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Chemical and biological characterization of antibacterial compounds present in *Ochna pretoriensis* (Ochnaceae) leaf extracts

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Declaration

The research presented in this report was carried out in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria under the supervision of Prof J.N. Eloff and Dr B.B. Samuel.

I declare that this thesis submitted is a result of my own investigations except where the work of others is acknowledged and has not been submitted to any other institution.

……

Tshepiso Makhafola.

Date

……..

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A person standing alone can be attacked and defeated, but two can stand back-to-back and conquer. Three are even better, for a triple-braided cord is not easily broken**.-Ecclesiastes 4 v 12**

I believe the choice to be excellent begins with aligning your thoughts and words with the intention to require more from yourself.-**Oprah Winfrey**

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List of abbreviations

ADA	antibiotic feed additives
AFA	antibiotic feed additives
ATCC	American Type culture collection
BEA	Benzene/Ethanol/Ammonia
BEA	Benzene/Ethanol/Ammonia
CC	Column Chromatography
COSY	Correlated spectroscopy
DEPT	Distortionless enhancement by polarization transfer
DNA	Deoxyribonucleic acid
EU	European Union
EMW	Ethylacetate/Methanol/Water
HMBC	Heteronuclear bond correlation
HMQC	Heteronuclear multiple quantum correlation
INT	p-Iodonitrotetrazolium violet
MIC	Minimal Inhibitory Concentration
MRSA	methicilin-resistant staphylococcus aureus
MEM	Minimal essential medium
MTT	3-(4, 5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide
M-H	Müller-Hilton
4-NQO	4-nitroquinoline 1-oxide
NMR	Nuclear magnetic resonance
OF	Ochnaflavone
OFME	ochnaflavone 7-O- methyl ether
SS	ß-Sitosterol
Rf	Retention factor
SD	Standard Deviation
S-S	Solvent-solvent
TA	Total Activity
TLC	Thin Layer Chromatography
UV	Ultra violet
WHO	World Health Organization

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Abstract

In preliminary work done in a tree leaf screening project in the Phytomedicine Programme (*www.up.ac.za/phyto*) *Ochna pretoriensis* acetone leaf extracts had good antibacterial activity against several important bacterial pathogens. The main aim of this study was to isolate and characterize antibacterial compounds present in the acetone leaf extract of *Ochna* species growing in South Africa. In a preliminary screening, the minimum inhibitory concentration of acetone leaf extracts of *Ochna natalitia*, *Ochna pretoriensis*, *Ochna pulchra*, *Ochna gamostigmata,* and *Ochna. serullata,* against *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* were determined by using a serial microplate dilution assay. The number of antibacterial compounds in the extracts was also determined by bioautography against the same bacteria. The MIC values of the five species ranged from 0.039 mg/ml to 1.25 mg/ml. The lowest average MIC values observed were for *O. pretoriensis* especially against *E. faecalis* and *E. coli*. The most sensitive organism to all the plants was *E. coli*. *O. pretoriensis* had the lowest average MIC value and the highest total activity value of 1538 ml/g. Based on bioautography some of the *Ochna* species had antibacterial compounds with similar R_f values. The thin layer chromatography chemical profiles of the five plant extracts may be useful in the taxonomy of the genus. *O. pretoriensis* was chosen for fractionation and isolation of antibacterial compound because it has the lowest average MIC values and highest total activity especially against *E. faecalis* and *E. coli*.

The acetone extract of *O. pretoriensis* was fractionated into seven fractions (hexane, carbon tetrachloride, chloroform, ethyl acetate, 35% water in methanol, 70% water in methanol and butanol) by solvent-solvent fractionation. Only three of the seven fractions (carbon tetrachloride, chloroform, ethyl acetate fractions) had clearly defined antibacterial spots/lines on bioautograms. The three fractions were further fractionated using column chromatography from which three compounds were successfully isolated. The chemical structures of the isolated compounds were determined using NMR spectroscopy as ß-Sitosterol (SS), ochnaflavone (OF) and ochnaflavone 7-O- methyl ether (OFME).

Compounds that are related to sitosterol have activity against neurodegenerative disorders as well as estrogenic, analgesic, anti-inflammatory, anthelminthic and antimutagenic activity. OF and OFME are biflavonoids which belong to the group ochnaflavones previously characterized from *Ochna obtusata*. These compounds have anti-atherosclerotic, anti-inflammatory, and anti-tumor activity. They also inhibit

lymphocytes proliferation, archidonic acid release and phospholipase activity. Moreover, OFME was reported to inhibit HIV-1 activity as well as HIV-1 reverse transcriptase activity.

The antibacterial activity, and potential cytotoxic, genotoxic and antigenotoxic effects of the isolated compounds were determined. The MIC values ranged from 31.3 to 250 µg/ml. SS was more active against *P. aeruginosa* with an MIC of 62.5 µg/ml, OF against *P. aeruginosa* and *E. faecalis* with MICs of 0.03 mg/ml and OFME against *P. aeruginosa* with an MIC of 31.3 µg/ml. The isolated compounds were much less active than the positive control gentamycin. The compounds had low cytotoxic activity, with LC_{50} values of 193.8 µg/ml for ß-Sitosterol, 125.9 µg/ml for OF and 125.9 µg/ml for OFME against Vero cells. The therapeutic indexes of the crude extract and the isolated compounds varied between 0.77 and 3.27, which is an indication of non-specific antibacterial activity i.e. general toxicity, thus the crude plant extract and compounds isolated from *O. pretoriensis* can only be recommended for external applications. e.g. topical treatments. None of the compounds tested had potential genotoxic and/or antigenotoxic effects. The number of revertants in the mutagenicity experiments was less than twice the number of revertants in the negative control. The percentage inhibition of 4NQO in the antimutagenicity experiments were less than 45%. The results obtained in this case may be principally associated with the general toxicity of the test samples to the bacteria used in this study.

Comparison of the total activity of the crude extract and the fractions gave a clear indication of synergic interaction of compounds in the crude extract to successfully inhibit the growth of the test pathogens. Approximately 76% of activity was lost in the 34% of dry mass lost during fractionation. Twelve percent of activity was present in the chloroform fraction and 6% in the carbon tetrachloride fraction. Despite the evidence for synergistic activity, the crude extract was also relatively toxic to the Vero cells with a therapeutic index of 0.8.

As far as could be established, the antibacterial activity of members of the *Ochna* genus and the cytotoxicity of ochnaflavones were determined for the first time in the current study. The two most active antibacterial compounds (ochnaflavone and ochnaflavone 7-O- methyl ether) are being reported from this species for the first time. The relative safety of the crude extract and the compounds isolated from this plant was relatively low. Preparations of *O. pretoriensis* may be safe in a topical application but internal use cannot be recommended for treating antibacterial infections before animal toxicity studies have been carried out. Caution is also required in using the isolated compounds or crude extracts for other applications.

CHAPTER 1

Introduction

1.1. Background

In animal breeding or production, the control and prevention of infectious diseases is of basic economical importance. To attain this, farmers have long used antibiotics in the form of antibiotic feed additives (ADA) as growth promoters where antibiotics are administered at sub-therapeutic dosages to control bacterial growth in the animal gut. This resulted in the development of resistant bacterial strains to the frequently used antibiotics. These resistant strains can affect human health if they are pathogenic or may pass on the resistant genes to other pathogens more especially in cases where the antibiotics used are in the same line or class with those used in human therapy (Wegener, 2003).

There have been searches for alternatives to replace the use of antibiotics in animal production with less success but the utilization of plant extracts with antimicrobial activity has proved to be one of the promising solutions. This is because plant extracts contains thousands of phytochemicals which sometimes acts synergistically to combat microbial growth and as a result, these compounds can be isolated and used to replace synthetic antimicrobials used in both the health and the farming sector. Most phytochemicals appear to have structures and mode of action distinct from those of the known antibiotics used in both the health and agricultural sector (Shand and Leyva, 2008). Cowan (1999) and Tedesco (2001) have listed some of the herbal remedies that are used in livestock production.

This study concentrates mostly on the isolation of antibacterial compound(s) with potential to add more information to the ongoing research to find replacements for the use of antibiotics in animal production. Different biological activity of the isolated compound(s) will also be investigated using *in vitro* biological models available in the Phytomedicine programme in the Department of Paraclinical Sciences of the University of Pretoria. Some work conducted within our Phytomedicine group have revealed that plant extracts have some synergistic antimicrobial activity and can also be used as potential feed additives to promote animal growth and health in this case broiler chickens (Zishiri, 2004, Chikoto, 2006).

1.2. Aim(s) and Objectives

The main aim of this project was to isolate and characterize antibacterial compounds from a medicinal plant that can be useful in human and animal health against bacterial infections. To achieve the aim, the objectives listed below will be addressed.

1.2.1 Objectives

The objectives of this study were to:

- Identify an *Ochna* species plant extract with high antibacterial activity
- Isolate antibacterial compounds present in the most active *Ochna* species using chromatographic methods
- Structure elucidation of the isolated compounds.
- Evaluate the presence of synergic antibacterial activity of the crude plant extract and fractions obtained by solvent-solvent fractionation.
- Determine the biological activity of the compounds isolated from the active *Ochna* species (antibacterial activity, cellular toxicity and genotoxicity)

1.3. Literature review

1.3.1. Medicinal plants and their uses.

Plants have always been considered a healthy source of life and therefore form the main ingredient of medicines in traditional systems of healing and have been the source of inspiration for several major pharmaceutical drugs. Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties. Although some of the therapeutic properties attributed to plants have been proven erroneous, medicinal plant therapy is based on the empirical findings of hundreds and thousands years as a result; there has been an increased interest in the study of ethnomedicine, ethnopharmacology and uses of medicinal plants as sources of pharmacologically active compounds worldwide. Natural crude plant extracts and biologically active compounds isolated from plant species used in traditional medicine can be prolific and inexhaustible resources for new drugs (Gurib-Fakim, 2006).

Indigenous people have relied on medicinal plants to combat ailments such as renal infections, abdominal pains, chest problems, headaches, toothaches, skin infections and many others and this information has been passed on from one generation to generation. A large proportion of these ailments occur due to the presence of microorganisms such as bacteria, fungi and viruses that infects the body. The search for biologically active compounds extracted from traditionally used medicinal plants is relevant due to the increasing resistance of bacteria to synthetic antibiotics and the occurrence of fatal opportunistic infections (Eloff, 1998a, Elgorashi *et al*., 2004). The antimicrobial properties of certain Indian medicinal plants have been reported based on folklore information and a few attempts were made on inhibitory activities of the plants against certain pathogenic bacteria and fungi (Sokmen *et al*., 1999). Plants also have a long history of use for the treatment of cancer and in Africa many people use plants for medicinal purposes as supplement to visiting Western health care practitioners (Cragg and Newton, 2005).

Medicinal plants are important in health care systems of developing countries. Throughout the ages, human have relied on plants as sources of medicines. This reliance on plants as medicines warrants the scientific validation of their safety and efficacy. The idea of using medicinal plants to treat livestock diseases is not new. This is because medicinal plants have been and are still an essential and important

component of ethnoveterinary medicine in most developing countries where plants are used in the treatment and prevention of livestock diseases (Masika and Afolayan, 2002).

1.3.2. Biological activity and antimicrobial compounds in plants

Plants have their own mechanisms of protection against invasion by pathogens. As a result, they are possibly on the edge for a comeback as sources of human and animal health products. The hopes of this comeback are rooted in the unique properties of plants to synthesize mixtures of structurally diverse bioactive compounds with multiple and mutually therapeutic effects [i.e. thousands of diverse chemical compounds with different biological activity]. Although all this properties have been known for a long time, the ability to better exploit the uniqueness of plant therapeutics was acquired only recently as a result of the dramatic advances in pharmaceutical screening (Loizzo *et al*., 2006). Many of these compounds referred to as secondary metabolites are highly toxic and help plants to protect themselves against pathogens in the soil, insects, herbivores and oxidative damage of free radicals. In the health sector, numerous drugs are of plant origin and used in human medicine. Most of these compounds have showed enormous activity against pathogenic microorganisms, infectious diseases and as potential cancer therapeutics (Hamburger and Hostettmann, 1991).

Secondary metabolites are the main source of the antimicrobial activity in plants. These compounds differ in structure and their main function or importance is of an ecological nature as they are generally derived by plants for protection. Antimicrobial compounds from plants to some extend mimic the growth inhibitory effects of synthetic antibiotics. These compounds include among others: simple phenols and phenolic compounds, quinolines ?, flavones, flavonoids and flavonols, tannins, coumarins, terpenoids and essential oils , alkaloids (Cowan, 1999).

1.3.2.1. Phenolic and polyphenols

These are some of the simplest bioactive phytochemicals consisting of single substituted phenolic ring. The mechanism thought to be responsible for the phenolic toxicity to microorganisms includes enzyme inhibition by the oxidative compounds (Cowan, 1999).

1.3.2.2. Quinones

These are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive and coloured. Quinines are known to complex irreversibly with nucleophillic amino acids in proteins. Their probable targets in the microbial cell are surface-exposed adhesion, cell wall polypeptides and membrane bound enzymes (Cowan, 1999).

1.3.2.3. Flavones, flavonoids and flavonols

Flavones are phenolic structures containing one carbonyl group. The addition of 3-hydroxyl group yields a flavonols. Flavonoids are hydroxylated phenolic substrates. This compounds re known to be synthesized by plants in response to microbial infection. These group of compounds posses a wide range of biological activity and have been found to be effective antimicrobial substances against a wide range of microorganisms. They posses anti-inflammatory, antiviral and antioxidant properties (Cowan, 1999)

1.3.2.4. Tannins

Tannins are a group of polymeric phenolic substances capable of tanning leather. They are divided into two groups (hydrolysable and condensed tannins) and have a high molecular weight. This class of compounds have a wide range of biological activities. The antimicrobial mode of action of tannins is similar to that of quinines and flavonoids (Cowan, 1999).

1.3.2.5. Coumarins

Coumarins are phenolic substances made of fused benzene rings and α-pyrone rings. They are reported to have anti-inflammatory, anti-thrombic and vasodilatory activities. Several other coumarins and hydroxycinnamic acids, a group of compounds related to coumarins have antibacterial activity (Cowan, 1999).

1.3.2.6. Terpenoids and essential oils

These are secondary metabolites that are enriched in compounds base on an isoprene structure. They are terpenes and can occur as diterpenes, triterpenes, and tetraterpenes, terpenoids are active against bacteria and have been reported to be have antifungal and anti-inflammatory activity (Cowan, 1999, Houghton et al., 2003).

1.3.2.7. Alkaloids

These are heterocyclic nitrogen compounds. Morphine is the first medically useful alkaloid. Alkaloids have antimicrobial and antiviral properties and are also effective against trypanoses and plasmodia. Their mode of action is attributed to their ability to intercalate DNA (Cowan, 1999). Almost all this compounds have distinctive biological activity which among others includes antimicrobial activity (Atsatt and Odowd, 1976).

Figure 1.1. Structures of common antimicrobial plant chemicals (Cowan, 1999).

1.3.3. Antibiotics: "Link between resistance development and use in animal production"

Antibiotics are molecules that stop microbes, both bacteria and fungi from growing or kill them outright. Antibiotics that stop bacterial growth are said to be bacteriostatic and those that cause bacterial cell death are called bactericidal. Antibiotic agents can either be natural or synthetic chemicals designed to block some crucial processes in microbial cells selectively (Walsh, 2003).

The emergence of antibiotic resistance/antimicrobial resistance which is the ability of microorganisms to withdraw the effects of antibiotics is an evolutionary process that is based on the selection for microbes that have enhanced ability to survive doses of antibiotics that would previously been lethal. In more than the past 70 years of the antibiotic era and the treatment of human infectious disease, pathogenic bacteria have developed relentlessly with the clinical significant resistance to one class of antibiotics after another. The more widely used the antibiotic, the more likely the resistance development. A lot of antibiotics have been produced and disseminated including those used for animal and agriculture suggesting a significant reservoir for the rise of resistant bacteria. Inappropriate antibiotic treatment and overuse are some of the many contributing factors to the emergence of resistant bacterial strains. The problem is furthermore exacerbated by self-prescribing of antibiotics by individuals without the guidelines of a qualified clinician and the non-therapeutic use of antibiotics as growth promoters in agriculture (Levy, 1998, Walsh and Amyes, 2004).

Even though bacterial antibiotic resistance is a natural phenomenon, societal factors also contribute to the problem. These factors include increased transmission coupled with inappropriate antibiotic use. Among others, excessive consumption of antibiotics by human also poses a potential risk to public

health by also increasing resistance. In veterinary practices and animal farming, the use of antibiotic feed additives (AFA) also called antibiotic growth promoters plays a major role in the development of resistant strains of microorganisms to currently available antibiotics. Evidence from some US and European studies suggest that these resistant bacteria can be pathogenic to humans and do not respond to commonly prescribed antibiotics. In response to these practices and the major problem of increase in the development of antimicrobial resistance, the use of antibiotics as growth promoters has been banned by the European Union and in addition to this, several organizations [e.g. The American Society for Microbiology (ASM) and many others, have called for restriction on antibiotic use in food animal production (EU commission, 2005). The World Health Organization recommends that antibiotics used in human health for therapy should not be used as growth promoters (WHO, 1997)
While hospitals are clearly fertile areas for selection of antibiotic resistant pathogenic bacteria, the complementary arena that has been understood for decades is the use of antibiotics in animal feed as growth promoters and for infectious diseases prophylaxis. In animal production, intensive farming in particular, the control and prevention of infectious diseases is of paramount importance based on the economical outcomes of every farm (Wierup, 2000). As a result, antimicrobials have been used and are still used in animal agriculture for therapeutic and non therapeutic purposes. The same antibiotics that are administered therapeutically have been included in animal feed and used continuously at sub-therapeutic levels to enhance animal growth and productivity (Wegener, 2003). More recently, it has been estimated that more than 70% of synthetic antibiotics are used in animal agriculture and veterinary practices (Sischo, 2006). The administration of antibiotics in animal agriculture has shown to be effective as growth promoters. The growth promoting effects of antibiotics is by the control of undesired intestinal bacteria which allows better nutrient absorption and preventing necrotic enteritis in poultry and diarrhoea in other animals (Doyle, 2001).

The resistance problem demands that a renewed effort be made to seek antimicrobial agents effective against pathogens resistant to current antibiotics. There are a number of suggested possible solutions to replace and eliminate antibiotic use in animal production. This includes isolation of sick animals to limit exposure of neighbouring animals to excreted pathogens, age segregation, sanitation and vaccination (Wierup, 2000). Good hygiene practices, in-feed enzymes, use of probiotics and immunologically active compounds have also been recommended (Doyle, 2001). Another approach that can be employed is the use of organic acids as feed additives. For microbes, organic acids can act as carbon and energy sources or as inhibitory agents depending on the concentration of the acid in the feed (Dahiya *et al*., 2006). All this measures have been tried but none of them proved to be to be effective and sufficient to completely eliminate the need for antibiotics in animal production.

One of the solutions to combat resistance is the development of pharmaceutical compounds that would revert antibiotic resistance. The only promising replacement of antibiotic feed additives to date is the rational localization of bioactive phytochemicals by the utilization of extracts from ethno-medicinal plants This is because plants have a limitless ability to synthesize secondary metabolites with diverse biological activity and it is thus recommended because of the occurrence of their synergistic antimicrobial activity i.e. the development of resistance to one compound is countered by the other (Conforti *et al*., 2006). Moreover, phytochemicals as opposed to synthetic antimicrobial appear to have different novel structures with different novel modes of action against most pathogenic organisms. Other suggested alternatives include the use of archaeocis, a different class/group of antibiotics from archae group of organisms (Stermitz *et al*., 1999, Shand and Leyva, 2008).

1.3.4. Pathogens used in this study

Staphylococcus aureus is a Gram-positive bacterium that is a common colonizer of human skin and mucous membranes. *S. aureus* can cause diseases particularly if there is an opportunity for the bacteria to enter the body. Illnesses such as skin and wound infections, urinary tract infections, pneumonia and bacteraemia may then develop (Ryan and Ray, 2004). This bacterium can survive on domesticated animals and can cause bumble-foot, a disease in chickens. Antibiotic resistance of *S. aureus* was almost unknown when penicillin was first introduced in 1943, but by 1950, the resistance had increased to 40% and to 80% by 1960 (Chambers, 2001). Most strains of this bacterium are sensitive to many antibiotics but the methicilin-resistant staphylococcus aureus (MRSA) has emerged as an epidemic that is responsible for rapidly progressive fatal diseases including necrotizing pneumonia and severe sepsis. The MRSA is the most frequent identified antimicrobial resistant pathogen in hospitals (Boyle-Vavra and Daum, 2007).

Enterococcus faecalis is also a Gram-positive bacterium that normally inhabits the gastrointestinal tract of humans and other mammals. Like other species in the genus *Enterococcus*, *E. faecalis* can cause life threatening infections in humans especially in environments like hospitals. *E. faecalis* is resistant to many commonly used antimicrobial agents because it contains the penicillin-binding proteins (PBP) (mechanism of resistance) (Ryan and Ray, 2004)

Pseudomonas aeruginosa is a Gram-negative bacterium which is a prevalent opportunistic pathogen of humans and plants. The pseudomonads are well known to plant microbiologists because they are one of the few groups of bacteria that are true pathogens of plants. In humans these bacteria causes respiratory system infections, dermatitis, soft tissue infections and a variety of other systematic infections One of the most troublesome characteristics of *P. aeruginosa* consists in its low antibiotic susceptibility due to the presence of the outer membrane with a low level of permeability. The low susceptibility is also attributed to a concerted action of multi-drug efflux pumps and this bacterium can also acquire resistance by mutations (Feng *et al*., 2002).

Escherichia coli is a Gram-negative bacterium inhabiting the lower intestinal tract of mammals. Some strains of this microorganism can cause serious infections in humans such as gastroenteritis, urinary tract infections and neonatal meningitis. Scientists have warned that *E. coli* is becoming resistant to antibiotics and the resistance problem could become as big as MRSA. One suspected source of resistance in *E. coli* is the use of antimicrobial drugs in agriculture with the high prevalence of the resistant strains in retail meat products, more especially poultry (Johnson *et al*., 2003).

1.3.5. Selection of plant species

The selection of plants used in the development of potential pharmaceuticals is dependent mostly on the importance of the plant species i.e. its utilization mainly in traditional medicine (ethno-pharmacological directed sampling approach) and the abundance of the plant. Nevertheless, approaches like random approach, which involves the collection of almost plants found in a given area, phytochemical targeting which entails the collection of all members of a plant family known to be rich in bioactive compounds, the chemotaxonomic approach which is based on specific plant parts such as seed, leaves etc. and lastly the ethno-directed sampling approach which is based on the traditional medicinal uses are also important for general screening of plats for different biological activities (Khafagi and Dewedar; 2000).

CHAPTER 2

Screening of five *Ochna* species for antibacterial activity and selecting the best for further work

2.1. Introduction

2.1.1. Need to screen medicinal plants for antibacterial activity

Long before mankind discovered the existence of microorganisms, the idea that certain plants had healing potential was accepted. These plants have always and still play an important role in the treatment of infectious diseases caused by a variety of pathogenic microbes and as a result, there is a need to study plants more intensively. Three general motives for performing screening studies on medicinal plants are to discover new leads compounds for developing pharmaceuticals, to validate the ethno-medicinal uses of the plants and to develop phyto-remedies for use as herbal medicine Eloff (2004).

2.1.2. *Ochna, "the genus"*

Ochna is a genus comprising 86 species of evergreen trees, shrubs and shrublets belonging to the family Ochnaceae. These species are native to tropical woodlands of Africa or Asia while some species are distributed in tropical and subtropical zones throughout the World (Hegnauer, 1969; Carvalho et al., 2000). Species of this genus are usually called *Ochnas* or Mickey-mouse plants, a name coming from the shape of their drupelets fruit. Some species, especially *Ochna serullata* are widely cultivated for decorative purposes. This family is characterized by the presence of flavonoids and biflavonoids and terpenoids as main secondary metabolites (Oliveira *et al*., 2002; Estevam *et al* 2005) and several studies on other *Ochna* species were conducted and revealed that the phytochemical contained within this genus constitutes mainly glycosides, saponims, steroids, flavones and fatty acids (Agra *et al*., 2007).

Figure 2.1. Pictures of the five *Ochna* species investigated in this study. A= *O. gamostigmata*, B= *O natalitia*, C= *O. pretoriensis*, D= *O. pulchra* and E= *O. serullata* (www.plantzafrica.com).

In preliminary work done in a tree leaf screening project in the Phytomedicine Programme (*www.up.ac.za/phyto*) *Ochna pretoriensis* acetone leaf extracts had good antibacterial activity against several important bacterial pathogens. This led to an interest in investigating the antibacterial activity of some of the *Ochna* species occurring in southern Africa. Hutchings *et al*., (1996) listed some ethnobotanical applications of few species from this genus. Some of the *Ochna* species are used traditionally by the Zulu tribe of South Africa for traditional and medicinal purposes. The root and bark infusion of *Ochna natalitia* (Meins.) Walp is taken to treat barrenness and the whole plant is used to bring prosperity. Root decoctions of *Ochna serrulata* (Hochst.) Walp. are used in enemas and applied topically for gangrenous rectitis. The plant is also used in some African countries for the treatment of bone diseases in children. *Ochna pulchra* is suspected of poisoning stock. Nonetheless, no toxic results were obtained from young leaves and shoots (Hutchings *et al*., 1996).

Although there is no traditional use for treating microbial infections recorded in southern Africa and *O. serrulata* did not have any antibacterial activity (Watt and Breyer Brandwijk, 1962*), O. macrocalyx* bark has been used to treat gastrointestinal disorders in Tanzania (Schlage et al. 2000). Tang et al (2003) isolated six compounds from the bark of *O. macrocalyx*. Two of these compounds had excellent

activities against multidrug resistant *S. aureus* with MIC's of as low as 8 µg/ml. These results supported the decision to investigate the antibacterial activity of *Ochna* spp.. There are twelve *Ochna* species growing in southern Africa (Germishuizen and Meyer, 2003). In this study, five *Ochna* species were selected for comparative antibacterial screening based on their availability.

2.2. Materials and methods

2.2.1. Collection and drying of plant material

Fresh leaves of the five plants investigated were collected in summer at the National Botanical Garden in Pretoria. Trees were identified from the tree labels. Voucher specimens were deposited at the Herbarium, Phytomedicine Laboratory, of the University of Pretoria. The leaves were dried in a dark at room temperature and milled into fine powders and stored in closed glass bottles in the dark until use.

2.2.2. Extraction

To extract phytochemicals from the leaves, a direct extraction method was employed. The dried plant materials were extracted with acetone following a ratio of 1:10 of plant material to extractant. Separate aliquots of 2 g of the powdered leaves were weighed into 50 ml polyester centrifuge tubes followed by the addition of 20 ml of acetone. The tubes were shaken vigorously on a labotec shaking machine for 30 minutes. The extracts tubes were then centrifuged at 300 x g for 15 minutes and the extracts were decanted into preweighed glass vials through Whatman No.1 filter paper and concentrated to dryness under a stream of cold air. After drying, the vials were weighed to determine quantity extracted.

2.2.3. Phytochemical analysis (Thin Layer Chromatography)

The dried plant extracts were resuspended in acetone to a stock solution of 10 mg/ml to be used in subsequent bioassays. From the stock solution, 10µl samples were loaded onto Thin Layer Chromatography (TLC) plates and developed in three mobile systems of differing polarities developed in the Phytomedicine laboratory of the University of Pretoria. The mobile systems used were:

- EMW (ethyl acetate/methanol/water): 40:5.4:4(v/v/v) polar
- CEF (chloroform/ethyl acetate/formic acid):5:4:1(v/v/v) intermediate polarity
- BEA (benzene/ethanol/ammonia hydroxide):90:10:1(v/v/v)) non polar

After development, the plates were visualized under UV light and thereafter sprayed with vanillin-sulphuric acid reagent spray. Other plates were sprayed with anisaldehyde-sulphuric acid and heated at 110°C until optimum colour development.

2.2.4. Test organisms (Bacterial cultures and strains)

Microbial cultures used in the determination of Minimal Inhibitory Concentrations (MIC) and bioautographic assays were obtained from the Microbiology unit of the Department of Veterinary Tropical Diseases. The bacterial cultures were maintained on Miller Hilton (M-H) agar at 4°C and in M-H broth at 37°C prior to use for MIC's and bioautography. The bacterial cultures (strain numbers included) used in this project were

- *Enterococcus faecalis* (ATCC 29212)
- *Staphylococcus aureus* (ATCC 29213)
- *Escherichia coli* (ATCC 27853)
- *Pseudomonas aeruginosa* (ATCC 25922)

2.2.5. Bioautography

Thin Layer Chromatography plates were prepared as described in 2.2.3. After development, the plates were left for two day under a stream of air to allow evaporation of solvents used in development. The plates were then sprayed with actively growing bacterial cultures until wet and incubated at 37°C under 100% relative humidity. Following incubation, the plates were sprayed with 2 mg/ml INT solution and incubated for 30 minutes and colour change was monitored to detect growth and inhibition of the test organisms. Clear zones against a pinkish background on the plates indicate inhibition of bacterial growth by antibacterial compounds present in the extracts (Beque and Kline, 1972).

2.2.6. Minimal Inhibitory Concentration (MIC)

To determine the minimal inhibitory concentration values of the plants extracts on the test organisms, the microplate dilution assay by Eloff (1998a) was used. This experiment was performed in triplicate and repeated twice for verification. The plant extracts were prepared to a concentration of 10 mg/ml in

acetone and 100 µl of the plant extracts were serially diluted two-fold with water and freshly prepared cultures of the test organisms. Acetone was used as negative control and gentamycin as the positive control. The plates were then incubated overnight at 37°C. After incubation, 40 µl of 0.2 mg/ml INT were added to each well as a growth indicator and incubated further until colour change at 30 minutes intervals. The MIC was recorded as the lowest concentration of the plant extract at which the bacterial growth was inhibited. The MIC values were read after 12 hours incubation and 24-36 hour incubation with the plant extracts to determine if the extracts possess bacteristatic or bactericidal activity. From the MIC values, total activity in ml/g was calculated as the total mass extracted from 1 g of plant material divided by MIC value i.e. TA= mass extracted from 1 g (mg)/MIC (mg/ml)

2.3. Results and Discussion

2.31. Percentage yield of plant extracts

Five Ochna species were selected for screening for antibacterial activity. Even though in traditional medicine water is used as an extractant, not all the compounds are extracted with water i.e. in case the bioactive compounds are non-polar, they are not extracted. As a result, acetone was chosen as an extractant in this experiment because of its ability to dissolve many hydrophilic and lipophilic components from many plants, its volatility and low toxicity in bioassays (Eloff, 1998b).

The highest quantity of material was extracted from *O. gamostigmata* (8%) followed by *O. pulchra* (7.5%), *O. serullata* (7%), *O. pretoriensis* (6.5%) and lastly *O. natalitia* (2.5%).

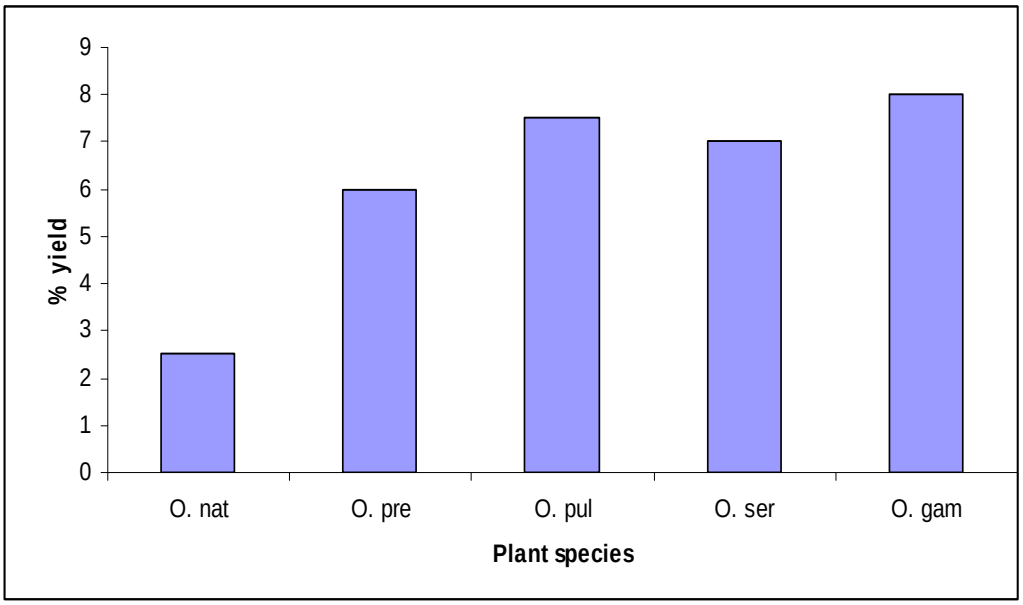


Figure 2.2: Percentage yield of samples/material extracted from five *Ochna* species with acetone.

2.3.2 Phytochemical analysis

To analyse the phytochemical constituents of the acetone extracts of the five *Ochna* species, thin layer chromatography (TLC) was used. This technique allows for the separation of compounds based on their polarities and is also useful for bioautography and the purification of complex plant extracts.

CEF was the best solvent system that separated compounds present in almost all the plant extracts more efficiently compared to EMW and BEA. The good separation in CEF is because of the intermediate polarity of the compounds extracted with acetone. This shows that the efficient extraction and separation of phytocompounds is largely dependent on the type of extractant used (Masoko *et al*., 2005).

Ochna gamostigmata had the largest number (30) of compounds that were separated and reacted with vanillin-sulphuric acid followed by *O. pretoriensis*, *O. serullata*, *O. pulchra* and lastly *O. natalitia*. It is clear that *O. pretoriensis* and *O. pulchra* had a similar chemical composition. Even though the five species differ in their chemical composition (Fig. 2.3 B), compounds with similar R_f values and vanillin colour were found in similar mobile phases across a number of species. This may indicate similar biogenetic pathways in the Ochna genus.

2.3.3. Bioautography

TLC based bioautography is a useful technique that allows the localization of bioactive compounds present in the crude plant extracts. The clear spots on the bioautograms indicate the location of the active compounds (Begue and Kline, 1972).

The bioautograms in figure 2.3(B) are a representative sample of the antibacterial activity of the five plant species against *S. aureus*. The bioautograms developed in CEF and EMW had defined clear lines of inhibition. *O. natalitia* extract did not show any well defined active spot(s). *O. gamostigmata* contains active spots in EMW which are also present in very small quantities in *O. pretoriensis* and *O. pulchra*. The presence of such compounds shows that this plant species contains compounds of intermediate polarity and polar compounds with antibacterial activity. These three species may contain similar compounds which may be observed in relatively related *Ochna* species. The plates developed in EMW had an active compound with an R_f value of 0.89 present in *O. pretoriensis*, *O. pulchra*, *O. serullata* and *O. gamostigmata*.

There are about 50 lines of inhibition in all the bioautograms. The active compounds are more concentrated in *O. pretoriensis* as opposed to *O. pulchra* because the number and the diameters of the active spots are higher in *O. pretoriensis*. In using another technique Fish and Codd, (1994) explained that the diameter of the inhibition zone is proportional to the growth inhibition. Even though the total number of active compounds from each bioautogram can be calculated and added, it [will be unnecessary] to do it because the active compounds will have different, R_f values in the different solvent systems. On the other hand counting only the number of active lines separated in one solvent system will underestimate the number of compounds present in the extract if all are not separated..

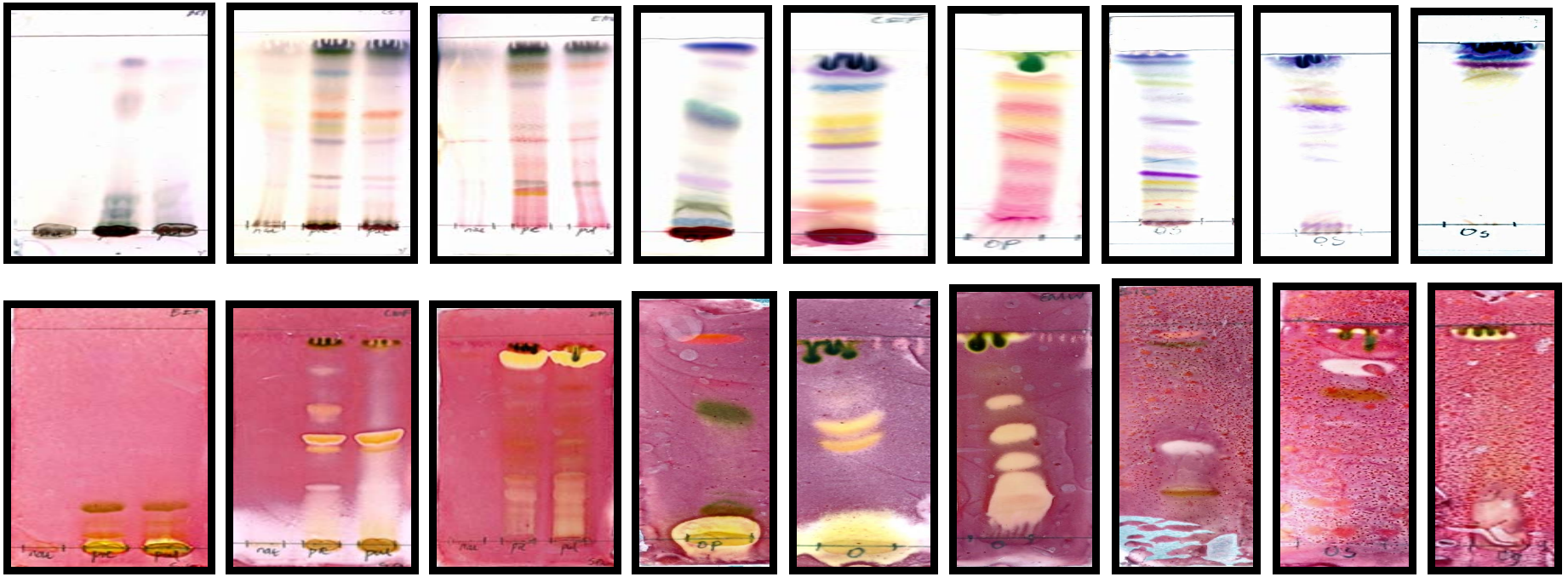


Figure 2.3: Chromatograms of the five *Ochna* species developed in BEA, CEF and EMW solvent systems and sprayed with vanillin-sulphuric acid reagent spray (A) to show the separation of compounds present in the extracts and (B) sprayed with actively growing S. aureus to indicate antibacterial compounds (no red colour)
O. nat= Ochna natalitia, *O. prêt= Ochna pretoriensis*, *O. pul= Ochna pulchra*, *O. gam= Ochna gamostigmata, O.ser= Ochna serullata*

2.3.4. Minimal Inhibitory Concentrations (MIC)

The bioautography results indicated the number of antibacterial compounds present, but do not provide any information on the quantitative antibacterial activity. The MIC values of the extracts were determined by the serial microplate dilution method (Eloff, 1998) and the values ranged from 0.039 to 1.25 mg/ml over 12 hours incubation and from 0.078 to 1.25 mg/ml over 36 hours of incubation. The lowest MIC was 0.039 mg/ml for the *O. pretoriensis* extract against *E. faecalis* and the highest 1.25 mg/ml for *O. natalitia O. serullata* and *O. gamostigmata* extracts against *P .aeruginosa* and *S. aureus* respectively.

The *O. pretoriensis* extract was more active against *E. coli* and *E. faecalis* with MIC values of 0.065 and 0.039 mg/ml respectively, The *O. pulchra* extract was more active against *P. aeruginosa* and *S. aureus* with MIC values of 0.065 and 0.078 mg/ml respectively. The *O. serullata* extract was more active against *P. aeruginosa* and *E. coli* with MIC values of 0.156 and 0.312 mg/ml, the *O. gamostigmata* extract against *P .aeruginosa*, *E. coli* and *E. faecalis* with MIC values of 0.104 and 0.312 mg/ ml respectively for both microorganisms. *O. natalitia* extract against *E. coli* and *S. aureus* with MIC values of 0.52 and 0.416 mg/ml. The *O. pretoriensis* extract was more active against almost all the tested bacteria, followed by, *O. pulchra, O. gamostigmata, O. serullata* and *O. natalitia.* In comparing the different pathogens, *E. faecalis* was the most sensitive followed by *E. coli, P .aeruginosa* and *S. aureus*.

The microtitre plates were incubated for 12 hours and examined 1 hour after INT was added, and again after 24 hours. The MIC values didn't change much from 12 to 24 hours of incubation. With the short generation time of the pathogens investigated, this probably means that the activity was largely bactericidal. When microplates were again investigated after 36 hours there were differences in some cases (Table 2.1).

The difference in activity of the extracts between 24 and 36 hours may probably be attributed to growth of other contaminants especially by fungal spores or a decomposition of the active compounds in the extract. It does appear as if there were hardly any difference in response to the Gram positive bacteria (Fig 2.4) but there were some differences in the response of the Gram negative bacteria. Even in this case the difference was never more than one dilution from the value after 12 or 24 hours. An alternative method to determine the bactericidality and/or

bacteristaticity effects of the plant extracts will be to plate the bacteria from the lowest MIC well on nutrient agar plates, incubate overnight at 37°C and observe colony growth.

Total activities together with the inverse of the MIC's of the six plant extracts were calculated from the MIC values. Total activity indicates the volume or extent to which the bioactive compound present 1 g of the dried plant material can be diluted and still inhibit growth of the test organism (Eloff, 2004). Masoko *et al*., (2005) explained that the inverse or reciprocal of the MIC values are also of importance as it shows the activity of compounds present in the plant extracts. The inverse of the MIC's (ml/mg) as shown in figure 2.4 graph B shows that approximately 25 ml of solvent can be added to1 mg of *O. pretoriensis* extract and it would still inhibit the growth of *E. faecalis*. In comparing the total activity of the different plant extracts (Eloff, 1999), *O. pretoriensis* had the highest total activity of 1538 ml/g against *E. faecalis*, followed by *O. pulchra* (1153 ml/g against *P. aeruginosa*), *O. gamostigmata* (769 ml/g against *P. aeruginosa*), *O. serullata* (448 ml/g against *P. aeruginosa*) and lastly, *O. natalitia* (213 ml/g against *E. faecalis*).

Table 2.1: MIC (mg/ml) and total activity (ml/g) values of the acetone extracts of five Ochna species against *E. coli*, *P. aeruginosa*, *S. aureus* and *E. faecalis*. In cases where SD not included, SD=±0.00

	Plant species 12 hours					Plant species 36 hours					
	MIC values (mg/ml)					MIC values (mg/ml)					
Organisms	*O .nat*	*O. pre*	*O. pul*	*O. gam*	*O. ser*	*O .nat*	*O. pre*	*O. pul*	*O. gam*	*O. ser*	Gentamycin
E. coli	0.520(±0.18)	0.065(±0.02)	0.312	0.312	0.312	1.250	0.312	0.625	0.312	0.312	0.0039
P .aeruginosa	0.833(±0.36)	0.104(±0.04)	0.065(±0.02)	0.104(±0.04)	0.156	1.250	0.156	0.104(±0.04)	0.312	0.312	0.0019
S. aureus	0.416(±0.18)	0.104(±0.04)	0.078	0.416(±0.18)	1.250	0.416	0.156	0.078	0.520(±0.18)	1.250	0.0048
E. faecalis	0.625	0.039	0.078(±0.02)	0.312	0.520(±0.18)	0.520(±0.18)	0.104(±0.04)	0.104(±0.04)	0.312	0.312	0.0039
	Total activity (ml/g)(12 hours)					Total activity (ml/g)(36 hours)					
E. coli	**48**	**923**	**288**	**256**	**224**	**30**	**192**	**120**	**256**	**224**	
P .aeruginosa	**30**	**576**	**1153**	**769**	**448**	**20**	**384**	**958**	**256**	**224**	
S. aureus	**60**	**576**	**958**	**192**	**56**	**60**	**384**	**958**	**153**	**56**	
E. faecalis	**213**	**1538**	**958**	**256**	**134**	**106**	**512**	**641**	**256**	**224**	

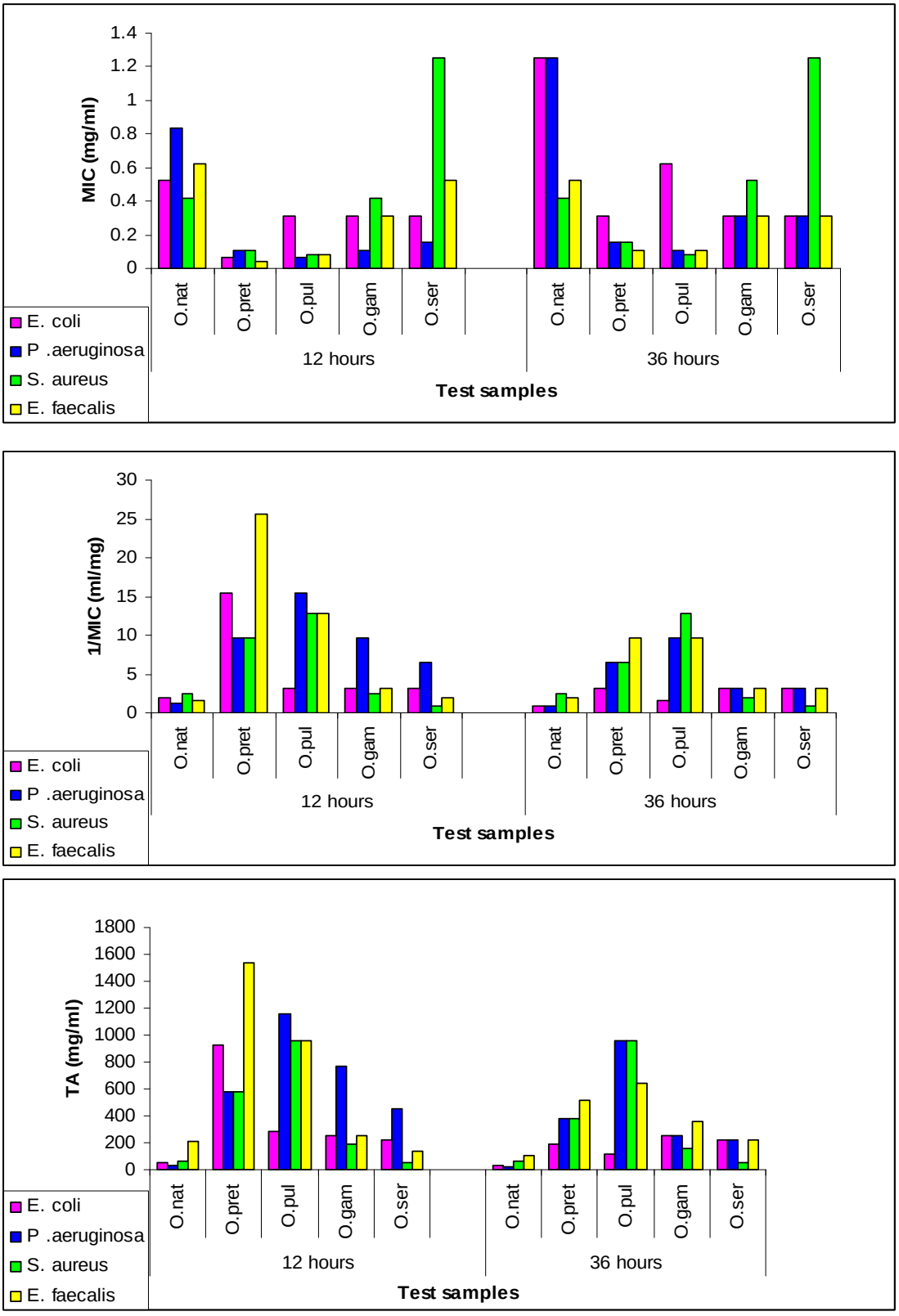


Figure 2.4. The average MIC values (mg/ml) to show the sensitivity/susceptibility of the test organisms to the acetone extracts of each plant species (A), average activity volume 1/MIC (ml/mg) (B) and total activity (ml/g of the five Ochna species.

2.4. Conclusions

Three of the five *Ochna* species (*O. pretoriensis, O. gamostigmata* and *O. pulchra*) investigated had considerable antibacterial activity when quantitatively evaluated. The plant extracts investigated had a varying degree of activity against the test organisms. *O. pretoriensis* and *O. pulchra* contained similar antibacterial compounds.

Some of the plants appear to contain similar compounds with antibacterial activity based on the R_f and vanillin sulphuric acid colour. If the antibacterial activity of these plants is only attributed to the compounds detected on the bioautograms, then the isolation of such compounds will be of interest since not many compounds were observed in each bioautogram. *O. pretoriensis* was chosen for fractionation and isolation of antibacterial compound because it has a high number of active compounds observed on bioautograms, has the lowest average MIC values and highest total activity especially against *E. faecalis* and *E. coli*. Moreover, there is not much research reported on this plant species.

In the next chapter the isolation of the antibacterial compounds by bioassay guided fractionation will be described.

Chapter 3

Isolation of antibacterial compounds from *Ochna pretoriensis*

3.1. Introduction

Natural products have long been a major source of new medicines. Just like drugs of synthetic origin, bioactive compounds present in crude plant extracts range from simple to complex structures as a result; the isolation of such compounds from complex crude plant extracts is of great importance as they may provide useful leads for the development of new pharmaceutical products (Hamburger and Hostettmann, 1991). The activity shown by a mixture of compounds such as extracts and fractions is due to the sum of the activities of the individual constituents. Fractionation leading to isolation of such individual compounds should result in products having a higher activity than the original extract. This approach forms the basis of lead compound discovery from naturally occurring sources and has led to the introduction of many important drugs (Houghton *et al*., 2007.).

The main difficulty in using natural products as sources for pharmaceutical leads involve separating the plethora of compounds from the active compound in crude extracts. The separation techniques leading to the isolation of bioactive compounds are important in the natural product discovery process (McRae *et al*., 2007). The complexity of crude plant extracts can be simplified through different separation techniques. Bioassay guided or activity-guided fractionation is the mostly frequently cited technique for separating plant compounds and isolating only those that exhibit the desired activity (Massiot *et al*., 2002).The techniques and processes used in the isolation of the active constituents are of importance in phytochemistry and biological research as they provide information on the correlation between biological activity of the crude extracts and distinctive compounds and the determination of the polarity window of the isolated compounds (Hamburger and Hostettmann, 1991)

The most widely used analysis and isolation techniques include among others Thin layer chromatography (TLC), Gas chromatography (GC), Paper chromatography (PC), Vacuum liquid chromatography (VLC), Column chromatography (CC), high performance liquid chromatography (HPLC)

and supercritical fluid chromatography (SFC). In the Phytomedine programme of the University of Pretoria, TLC and CC are the most widely used techniques. In the isolation of antimicrobial compounds, bioautography is critically important to identify the R_f value of antimicrobial compounds present in crude extracts.

Thin Layer Chromatography is a chromatographic technique used for the separation of various components of mixtures to guide the separation process. It is performed on a sheet of glass, plastic or aluminium foil which is coated with a thin layer of adsorbent material usually silica gel or cellulose known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture also referred to as the mobile phase is drawn up the plate via capillary action. Because different analytes ascend to the TLC plates at different rates, separation is achieved. This method of separation is important to analyze fractions during the isolation and purification processes; it is very useful in the fingerprint of natural products and it is also the basis for bioautography (Stoddard *et al*., 2007).

The principles of column chromatography are similar to those of TLC except that in column chromatography, the stationary phase is within a vertical tube hence the word “column”. The choice of the stationary phase in CC depends on whether the constituents are separated based on their polarity, affinity to the stationary phase or their size. The individual components are retained by the stationary phase differently and separate from each other while they are separated at different speeds through the column with the eluent (Stoddard *et al*., 2007).

The mixtures may be simplified by a preliminary separation using a series of extractants of increasing polarity. After extraction of plant material with a single extractant, a preliminary separation may include solvent–solvent fractionation, vacuum liquid chromatography or flash chromatography. The fraction with the highest activity is separated by column chromatography usually with one or more than one solid phases to yield pure compounds. If required as a final step Sephadex LH-20 gel filtration was used to purify compounds based on their molecular sizes.

Based on experience in the Phytomedicine Programme in isolating antibacterial compounds the procedures we will use involve direct extraction of plant material with acetone, solvent-solvent fractionation of the acetone extract and column chromatographic separation of the active fractions to obtain pure active compounds.

3.2. Materials and Methods

3.2.1. Extraction, Solvent-Solvent fractionation and antibacterial activity

The dried milled leaves (300 g) of *O. pretoriensis* were extracted three times with 3 litres of acetone. The extract was concentrated with a rotary evaporator to dryness and decanted into a preweighed container. The dried extract was fractionated using the solvent-solvent fractionation procedure (Suffness and Douros, 1979) with minor modifications (where the 35% water in methanol fraction was fractionated further into 70% water in methanol and ethylacetate). The various fractions obtained from solvent-solvent fractionation were assayed for qualitative antibacterial activity using bioautography and as described in Chapter 2, 2.6. The active fractions were then selected for the isolation of antibacterial compounds using column chromatography.

3.2.2. Isolation of C1 and C2 from chloroform and ethyl acetate fraction

For the isolation of antibacterial compounds from the chloroform fraction, 120 g silica gel 60 was mixed with hexane to form a slurry. The slurry was packed in a 5 cm diameter glass column to a height of 40 cm. The chloroform fraction (1.2 g) was dissolved in a small volume of ethyl acetate, mixed with 4 g of silica gel, allowed to dry under a stream of air and loaded onto the packed column. The column was initially eluted with 400 ml of 100% hexane subsequently increasing the polarity of the eluting solvent with ethyl acetate from 10-100 % ethyl acetate. A total of 50 fractions were collected and analyzed by TLC.

To isolate the active compounds from the ethyl acetate fraction, 20 g of silica gel was mixed with ethyl acetate to form slurry and packed in a 5 cm diameter column to a height of 40 cm. The ethyl acetate fraction (250 mg) was dissolved in a small volume of acetone, mixed with 2 g of silica gel, allowed to dry under a stream of air and loaded onto the packed column. The column was initially eluted with 400 ml of 100% chloroform followed by equal volume of 10% methanol in chloroform, subsequently increasing the polarity to 100% methanol. A total of 40 fractions were collected and analyzed by TLC. Fractions with similar composition were combined and tested for antibacterial activity using bioautography and determining the MIC values. For the final stage separation of C1 and C2, column chromatography using Sephadex-LH 20 was used.

3.2.3. Isolation of C3 from the carbon tetrachloride fraction

For the isolation of antibacterial compounds from the carbon tetrachloride fraction, 20 g of silica gel 60 was mixed with hexane to form a slurry. The slurry was packed in a 5 cm diameter glass column to a height of 45 cm. The carbon tetrachloride fraction (680 mg) was dissolved in a small volume of ethyl acetate, mixed with 2 g of silica gel, allowed to dry under a stream of air and loaded onto the packed column. The column was initially eluted with approximately 400 ml of 100% hexane, then the polarity of the eluting solvent was successively increased with ethyl acetate from 10% EtAc in hexane, followed by, 20, 30, 40, 50, 60, 70, 80, 90% Etac in hexane and finally 100% ethyl acetate. A total of 70 fractions of 100 ml were collected and spotted for phytochemical analysis (TLC). Similar fractions were combined to obtain compound C3.

Fig 3.1. Schematic representation showing the stepwise procedure followed in the isolation of antibacterial compounds from *O. pretoriensis*.

3.3. Results and discussion

3.3.1. Percentage yield of fractions

The solvent-solvent procedure outlined by Suffness and Douros (1979) resulted in seven fractions. Hexane fraction collected the most quantity of material from the crude extract followed by water, butanol, 70% water in methanol, carbon tetrachloride and lastly ethyl acetate. This shows to some extent that the crude extract contained a lot of non-polar material.

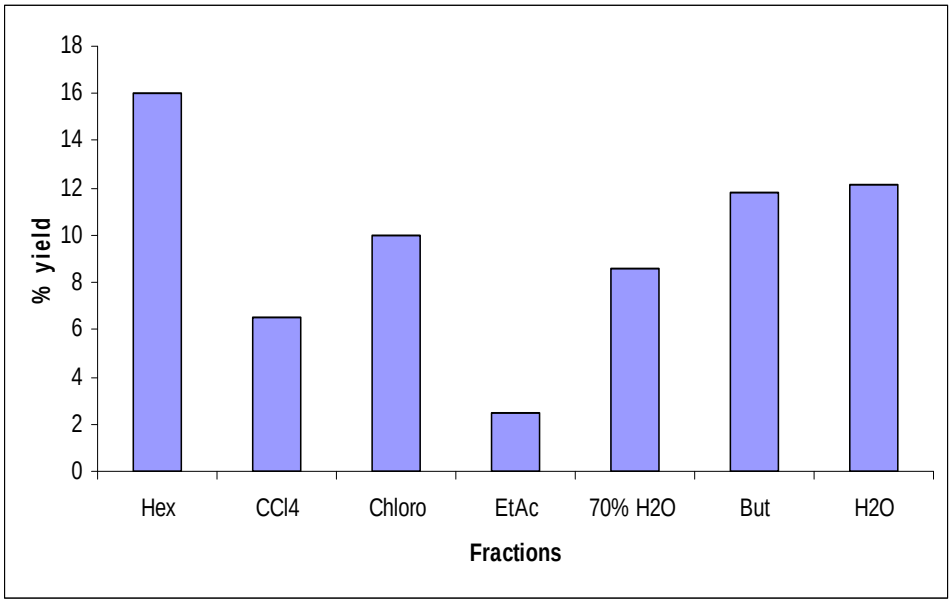


Figure 3.2. Percentage yield of fractions from *Ochna pretoriensis* acetone extract.

3.3.2. Phytochemical analysis and Bioautography S-S fractions

The compounds present in the fractions were separated better by CEF than EMW. This implies that the compounds in the fractions may be of intermediate polarity. The compounds had different Rf values in each fraction but, there were similar compounds present in the chloroform and the ethyl acetate fraction, with equal Rf values. Three of the seven fractions had most of the active compounds in bioautography i.e. the carbon tetrachloride fraction, chloroform fraction and ethyl acetate fraction. Bioautograms developed in CEF had a better separation that showed that the three fractions do have antibacterial compounds. The chloroform and ethyl acetate fractions contained four similar antibacterial compounds as indicated by their Rf values (0.28, 0.6, 0.76 and 0.92). The carbon tetrachloride fraction had a simple

chemical composition with four antibacterial compounds with R_f values of 0.52, 0.64, 0.76 and 0.88 with CEF as solvent system.

The aim was now to isolate the compounds with antibacterial activity from the three fractions. The two major antibacterial compounds in the carbon tetrachloride fraction had a bluish colour when sprayed with vanillin-sulphuric reagent spray. Three of the antibacterial compounds in the chloroform and the ethyl acetate fraction gave an orange to reddish colour and one had a purplish colour upon reaction with vanillin-sulphuric reagent spray.

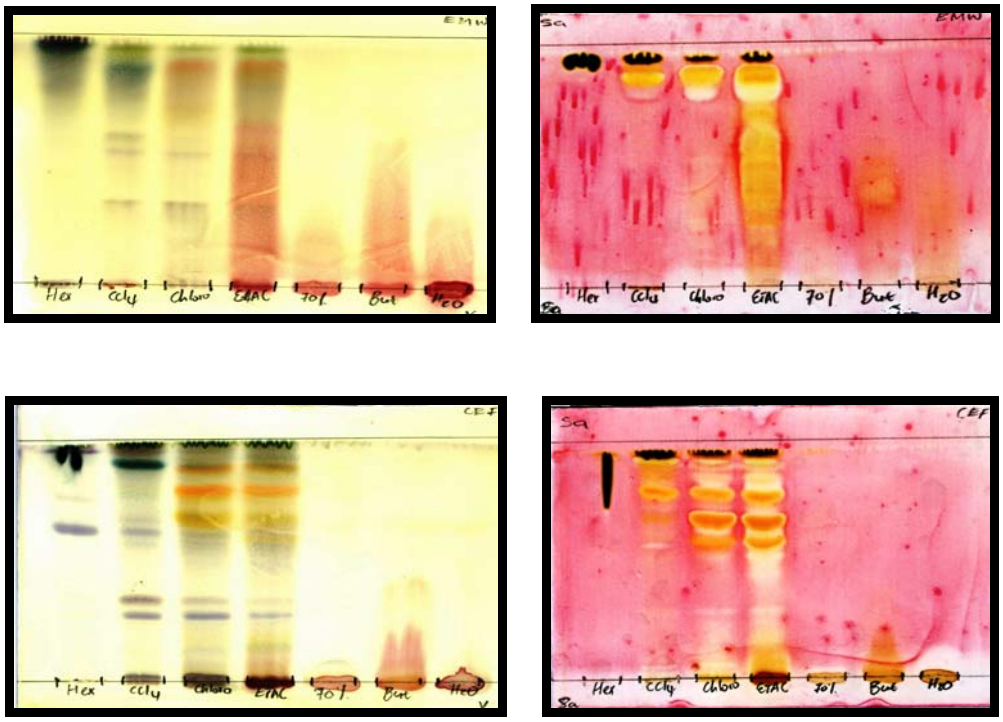


Figure 3.3. Chromatograms developed in EMW (1) and CEF (2) and sprayed with vanillin-sulphuric acid reagent spray (A) and *S. aureus* (B). The clear spots on the pinkish background in B indicate zones of inhibition.

3.3.3. Isolation

The carbon tetrachloride fraction was subjected to open column chromatography and 70 fractions were collected. After the TLC analysis, fractions 9-11, fractions 18-23, fractions 38-40, fractions 41-48, fractions 57-61, and fractions 67-69 were combined to yield seven sub-fractions labelled 1A, 2A, 1B, 2B, 3, 4 and 5 respectively. The pooled fractions were tested for antibacterial activity using bioautography. From the seven fractions tested, only three fractions were active i.e. fractions 2A, 2B and 3.

From the ethyl acetate fraction, 50 fractions were collected and analyzed by TLC. After TLC analysis, fractions 1-4, fractions 5 and 6, fractions 7-10 and fraction 11 and 12 were pooled together individually and labelled 1, 2, 3, F1 and F2. The combined fractions were tested for antibacterial activity and fractions 1, 2, and 3 were active. The methanol wash from the chloroform fraction was mixed with the fraction labelled 3.

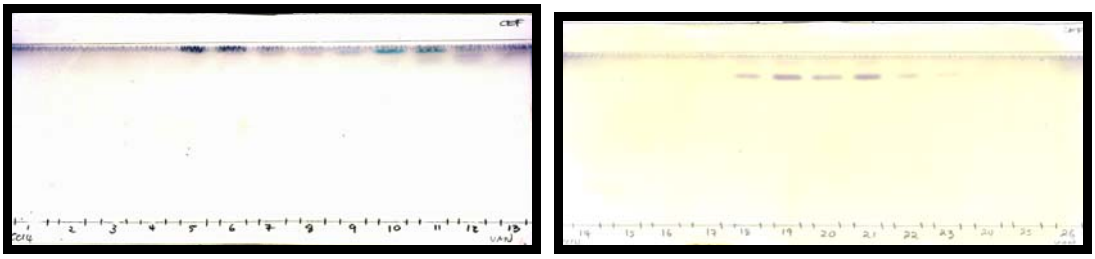


Fig 3.4. Chromatograms developed in CEF of fractions from the carbon tetrachloride fraction after open column chromatography and sprayed with vanillin sulphuric acid.

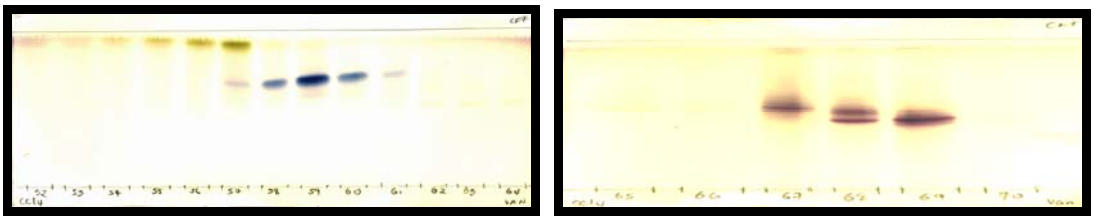


Fig 3.5. Chromatograms developed in CEF of fractions from the carbon tetrachloride fraction after open column chromatography and sprayed with vanillin sulphuric acid.

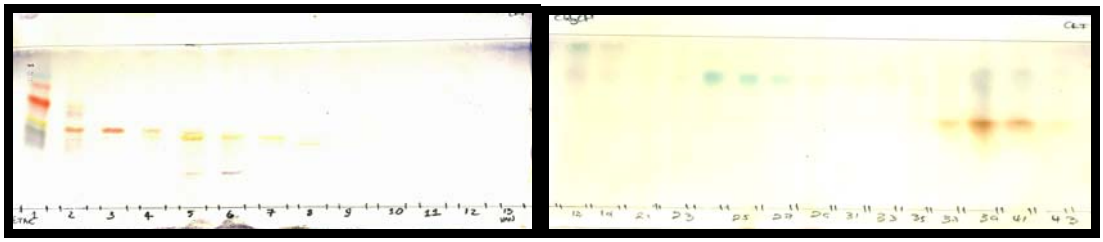


Fig 3.6. Chromatograms developed in CEF of fractions from the ethyl acetate fraction and chloroform fraction after open column chromatography and sprayed with vanillin sulphuric acid

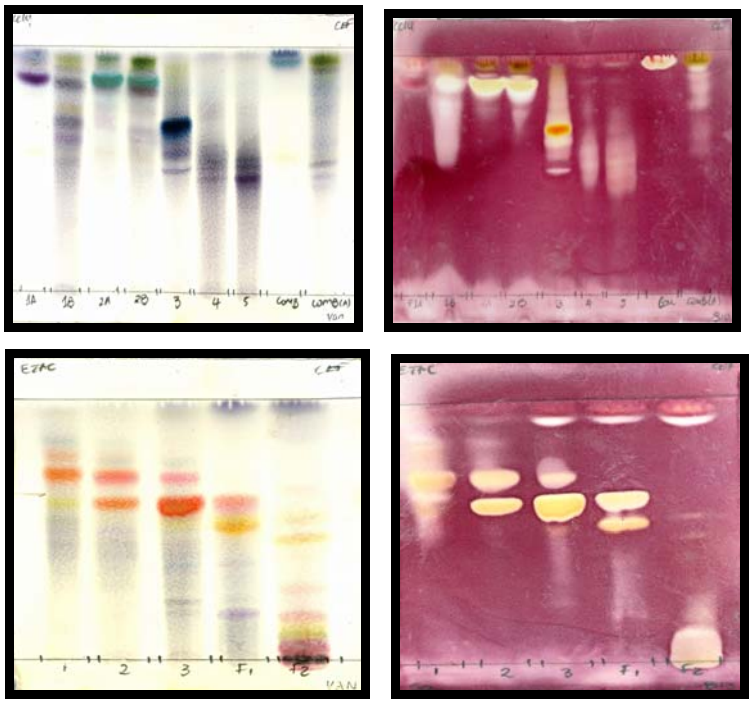


Figure 3.7. Chromatograms developed in CEF of the pooled carbon tetrachloride (1) and ethyl acetate fractions (2), sprayed with vanillin-sulphuric acid reagent spray (A) and *S. aureus* (B).

Fractions 1, 2 and 3 from the ethyl acetate and the chloroform fraction were combined and separated on sephadex. A total of 6 fractions were collected and compound 1 (C1) (30 mg) was isolated as yellow crystals and compound 2 (C2) (10 mg) as yellow crystals.

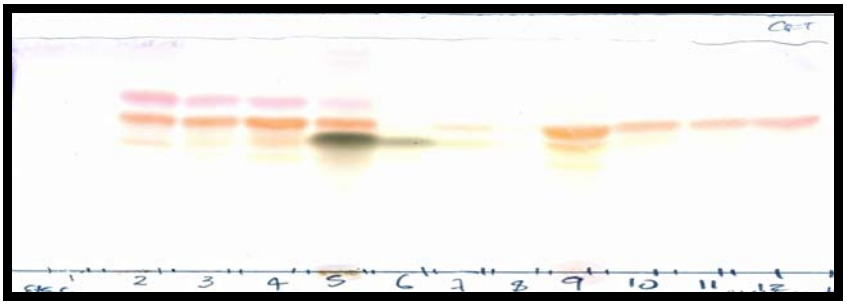


Figure 3.8. Chromatograms developed in CEF of the ethyl acetate fraction separated with sephadex to separate compound 1 and 2 and sprayed with vanillin-sulphuric acid.

Fractions 2A and 2B from the carbon tetrachloride were combined and washed with hexane. During washing, compound 3 (C3) (12 mg) precipitated out as a white powder from a mixture of ethyl acetate and hexane.

To ensure the purity of the isolated compounds, 10 mg/ml concentration of all the compounds in acetone was prepare and 10 µl of each was loaded on separate TLC plates and developed in different mobile systems. For C1 and C2, CEF (1:4:2) was used and for C3 chloroform ethyl acetate (1:4) was used to develop the plate. After developing, the plates were sprayed with vanillin-sulphuric acid reagent spray and heated at 110 until maximum colour development. Compound 1 gave an orange colour upon reaction with vanillin-sulphuric acid, compound 2 also gave an orange colour when it reacted with vanillin-sulphuric acid and lastly, compound 3 gave a purple colour upon reaction with vanillin-sulphuric acid

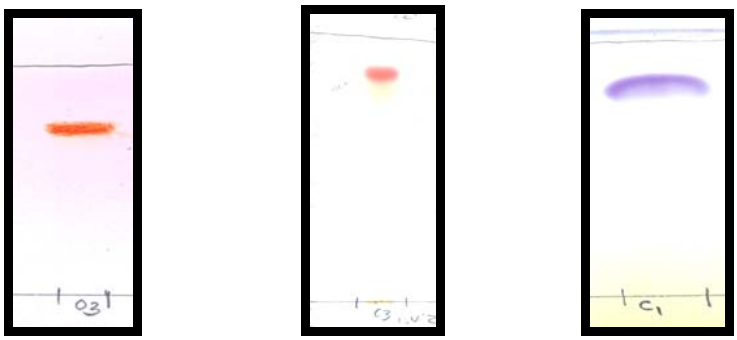


Figure 3.9. TLC fingerprints of four pure compounds isolated from *O. pretoriensis*.

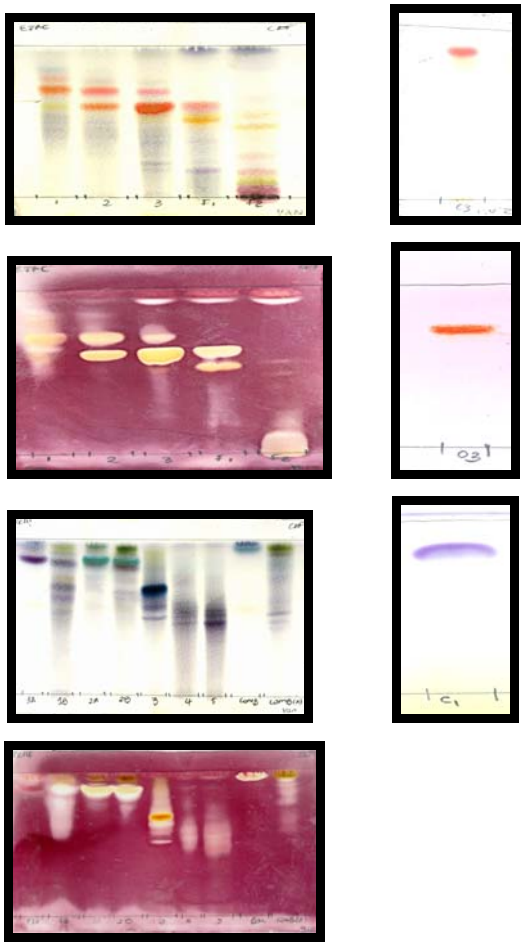


Figure3.10. Diagram indicating the pure active compounds from the pooled fractions.

3.4. Conclusion

Three antibacterial compounds were successfully isolated from the leaves of *Ochna pretoriensis* using column chromatography. Based on the different polarities of solvents used in the separation of these compounds and their different Rf values, it is evident that these compounds have a varying range of polarities although compound 1 and compound 2 may be closely related structurally. The two compounds gave the same colour when sprayed with vanillin-sulphuric acid but had different Rf values with the TLC solvent system used. The compounds were separated by a combination of silica gel and Sephadex chromatographic systems. The structural elucidation and chemical characterization of the isolated compounds is discussed in the next chapter.

Chapter 4

Chemical characterization and structure elucidation of compounds isolated from *O. pretoriensis*

4.1. Introduction

The ability of chemists to synthesize purely artificial medicinal compounds leads to the production of many effective medicines. The promise of wholly synthesized compounds with relatively simple structures and known modes of action is starting to diminish as new diseases and drug resistant strains of existing pathogens continue to emerge. As such, attention is focused on natural sources of lead compounds in which exists a wealth of more complex compound structures and novel modes of action (Lesney, 2004). While there are many different compounds that can be found within the 250 000 flowering plant species worldwide, they can be classified into distinct classes of compounds based on similar characteristics. The main classes of bioactive compounds from plants include flavonoids, terpenoids, terpenes, alkaloids, saponins and coumarins (Cowan, 1999).

Isolation and characterization of biologically active compounds from medicinal plants is an important tool in drug discovery which led to the isolation of pharmacologically active drugs such as cocaine, codeine, digitoxin, quinine and morphine (Newman et al., 2000). It is after the compound has been characterised (both chemically and biologically) that it can be assessed in terms of its potential as a lead compound and indeed if the compound is actually novel and worthy of further investigations. To be considered as a pharmaceutical lead, the active compound must have a reasonably simple structure so it can be easily synthesized or have a novel mode of action that is more efficient than current drugs. Since activity and mode of action are the most critical characteristics of the lead compounds in phytochemistry and pharmaceutical science research, and because they are intrinsically linked to the structure of the compound, accurate structural elucidation is a key factor in obtaining lead compounds from plants (McRae *et al*., 2007).

The structures of pure active compounds isolated from plants can be determined using information obtained from various spectroscopic techniques like Nuclear Magnetic Resonance (NMR), Mass Spectroscopy (MS), infrared (IR), Ultraviolet (UV).

4.2. Materials and methods

4.2.1. Nuclear Magnetic Resonance (NMR) analysis

The nuclear magnetic resonance spectrum of the isolated compounds was determined using the Varian Unity Inova 300 MHz (University of Kwa-Zulu Natal). Both the one dimension and two dimensional spectra were generated. Approximately 15-20 mg of each of the compounds were dissolved in deuterated acetone and then pipetted into NMR tubes and analysed based on the under listed experiments. The spectral results were then used to elucidate the structures of the isolated compounds.

4.2.2 ^{1}H and ^{13}C NMR (One dimensional spectra)

One dimensional NMR spectra were recorded for the isolated compounds on Varian 300 Spectrophotometer using deuterated methanol as solvent. 2D NMR (COSY, HMBC and HMQC) data were also obtained.

4.2.3 HMQC ^{13}C - ^{1}H correlation.

Heteronuclear multiple quantum correlation (HMQC) was used for the determination of substitution patterns at the different carbon atoms, correlation of carbon shifts, multiplicity and proton shifts were compiled from a two dimensional heteronuclear correlation spectrum. The combined spectral information allowed a view of carbon atoms showing different functionalities and substitutions.

4.2.4 COSY- ^{1}H-^{1}H 2D

Two dimensional spectra were obtained by recording a series of conventional NMR in which two parameters are changed incrementally. The most readily established connections between the individual carbon atoms were derived from connectivities through couplings between the protons as compiled from a COSY spectrum. From these correlations, the main parts of the proton spin systems were outlined and several structural fragments were identified.

4.2.5 HMBC long range ^{1}H-^{13}C correlation.

Heteronuclear bond correlation (HMBC) experiment provided information on the direct ^{13}C and ^{1}H heteronuclear connectivity. The method relies on the indirect detection of the ^{13}C by observing their effects on the more sensitive proton nuclei to which they are coupled. It not only shows the connection of unprotonated carbon atom to the proposed elements, but also indicated vicinal proton relationships not resolved in the COSY spectrum. Analysis and interpretation of the spectroscopic data obtained led to the structures proposed for the active compounds are presented below.

4.3. Results and discussion

Structural elucidation and characterization of isolated compounds

4.3.1 Spectroscopic analysis of isolated compounds.

The different interactions of electromagnetic radiation with organic compounds based on their structural features form the basis of the application of spectroscopy in the structural elucidation of organic compounds. The identification of compounds involves a diverse range of analytical techniques and methods such as nuclear magnetic resonance (NMR), ultraviolet (UV) and infrared (IR) spectroscopy, and mass spectroscopy (MS). In this study NMR technique was used for the identification and structural elucidation of the compounds isolated from *Ochna pretoriensis.*

4.3.2 Compound 1

Compound 1 was isolated as yellow amorphous solid. The NMR spectra of this compound are presented in Appendix, A1-F1. The spectra data are consistent with a biflavonoid which consist of a flavonone and a flavone unit linked through a C-O-C linkage between the C-4'' of a flavone ring to the C-3' of the flavonone ring.

This class of compound consists of two sets of three ring systems A, B, and C. Ring B which has a hydroxy substitution at C-4 often gives a typical 4 peak pattern of two doublets (AA'BB' system) with a characteristic coupling constant; this pattern was clearly shown in C-3, C-5, C-2 and C-6 with resonance at 7.99 ppm and 7.07 ppm respectively and a coupling constant of 9 Hz at ring IIB of the flavone ring. The protons at H-6 and H-8 in ring A consist of 2 doublets with signal at 5.99 ppm and 5.96 ppm respectively with a coupling constant of 2.1 Hz; this is due to the meta-coupling of these two related protons. This is often characteristic of flavonone which contain the 5, 7 dihydroxy substitution as observed in compound A. This is differentiated from the substitution pattern in flavone because the metacoupled proton at 5, 7 dihydroxy substitution occurs further downfield in the flavone component when compared with flavonone.

The flavonone component is further confirmed by the two doublets at 5.54 and 5.50 ppm; this is due to the proton at C-2 positioned which is coupled to the two protons at the C-3 positions. The two proton at the C-3 also showed characteristic signal at 3.31 and 2.82 ppm which exist as doublet of doublet because the two protons are coupled to each other and the proton at the C-2 position. Two ^{1}H signals at 12.92 and 12.13 ppm are consistent with two chelating protons (H-5' and H-5'' respectively) in the biflavonoid structure of the isolated compound. One distinct singlet was observed at 6.66 and 6.83 ppm due to the singlets at II-H-3, this clearly differentiated the isolated compound from the amentoflavone series with a second singlet at the I-H-3 position.

Compound 1 was isolated in its aglycone form as the absence of glycoside derivative is confirmed by the complete absence of glucosyl signals in the chemical shift range of 4–5 ppm. The occurrence of the signals of H-2' and H-6' at 8.12 ppm and 8.04 ppm was affected by the nature of substitution on the C-ring. The observed resonances differentiated the isolated compound from other classes of flavonoids with different oxidation level on the C-ring such as chalcones, aurones, dihydroflavonols, isoflavones and flavonols. While the coupling constant of these classes of flavonoids is similar for the C-2 and C-6, their chemical shift signals differ by the substitution pattern on the C-ring. This is differentiated from the isoflavones class with C-2 substitution which occurred further downfield because it's in a beta position to the ketone group at the fourth carbon position; it is also differentiated from the chalcones which exist as a doublet due to the double bond between C-2 and C-3.

Table 4.1: ^{1}H and ^{13}C NMR data for compound 1.

Position	^{13}C (ppm)	^{1}H (ppm)	Rao *et al* (1997) ^{13}C
2	80.22	5.52	79.4
3	44.14	3.22; 2.82	43.4
4	197.54		197.3
4a	103.90		103.7
5	165.70	12.13 (0H)	165.1
6	96.64	5.96	95.5
7	168.17		168.8
7-OMe (**C2**)	55.86	3.802	56.0
8	95.51	5.99	94.6
8a	164.02		163.9
1'	134.88		132.1
2'	121.71	7.83	121.5
3'	144.77		142.7
4'	154.60		150.6
5'	118.11	7.23	118.4
6'	125.04	7.85	125.5
2''	164.84		164.4
3''	105.47	6.66	105.0
4''	183.71		183.0
5''	105.47	12.92 (OH)	105.3
6''	125.99	6.25	163.3
7''	165.99		164.9
8''	95.51	6.51	94.8
8a''	159.46		158.8
1''	125.99		125.9
2''	129.73	7.77	129.1
3''	118.11	7.03	117.4
4''	164.02		162.1
5''	118.11	7.03	117.4
6''	129.73	7.77	129.1

HMQC

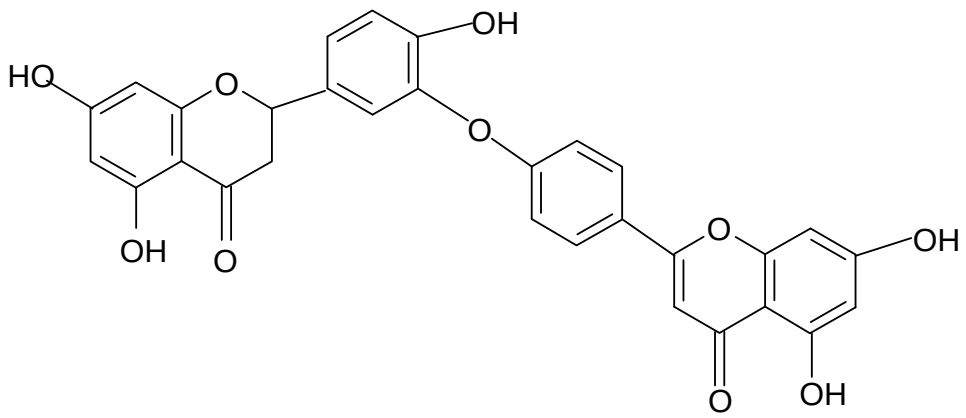
From the HMQC spectra in Appendix C1, which showed the substitution pattern directly on the carbon atom, the following prominent linkages were clearly observed.

Table 4.2: Carbon-Hydrogen linkages for compound 1

Carbon number	Hydrogen linkage
C-3 (44.14)	H-3a (2.8), H-3b (3.2)
C-2(80.22)	H-2 (5.6)
C-6(95.51)	H-6 (5.99)
C-2" (129.73)	H-2" (7.77)
C-3" (118.11)	H-3" (7.03)
C-5" 118.11	H-5" (7.03)
C-6"129.73	H-6" (7.77)

COSY spectra (Appendix D1) which indicate $^{1}H - {}^{1}H$ direct correlation clearly showed the proton at H-3a with a chemical shift of 3.22 ppm is coupled to the proton at H-3b with a chemical shift of 2.82; this confirm the flavonone component of the biflavonoid.

The ^{1}H NMR, 13C NMR and 2DNMR (HMQC, HMBC, COSY and DEPT) data showed compound 1 is ochnaflavone; this was further confirmed because the spectra data for isolated compound was similar to the data in the first isolation of this compound (Rao *et al*, 1997). The structure is as shown below



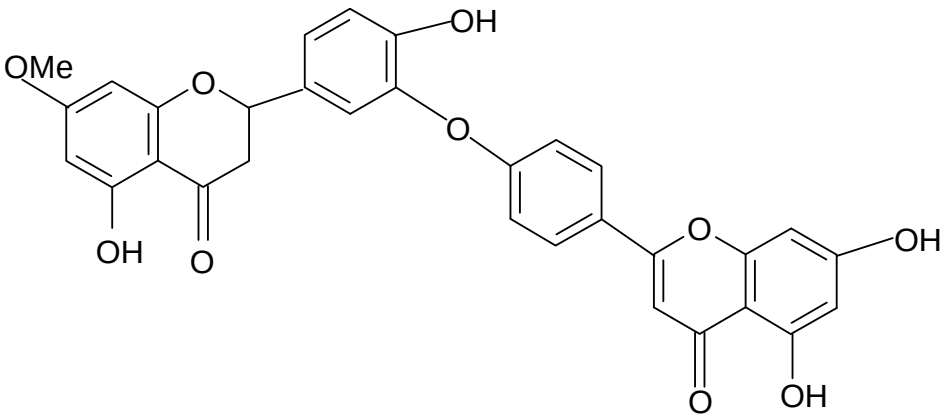
Ochnaflavone

Figure 4.1. Chemical structure of C1

4.3.3 Compound 2

Compound **2** was isolated as a yellow solid which showed a similar spectral pattern to compound **1** as shown by all the proton and carbon spectra for the two compounds (Appendix A2-F2). The only major difference was the observation of a methoxy signal in both proton and carbon spectra in compound 2. There was 3.802 ppm in ^{1}H NMR and 55.86 ppm signal in its ^{13}C NMR spectrum (Appendix A2 and B2). Correlation of the obtained data with a previously isolated methoxy derivative of ochnaflavone confirmed compound **2** to be ochnaflavone 7-O- methyl ether (Rao *et al*, 1997).

The proposed structure of the compound 2 is as shown below.



Ochnaflavone 7-O- methyl ether

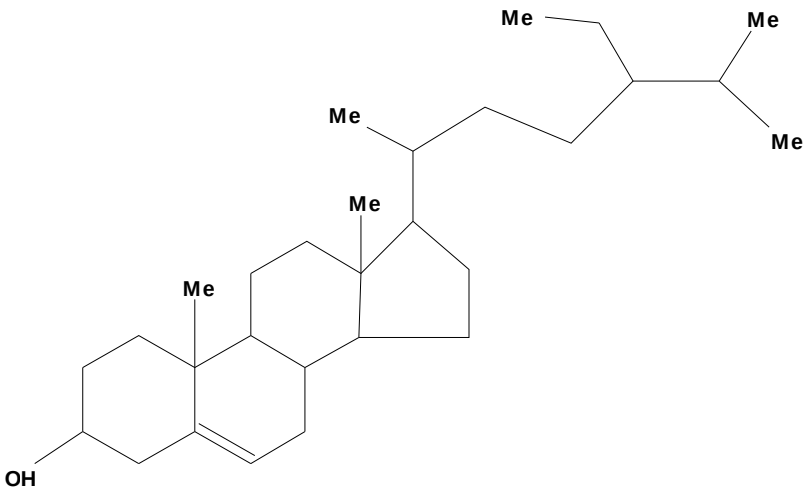
Figure 4.2. Chemical structure of C2

4.3.4. Compound 3

The NMR Spectrum of compound 3 showed the chemical shift for six methyl protons, an olefinic proton with a complex upfield signal which showed high level of saturation in the Carbon-Proton linkages in its structure. The major characteristic signal for this class of compound is the chemical shifts of the C-6 olefinic signal and the C-29 Methyl signal. This confirmed the isolated compound to be 24-ethyl steroid derivative. Comparison of the proton and carbon spectra data for compound 3 with the compiled data of previously isolated steroids showed compound 1 to be *ß* -Sitosterol- a common plant sterol (Rubinstein et al., 1976, Kovganko *et al*., 2000).

Table 4.3. ^{13}C and ^{1}H NMR spectra data for compound 3.

Carbon position	Chemical shift	Major proton chemical shift	Kovganko *et al*., 2000 (^{13}C)
1	37.23		37.3
2	31.9		31.6
3	71.80	3.51 m	71.8
4	42.29		42.3
5	140.7		14.08
6	121.7	5.33 m	121.7
7	31.65		32.1
8	31.9		32.1
9	50.11		50.2
10	36.5		36.5
11	21.07		21.1
12	40.48		39.8
13	42.3		42.3
14	56.75		56.8
15	24.3		24.3
16	28.2		28.3
17	56.04		56.1
18	12.0	0.72 CH_3	12.0
19	19.4	1.0 CH_3	19.1
20	36.5		36.2
21	19.1	0.93 CH_3	18.8
22	34.0		34.0
23	26.1		26.2
24	45.9		45.2
25	29.6		29.2
26	19.4	0.83 CH_3	18.9
27	19.7		19.1
28	23.1	0.82 CH_3	23.1
29	11.9	0.86 CH_3	11.9



ß-Sitosterol

Figure 4.3. Chemical structure of C3

4.4. Conclusion

The NMR spectral data was used to chemically characterize and elucidate the structures of three compounds isolated from O. pretoriensis. The three compounds were identified as (Chemical names) *ochnaflavone,* ochnaflavone 7-O- methyl ether *and ß-Sitosterol*. The *Ochna* genus is reported to contain this group of compounds in abundance. Even though these compounds have been previously isolated from other plant species from different genus, this is the first report on the isolation of this three compounds form *O. pretoriensis*.

Chapter 5

Evaluation of recovery of activity during fractionation and evidence for synergism in the crude extract

5.1. Introduction

As previously stated there is an increasing concern about the problems of antimicrobial resistance in human and animal health; as a result, there is a need to search for or create novel classes of antibacterial drugs (Wierup, 2000). Secondary metabolites/products involved in plant defence through cytotoxicity towards microbial pathogens could prove useful as antimicrobial medicine in human and animal medicine if not toxic. Potential antimicrobial compounds should inhibit or kill the target microorganism but not be toxic to the host cell i.e. antimicrobial compounds should be selective (Cragg and Newton, 2005).

The isolation and use of natural products has resulted in replacement of plant extracts used before the antibiotic era with single chemical entities. There is a basic supposition that any plant possessing clinical effectiveness must contain an active principle which can completely replace the plant extracts (Phillipson, 1995). Many of the most effective phytomedicines are on the drug market as whole plant extracts and practitioners believe that synergistic interactions between the components of individual or mixtures of herbs are a vital part of their therapeutic efficacy (Williamson, 2001).

In theory, the activity shown by extracts and fractions is due to the sum of the activities of the individual constituents. Thus fractionation leading to isolation of the individual compounds should result in fractions having a higher activity than the original extract (Houghton *et al.,* 2007). If the active compound was present at a concentration of 0.1% in the crude extract one would expect the pure compound to have an activity 1000 times higher than that of the crude extract if there was a 100% recovery. It has been stated before that if the quantity of a fraction and the activity is brought into consideration closer examination should indicate any changes in total activity during the isolation process (Eloff, 2004). In this chapter, the quantitative antibacterial activity of the crude extract and fractions obtained by solvent-solvent fractionation was determined and by taking relevant quantities into consideration the total activity was calculated in an attempt to investigate the presence of synergy in the crude extract or loss during the fractionation.

5.2. Material and Methods

5.2.1. Determination of total activity

The microdilution assay by Eloff 1998a, was used as described in chapter 2, section 2.6. From the MIC values and mass present for each fraction, the total activity of all the fractions was determined by dividing the mass in mg by the MIC in mg/ml (Eloff, 2004). This gives the volume in ml to which that fraction can be diluted and still kill the test organism.

5.3. Results and discussion

The MIC and the total activity of *O. pretoriensis* crude acetone extract and fractions obtained by solvent-solvent fractionation were determined (Table 5.1 and Table 5.2). The calculation of total activity after every step of bioassay-guided fractionation is of great importance to determine if there is a loss of biological activity during fractionation of active plant extracts (Eloff, 2004). The total activity values of the crude extract and the fractions obtained by solvent-solvent fractionation were compared in order to establish if there was any loss or gain/increase in antibacterial activity during fractionations and subsequent purification of compounds. In addition, the percentages of activity recovered were calculated. This is important in investigating the presence of synergism which is an important aspect in phytomedicine as it explains the need for the isolation of single active constituents from plant extracts and helps to explain the efficacy of apparently low doses of the active constituents in herbal products (Williamson, 2001).

5.3.1. MIC values of crude extract and fractions

Based on the MIC values only three of the seven fractions (carbon tetrachloride fraction, chloroform fraction and the ethyl acetate fraction) representing compounds with intermediate polarity had substantial antibacterial activity (Table 5.1, Figure 5.1). There appeared to be no difference in the activity against Gram-positive and Gram-negative bacteria. The MIC values obtained correlates well with the bioautography results obtained in Chapter 3 (figure 3.3) where defined lines of bacterial inhibition were observed only in three fractions with low MICs. The results also support the selection of these fractions to isolate antibacterial compounds based on bioautography.

Table 5.1. MIC (mg/ml) of the crude extract and seven fractions from the acetone leaf extract of *O. pretoriensis* against test organisms and quantity in mg present in each fraction. (In cases where Standard Deviation not included, SD=±0.00.)

Fractions/ organisms	Crude extract	Hex	CCl_4	Chloro	EtAc	70%H_2O	But	H_2O
E. coli	0.065 (±0.02)	2.50	0.156	0.156	**0.039**	2.50	2.50	2.50
P. aeruginosa	0.104 (±0.04)	1.25	0.078	0.078	**0.039**	1.67(±0.72)	1.67(±0.72)	2.50
S. aureus	0.104 (±0.04)	2.50	0.078	**0.039**	**0.039**	2.50	2.50	2.50
E. faecalis	**0.039**	1.67(±0.72)	**0.039**	**0.039**	**0.039**	1.67(±0.72)	1.67(±0.72)	2.50
Average MIC	**0.078**	1.98	**0.088**	**0.078**	**0.039**	2.08	2.08	2.50
Quantity in mg	10 480	1720	680	1070	250	900	1239	1268

The difference in the bacterial inhibitory effects of the crude extracts and the fractions after fractionation may be explained to be a result of either synergy and/or antagonism. In the case where the MIC values decreased upon fractionation, the increased inhibitory effects may be due to the removal of antagonistic or inhibitory compounds present in the crude extract which interferes with the activity of antibacterial compounds present in the crude extract. These inhibitors act by affecting the absorption, assimilation and subsequent metabolism of the active constituents by the organism or form inactive complexes by coupling with the active compounds. In the case where the MIC values of the fractions are higher than those of the crude extracts, the decreased inhibitory effects may be associated with the synergistic antibacterial activity of all the compounds in crude extract because it is expected that upon fractionation and isolation of active constituents, the activity of the isolated constituents should be enhanced.

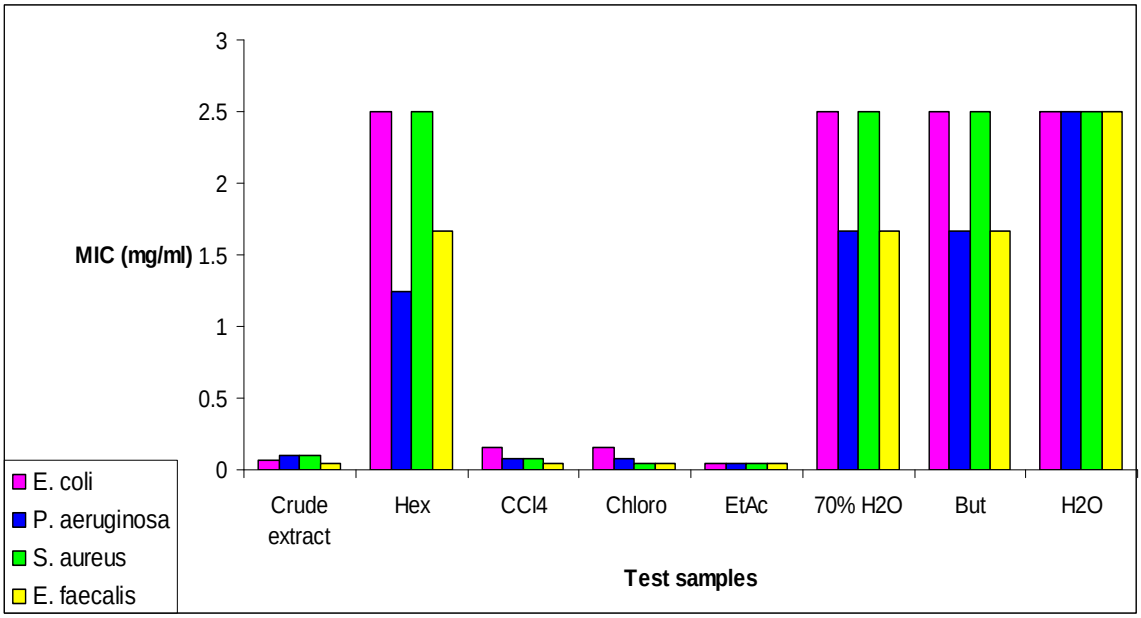


Figure 5.1. Average MIC values of the crude acetone extract of *O. pretoriensis* and seven fractions obtained by solvent-solvent fractionation against *E. coli, P. aeruginosa, S. aureus* and *E. faecalis*

5.3.2. Evaluation of the total activity of the crude extract and the fractions

The quantity and activity of the antibacterial compounds present in the crude extracts and the fractions was determined by calculating the total activity for each test material against every bacterium (Figure 5.3). Total activity indicates the volume to which the biologically active compounds present in the fraction can be diluted and still kill the test organisms. In this study, the total activity of the crude extract was calculated using the initial mass of the extract in comparison to the yields of the fractions. In general the main purpose of determining the total activity of each fraction was to identify fractions with high total activities which can then be selected for further investigation. The comparison of the total activity of crude extracts and fractions obtained after every step of fractionation during isolation of bioactive compounds by bioassay-guided fractionation makes it easy to determine if there is a change in biological activity along the way (Eloff, 2004).

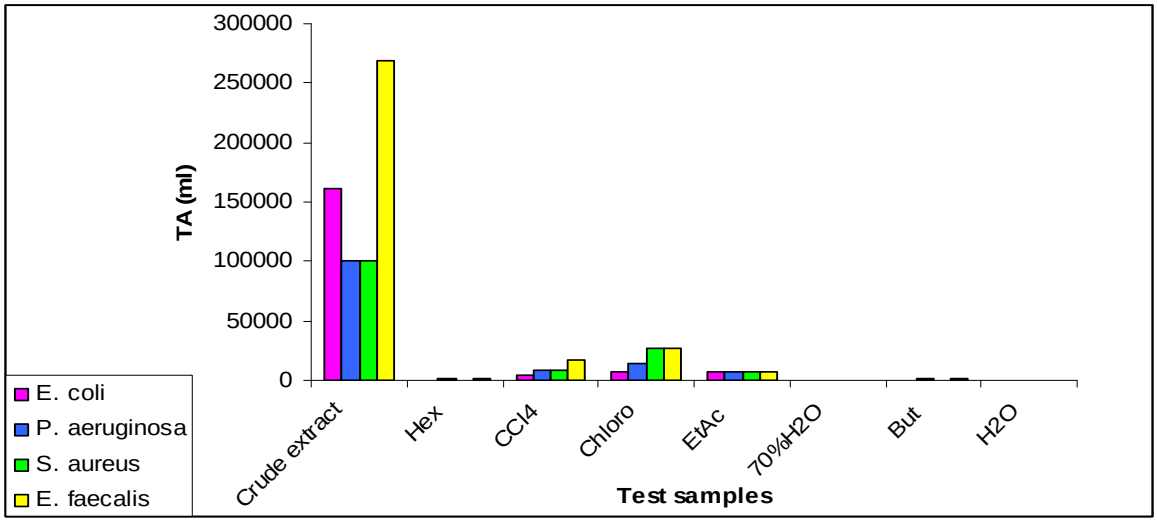
As expected the fractions with a low MIC (carbon tetrachloride, chloroform and ethyl acetate fractions) had the highest total activities against all the test organisms. The chloroform fraction had the highest average total activity compared to all the other fractions (figure 5.2(B). On average, the chloroform had the highest total activity against all the test organisms. The average total activity of the chloroform fraction was 18 862 ml followed by 9808 ml for the carbon tetrachloride fraction and 6410 ml for the

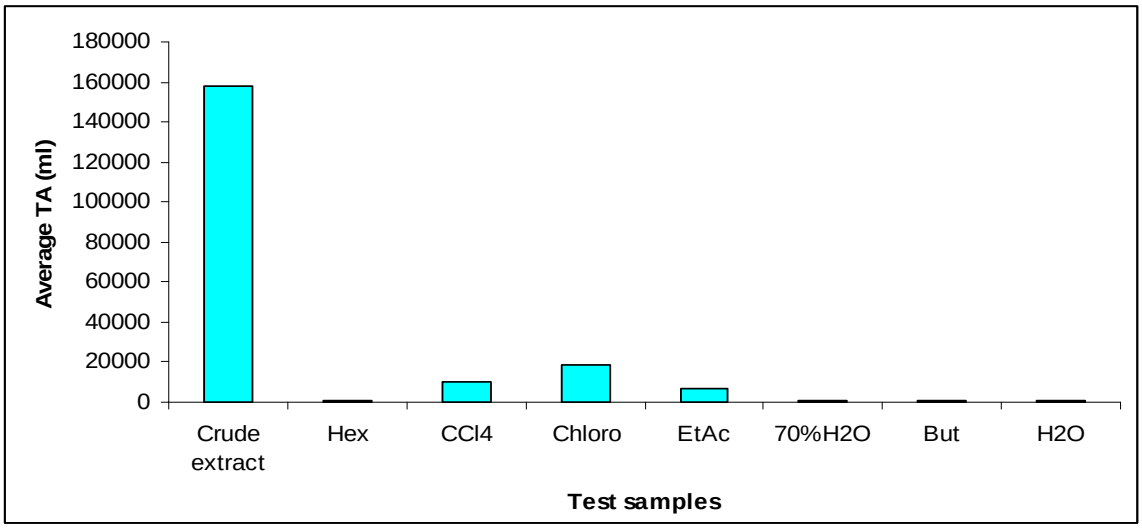
ethyl acetate fraction. These results indicate the importance of determining the total activity. If only MICs were considered, the ethyl acetate fraction would have been the only fraction selected for further analysis because the average MIC was two times lower than the chloroform fraction (i.e. two times as active). When total activity was determined the chloroform fraction had close to three times higher activity than the ethyl acetate. All the other fraction had combined total activities less than 1000 ml.

E. faecalis was the most sensitive followed by *S. aureus*, *P. aeruginosa* and *E. coli* (Figure 5.2 (C). The average total activity of the crude extract (157 872 ml) was much higher than to the combined average total activity of the fractions (37 600 ml). The loss of more than three quarters in the total activity of the crude extract compared to the fractions is a strong indication for the presence of synergic interactions of the different compounds in the crude extract which are separated in the fractions. During fractionation, approximately 3.4/10.48 g of the initial mass of the crude extract was lost as pellicles in solvent interfaces and this may imply that the interfaces did contain some compounds with antibacterial activity. Even when this loss is taken into consideration, there is still strong evidence for synergism.

Table 5.2. Total activity in ml of crude extract and fractions obtained by solvent-solvent fractionation against *E. coli*, *P. aeruginosa*, *S. aureus*, and *E. faecalis*

Fractions/ organisms	Crude extract	Hex	CCl_4	Chloro	EtAc	70% H_2O	But	H_2O
E. coli	161 231	688	4359	6859	6410	360	496	507
P. aeruginosa	100 769	1376	8718	13 718	6410	539	742	507
S. aureus	100 769	688	8718	27 436	6410	360	496	507
E. faecalis	268 718	1030	17 436	27 436	6410	539	742	507
Average	**157 872**	**945**	**9808**	**18862**	**6410**	**449**	**619**	**507**





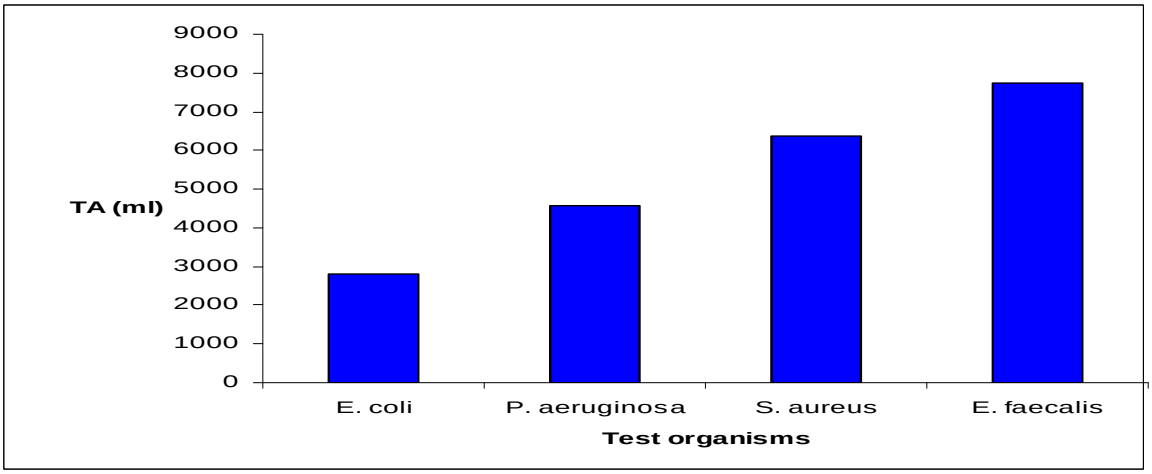


Figure 5.2. Total activity of crude extract and fractions obtained by solvent-solvent fractionation from the acetone leaf extract of *O. pretoriensis* (A), average total activity of the test samples to confirm the most active test sample (B), and average total activity of fractions to reveal the sensitivity of the test organisms (C) (TA averages were calculated from total activity values of all the fractions against each of the bacterial species).

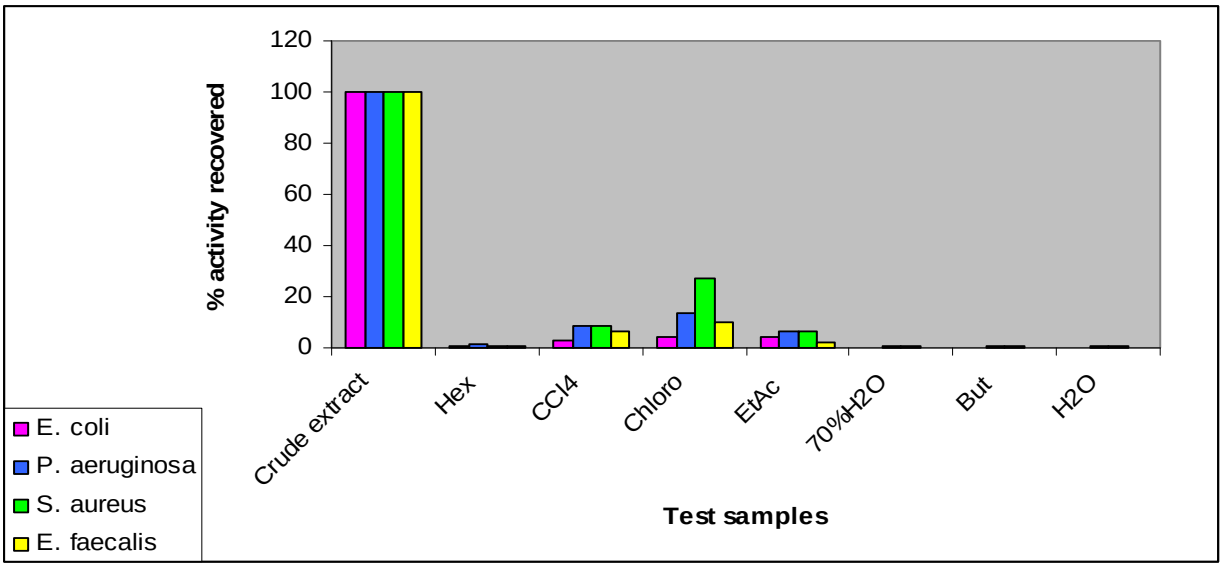
5.3.3. Evaluation of the percentage of activity recovered upon fractionation

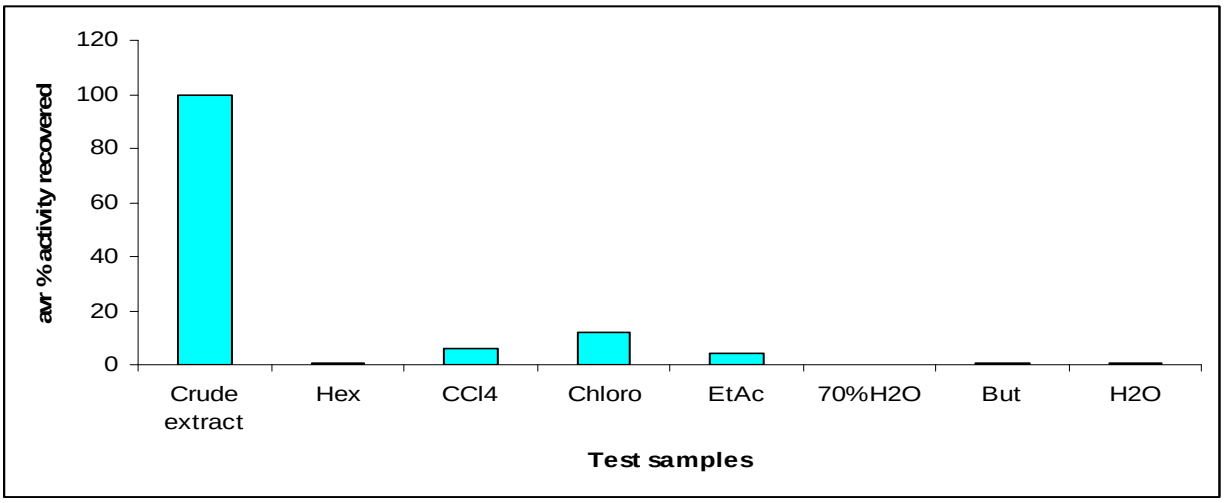
To further quantify the presence of synergistic antibacterial inhibitory effects of compounds present in the acetone crude extract of *O. pretoriensis*, I calculated the percentage of activity recovered for every fraction against the test organisms (Figure 5.3).

The highest average percentage of activity recovered was for the chloroform fraction and the carbon tetrachloride fraction with 12% and 6% activity recovered respectively. Less than 1% of average activity was recovered in the hexane fraction, 70% water in methanol, butanol and the water fraction. Most of the activity was recovered in the chloroform fraction with approximately 28% activity recovered against *S. aureus*, 14% against *P. aeruginosa*, 10% against *E. faecalis* and 5% against *E. coli*. Equal percentages of activity were recovered in the carbon tetrachloride and the ethyl acetate fraction against *P aeruginosa* and *S. aureus* (9% and 6.5%) respectively.

The experience in the Phytomedicine Programme is that in practically all cases the most active compounds were found in the intermediate polarity fractions. In using the mild solvent-solvent fractionation procedure described by Suffness and Douros, (1979) loss of activity by chemical changes should be minimal. The activity recovered in the chloroform fraction was 2 times more than the activity recovered in the carbon tetrachloride fraction. This indicates that the chloroform fraction is the best fraction from which antibacterial compounds can be isolated. Approximately 24% of the activity of the crude extract was regained after fractionation i.e. approximately 76% of activity was lost.

One alternative explanation to synergism is that the 32% (3.4 g/10.48 g) loss of dry mass in solvent interfaces during fractionation or loss of volatiles during drying could have been very active compounds. The 3.4 g should have been responsible for a loss of 120 272 ml. This means that the MIC of these lost compounds should have been 0.028 mg/ml. This is not impossible, but if a major part of the loss was due to evaporation of non-polar volatile compounds it appears to very unlikely because volatile compounds have low activity based on the experience in the Phytomedicine Programme. Even though *E. faecalis* was the most sensitive test organism, followed by *S. aureus, P. aeruginosa* and *E. coli*, the highest percentage of activity regained was against *S. aureus*, followed by *P. aeruginosa*, *E. faecalis* and *E. coli*.





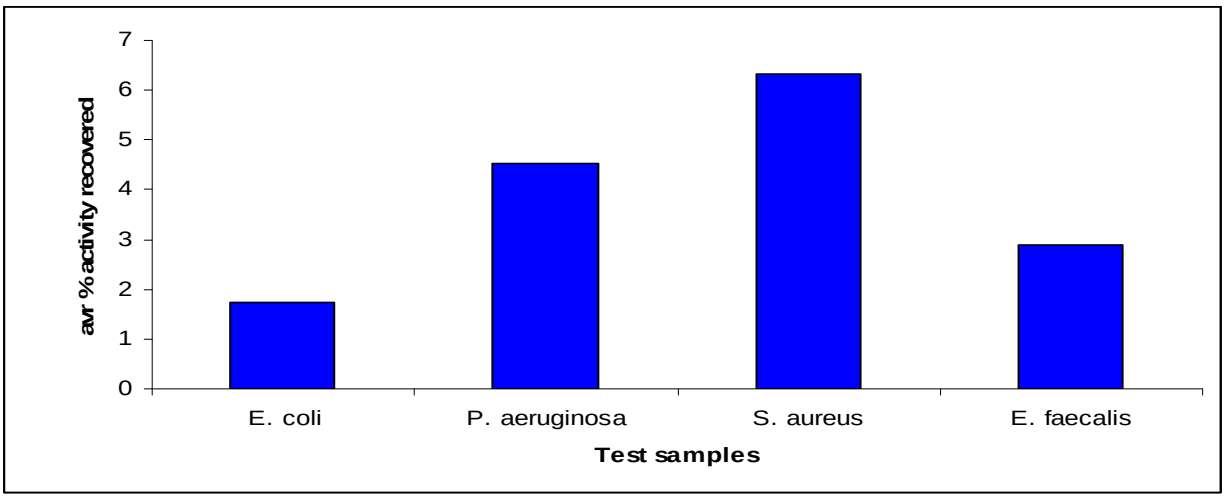


Figure 5.3. Percentage activity recovered from the fractions obtained by solvent-solvent fractionation from the crude acetone leaf extract of *O. pretoriensis* (A), average percentage activity of the fractions (B) and average percentage sensitivity of the test organisms to the fractions (C) .

5.4. Conclusion

The importance of working quantitatively in fractionation of antibacterial compounds is demonstrated by these results. Of the total quantity separated by solvent-solvent fractionation about 32% of the mass was lost. The loss may be ascribed to compounds present in the interphase of pellicles formed during the solvent-solvent fractionation or more likely evaporation of compounds during the drying of the extracts. Yet when the recovery of biological activity was calculated it appeared that more than 76% of activity was lost. This enormous loss can be explained by inactivation during the fractionation by chemical processes. It is unlikely because solvent-solvent fractionation is generally considered to be a very mild procedure. A more likely explanation is that there was a surprising degree of synergism between compounds separated during the process. This hypothesis could be tested (a) by repeating the work and noting carefully losses in quantity at different stages or more elegantly, (b) by combining fractions to determine if activity could be regained.

In the next chapter, compounds isolated from the fractions will be assayed for antibacterial activity, potential cytotoxicity and genotoxicity effects.

Chapter 6

In vitro evaluation of the biological activity of crude extract and compounds isolated from *O. pretoriensis*

6.1. Introduction

Medicinal concoctions prepared from medicinal plants and secondary metabolites obtained from plants are not necessarily benign chemical substances. Plants have evolved such chemicals in order to deter, poison, kill threatening species or attract symbiotic species. It would therefore be incorrect to assume that plant extracts and isolated compounds in general are inevitably safe. As a result there is a need to investigate the cytotoxicity of phytocompounds on a number of cell lines. This provides more information on the understanding of issues concerning the safety, cytotoxicity, and side-effects of synthetic and naturally occurring compounds, and the need to find new medicines, to treat chronic diseases (Gurib-Fakim, 2006).

In all cases the benefit risk ratio plays a very important role. If we could only use pharmaceutical products that are completely safe, may people would die from diseases that could be controlled by toxic substances at an appropriate dose. It is therefore important to determine the activity and toxicity of the isolated compounds to evaluate their potential use. The three compounds isolated from the acetone extract of *O. pretoriensis* were tested for antibacterial activity against four nosocomial pathogenic bacteria. The volume of activity was determined for each compound against every bacterium by calculating the inverse of the MIC values. For ease of reference the compounds were labelled ochnaflavone (OF), ochnaflavone 7''-O-methyl ether (OFME) and β-sitosterol (SS).

In addition to the evaluation of the antibacterial activity of the isolated compounds, to assess the potential cytotoxic effects of the crude extract and the isolated compounds, MTT (3-(4, 5-dimethylthiazonyl-2)-2, 5-diphenyltetrazolium bromide) based cytotoxicity assay described by Mosmann (1983) was used. MTT (3-(4, 5-dimethylthiazonyl-2)-2, 5-diphenyltetrazolium bromide) based cytotoxic assay is one of the most frequently used methods for measuring cell proliferation and cytotoxicity. MTT is a yellow water-soluble tetrazolium dye that is reduced by live metabolically active cells to a purple formazan that can be measured using a spectrophotometer. The MTT assay is based on the ability of a

mitochondrial dehydrogenase enzyme from metabolically active cells to cleave the tetrazolium ring of the pale yellow MTT and form purplish formazan crystals.

Genotoxicity testing is of great importance for the detection of compounds that induce genetic damage directly or indirectly by various mechanisms. The study of DNA damage is also an essential part of genetic toxicology because it is an important event in carcinogenesis. Epidemiological studies indicate that many cancers are dependent on multiple mutational etiologies. Compounds which are positive in tests that detect such kind of damage have the potential to be human carcinogens and/or mutagens (Verschaeve and Van Staden, 2008)

For genotoxicity testing, the Ames test described by Maron and Ames (1983*)* was used. The Ames test assesses mutagenic potential of compounds and chemicals in bacterial reverse mutation test. This test has shown to detect relevant genetic changes and the majority of carcinogens (McCann and Ames, 1967). The Ames test uses a set of histidine requiring strains (amino acid-dependent strains of *S. typhimurium* and *E. coli*). The reversion of histidine dependent bacteria to histidine independent, caused by mutations of the test samples are visualized by plating *S. typhimurium* bacteria in a histidine poor growth media. Only histidine independent mutants are able to grow and form visible colonies. However, the same procedures can also be used with minor modifications to test the effects of the inhibition of mutagenic effects of known mutagenic substances, thus detecting the antimutagenic potential of test samples. This is also an important tool towards the discovery of anticarcinogenic agents.

6.2. Materials and methods

6.2.1. Minimal Inhibitory Concentrations (MIC's)

The microdilution assay by Eloff 1998a, was used as described in chapter 2, section 2.6.

6.2.2. Cytotoxicity testing "Cell culture and treatments"

The cytotoxicity of the crude acetone extract and the compounds isolated from *O. pretoriensis* was determined using the MTT assay. Vero cells (African Monkey kidney cells) obtained from the Department of Veterinary and Tropical Diseases of the University of Pretoria were used in this experiment. The cells were maintained in Minimal Essential Medium (MEM, Highveld, Biological)

supplemented with 0.1% gentamycin and 5% foetal calf serum (Adcock-Ingram). The cells of a subconfluent culture were harvested and centrifuged at 200 x g for 5 minutes and resuspended in growth medium to 2.4 x 10^3 cells /ml. A total of 200 µl of the cell suspension was pipetted into each well of columns 2 to 11 of a 96 well culturing plate. The same amount of the growth medium was added to wells of column 1 and 12 to maintain humidity and minimize the edge effect. The plates were incubated at 37°C in a 5% CO_2 incubator until the cells were in the exponential phase of growth. After incubation, the MEM was aspirated from the cells and replaced with 200 µl of different concentrations of the test samples. Each dilution of the test sample performed in quadruplicate. The plates were again incubated for 5 days at 37°C in a 5% incubator. A negative control (untreated cells) and positive control (cells treated with different concentrations of berberine chloride (Sigma)) were included. After incubation, 30 µl of 5 mg/ml MTT (Sigma) in phosphate-buffered-saline PBS was added to each well and the plates were incubated for a further 4 hours at 37°C. After incubation with MTT, the medium in each well was removed and the formazan crystals formed were dissolved by adding 50 µl of DMSO to each well of the plates. The plates were gently shaken until the crystals were dissolved. The amount of MTT reduction was measured immediately by detecting the absorbance using a microplate reader at a wavelength of 570 nm (VersaMax, Molecular Devices). The wells in column 1 and 12, containing medium and MTT but no cells was used to blank the microplate reader. The percentage of cell viability was calculated using the formula below:

$$\%\text{cell viability} = \frac{\text{Mean Absorbance of Sample}}{\text{Mean Absorbance of control}} \times 100$$

The LC_{50} values were calculated as the concentration of the test sample that resulted in 50% reduction of absorbance compared to untreated cells. The intensity of the of the MTT formazan produced by living metabolically active cells is directly proportional to the number of live cells present

6.2.3. Genotoxicity testing (Ames test)

The potential genotoxic effects of three of the four compounds isolated from *O. pretoriensis* was determined using the well-known bacterial mutagenicity test. The Ames test (Maron and Ames, 1983) was performed with *Salmonella typhimurium* strain TA98. This bacterial strain allows for the detection of frame shift mutations. The well-known plate incorporation procedure described by Maron and Ames (1983) was used. One hundred microliters of bacterial stock were incubated in 20 ml of Oxoid Nutrient

broth for 16h at 37 8C on a rotative shaker. Of this overnight culture 0.1 ml were added to 2.0 ml of top agar (containing traces of biotin and histidine) together with 0.1 ml test solution (test sample, solvent control or positive control) and 0.5 ml phosphate buffer The top agar mixture was poured over the surface of a minimal agar plate and incubated for 48 h at 37 8C. After incubation the number of revertant colonies (mutants) was counted. All cultures were made in triplicate (except the solvent control where five replicas were made). Toxicity can be checked by investigation of the background layer of bacteria. Absence of toxicity was examined by observing the background bacterial growth, which should be normally present. The positive control used in this study was 4-nitroquinoline 1-oxide (4-NQO) at a concentration of 2 µg/ml.

6.3. Results and Discussion

6.3.1. Antibacterial activity

The MIC values of the three compounds against *E. coli, P. aeruginosa, S. aureus* and *E. faecalis* were determined and recorded (table 6.1). The minimal inhibitory concentrations ranged from 31.3 to 250 µg/ml across the all test microorganisms. The MIC values didn't change over 12, 24 and 36 hours of incubation. This indicates that the isolated compounds reflected bactericidal and not bacteriostatic effects. The activity is not really exciting for pure compounds and there is hardly any opportunity for further investigation. The total yield of the pure compounds could not be calculated due to the loss of material during isolation. Another way of expressing the biological activity is to calculate the activity volume as the inverse of the MIC values. This expresses the volume in ml to which 1 mg of the isolated compound can be diluted and still inhibit the growth of the test organism.

Two of the three isolated compounds (ochnaflavone **(OF)** and ochnaflavone 7''-O-methyl ether **(OFME)**) were equally active against *P. aeruginosa* with MIC values of 31.3 µg/ml whereas β-sitosterol **(SS)** had an MIC of 62.5 µg/ml. OF was also equally effective against *P. aeruginosa* and *E. faecalis* with MIC's of 31.3 µg/ml, OFME against *P. aeruginosa* (31.3 µg/ml) and *E. faecalis* (62.5 µg/ml) and SS against *P. aeruginosa* with MIC of 62.5 µg/ml. This is an indication that *P. aeruginosa* was generally sensitive to the all three compounds tested. Overall, there was no change in the bacterial inhibitory effects of the isolated compounds compared to the crude extract. The MIC values of OF and OFME were almost 3

and a half times less than the MIC of the crude extract against *P. aeruginosa*. The crude extract was more active against *E. coli* compared to all the compounds. This may explain that *E. coli* was more sensitive to the synergistic effects of all the compounds present in the crude extract as opposed to the individual isolated compounds. Ochnaflavone **(OF)** and ochnaflavone 7''-O-methyl ether **(OFME)** can be used effective against infections caused by pseudomonads. One of the most troublesome characteristics of *P. aeruginosa* consists in its low antibiotic susceptibility due to the presence of the outer membrane with a low level of permeability (Feng *et al*., 2002).
Two biflavonoids isolated from the bark of *O. macrocalyx* (calodenin and dihydrocalodenin) were much more active than the compounds isolated here. The MICs against multidrug resistant strains of S aureus were low as 8 µg/ml (Trag et al, 2002).

On average, all three compounds tested were more active against *P. aeruginosa*, followed by *E. faecalis, S. aureus* and lastly *E. coli*. It is clear that the activity of the compounds is not associated with Gram positive or Gram negative classification of the test organisms. This may suggest that the activity of the compounds may not be cell wall related. From this information, one cannot confidently formulate a strong structure-activity relationship since this is different for the different pathogens. ANOVA single factor was used to compare and confirm that there was no significant difference in the activity of the compounds on either Gram positive or Gram negative bacteria used in this study ($p=0.66$ at $p \leq 0.05$). This means that the compounds may have a broad spectrum of antibacterial activity.

Approximately 24 ml of water and/acetone can be added to 1 mg of ochnaflavone and still inhibit the growth of *E. coli*, 32 ml against *P. aeruginosa*, 16 ml against *S. aureus* and finally, 32 ml against *E. faecalis*. The antibacterial activities of ochnaflavone and ochnaflavone 7''-O-methyl ether against *P. aeruginosa* is similar since in each case, approximately 32 ml of solvent can be added to 1 mg of any of these compounds and inhibit the growth of *P. aeruginosa*. Ochnaflavone was the most active compound against all the test compounds. The higher the amount, the more active the compound. The activity volume of the crude extract (Figure 2.4 (B)) indicates that the isolated compounds are almost one and a half times more active than the crude extract.

Gentamycin, the positive control used in this study was more active than the isolated compounds and the crude extract. Based of the activity volume values for the crude acetone extract, 1 mg of the crude extract can be diluted in 26 ml of solvent and inhibit the growth of *E. faecalis*, 16 ml for *E coli*, and 9.6 for both *S. aureus* and *P. aeruginosa*.. The crude extract and the compounds were more active against *E. faecalis, P. aeruginosa, E. coli*, and *S. aureus*

Table 6.1. Minimal inhibitory concentrations (MIC) (µg/ml) of three compounds isolated from *O. pretoriensis* against *E. coli, P. aeruginosa, S. aureus* and *E. faecalis*, and average activity volume (1/MIC) (ml/mg) values.

Bacteria	12 hours			36 hours			Gentamycin
	OF	OFME	SS	OF	OFM	SS	
E. coli	41.6(±0.02)	250	250	62.5	125	250	7
P. aeruginosa	31.3	31.3	62.5	31.3	62.5	62.5	8
S. aureus	62.5	125	104(±0.04)	62.5	125	104(±0.04)	8.3
E. faecalis	31.3	62.5	125	62.5	31.3	125	10
Activity volume (ml/ mg)							
E. coli	24	8	4	16	8	4	142
P. aeruginosa	32	32	16	32	16	16	125
S. aureus	16	8	9	16	8	9	121
E. faecalis	32	16	8	16	32	8	100

6.3.2. Cellular toxicity

The effects of berberine, three compounds isolated from *O. pretoriensis* and the acetone crude extract on Vero cell viability are represented in figure 6.1. There was a dose related toxicity. The dose that kills 50% of the cells are frequently used to depict toxicity. The lower the value is the more toxic the compound tested. The crude extract was the most toxic with and LD_{50} of 55.2 µg/ml, but it was less toxic than the positive control berberine (LD_{50} of 39.0 µg/ml). β-sitosterol was the least toxic and the methylation of ochnaflavone decreased the toxicity slightly. (Table 6.2). It has been reported that increased hydroxylation of compounds results in increased toxicity (Geissman, 1963). However, in this case, ochnaflavone and ochnaflavone 7''-O-methyl ether differ only in the methoxy substitution at position 7'' which had little effect on the toxicity. .

TABLE 6.2. LC_{50} of the crude acetone extract of *O. pretoriensis* three compounds isolated from *O. pretoriensis*.and the positive control berberine

Test sample	Crude extract	OF	OFME	SS	Berberine
LC_{50} (µg/ml)	55.2	125.9	162.0	193.8	39.0

The crude extract and all the compounds tested were less toxic compared to berberine with LC_{50} of 39 µg/ml. However, the crude extract was more toxic compared to all the compounds. This may be due to the synergistic effect of all the compounds in the extract, some of which were not isolated in this study. Given the low cytotoxic effects of the isolated compounds, it could be of interest to investigate their cytotoxic effects on cancer cell lines in order to establish if they possess selective toxicity. The comparison between the ratio percentage cell viability of Vero cells and tested cancer cell lines will give an indication of selective activity.

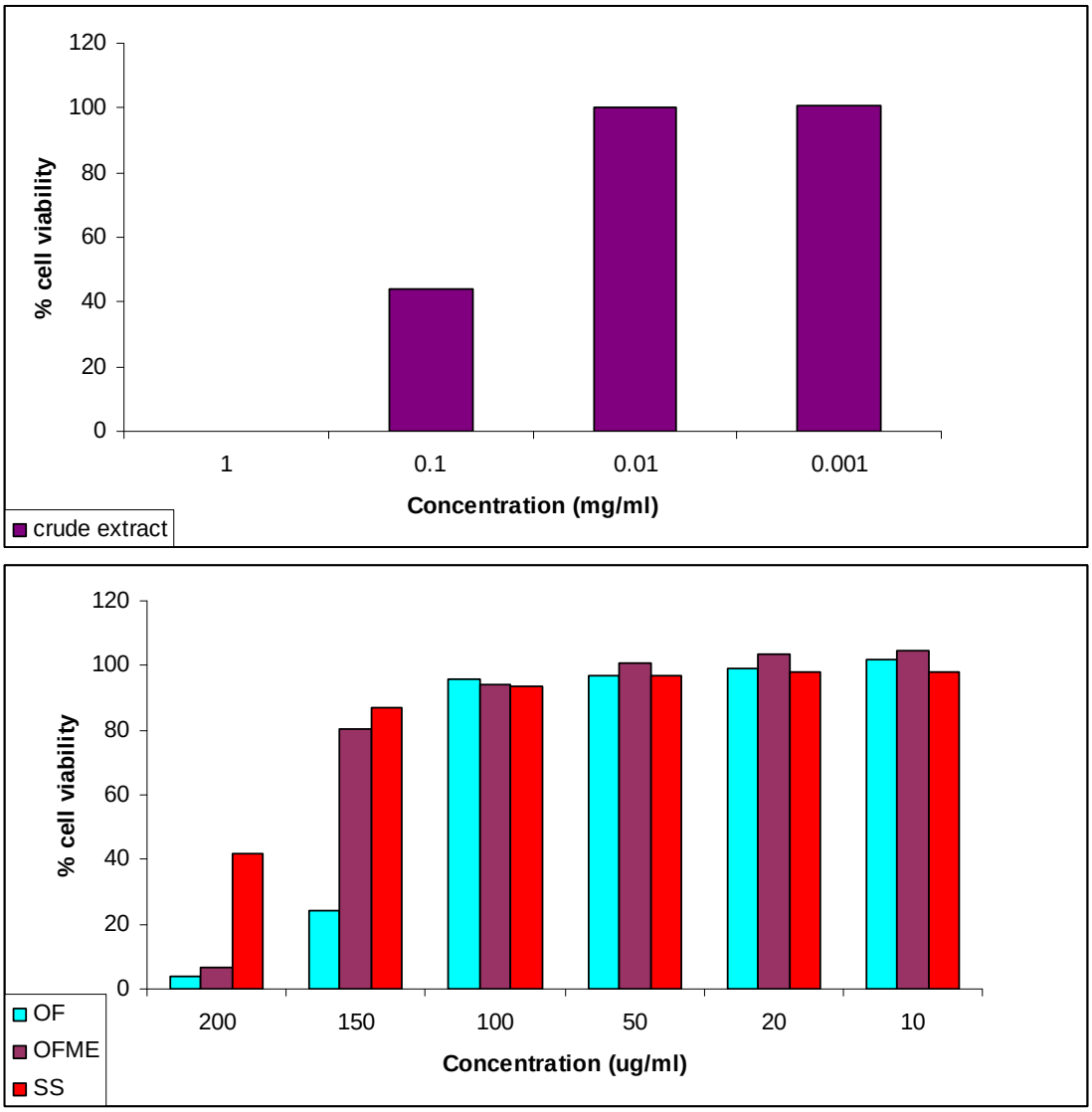


Figure 6.1. Percentage viability of Vero cells after exposure to different concentrations of the acetone crude extract and three compounds isolated from *O. pretoriensis*

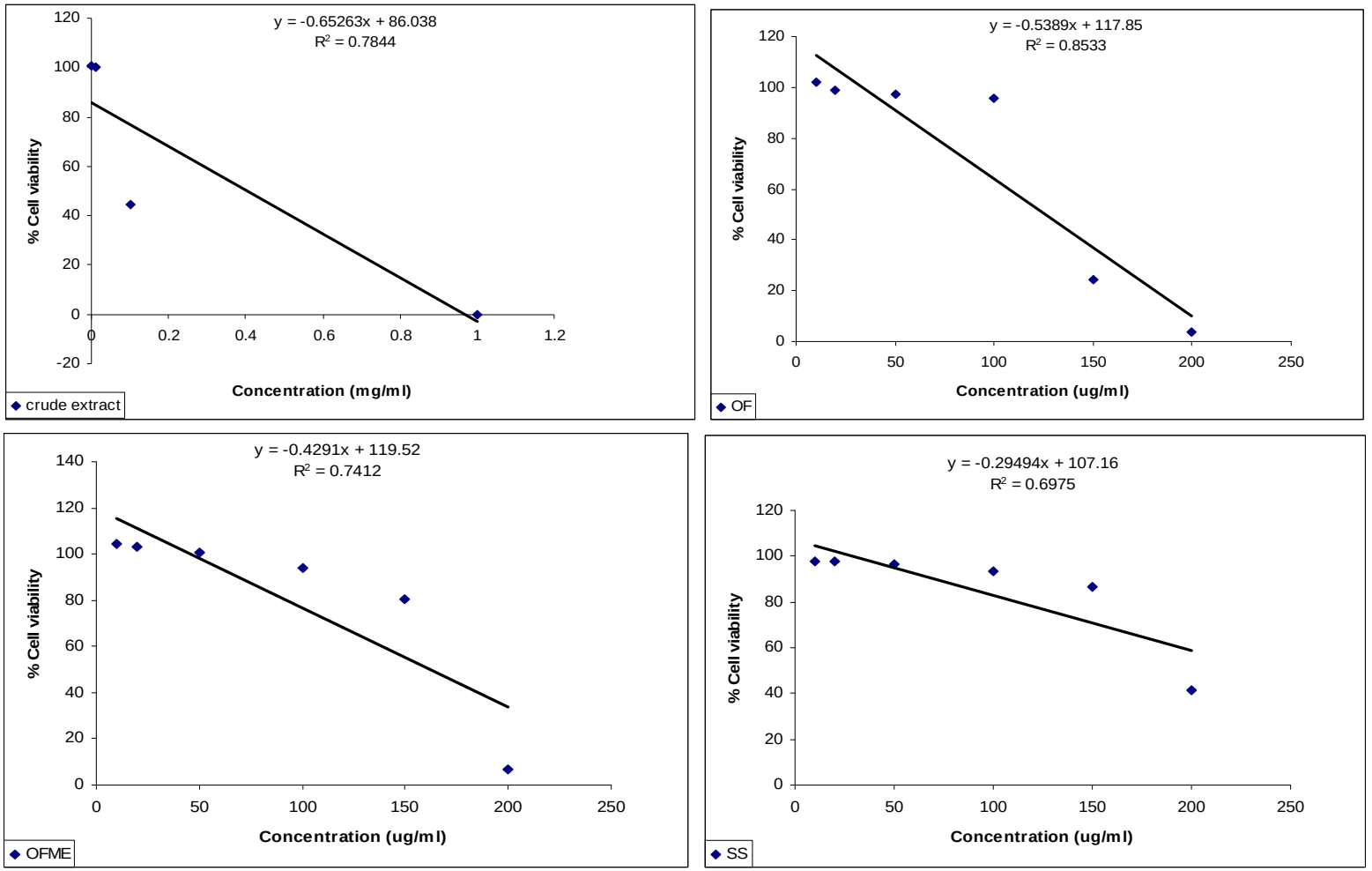


Figure 6.1. Cytotoxicity of the acetone crude extract (LC_{50} = 55.2 µg/ml), OF (LC_{50} = 125.9 µg/ml), OFME (LC_{50} = 162.0 µg/ml) and SS (LC_{50} = 193.8 µg/ml) against Vero cells.

Many people consider herbal medicines to be safer that pure compounds, but this is a dangerous assumption. As a result, there is a need to develop standardized biological and mathematical systems to prove selective biological activity of natural products (Suffness and Pezzuto, 1990). The cytotoxicity results can be useful in the exploration of the selective activity of the test material against test bacteria used in this project. This is by the calculation of the therapeutic index (TI) which is a concept that is used to gauge the relationship between the activity and toxicity of the compounds depending on the nature of activity investigated. Since the compounds investigated in this case are antibacterial, the therapeutic index of the crude extract and the isolated compounds was calculated using MIC values against bacteria and LC_{50} concentrations against Vero cells using the formula below

$$TI = \frac{LC_{50} \text{ against Vero cells}}{MIC}$$

Overall, the compounds isolated had a low therapeutic index. A therapeutic index of higher than 10 is preferred to ensure that an overdose does not harm the host. At these low therapeutic indexes the crude extract or isolated compounds would only be useful in topical applications. The first assumption is that cellular toxicity approximates in vivo toxicity, but this may not necessarily be true. Nevertheless it appears that the crude extract or the compounds isolated have very limited application and one would not advise traditional healers to recommend the extracts for internal use.

Table 6.3. Therapeutic Index (TI) values of three compounds isolated from *O. pretoriensis*.

Compound	*E.coli*	*P aeruginosa*	*S. aureus*	*E. faecalis*	Average
Crude extract	0.88	0.50	0.50	1.33	**0.80**
OF	3.02	4.03	2.10	4.02	**3.27**
OFME	1.29	5.18	1.29	2.59	**2.58**
SS	0.77	3.23	1.86	1.55	**1.85**

6.3.3. Genotoxicity (potential mutagenic and antimutagenic effects

The potential mutagenic and antimutagenic activity of the three compounds isolated from *O. pretoriensis* was determined using the Ames test. Table 6.2 gives the average number of revertants per plate in the mutagenicity testing whereas figure 6.2 gives the percentage antimutagenic activity of the test samples on *Salmonella typhimurium* strain TA98. Verschaeve *et al*. (2004) reported that this strain detects most of the mutagenic compounds thus it's the most appropriate strain to be used in the case of shortage of test samples For a substance to be considered genotoxic in the Ames test, the number of revertant colonies on the plates containing the test compounds must be more than twice the number of colonies produced on the solvent control plates (i.e., a ratio above 2.0). Furthermore, a dose–response should be evident for the various concentrations of the mutagen tested.

There were no significant differences in the mutagenic effects of all the compounds in comparison to one another. This is because the number of revertants is almost in the same rage for each compound at all the tested concentrations. Even though the number of revertant colonies in each case (i.e. each compound at differing concentrations), are more than that in the negative control, the compounds tested are considered to be non-mutagenic based on the statement made above. At the highest concentration tested for each compound, the number of revertants seemed to decrease compared to the lower concentrations tested. This may be a result of toxicity to the bacteria since the compounds being tested have antibacterial activity in chapter 5.

Table 6.4. Mutagenic activity of compounds isolated from *O. pretoriensis* in the frame shift *Salmonella typhimurium* strain TA98

Compound	50 µg/ml	500 µg/ml	1000 µg/ml
OF	32.67 ± 3.05	37.33 ± 4.93	32.00 ± 2.64
OFME	29.00 ± 4.00	40.67 ± 2.08	30.67 ± 2.08
SS	35.67 ± 4.04	32.67 ± 3.78	33.66 ± 5.51
4NQO		**210.00 ±12.28**	
Spontaneous revertants		**28.80 ± 4.17**	

In addition to the negative mutagenicity results, none of the compounds significantly inhibited the mutagenic effects of 4NQO (a positive known mutagen) (figure 6.2). The antimutagenic activity of the compounds was less than 25% in each case, thus all the compounds may be considered to have weak or no antimutagenic activity. Verschaeve and Van Staden (2008) explained that when the antimutagenic effects of a substance is more than 25%, then the test sample may be considered to have moderate antimutagenic effects and that it can be concluded that a test sample has a strong antimutagenic effect when the percentage antimutagenicity is higher than 40%. Since the compounds tested in this experiment have demonstrated antibacterial activity, if this compounds exhibit toxicity towards the test strain used, then the toxicity may mask the genotoxic response.

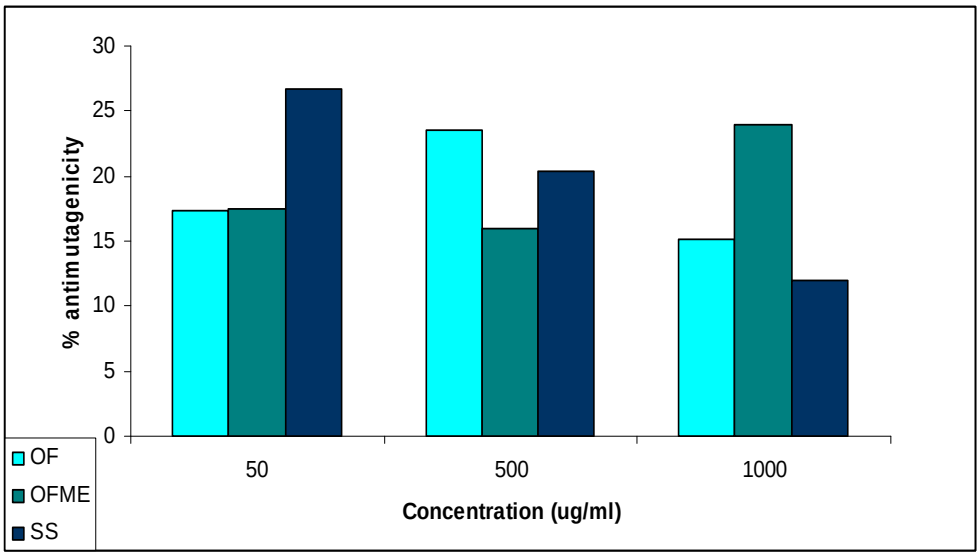


Figure 6.2. Percentage inhibition of 4-NQO demonstrating antimutagenic activity of three compounds isolated from *O. pretoriensis*.

6.4. Known biological activities of ochnaflavones and β-sitosterol

Ochnaflavone and ochnaflavone 7''-O-methyl ether are biflavonoids that belongs to the ochnaflavone group of biflavonoids. This class of compounds were first identified by Rao *et al*., (1997) in *Ochna obtusata*. Generally, biflavonoids have diverse biological and pharmacological properties. This includes among others, antibacterial, antifungal, antiallergic, antiviral, antihepatotoxic, anticancer and immune suppressive activities (Kim *et al*., 2004). Ochnaflavones have been reported to have anti-inflammatory activity, mast cell histamine release-inhibitory activity, antitumour activity, neuroprotective effects and anti-atherosclerotic activity (Kang *et al*., 2005 and Suh *et al*., 2006). Recently, ochnalavone 7''-O-methyl ether (OFME) and 2'', 3, dihydro ochnaflavone 7''- O-methyl ether were found to inhibit HIV-1 as well as

HIV-1 reverse transcriptase activity (Reutrakul *et al*., 2007). These results indicate that *O pretoriensis* extracts may also be active in these areas. No reports on the quantitative antibacterial activity of ochnaflavones were found.

The two ochnaflavones isolated in this study have lower MIC's against Gram-negative bacteria than to Gram positive bacteria. In most cases, plant extracts and compounds isolated from plants are more active against Gram-positive bacteria compared to Gram negative bacteria. Methylation of flavonoids is considered a structural feature essential for antimicrobial activity. It is thought to play a role in altering the solubility and intracellular compartmentalization (methylation renders flavonoids more hydrophobic, thus act better on cytomembranes) (Williamson *et al*., 2000). This phenomenon was not evident in this study as, methylation of ochnaflavone led to a lower activity

Structure-activity relationship may not appear to play a role in the antibacterial activity of most biflavonoids. In almost all the cases in the antimicrobial activity of flavonoids, hydroxyl-groups opposed to methyl-groups are needed for effective and enhanced activity (Teffo *et al*., 2009) i.e. opposed to a reports that methylation is essential for antimicrobial activity. The report by Teffo *et al*., (2009) suggests that hydroxylation is important for activity. Generally, the pattern of selectivity amongst Gram-positive and Gram-negative bacteria is not restricted only to compounds from plants, but is an observation amongst most antibiotics (Basile *et al*., 1999, Martini *et al*., 2004).

Compound SS, β-sitosterol, belongs to the class of phytosterols with chemical structure similar to that of cholesterol and had less antibacterial activity in comparison to the other two compounds. β-sitosterol had MIC' of 270 and 350 µg/ml against *S. aureus* and *E. coli* respectively after 48 hours of incubation (Mokbel *et al*.,2005). In our study, the MIC's for β-sitosterol against the two organisms were 104 and 250 µg/ml respectively after 12 and 36 hours of incubation. The data obtained in this study does not agree with Mokbel's *et al* (2005) observation. Even though the compound had bactericidal effects in our study, the higher MIC observed in the referred study may be attributed to the longer incubation time. Nonetheless, SS was more active against *P. aeruginosa*, with MIC value of 62.5 µg/ml. Overall, sitosterols have been reported to have a variety of biological activity including estrogenic effects (Rosenblum *et al*., 1995), activity against neurodegenerative disorder (Khabazian *et al*., 2001), analgesic activity, anti-inflammatory, anthelminthic and antimutagenic activity (Villasenor *et al.,* 2002). This class of compounds also possess chemopreventive and protective effects and are used in the treatment of hypercholesterolemia (Oregna *et al*., 2004).

6.5. Conclusion

The three compounds isolated from *O. pretoriensis* were much less active than gentamicin, the positive control. Methylation of the OH at the 7th position of OFME as opposed to OF did not increase the antibacterial activity of the compounds as reported for other compounds. Nonetheless, the combination of the isolated compounds for effective bacterial growth inhibition may also enhance their activity as a result of combined activity.

The compounds did not have substantial toxicity towards Vero cells. However, they had specific/selective antibacterial activity with selective indexes ranging from 0.77 to 3.27. Given the therapeutic indexes of the isolated compounds and the crude extract, herbal preparations from this plant should be used only as topical applications for external microbial treatments because of their low selective indexes. On average, the best compound that can be considered for further investigation is ochnaflavone **(OF)** with the highest average SI, followed by ochnaflavone 7''-O-methyl ether **(OFME)** and β-sitosterol **(SS)**. None of the compounds isolated were mutagenic or antimutagenic in the Ames test. The results obtained were not entirely conclusive because the activity observed in the Ames test may be attributed mostly to antibacterial activity of the compounds rather than their anti-genotoxic effect since the genotoxicity test used was bacterial based. Thus other genotoxicity assays like comet assay and micronucleus assay etc. can be used to shed more light on the genotoxic effects of the compounds tested.

The next chapter covers the overall conclusions of the results obtained from this project.

Chapter 7

Overall discussion and conclusions

It is widely accepted that there is an urgent need for new antibiotics to address the emergence of resistant microbes. One approach that is used widely is to investigate plants for antibacterial activity. In many cases plant extracts have been used traditionally to treat infections, apparently with some success. To date there has been very little success in isolating highly active molecules from traditionally used plants. The Phytomedicine Programme has followed a different approach by determining the activity of leaf extracts of more than 600 trees against 8 important bacterial and fungal pathogens. *Ochna* species apparently have not been used traditionally to treat infections in South Africa, yet the acetone leaf extract of *Ochna pretoriensis* had good activity. Consequently it was decided to investigate the *Ochna* species occurring in southern Africa especially because two biflavonoids with high activity were isolated from *O. macrocalyx* growing in Tanzania (Tang et al., 2003)..

The main aim of this study was therefore to isolate and characterize antibacterial compounds present in the most active *Ochna* species, followed by the investigation of the toxicity of the isolated compounds. Five *Ochna* species (O*. pretoriensis, O. natalitia, O. pulchra, O. serullata* and *O. gamostigmata*) were screened for antibacterial activity against *E. coli, E. faecalis, P. aeruginosa* and *S. aureus*. The bacteria used in this study were selected because they are the most important nosocomial bacterial pathogens and the strains used are those recommended for antibacterial testing by the United States National Committee for Clinical Laboratory Standards (NCCLS, 1990). Of all the species investigated, *O. pretoriensis* was the most active with MIC values ranging from 0.039 to 0.104 mg/ml. Antibacterial compounds with similar R_f values were present in all the species analyzed by bioautography. *Ochna pretoriensis* was selected for further investigation because of its low MIC values, presence of better defined zones/lines of inhibition on bioautograms and high total activity. The choice was also influenced by the availability of the plant material.

Bioassay-guided fractionation using a combination of column chromatography and bioautography was used in the isolation of antibacterial compounds from *O. pretoriensis*. Three compounds were successfully isolated and their chemical structures were determined using the ^{13}C and ^{1}H NMR (1 and 2 dimensional NMR) spectral data. The compounds were identified as, OF *(*Ochnaflavone), OFME

(ochnaflavone 7-O- methyl ether) and SS (*ß-Sitosterol)*. Flavonoids may represent a novel set of lead compounds in the search of antimicrobial phytochemicals. Future optimization of these compounds through structural alterations may allow the development of pharmacologically acceptable antimicrobial compounds/agents.

In theory, the activity shown by extracts and fractions is due to the sum of the activities of the individual constituents. Thus fractionation leading to isolation of the individual compounds should result in fractions having a higher activity than the original extract (Houghton *et al.,* 2007). The MIC's of the crude extract and seven fractions obtained by solvent-solvent fractionation were determined and the total activity (Eloff, 2004) was calculated. The main aim of this experiment was to evaluate the presence of synergism in the acetone crude extract. Only three fractions had low MIC's of 0.039-0.078 mg/ml (carbon tetrachloride, chloroform and ethyl acetate fractions). As expected the fractions with the lowest MIC's had the highest total activities. Approximately 76% of the total activity of the crude extract was lost during fractionation .i.e. only 24% was recovered in the seven fractions. The highest percentage of activity was present in the chloroform fraction (12%) and the carbon tetrachloride fraction (6%). Less than 1% was present in the other solvent-solvent fractions. Of the10.48 g of crude extract fractionated, 3.4 g was lost as pellicles in solvent interfaces or during filtration of insoluble components. The 32% loss in the dry mass does not edequately explains the 76% loss in activity. From the results obtained in this study, based on quantitative analysis of the antibacterial activity of the acetone crude extract and fractions from *Ochna pretoriensis* there seem to be some advantages in the use of extracts and/or fractions as opposed to single isolated compounds. This is because the crude extract had higher total antibacterial activity compared to the fractions which is probably attributed to synergistic interaction of compounds present in crude extracts for effective antibacterial activity.

The aim of the pharmaceutical industry is to develop novel drugs for the treatment of diseases. Such drugs require specificity of action aimed at a particular subset of receptors. Although natural products continue to supply loads of compounds or new screens, the focus of industry is currently on combinatorial synthesis for new drug development. This brings into consideration the toxicology profiles of new leads, where in each case, the selective activity of the compounds should be identified. In this study, the crude extract and the compounds isolated from *O. pretoriensis* in this study were assayed for antibacterial activity, cellular toxicity, and genotoxicity effects.

Ochnaflavone was the most active compound with an MIC of 31.3 µg/ml against *P. aeruginosa* and *E. faecalis*, 62.5 µg/ml against *S. aureus* and 41.6 µg/ml against *E.coli*, followed by, ochnaflavone 7-O-

methyl ether with an MIC of 31.3 µg/ml against *P. aeruginosa*, 62.5 µg/ml against *E. faecalis*, 125 µg/ml against *S. aureus* and 250 µg/ml against E. coli. ß-Sitosterol had an MIC of 62.5 µg/ml against *P. aeruginosa*, 104 µg/ml against *S. aureus*, 125 µg/ml against *E. faecalis* and 250 µg/ml against *E. coli*. Generally, *P. aeruginosa* was the most sensitive and *E. coli* the least sensitive test organism. The isolated compounds exhibited bactericidal effects on the test organisms.

The antibacterial activity of the compounds isolated from *O. pretoriensis* is not associated with Gram positive of Gram negative classification of the test organisms. The cytotoxic effects of the crude extract and all the compounds were low compared to berberine. Berberine (39.0 µg/ml) crude< (55.2 µg/ml) <ochnaflavone (125.9 µg/ml) < ochnaflavone 7-O- methyl ether (162.0 µg/ml) < *ß-Sitosterol* (193.8 µg/ml). The therapeutic indexes of the crude extract and the isolated compounds varied between 0.77 and 3.27, which is indication that the compounds did not have specific antibacterial activity. In most cases, the antibacterial activity is closely associated with toxicity to the host cells, which is likely to be in this case. The crude extract and the compounds were almost equally toxic to mammalian cells used for cellular toxicity and the bacteria. Based on the low therapeutic indexes of the test samples, the plant has very limited applications as an ingestible/intravenous therapeutic agent (the plant as a whole is not a good candidate for development of medicinal products for internal use i.e. ingestion) but can be useful for the treatment of topical bacterial infections. The tested compounds did not indicate mutagenic or antimutagenic properties when using the Ames test.

In conclusion, screening of plant products (crude extracts, fractions and pure compounds) may lead to the identification of products that may be sufficiently potent to be useful as antimicrobial chemotherapeutics. Information obtained from the screening of medicinal plants for diverse biological activity may assist in the optimization of the activity of the lead products, provide a focus for toxicological attention and aid in the anticipation and prevention of resistance. The potential antibacterial activity of the *Ochna* genus has been demonstrated for the first time in the current study. The two most active compounds isolated from *O. pretoriensis* (ochnaflavone and ochnaflavone 7-O- methyl ether) are being reported from this species for the first time. Their relative safety was established. Further studies on possible synergistic effects of the bioactive compounds and extensive *in vivo* investigations are hereby proposed before the plant is certified for human or animal usage.

Chapter 8

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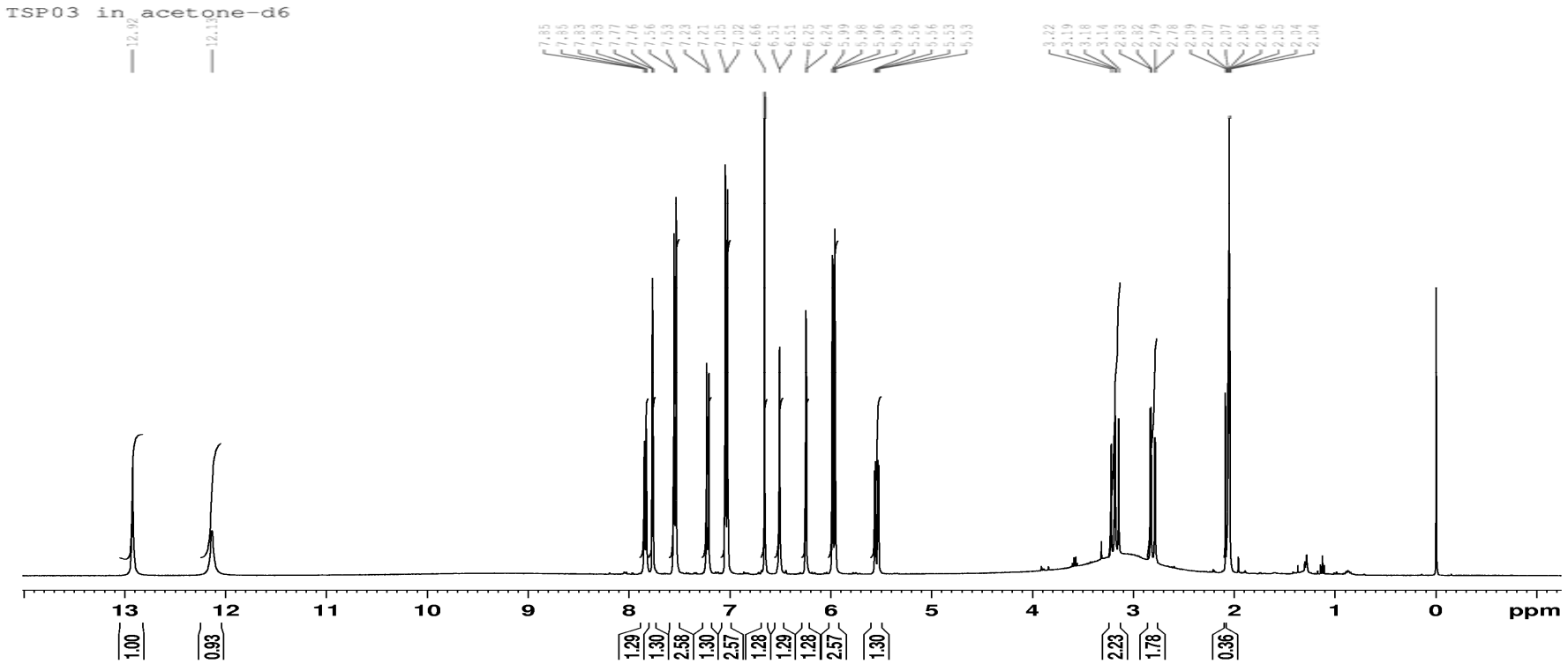
Appendix



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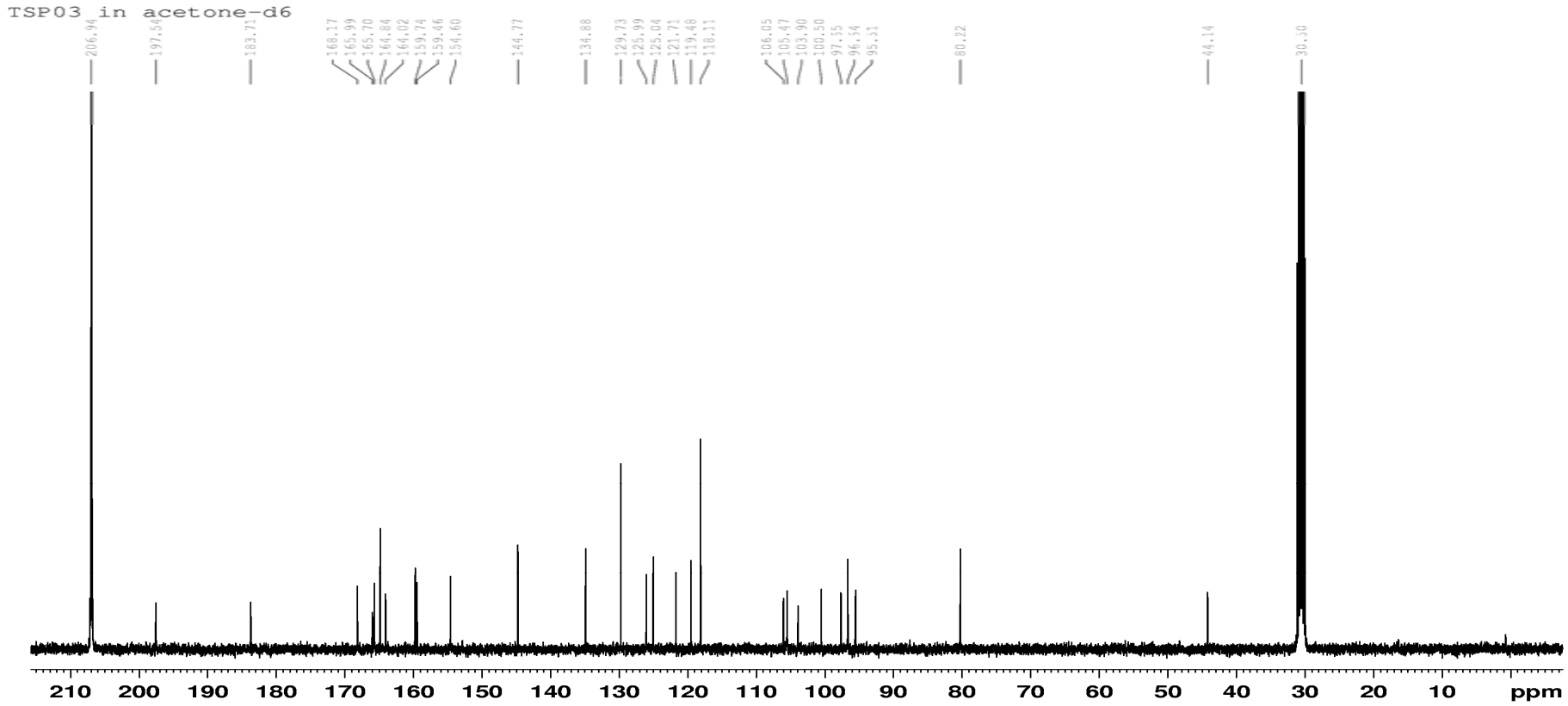

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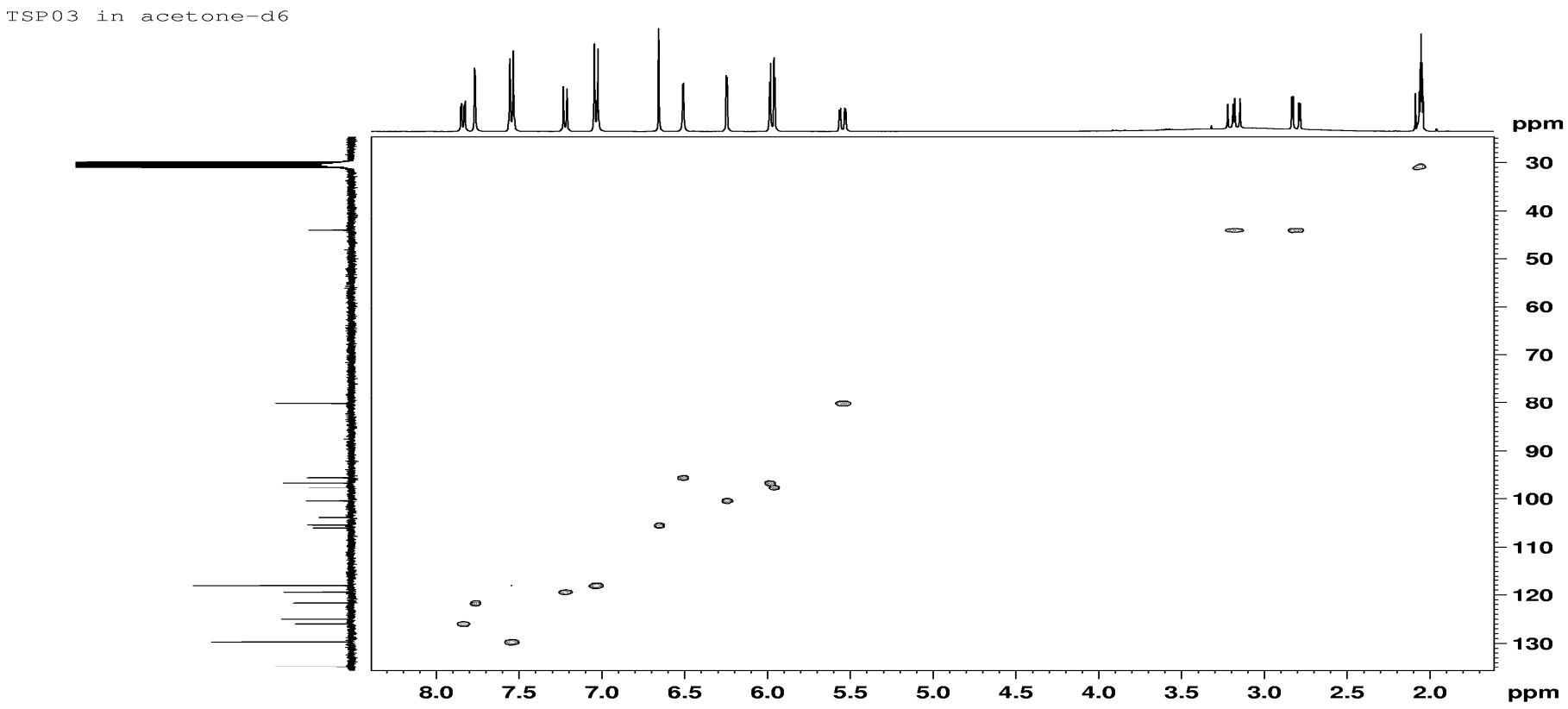
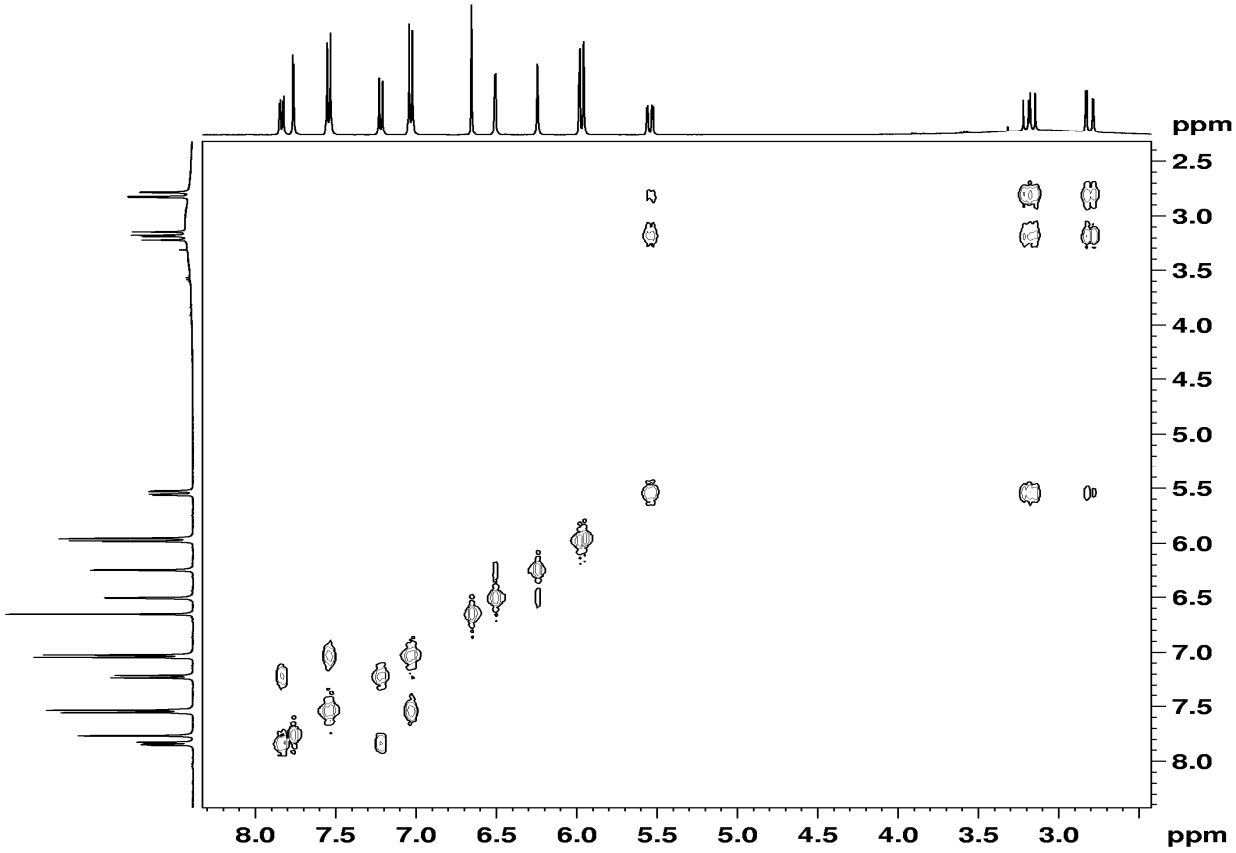


C1. HMQC-NMR Spectroscopy of C1

TSP03 in acetone-d6



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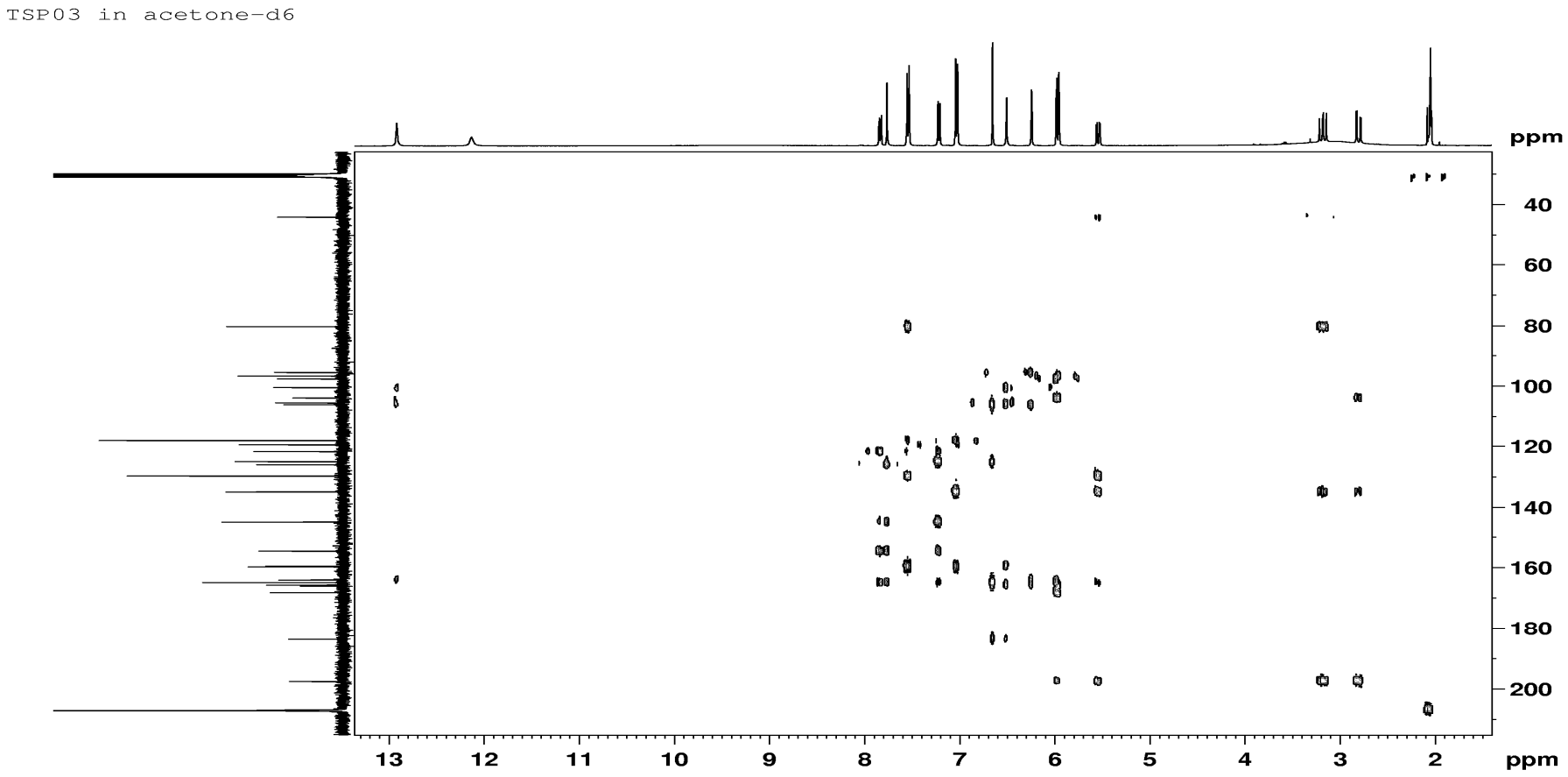

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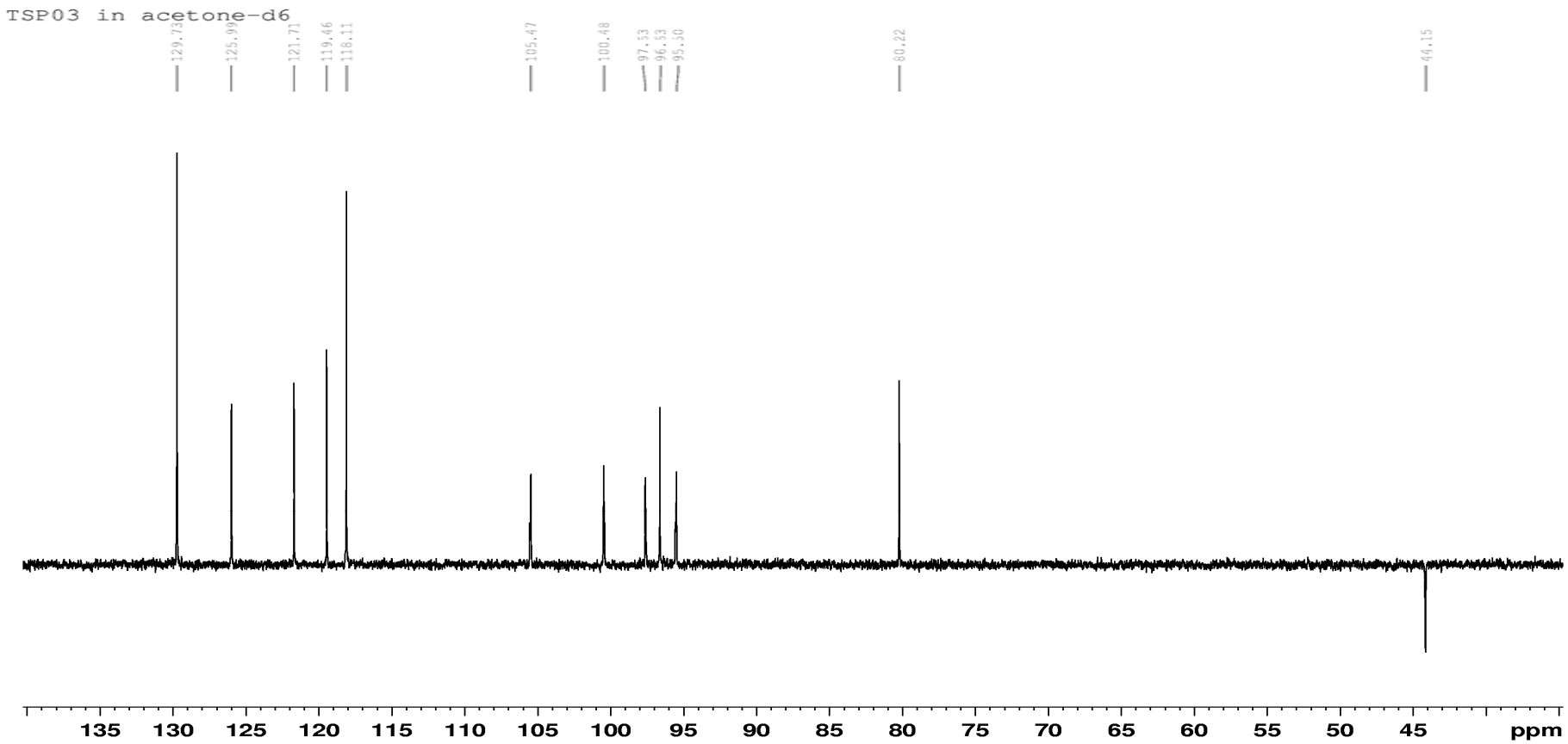
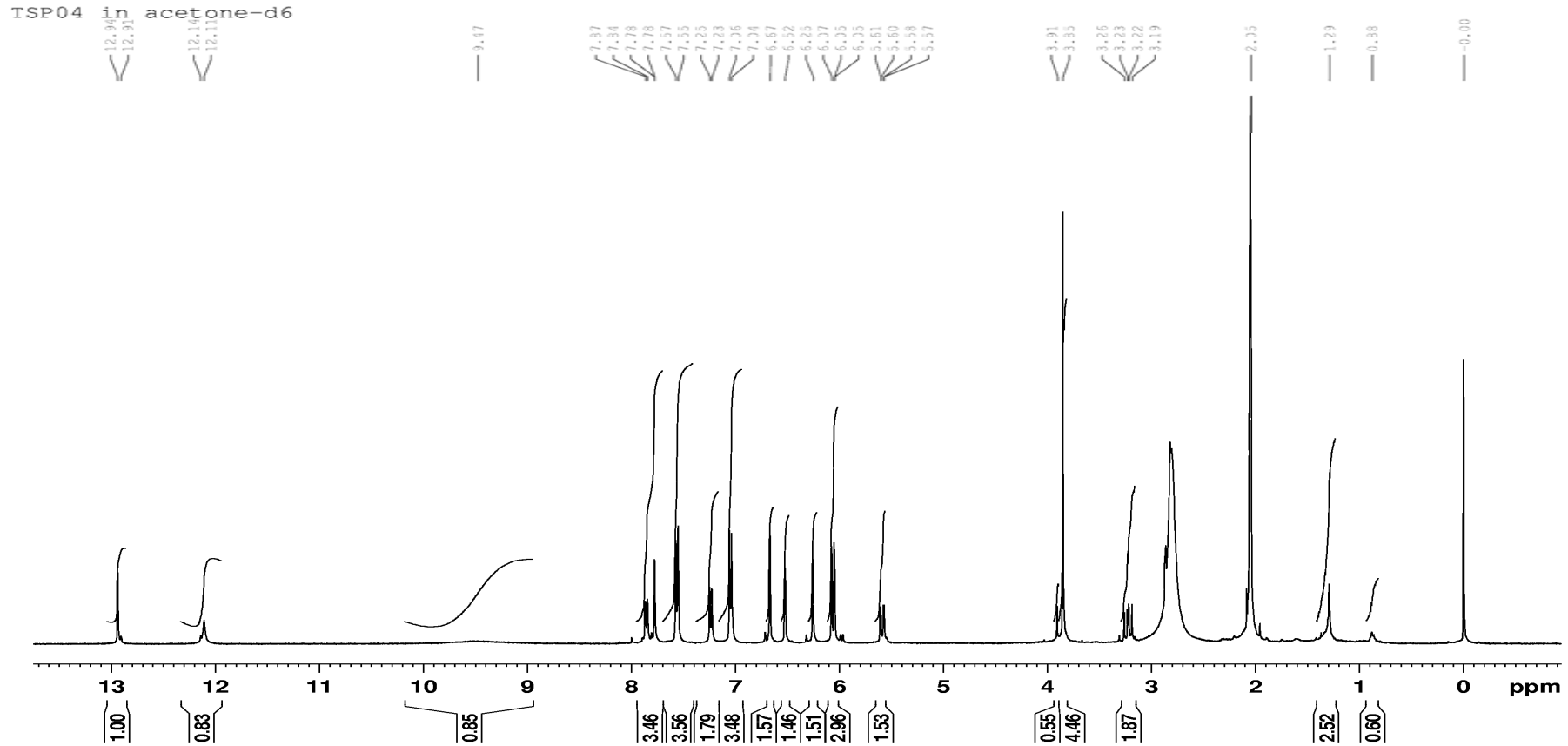
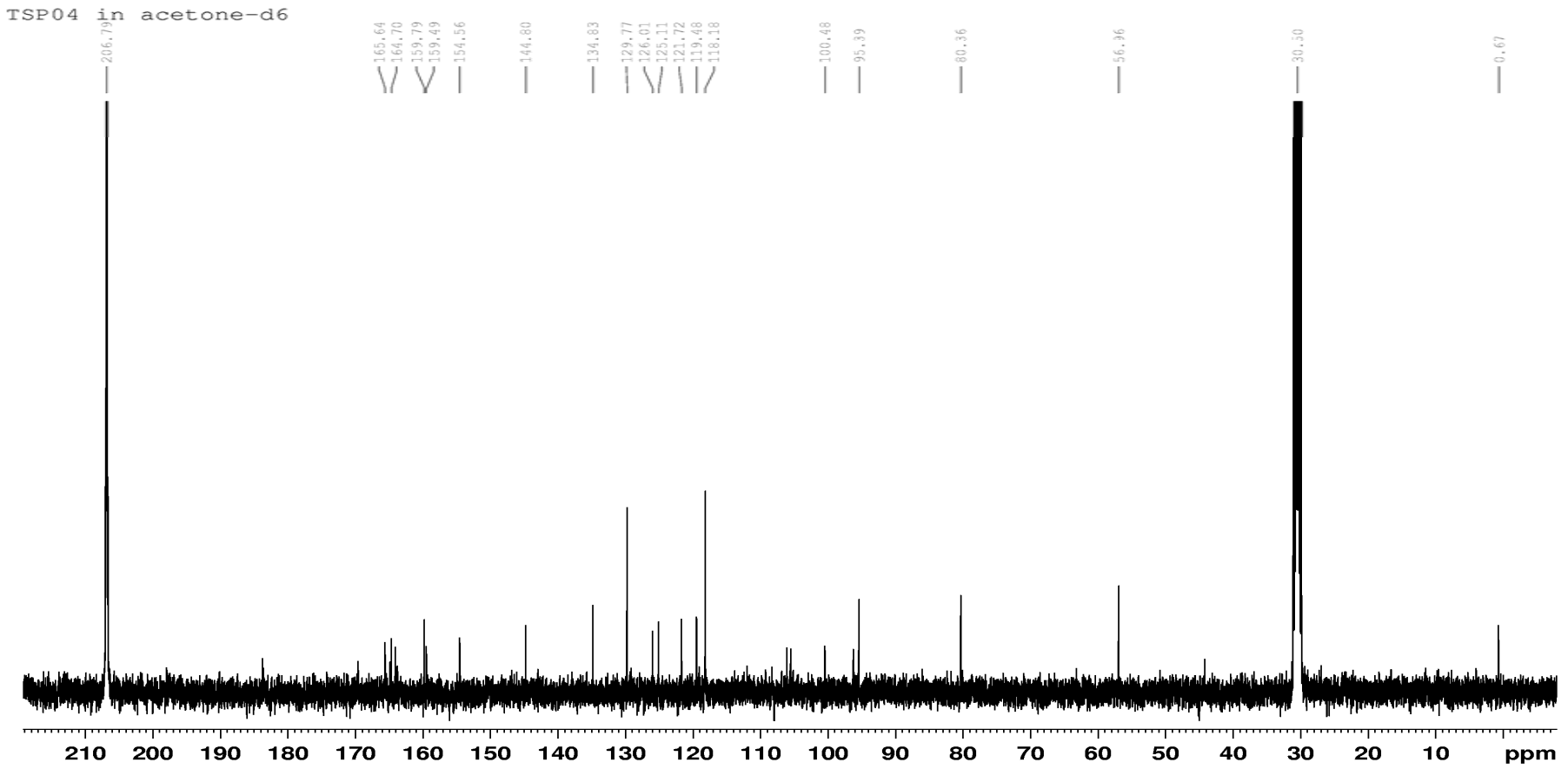


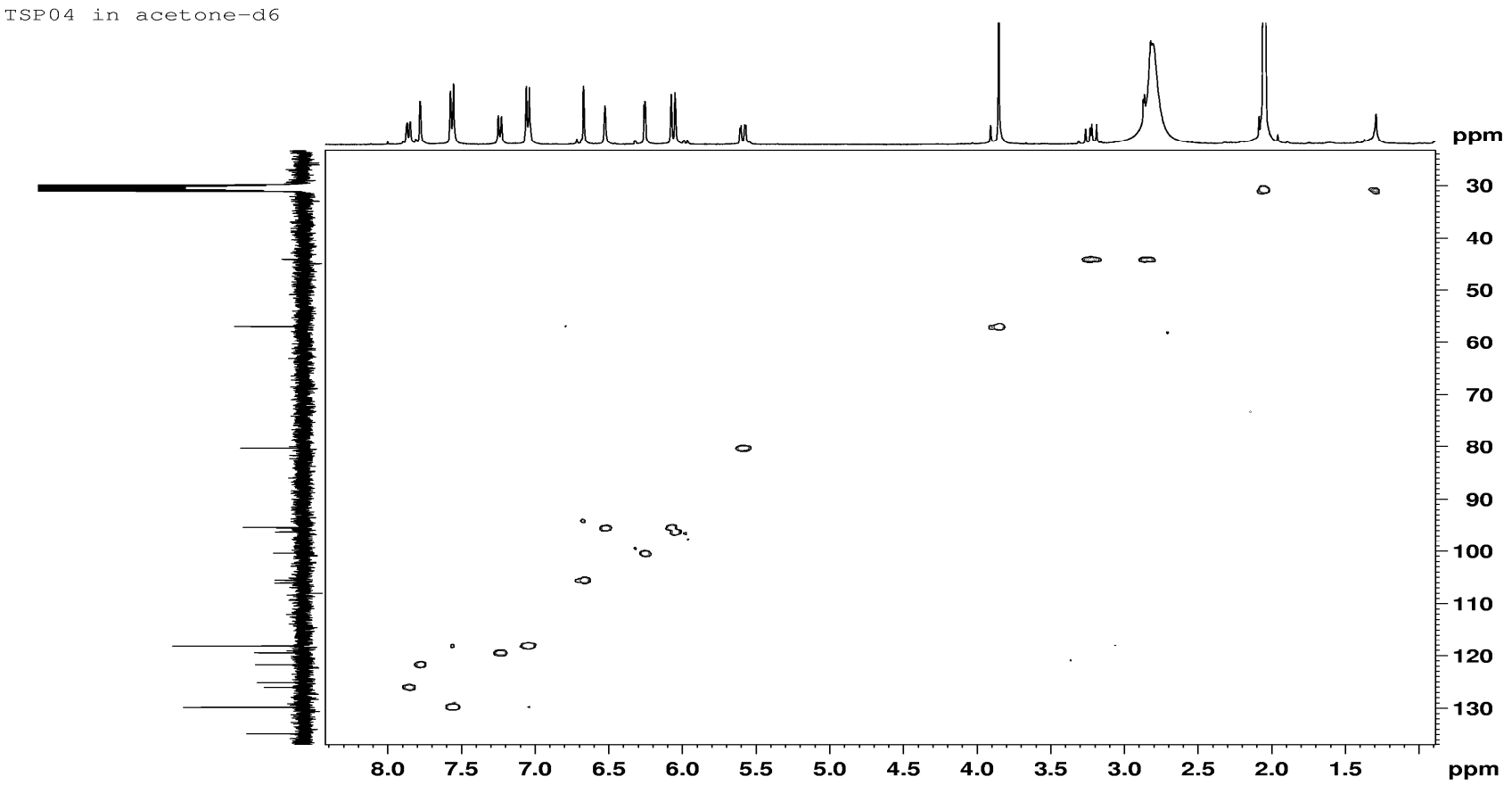
F1. ^{13}C-DEPT-NMR Spectroscopy of C1



A2. ^{1}H-NMR Spectroscopy of C2

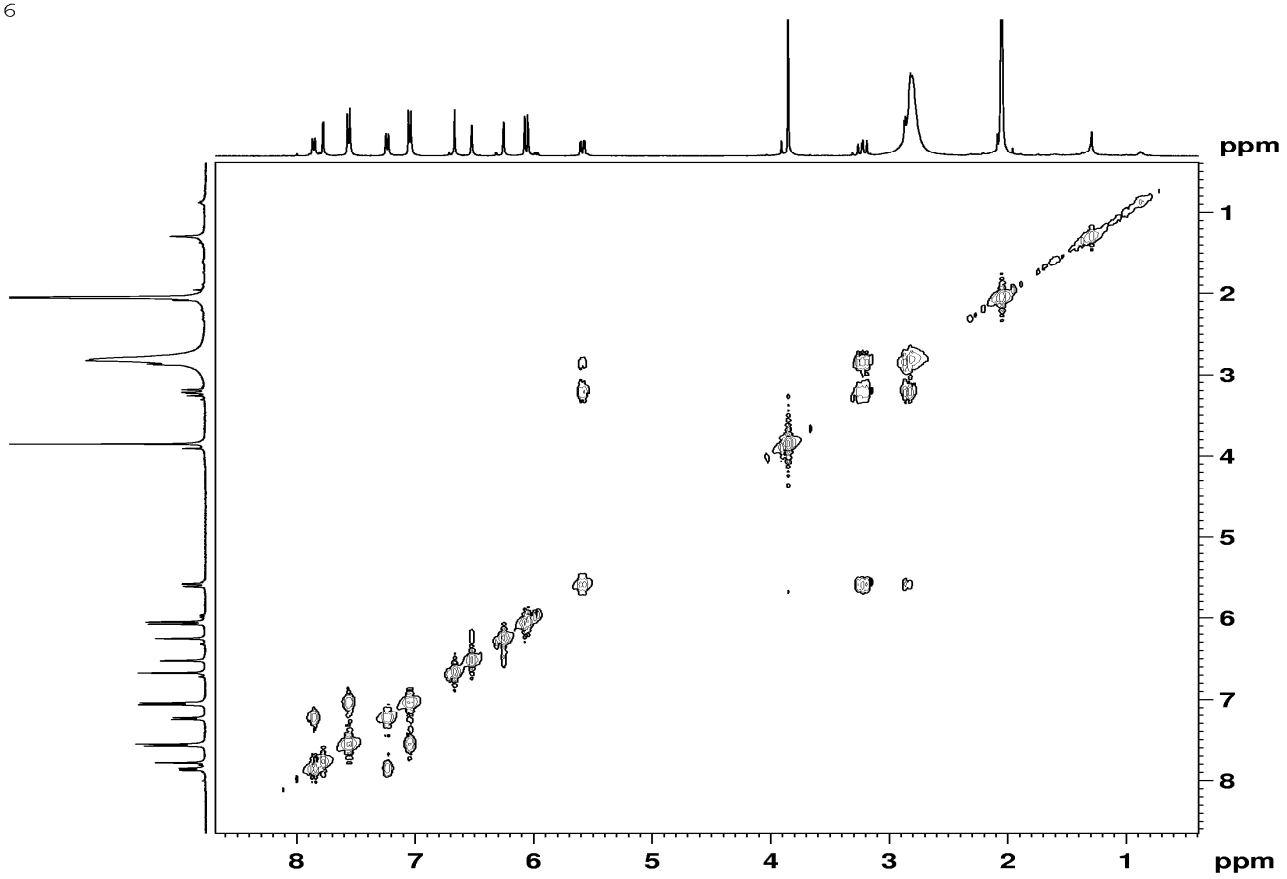


B2. ^{13}C-NMR Spectroscopy of C2

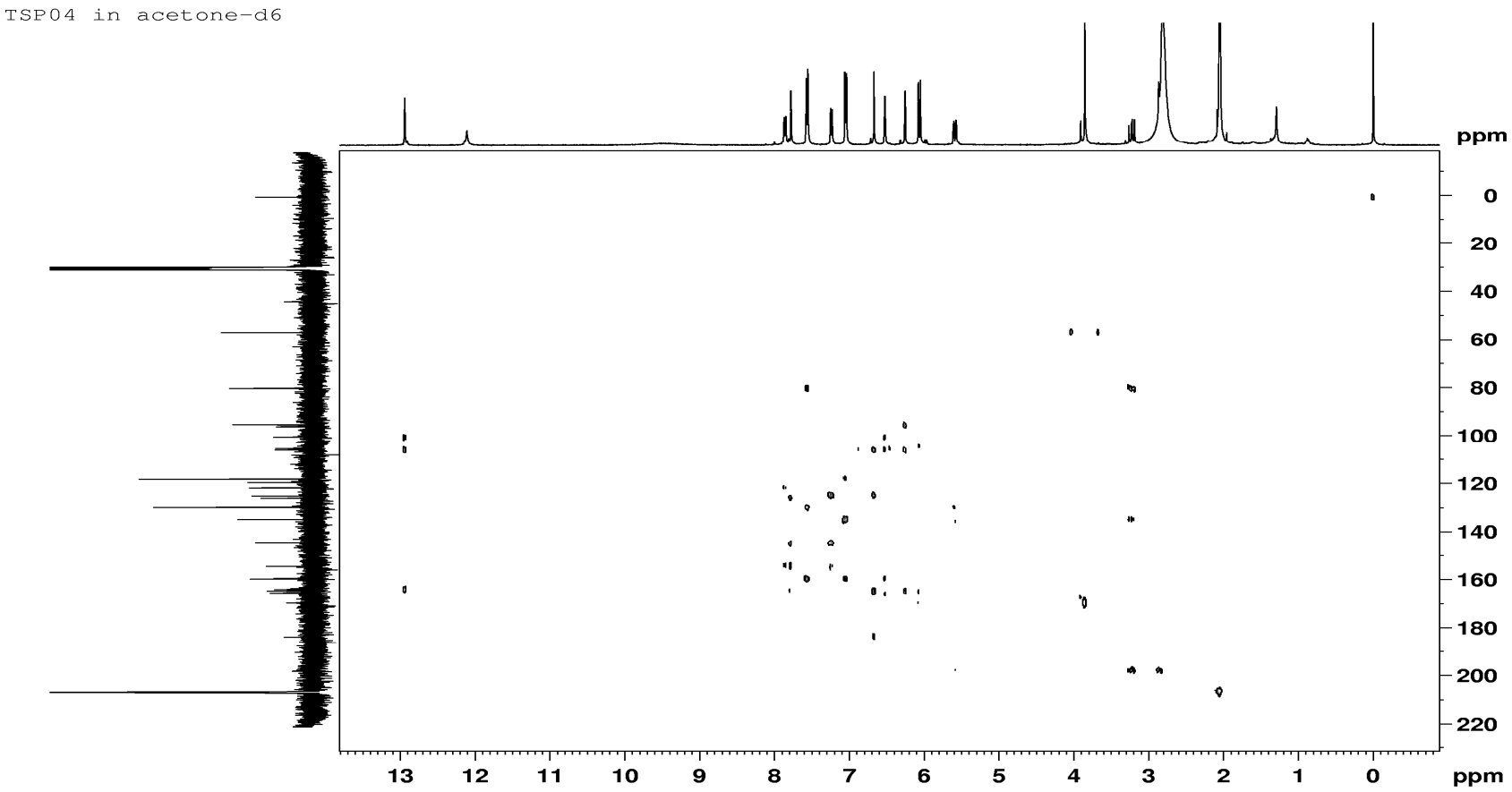


C2. HMQC-NMR Spectroscopy of C2

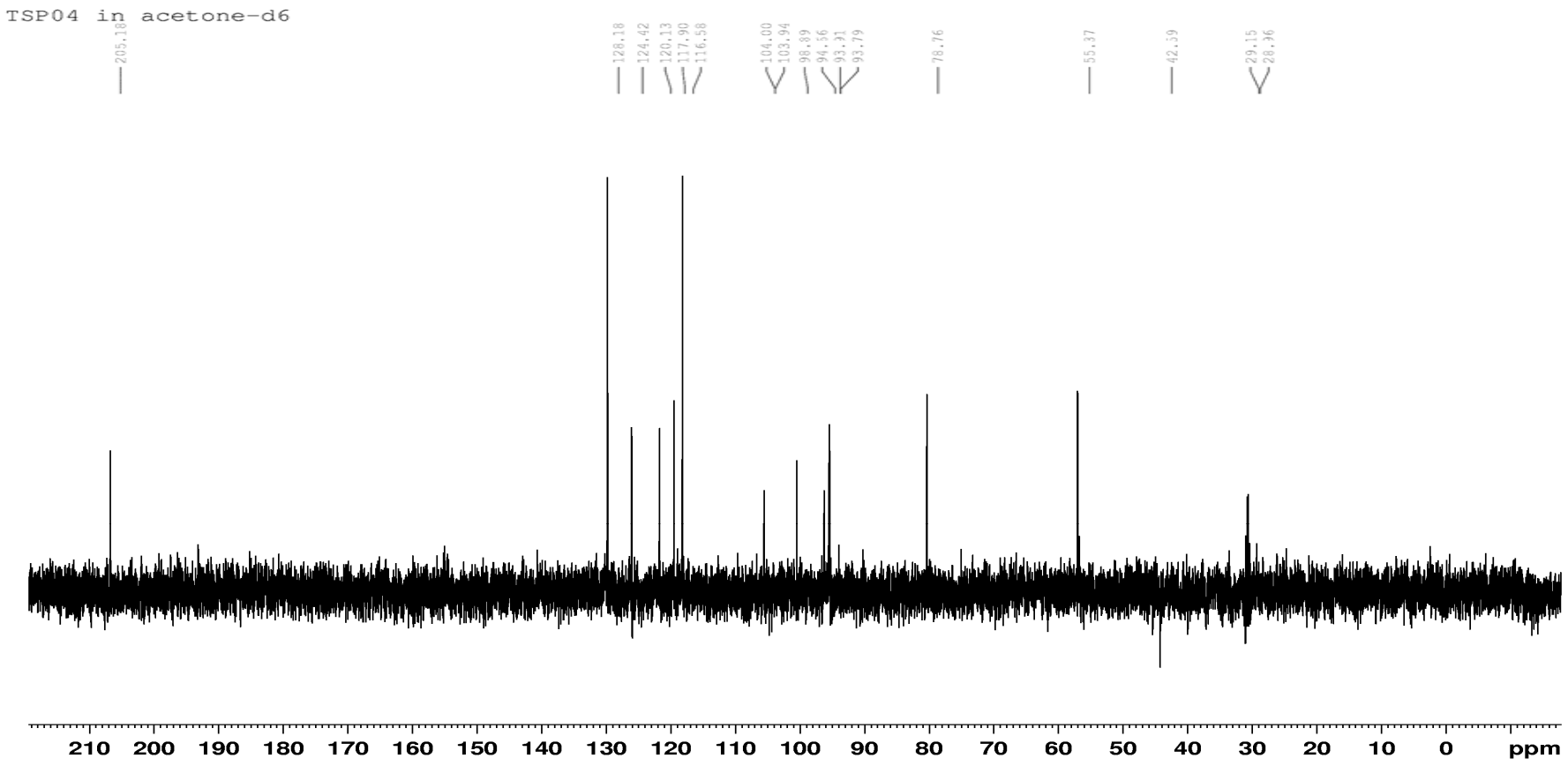
TSP04 in acetone-d6



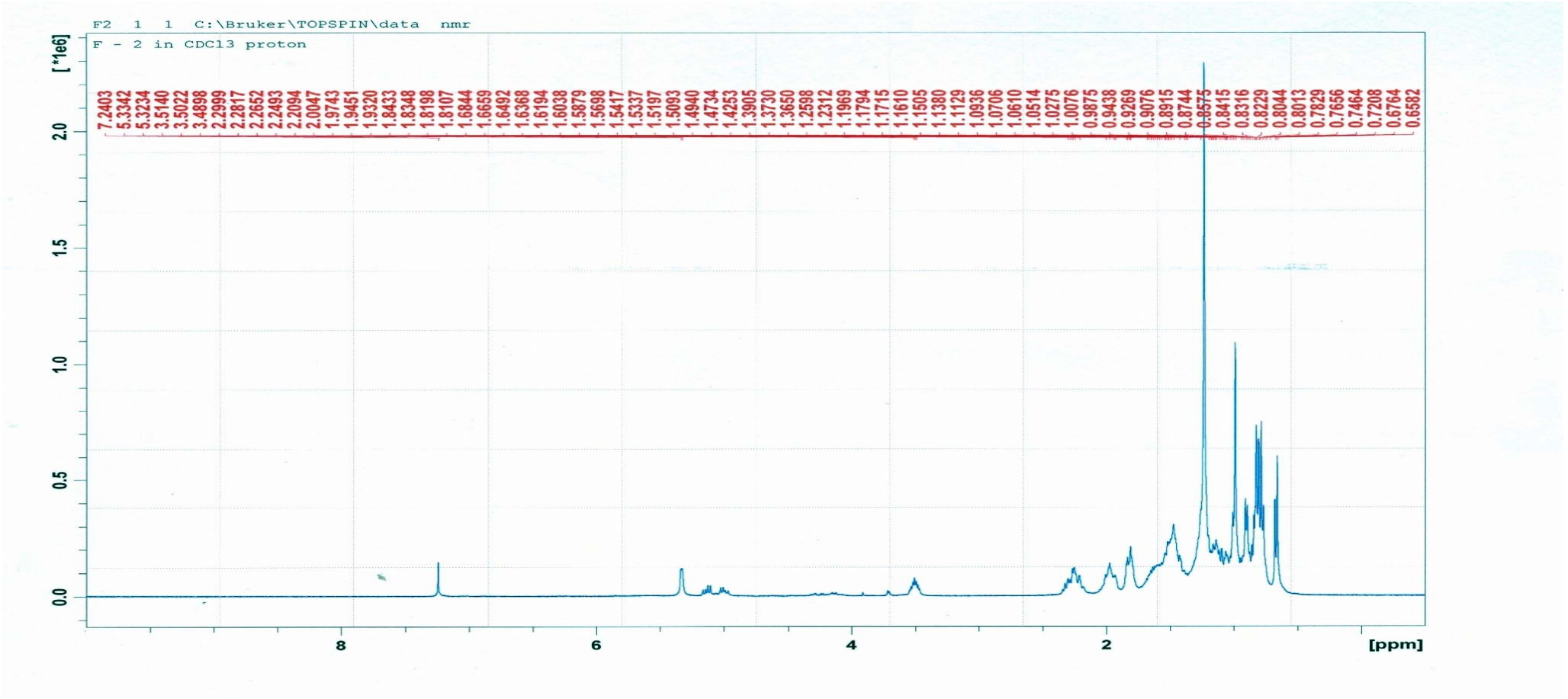
D2. COSY-NMR Spectroscopy of C2



E2. HMBC-NMR Spectroscopy of C2

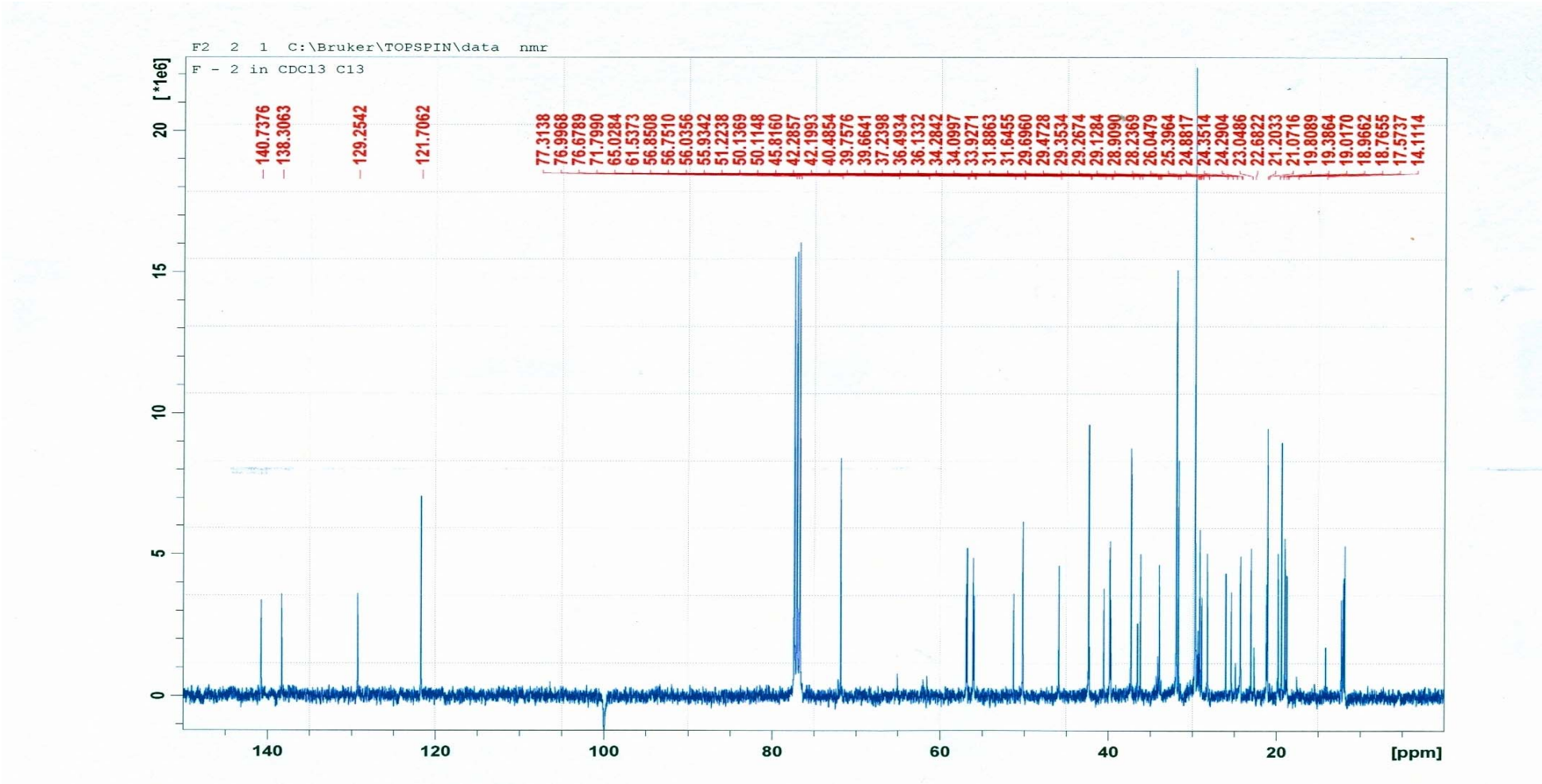


F2. ^{13}C-DEPT-NMR Spectroscopy of C2



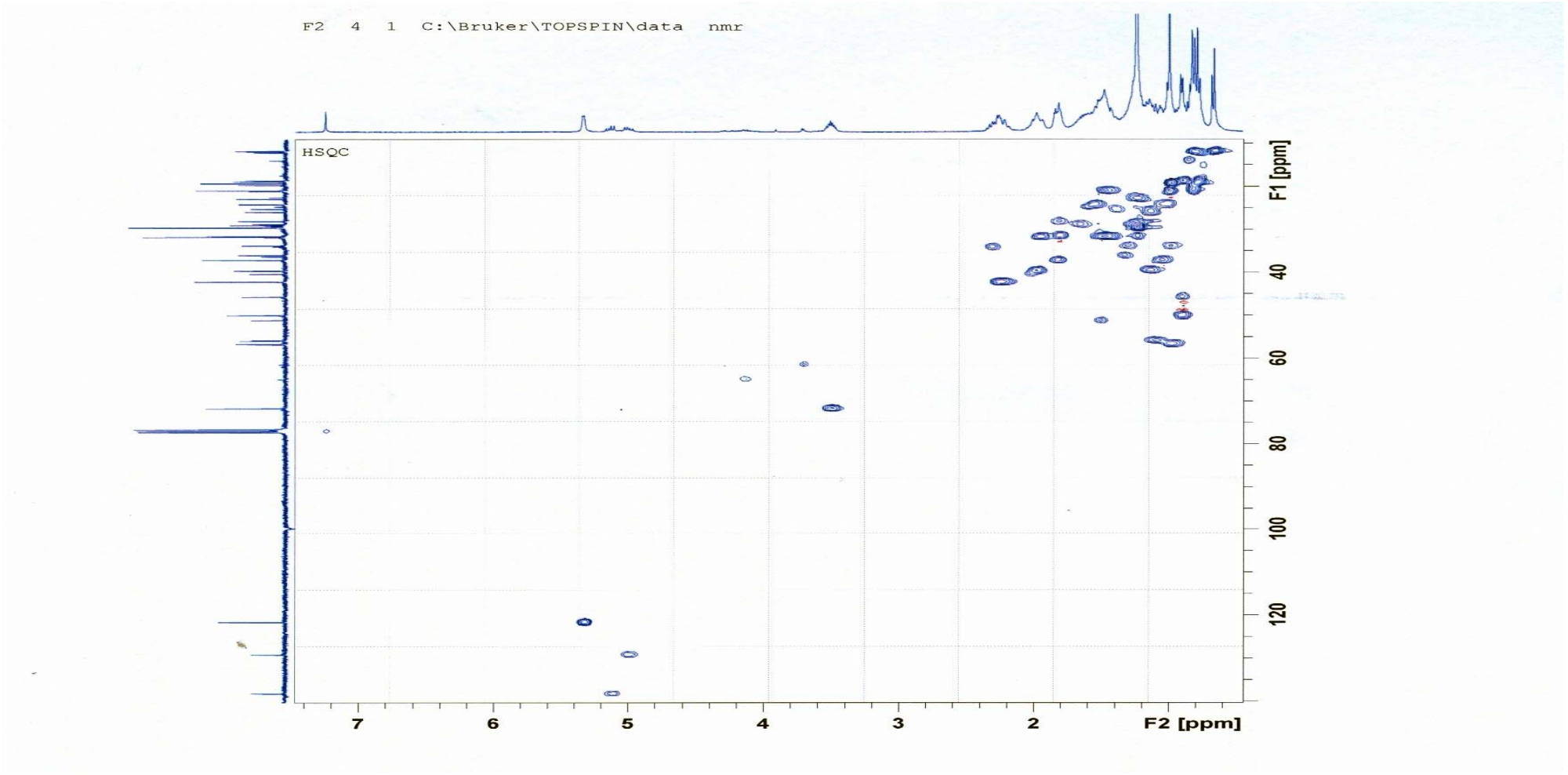
A3. ^{1}H-NMR Spectroscopy of C3

M



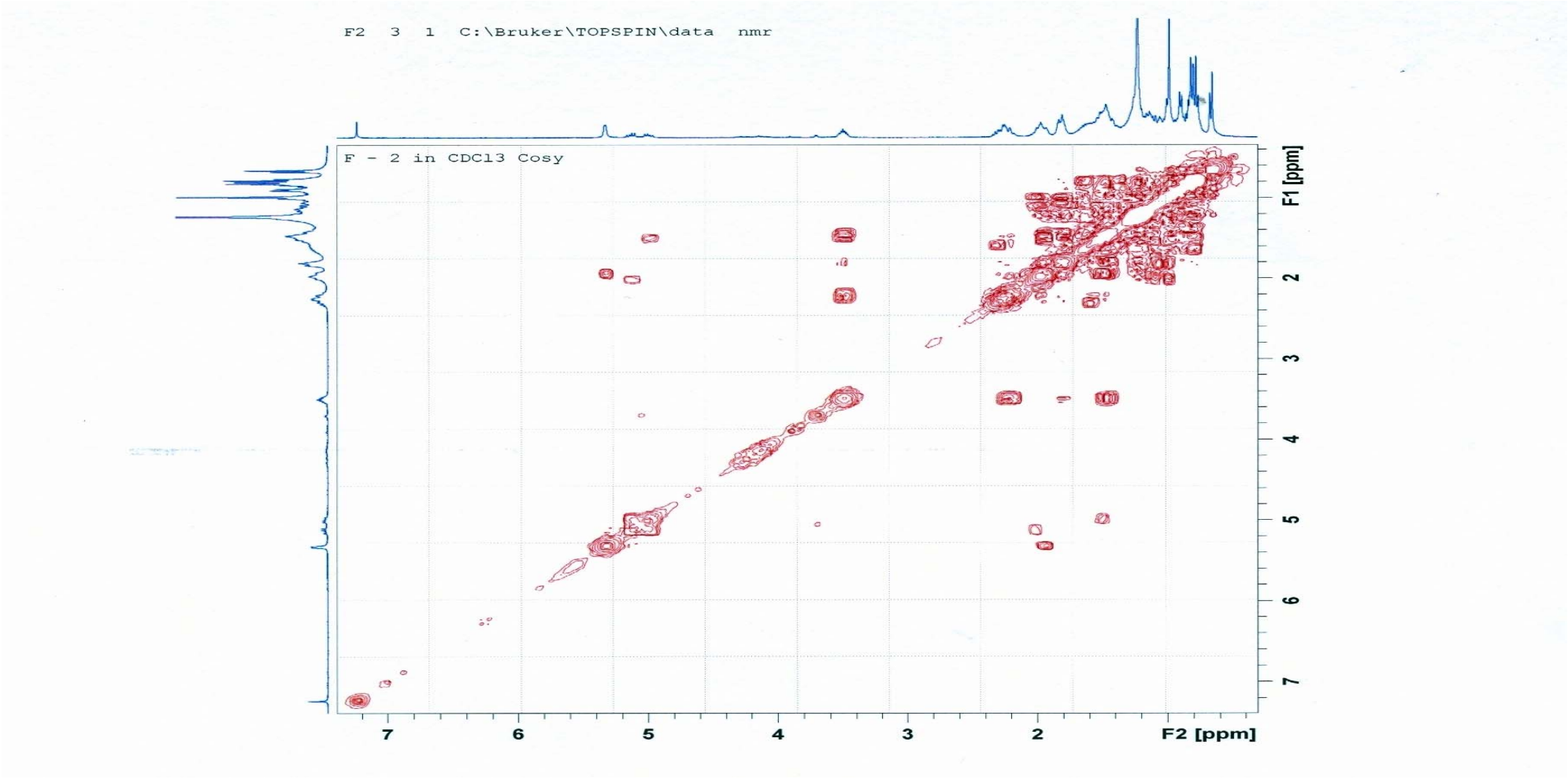
B3. ^{13}C-NMR Spectroscopy of C3

N



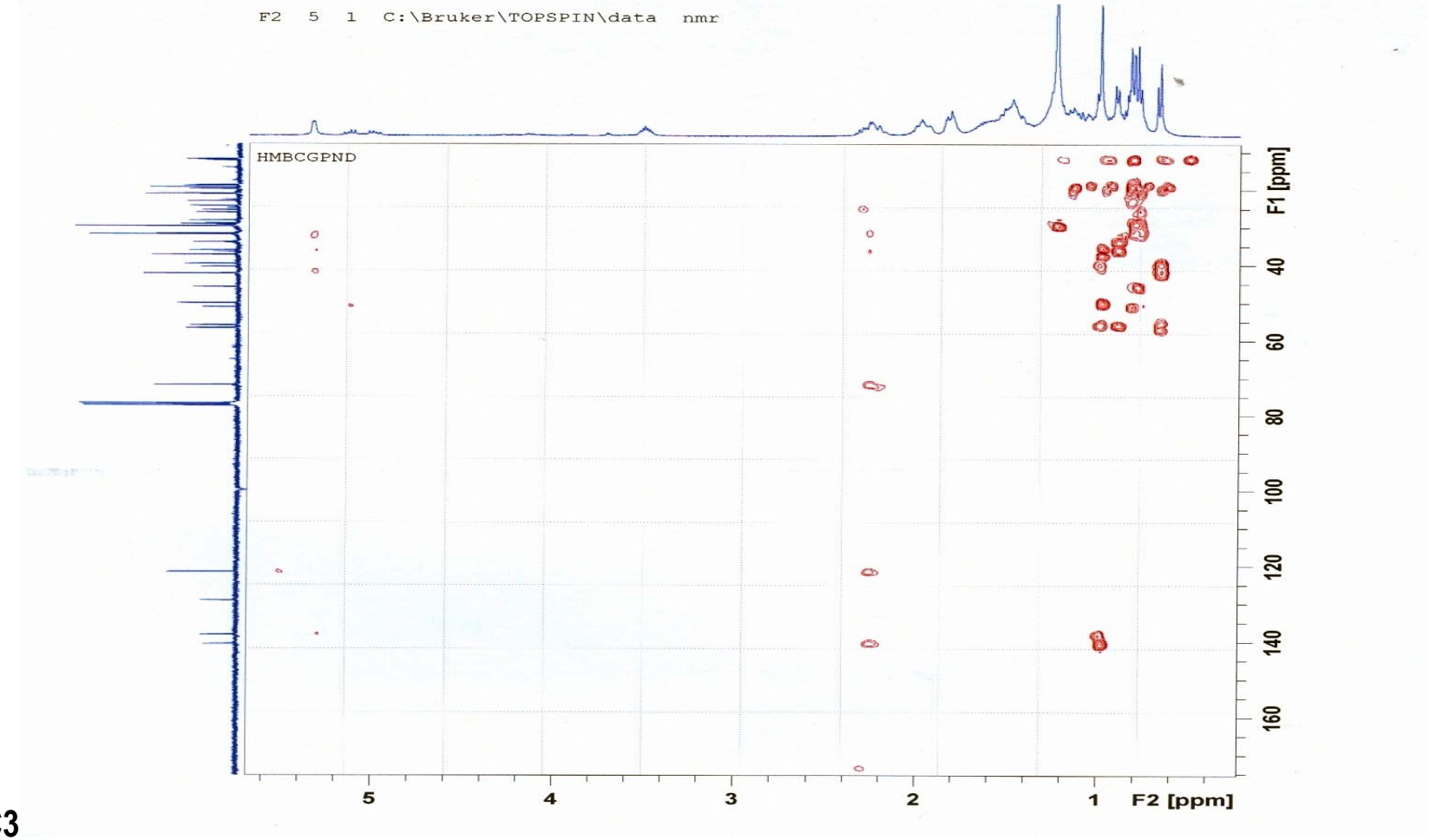
C3. HMQC-NMR Spectroscopy of C3

O


F2 3 1 C:\Bruker\TOPSPIN\data nmr
F - 2 in CDCl3 Cosy
F1 [ppm]
F2 [ppm]
1
2
3
4
5
6
7

P

D3. COSY-NMR Spectroscopy of



C3

E3. HMBC-NMR Spectroscopy of C3

Q

F3. ^{13}C-DEPT-NMR Spectroscopy of C