Phytochemical and antimicrobial studies on *Rhus natalensis*

By

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ABSTRACT

Extracts from the root bark, stem bark, and leaves of *R. natalensis* were screened for antibacterial activity against standard bacterial strains; *Staphylococcus aureas, Escherichia coli* and *Pseudomonas aureginosa,* and fungi; *Candida albicans*, *Trichophyton mentagrophytes* or *Microsporum gyseum.* Chromatographic techniques were utilized to isolate pure compounds. This study validates and documents, in a systematic way, the antimicrobial properties of the *R. natalensis* used for many years by many people of the world. It also provides valuable information for further phytochemical isolation and characterization studies of active compounds, necessary for the development of new drugs. The extractions were carried out using broad spectrum of solvents (hexane, dichloromethane, ethyl acetate, and methanol). Fractionation was done using standard chromatographic techniques. A total of seven (7) compounds were isolated from *R. natalensis.* Three of the isolates were characterized and their structures were unambiguously established by detailed spectroscopic analysis that involved high resolution mass spectrometry, 1D and 2D-NMR spectral data experiments ^{1}H, ^{13}C, DEPT, COSY, HMBC, and NOESY. These compounds are: **3-(1-(2,4-dihydroxyphenyl)-3,3-bis(4-hydroxyphenyl)-1-oxopropan-2-yl)-7-methoxy-4H-chromone-4-one (39),** Rhuschromone, a novel compound isolated for the first time, **2',4'-dihydroxychalcone-(4-O-5''')-4'',2''',4'''-trihydroxychalcone (40)** and **3-((Z)-heptadec-13-enyl) benzene-1,2-diol (41)**, an urushiol. Compound **39** recorded the highest activity zone of inhibition (21mm) against *S. aureas*, which was found to be 50% as active the chloramphenicol standard used. The traditional use of the extracts in infections and inflammatory conditions is rationalized based on the content of the isolated compounds, and it has been proposed that the total crude extract, with its contents of so many bioactive compounds, could be formulated for use in many infections, microbial or fungal. Furthermore, not all of the species studied to date have been fully characterized for potential bioactivities. Thus, there remains a significant research gap spanning the range from lead chemical discovery through process development and optimization in order to better understand the full bioactive potential of many of these plants.

DECLARATION

I, Henry Maina Mwangi hereby declare that this work is my original dissertation and to my knowledge, it has not been submitted anywhere else for the award of a degree at any other University.

Date …… Signed ….....

ACKNOWLEDGEMENT

My sincere gratitude to my supervisor Prof. Wilfred T. Mabusela for his tireless and persistent effort and great guidance without which this work could not have been accomplished. Special thanks to Prof. Berhanu Abegaz formerly from the University of Botswana for guiding me through the characterization process of the novel compound obtained. My most humble gratitude to Dr. Martin Onani at the University of Western Cape for carrying out the initial running of NMR spectra. To Prof. Paul K. Tarus of Moi University who started me off so strongly through the research techniques. He was very supportive through my initial laboratory work despite serious challenges. I am greatly indebted to them all. To my fellow post graduate students during the whole period of my research Stanley, Paul, Alloys, all at the University of the Western Cape, each one of them contributed greatly towards the completion of this work.

Ultimately but not the least, I would not have made it this far were it not for the hand of the Lord God. I thank the Almighty for the gift of good health, strength, endurance, inquisitive and critical thinking mind.

DEDICATION

To my loving wife Monica and my kids Edward and Winnie, who have been an inspiration to my struggle. To my loving Mum and Dad for their moral support in my endeavors into this study. May the Lord God bless them in a mighty way.

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List of abbreviations

AIDS- Acquired Immune Deficiency Syndrome

ATCC- American Type Collection Centre

AZT- Azidothymidine

BCG- Bacillus of Camette Guerin

COSY- Correlation Spectroscopy

CD_3OD- Deuteriated Methanol

DCM- Dichloromethane

DEPT- Distortion Enhanced by Polarization Transfer

DMSO- Dimethylsulfoxide

EtOAc- Ethyl acetate

FDA- Food and Drug Administration

FDC- Food, Drug and Cosmetic

HBV- Hepatitis B Virus

HMBC- Heteronuclear Multiple Bond Correlation

HMQC- Heteronuclear Multiple Quantum Coherence

HIV- Human Immune Virus

HSV- Herpes Simplex Virus

HSQC- Heteronuclear Single Quantum Coherence

IR- Infra Red

LAV- Low Activity Virus

MDR- Multiple Drug Resistance

MeOH- Methanol

MIC- Minimum Inhibitory Concentration

MS- Mass Spectrometry

NMR- Nuclear Magnetic Resonance

NOESY- Nuclear Overhauser Enhancement Spectroscopy

PHL- Public Health Laboratory

TLC- Thin Layer Chromatography

TMS- Tetramethylsilane

US- United States

UV- Ultra Violet

VLC- Vacuum Liquid Chromatography

WHO- World Health Organization

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CHAPTER 1

INTRODUCTION

1.1 Background

Plant chemistry has developed over the years as a distinct discipline between natural product organic chemistry and plant biochemistry. It is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, their biosynthesis, metabolism, natural distribution and biological function.

Medicines from plants contributed largely to human health. A lot of research on plants has led to the development of many drugs and plants are a good source for the present and the future. However, continued use of crude extracts without scientific evaluation may lead to serious complications such as overdose, and lack of awareness about the presence of toxic substances that would otherwise poison the body cells. The manner in which drugs are handled is important to ensure that they do not lose their potency. Upon expiration, harmful products may be formed and as such, it is necessary to establish a proper scientific procedure for preparation and use of such herbal medicines through toxicological, microbial, pharmacological and chemical studies (Nkunya *et al*., 1990). New antimicrobials with related activities and structures but different from those currently in use, can be obtained from plants. This requires a follow up with attempts to isolate and identify those active compounds. The plant kingdom is rich in, and has abundant sources of many remedies for the prevention and cure of various human ailments with minimum side effects, as well as maintenance of healthy and normal physiological state (Goswan, 2002).

The African continent is known to have a wide variety of indigenous species of plants. By making appropriate approaches and investigations of these indigenous resources, very important biologically-active compounds could be discovered from the traditional medicinal plants people use (Rosenthal, 2001). Up to 70% of our modern drugs are obtained from these natural sources, using either the natural substance directly or those synthesized through modern technology (Jassim *et al.*, 2003).

Plants which have medicinal activities are known to have an advantage of possessing a range of chemical diversities of secondary metabolites. Such medicinal properties are often of superior, and if not, of similar activity as modern synthetic drugs. A wide range of phytochemicals that includes flavonoids, anthocyanins, alkaloids, and coumarins among others, has been discovered (Jassim *et al*., 2003). In Africa, reliance on herbal medicine is very high, as indicated by the fact that 70% of the population depends on traditional medicines for their primary health care, despite the lack of scientific information on efficacy and side effects (Kokwaro, 1996). This is generally due to limited access to the modern health facilities. Poverty further aggravates the situation as available medicine is sometimes unaffordable. This situation is true in parts of Africa and a few Asian and Latin American countries. Traditional medicine, particularly herbal medicine (Nsowah-Nuamah, 2002) has found broad use in these regions.

1.2 Microbial infections

The bodies of mammals act as a favourable environment for the growth of numerous micro-organisms. The multiplication of these micro-organisms result in an unfavourable interaction leading to diseases. Further complications arise from those which are able to make harmful chemicals that act like acids or poisons. At times microbial infections are so irritating to the body systems such that our own body's defense overcompensates and attack not only the bacteria but also the tissues that host them. Bacterial infections usually make one to be noticeably sick, whether it's an infected toe nail or bloody diarrhoea (Hensley *et al*., 2004).

As much an infection may be obvious, it requires a modern laboratory to identify the exact germ such that the correct treatment may be prescribed. Most microbes can be grown in the laboratory and tested for their reaction to different antibiotics. This is why a specimen must be collected from an infected individual (Shlaes *et al.,* 2002). *Pneumococcal* and *Influenza* infections are the most common causes of acute respiratory infections in children. Except for a few cases like BCG (Bacillus of Camette and Guerin), vaccines have not been developed for all bacterial infections and so far, no anti-fungal vaccines are known. This is the reason why chemotherapeutic agents are necessary (WHO, 1996). It is to be appreciated that the introduction of antimicrobial agents into general clinical use represents one of the landmark medical advances of modern medicine. However, the issue of antimicrobial resistance has become a concern recently. The resistance of bacteria and fungi to current antimicrobial agents is a worldwide medical problem (WHO 2004). Currently, there are recurring multiple drug resistance (MDR) outbreaks in the developing worlds (WHO 2004).

More than 70% of the bacteria causing infections have become resistant to some particular drugs (WHO 2004). The situation has been made worse by the prevalence of the HIV/AIDS pandemic, poverty and an emergence of new infectious diseases like swine flu and Ebola, high costs and side effects of the available drugs. This has resulted in increased severity of such infectious diseases and the high mortality rate from certain infections. All these factors have necessitated studies on the potential sources of effective, safe and cheap antimicrobial alternatives. Such have been found to originate from plants and these have not been exhaustively exploited (Thangadurai *et al*., 2004). This review will examine the history of antimicrobial drug development, how it has influenced the present situation, where we stand today as in terms of turning into natural medicine from plants, and it shall also address some of the potential solutions for spurring future antimicrobial development.

1.3 Antibacterial drugs

The past uses of antibacterial drugs have generated issues associated with their safety. In fact, safety issues associated with antimicrobials have changed the face of public health. The deaths associated with use of some types of sulfonamides resulted in the establishment of the Food, Drug and Cosmetic (FD&C) Act in 1938 (Munson *et al*., 1995). During the 30 years following the introduction of penicillin and sulfonamides, scientists discovered and developed a wide range of antimicrobials to treat bacterial diseases, presenting clinicians with a number of treatment options for most infectious diseases. The method of discovery of new agents was largely based on the methods of scientists working and evaluating naturally occurring compounds.

Many new antimicrobials have been discovered serendipitously, and by observation of various experimental works, many other such drugs have been developed by chemical modification of existing agents (US FDA., 2004). The discovery of new classes of antibacterial drugs, defined here as drugs with a completely novel mechanism of action, had slowed down. For the last novel class of antibacterial drug discoveries during the modern times, the majority of these have consisted of chemical modifications of previously discovered classes of drugs. This is testament to the challenges inherent in the discovery of new agents.

In spite of a lack of new classes of agents, drug developers have continued to introduce new agents within existing classes. Some of these agents have efficacy against diseases caused by pathogens that were resistant to previous 'generations' of the same class. In addition, some of the drugs have demonstrated improved efficacy in certain diseases compared with previous drugs. For instance, third generation cephalosporins (Williams, 1987) are effective in diseases caused by Gram-negative organisms and in diseases, such as acute bacterial meningitis, where first-generation cephalosporins were less effective. Other agents had similar efficacy but manifested a different safety profile or a more convenient dosing schedule. Other agents introduced during this time represent major advances over previous agents within the class and do present clinicians with an expanded range of treatment options.

Antibacterial drugs should be used only if clinical or laboratory evidence suggests bacterial infections. Use of these drugs for viral illnesses is inappropriate; it subjects the patients to drug complications without any benefit and contributes to bacterial resistance. Certain bacterial infections (abscesses, infections with foreign bodies) require surgical intervention and do not respond to antibiotics alone (Reichert *et al*., 2003).

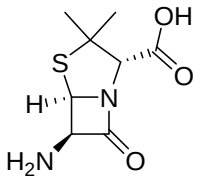
Culture and antibiotic sensitivity tests are essential for selecting a drug for serious infections. These are diagnostic laboratory procedures used to identify the type of bacteria and to determine which antibiotics can successfully fight an infection. The absence of bacteria does not mean there is no infection, since it could be a virus that will not grow in a specific culture medium. Treatment, however, must begin once culture results are available, necessitating selection according to the most likely infecting organisms. Depending on the culture results, drugs used (DiMasi *et al*., 2003) should possess the narrowest spectrum of activity that will control the infection. Treatment of a serious infection that may involve any one of several pathogens or due to multiple pathogens like polymicrobial anaerobic infection, a broad spectrum of activity is desirable. The most likely organisms and their susceptibility to antibacterial effects vary according to their geographical location.

Antibacterial drugs kill bacteria *in vitro*. Bacteriostatic drugs slow or stop *in vitro* bacterial growth but recovery depends on body defenses to kill them. Quantitative methods identify the minimum *in vitro* concentration at which an antibiotic can inhibit growth or kill such bacteria. However, *in vivo* antibacterial effectiveness involves other factors including pharmacology (Boggs *et al*., 2004) i.e. absorption, distribution, concentration in fluids and tissues, protein binding and rate of excretion or metabolism, presence or absence of drug interactions or inhibiting substances and host defense mechanisms. Usually greater *in vitro* killing power is important only if local or systemic host defenses are weak, for example in endocarditis, meningitis, serious infections in netropenic or other immune–compromised patients.

The predominant determinant of bacteriological response to antibiotics (Spellberg *et al*., 2002) is either the duration that blood levels of the antibiotic exceed minimum inhibitory concentration (time dependence) or the peak blood level relative to minimum inhibitory concentration (concentration dependence). This avoids subjecting a patient to drug complications and contribution to bacterial resistance. Many antimicrobials are prescribed for treatment durations ranging from a single dose to 10 days of treatment. This short-term use limits the potential profitability of antibacterial drugs compared with other classes of drugs. Some of these drugs include the following

1.3.1 Penicillin

Penicillins refer to a group of beta-lactam antibiotics used in the treatment of bacterial infections caused by Gram–positive bacteria. They inhibit bacterial growth by interfering with the transpeptidation reaction of bacterial cell wall synthesis. Penicillin is used in reference to a specific member of the penicillin group Penam skeleton. The basic nucleus of penicillin is 6-aminopenicillanic acid (**1**)



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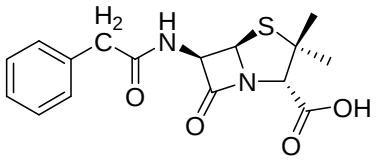
An example of narrow spectrum penicillin along with a poor oral activity is penicillin G (**2**). These led to the search for derivatives of penicillin that could treat a wider range of infections.



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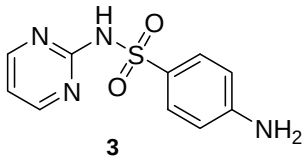
1.3.2 Sulphonamides

Sulphonamides are one of the oldest groups of antimicrobial compounds. These were the first antimicrobial agents to be used in the treatment of human systemic infections (Mandell, *et al*., 2003). Chemically useful sulphonamides are derived from sulphonamide by substitution of the amide moiety such as in sulphadiazine (**3**). There have been human body side effects resulting from sulphonamide residues. Some of these are headaches, mental depression and vomiting.



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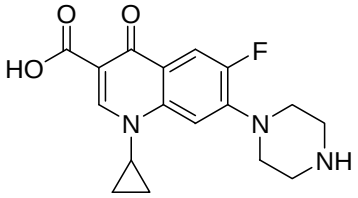
1.3.3 Fluoroquinolones

These antimicrobials are used to treat infections caused by bacteria. Physicians prescribe these drugs for bacterial infections in bones and joints, skin, urinary tract and some sexually transmitted diseases, and opportunistic attacks that affect people with HIV/AIDS among other diseases.

Continued attempts to produce compounds with significant chemical novelty and patentability have become extremely a great challenge. A number of avenues have been explored in order to cover new ground including discoveries from plant medicine. There are, however, some available drugs, both in tablet or injectable forms. Some examples of such chemical structures of the fluoroquinolone antibiotics are ciprofloxacin (**4**), moxifloxacin (**5**) and ofloxacin (**6**) (Knowles, 1997).



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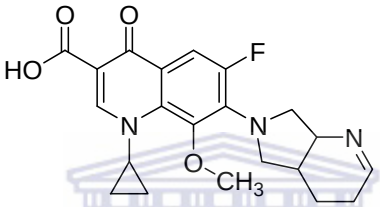

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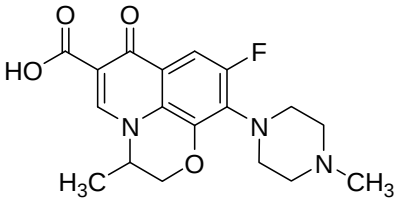

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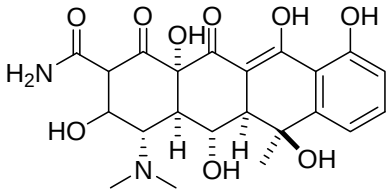
The most common side effects of fluoroquinolones are nausea, skin rashes, vomiting, insomnia, diarrhoea, dizziness, photosensitivity and abnormal liver function. Due to some serious side effects that lead to fatalities, some drug varieties under this category of antimicrobials have been banned (Frothingham, 2004).

1.3.4 Tetracyclines

Tetracyclines act by inhibiting protein synthesis after uptake by active transport of the susceptible organism (Catlin *et al.,* 1982). Tetracyclines chelate calcium and deposit in growing bones and teeth causing staining and sometimes dental hyperplasia. High doses of tetracyclines are known to decrease protein synthesis in host cells.

Examples of tetracycline broad-spectrum of antibiotics include oxytetracycline (**7**), deoxycycline (**8**), and minocycline (**9**).



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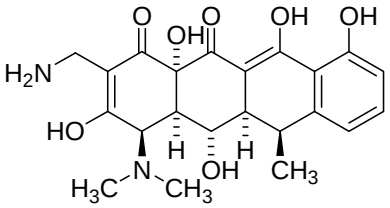

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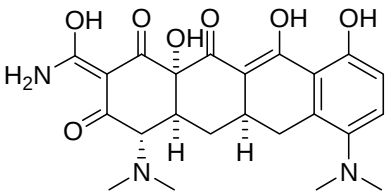

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1.4 Antifungal drugs

Antifungal drugs are of two kinds, systemic and topical. Systemic antifungal drugs are medicines taken orally or by injection to treat infections whereas topical antifungal drugs are those applied to the skin to treat skin infections (Espinel-Ingroff *et al.*, 1989). Common fungal infections include athlete's foot, jock itch, candidiasis (also called thrush or yeast infection).

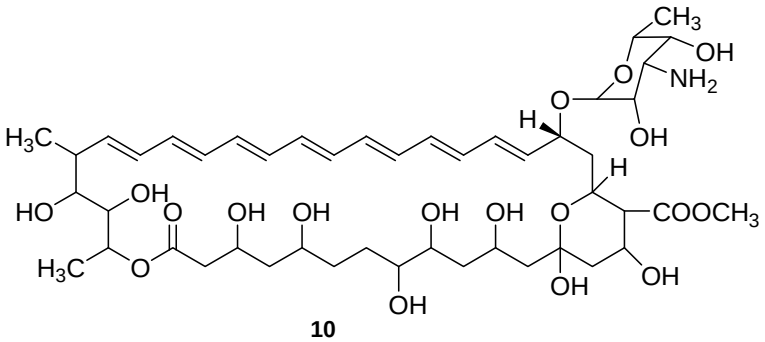
Topical antifungal drugs not only relieve the symptoms of fungal infection, such as itchiness, burning, and cracked skin, but they also eliminate the fungus. However, fungi which do not clear up after treatment with creams or ointments may need to be treated with systemic antifungal drugs (Groll *et al.*, 1998). This category of drugs is available even without a physician's prescription and come in many forms, including creams, ointments, liquids, powders, aerosol sprays, and vaginal suppositories (Cuenca *et al.*, 2007). Creams and liquids are usually the most effective for treating fungal infections on the skin, because they can get into the cracks and crevices where fungi grow.

On the other hand, powders absorb moisture and hence they are good to use in moist areas of the body, such as between the toes (Fitzpatrick *et al*., 2006). Systemic antifungal drugs work best when the amount of medicine in the body is kept constant. This means that the medicine has to be taken regularly. Medicine should be taken at the same time every day, and one should not miss any doses. During the course of taking such medicines, it is recommended that treatment be reviewed as often as possible. There is every need to keep checking for side effects throughout the antifungal therapy. Taking systemic antifungal drugs with certain other drugs may affect the way the drugs work or may increase the chances of side effects. The most common minor side effects of systemic antifungal drugs are constipation, diarrhoea, nausea, vomiting, headache, drowsiness, dizziness, and flushing of the face or skin. These problems usually go away as the body adjusts to the drug and do not require medical treatment (McCullough *et al*., 1999). The recommended dosage depends on the type of antifungal drug and the medical problem for which the drug is being taken. Doses may also differ from one patient to the next. Fungal infections can take a long time to clear up, so it may be necessary to take the medicine for several months, or even for a year or longer (Bougnoux *et al*., 2004). Commonly used antifungal drugs are in the following sections (1.4.1 and 1.4.2)

1.4.1 Amphotericin B

This is a macrolide of broad spectrum activity. It is very active against most fungi and yeasts, both superficial on exposed organs and systemic infections, affecting deeper tissues and organs. Orally administered amphotericin B is poorly absorbed (Bunow *et al*., 1997). Serious side effects of amphotericin B are its real toxicity towards the reduction of renal function, impaired hepatic function and anaphylactic reactions. Unfortunately, the drugs which suppress these adverse reactions are very expensive (Barnes *et al*., 2003).

The recently discovered amphotericin B **(10)**, a lipid complex, is less toxic especially towards renal organs.



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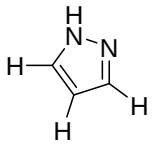
1.4.2 Flucytosines

These are used only in combination with amphotericin B or the azoles because of the high rate at which the target microbes may develop resistance. Extreme caution is needed in their usage since they cause renal impairments (90% excreted unchanged in urine). Examples of these drugs include imidazoles like; 1, 2-diazole (**11**) and 1, 3-diazole (**12**) among others (Cuenca *et al*., 2007).



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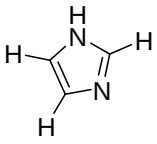

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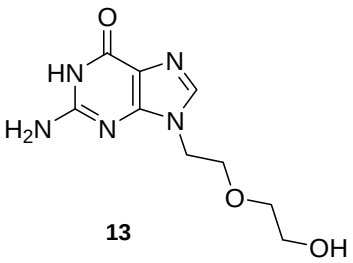
Emergence of some limitations of these azoles involves their susceptibility to resistance by fungal organisms like *Candida* species. Medicinal plant research studies are being undertaken worldwide in order to help overcome these limitations by providing alternative drugs (Jassim *et al.*, 2003).

1.5 Antiviral drugs

The development of antiviral drugs may be looked at on a comparative basis with that of antibacterial (Mandel *et al*., 2003). Since there are far fewer antiviral drugs already available than antibacterial, there is less competition. Many antiviral drugs are for chronic illnesses like HIV/AIDS, and are prescribed on a long-term basis for the life of the patient. There is less likelihood of inappropriate prescriptions, at least with some antiviral, such as anti-HIV drugs, since diagnostic tests can accurately select patients who require treatment, and the need for immediate treatment is less in a chronic illness than in a bacterial disease that may be rapidly fatal. Consequently, there has been an increase over time in the number of FDA approved antiviral drugs (Srinivasan *et al.*, 2004).

The discovery of antiviral drugs has not been very easy, but lead compounds have been modified in relation to the target in order to come up with varieties having improved bioactivity. Examples of some of the antiviral drugs are: Acyclovir (**13**), and Gancyclovir (**14**).



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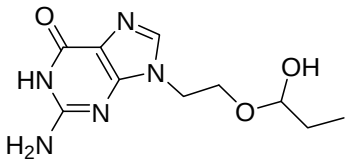

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The problems associated with most of these drugs are their toxicity levels in that they have many side effects, for example, Acyclovir (**13**) that causes anaemia. Resistance to antiviral action also increases with prolonged use. Another factor that leads to the decline in their efficiency is increased viral load as a result of reduction in the immune mechanisms (Piscitelli *et al.,* 2003).

Due to rapid mutation of the virus, more complications arise towards their control (Rocco *et al*., 1999). Attempts to obtain more curative drugs with minimal resistance or none at all may involve working on medicinal plants found in nature.

1.6 Statement of research problem

Medicinal plants are important elements in traditional medicine in all cultures of the world. The idea that certain plants had healing potential was known long before human beings discovered the existence of pathogens. Medicinal plants which have been used by man to treat common diseases are important elements of traditional medicine. In recent years, traditional medicine has not been limited to a specific culture. It has been used in developing countries as well as in developed countries. Effects of HIV/AIDS, HBV, Ebola and other microbial attacks have worsened the situations and there is need to explore those plants that can offer medicinal value. The proposed investigation here seeks to isolate and determine structures of various bioactive metabolites from some species of the genus *Rhus* in the family of Anacardiaceae.

1.7 Justification

In various countries both in Africa as well as Europe, medicinal plants are important elements of indigenous medical systems. 70-80% of Africans depend on herbal drugs and traditional medicines for primary health care. Africa possesses a large number of plants, which have the potential for therapeutic applications in modern medicine. Varieties of the plants are known to traditional healers in many communities and are being used by them in therapy. Many plants used in our traditional therapy have not been well studied in order to understand their efficiency, safety and activity.

When such active components are known, they may be isolated and their structural modification performed so as to synthesize more derivatives. The bioactive compounds can thus be used as templates for synthesizing novel drugs for clinical use (Murray, 1995). The potential for side effects and development of drug resistance make research based on natural resources such as medicinal plants ever more important.

1.8 Hypotheses

i) Plants from the genus *Rhus* may contain unique compounds with antibacterial and antifungal activity.

ii) Such compounds may be stable and can be isolated and identified.

iii) Compounds isolated may remain bioactive once isolated from the plant sources.

1.9 Objectives

1.9.1 General objective

Isolation and structural elucidation of secondary metabolites from selected species of the genus *Rhus*

1.9.2 Specific objectives

i) To perform sequential extractions on a selected species of the genus *Rhus*; *R. natalensis.*

ii) To determine the antimicrobial activity of the crude extracts.

iii) To isolate pure compounds from the crude antimicrobial fraction using chromatographic techniques.

iv) Determine the structures of the compounds using spectroscopic techniques (e.g. UV, NMR, COSY techniques).

v) Determine antimicrobial activity of the isolated pure compounds

CHAPTER 2

LITERATURE REVIEW

2.1 The family Anacardiaceae

This research focuses on the Anacardiaceae family which includes plants that produce gums, resins or latex from which antimicrobial compounds may be isolated. The special focus for this research is the *Rhus* genus. These are plants having trifoliate leaves with small flowers that eventually produce fruits which are in the category of drupes. *Rhus natalensis* is a shrub with a smooth dark brown bark. The leaflets are elliptic or obvate with rounded apex. Their leaf margins do crenate at times. They produce cream or greenish yellow flowers that eventually bear green fruits, round in shape. Plate 1 shows a part of the plant under study. The plant is common in wooded grassland thickets or grasslands with rocky sites. In the different regions of Kenya, the plant has different traditional names, and it is widely recognized by the indigenous people for treating various conditions. The vernacular names include Mutheo (Kamba), Muthigiu (Kikuyu), Siriat (Kipsigis), Sangala–madongo (Luo), Olmisigiyioi (Maasai), Murimuthu (Meru). The fruits are edible; a decoction of the fruit is employed against diarrhoea (Henk, 1991).

Plate 1. Photograph of *Rhus natalensis*.

2.2 The genus *Rhus*

Rhus plants are flowering plants in the family Anacardiaceae (USDA, 2007) found in temperate and tropical regions worldwide. Representative members by their geographical locations are listed in Table 1. In general, *Rhus* plants can grow in non-agriculturally viable regions, and various species have been used by indigenous cultures for medicinal and other purposes (Van Wyk *et al*., 2004). They often grow in areas of marginal agricultural capacity, and have a long history of use by indigenous people for medicine and other applications without necessarily competing with food production in terms of land use. Research undertaken on *Rhus* extracts to date indicates a promising potential for this plant group to provide renewable bioproducts with the following reported desirable bioactivities; antimicrobial, antifungal, antiviral, antimalarial, antioxidant, antifibrogenic, antiinflammatory, antimutagenic, antithrombin, antitumorigenic, cytotoxic, hypoglycaemic, and leukopenic. Furthermore, the bioactive components can be extracted from the plant material using environmentally benign solvents that allow for both food and industrial end-uses. The favorable worldwide distribution of *Rhus* also suggests that desirable bio-products may be obtained at source, with minimal transportation requirements from that source, through processing to end consumer. However, not all of the species studied to date have been fully characterized for potential bioactive components and bioactivities.

Table 1. **Geographic distribution of member representatives of the genus *Rhus*.**

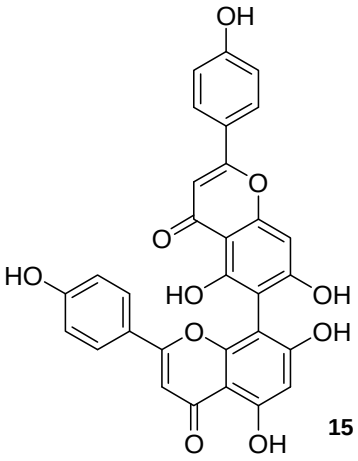
Asia	**Africa cont'd**
R. chinensis (Chinese Sumac)	*R. lucida*
R. hypoleuca	*R. macowanii*
R. javanica	*R. magalismontana*
R. punjabensis (Punjab Sumac)	*R. maricoana*
R. verniciflua	*R. marlothii*
Australia	*R. microcarpa*
R. taitensis	*R. montana*
Mediterranean	*R. natalensis*
R. coriaria (Tanner's Sumac)	*R. nebulosa*
R. pentaphylla	*R. pallens*
R. tripartita	*R. pendulina*
Mexico and Central America	*R. pentheri*
R. muelleri (Müller's Sumac)	*R. pondoensis*
Pacific Ocean	*R. populifolia*
R. sandwicensis	*R. problematodes*
Africa	*R. pterota*
R. acocksii	*R. pygmaea*
R. albomarginata	*R. pyroides*
R. angustifolia	*R. quartiniana*
R. batophylla	*R. refracta*
R. baurii	*R. rehmanniana*

Africa cont'd	**Africa cont'd**
R. bolusii	*R. rigida*
R. burchellii	*R. rimosa*
R. carnosula	*R. rogersii*
R. chirindensis	*R. rosmarinifolia*
R. ciliata	*R. rudatisii*
R. crenata	*R. scytophylla*
R. cuneifolia	*R. sekhukhuniensis*
R. dentata	*R. stenophylla*
R. discolor	*R. tenuinervis*
R. dissecta	*R. tomentosa*
R. divaricata	*R. transvaalensis*
R. dracomontana	*R. tridactyla*
R. dregeana	*R. tumulicola*
R. dura	*R. undulata*
R. engleri	*R. volkii*
R. erosa	*R. wilmsii*
R. fastigiata.	*R. zeyheri*
R. gerrardii	*R. gueinzii*
R. glauca	*R. harveyi*
R. gracillima	*R. horrida*
R. keetii	*R. grandidens*
R. krebsiana	*R. kirkii*

Africa cont'd	**North America cont'd**
R. laevigata	*R. ovata* (Sugar Sumac)
R. lancea	*R. trilobata* (Skunkbush Sumac)
R. leptodictya	*R. typhina* (Staghorn Sumac)
R. longispina	*R. toxicodendron*
R. lucens	*R. vernix*
R. incisa	*R. virens* (Evergreen Sumac)
North America	*R. glabra* (Smooth Sumac)
R. aromatica (Fragrant Sumac)	*R. integrifolia* (Lemonade Sumac)
R. choriophylla (Mearns Sumac)	
R. copallina (Winged Sumac)	
R. lanceolata (Prairie Sumac)	
R. incisa R. laurina (Laurel Sumac)	
R. michauxii (Michaux's Sumac)	
R. microphylla (Desert Sumac)	

The genus *Rhus* is of great interest since its members have been found to possess chemical compounds that are toxic to micro–organisms. The compounds include flavonoids, biflavonoids, and isoquinolines among others. The genus *Rhus* consists of over 200 species and is known to be rich in biflavonoids and urushiols. Interest in biflavonoids has increased in recent times due to realization of a variety of biological activities manifested by them (Lin *et al*., 1999).

The root methanol extract from *Rhus succedonea* has been reported to contain compounds such as agathisflavone (**15**) robustaflavone (**16**) hinokiflavone (**17**) volkensiflavone (**18**) rhusflavone (**19**) succedaneflavone (**20**) and their methyl ethers and acetates.



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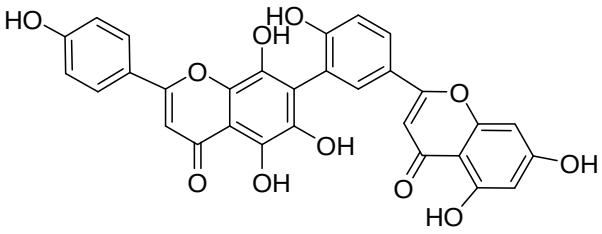

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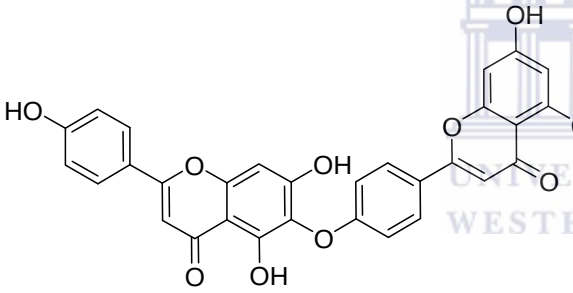

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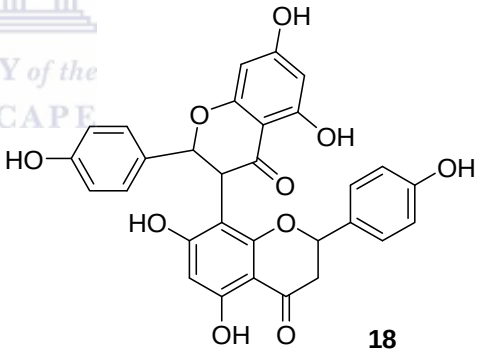

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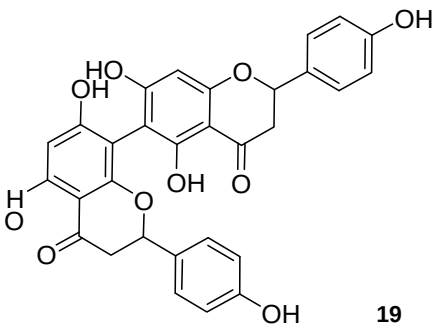

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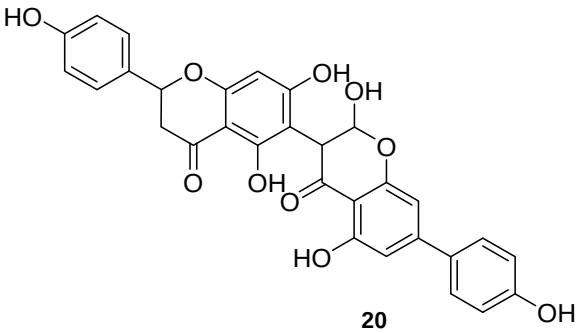

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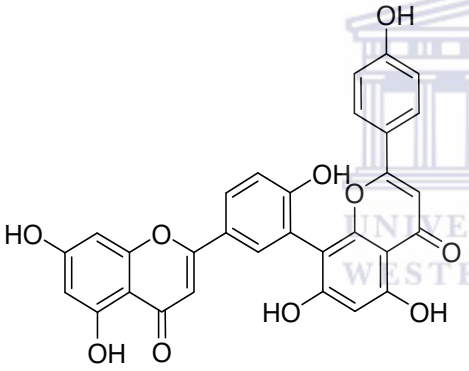
Compounds **16**, **18**, **19**, and **20** were evaluated for their antiviral activities and **16** exhibited strong inhibitory effects against influenza A and influenza B viruses (Lin *et al*., 1999). The same activity was demonstrated by compounds **15** and amentoflavone **(21)** which also exhibited moderate anti-Herpes Simplex Virus (HSV)-I and anti-Herpes Simplex Virus (HSV)-2 activities (Lin *et al.*, 1991). Some biflavonoids from *Rhus succedonea* as well as their methyl ethers have shown anti HIV-I reverse transcriptase activity. The biflavonoids responsible for this are compounds **16** and **17**. Morelloflavone **(22)** has also demonstrated significant antiviral activity against HIV-1 (strain LAV-1) in phytohemagglutinin-stimulated primary human peripheral blood mononuclear cells (Lin *et al*., (1999).



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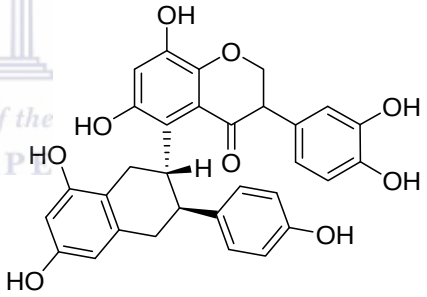

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Recent studies have examined a broad spectrum of antiviral properties of *Rhus* extracts, and the work focused on biflavonoids isolated from the seed kernels of *R. succedanea* (Lin *et al.*, 1999). Six of the biflavonoid compounds **14**, **15**, **18**, **19**, **20** and **21** were isolated from *R. succedanea* seeds and tested for inhibitory activities against a number of viruses namely respiratory viruses (influenza A, influenza B, respiratory syncytial, para-influenza type 3, adenovirus type 5), measles and herpes viruses (HSV-1, HSV-2, HCM, and VZV). The results indicated that compound **16** exhibited strong inhibitory effects against influenza A and influenza B viruses whereas compounds **15** and **21** had only an insignificant activity.

Compounds **16** and **21** had moderate anti-HSV-1 and anti-HSV-2 activities. Compound **19** inhibited influenza B measles and HSV-2 viruses, while compound **20** inhibited influenza B virus and VZV. It is also noteworthy that compound **21** has been reported in *R. retinorrhoea* leaves (Ahmed *et al*., 2001) and compound **15** in *R. semialata* leaves (Bagchi et al., 1985), suggesting that other *Rhus* species may contain antiviral constituents. The literature strongly suggests the potential for useful antimicrobial, antifungal, and antiviral agents obtained from *Rhus* extracts, but much work to date has been focused on one species and plant part, that is, the fruits of *R. coriaria,* given its use as a spice. This focus is understandable, but as with the other bioactive properties highlighted above, future efforts should include a worldwide survey of *Rhus* genus in order to determine if these properties are generalizable across the genera. Furthermore, since the bioactivities appear to be ascribed to polar compounds extractable with protic solvents, additional studies are required in order to determine whether these properties occur in extracts from other plant parts such as the stem barks, root barks, and the leaves, and what the optimum extraction and storage conditions are to obtain the highest quality yields of desired functionality.

Among the over 200 *Rhus* species, six are common in Kenya. These are *R. natalensis, R. longipes, R.quartinana, R. tenuinorvis, R. ruspolii and R. vulgaris* (Kokwaro 1993). In Tanzania, an extract of pounded *R. natalensis* roots steeped in hot or cold water is taken for influenza, abdominal pain and gonorrhea. The root decoction also forms part of a medicine for hookworm infestation. The leaves are used as a cough syrup; they are pounded and steeped in hot water and the patient drinks the extract. Sometimes the steam from the hot water leaf extract is inhaled to cure colds (Kokwaro, 1993). The stem bark methanol extract of *R. quartiniana* has antibacterial activity against gonorrhea and *Staphylococcus aureus* (Chhabra *et al.*, 1991).

Some of the compounds that have been isolated from the plant include anthocyanins, coumarins, essential oils, flavonoids, quinines, saponins, sterols and tannins. In Tanzania, dried stem bark is used to treat influenza (Chhabra *et al*., 1991). In Kenya and Tanzania, a decoction of boiled roots of *R. longipes* is used for the treatment of abdominal pain and indigestion. Also escaping vapour from boiled roots is inhaled as a treatment for influenza (Kokwaro, 1993). In East Africa, fruits of *R. vulgaris* are boiled in water and the extract is drunk to stop diarrhoea while stems are boiled and the extract applied to wounds. The roots mixed with other plants are known to make a viable drink for expectant mothers, which are believed to make delivery easy, while it is also used for the treatment of infertility (Kokwaro, 1993).

In China, the decoction of all parts of *R. chinensis* is used for treating *Diabetes mellitus*. A decoction from the seed is used for malaria and rheumatism management (Duke *et al*., 1985). In Israel, an infusion of dried fruit is used for the treatment of diarrhoea, aching gums, toothaches and swollen legs (Dafini *et al*., 1984), while in Turkey the leaf extract is used for treating animal bites. The leaves are boiled in water and used as part of medicine for peptic ulcers, kidney stones, eruptions and bruises (Yeslanda *et al.*, 1995). *R. glabra* is traditionally used by the indigenous people of North America for the treatment of bacterial diseases such as syphilis, gonorrhea, dysentery, and gangrene (Erichsen-Brown, 1989). *R. coriaria,* which grows wildly in the region of the Canary Islands over the Mediterranean region to Iran and Afghanistan, is commonly used as a spice by grinding the dried fruits with salt, and is also widely used as a medicinal herb in the Mediterranean and Middle East, particularly for wound healing (Sezik *et al*., 1991). Among the six species of the *Rhus* genus commonly found in Kenya, there is not much phytochemical studies that have been done.

Since these plants have been used traditionally to cure certain ailments, there exists a possibility of finding new compounds responsible with the relevant pharmacological properties.

2.3 Bioactivity of *Rhus* Extracts

Rhus extracts have been shown to exhibit a wide range of biological activities, as summarized in Table 2 in relation to antibacterial, antifungal, and antiviral activities. *Rhus* extracts are most notable for their antibacterial activities, although not much information is available on their antifungal and antiviral activities. As part of a screening of over 100 medicinal plants in British Columbia (Canada), crude methanolic extracts of *R. glabra* leaves exhibited both the widest zones of inhibition in a disc assay, and the broadest spectrum of inhibition was reflected against some of the following bacterial species tested: *Bacillus subtilis, Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Mycobacter phlei, Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus, and Salmonella typhi* (Saxena *et al.,* 1994). Similarly, in a follow-up study, the same methanolic extracts inhibited the growth of the following fungal strains: *Aspergillus flavus, A. fumigatus, Candida albicans, Fusarium tricuictum, Microsporum cookerii, M. gypseum, Saccharomyces cerevisiae, Trichoderma viridae, and Trichophyton mentagrophytes* (McCutcheon *et al.,* 1994). To the best of our knowledge, work on antiviral activities of *Rhus* extracts has focused on biflavonoids isolated from the seed kernels of *R. succedanea* (Lin *et al.*, 1999) as alluded to earlier in this review.

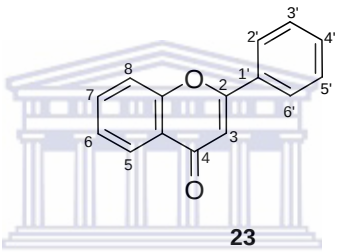
Table 2. Reported biological activities of compounds and fractions extracted from *Rhus*

Biological activity	**Species**	**Plant part**	**Compound(s) and/or extract type**	**References**
Antimicrobial	*R. retinorrhoea* *R. glabra* *R. coriaria*	Leaves Stem Seeds	(a)Ethanol and methanol extracts (b) Water extract	Nasar-Abbas *et al,.* 2004. Ahmed *et al*., 2001
Antifungal	*R. glabra*	Stem bark	Methanol extract	Towers *et al*., 1994
Antiviral	*R. succedanea*	Seeds	Robustaflavone (**16**) Amentoflavone (**21**) Volkensiflavone (**15**) Succedaneaflavone (**20**)	Lin *et al*., 1999
Cytotoxic	*R. verniciflua*	Stem bark	Ethanol extract	Kitts *et al*., 2001
Antimalarial	*R. retinorrhoea*	Leaves Seeds	Methanol extract Ethanol extract	Ahmed *et al*., 2001
Antioxidant	*R. coriaria*	Leaves	Methanol extract	Ozcan., 2003
Anti-inflammatory	*R. undulate*	Root bark	Dimethyl ether	Fourie *et al*., 1984

Biological activity	**Species**	**Plant part**	**Compound(s) and/or extract type**	**References**
Antifibrogenic	*R. verniciflua*	Stem bark	Butein	Lee *et al*., 2003
Antimutagenic	*R. verniciflua*	Heartwood stem bark	Garbanzol, sulfuretin, fisetin, fustin, and mollisacasidin	Park *et al.,* 2004
Antithrombin	*R. verniciflua*	Stem bark	6-pentadecylsalicylic acid	Kuo *et al*., 1991
Antitumorigenic	*R. verniciflua*	Branches and leaves	Ethanol extract fractionated on Sephadex G-150	Lee *et al.,* 2004
Hypoglycaemic	*R. coriaria*	Fruits	Methanol extract further fractionated with ethyl acetate and hexane	Giancarlo *et al*., 2006
Leukopenic	*R. vernificera*	Plant sap	Polysaccharide extracts	Yang *et al*., 2003

2.4 Flavonoids

Flavonoids may be classified into three main categories, namely: flavonoids, isoflavonoids, and neoflavonoids. Flavonoids (both flavonols and flavanols) are most commonly known for their antioxidant activity. They are the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants (Spencer, 2008). Both flavonols and flavanols have shown evidence of health-modulating effects in animals which consume them. These are compounds for whose basic skeleton is a 2-phenyl-4-benzopyrone (**23**), a flavone.



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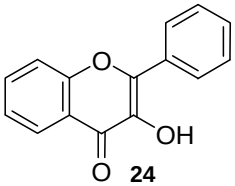
Flavones are the most abundant of the naturally occurring oxygen containing heterocyclic compounds. They form the important group of yellow natural pigments; other closely related compounds are the 3-hydroxyflavone (**24**), a flavonol, 2, 3-dihydroxyflavanone (**25**), a flavanone and a flavylium ion (**26**) for anthocyanins which often occur together with flavones in the same plant.



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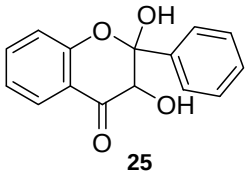

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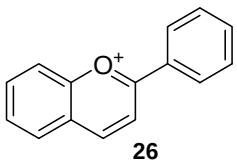

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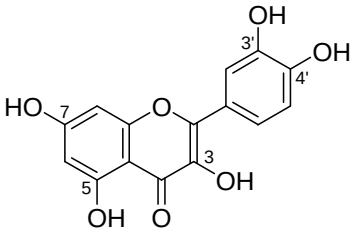
A collective term for all these groups of compounds are flavonoids. They occur in higher plants as well as in ferns. They carry hydroxyl groups which are usually located at positions 3, 5, 7, 3' and 4', for example, quercetin (**27**). There is also a high tendency for flavonoids to occur as glycosides (sugar containing molecules) e.g.Kaempherol-7-Rhamnoside (**28**).



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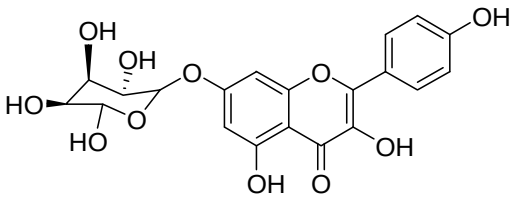

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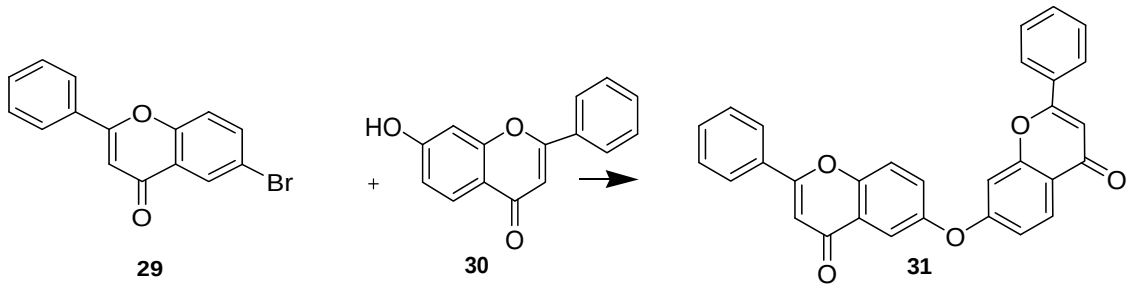
2.4.1 Biflavonoids

Biflavonoids are dimers of monomeric flavonoids. Biological activity of many biflavonoids isolated from plants has been found to be largely of an anti-inflammatory nature both *in vivo* and *in vitro*. The mechanism for their anti-inflammatory action includes inhibition of mast cell histamine release (Amella, *et al.,* 1985) and lymphocyte proliferation (Lee *et al*., 1995).

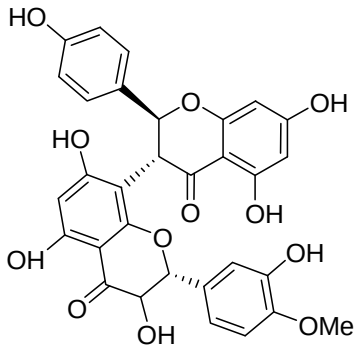
Recently, several C-C biflavones were chemically synthesized and some have shown anti inflammatory activity.

To extend these findings, structurally optimized derivatives such as the C-*O*-C biflavone (**31**) having a 6-*O*-7''linkage have been prepared. Such biflavones are synthesized via intermolecular coupling of 6-bromoflavone (**29**) analogs and 7-hydroxyflavone (**30**) analogs as key intermediates.



Scheme1. Formation of 6-*O*-7'' linkages

There also exist other forms of biflavonoids where the coupling of the monomers occurs through the 3 and 8'' positions. The 3, 8''-biflavonoids were mainly isolated from *Garcinia, Rheedia*, and *Allanblackia* species (Clusiaceae), and their remarkable pharmacological activities, such as antihepatotoxic (Iwu *et al*., 1987) and bactericidal properties, have been intensely studied and reported. Furthermore, manniflavanone (**32**) was patented for the treatment of diseases resulting from disorders of vascular permeability and fragility, and for the prevention of complications of diabetes mellitus (Offner *et al*., 1981). This type of secondary metabolite also exhibits very promising analgesic properties, which have to be further investigated (Bittar *et al*., 2000). This class of biflavonoids consists of a flavanone and 3-hydroxyflavanone units and are represented by compound **32** and kolaflavanone (**33**), respectively. Their structures differ only in the substitution pattern of the B rings.



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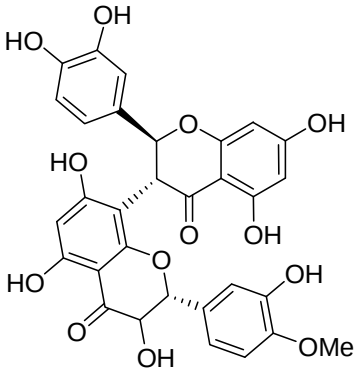

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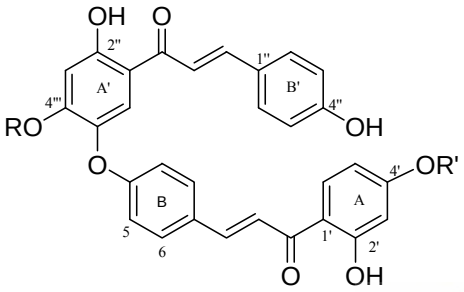
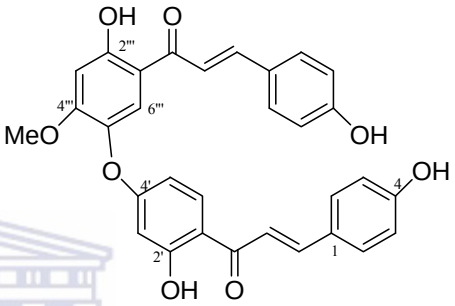
A great number of biflavonoids reported in the most recent literature are rated for great activity against a range of pathologically significant viruses (Lin *et al*., 1999). For example hinokiflavone (**17**) amongst other natural flavonoids inhibits the pro-coagulant activity of adherent human monocytes stimulated by endotoxin and interleukin-1 *in vitro* (Lale *et al*., 1996) while others have shown cytotoxicity and antimalarial activities (Ahmed *et al*., 2001). Another class of compounds found in the genus *Rhus* is the bichalcones. For example *R. pyroides,* a shrub that grows to a medium-sized tree and is widely distributed in the eastern parts of Botswana had, its twigs investigated, where isolation and characterization of new bichalcones was achieved and given the name rhuschalcone (Masesane *et al*., 2000).

In a recent report, five new bichalcones namely: rhuschalcone I (**34**), rhuschalcone II (**35**), rhuschalcone III (**36**), rhuschalcone IV (**37**), and, rhuschalcone V (**38**) have been recorded. All of their structures were determined by spectroscopic and chemical methods. Upon testing, they exhibited selective cytotoxicity against some colon tumor cells.



34 R=R'=Me
35 R=R'=H
36 R=Me,R'=H



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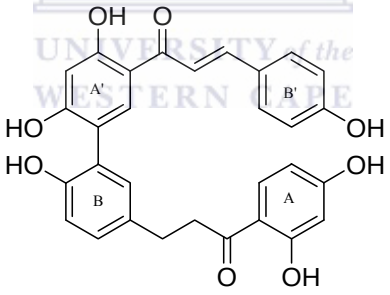

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2.4.2 Biological activity

Flavonoids are widely distributed in plants fulfilling many functions including producing yellow or red/blue pigmentation in flowers and protection from attack by microbes and insects. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant compounds (for instance alkaloids) suggest that many animals, including humans, ingest significant quantities in their diet.

Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens. They show anti-allergic, anti-inflammatory (Yamamoto *et al*., 2004), anti-microbial (Cushnie *et al*., 2005) and anti-cancer activity. Consumers and food manufacturers have become interested in flavonoids for their medicinal properties, especially their potential role in the prevention of cancer and cardiovascular diseases. The beneficial effects of fruits, vegetables, and tea or even red wine have been attributed to flavonoid compounds rather than to known nutrients and vitamins (Félicien, 2008).

2.4.3 Health benefits besides antioxidant properties

In recent times, research conducted on Free Radical Biology and Medicine indicates that inside the human body, flavonoids themselves are of little or no direct antioxidant value (Lotito, 2006). Body conditions prove to be unlike controlled test tube conditions, and the flavonoids are poorly absorbed (less than 5%), with most of what is absorbed being quickly metabolized and excreted. The huge increase in antioxidant capacity of blood seen after the consumption of flavonoid-rich foods is not caused directly by the flavonoids themselves, but most likely is due to increased uric acid levels. An interesting quotation from David, (2007) reads as follows: - "we can now follow the activity of flavonoids in the body, and one thing that is clear is that the body sees them as foreign compounds and is trying to get rid of them."

The process of gearing up the body to get rid of unwanted compounds induces the formation of the so-called Phase II metabolic enzymes that also help eliminate mutagens and carcinogens, and therefore may be of value in cancer prevention. Flavonoids can induce mechanisms that help kill cancer cells and inhibit tumor invasion (Ladislaus, *et al*., 2003).

Cancer researchers have found that human subjects who ate foods containing certain flavonoids seemed to be protected from developing lung cancer (David, 2007). Some of the flavonoids that have appeared to be the most protective include quercetin (**27**) and kaempferol (**28**). Researchers have indicated that only small amounts of flavonoids are necessary to see these medical benefits. Taking large dietary supplements provides no extra benefit and may pose some risks.

CHAPTER 3

MATERIALS AND METHODS

3.1 Reagents, equipment and materials

3.1.1 Reagents

Sulphuric acid, acetic acid, *p*-anisaldehyde, vanillin were bought from Kobian chemicals, Nairobi Kenya; Solvents: Dichloromethane, *n*-hexane, ethyl acetate, chloroform, petroleum ether, acetone, toluene and methanol bought from Kobian chemicals, Nairobi Kenya. Analytical grade or double distilled solvents were bought from Fluka AG Switzerland. Deuteriated solvent MeOH used for spectroscopic analysis was bought from Sigma-Aldrich South Africa.

3.1.2 Equipment

3.1.2.1 Solvent evaporation

Solvent evaporation was performed on a Buchi Rotavapor RE 111 with a water bath at 40^0C and 14 mbar.

3.1.2.2 Chromatography

3.1.2.2.1 Thin layer chromatography (TLC)

Analytical thin layer chromatography was performed on both aluminium and plastic pre-coated plates of silica gel 60 F_{254} with a 0.2 mm layer thickness. Visualization of the TLC spots was achieved under UV light at 254nm or 366nm and by spraying with the *p*-anisaldehyde reagent prepared by mixing 0.5ml anisaldehyde mixed with 10ml glacial acetic acid, followed by 85ml of chilled methanol and 5ml of 98% sulphuric acid (Randerath *et al*., 1968).

Preparative thin layer chromatography (PTLC) was performed using normal phase silica gel 60 F_{254} pre-coated on glass plates (20 x 20), with varying thickness (0.25, 0.5, 1.0, or 2.0 mm). Detection was done under UV light at 256 or 366 nm.

3.1.2.2.2 Column chromatography (CC)

Glass columns (20-25 mm diameter) wet packed with silica gel 60 (0.040-0.063mm) (230-400 Mesh ASTM, Merck) were used for column chromatography. Size-exclusion chromatography was carried out using Sephadex® LH-20 (Pharmacia), eluting with either MeOH or MeOH-$CHCl_3$.

3.1.3 Spectroscopy

3.1.3.1 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were measured on Varian-600 NMR machine based at the Department of Chemistry at the University of the Stellenbosch, South Africa and a Bruker-300NMR at the University of Botswana. Deuteriated methanol (CD_3OD) was used as the solvent. Chemical shifts were recorded in δ (ppm).

3.2 Plant material

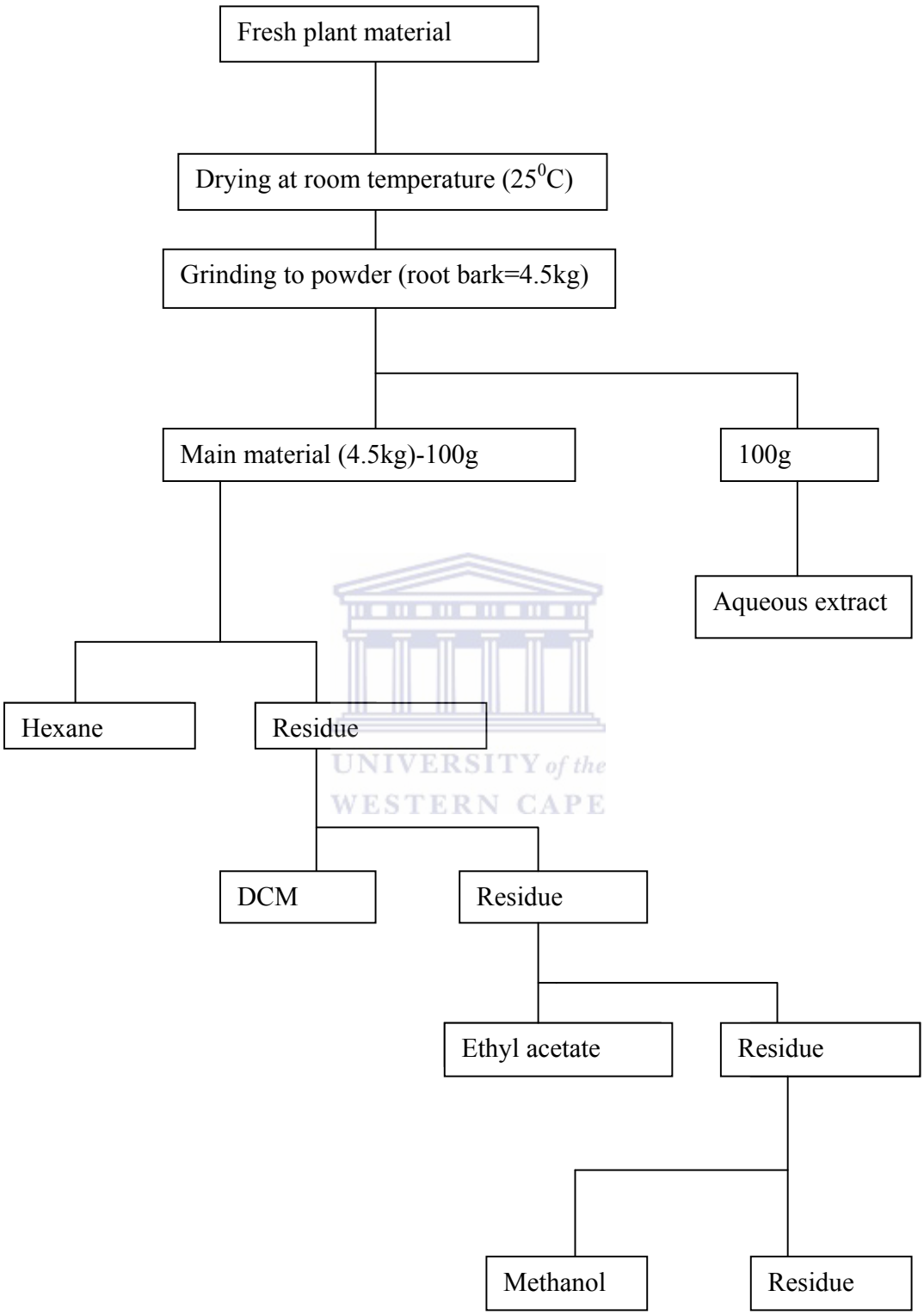
3.2.1 Plant samples collection and identification

Plant material was collected in Kenya, near the Thika river within Gatanga division in June 2008. The three plant parts namely; root bark, stem bark and leaves were dried for a period of three months, stored under controlled conditions in order to minimize undue chemical changes. The material was ground into a powder prior to extraction.

The plant was taxonomically identified by Mr. Simon Mathenge of the Department of Botany, University of Nairobi, Kenya. The voucher specimen (HM 2008/01) was deposited at the Department of Botany Herbarium, University of Nairobi, Kenya.

3.2.2 Extraction

The powdered material was extracted sequentially with solvents of increasing polarity starting with hexane (C_6H_{14}), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and finally methanol (MeOH). Each extraction involved maceration for a period of about 48 hours, with three repetitions for each solvent except for methanol which was done twice. Occasional swirling was done to ensure thorough extraction. The extracts were decanted and filtered using Whatman filter paper. The extracts were concentrated under reduced pressure using a rotary evaporator at temperatures less than50 ^{o}C. Separation and purification of the plant constituents was carried out using one or the other of a combination of chromatographic techniques.



Scheme 2. A flow chart for the preparation of the plant extracts

3.3 Antibacterial and anti-fungal screening tests

Bioassay of the crude extracts against test organisms was carried out using the agar diffusion technique. Micro-organisms used in these tests were obtained from the National Public Health Laboratories (NPHL). These included local and standard clinical isolates. The micro-organisms used included Gram positive bacteria, *Staphylococcus aureas* (ATCC 25923), *Escherichia coli* (ATCC 25922) Gram negative bacteria, *Pseudomonas aureginosa* (clinical isolate). The fungi used *Cryptococcus neoformans* (clinical isolate), *Trichophyton mentagrophytes* (clinical isolate), *Penicillium spp* (environmental isolate) and *Candida albicans*. These test strains of bacteria were kept refrigerated at 4^{o}C in Muller-Hutton agar slants during the storage period. They were then tested biochemically for purity before use (Elgayyar *et al.,* 2000). A colony of each bacterial strain was put in some agar slants and incubated for 18 hours at normal body temperature.

Sterile Petri-dishes containing about 20ml of sterile medium of yeast, malt extracts and glucose agar for fungi and nutrient broth agar for bacteria were inoculated with an 18 hour culture of the appropriate test organism. After 6 hours, a diluted sub-culture was prepared with a similar broth. The plant extract was then dissolved in dimethylsulphoxide (DMSO) and 20µl were loaded onto a 6mm filter paper. A standard antibiotic, chloramphenicol 2mg and fluconazole 2mg for the fungi were used as controls. The various Petri-dishes were then inoculated with 0.1ml of the diluted bacteria or fungi culture directly from the 18 hour broth. The inoculations were then spread using a sterile glass rod to avoid contamination and allowed to stand for 2 – 3 minutes in a refrigerator which was set at 4 ^{o}C. Discs loaded with the plant extracts were then placed onto the seeded plates. All the Petri-dishes were then incubated at 37 ^{o}C for 24 hours for the bacterial pathogens and 3 days for the fungal pathogens.

After that period of incubation, antibacterial or antifungal activity was assessed by measuring the diameter of all the zones of inhibition which were measured in millimeters, taking triplicate values of each by measuring in different directions and then calculating the average, using a standard laboratory ruler (Elgayyar *et al.,* 2000).

3.4 Isolation of the pure compounds

3.4.1 Fractionation of the root hexane extract

The hexane extract (10.6g) was adsorbed on silica gel and fractionated on a column by gravity elution using the following solvent systems; 500ml of 100% hexane, then 250ml volumes of mixtures with DCM at the following ratios (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90) and finally 100%DCM. This was further followed by 250 ml volumes of DCM-EtOAc mixtures using similar ratios until 100% EtOAc. Finally small portions of MeOH were introduced in the mixture with EtOAc up to 15% MeOH (98.5:2.5), (95:5), (92.5:7.5), (90:10), (95:15). Fractions collected were analyzed by TLC using hexane-EtOAc (7:3). Fractions with similar Rf values were pooled together. The combined fraction from vials 26-29 (400mg) was loaded onto a sephadex column for further separation. DCM-MeOH (50:50) 500ml was used as eluent leading to the isolation of compound **41** as a clear liquid (171mg).

3.4.2 Fractionation of the EtOAc extract of the root

The EtOAc extract (69.1g) was adsorbed on silica gel and fractionated on a column by gravity elution using the following solvent system; 250ml of 100% hexane, then 250ml volumes of mixtures with DCM at the following ratios (90:10), (80:20), (70:30), (60:40), (50:50), (40:60), (30:70), (20:80), (10:90) and finally 100%DCM. This was further followed by 500 ml volumes of DCM-EtOAc mixtures using similar ratios until 100% EtOAc. Finally, small portions of MeOH were introduced at 5% intervals in the mixture with EtOAc of up to 50% MeOH; (95:5), (90:10), (85:15), (80:20), (75:25), (70:30), (65:35), (60:40), (50:50).

Fractions collected were subjected to TLC analysis using DCM-MeOH (9.5:0.5) ratio. Pooling of fractions with similar TLC profiles was done. The combined fraction from vials 39-51 (800mg) upon drying was chromatographed on Sephadex LH-20 using the DCM-MeOH (50:50) ratio. Fractions collected were analyzed by TLC in DCM-MeOH (9:1). Preparative TLC using DCM-MeOH (9:1) gave compounds E2 (10.5mg) and **40** (14.9mg). Re-crystallization and subjecting other portions to sephadex column chromatography using DCM-MeOH (50:50), and further analysis of the fractions by TLC using DCM-MeOH (8:2) gave a yellow powder (321mg). This was further purified by preparative TLC using DCM-MeOH (8:2) ratio. Compounds E3 (11.2mg) and **39** (13.5mg) were obtained as yellow products. Pooling of combined fractions from vials 72-78 (400mg) and subjecting the collection to sephadex column chromatography using DCM-MeOH (7:3) as eluent; compounds E5 (12.9mg), a crystalline yellow powder and E6 (9.9mg), deep yellow crystals, were obtained.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Crude plant extracts

The finely ground *R. natalensis* leaves, stem and root barks were available as shown in table 3. Only the root bark and stem bark materials that were subjected to sequential extraction with the solvents hexane, DCM, EtOAc and finally MeOH.

Table 3. Initial weights of the plant collection.

Part of the plant	Weight in Kgs
Root bark	4.5
Stem bark	4.0
Leaves	2.0

Some of the crude concentrated extracts deposited crystals on standing while others remained in the form of a paste. The yields obtained are presented in table 4. About 1g of each of these crude extracts was set aside for use in bioassays.

Table 4. Summary data for the crude yields.

Part of plant extracted	Hexane	DCM	Ethyl acetate	Methanol
Root bark	10.6g	13.9g	69.1g	85.2g
Stem bark	9.6g	16.6g	32.5g	59.6g

4.2 Bioassays

4.2.1 Bioassay of the crude extracts

Bioassay of the crude extracts against selected test organisms was carried out as described previously. The data obtained was used to determine MIC breakpoints. The antibacterial and antifungal activities of the extracts in terms of diameters of inhibition zones were reported as in Table 5

Table 5. Inhibition zones of micro-organisms.

EXTRACTS/ CONC 0.5mg/ml	**TEST MICROORGANISMS/INHIBITION ZONE IN MM**						
	Bacteria			**Fungi**			
	Sau	*Eco*	*Pseudo*	*Penici*	*TM*	*Can*	*Crypto*
1	7	6	9	7	6	6	0
2	18(0.5)	6	9	6	6	6	7
3	15(0.03)	7	15(0.03)	7	8	8	7
4	17(0.015)	6	14(0.015)	10(0.5)	10(0.5)	7	7
5	6	7	15(0.03)	11(0.5)	7	7	6
6	14(0.25)	6	10(0.5)	8	6	6	6
7	7	7	13(0.25)	8	8	7	7
8	6	6	12	6	6	7	6
9	20	18	17	-	-	-	-
10	-	-	-	18	19	15	16

Each value is the mean of three replications ± standard deviation in bracket measured in 6mm disc diameter.

KEY:

1. Root bark DCM
2. Root bark hexane
3. Stem bark EtOAC
4. Stem bark MeOH
5. Root bark EtOAC
6. Stem bark DCM
7. Root bark MeOH
8. Stem bark Hexane
9. Chloramphenicol standard
10. Fluconazole standard

Test Micro-organisms

- Sau-*Staphylococcus aureus* ATCC 25923
- E. coli-*Escherichia coli* ATCC 25922
- Pseudo- *Pseudomonas aureginosa* clinical isolate
- Can- *Candida albicans* ATCC 90028
- Crypto-*Cryptococcus neoformans* clinical isolate
- Penici-*Penicillium spp* environmental isolate
- TM-*Trichophyton mentagrophytes* clinical isolate

The method of testing the efficacy of the extracts was done by introducing a sample into the middle of a bacteria-laden or fungi Petri-dish. Standards were also used in order to compare the results. A clear zone indicated bactericidal activity. The larger the diameter of the zone of inhibition, the higher the efficacy of that extract. The average data was determined and compared to the positive controls used. The inhibition zones against microbial growth are presented in Table 5. The three bacterial test organisms used showed a wide range of activity; *P. aureginosa* (gram negative bacterium) was the most sensitive to all the extracts. *S. aureus* (gram positive bacterium) that plays a considerable role in skin infections was highly sensitive to the root hexane extract with an inhibition zone of 18mm. It showed low sensitivity to the stem hexane extract, an indicator that the roots had greater potency. *E. coli* showed intermediate resistance to all the extracts in comparison to the standard chloramphenicol. Values greater than 10mm could be considered as reflecting moderate activity. However the differences in susceptibility between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between these classes of bacteria. The Gram-negative bacteria cell wall outer membrane appears to act as a barrier to many substances including antibiotics (Tortora *et al*., 2001).

The choice of the fungus *C. albicans* was made due to the serious systemic infections, it causes including the opportunistic infections that are common with HIV positive patients. These various crude extracts demonstrated limited antifungal activity. The activity on fungi was much lower than that on bacteria. There was no activity recorded at all for the root DCM extract against *C. neoformans*. Moderate activities of the extracts against the fungi were recorded for *Penicillium ssp* at 11mm (0.5) and 10mm (0.5) as well as *T. mentagrophytes* at 10mm (0.5) which were clinical isolates. *C albicans* and *C. neoformans* displayed intermediate resistance.

However, the antifungal activity was far from comparable (as determined by the zones of inhibition) to that of fluconazole used as the standard. The observed biological activities could be due to the flavonoids present in the plant under study, as these compound types have been known to posses antibacterial and antifungal activity. Traditionally this plant is used in the treatment of oral candidiasis and athlete's foot. The results show that the methanol and ethyl acetate extracts have a greater potential as a source of antibacterial or antifungal agents of natural origin. Preliminary phytochemical studies have shown that the aerial and root parts of the genus *Rhus* contain biflavonoids responsible for these antimicrobial activities.

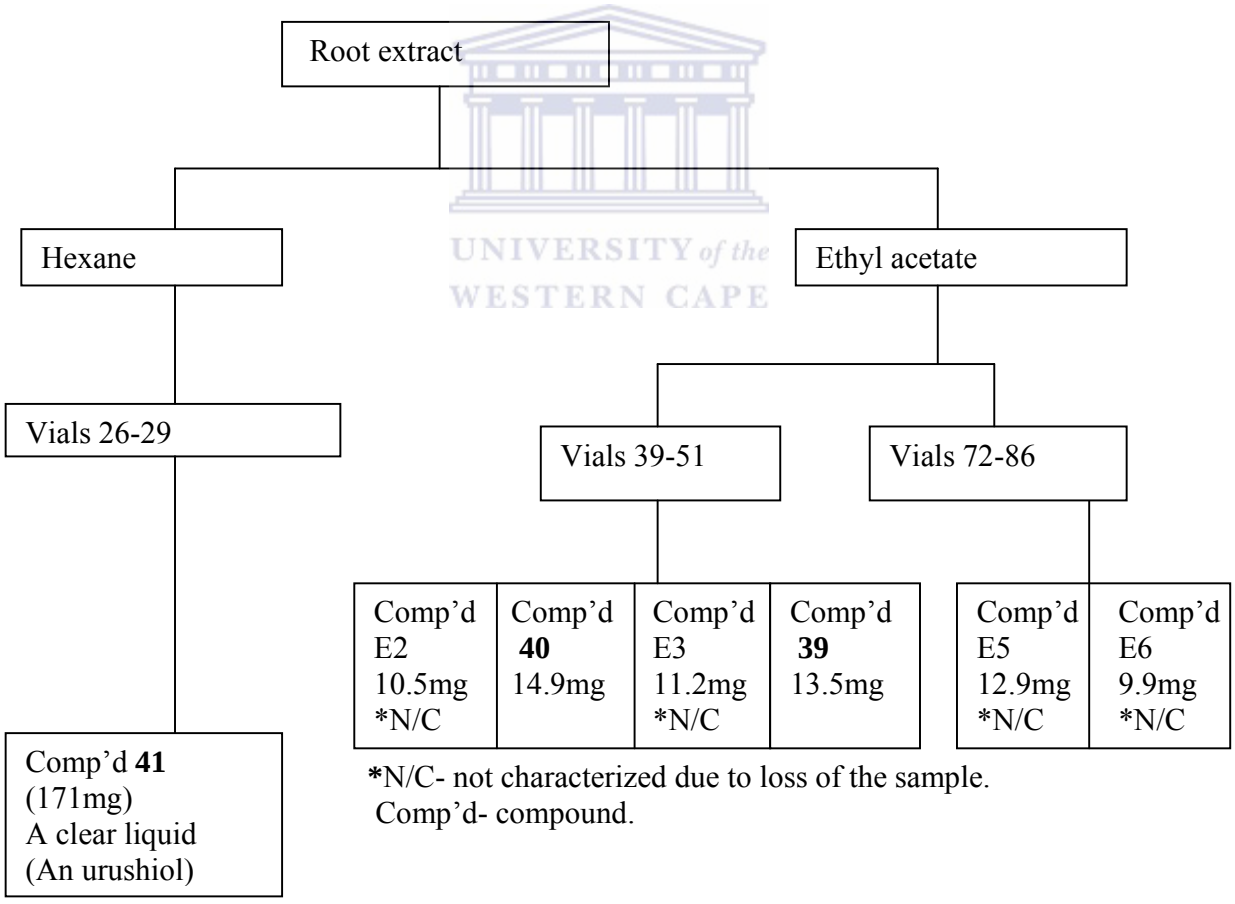
Table 6. Antimicrobial activity of isolated compounds

Name of extracts	Wt	Volume of DMSO used	Amount /disc	Antibacterial assay			Antifungal Assay		
				Sau	Eco	Pseudo	Can	TM	Mg
HM/RN/H/1(**41**)	5mg	400µl	20µl	9	0	0	0	0	0
HM/RN/E/2a(**40**)	5mg	400µl	20µl	17	0	0	0	0	0
HM/RN/E/4 (**39**)	5mg	400µl	20µl	21	0	0	0	0	0
Chloramphenicol	2mg	400µl	20µl	20	18	17	-	-	-
Fluconazole	2mg	400µl	20µl	-	-	-	15	12	14

KEY:

1. Sau-*Staphylococcus aureus* ATCC 25923
2. Eco-*Escherichia coli* ATCC 25922
3. Pseudo-*Pseudomonas aureginosa* clinical isolate
4. Can-*Candida albicans* ATCC90028
5. Tm-*Trichophyton mentagrophytes* clinical isolate
6. Mg-*Microsporum gyseum* clinical isolate

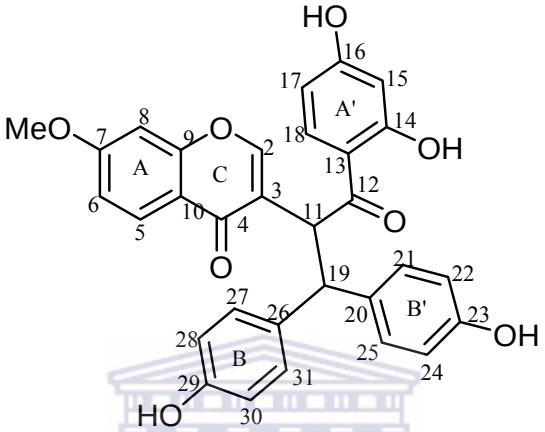
The results in table 6 showed that the isolated compounds expressed some level of activity against the bacterium *S.aureus* in comparison to the chloramphenicol. Compound **39** showed a more pronounced zone of inhibition (21mm) against *S. aureus*. The compounds did not register any activity with *E. coli* and *P. aureginosa.* No activity on the fungi was recorded for the *C. albicans*, *T. mentagrophytes* or *M. gyseum* as compared to fluconazole standard. Comparing these results with those of the crude extracts may suggest that the potency of individual compounds is compromised by their isolation or that they were not used at sufficient concentrations. On the other hand, it is possible that the more potent compounds evaded the isolation process.



Scheme 3. A flow diagram for the compounds extracted.

4.3. **3-(1-(2,4-dihydroxyphenyl)-3,3-bis(4-hydroxyphenyl)-1-oxopropan-2-yl)-7-methoxy-4H-chromen-4-one (39)**

Compound (**39)**, was obtained as an amorphous yellow powder, revealing a pseudo molecular ion $[M+H]^+$ at m/z 524, corresponding to the molecular formula $C_{31}H_{24}O_8$.



39

The structure was established on the basis of 1D (^{1}H, ^{13}C and DEPT) and 2D-NMR experiments (COSY, NOESY, HSQC and HMBC) (Table 7, Appendix 1a-k). The ^{1}H NMR spectrum assigned with the aid of the COSY experiment enabled the distinction of two 1, 4-disubstituted aromatic rings that were identical (B, B') and two 1, 2, 4-trisubstituted (A, A') aromatic rings. Long range interaction of two *meta*-protons H-6 (δ 6.44, *dd, J*= 2.1, 2.1Hz) and H-8 (δ 6.28, *d*, *J*= 2.1Hz) as well as H-15 (δ 6.69, *d*, *J*=2.1Hz) and H-17 (δ 6.83, *dd*, *J*=2.1, 2.1Hz) were confirmed by COSY. A typical singlet for proton H- 2 of the isoflavone moiety was observed at δ 8.20, (*s*) and an AB system with a bridge between H-11 (δ 6.02, *d*, *J*=12Hz) and H-19 (δ 4.67, *d*, *J*= 12.0 Hz) were detected. There was long range coupling between the methine proton at H-19 (δ 4.67, C- δ 54.5) and H-21/H-25 (δ 7.15, C-129.9) of the two aromatic identical rings B and B' which are interchangeable, hence the rings are related to that methine group by ^3J (**figure 1a**). The upfield part of the ^{1}H NMR spectrum showed one methoxy resonance at δ3.76.

The location of this methoxy substituent in the A- ring was deduced on the basis of NOESY enhancement between proton H-6 (δ 6.4, *dd*, *J*=2.4, 2.4Hz) and H-8 (δ 6.28, *d*, *J*=2.4Hz). The methoxy group showed through-space interaction with two protons only, and these were meta-coupled to each other (**figure 1b**). Proton H-6 (δ 6.44, *dd*, *J*=2.4, 2.4Hz) showed a NOESY relationship with the proton H-5 (δ 8.21, *d*, *J*=3.9Hz). Another important NOESY was observed between H-2 (δ 8.20, *s*) and H-11 (δ 6.02, *d*, *J*= 12.0Hz) then H-19 (δ 4.67, *d*, *J*= 12Hz) with H-21/25 (δ 7.15, *d*, *J*= 9.0Hz). Proton H-21/25 (δ 7.15, *d*, *J*= 9.0Hz) with H22/24 (δ 6.57, *d*, *J*= 8.4Hz). Aromatic protons were observed at; δ 8.21 (*d*, *J*=3.9Hz, H-5), δ 6.44 (*dd*, *J*=2.4, 2.4Hz, H-6) and δ 6.28 (*d*, *J*=2.4Hz, H-8). The 2, 4-dihydroxyphenyl signals in ring A' were observed at δ 6.69 (*d*, *J*=2.1Hz, H-15), δ 6.83 (*dd*, *J*=2.1, 2.1Hz, H-17), and δ 7.87 (*d*, *J*=8.7 Hz, H-18). The protons H-11 and H-19 were found to be in a trans-conformation due to their *J* value of 12.0 Hz (Ghogomu *et al* 1987). The assignment of H-11 was confirmed from HMBC's with C-2 (δ 157.5), C-3 (δ 122.6), C-4(δ 177.2), C-12 (δ 205.4), and C-13(δ 135.9). Similarly H-19 was confirmed from HMBC's with C-11(δ 45.1), C-12(δ 205.4), and C-21/25 (δ 129.9).

^{13}C NMR spectrum showed the typical methine signals for C-11, C-19 at δ 45.1 and δ 54.5 respectively and this was confirmed by HMQC. There were two carbonyl signals at δ205.4 and δ 177.0. Other carbons bearing oxygen atoms appeared between δ 156.8 to δ 168.9. H-11 (δ 6.02, *d, J*= 12Hz) showed connectivity to the A, C isoflavone ring moiety. The B, B' identical rings were bound together to C-19 (δ 54.5). The carbonyl group at δ 205.4 was linked to ring A' and the methine at C-11. The ^{13}Cspectrum displayed signals for 31 carbons which were edited by DEPT into 1 methyl, 17 methines and 13 non-protonated carbons consisting of some two carbonyls and 9 oxygenated carbons. Detailed analysis of the HMBC and HMQC data enabled the complete assignment of the proton and carbon signals leading to the new structure **39**.

Table7. ^{1}H NMR and ^{13}C NMR data of compound 39 in CD_3OD at 300MHz

Position	^{1}H NMR (δ multiplicity, J)	^{13}C NMR (δ)	COSY	HMQC	HMBC
2	8.20 (*s*)	157.5		8.20	
3		122.6			
4		177.2			
5	8.21 (*d*, *J*= 3.9)	134.1	H-6	8.21	C-4, C-7, C-9
6	6.44 (*dd* , *J*= 2.1, 2.1)	108.6	H-5,8	6.44	C-8, C-10
7		166.9			
8	6.28 (*d*, *J*= 2.1)	101.9	H-6	6.28	C-6, C-7, C-10
9		159.5			
10		115.2			
11	6.02 (*d*, *J*= 12.0)	45.1	H-19	6.02	C-2, C-3, C-4, C-12, C-13
12		205.4			
13		135.9			
14		168.2			
15	6.69 (*d*, *J*=2.1)	103.4	H-17	6.69	C-14, C-16, C-17
16		164.9			
17	6.83 (*dd*, *J*=2.1,2.1)	116.7	H-15,18	6.83	C-13, C-15
18	7.87 (*d*, *J*=8.7)	128.3	H-17	7.87	C-14, C-16
19	4.67(*d*, *J*= 12)	54.5	H-11,21	4.67	C-11, C-12, C-21/C-25
20		134.9			
21	7.15 (*d*, *J*= 9.0)	129.9	H-22	7.15	C-19, C-23, C-25
22	6.57 (*d*, *J*= 8.4)	116.2	H-21	6.57	C-20, C-23
23		156.8			
24	6.57 (*d*, *J*= 8.4)	116.3			
25	7.15 (*d*, *J*= 9.0)	129.9			
26		134.9			
27	7.15 (*d*, *J*= 9.	130.6			
28	6.57 (*d*, *J*=8.4)	116.2			
29		156.9			
30	6.57 (*d*, *J*=8.4)	116.3			
31	7.15 (*d*, *J*= 9.0)	130.6			
-OCH_3	3.76 *s*	56.2		3.76	

B and B’ values may be reversed

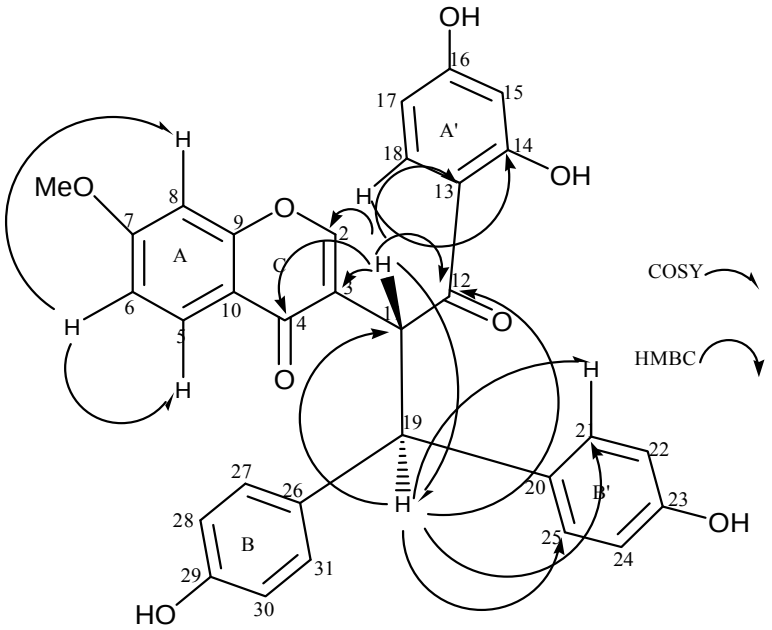


Figure 1a. Important HMBC's (full arrow) and COSY (half arrow) correlations for compound **39**

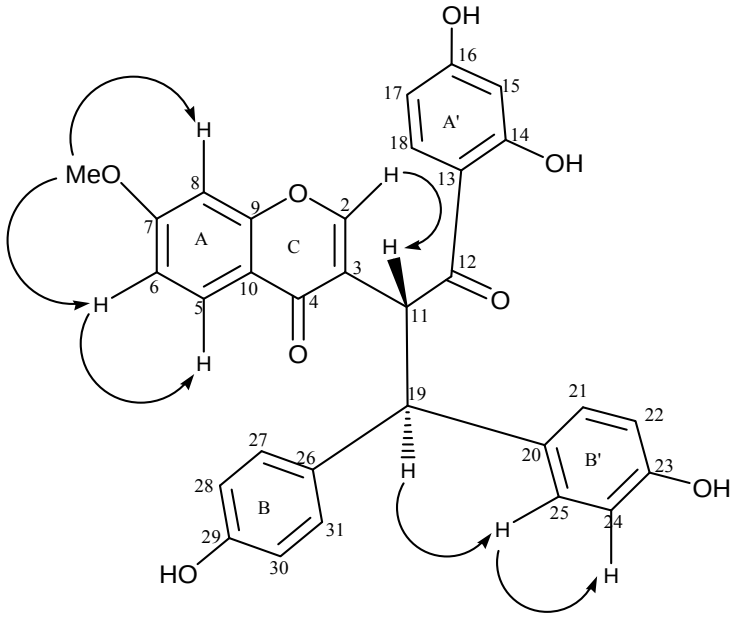
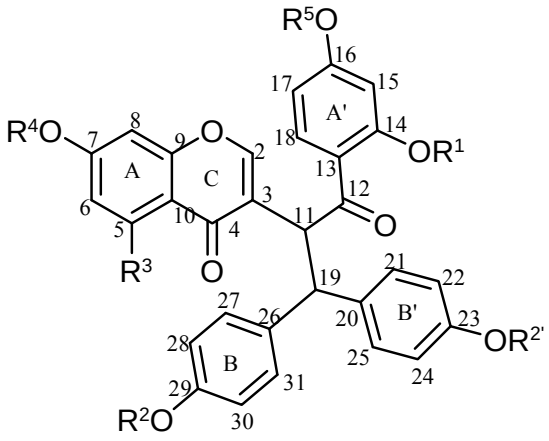


Figure 1b. Important NOESY correlations for compound **39**

Such types of biflavonoids are relatively rare in nature, and 3-(1-(2,4-dihydroxyphenyl)-3,3-bis(4-hydroxyphenyl)-1-oxopropan-2-yl)-7-methoxy-4H-chromen-4-one (Rhuschromone) is, to our knowledge, the first hydroxyphenyl chromen-4-one to be reported from *Rhus natalensis* and possibly within the entire *Rhus* genus. The other phenoxychromone to be isolated was chamaechromone (**42**) from the roots of *Stellera chamaejasme L* (Thymelaeaceae) (Masatake *et al*., 1984). Subsequently, R. Ghogomu *et al*., (1987) isolated Lophirone A (**43**), a phenoxychromone from the stem bark of *Lophira lanceolata* (Ochnaceae) and Calodenone (**44**) (Messanga, et al., 1992) isolated from the stem bark of *Ochna calodendron* (Ochnaceae). The reported biological activity of the latter did not include any significant antibacterial activity. On the contrary Rhuschromone was found to possess a relatively high activity as an antimicrobial zone of inhibition (21mm). It was speculated that the activity observed may be associated with the unique positioning of the 7-OCH_3 group in ring A. From a biogenetic point of view, Rhuschromone differs from known biflavonoids in having involved an aryl shift of ring B from one flavone unit to the next resulting to an isoflavone. The term 'isobiflavonoid' would best suit this class of compounds.



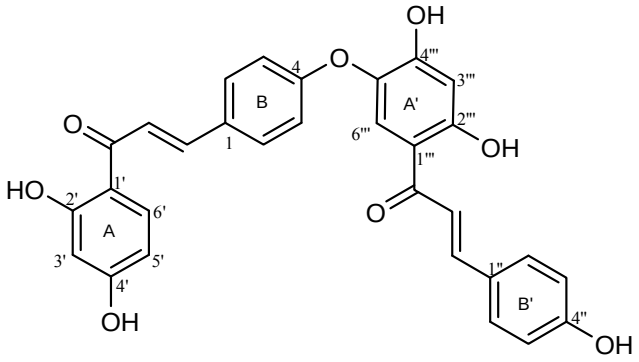
42 R^1=R^2=$R^{2'}$=R^3=OH,R^4=R^5=H

43 R^1=R^2=$R^{2'}$=R^3=R^4=R^5=H

44 R^1=CH_3,R^2=$R^{2'}$=R^3=R^4=R^5=H

4.4. **2', 4'-dihydroxychalcone-(4-*O*-5''')-4'',2''',4'''-trihydroxychalcone (40)**

Compound **40**, was obtained as a yellow solid. MS indicated an ion at m/z 510.1212 (M+, calculated 510.1314) consistent with a molecular formula of $C_{30}H_{22}O_8$.



40

The structure was established on the basis of 1D (^{1}H, ^{13}C and DEPT) and 2D-NMR experiments (COSY, NOESY, HSQC and HMBC) (Table 8, Appendix 2a-i). The ^{1}H NMR spectrum (300MHz, CD_3OD-d_6) showed signals of a three ABC proton spin system observed at H-3'(δ 6.31, *d, J*= 2.4 Hz) that had a long range meta-coupling with signal at H-5' (δ 6.42, *dd, J*=9.0,2.4Hz), and H-6' (δ 8.01,*d*, *J*=9.0 Hz) characteristic of a 1,2,4-trisubstituted benzene unit. There were two singlet signals integrating for one proton each observed at H-6''' (δ 7.94, *s*) and H-3''' (δ 6.61, *s*). Two pairs of the AA'BB'-type signals, where one pair was at H-2/6 (δ 7.75, *d, J*=8.41Hz) and H-3/5 (δ 6.96, *d, J*=8.8 Hz) and the other pair at H-2''/6'' (δ 7.67, *d, J*=8.4Hz) and H-3''/5'' (δ 6.87, *d, J*=8.7Hz), were observed for the two *para*-substituted phenolic moieties. The given data strongly suggested compound **40** to be a bichalcone. Signals of *trans*-alkene protons of the bichalcone system were not clearly observed due to weak resolution of some peaks. Multiplets at H-α (δ 7.57 *m*) and H-α' (δ 7.68 *m*) for hydrogen protons connected to α and α' carbons (δ_{CH} 118.3) could only be tentatively assigned.

The ^{13}C NMR spectrum of compound **40** displayed signals for 30 carbon atoms which were edited by DEPT into 17 methines and 13 non-protonated carbons amongst which were two carbonyls and seven oxygenated carbons. The HMBC spectrum showed the chemical shift of a carbonyl group whose carbon signal had ^{3}J interactions with the H-6''' singlet at (δ 7.94 *s*) and the H-β' signal at (δ 7.85 *d*, *J*=15.3 Hz) and this was assigned to with the upfield C=O' signal at δ_C 192.4. Likewise, the second H-β signal at (δ 7.83 *d*, *J*=15.3Hz) was correlated to the other C=O resonance at δ_C 192.0 (**figure 2a**). The presence of the two C=O at δ 192.0 and δ 192.4, allowed the proposal of the bichalcone structure (Table 8). NOESY irradiation gave some important information in establishing the inter-chalcone linkage in that the signals belonging to H-5' and H-6' of ring A of one of these chalcone moieties had no corresponding correlation with the signals for H-3''' and H-6''' of ring A' of the other. The linkage was determined to be at C-4 (δ 158.4) and C-5''' (δ 127.3) forming the C-4-*O*-5'''connection. The ^{13}C (DEPT), COSY and HMBC spectra results were in agreement with those of a chalcone reported on, to a great extent in the literature namely, Rhuschalcone II (Ladislaus *et al*., 2003) from the root bark of *Rhus pyroides*. The accompanying NMR data facilitated the proposal of a similar bichalcone as a constituent of the root bark of *Rhus natalensis*. The tentative structure of the chalcone was therefore deduced to be the 2', 4'-dihydroxychalcone-(4-*O*-5''')-4'',2''',4'''-trihydroxychalcone (**40**)

Table 8. ^{1}H NMR and ^{13}C NMR data of compound 40 in CD_3OD at 300MHz

Position	^{1}H NMR (δ multiplicity, J)	^{13}C NMR (δ)	COSY	HMQC	HMBC
1		129.9			
2	7.75 (*d*, *J*= 8.4)	128.8	H-3	7.75	C-β, C-3/5, C-4
3	6.96 (*d*, *J*= 8.7)	115.6	H-2	6.96	C-1, C-2/6, C-4
4		158.4			
5	6.96 (*d, J*= 8.7)	115.6	H-6	6.96	C-1, C-2/6, C-4
6	7.75 (*d*, *J*= 8.4)	130.6	H-5	7.75	C-β, C-3/5, C-4
1’		165.2			
2’		113.9			
3’	6.31 (*d*, *J*= 2.4)	102.4	H-5’	6.31	C-1’, C-2’, C-5’
4’		166.2			
5’	6.42 (*dd*, *J*= 9.0,2.4)	108.1	H-3’, H-6’	6.42	C-1’, C-3’
6’	8.01 (*d*, *J*= 9.0)	132.1	H-5’	8.01	C=O, C-2’, C-4’,
1’’		129.1			
2’’	7.67 (*d*, *J*= 8.4)	130.2	H-3’’	7.67	C-β’, C-3’’/5’’, C-4’’
3’’	6.87 (*d*, *J*= 8.7)	116.7	H-2’’	6.87	C-1’’, C-2’’/6’’
4’’		160.3			
5’’	6.87 (*d*, *J*= 8.7)	115.3	H-6’’	6.87	C-1’’, C-2’’/6’’
6’’	7.67 (*d*, *J*= 8.4)	128.0	H-5’’	7.67	C-β’, C-3’’/5’’C-4’’
1’’’		113.3			
2’’’		160.4			
3’’’	6.61 (*s*)	107.8		6.61	C-1’’’, C-5’’’
4’’’		157.9			
5’’’		127.3			
6’’’	7.94 (*s*)	126.4		7.94	C-2’’’, C-4’’’, C=O
α	7.57 (*m*)	118.3	H-β	7.57	C-1, C-β
β	7.83 (*d*, *J*=15.3)	143.6	H-α	7.83	C-1, C-α, C2/6, C=O
α’	7.68 (*m*)	118.3	H-β’	7.68	C-1’’, C-β’
β’	7.85 (*d*, *J*=15.3)	144.8	H-α’	7.85	C-α’, C=O’, C-2’’/6’’
C=O		192.0			
C=O’		192.4			

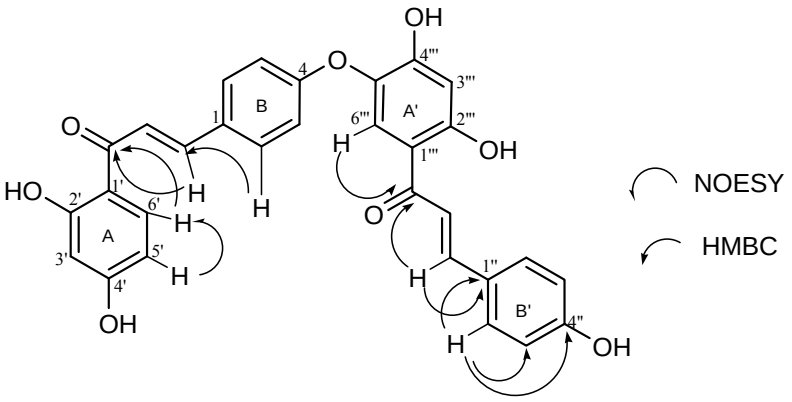


Figure 2a.Important HMBC correlations (full arrow) and NOESY (half arrow) for compound **40**

This is another biflavonoid that is of a relatively rare bichalcone class in nature, and is being reported for the first time as isolated from *Rhus natalensis*. On the part of the biological activity, 2', 4'-dihydroxychalcone-(4-*O*-5''')-4'',2''',4'''-trihydroxychalcone (**40**), exhibited significant antibacterial activity (Table 6). It showed relative activity as an antimicrobial, zone of inhibition (17mm) though much lower than the standard chloramphenicol on *Staphylococcus aureus*. Reported biological activity of rhuschalcone II was carried out for *in vitro* primary cytotoxic screening using human tumor cell lines (Ladislaus, *et al*., 2003). Varying degrees of cytotoxicity activity were recorded.

4.5. 3-((Z)-heptadec-13-enyl) benzene-1, 2-diol (41)

Compound **41,** was obtained as a clear liquid, revealing a pseudo molecular ion $[M+H]^+$ at m/z 347.2945, corresponding to the molecular formula $C_{23}H_{38}O_2$ (calculated 347.2945).

41

The structure was established on the basis of 1D (^{1}H, ^{13}C and DEPT) and 2D-NMR experiments (COSY and HMBC) (Table 9, Appendix 3a-h). The ^{1}H and ^{13}C NMR showed the typical pattern of a 5-alkenyl substituted catechol with a linear 17-carbon side chain containing one double bond at δ 5.33 (H-13'/H-14'), δ130.8 and 130.2 (C-13'/ C-14'), 14 methylene groups between δ_C 23.7 and δ_C 36.9 and one methyl group resonating at H-17' (δ 0.89 *t*) and C-17' (δ 14.4) (Valcic *et al*., 2002; Jin *et al*., 2006). The existence of a phenol ring originating from catechol could be identified with the signals between 6ppm and 7 ppm. Six aromatic carbons at C-1 (δ 158.3), C-2 (δ 145.6), C-3 (δ 130.9), C-4 (δ 116.2), C-5 δ 120 and C-6 (δ 113.5) as well as three proton signals at H-4 (δ 6.63, *d*, *J*=7.2), H-5 (δ 7.05 *t*) and H-6 (δ 6.57, *d, J*=7.8) indicating a catechol moiety in compound **41**. δ_H (600MHz, CD_3OD), 0.89 (3H, *t*, Me), 1.29 (18H, $(CH_2)_9$), 2.03 (4H, *m*, $2CH_2CH$=), 2.52 (2H, *t*, CH_2Ar), 5.33 (2H, *dd*, CH=CH, *J*=5.4,9.6Hz), 6.63 (*d, J*=7.2, H-4), 6.57 (*d*, *J*=7.8, H-6) and 7.05 (*t*, H-5).

The first four positions 1'-4' of the side chain were assigned by ^{1}H, ^{13}C (HMBC)-long range correlations, COSY and NOESY (**Figure 3a** and 3**b**).There was long range coupling between the proton H-14' (δ5.33) and H-12' (δ2.03).

The ^{13}C peak selection offered that possibility of determining HSQC and the HMBC spectra for the crowded region of the methylene carbons of compound **41** (δ10-40ppm) with high resolution. HMBC correlations that are there between the protons of the terminal methyl group at δ0.89 and the carbons at δ_C23.7 and δ_C30.8 assisted in establishing the C-16' and C-15' positions respectively. There was a $^{2}J^{1}H$-^{13}C- connectivity between the latter carbon and the proton signal of H-14' at δ5.33 which shows a ^{3}J HMBC correlation with the allylic C-12' (δ 28.1) that assisted locate the double bond at C-13' (δ 130.8). This assignment was confirmed by HMBC correlation between both the olefinic resonance H-14' (δ5.33) and C-16'at δ23.7 and the latter with the methyl group H-17' (δ 0.89). Homo-nuclear decoupling experiments on the allylic protons as described in previous literature enabled establish the position of the double bond (Gonzales *et al*, 1996) and determination of the Z-configuration (J=9.6).

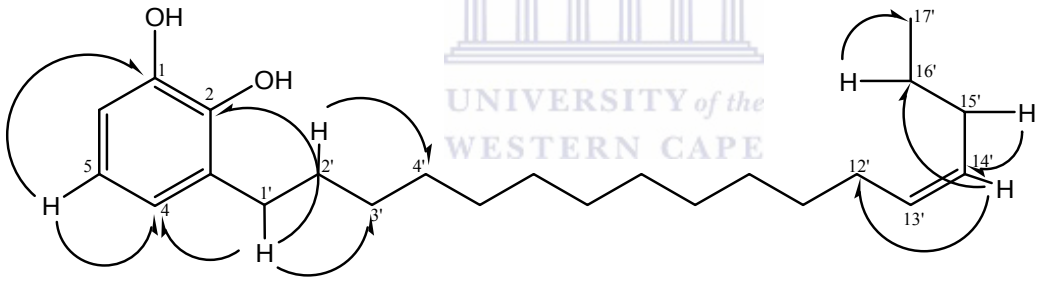


Figure 3a. Important HMBC

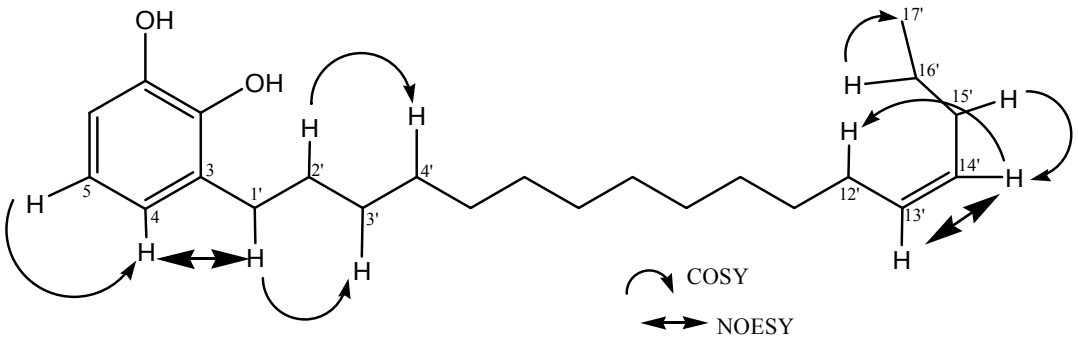


Figure 3b. Important COSY and NOESY (double arrow)

Table 9. ^{1}H NMR and ^{13}C NMR data of compound 41 in CD_3OD at 600MHz

Position	^{1}H NMR	^{13}CNMR	HSQC	HMBC
	δ, multiplicity, J	δ (ppm)		
1		158.3		
2		145.6		C-4, C-6
3		130.9		
4	6.63 *d, J*=7.2	116.2	6.63	C-2, C-6
5	7.05 t	120.7	7.05	C-1, C-3
6	6.57 *d*, *J*=7.8	113.5	6.57	C-2, C-4
1’	2.52 *t*	36.9	2.52	C-2. C-3, C-4, C-2’
2’	1.58, *q*	33.0	1.58	C-1’, C-3
3’	1.29 *ov*	30.3		
4’	1.29 *ov*	30.4		
5’	1.29 *ov*	30.6		
6’	1.29 *ov*	30.7		
7’	1.29 *ov*	30.7		
8’	1.29 *ov*	30.7		
9’	1.29 *ov*	30.7		
10’	1.29 *ov*	30.7		
11’	1.29 *ov*	32.6		
12’	2.03 *m*	28.1		C-11’, C-13’, C-14
13’	5.33 *dd, J*=5.4, 9.6	130.8	5.33	C-12’, C-15
14’	5.33 *dd, J*=5.4, 9.6	130.2	5.33	C-12, C-15, C-16
15’	2.03 *m*	30.8		
16’	1.37 *ov*	23.7		
17’	0.89, *t*	14.4		C-15’, C-16’

Ov- overlapped by other signals

3-((Z)-heptadec-13-enyl) benzene-1, 2-diol (**41**) is a major constituent of the resulting monomeric urushiols extracted from the roots of *Rhus natalensis*. In addition to other compounds extracted from the plant, this catechol is the only one of its kind obtained from the hexane extract. Biological activity of the compound did not exhibit any major antibacterial activity. It was found to possess only a moderate to low activity as an antimicrobial zone of inhibition (9 mm) as compared to the standard chloramphenicol. Previous literature reports that meta-substituted catechol functionalities are present in a large number of natural compounds with beneficial effects on the human body. They have been used in the production of some anti-hypertensive pharmaceuticals (Kieboom *et al*, 2001). Compound **41** is one of such natural products.

In this study, a number of the crude extracts were active against both Gram-positive and Gram-negative bacteria, but for the pure compounds, activity was observed only against Gram-positive bacteria. The activity against both types of bacteria may be indicative of the presence of a broad spectrum of antibiotics or simply general metabolic toxins in these extracts. The antibacterial activity of biflavonoids against both Gram-positive and Gram-negative bacteria has been reported. Activity against bacteria like *S. aureus* was demonstrated mainly by compounds that contained hydroxyl groups in ring B. Biflavonoid compounds having two to three hydroxyl groups in rings A or B were more active against such Gram-positive bacteria. It has been documented (Grosvenor, *et al*., 1995; Martinez, *et al*., 1996; Chariandy, *et al*., 1999; Stickler and King, 1992) that *S. aureus* is one of the bacteria most susceptible to plant extracts. Other biflavonoids are known to be active against antibiotic-resistant bacteria.

Such bacteria are inhibited by those compounds carrying hydroxyl groups in ring B, for example quercetin (**27**), and kaempherol (**28**).

The results of the present investigation have clearly indicated that the antibacterial and antifungal activity vary with the different plant parts (Hoffman *et al*., 1993). This study is a preliminary evaluation of antimicrobial activity of *R. natalensis*. It indicates that the plant has the potential to generate novel metabolites. The crude extracts demonstrating anti-candidal activity could lead to the discovery of novel anti-candidal agents. The plant has demonstrated a broad spectrum of activity that may help discover new chemical classes of antibiotics that could serve as selective agents for the maintenance of animal or human health and provide biochemical tools for the study of infectious diseases. These findings provide yet another example of the validity of making more advantageous structural modifications of seemingly rather thoroughly examined anti-infective drug in further researches. Compound **39** with the name Rhuschromone, is a new biflavonoid that is naturally isolated and with high antibacterial activity.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The research efforts on *Rhus* extracts indicate a promising potential for the plant family to provide renewable bio-products with the following desirable bioactivities: antimicrobial, antifungal, antimalarial, antiviral, antifibrogenic, antiinflammatory, antimutagenic, antioxidant, antithrombin, antitumorigenic, cytotoxic, hypoglycaemic, and leukopenic. More bioactive components can be extracted from the plant material using environmentally benign solvents like methanol, and water that allow for both food and industrial end-uses. A substantial opportunity exists to investigate the use of other good solvents like the sub-critical and super-critical liquids, ionic liquids among others for extracting bioactive components plus other phytochemicals in processing the residue for complete biomass conversion.

This study validates and documents, in a systematic way, the antimicrobial properties of the genus *Rhus* used for many years by many people of the world. It also provides valuable information for further phytochemical isolation and characterization studies of active compounds, necessary for the development of new drugs. At present the search for compounds active against antibiotic-resistant strains of bacteria is continuing among the flavonoids, compounds which are non-toxic or have low toxicity (Narayana, *et al*., 2001). However, as this overview demonstrates, the previous work had focused on only a few members of this large plant family. In addition, not all of the species studied to date have been fully characterized for potential bioactivities.

Thus, there remains a significant research gap spanning the range from lead chemical discovery through process development and optimization in order to better understand the full bioactive potential of the *Rhus* genus as part of the global green technology based bio-product and bio-process research programs.

5.2 Recommendations

- The results obtained from the parts of *Rhus natalensis* plant should be considered for further studies aimed at isolating and identifying more active compounds having discovered the antimicrobial activities observed in this research work.
- Strong activity against Gram-positive bacteria particularly by the compounds extracted should make this plant a target for further studies aimed at discovering new and more potent antimicrobial drugs which are effective.
- Further research could be carried out to screen for any immune boosting in the human body especially against viral attacks by drugs from this plant.
- *In vivo* activities should be initiated as soon as *in vitro* results have provided clear evidence for proof of activity.
- The dispensary details should be determined in order to provide the proper dosage for correct administration of such drugs.
- The Government should offer support to sustainable harvesting of these medicinal plants and put systems in place enabling the marketing of such drugs obtained from this kind of plants.
- Conservation measures of such plants, having proven antimicrobial activity, are very important. This is by conserving all the major forests from extinction.

- Further studies should be extended to other related species of the plant from the different geographical regions to evaluate their activity against microbials.

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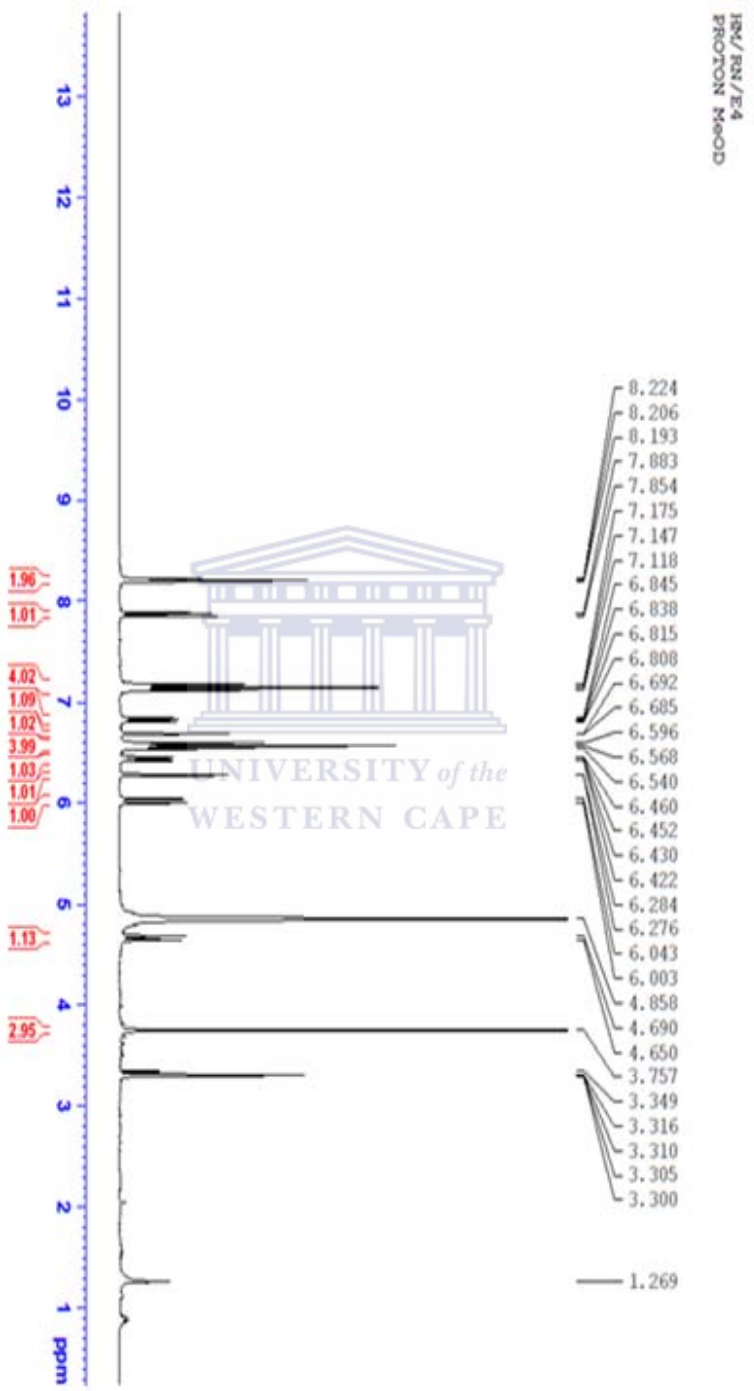
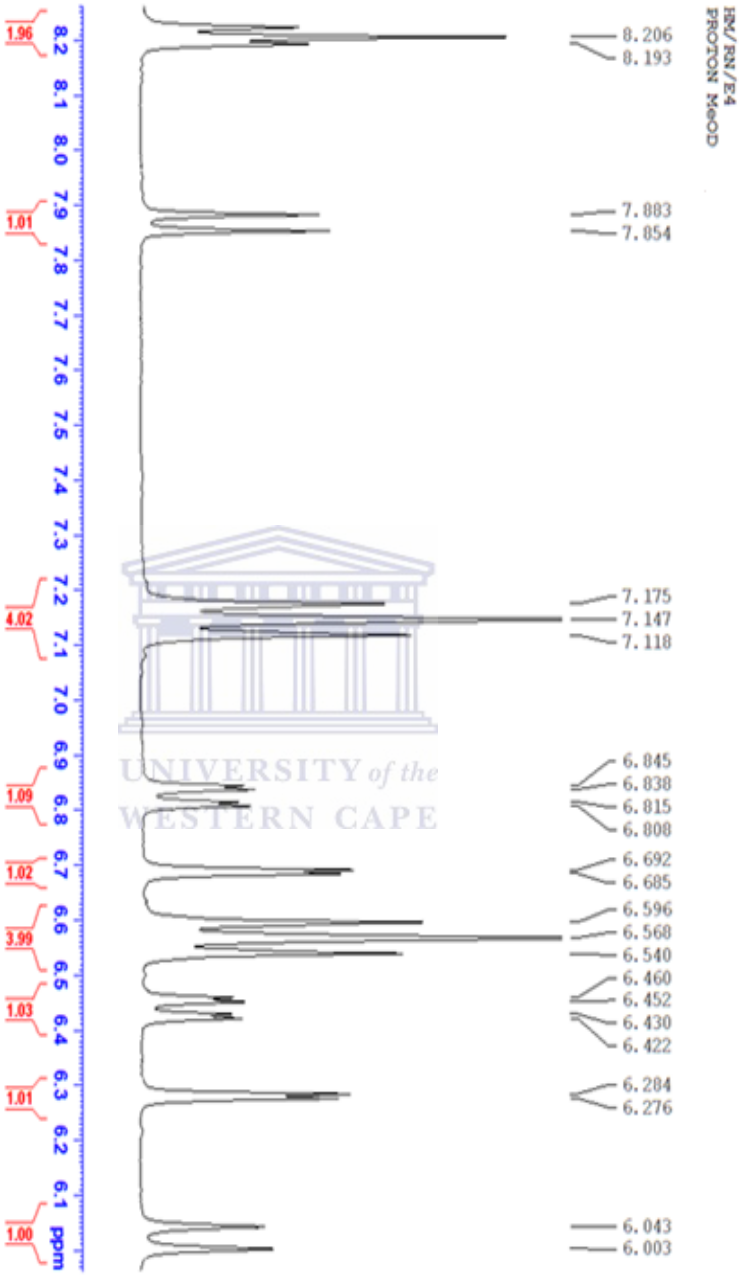
APPENDIX

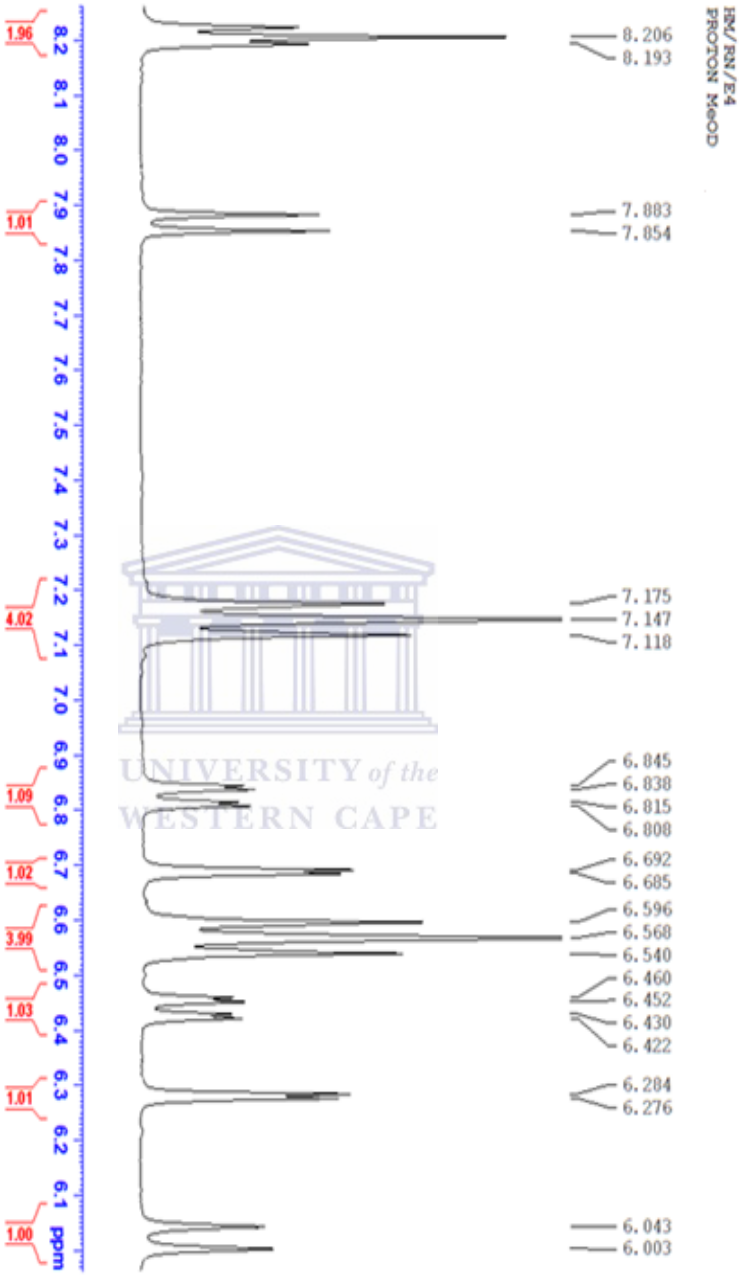
Appendix 1a ^{1}H NMR of **39**



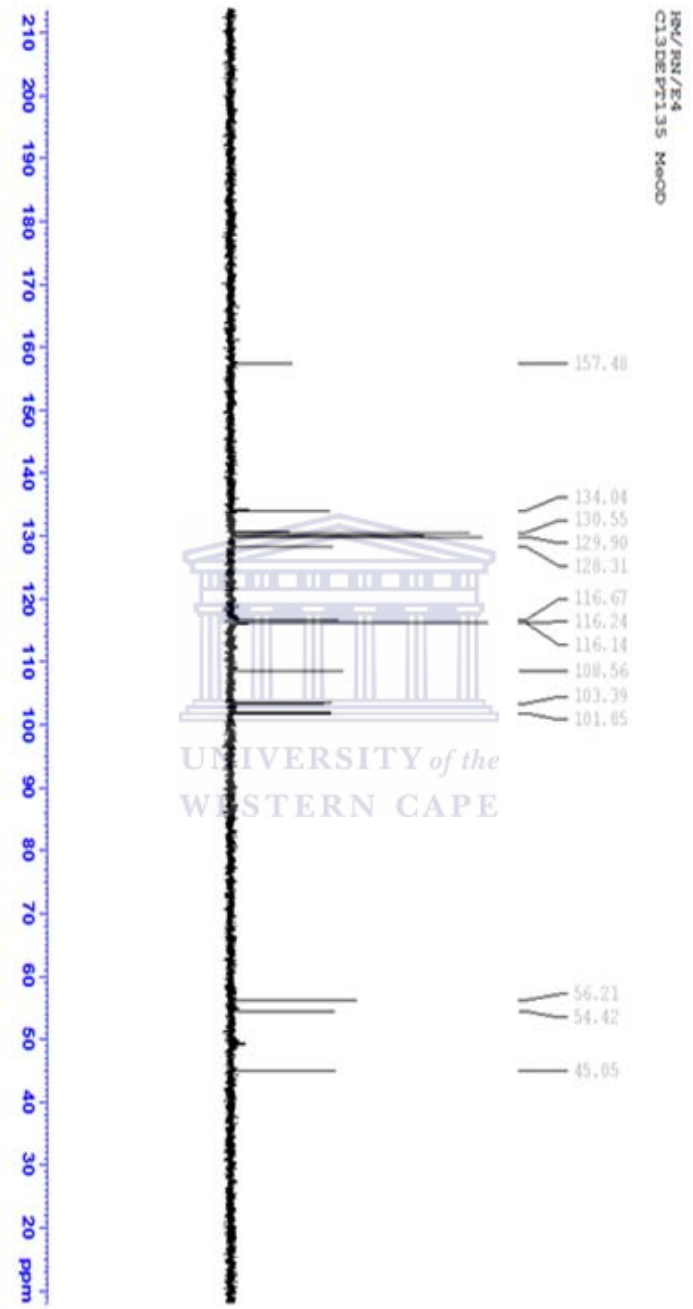
Appendix 1b ^{1}H NMR of **39**



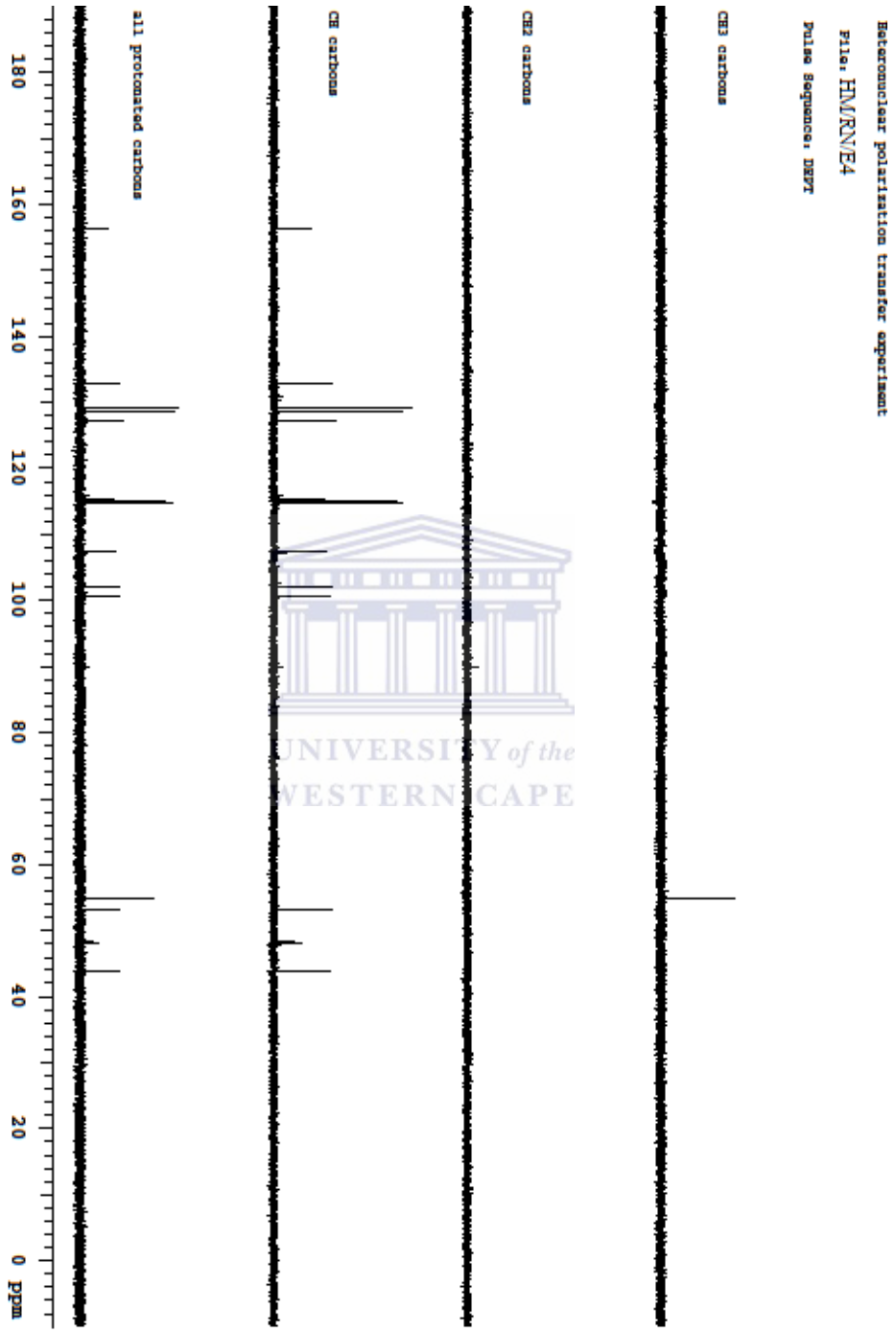
Appendix 1c ^{1}H NMR of **39**



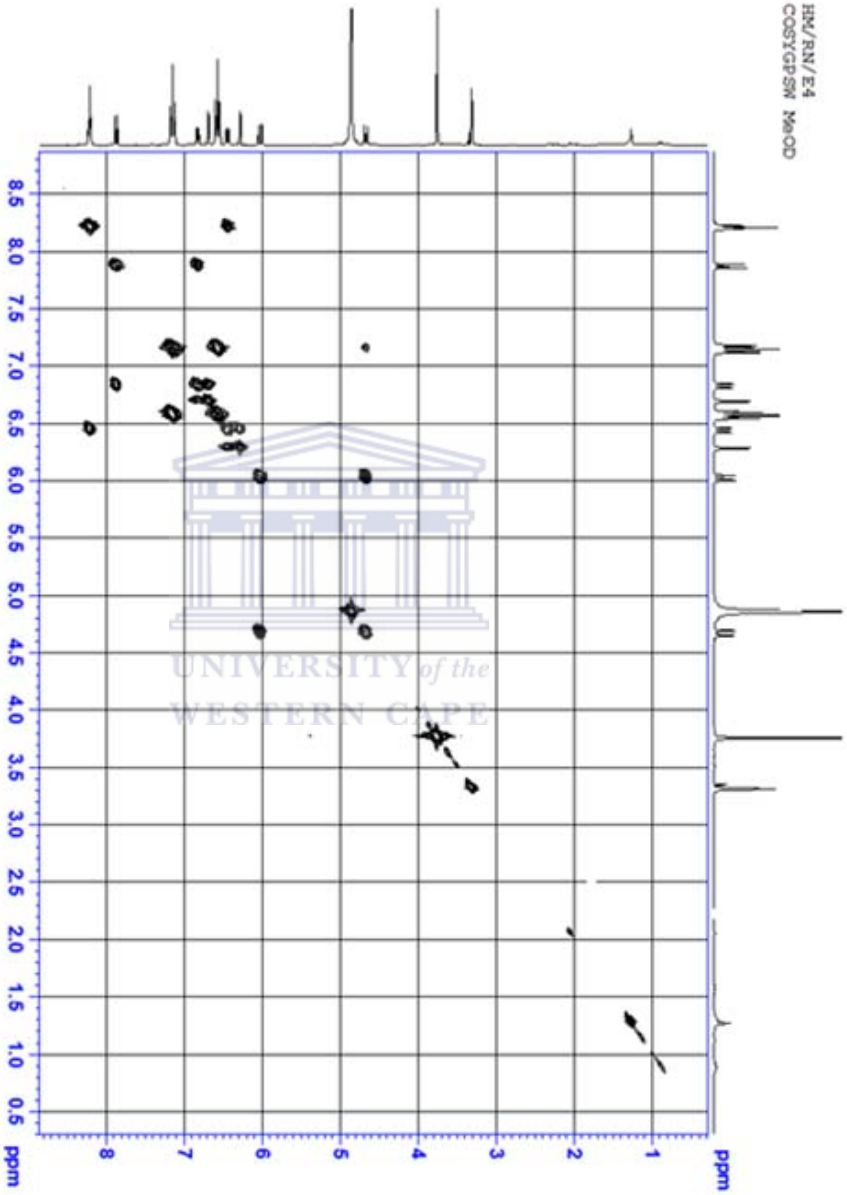
Appendix 1d DEPT 135 of **39**



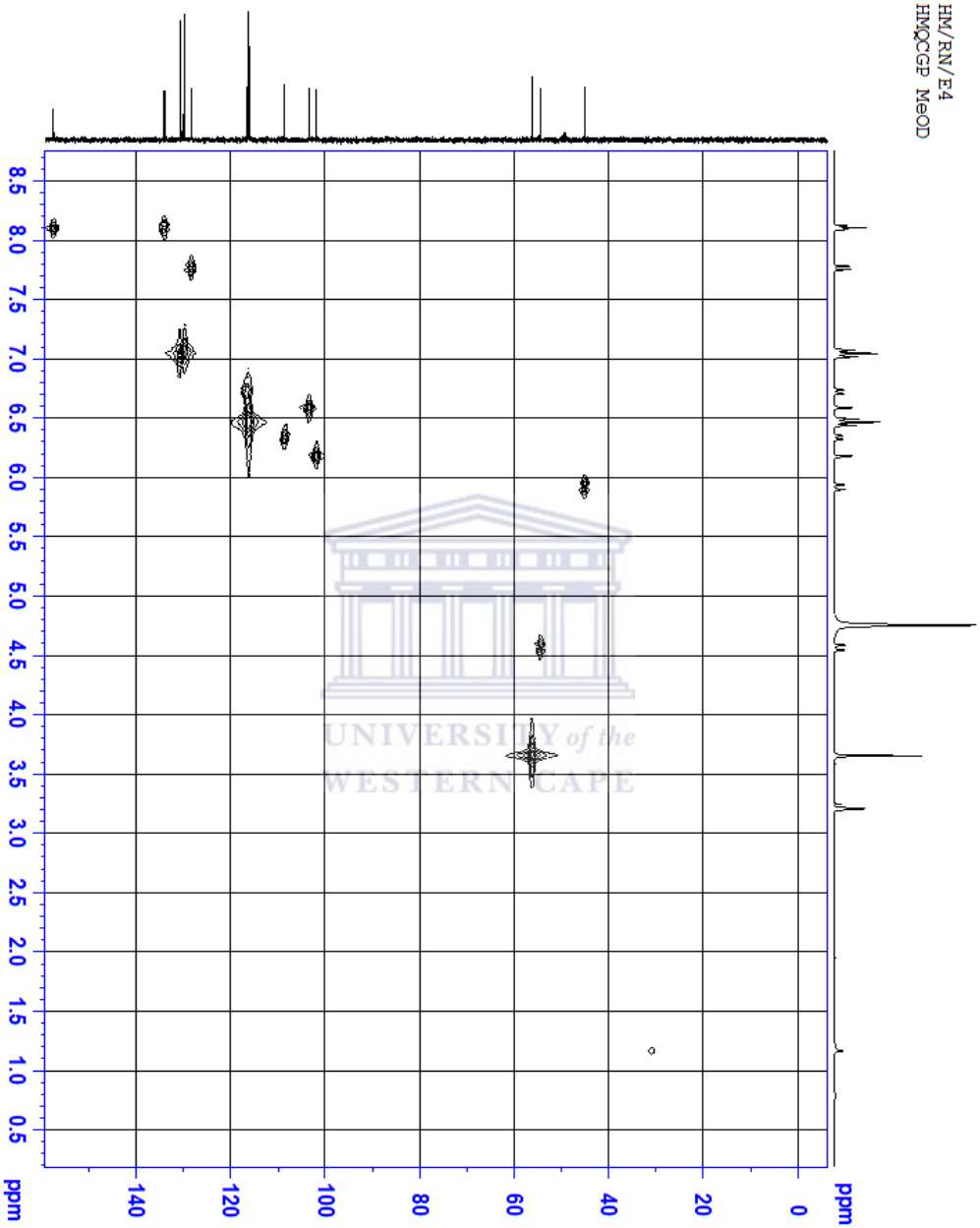
Appendix 1e DEPT of **39**



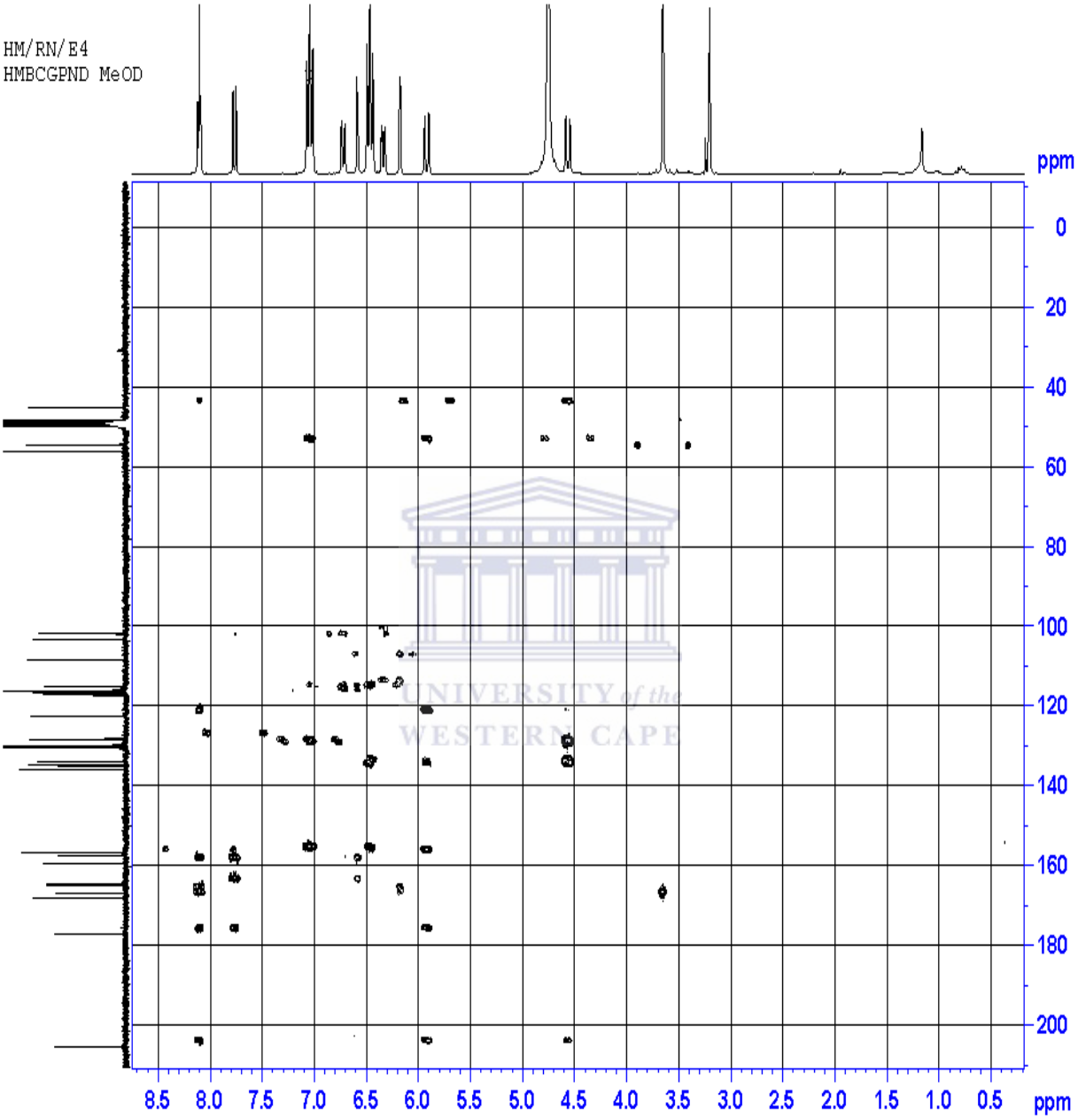
Appendix 1f COSY of **39**



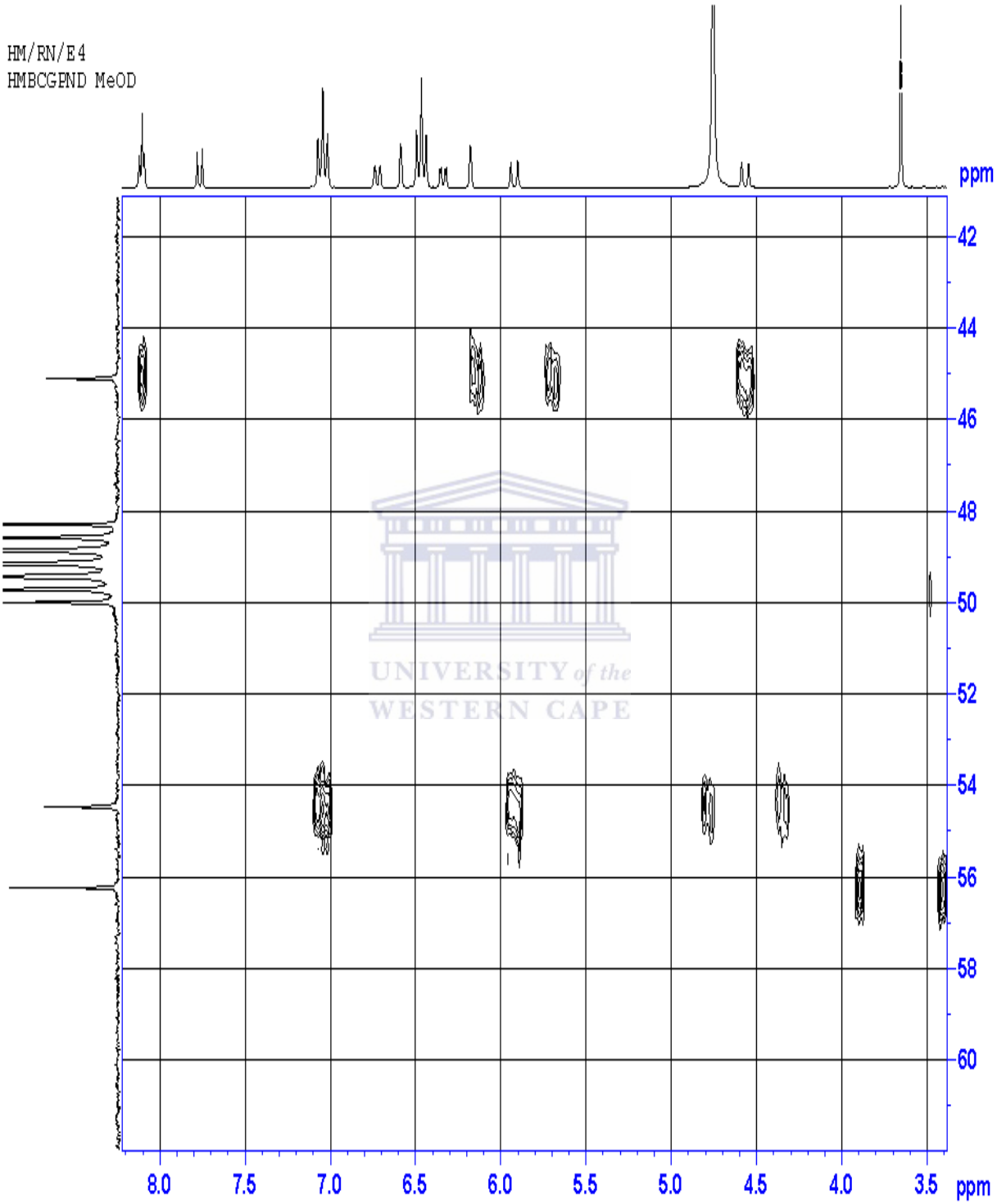
Appendix 1g HMQC of **39**



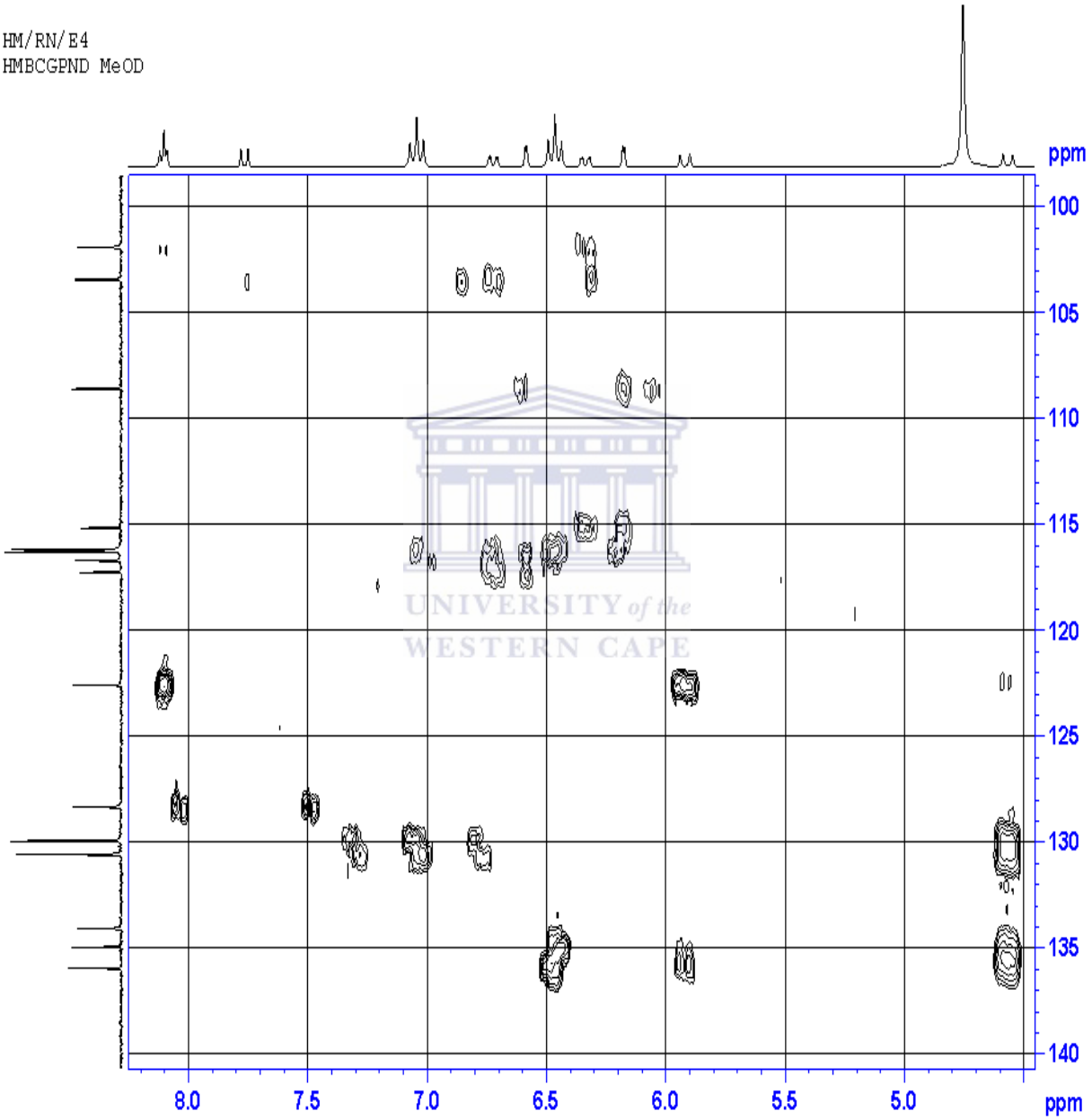
Appendix 1h HMBC of **39**



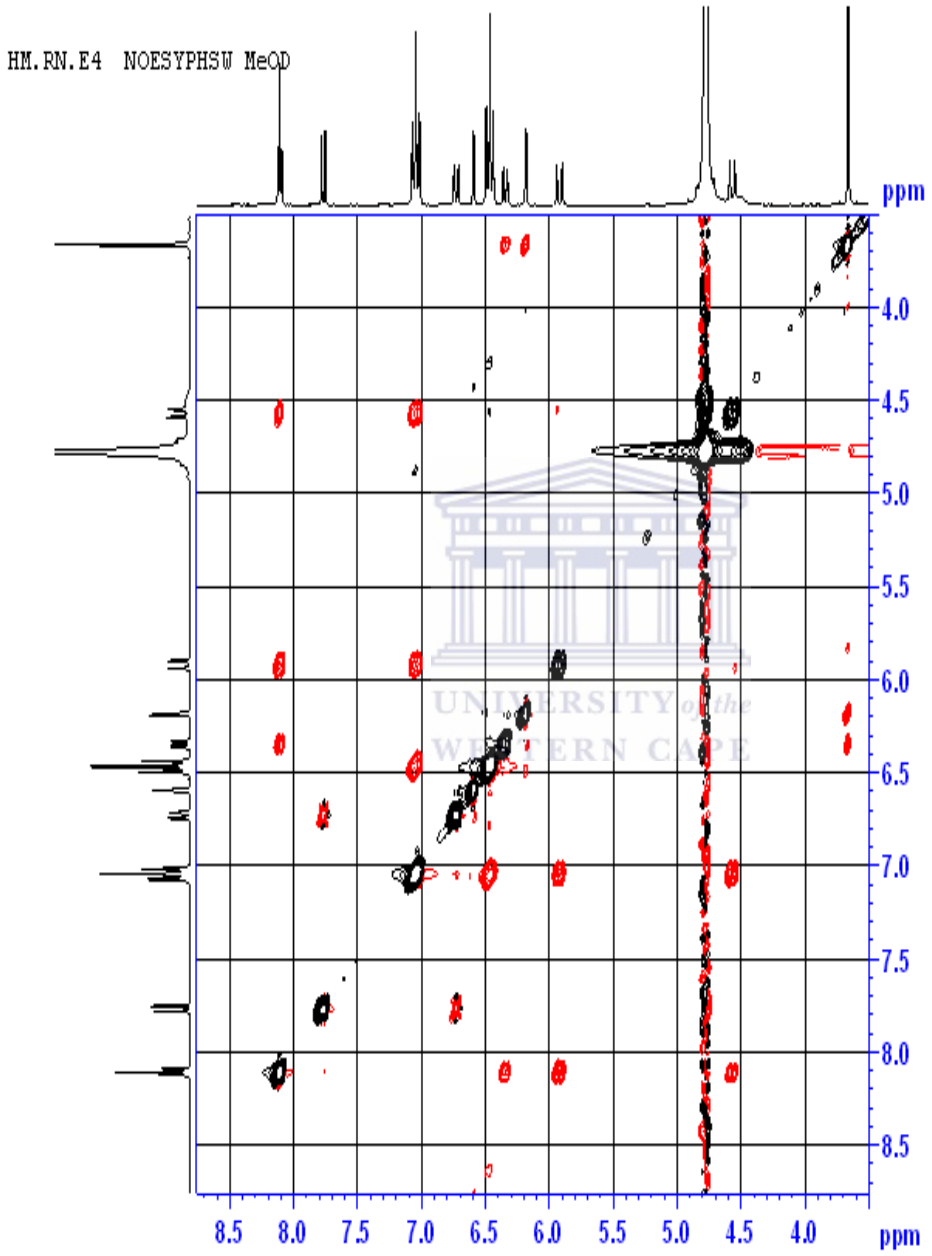
Appendix 1i HMBC of **39**



Appendix 1j HMBC of **39**



Appendix 1k NOESY of **39**

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PROCNO                       1

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TD                        2048
SOLVENT                   MeOD
NS                          32
DS                           4
SWH                  2587.992 Hz
FIDRES               1.263668 Hz
AQ                   0.3957236 sec
RG                       161.3
DW                     193.200 usec
DE                       6.00 usec
TE                      299.2 K
d0                   0.00018047 sec
D1                   1.93896902 sec
D8                   1.00000000 sec
IN0                  0.00038640 sec
TSCNT                      128

======== CHANNEL f1 ========
NUC1                       1H
P1                      10.00 usec
PL1                      0.00 dB
SFO1              300.1313714 MHz

F1 - Acquisition parameters
NID                          1
TD                         320
SFO1                300.1314 MHz
FIDRES               8.887474 Hz
SW                       8.623 ppm
FnMODE            States-TPPI

F2 - Processing parameters
SI                        1024
SF                300.1300353 MHz
WDW                     QSINE
SSB                          2
LB                        0.00 Hz
GB                           0
PC                        1.00

F1 - Processing parameters
SI                        1024
MC2               States-TPPI
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WDW                     QSINE
SSB                          2
LB                        0.00 Hz
GB                           0
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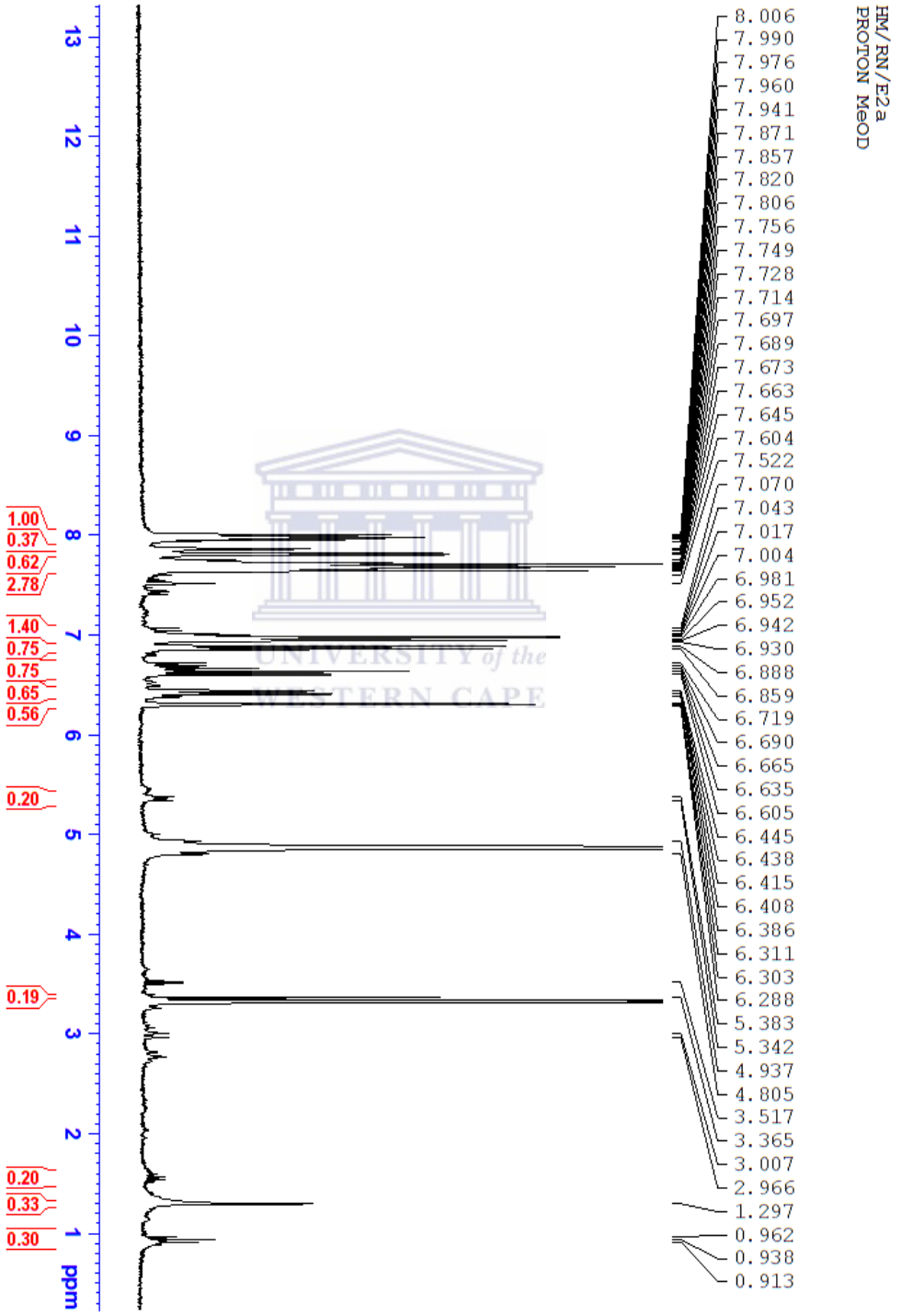
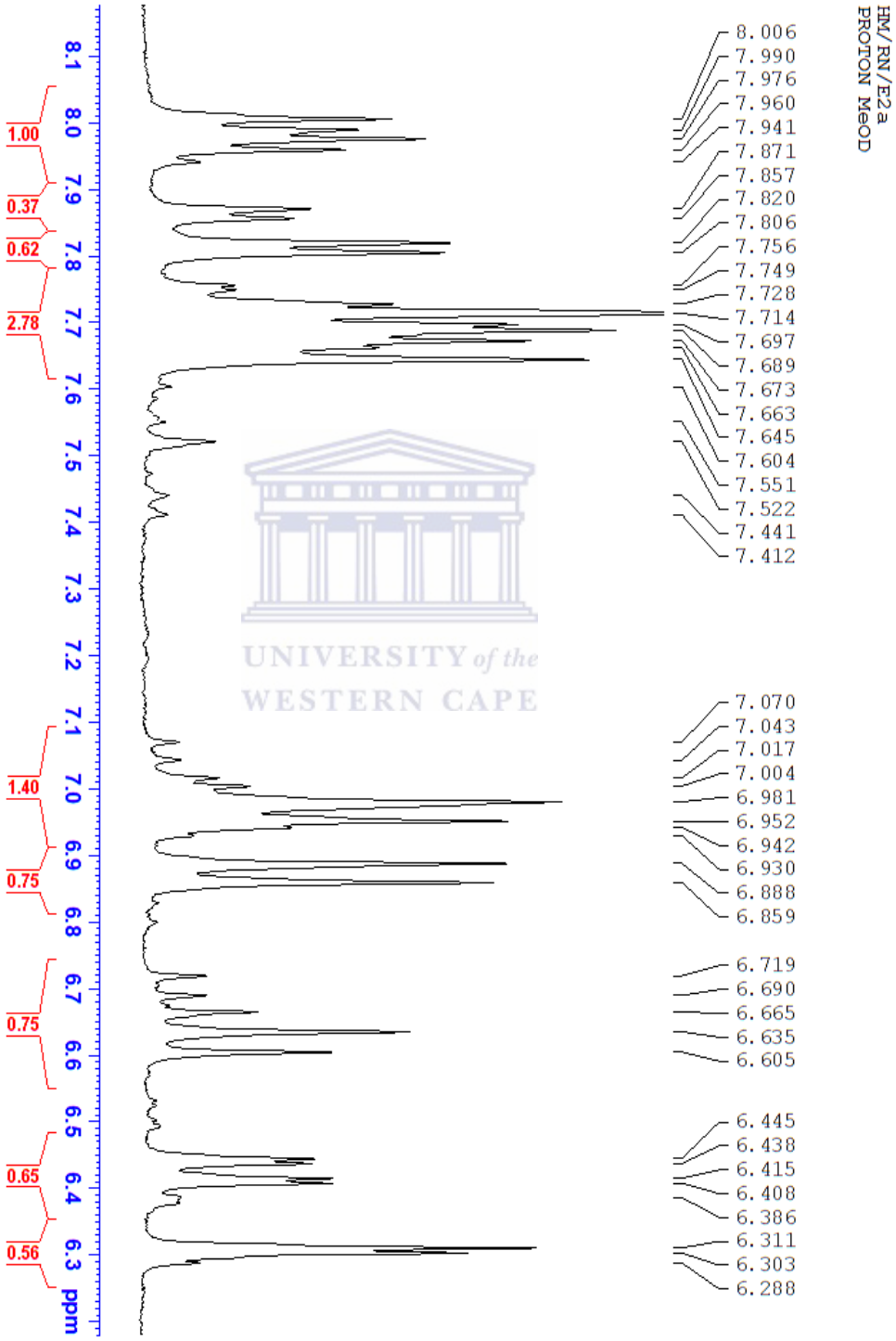
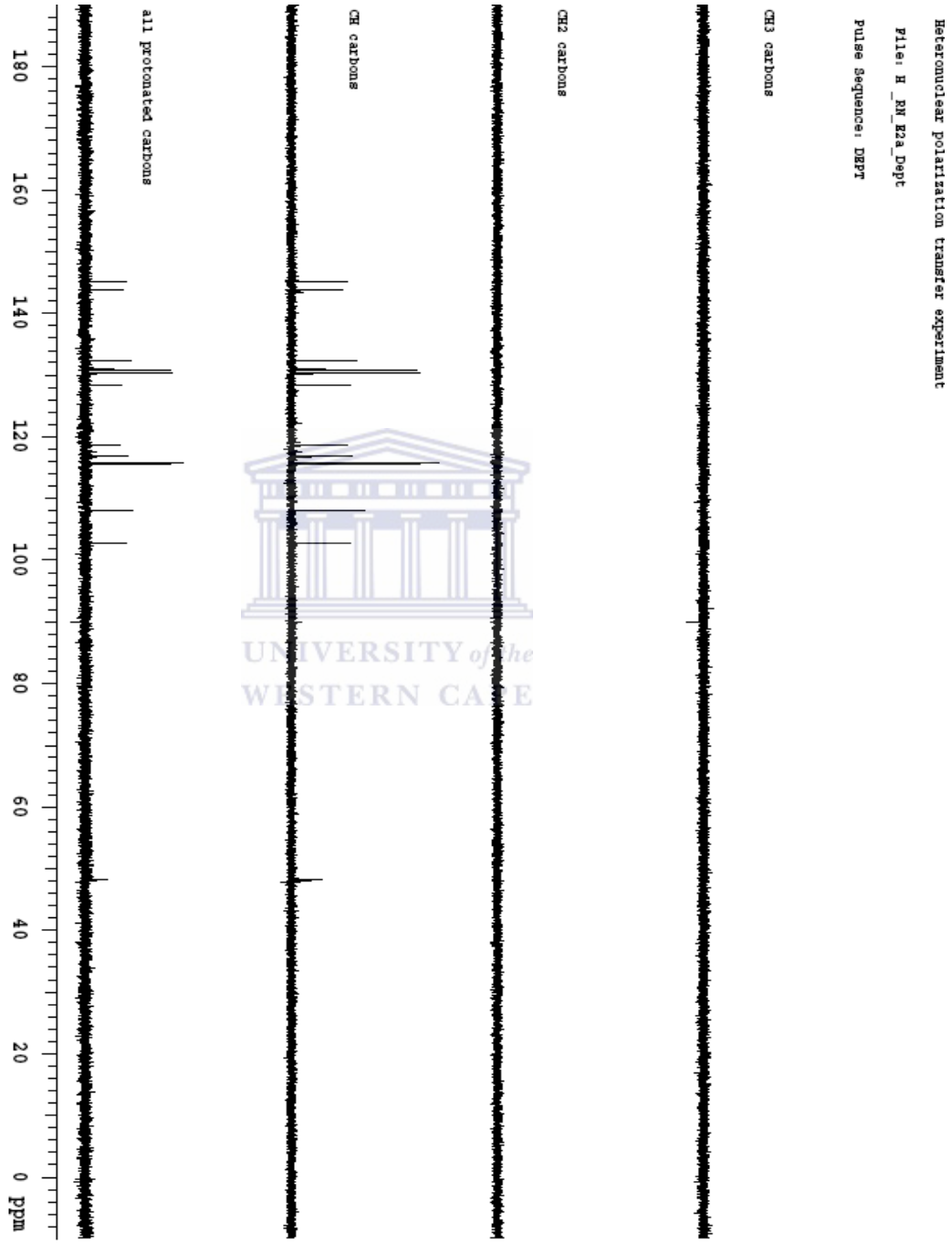
Appendix 2a ^{1}H NMR of **40**



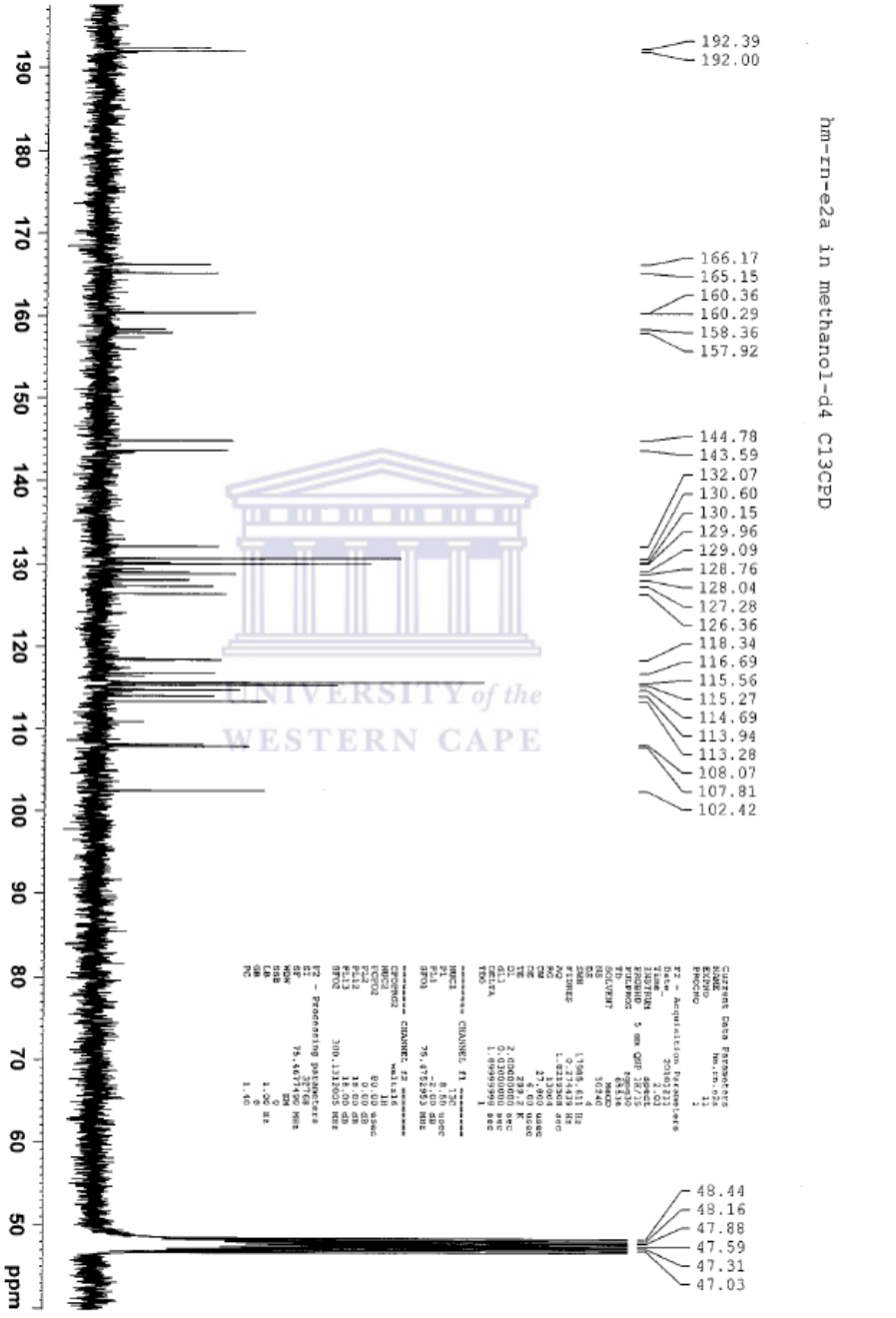
Appendix 2b ^{1}H NMR of **40**



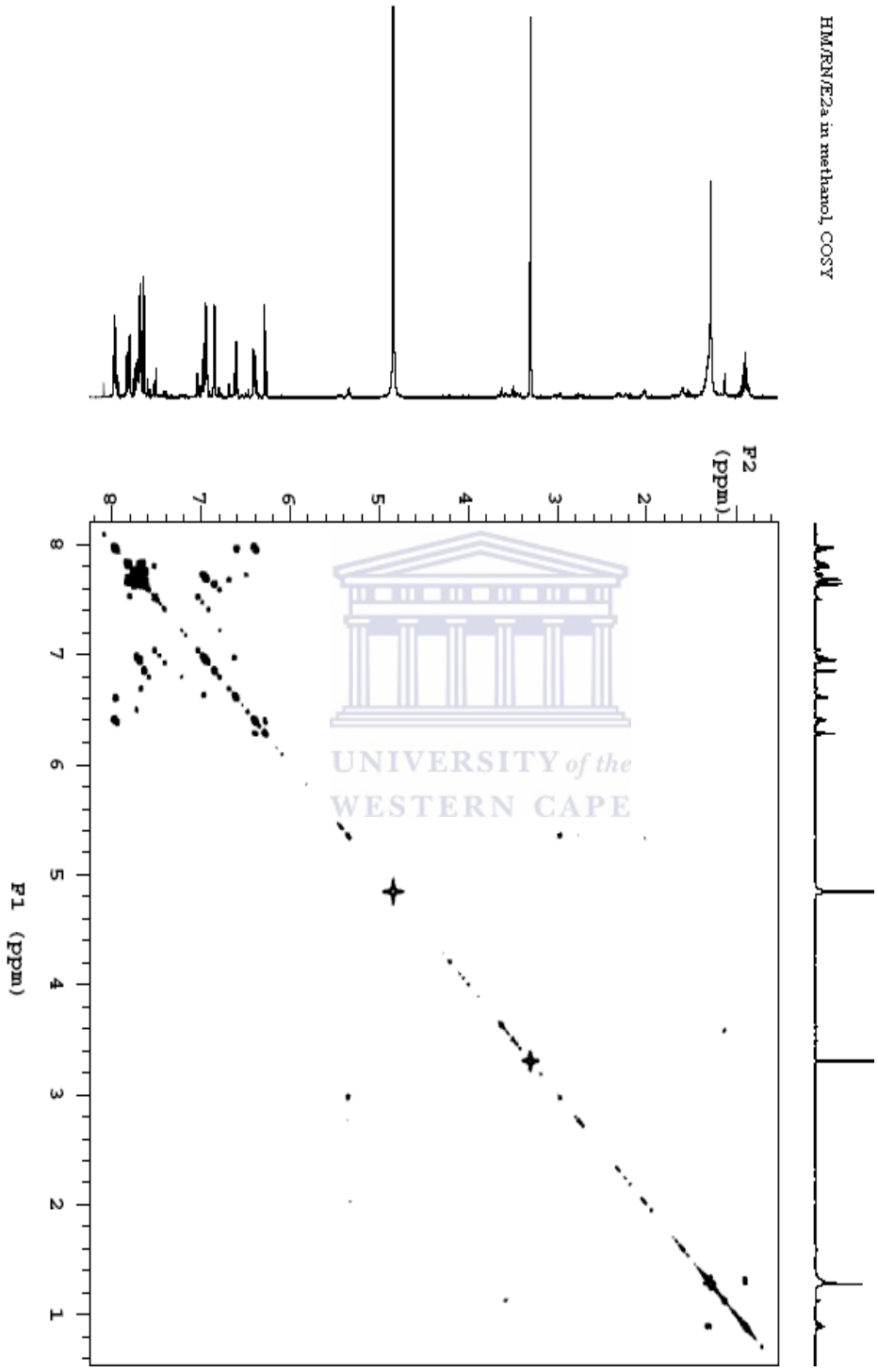
Appendix 2c DEPT of **40**



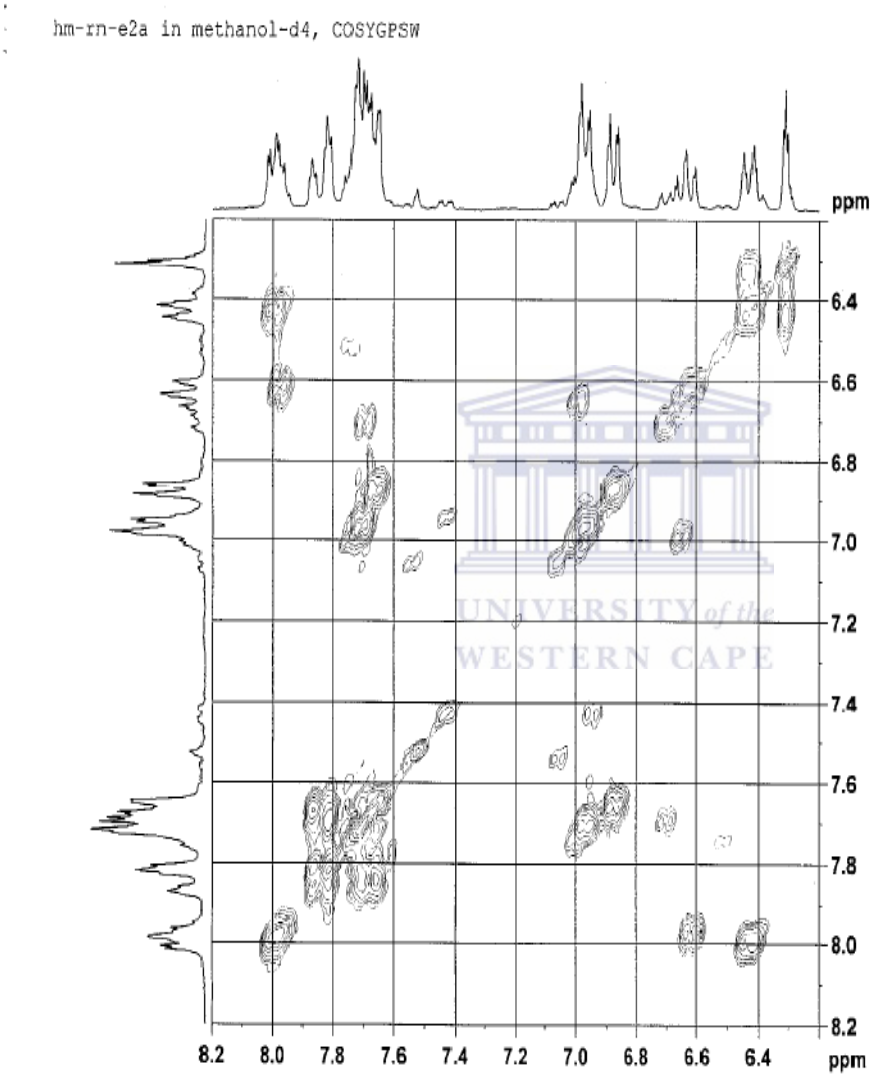
Appendix 2d ^{13}C in CD_3OD of **40**



Appendix 2e COSY of **40**



Appendix 2f COSY of **40**

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PROCNO                    1

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TD                   2048
SOLVENT              MeOD
NS                     24
DS                      8
SWH              2490.159 Hz
FIDRES           1.211015 Hz
AQ               0.4129268 sec
RG                     256
DW                201.600 usec
DE                  6.00 usec
TE                299.2 K
d0               0.00000300 sec
d1               1.33000400 sec
d13              0.00000400 sec
d16              0.00020000 sec
INO              0.00040320 sec

======== CHANNEL f1 ========
NUC1                   1H
P0                  10.00 usec
P1                  10.00 usec
PL1                  0.00 dB
SFO1          300.1313429 MHz

======== GRADIENT CHANNEL =====
GPNAM1          SINE.100
GPNAM2          SINE.100
GPZ1               10.00 %
GPZ2               10.00 %
P16              1000.00 usec

F1 - Acquisition parameters
NDO                    1
TD                   256
SFO1          300.1313 MHz
FIDRES           9.680120 Hz
SW                8.264 ppm
FnMODE                QF

F2 - Processing parameters
SI                  1024
SF          300.1300000 MHz
WDW                 SINE
SSB                    0
LB                  0.00 Hz
GB                     0
PC                  1.40

F1 - Processing parameters
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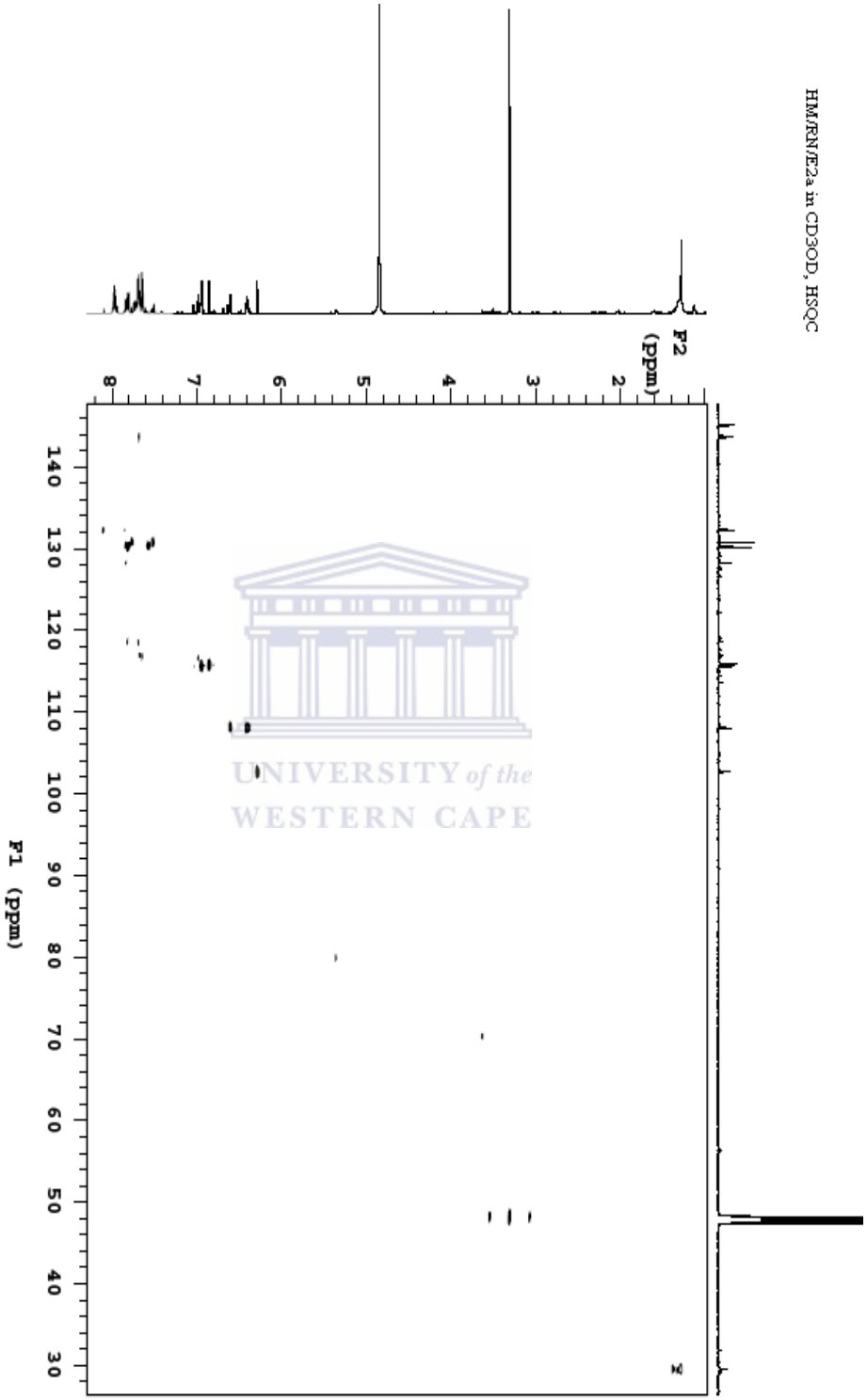
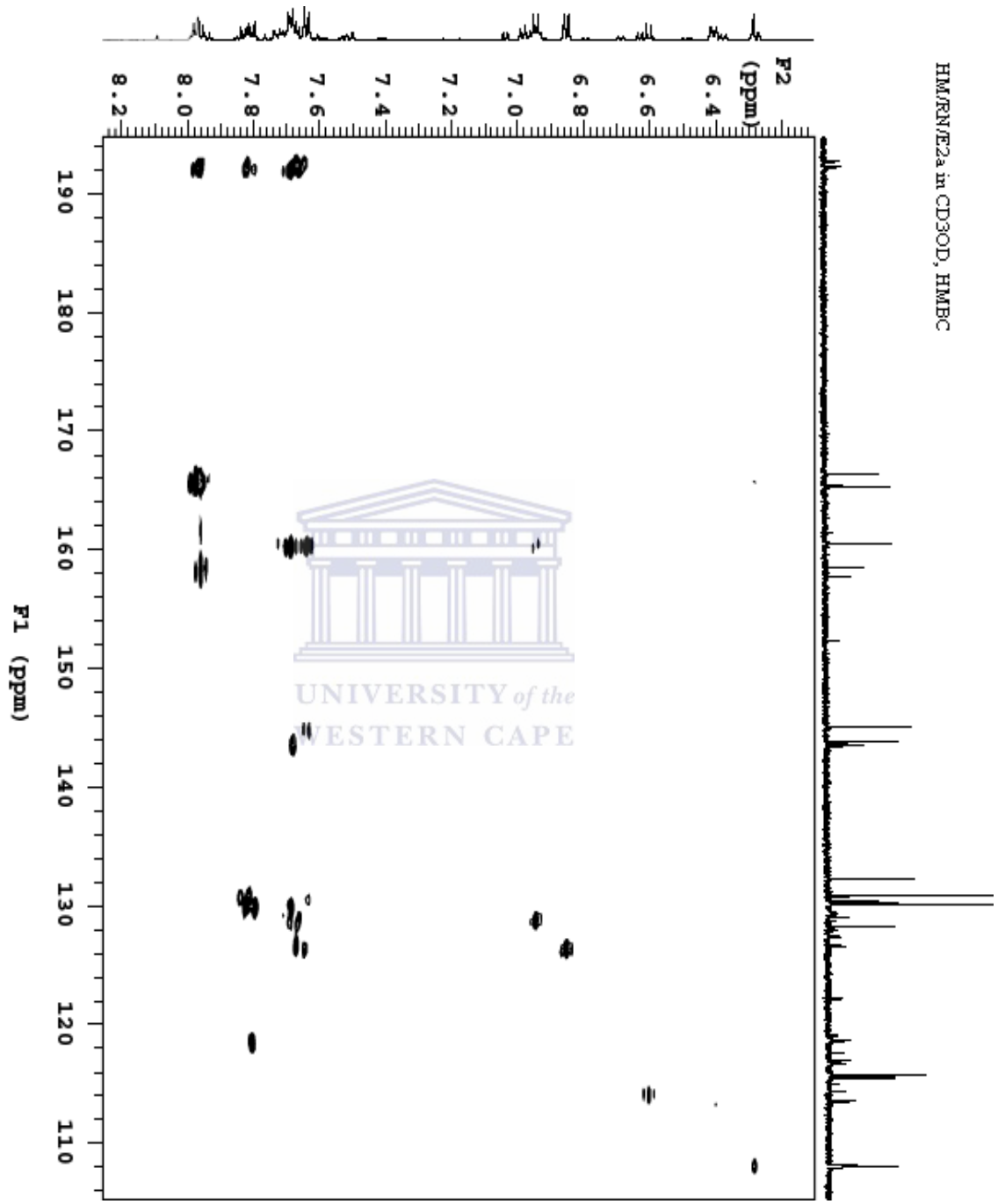
MC2                   QF
SF          300.1300000 MHz
WDW                 SINE
SSB                    0
LB                  0.00 Hz
GB                     0
```

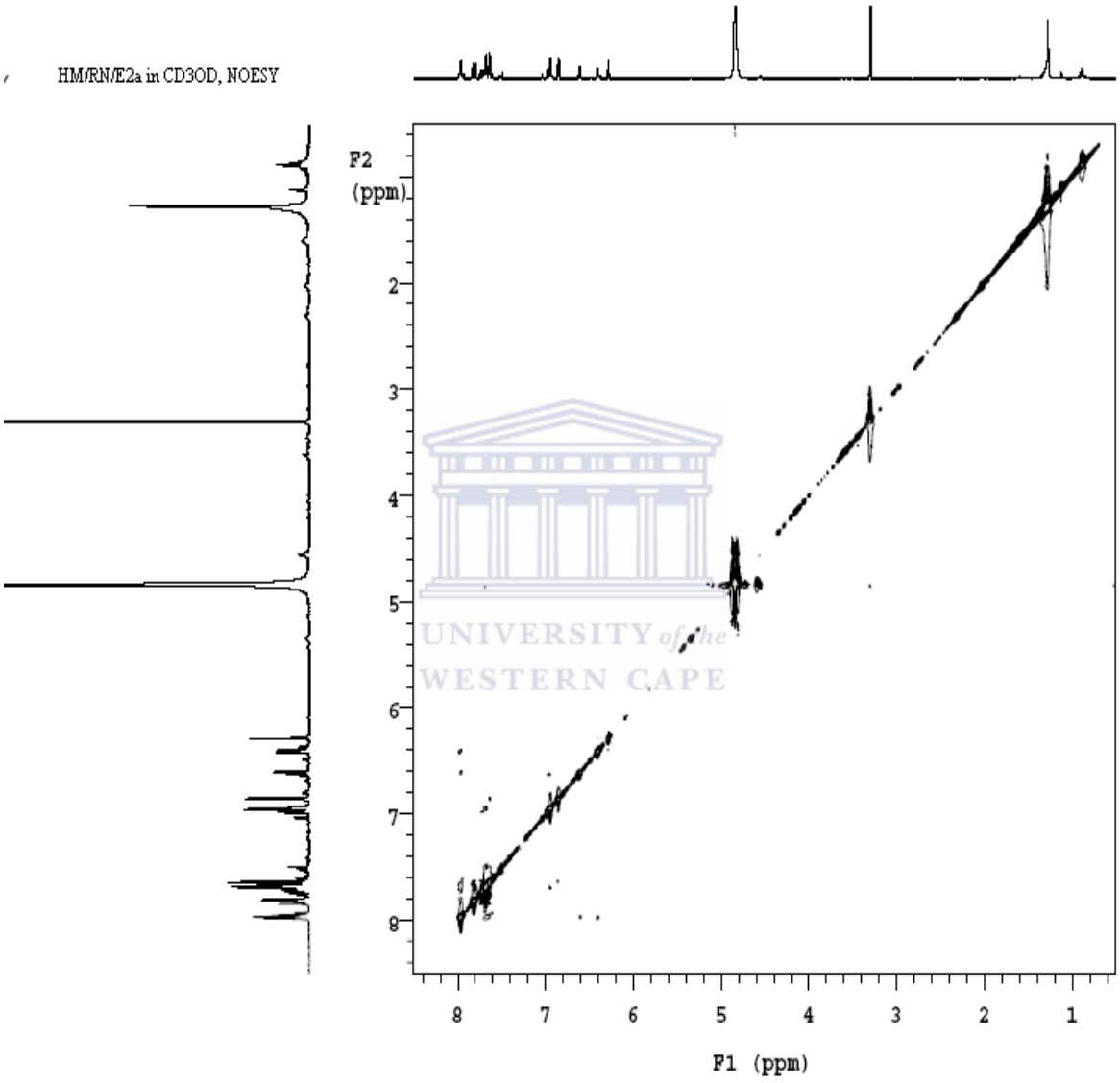
Appendix 2g HSQC of **40**



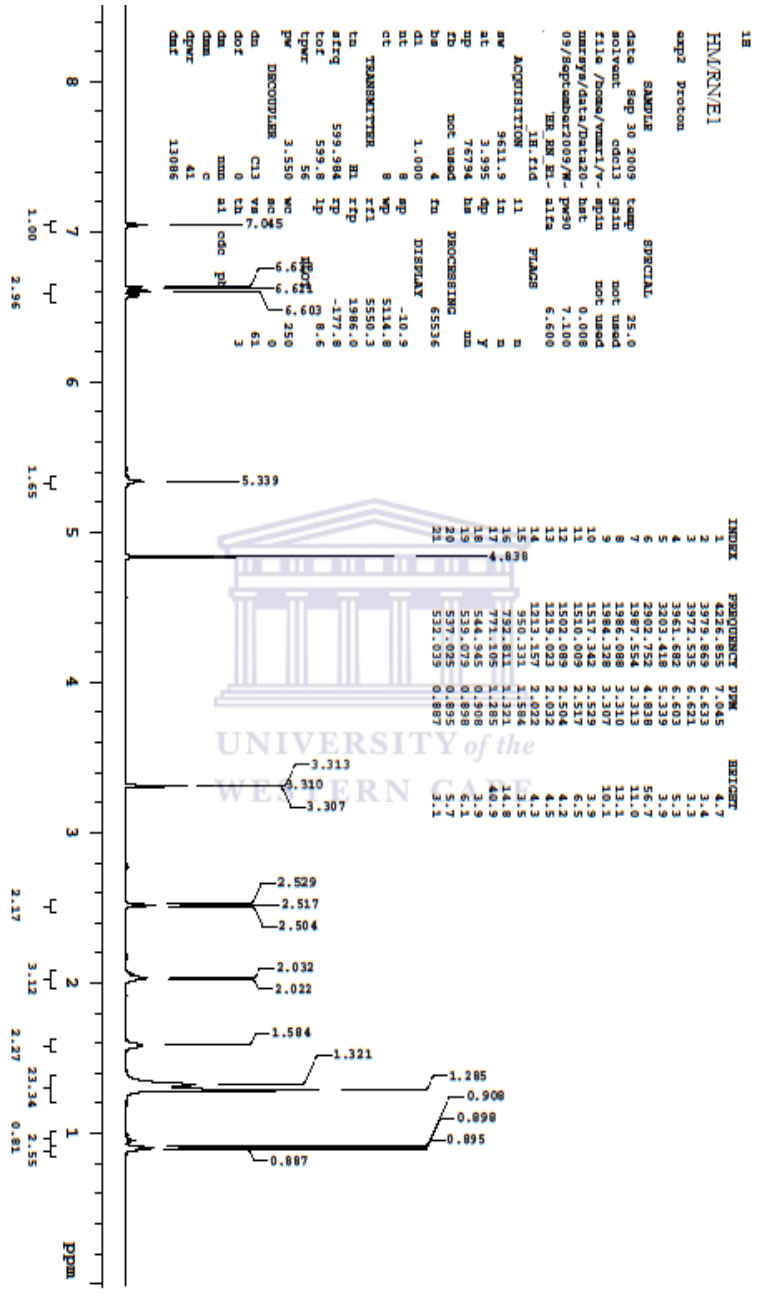
Appendix 2h HMBC of **40**



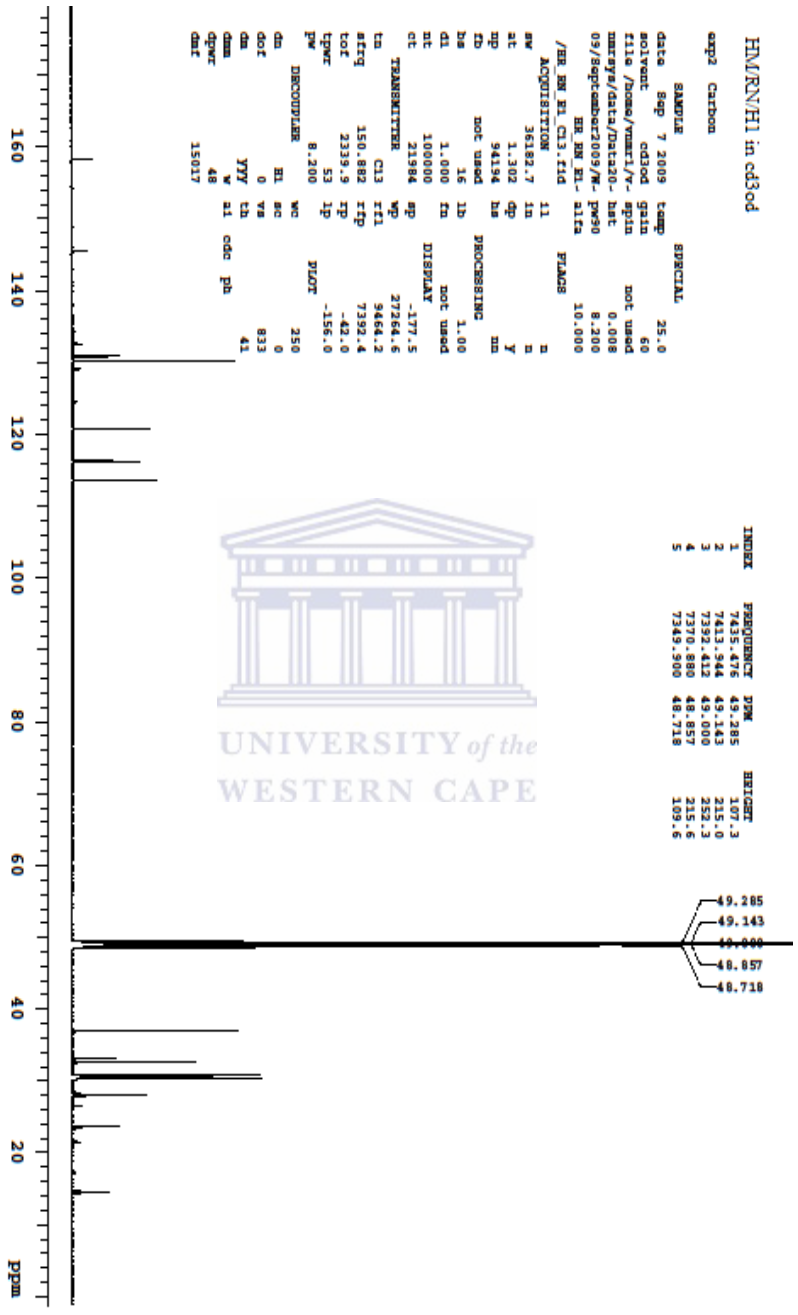
Appendix 2i NOESY of **40**



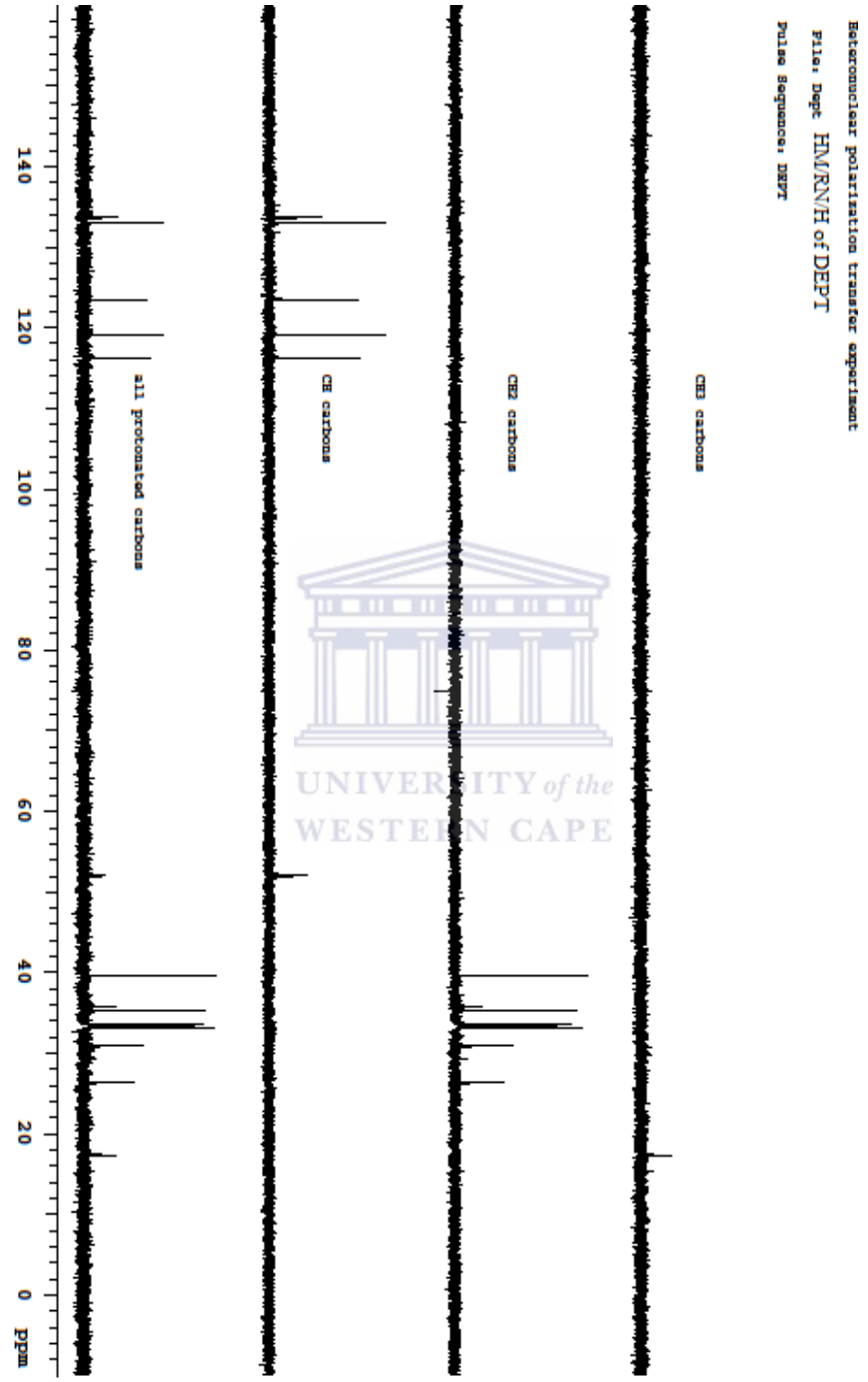
Appendix 3a ^{1}H NMR of **41**



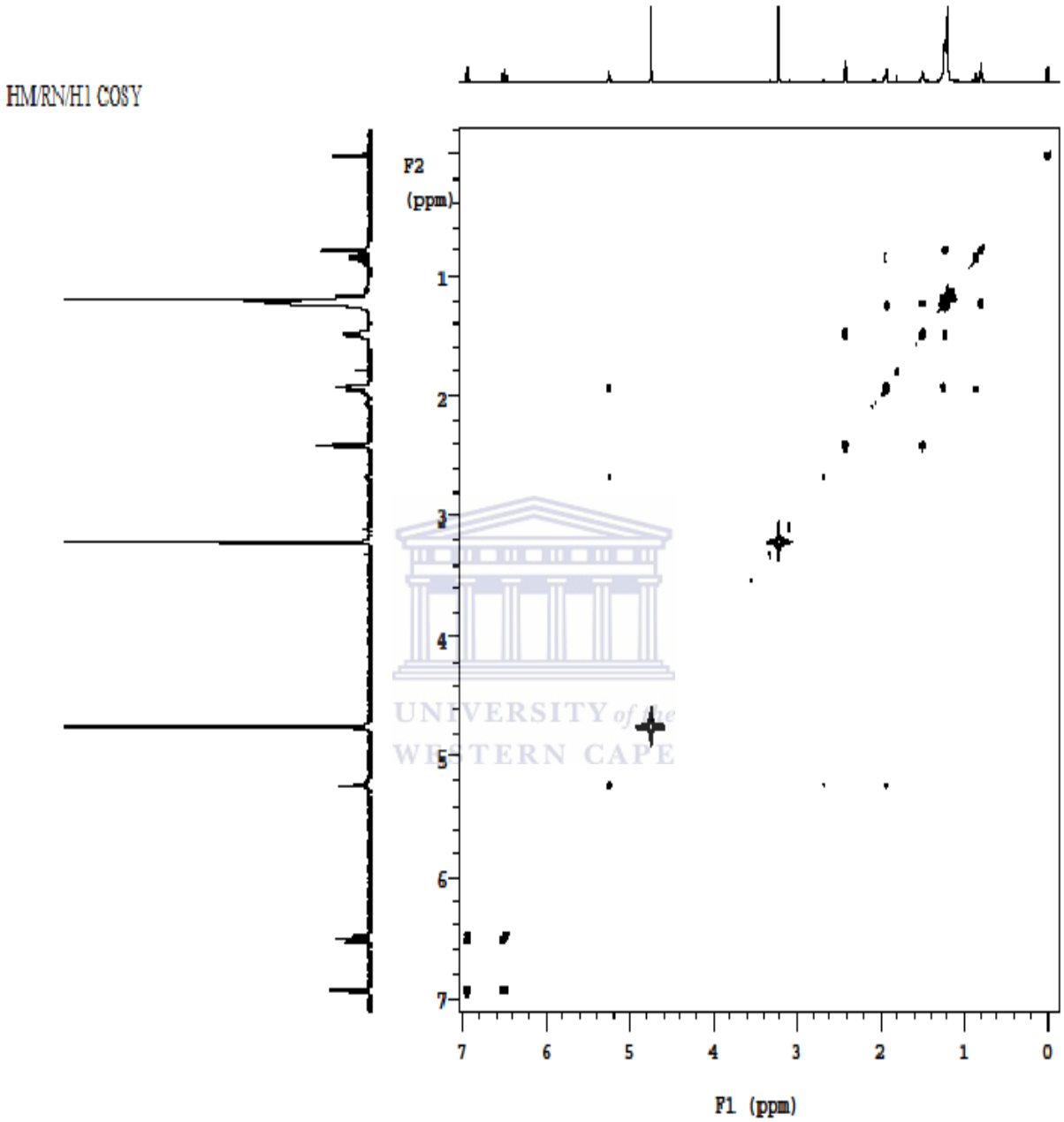
Appendix 3b ^{13}C NMR of **41**



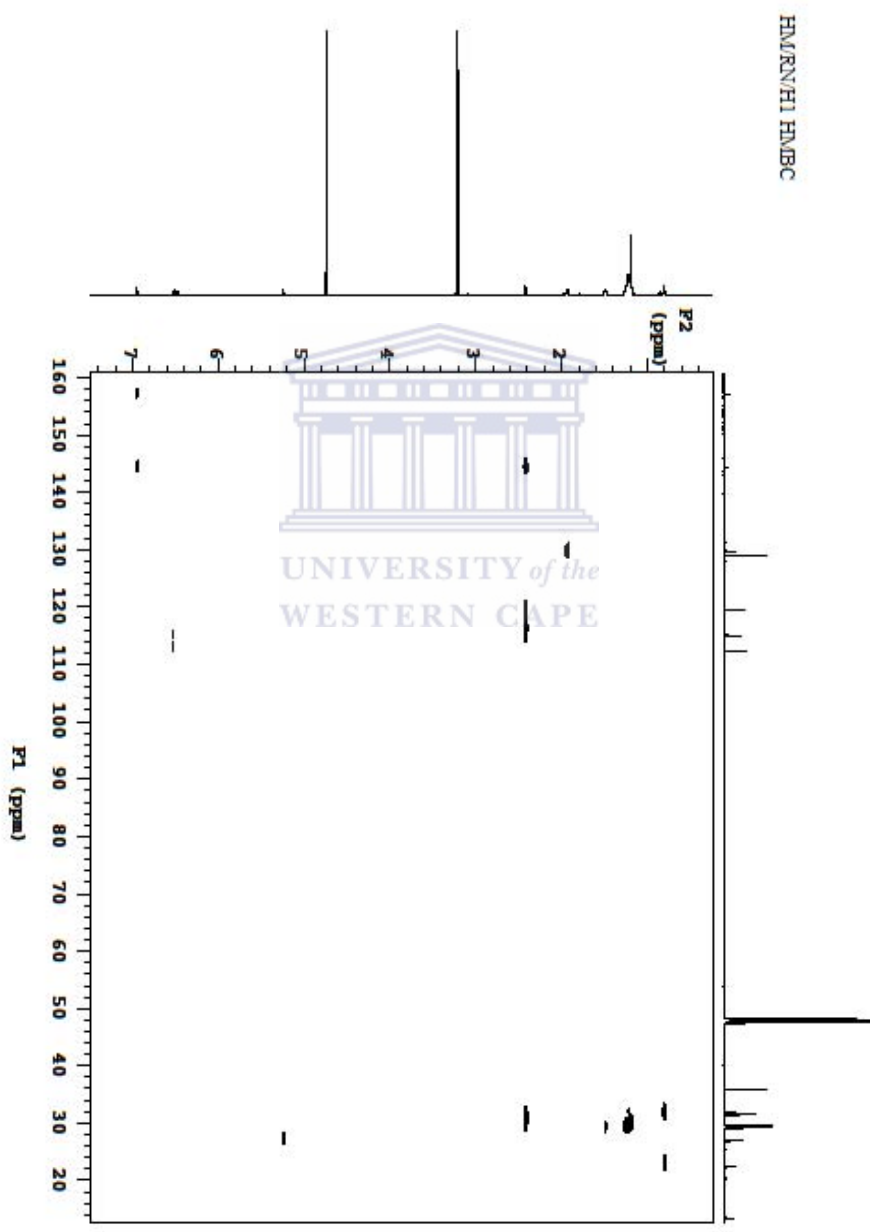
Appendix 3c DEPT NMR of **41**



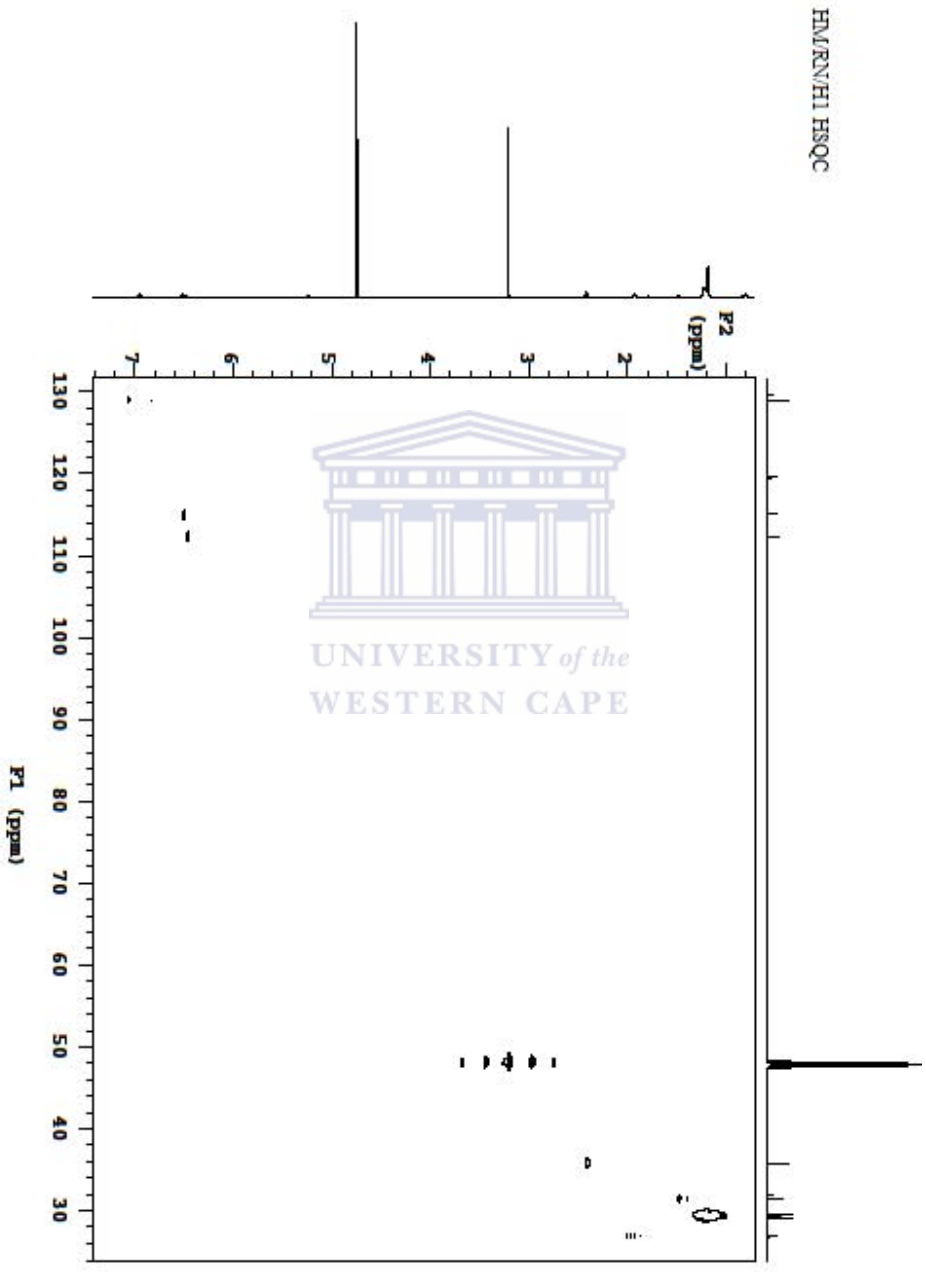
Appendix 3d COSY NMR of **41**



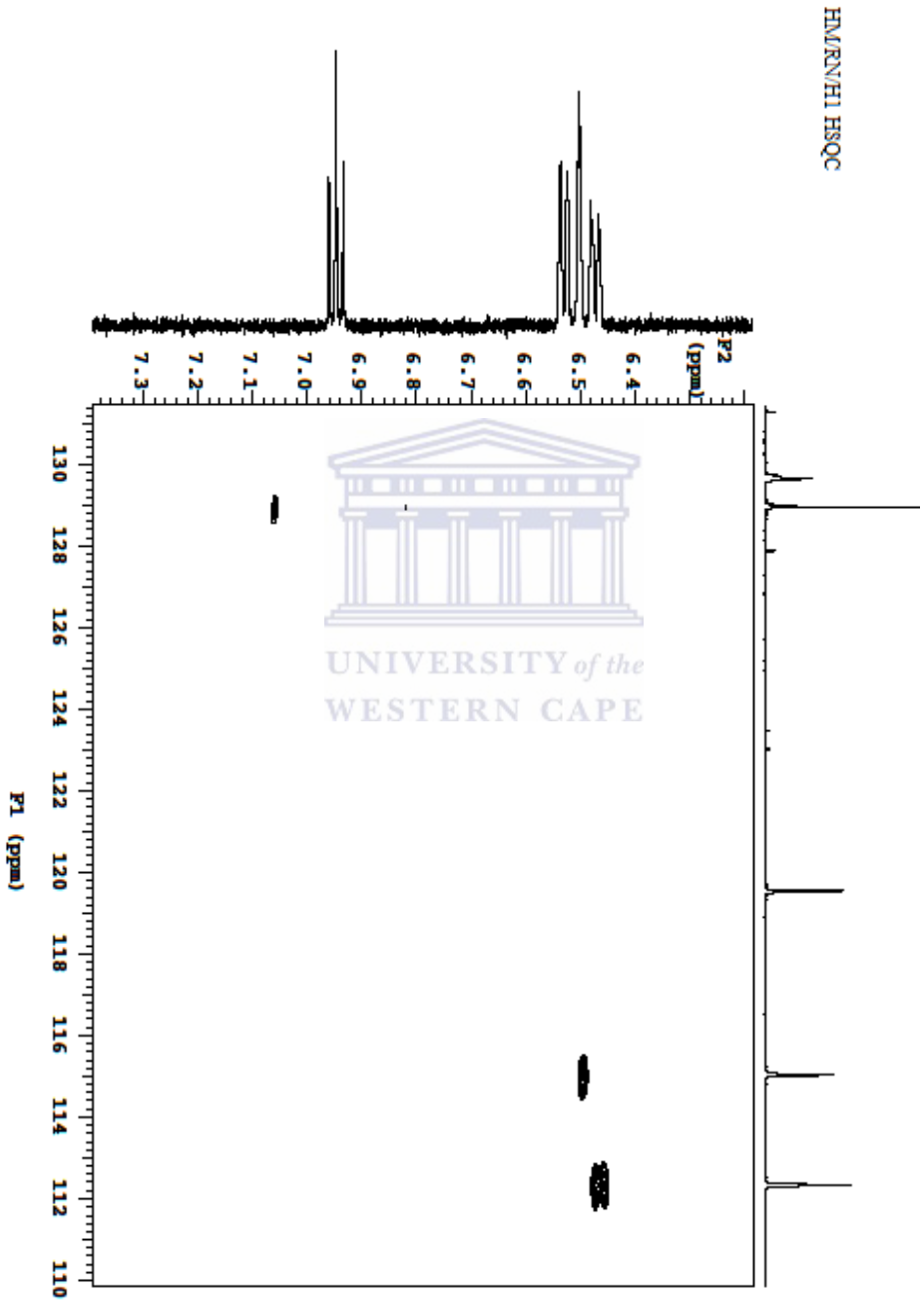
Appendix 3e HMBC NMR of **41**



Appendix 3f HSQC NMR of **41**



Appendix 3g HSQC NMR of **41**



Appendix 3h NOESY NMR of **41**