) from 100% to 20% EtAC in H at an interval of 5%.

TLC fingerprinting of the second column hexane fraction developed in H:EtAC (7:3) solvent system, sprayed with vanillin-sulphuric acid are shown in Figure 4.9. Fractions 1-6 and fractions 7-28 were combined. Fraction 7-28 (320 mg) was investigated further because it contained the bioactive compounds.

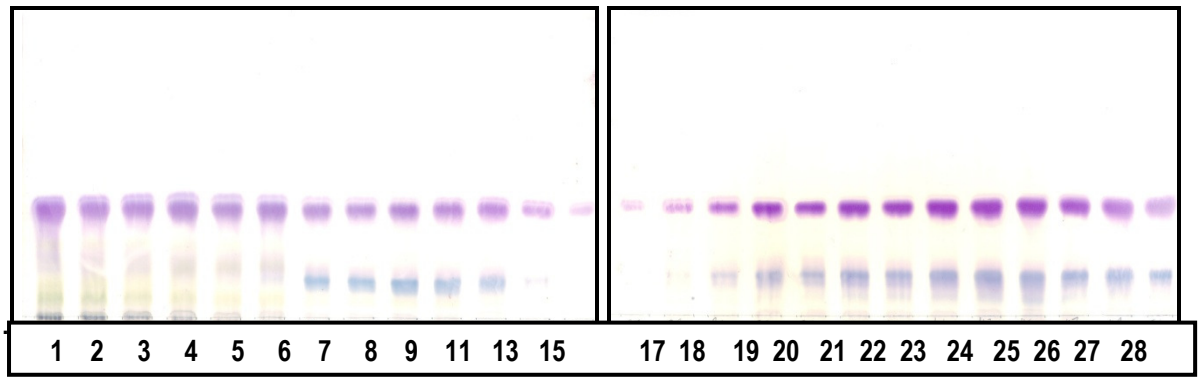


Figure 4.9: TLC fingerprinting of combined fractions$_{6\text{-}7}$ developed in H:EtAC (7:3).

Column 3 of the hexane fraction developed in H:EtAC (7:3), sprayed with vanillin-sulphuric acid are shown in figure 4:10. Fractions 1-17 and fractions 18-27 were combined and dried under a stream of cold air. Fractions 18-27 formed white precipitates (112 mg) which were washed with hexane to remove impurities. The final mass

of the white precipitate was 101 mg. An attempt to isolate other active compounds from combined fractions (1-17) was unsuccessful.

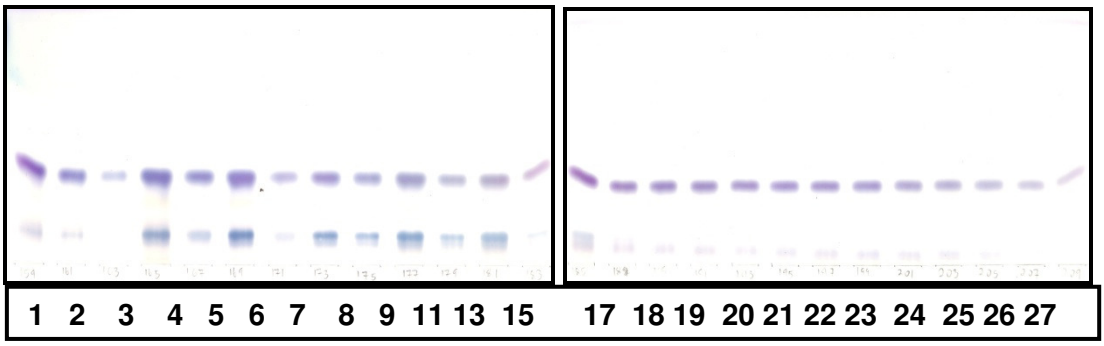


Figure 4.10: TLC fingerprinting of fraction 7-28 (combined), developed in H:EtAC (7:3).

4.4 CONCLUSION

One antibacterial compound was isolated from the hexane fraction of *F. africana* using column chromatography with silica gel as the stationary phase. Attempts to isolate other bioactive compounds from other active fractions were unsuccessful. Some phytochemicals may be lost during each step of isolation when using silica gel in column chromatography.

The only traditional uses we could find for *Funtumia africana* was for treating burns and incontinence *(*Wagner *et al.*, 1987). In treating burns the indication could be for pain relief or for promoting healing/reducing scarring. In the next chapter the anti-inflammatory activity will be examined as a measure for pain relief.

CHAPTER 5

ANTI-INFLAMMATORY ACTIVITY OF THE LEAF EXTRACTS AND FIVE FRACTIONS OF *F. africana*.

5.1 INTRODUCTION

Inflammation occurs in response to tissue damage, characterized by erythema (redness), oedema (swelling), heat and pain. The inflammatory response involves three major events: the dilation of blood capillaries or vasodilation, resulting in increased blood flow to the site of damage; the increase in capillary permeability, causing the leakage of plasma proteins and soluble cell mediators from blood circulation; and the migration of leukocytes through capillary endothelium into the surrounding tissues (Gale *et al*., 2007).

Diseases and disorders are manifested through inflammatory responses as the body recognizes the injury and prepares to repair the damage; as a result the body releases inflammatory mediators. These mediators are substances released as plasma proteins or other cells like mast cells, platelets, neutrophils and monocytes/macrophages (Iwalewa *et al*., 2007). These substances bind to specific target receptors on the cells and may increase vascular permeability, promote neutrophil chemotaxis, stimulate smooth muscle contraction, increase direct enzymatic activity, induce pain and/or mediate oxidative damage (Coleman, 2002).

Examples of chemical mediators are prostaglandins, nitric oxide, leukotrienes, vasoactive amines (histamine, serotonin), and cytokines (tumour necrosis factor and interleukins-1, 12). Prostaglandins are a family of C_{20} fatty acids found widely distributed in almost all living animal cells, tissues and glands (Hickock *et al*., 1985; Rang and Dale, 1987). Many inflammatory diseases are associated with synthesis of prostaglandins, which are responsible for sensation of pain. The principal substrate is arachidonic acid, found as constituent of phospholipids. Arachidonic acid is oxidised by enzyme cyclooxygenase (COX) to prostaglandins (Rang and Dale, 1987). COX occurs in three forms, COX-1, COX-2 and COX-3. COX-1 is constitutively expressed, whereas COX-2 is induced in inflamed tissues. Another catalytically active COX variant, COX-3, derived from an alternative splicing of the COX-1 gene, has been identified in the brain (Chandrasekharan *et al*., 2002).

A number of non-steroidal anti-inflammatory drugs (NSAIDs) have been used to treat pain, fever, and inflammatory conditions such as rheumatoid arthritis, osteoarthritis and other inflammatory syndromes (Gale *et al*., 2007). Since the discovery of the first NSAID, acetylsalicylic acid or aspirin in the nineteenth century, a lot of progress has been made. NSAIDs such as ibuprofen, indomethacin, meclofenamic acid, diclofenac, piroxicam, etadolac, meloxicam are widely available. Some NSAIDs show preferential inhibitory effect towards COX-1 isoform. The selectivity appears to be influenced by the mechanism in which they inhibit COX. NSAIDs are often classified according to the kinetics of interaction with the active site of the COX enzymes that is COX-1 versus COX-2 inhibitors (Ouellet and Percival, 1995; Gierse *et al*., 1999).

In spite of the immense contribution that NSAIDs has made over the years, NSAIDs are associated with a number of side effects. These include damage of the kidneys, worsening asthma, and damage to the upper gastrointestinal tract (Gale *et al*., 2007).

Prostaglandins synthesis-inhibition can be evaluated using an *in vitro* assay originally described by White and Glassman (1974). This assay measures the degree of the COX enzyme activity in prostaglandins synthesis (Mantri and Witiak, 1994; Vane, 1994). The assay has been used widely to evaluate medicinal plant extracts.

Medicinal plants have been used traditionally to treat many conditions including inflammation and pain. *Funtumia africana* has been used traditionally to treat burns in West Africa (Wagner *et al*., 1987). The aim of this study was to evaluate the activity of the crude extract and fractions of *F. africana* on COX1 and COX2 inhibition.

5.2 MATERIALS AND METHOD

5.2.1 Plant material and extraction

The powdered leaves materials of *F. africana* were extracted as described in section 4.2.1. The crude extract was then fractionated into five fractions (water, butanol, hexane, 30% water and chloroform) as shown in Chapter 4, Figure 4.2. All the fractions and crude extract were reconstituted in ethanol.

5.2.2 Anti-inflammatory activity of the crude extract of *F. africana* and its five subfractions.

Anti-inflammatory activity was determined using both the COX-1 and COX-2 assays. The COX-1 bioassay was performed as described by White and Glassman (1974) with slight modifications (Jäger *et al*., 1996). Cyclooxygenase enzyme (3 units protein prepared from ram seminal vesicles [Sigma Aldrich] was mixed with co-factor solution (0.3 mg/ml adrenalin and 0.3 mg/ml reduced glutathione in 0.1 M Tris buffer, pH 8.2) and incubated on ice for 5 minutes. Sixty microlitres of the enzyme and co-factor solution was added to 20 µl of sample (2.5 µl of ethanolic extract and 17.5 µl water) and incubated at room temperature for 5 minutes. Twenty microlitres of [^{14} C] arachidonic acid was added to the assay and incubated in a water bath for 10 minutes at 37ºC. The reaction was terminated by adding 10 µl of 2 N HCl. After incubation, 4 µl of a 0.2 mg/ml carrier solution of unlabelled prostaglandins was added.

The ^{14}C-labelled prostaglandins synthesized during the assay were separated from the unmetabolised [^{14}C] arachidonic acid by column chromatography. Silica gel in eluent 1 (hexane: 1.4-dioxan:acetic acid glacial 350:150:1) was packed in pasteur pipettes to a height of 3 cm. One ml of eluent 1 was added to each of the assay mixtures and the mixture was applied to separate columns. The arachidonic acid was eluted with 4 ml of eluent 1 and discarded. The prostaglandins were subsequently eluted with 3 ml of eluent 2

(ethylacetate:methanol 85:15) into scintillation vials. After mixing with scintillation liquid, the radioactivity was counted using a scintillation counter (Wallac 1409). The same protocol was followed for COX-2 except that in the preparation of co-factor solution, 0.6 mg adrenaline was used. All the five fractions and crude extract were tested at a concentration of 250 µg/ml with double determinations for each extract per assay. In each test assay, four controls were run. Two were background in which the enzyme was inactivated with HCl before the addition of [^{14}C] arachidonic acid, and two were solvent blanks (2.5 µl ethanol and 17.5 µl water). Positive control measurements were carried out by determining the IC_{50} of indomethacin. The percentage inhibition of the fractions and crude extract was calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank. The percentage inhibition (% *I*) of prostaglandin synthesis was calculated as follows:

% *I* = [1- DPM of sample

- DPM of background÷ DPM of blank- DPM of background]
  ×100

Where DPM is the disintegrations min^{-1} (Jin *et al*., 1999).

5.3 RESULTS AND DISCUSSION

The percentage inhibition of prostaglandin synthesis by COX-1 and COX-2 of *F. africana* crude extract and its five fractions with indomethacin as the control are shown in Table 5.1. To classify a plant extract tested at a final concentration of 250 µg/ml as active the inhibition by aqueous extracts tested must be at least 59% and for ethanolic extracts 70% per solution (Fennell *et* al., 2004). The crude extract had a moderate activity for both COX-1 (59.7 ± 1.4) and COX-2 (54.3 ± 0.3). Of the five *F. africana* fractions obtained after solvent-solvent fractionation; hexane and chloroform fractions had moderate activity for both COX-1 and COX-2. The other three fractions had no inhibitory effect on COX-1 or COX-2. The presence of polyphenols, saponins, certain pigments or fatty acids in crude extracts can lead to false positive results in enzyme assays (O'Neill and Lewis, 1993). The constituents of a plant extract may have an inhibitory effect in the assay by denaturing the enzyme, or by acting on the prosthetic group thus inactivating the enzyme. Tannins and phenols have such effects in *in vitro* test (Van Wyk *et al*., 1997). Because only the non-polar fractions were active and polyphenols or saponins would not be expected in the non-polar fractions, it is unlikely that these compounds could have been responsible for the activity. Long chain fatty acids would have been expected in the hexane fraction and may be responsible for the activity in this fraction.

During a bioassay guided fractionation it is important to take the quantity present in each fraction into account and calculate the total activity otherwise incorrect conclusions may be drawn (Eloff, 2004). This approach also makes it possible to determine if there was any loss or increase in total activity of the fractions during the

process. A substantial loss of activity may indicate synergism or inactivation of biologically active compounds. An increase in activity may indicate removal of antagonistic compounds during the process. To calculate the relative total activity of the fractions the percentage of the mass of the fractions on the crude extract was multiplied with the % inhibition (Table 5.1) In the case of COX-1 the two active fractions represented 68% ([2327+1739]/5970 x 100) and in the case of COX-2 75% of the activity of the crude extract (Eloff, 2004). It does therefore appear as if there may have been a loss of activity during the fractionation process. In section 4.3.1 it was shown that there was no significant loss of mass in the solvent-solvent fractionation process. It is possible that some compounds may have been chemically inactivated during fractionation. Because solvent-solvent fractionation is such a mild process, this may not be likely. Another explanation may be that there was a degree of synergism between different components present together in the crude, but not present together in the fractions. When the antibacterial activities of the different fractions were determined however, there was no evidence of synergism.

Table 5.1: Percentage inhibition of COX-1 and COX-2 prostaglandin synthesis by, a crude leaf extract of *F. africana* and its five solvent-solvent fractions (250 µg/ml) from two experiments. Indomethacin was used as a positive control. Relative total activity (RTA) calculated by multiplying the % of crude extract with % inhibition.

Sample	% crude extract	COX-1 % inhibition	COX-2 % inhibition	RTA COX 1	RTA COX 2
Crude extract	100	59.7 ± 1.4	54.3 ± 0.3	5970	5430
Hexane fraction	50.7	45.9 ± 3.2	50.4 ± 9.9	2327	2555
Chloroform fraction	25.5	68.2 ± 6.6	59.1 ± 4.5	1739	1507
30% H_2O fraction	10.0	0	0	0	0
Butanol fraction	7.7	0	0	0	0
H_2O fraction	3.5	0	0	0	0
Indomethacin µM (IC_{50})	-	3.3 ± 0.008 (1.2 ± 0.002 µg)	122 ± 5.7 (43.6 ± 2.0 µg)	-	-

Values are the mean ± S.E.M percentages of results obtained from two experiments

Although the crude extract together with hexane and chloroform fractions had moderate activity, neither the crude extract nor the two fractions had selective activity against either COX-1 or COX-2 as shown in Table 5.1. More emphasis is on finding NSAIDs which selectively inhibit COX-2 with little interference of COX-1. Compounds which are selective inhibitors of COX-2 may have anti-inflammatory and nonulcerogenic activity and are therefore of considerable interest for therapeutic use (Mantri and Witiak, 1994). However, certain

precautions must be taken because COX-2 selective inhibitors can cause thrombosis and hence heart attack (Bombardier *et al*., 2000; Mukherjee *et al*., 2001; Mamdani *et al*., 2004).

5.4 CONCLUSION

The anti-inflammatory activity of extracts is considered highly active with values between 70-100% and moderately active between (40-70%) (Taylor and Van Staden, 2002). The leaf extract of *F. africana* together with hexane and chloroform fraction had moderate activity against both COX-1 and COX-2. The chloroform fraction was more active than the crude extract. The highest percentage inhibition was obtained with the chloroform fraction for COX-1 (68. 2 ± 6.6). This indicates that the compounds with intermediate polarity are responsible for the activity. It is unlikely that the use of *F. africana* for pain relief can be ascribed to COX-1 or COX-2 inhibition because traditional healers usually only have water available as an extractant and the polar fractions of the crude extract had no activity against these enzymes. It is however possible that plant extracts without high activity in the cyclooxygenase assay might still exert their anti-inflammatory activity through other mechanism such as nitric oxide, leukotrienes, vasoactive amines and cytokines (McGaw *et al*., 1997).

CHAPTER 6

STRUCTURE ELUCIDATION

6.1 INTRODUCTION

The structure of a purified biologically active compound can be determined using information from various spectroscopic techniques. The most common techniques for elucidating natural products involve nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS) to determine the molecular weight of the isolated compounds (McRae *et al.,* 2007). The NMR has been a major advantage in determining the structure of unknown compounds since samples can generally be recovered after analysis (McRae *et al.,* 2007). The MS alone is unable to characterize complex natural products due to the occurrence of complex mixtures of conjugates (glycosides) and aglycones present in natural products (Yang, 2006). The other spectroscopic techniques used in determining the structure of isolated compounds includes infrared (IR) spectroscopy to ascertain functional groups, High performance liquid chromatography (HPLC), Ultraviolet (UV) together with other spectroscopic methods.

6.2 MATERIALS AND METHODS

The structure of the antimicrobial isolated compound from the leaves of *F. africana* was elucidated using Varian-NMR-vnmrs 600 from CSIR, BIO-Chemtek for both ^{1}H and ^{13}C, using dimethyl sulphoxide (DMSO) as the solvent and MS. The spectroscopic data for the structure was interpreted by Dr N Moodley from CSIR and Mr A. Aroke from University of Pretoria (Phytomedicine Programme).

6.3 RESULTS AND DISCUSSION

6.3.1 Identification of the isolated compound

The ^{13}C NMR spectrum showed the presence of 30 resonances suggesting that the molecule is a 5- ring membered triterpenoid (Table 5.1). The resonance at δ_C 178.7 is characteristic of an acetyl group occurring at C-28. The resonances at δ_C 125.0 and δ_C 138.6 are characteristic of C-12 and C-13 respectively for ursane type triterpenoids. The DEPT spectrum was not clear and therefore the carbon resonances could not be resolved.

The methine carbon resonance at δ_C 77.3 showed HMBC correlations to two methyl resonances at δ_H 0.66 and δ_H 0.88. Both these methyl resonances showed HMBC correlations to another methine carbon at δ_C 55.2. The methyl groups were therefore assigned to 3H-23 (δ_H 0.88) and 3H-24 (δ_H 0.66) upon comparison with literature values. Both the 3H-23 and 3H-24 showed a correlation to a fully substituted carbon resonance at δ_C 38.8,

which was assigned to C-4. The methine resonance at δ_C 55.2 also showed a correlation to the methyl resonance at δ_H 0.85. This resonance at δ_C 55.2 was therefore assigned to C-5, the methyl resonance at δ_H 0.85 to 3H-25 and the other methine resonance at δ_C 77.3 was therefore assigned to C-3.

The 3H-25 resonance showed a correlation to a methine resonance at δ_C 47.5 and a fully substituted carbon at δ_C 37.0, which were assigned to C-9 (δ_C 47.5) and C-10 (δ_C 37.0) respectively. The C-9 resonance showed a correlation with a methyl resonance at δ_H 0.72 which showed a further correlation to a fully substituted carbon resonance at δ_C 42.1 and a methylene carbon resonance at δ_C 33.1. The methyl resonance was assigned to 3H-26 (δ_H 0.72) and the methylene resonance to C-7 (δ_C 33.1). The fully substituted carbon resonance at δ_C 42.1 also showed a correlation to a methyl proton resonance at δ_H 1.02 which showed a correlation to a methylene carbon resonance at δ_C 28.0. The methyl resonance was assigned to 3H-27 (δ_H 1.02) and the methylene to C-15 (δ_C 28.0). The fully substituted carbon resonance can be assigned to C-8 or C-14. This resonance was assigned to C-14 (δ_C 42.1) upon comparison with similar compounds.

The methyl resonance at δ_H 0.80 showed a correlation to a methine carbon resonance at δ_C 52.8 and a methine resonance at δ_C 38.9. This methyl resonance was assigned to 3H-29 (δ_H 0.80) and the methine resonances to C-18 (δ_C 52.8) and C-19 (δ_C 38.9). The remaining methyl proton resonance at δ_H 0.90 was assigned to 3H-30 by the process of elimination because this was the only unassigned methyl resonance of the seven.

All methyl resonances, two methylene, six methine and five fully substituted resonances were fully assigned using HMBC correlations. The remaining resonances were assigned upon comparison with available NMR data for methyl ursolate (*Seo et al*., 1975). Seo *et al*. (1975) used ^{1}H single-frequency off-resonance decoupling experiments to assign the ^{13}C resonances. This is the reason why the chemical shifts of C-19 and C-20 and C-24 and C-25 were reported to be interchangeable. Despite the differences in the methods used in elucidating the structure of this compound, the values compare very well with only two carbon resonance (C-3 and C-24) differing by approximately 1 ppm.

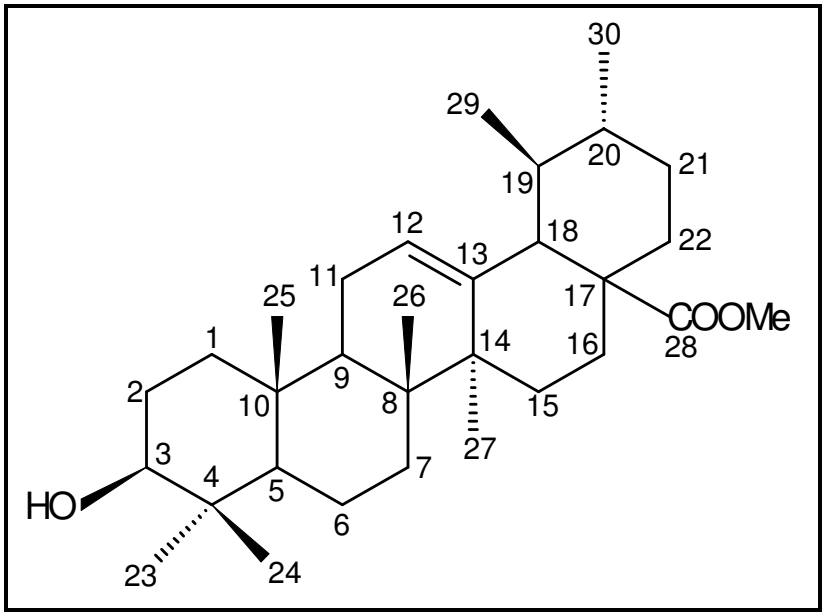


Figure 6.1: The structure of methyl ursolate isolated from the leaves of *Funtumia africana.*

The mass spectrometry revealed that the methyl ursolate isolated had the molecular weight of 470.330^{+} which corresponds to molecular formula of $C_{31}H_{50}O_3$ as shown in Figure 6.4, which corresponded to the mass spectrum of methyl ursolate isolated from the almond hulls (Takeoka *et al*., 2000). Furuya *et al* (1987) also reported methyl ursolate with mass spectrum which closely matched the 5-ring membered triterpenoid isolated from the leaves of *F. africana*.

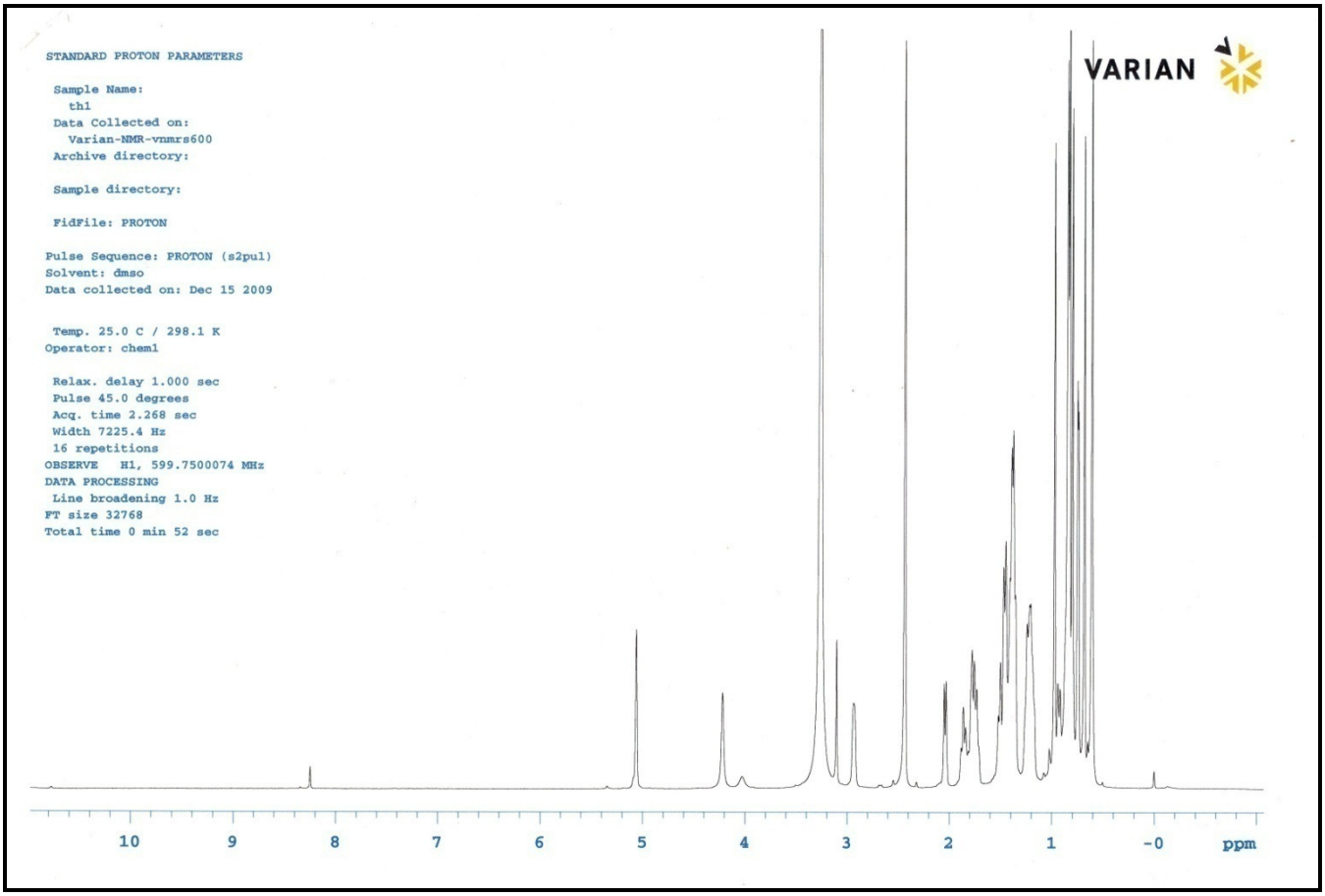


Figure 6.2: ^{1}H NMR spectrum of the methyl ursolate isolated from the leaves of *F. africana*.

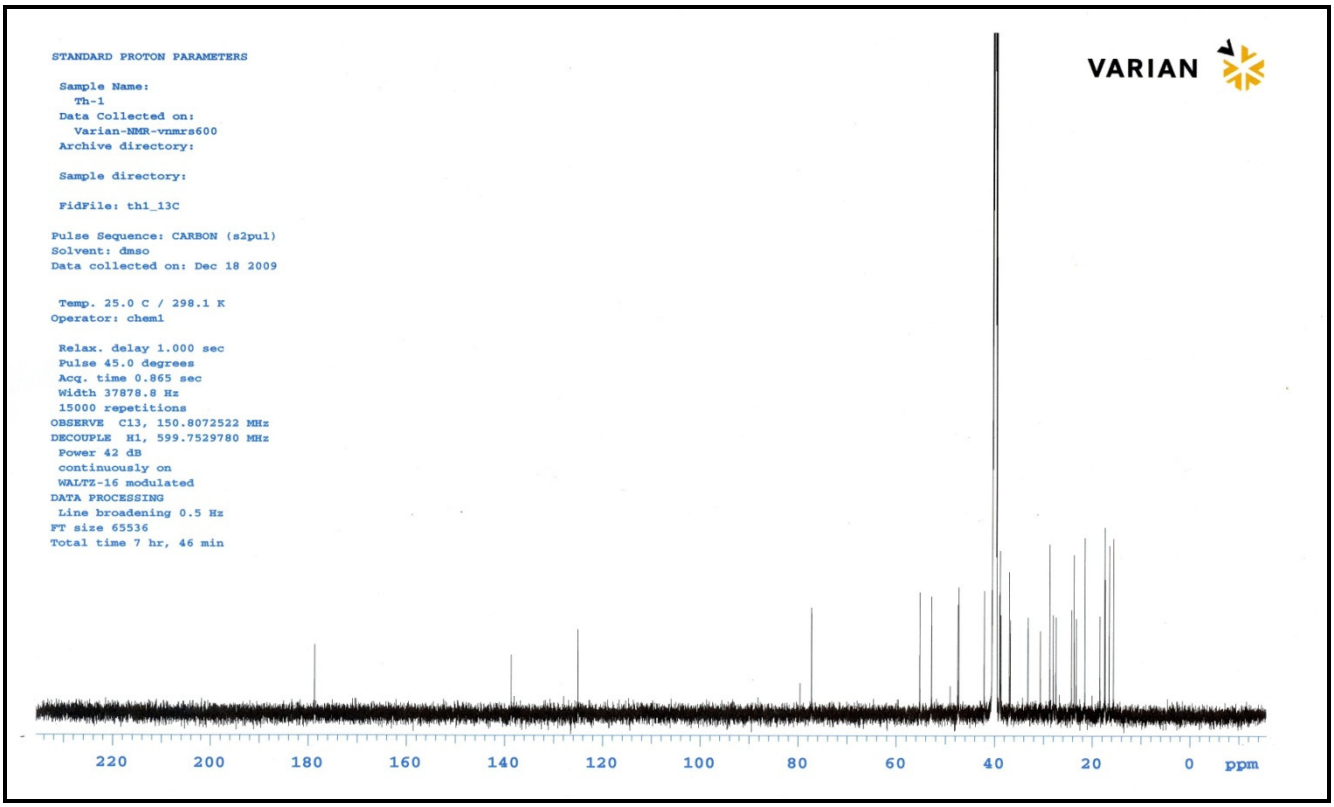


Figure 6.3: ^{13}C NMR spectrum of the methyl ursolate isolated from the leaves of *F. africana*.

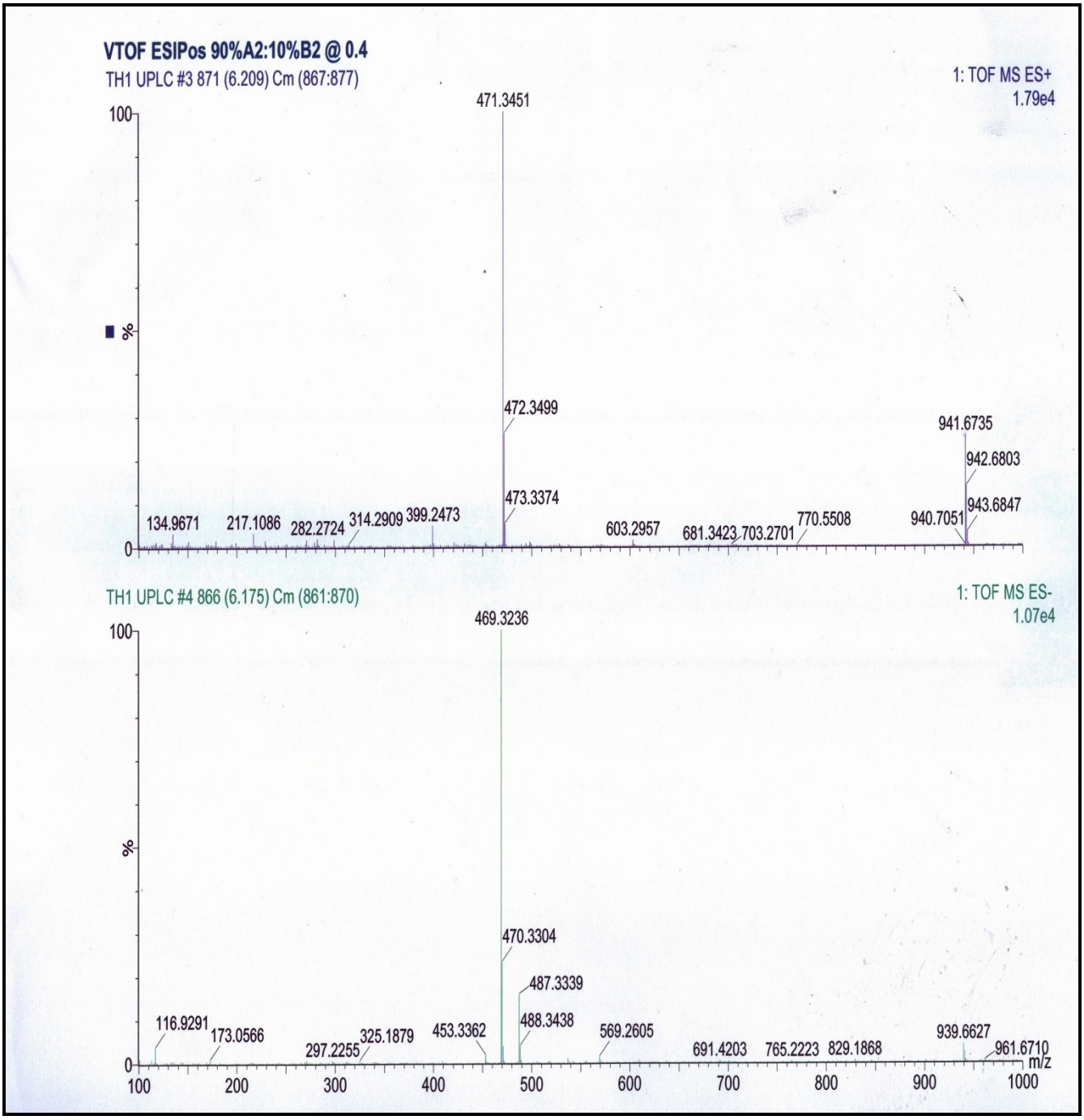


Figure 6.4: Mass spectrum of the methyl ursolate isolated from the leaves *F africana*.

Table 6.1: ^{1}H and ^{13}C NMR data for methyl ursolate (600 M*Hz*, DMSO, room temperature).

No.	^{1}H (ppm)	^{13}C (ppm)	$^{13}C^{*}$ (ppm) (Literature)	HMBC (C ⟶ H)
1		38.8 (CH_2)	38.8	
2		27.4 (CH_2)	27.3	
3	2.98 (*s*)	77.3 (CH)	78.8	23, 34
4		38.8 (C)	38.8	23, 24
5		55.2 (CH)	55.4	23, 24
6		18.4 (CH_2)	18.4	
7		33.1 (CH_2)	33.0	26
8		38.9 (C)	39.6	
9		47.5 (CH)	47.5	25, 26
10		37.0 (C)	37.0	25
11		23.7 (CH_2)	23.3	
12	5.20 (*s*)	125.0 (CH)	125.5	
13		138.6 (C)	138.0	
14		42.1 (C)	42.0	26, 27
15		28.7 (CH_2)	28.2	27
16		24.2 (CH_2)	24.3	
17		47.3 (C)	48.1	
18	2.08 (*d*, *J* = 11.46 *Hz*)	52.8 (CH)	52.8	29
19		38.9 (CH)	39.1	29
20		38.8 (CH)	38.8	
21		30.6 (CH_2)	30.7	
22		36.8 (CH_2)	36.7	
23	0.88 (*s*)	28.7 (CH_3)	28.2	24
24	0.66 (*s*)	16.5 (CH_3)	15.5	23
25	0.85 (*s*)	15.6 (CH_3)	15.7	
26	0.72 (*s*)	17.3[a] (CH_3)	16.9	
27	1.02 (*s*)	23.7 (CH_3)	23.6	
28		178.7 (C)	177.7	
29	0.80 (*s*)	17.4[a] (CH_3)	16.9	
30	0.90 (*s*)	21.5 (CH_3)	21.2	

* Seo *et al*. (1975), (15.09 M*Hz*, $CDCL_3$ at room temperature), [a] values are interchangeable.

CHAPTER 7

ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES OF THE ISOLATED METHYL URSOLATE

7.1 INTRODUCTION

Medicinal plants generally contain mixtures of different chemical compounds that may act individually or in synergy to improve health (Gurib-Fakim, 2006). Synergy is an adaptive strategy in plant defence that may provide insight into the diversity and function of secondary metabolites. Synergy is defined as an interaction among compounds which produces a greater effect based on individual activities (Mckey 1979; Berenbaum, 1985). Most effective phytomedicines on the drug market are available as whole extracts of plants, and practitioners have always believed that synergistic interactions between the components of individual or mixtures of herbs are a vital part of their therapeutic efficacy (Williamson, 2001).

Although phytochemicals are often less effective than retained in the mixtures present in plant (Cott and Misra, 1999), isolated compounds have made an important contribution in drug discovery. Isolated compounds from plants, either new or known can also provide new biological leads for drug discovery (Kramer and Cohen, 2004), especially for plant species which were not investigated for a wide range of biological activity. The search for compounds exhibiting a spectrum of activity complementary to existing drugs remains a task to be pursued, even though no plant derived compounds has been found, which can compete with antibiotics (Taylor *et al*., 2001).

7.2 MATERIALS AND METHODS

7.2.1 Phytochemical analysis of the isolated compound

TLC fingerprinting was performed as described in chapter 3, section 3.2.2. The isolated methyl ursolate was dissolved in acetone to a concentration 2.5 mg/ml and 1.25 mg/ml and then 10 µg was loaded on the TLC plate. The plate was then developed in H:EtAC (7:3) solvent system before sprayed with vanillin-sulphuric acid.

7.2.2 Bioautography of the isolated compound

Bioautography was carried out as in chapter 3, section 3.2.4. The isolated methyl ursolate was dissolved in acetone to concentrations of 2.5 mg/ml and 1.25 mg/ml, and 10 µg was loaded on TLC plates. The plates were developed in H:EtAC (7:3) solvent system and allowed to dry overnight before spraying with overnight culture suspensions of the bacteria. The compound was tested against the four bacteria (*E. coli*, *E. faecalis*, *P. aeruginosa,* and *S. aureus)*. The red colour indicated the growth of the bacteria and clear zones indicated inhibition of bacteria by the compound.

7.2.3 Minimal inhibitory concentration (MIC) of the isolated methyl ursolate

7.2.3.1 Antibacterial assay

The MIC values were determined using the same method described in chapter 3, section 3.2.5. However, the isolated compound dissolved in acetone to a concentration of 2.5 mg/ml.

7.2.3.2 Antifungal assay

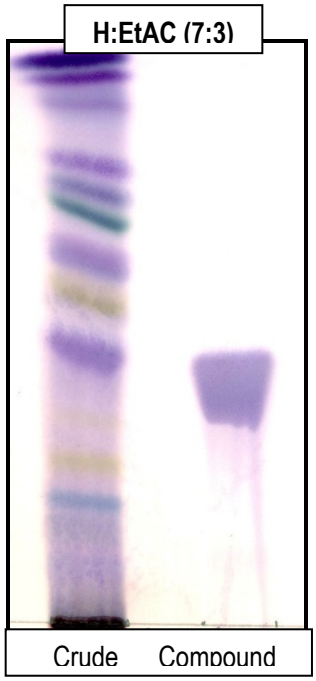
A serial microdilution assay (Eloff, 1998) with slight modification (Masoko *et al.,* 2005) was used to determine the minimum inhibitory concentration value of the compound using tetrazolium violet reduction as an indicator. Five pathogenic fungi obtained from the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science were used as test organisms namely; yeasts (*Candida albicans* and *Cryptococcus neoformans*) and moulds (*Fusarium oxysporum, Penicillium janthinellum,* and *Rhizoctonia solani*).

Residues of the isolated compound and the crude extracts were dissolved to a concentration of 1 mg/ml and 10mg/ml respectively. Exactly 100 µl of the compound and the extracts were serially diluted with 50% water in 96-well microtitre plates. Fungal cultures were transferred into fresh Sabouraud dextrose broth, and 100 µl of this was added to each well. Amphotericin B was used as the positive control. As an indicator of growth, 40 µl of 0.2 mg/ml of INT dissolved in water was added to each of the microplate wells. The covered microplates were incubated for 2 to 3 days at 35 ºC and 100% relative humidity. The MIC was recorded as the lowest concentration of the compound and extracts that inhibited antifungal growth after 24 and 48 hours.

7.3 RESULTS AND DISCUSSION

7.3.1 Phytochemical analysis

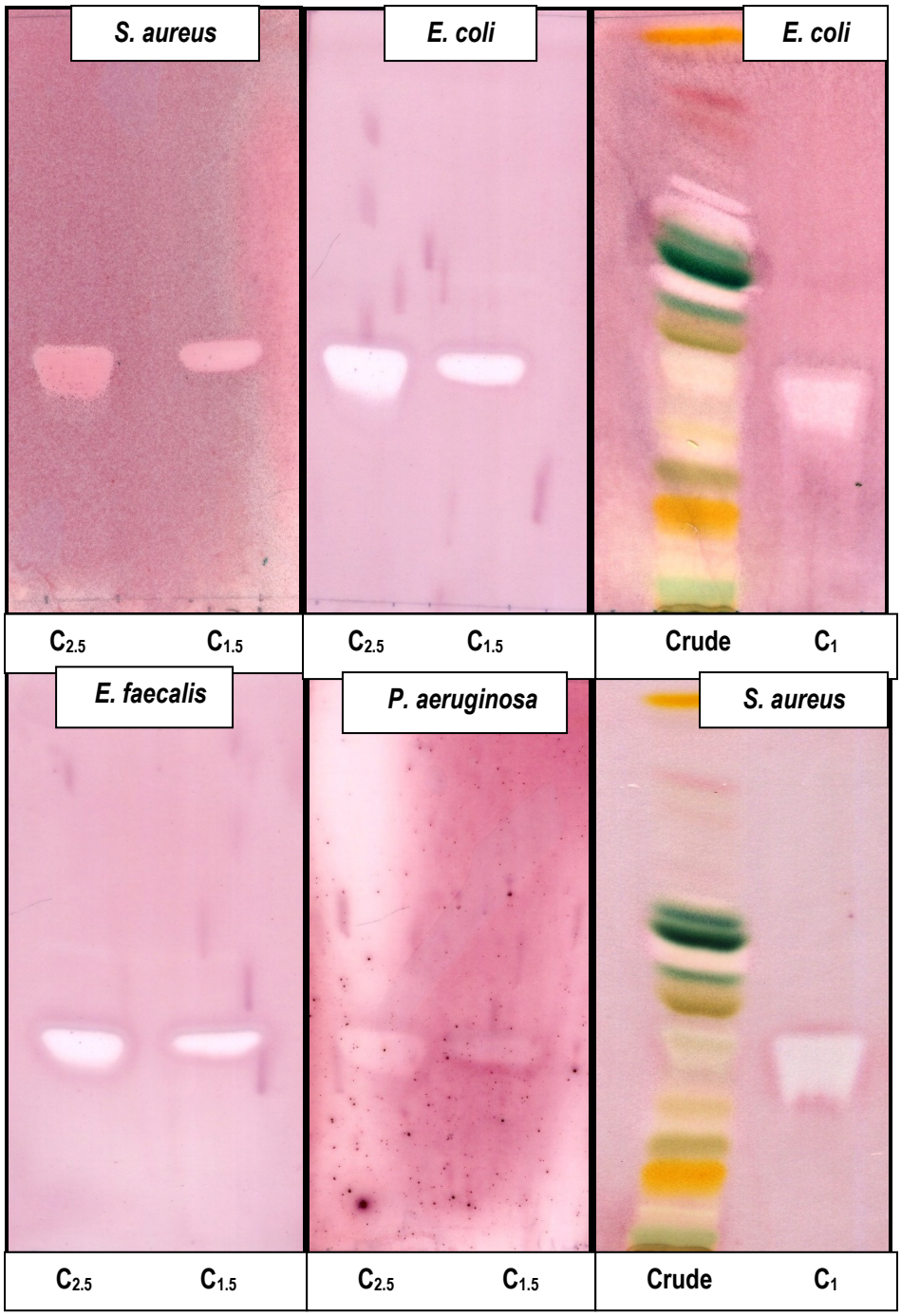
The TLC fingerprinting of the extracts and methyl ursolate as shown on Figure 7.1 was used to localize the compound in the extract. The isolated white powdered compound was not visible under the ultraviolet light at both 254 nm and 360 nm wavelengths.



7.1: TLC fingerprinting of crude extract of *F. africana* and the isolated methyl ursolate developed in H:EtAC (7:3) solvent system and sprayed with vanillin sulphuric-acid.

7.3.2 Bioautography of the methyl ursolate

The TLC bioautograms of the isolated methyl ursolate tested against *E. coli, E. faecalis*, *P. aeruginosa* and *S. aureus* compared to the crude extract of *F. africana* are shown in Figure 7.2. The isolated compound had activity against all the four tested bacteria when 12.5 µg was used for bioautography. The same R_f values of the isolated methyl ursolate with the active compound in the crude extract indicates that the compound isolated with clear white zones of inhibition is not an artefact of the isolation process.



7.2: TLC bioautograms of the isolated methyl ursolate at different concentrations ($C_{2.5}$, 25 µg and $C_{1.5}$, 12.5 µg), and sprayed with four different bacterial suspensions compared to the bioautography of the crude extract of *F. africana* developed in the same eluent system [H:EtAC (7:3)], The red colour indicates the growth of the bacteria, while clear zones indicate the bacterial inhibition by the compound.

7.3.3 Minimal inhibitory concentration of the isolated compound

7.3.3.1 Antibacterial and antifungal activity of methyl ursolate isolated from *F. africana*

The MIC values of the isolated methyl ursolate tested against four bacteria are shown in Table 7.1. Isolated compounds with antimicrobial activities of 64-100 µg/ml are accepted as having clinical relevance (Gibbons, 2004). Furthermore, compounds MIC values of 10 µg/ml or less are noteworthy (Rios and Recio, 2005). Ojinnake and Kenne (1985) also reported the isolation of methyl ursolate from the stem of *Myrianthus arboreus.* The isolated methyl ursolate from the leaves of *F. africana* was less active with MIC values of >250 µg/ml against all the four tested bacteria. Haraguchi *et al* (1999) reported the antibacterial activity of ursolic acid for *S. aureus* (>200 µg/ml), *E. coli* (>200 µg/ml), *P. aeruginosa* (100 µg/ml). The crude extract of *Funtumia africana* had other antibacterial compounds in small concentration as shown in Chapter 3, Section 3.3.3, and Figure 3.3. It is important to keep in mind the complexity of plant extracts and that a single compound may not be responsible for the activity but rather a combination of compounds (either major or minor) interacting in an additive or synergistic manner (Van Vuuren, 2007). As a result, the possibility that the higher antibacterial activity on the crude extract compared to the isolated methyl ursolate may be due to synergistic interactions of different compounds in the crude extract should not be ruled out. Ursolic acid has a low water-solulibility, which results in low biological activity (Baglin *et al*., 2003). Three alkaloids have been isolated from the stem bark of the same *F. africana* (Wagner *et al*., 1987).

The triterpenoids are large and structurally diverse group of natural products derived from squalene or related acyclic 30-carbons precursors (Connolly and Hill, 2002). Most triterpenoids are tetracyclic, pentacyclic, or pentacyclic, but acyclic, monocyclic, bicyclic, and hexacyclic triterpenoids have also been isolated from natural sources (Xu *et al*., 2004).

Ursolic acid (also known as (3β)- hydroxyous- 12- en-28-oic acid) has relative similar chemical structure to other terpenes and particularly hydroxyl pentacyclic triterpenoic acids such as oleanolic acid (3β- 3- hydroxyolean- 12- en- 28- oic acid) and betulinic acid (3β- 3- hydroxylup- 20(29)- en- 28- oic acid) (Fontanay *et al*., 2008). It is reported that both oleanolic and ursolic acid have beneficial and notable effects on hepatoprotection, anti-inflammation, anti-tumour promotion, and anti-hyperlipidemia (Liu, 1995). The antibacterial, antifungal and cytotoxicity activity of ursolic acid has also been reported (Haraguchi *et al*., 1999; Fontanay *et al*., 2008; Shai *et al*., 2008). Ursolic acid had moderate to good antibacterial activity against *E. coli* ATCC 25922 (>256 mg/L), *P. aeruginosa* ATCC 27853 (256 mg/L), and *E. faecalis* ATCC (4 gm/L), and *S. aureus* ATCC 29213 (8 mg/L) (Fontanay *et al*., 2008). Methyl ursolate differs to ursolic acid only due to the presence of a methyl group at C-28, instead of the COOH carboxyl group. The esterification of 28-COOH is essential for activity of triterpenoids (Ma *et al*., 2003).

Table 7.1: The minimal inhibitory concentration (MIC) in µg/ml of the isolated methyl ursolate (MU) from the leaves of *F. africana* tested against the four bacterial strains, with gentamicin as the positive control.

Microorganisms	Crude extract	MU	Gentamicin
E. coli	240	> 250	8.0
E. faecalis	120	> 250	1.6
P. aeruginosa	80	> 250	0.2
S. aureus	80	> 250	0.3

MIC values of the isolated methyl ursolate from *F. africana* against five pathogenic fungi are recorded in Table 7.2. The methyl ursolate was generally less active than the crude extract in some cases against the tested fungi with MIC values ranging from 63 µg/ml to 250 µg/ml. The isolated compound had a higher activity on *F. oxysporum* with MIC value of 63 µg/ml. The MIC value for *C. albicans* of 250 µg/ml is consistent with the results of Haraguchi *et al* (1999) for the same fungal pathogen with MIC value of >200 µg/ml. The crude extract had good antifungal for *C. neoformans* compared to the pure isolated compound, which might be due to synergistic effect of the active and non-active compounds in the crude extract. The results are consistent with others reported on other species of the same genus, such as *F. elastica* which has been found to posses antifungal activity (Adekunle and Ikumapayi, 2006).

Table 7.2: MIC in µg/ml of methyl ursolate (MU) isolated from *F. africana* tested against five pathogenic fungi (*Candida albicans*, *Cryptococcus neoformans, Fusarium oxysporum, Penicillium janthinellum,* and *Rhizoctonia solani*).

Microorganisms	MIC values (24 hours)		MIC values (48 hours)		
	Crude	MU	Crude	MU	Amphotericin B
C. albicans	160	125	160	250	0.16
C. neoformans	80	125	320	125	0.16
F. oxysporum	80	62.5	320	125	0.2
P. janthinellum	320	> 250	630	> 250	0.2
R. solani	320	> 250	1250	> 250	0.2

Because the antibacterial activity of the methyl ursolate was so low, it did not appear to make sense to determine the cellular toxicity of this compound.

7.3 CONCLUSION

The methyl ursolate isolated from the leaves of *F. africana* had antibacterial activity with clear zones of inhibition against all the tested bacteria on bioautography. However, the isolated compound had very low antimicrobial activity with MIC values of >250 µg/ml against four bacteria and MIC values ranging from 63 µg/ml to 250 µg/ml against the fungi.

The MICs of the closely related ursolic acid against *S. aureus* (63 µg/ml), *E. coli* (250 µg/ml), *E. faecalis* (47 µg/ml), and *P. aeruginosa* (4 µg/ml) were much better than the values of methyl ursolate (Shai et al. 2008). The antifungal activity of ursolic acid against the same strains of *C. albicans* (63 µg/ml) and *C. neoformans* (63 µg/ml) were also higher than the activity of methyl ursolate. The methyl group attached to C-28 ($COOCH_3$) instead of the carboxylic group (COOH) therefore leads to a strong reduction in antibacterial activity.

Based on the data from the total activity of the different fractions it does not appear as if there was any loss of activity during the preliminary fractionation process. The situation was drastically different when the activity of the chloroform crude fraction (20 µg/ml) was compared to the very low (>250 µg/ml) activity of the isolated methyl ursolate. This provides strong evidence for synergistic interactions between different active and/or inactive compounds in the crude extract. Compounds that are inactive when separated from others in bioautography may have an effect on the absorption and/or metabolism of the active compound in the microorganism. In this study bioautography was used as a measure of antibacterial activity in the bioassay guided fractionation. If the MIC values of fractions were determined and total activity was calculated, the large loss of activity would have been noticed. Although there are some difficulties to determine the MIC values of many fractions, this appears to be a more reasonable approach to isolate antimicrobial compounds.

In this study the MIC of the chloroform fraction was very promising 20 µg/ml and the compound isolated from this fraction had an MIC more than 10 times lower (>250 µg/ml). This system may therefore be a good model to evaluate the mechanism of synergism.

CHAPTER 8

GENERAL CONCLUSIONS

The main aim of the study was to evaluate the antibacterial activity of the leaf extracts of nine different tree species selected from Phytomedicine Programme Database and then isolate and characterize the bioactive compounds from the most promising plant species. To attain this aim a number of objectives were formulated. The results obtained in addressing the different objectives of the study are discussed below.

8.1 Preliminary screening of leaf extracts of nine plant species for antibacterial activity

In the course of the study, acetone leaf extracts of nine different plant species were investigated for antibacterial activity using a serial microdilution method and direct bioautography. Bacterial strains used for the study were *E. coli*, *E. faecalis*, *P. aeruginosa* and *S. aureus*. *F. africana* and *O. tenuiloba* had the best activity against the four tested bacteria with MIC values as low as 0.08 mg/ml against *S. aureus*. On bioautograhy assay, *F. africana* had consistent activity with clear zones of inhibition on TLC plates against the four tested bacteria while *O. tenuiloba* had selective activity on *P. aeruginosa*. *F. africana* was then chosen for further studies.

8.2 Isolation of antibacterial compound(s) from the leaf extracts of *F. africana*

Bulk powdered leaves materials of *F. africana* were extracted with acetone. The acetone extract was fractionated using liquid-liquid fractionation to group the compounds based on polarity. Five fractions namely: hexane, chloroform, 30% H_2O in methanol, H_2O and butanol were collected. All the fractions were tested for antibacterial activity. The hexane and chloroform fractions had clear zones of inhibition on bioautography against *S. aureus*. The chloroform fraction was very active with an MIC value of 0.02 mg/ml. Based on TLC fingerprinting and bioautography the active compounds for both hexane and chloroform appeared to be the same. One antibacterial compound was isolated from hexane fraction using column chromatography with silica gel as the stationary phase. The mobile phase used was a gradient of hexane:ethylacetate with an increasing polarity.

8.3 Anti-inflammatory activity of the leaf extracts and five fractions of *F. africana*

The only traditional uses of *F. africana* we could find were to treat burns and incontinence. In treating burns the indication could be for pain relief or for promoting healing/reducing scarring. As a result, the anti-inflammatory activity of the *F. africana crude* extract and its five sub-fractions were determined using the both the COX-1 and COX-2 cyclooxygenase assay (White and Glassman, 1974). The crude leaf extract together with hexane and chloroform had moderate activity against both COX-1 and COX-2.

This indicates relatively non-polar compounds are responsible for the activity. Traditional healers usually only have water available as an extractant and the polar fractions of the crude extract had no activity. It is therefore unlikely that the use *F. africana* for pain relief can be attributed to COX-1 and COX-2 inhibition. Because pain relief is based on a quick response one would have expected effectivity in determining anti-inflammatory activity. However, plant extracts might still exert anti-inflammatory activity through other mechanisms.

8.4 Structure elucidation

With the help of colleagues of the Phytomedicine Programme and the CSIR the structure of the isolated compound from the leaves of *F. africana* was identified using NMR and mass spectroscopy as methyl ursolate. Methyl ursolate is a derivative of ursolic acid that differs by the presence of methyl group at C-28, instead of the carboxylic group. My results confirm the conclusion that the carboxylic acid is required for enhanced pharmacological activity of ursolic acid (Kashiwada *et al.,* 2000; Ma *et al.,* 2000).

8.5 Antibacterial and antifungal activities of the isolated methyl ursolate

The isolated methyl ursolate was tested for antibacterial activity against four bacteria *(E coli*, *E. faecalis*, *P. aeruginosa* and *S. aureus)* and antifungal activity against five pathogenic fungi (*C. albicans, C. neoformans, F. oxysporum, P. janthinellum* and *R. solani)*. Bioautography was used to localize the isolated methyl ursolate.

The isolated methyl ursolate had consistent activity against all the tested bacteria in bioautography with clear zones of inhibition at a concentration of 12.5 µg /ml. However, the compound was less active quantitatively with MIC values >250 µg/ml against all the tested bacteria. The isolated compound was also relatively inactive against the tested pathogenic fungi with MIC values ranging from 63 µg/ml to 250 µg/ml. The only noteworthy activity of methyl ursolate was against *F. oxysporum* with MIC value of 63 µg/ml.

Although three steroidal alkaloids have been isolated from the stem bark of *F. africana,* there was apparently no work done on leaves of the species. As far as our literature survey could ascertain this is the first report for *in vitro* antibacterial, antifungal and anti-inflammatory activity of *F. africana.* For sustainable utilization, leaves may be used especially if their activity is not significantly different from the activity of the bark and roots of the same plant species.

By determining the mass and total activity of the crude extract and the different solvent-solvent fractions it was clear that there was no substantial loss during the preliminary fractionation process. When the R_f value of the methyl ursolate was compared to the active compound present in the crude extract, it was clear that the methyl ursolate was present in the original crude extract as one of the few major antimicrobial compounds. Yet the MIC of the chloroform fraction (20 µg/ml) was more than a hundred times lower than the MIC of methyl ursolate

(>250 µg/ml). This provides very strong evidence for synergistic activities and this may be a good model system to investigate synergism in antimicrobial activities.

CHAPTER 9

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