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LIST OF ABBREVIATIONS

ABTS: 2, 2′-azino-bis (3-ethylbenzthialzoline-6-sulfonic acid)

AIDS: Acquired immune deficiency syndrome CFU: Colony forming units CD: Circular dichroism

Cosy: Correlated spectroscopy

^{13}C-NMR: Carbon-nuclear magnetic resonance

DEPT: Distortionless enhancement by polarization transfer

DIG-POD: anti-digoxigenine-peroxidase

DIG-dUTP: digoxigenine-deoxyuridine triphosphate

dTT: deoxythymidine triphosphate

DMEM: Dulbecco-modified Eagle's Medium

DMSO: Dimethyl sulphoxide

ds: double-stranded

EDTA: Ethylendiaminotetra acetic acid

ELISA: Enzyme- Linked Immunosorbent Assay

GC: Gas chromatography

GC/ MS: Gas chromatography/ Mass spectra

GP: Glycoprotein

HIV: Human immunodeficiency virus

HMBC: Heteronuclear multiple bond correlation

HMQC: Heteronuclear multiple quantum correlation

^{1}H-NMR: Nuclear magnetic resonance

IN: Integrase

IR: Infra red

MIC: Minimal inhibitory concentration

MRC: Medical Research Council

NOESY: Nuclear overhauser effect spectroscopy

RNA: Ribonuclease

RT: Reverse transcriptase

TLC: Thin layer chromatography

UV: Ultra violet

WHO: World Health Organization

SUMMARY

Antimicrobial activity of compounds isolated from *Lippia javanica* (Burm.f.) Spreng and *Hoslundia opposita* against *Mycobacterium tuberculosis* and HIV-1 Reverse transcriptase

by

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Degree: PhD Plant Science

For centuries medicinal plants have been used all over the world for the treatment and prevention of various ailments, particularly in developing countries where infectious diseases are endemic and modern health facilities and services are inadequate. In recent years the use of and search for drugs derived from plants have been accelerated. Ethnopharmacologists, botanists, microbiologists, and natural-product chemists are trying to discover phytochemicals and "leads" which could be developed for the treatment of infectious diseases. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties**.** The evaluation of these plants for biological activity is

necessary, both to substantiate their use by communities, and also to discover possible new drug or herbal preparations.

Twenty five plants selected through ethno-botanical surveys in Mozambique which are used to treat respiratory diseases, wounds, viruses, stomach ailments and etc., were collected and investigated for antimicrobial activity. Acetone extracts of selected plants were tested for antibacterial, antimycobacterial and anti-HIV-1 activity. Antibacterial activity was evaluated using the agar diffusion method. Five Gram-positive (*Bacillus cereus, Bacillus pumilus, Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis*) and five Gram-negative (*Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Serratia marcescens*) bacterial species were used in this study.

The extracts of each plant were tested at concentrations ranging from 0.125 to 5.0 mg/ml. Most of the plant extracts inhibited the growth of the Gram-positive microorganisms. The minimum inhibitory concentration of eight plants (*Cassia abbreviata, Elephanthorrhiza elephantina, Hemizygia bracteosa, Hoslundia opposita, Momordica balsamina, Rhoicissus tomentosa* and *Salvadora australis*) against Gram-positive bacteria was found to be 0.5 mg/ml. Gram-positive bacteria were found to be susceptible to extracts of *Lippia javanica* at concentration of 0.125 mg/ml. Among the 22 acetone extracts tested, two were found to have activity against Gram-negative bacteria at a concentration of 5.0 mg/ml (*Adenia gummifera* and *Momordica balsamina*). *Rhoicissus revoilli* inhibited *E. cloacae*, a Gram-negative strain, at a concentration of 2.5 mg/ml.

To evaluate antimycobacterium activity ten plants species were tested against H37Rv, a drug-sensitive strain of *Mycobacterium tuberculosis* at concentrations ranging from 0.5 to 5.0 mg/ml using BACTEC radiometric method. Four of the plant species tested (C*assia abbreviata, Hemizigya bracteosa, Lippia javanica* and *Melia azedarach*) were observed to be active against the H37Rv. (ATCC 27294) strain of TB at a concentration of 0.5 mg/ml which was the lowest concentration used in this study.

Seventeen plant species, were screened for anti-HIV bioactivity in order to identify their ability to inhibit the enzymes glycohydrolase (α-glucosidase and β- glucuronidase) and eleven species were further tested against Reverse transcriptase. It was found that 8 plant species (*Cassia abbreviata, Elephantorrhiza elephantina*, *Rhoicissus tomentosa, Pseudolachnostylis maprouneifolia, Lippia javanica*, *Litogyne gariepina, Maerua junceae and Momordica balsamina*) showed inhibitory effects against α-glucosidase and β-glucuronidase at a concentration of 200 µg/ml. The results of the tests revealed that the plant extracts of *Melia azedarach* and *Rhoicissus tomentosa* appeared to be active, showing 49 and 40% inhibition of the enzyme activity respectively.

Lippia javanica was found to have the best activity exhibiting a minimum inhibitory concentration of 0.125 mg/ml. The extracts also showed positive activity against *Mycobacterium tuberculosis* at concentration of 0.5 mg/ml and HIV-enzyme glycohydrolase was (α-glucosidase and β-glucuronidase) inhibited by 62 % and 73 % respectively. Considering its medicinal use local for HIV and various infections, it was therefore, selected for identifying its bioactive constituents. In the initial screening of

plants used in Mozambique *Hoslundia opposita* demonstrated good antitubercular activity. It was therefore, selected to identify its bioactive constituents.

A Phytochemical investigation of *L. javanica* led to the isolation of eight compounds, 4-ethyl-nonacosane (**1**), (*E*)-2(3)-tagetenone epoxide (**2**), myrcenone (**3**), piperitenone (**4**), apigenin (**5**), cirsimaritin (**6**), 6-methoxyluteolin 4'-methyl ether (**7**), 6-methoxyluteolin and 3',4',7-trimethyl ether (**8**). Three known compounds, 5,7-dimethoxy-6-methylflavone (**9**), hoslunddiol (**10**) and euscaphic acid (**11**) were isolated from *H. opposita*. This is the first report of compounds (**1**), (**2**), (**5-8**) from *L. javanica* and of compound (**9**) from *H. opposita*. The compounds were tested against *Mycobacterium tuberculosis* and HIV-1 reverse transcriptase for bioactivity. It was found that compounds (**2**), (**4**) and (**9**) inhibited the HIV-1 Reverse transcriptase enzyme by 91%, 53% and 52% respectively at 100 µg/ml. Of all the compounds tested against a drug-sensitive strain of *Mycobacterium tuberculosis*, euscaphic acid (**11**) was found to exhibit a minimum inhibitory concentration of 50 µg/ml against this strain.

The present study has validated scientifically the traditional use of *L. javanica* and *H. opposita* and a few other Mozambican medicinal plants to some extent.

LITERATURE REVIEW

1.1 Introduction

Herbal medicine has a long history in the treatment of several kinds of disease (Holm *et al*., 1998). Their use for the treatment of disease has been practised by man for many years and is still being widely practised even today (Kokwaro, 1993). For many years, people have developed a store of empirical information concerning the therapeutic values of local plants before orthodox medical practice appeared. Through periods of trial, error, and success, these herbalists and their apprentices have accumulated a large body of knowledge about medicinal plants. According to Iwu *et al.* (1999) the first generation of plant drugs were usually simple botanicals employed in more or less their crude form. Several effective medicines used in their natural state were selected as therapeutic agents based on empirical study of their application by traditional societies from different parts of the world.

Following the industrial revolution, a second generation of plant drugs emerged based on scientific processing of the plant extracts to isolate ″their active constituents″. Plant materials remain an important component in combating serious diseases in the world; for the therapeutic approach to several pathologies. Interest in medicinal plants has been overwhelming in the recent times especially as an important source of medication/health care. Currently, the global market for medicinal plants has been estimated to be around US $62 billion and the demand is growing rapidly (Indian Council of Medical Research, 2003). It is globally recognised that medicinal plants

play a significant role in providing health benefits to human beings. The World Health Organization (2000) has estimated that 80 % of the inhabitants of the world rely mainly on traditional medicines for their primary health care needs, and it may be presumed that a major part of traditional healing involves the use of plant extracts or their active principles**.**

Infectious diseases account for approximately one-half of all deaths in tropical countries (Iwu, 1999). Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties. Historically, plants have provided a good source of anti-infective agents. The isoquinoline alkaloid, emetine, obtained from the underground part of *Cephaelis ipecuanha*, and related species, have been used for many years as an amoebicidal drug for the treatment of abscesses due to the spread of *Escherichia histolytica* infections. Quinine, an alkaloid that occurs naturally in the bark of the *Cinchona* tree, is another important drug of plant origin with a long history of usage against malaria. The higher plants have made important contributions in areas beyond anti-infective, such as cancer therapies. Scientists from divergent fields are investigating plants with an intention to discover valuable phytochemicals. Laboratories all over the world have found literally thousands of phytochemicals which have inhibitory effects on all types of microorganisms *in vitro* (Cown, 1999).

1.2 The value of plants used in ethnomedicine for drug discovery

Medicinal plants provide a rich source of raw materials for primary health care in Africa and other parts of the developing world. According to Fabricant & Farnsworth (2001) the goals of using plants as sources of therapeutic agents are: 1) to isolate bioactive compounds for direct use as drugs; 2) to produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity and/ or lower toxicity**;** 3) to use agents as pharmacologic tools; 4) to use the whole plant or part of it as a herbal remedy. Notable examples were quinine from *Cinchona pubescens*, reserpine from *Rauvolfia serpentine* and taxol from *Taxus spp*. Various other plant based drugs are listed in Table 1.1. The sequence for development of pharmaceuticals usually begins with the identification of active lead molecules, detailed biological assays, and the formulation of dosage forms. This is followed by several phases of clinical studies designed to establish safety, efficacy and the pharmacokinetic profile of the new drug (Iwu *et al.*, 1999).

During the last few decades, there has been a resurgence of interest in plants as source of medicines and of novel molecules for use in the elucidation of physiological/biochemical phenomena. There is the worldwide **g**reen revolution, which is reflected in the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs. Furthermore, underlying this upsurge of interest in plants is the fact that many important drugs in use today were derived from plants or from starting molecules of plant origin: digoxin/digitoxin, the vinca alkaloids, reserpine and tubocurarine are some important examples (Iwu *et al.*, 1999).

Table1.1 Drugs from plants (Ali & Azhar, 2000)

Drug	**Disease**	**Plant species**	**Family**
Ajmaline	Arrhytmia	*Rauvolfia* spp.	Apocynaceae
Vinblastine	Hodgkin's disease	*Catharanthus roseus*	Apocynaceae
Strophanthin	Congestive heart failure	*Strophanthus gratus*	Apocynaceae
Deserpidine	Hypertension	*Rauvolfia canescens*	Apocynaceae
Rescinnamine	Hypertension	*Rauvolfia serpentina*	Apocynaceae
Reserpine	Hypertension	*Rauvolfia serpentina*	Apocynaceae
Proscillaridin	Cardiac malfunction	*Drimia maritima*	Liliaceae
Protoveratrine	Hypertension	*Veratrum album*	Liliaceae
Colchicine	Gout	*Colchicum autumnale*	Liliaceae
Demecolicine	Leukemia, limphomata	*Colchicum autumnale*	Liliaceae
Atropine	Ophthalmology	*Atropa belladona*	Solanaceae
Scopolamine	Motion sickness	*Datura stramonium*	Solanaceae
Ipratropium	Bronchodilator	*Hyoscyamus niger*	Solanaceae
Hyoscyamine	Anticholinergic	*Hyoscyamus niger*	Solanaceae
Stigmasterol	Steroidal precursor	*Physostigma venonosum*	Fabaceae
Dicoumarol	Thrombosis	*Melilotus officinalis*	Fabaceae
Psoralen	Vitiligo	*Psoralea corylifolia*	Fabaceae

Table1.1 (continued)

Drug	Disease	Plant species	Family
Physostigmine	Glaucoma	*Physotigma venenosum*	Fabaceae
Morphine	Analgesic	*Papaver somniferum*	Papaveraceae
Noscapine	Antitussive	*Papaver somniferum*	Papaveraceae
Cocaine	Analgesic, antitussive	*Papaver somniferum*	Papaveraceae
Papaverine	Antispasmodic	*Papaver somniferum*	Papaveraceae
Quinidine	Cardiac arrhytmia	*Cinchona pubescens*	Rubiaceae
Quinine	Malaria prohylaxis	*Cinchona pubescens*	Rubiaceae
Emetine	Amoebic dysentery	*Cephaelis ipecacuanha*	Rubiaceae
Ipecac	Emetic	*Cephaelis ipecacuanha*	Rubiaceae
Aspirin	Analgesic, inflamation	*Filipendula ulmaria*	Rosaceae
Benzoin	Oral disinfectant	*Styrax tonkinensis*	Stracaceae
Camphor	Rheumatic pain	*Cinnamomum camphora*	Lauraceae
Ephedrine	Bronchodilator	*Ephedra sinica*	Ephedraceae
Eugenol	Toothache	*Syzygium aromaticum*	Myrtaceae
Papain	Attenuates	*Carica papaya*	Caricaceae
Picrotoxin	Barbiturate antidote	*Anamirta cocculus*	Menispermaceae
Picrotoxin	Glaucoma	*Pilocarpus jaborandi*	Rutaceae
Sennoside A, B	Laxative	*Cassia angustifolia*	Fabaceae

Table1.1 (continued)

Drug	Disease	Plant species	Family
Teniposide	Bladder neoplasms	*Podophyllum peltatum*	Berberidaceae
Xanthotoxin	Vitiligo	*Ammi majus*	Apiaceae
Caffeine	Stimulant	*Camellia sinenis*	Theaceae
Theophylline	Diuretic, asthma	*Camellia sinenis*	Theaceae
Digitoxin	Atrial fibrillation	*Digitallis purpurea*	Scrophulariaceae

Laboratories around the world are engaged in the screening of plants for biological activity with therapeutic potential. The potential of higher plants as sources for new drugs is unexplored (Hostettman *et al*., 1996). Among more than 250 000 species of higher plants, only about 5-10 % has been investigated chemically for the presence of biological active compounds (Balandrin *et al*., 1993; Ayensu and De Filipps, 1978).

1.3 Antiviral compounds from plants

Many antiviral agents have been isolated from plant sources and have been partly or completely characterised. An antiviral may be defined as a product that is able, *in vitro* or *in vivo*, to directly or indirectly reduce the infectious viruses in the host cell. The discovery of antiviral agents from plants and other natural sources has assumed a sense of urgency (Hudson & Towers, 1991). Table 1.2 summarizes isolated antiviral compounds studied, as well as their targets of action**.** Since a retrovirus, designated Human Immunodeficiency Virus (HIV), was isolated and identified as the etiologic

agent of the Acquired Immune Deficiency Syndrome (AIDS), numerous compounds have been evaluated for their inhibitory effects on HIV replication *in vitro* (Ito *et al*., 1987). Effective therapies for HIV infection are being sought far and wide, in the natural world as well as in laboratories (Cown, 1999). For example, benzylisoquinoline alkaloid, 'papaverine', has been shown to have a potent inhibitory effect on the replication of several viruses including HIV.

A traditionally used tuber found growing along the banks of the Zambezi River and used commonly throughout southern Africa has become a popular traditional treatment for HIV-related illnesses. It is widely called the 'African potato', but the botanical name is *Hypoxis hemerocallidea* (formerly *H. rooperii*) and it has been traditionally used as food and medicine. The tuber is reported to help maintain or increase CD4-cells and boost cellular immunity in the body. Traditional health practitioners in southern Africa use it for managing HIV infections, cancer, TB, influenza, arthritis, psoriasis and common cold (Bodeker, 2003).

1.4 Mozambican traditional medical practice

In Mozambique, as in most developing countries, human health services are still very poor and are compounded by many people living in rural areas several kilometers from a health center. Modern health services have not been provided to the greater part of the rural areas of the country. What are available to this sector of the population are their own indigenous medicines, especially the folk herbal medicines. These remedies are fairly well accepted, easily available and bear at minimal cost.

Table 1.2 Compounds isolated from higher plants with antiviral activity against animal or human viruses[a] (Vanden Berghe *et al*. 1993)

Plant derived compounds	**Origin**	**Target (s)**
Methylgallate	*Sapium sebiferum*	Herpes simplex
Gallotannins	*Spondias mombia*	Coxsackie B virus Herpes simplex virus
Tetragalloyl quinic acids	*Turkish and Chinese galls*	HIV reverse transcriptase
Quinovic acid glycosides (triterpenes)	*Uncaria tomentosa*	Vesicular stomatitis virus
Quinovic acid glycosides (triterpenes)	*Guettarda platypoda*	Rhino virus type1 B
Glycyrrhizin	*Glycyrrhiza radix*	Polypeptide phosphorylation, HIV
Castanospermine (alkaloids)	*Castanospermum australe*	Cytomegalo virus HIV
5, 7, 4'- Trihydroxy- 8-methoxyflavone and others	*Scutellaria baicalensis*	Influenza A virus
Isoflavonic glycoside	*Ulex europaeus*	Herpes simplex virus Polio virus
Triterpenes	*Euptelea polyandra*	Epstein Barr virus activation
Gossypol (polyphenols)	Cotton seed	HIV reverse transcriptase
Dextro-odorinol (alkaloids)	*Aglaia roxburghiana*	Ranikhet disease virus
Alkaloids	Amaryllidaceae	*Herpes simplex virus*
Citrusinine I (acridone alkaloid)	Citrus	*Herpes simplex virus* Cytomegalo virus
Alkaloids	*Chelidonium majus*	Adenovirus 12 and 5 *Herpes simplex virus*
Sesquiterpene glycosides	*Calendula arvensis*	Vesticular stomatitis virus Rhinovirus type I B

Aloe emodin (Anthroquinones)	*Aloe barbadensis*	Enveloped virus (virucidal)
Hypericin and pseudohypericin	Species of *Hypericum*	Retroviruses
Hypericin	*Hypericum triquetrifolium*	Herpes simplex virus Influenza A virus
Lignins	*Pinus parviflora*	Influenza A virus
α-(-) Peltatin (lignans)	*Amanoa oblongifolia*	Sindbis virus Murine cytomegalo virus
Lectins	*Narcissus pseudonarcissus* *Listeria ovata*	Cytomegalo virus
Plant proteins	*Gelonium multiflorum* *Dianthus caryophyllus*	HIV
Trichosanthin and other proteins	*Trichosanthes kirilowii*	HIV
Fulvoplumierin (iridoids)	*Plumeria rubra*	HIV reverse transcriptase
Allicin (sulfur compounds)	*Allium sativum*	Virucidal activity
Prunellin (sulfated polysaccharides)	*Prunella vulgaris*	HIV
Phloroglucinol derivates (polyphenols)	*Mallotus japonicus*	Herpes simplex virus
Catalpol (iridoids)	*Picrorrhiza kurroa*	Hepatitis B virus
Epilupeol (triterpenes)	*Vicoa indica*	Ranikhet disease virus

[a] All compounds were isolated or studied after 1987.

The traditional use of medicinal plants in Moçambique has been well documented (Yansen & Mendes, 2001). However, the effectiveness of these plants has not been scientifically evaluated. There is a lack of scientific validation and no documented evidence of efficacy is found in particular with reference to use against microbial and viral complaints. The present study was undertaken to test a few medicinal plants

collected in Mozambique for their activity against a variety of human pathogens namely: Gram-positive and Gram-negative bacteria, Human Immunodeficiency Virus (HIV) and *Mycobacterium tuberculosis.*

1.5 Hypothesis and motivation of study

Natural product research continues to provide a tremendous variety of lead structures, which are used as templates for the development of new drugs by the pharmaceutical industry. Many of the plants studied have shown very promising activity in the area of antiviral agents (Table 1.2). Also many species of plants have been found to be active against a wide variety of micro-organisms. Among the more than 250 000 species of higher plants, only a small percentage of about 5-10 % have been phytochemically investigated (Nahrsted, 2002; Ayensu and De Filipps, 1978) and an even smaller fraction has been submitted to biological or pharmacological screenings (Hostettmann, 1991). The plant kingdom still represents an enormous reservoir of new molecules to be discovered. There should be an abundance of drugs remaining to be discovered from plants.

The discovery of new antibacterial, anti-HIV and antituberculosis compounds from herbal remedies would assist in the development of new preparations to combat infectious diseases. Infectious diseases, TB and HIV cases are quite prevalent in Mozambique, particularly in rural areas where an astounding number and variety of plants are used by communities to treat these diseases without prior scientifically determined information. In this study, the antibacterial, antituberculosis and anti-HIV activities of the medicinal plants collected in Mozambique were examined. The

evaluation of these plants for biological activity is necessary, both to substantiate the use of these plants by healers, and also as a possible lead for new drugs or herbal preparations. This study will provide valuable information for further isolation of bioactive compounds from the studied plant species.

1.6 Objectives of the study

The primary objectives of this study were to investigate the antimicrobial and antiviral properties of medicinal plants collected in Mozambique by in *vitro screening* and secondly to isolate bioactive compounds from selected plants with antituberculosis, anti-HIV and antibacterial activity.

The specific objectives of this study were to:

- Determine antibacterial, antitubercular and antiviral (anti-HIV) activities of the crude extracts of selected medicinal plants from Moçambique.
- Isolate, identify and determine the structures of the active principles from the one or two samples which exhibit potent antimicrobial activity.
- Determine the antibacterial, antitubercular and anti-HIV activity of the purified compounds.
- Determine the cytotoxicity of selected extracts and purified compounds.
- Establish a scientific basis for the use of these plants.

1. 7 Scope of this thesis

The importance of plant- based drugs has been discussed in **Chapter 1**.

In **Chapter 2** the antibacterial activity of acetone extracts of 22 Mozambican medicinal plants against Gram-positive and Gram-negative bacteria species, using the agar diffusion methods has been reported. **Chapter 2** further describes the antimycobacterial activity of 10 selected medicinal plants against *Mycobacterium tuberculosis***.**

In **Chapter 3** the *in vitro* activity of Mozambican medicinal plants against the human immunodeficiency virus has been documented. Determination of activity against HIV was based on inhibition of the enzymes α-Glucosidase, β-Glucuronidase and Reverse transcriptase (RT).

The isolation and identification of compounds from *Lippia javanica* and *Hoslundia opposita* is described in **Chapter 4** and **5**, respectively**.**

Chapter 6 describes the antibacterial activity of isolated compounds from *Lippia javanica* and *Hoslundia opposita*. **Chapter 7** describes the antimycobacterial bioassay of compounds isolated from *Lippia javanica* and *Hoslundia opposita*. **Chapter 8** documents the antiviral activity of isolated compounds from *Lippia javanica* and *Hoslundia opposita*. In **Chapter 9** the cytotoxicity of *Lippia javanica* and *Hoslundia opposita* plant extracts and bioactive isolated compounds is discussed.

Finally **Chapter 10** summarises the entire project, the importance of medicinal plants folkloric use and entails the recommendations from the findings of this study.

1.8 References

ALI MUHAMMAD SHAIQ & AZHAR IQBAL 2000. Treatment by natural drugs in Hamdard Medicus, vol. XLIII, no 2, Bait al-Hikmah at Madinat al-Hikmah**.**

AYENSU, E.S. & DE FILIPPS, R.A. 1978. Endangered and threatened plants of the United States. Washington, DC: Smithsonian Institution.

BALANDRIN, M.F., KINGHORN, A.D & FARNSWORTH, N.R. 1993. Plant-derived natural products in drug discovery and development: an overview. IN: Human medicinal agents from plants. Kinghorn, A.D. & Balandrin, M.F. (Eds.) American Chemical Society, Washington, D.C. ISBN 0-8412-2705-5. PP. 2-12.

BODEKER, G. 2003. Traditional medical knowledge, intellectual property rights & benefits sharing. University of Oxford Medical School & Chair, Global initiative for traditional systems (GIFTS) of Health, Oxford, UK.

COWN, M.M. 1999. Plant products as antimicrobial agents. Journal of Clinical Microbiology Rev. Vol. **12 (4):** 564-582.

FABRICANT, D.S. & FARNSWORTH, N.R.2001. The Value of plants used in Medicine for Drug Discovery. Environmental health perspectives. Volume 109/ Supplement 1/ March.

HOSTETTMAN, K., WOLFENDER, J.L., RODRIGUEZ, S. & MARSTON. A. 1996. Strategy in the search for bioactive plant constituents. IN: Chemistry, biological and pharmacological properties of African medicinal plants.

Proceedings of the First International IOCD Symposium. Hostettman K, Chinyanganga F, Maillard M, Wolfender, J L. (Eds.). ISBN 0-908307-59-4. pp. 21-42.

HOSTETTMAN, K. 1991. Methods in Plant Biochemistry. Assays for Bioactivity, Volume 6. Academic Press Limited, 24-28 Oval Road, London New1 7DX.

HOLM, G., HERBST, V. & TEIL, B. 1998. Brogenkunde. IN: Planta Medica (2001) **67:** 263-269.

HUDSON, J.B. & TOWERS, G.H.N., 1991. Therapeutic potential of plant photosensitizers. Pharmacol. Ther. 49, 181-222.

KOKWARO, J.O. 1993. Medicinal Plants of East Africa, Second Edition, Kenya Literature Bureau, Nairobi.

INDIAN COUNCIL OF MEDICAL RESEARCH 2003. Quality Standards of Indian Medicinal Plants, Volume 1. Ansari Nagar, New Delhi-110029, India.

ITO, M., NAKASHIMA, H., BABA, M., PAUWELS, R., DE CLERCQ, E. & SHIGETA, S. 1987. Inhibitory effect of glycyrrhizin in the in vitro infectivity and cytopathic activity of the human immunodeficiency virus HIV (HTLV-III/LAV) – Antiviral Res**.7:** 127

IWU, M.M., DUNCAN, A.R. & OKUNJI, C.O. 1999. New Antimicrobials of Plant. Origin. J. Janick (ed), ASHS Press, Alexandria, VA. Egypt.

YANSEN, P.C.M. & MENDES, O. 2001. Plantas medicinais. Seu uso tradicional em Moçambique tomos 1, 2, 3, 4, 5. Ministério da Saúde, Moçambique.

NAHRSTEDT, A. 2002. Screening of African medicinal plants for antimicrobial and enzyme inhibitory activity. Journal of Ethnopharmacology 80: 23-35.

WHO, 2000. Integration of traditional and complimentary medicine Into National Health care systems, 23 J. Manipulative & Physiological Therapeutics 139, 140.

ANTITUBERCULOSIS AND ANTIBACTERIAL ACTIVITY OF MEDICINAL PLANTS FROM MOZAMBIQUE

Abstract

Twenty two medicinal plants selected through a literature survey in Mozambique were investigated using the agar diffusion method for their antibacterial activity. Five Gram-positive and five Gram-negative bacterial species were used. Acetone extract of *Lippia javanica* showed inhibitory activity against Gram-positive bacteria, at a concentration of 0.125 mg/ml. The minimal inhibitory concentrations (MIC) of six other plant extracts were found to be 0.5 mg/ ml. Only extracts of *Adenia gummifera* and *Momordica balsamina* were found to have activity against Gram-negative bacteria at a concentration of 5.0 mg/ ml. Acetone extracts of ten plants species used for respiratory diseases were also tested against *Mycobacterium tuberculosis* using the BACTEC radiometric method. Four extracts showed activity against *M. tuberculosis* at 0.5 mg/ml.

2.1 Introduction

Man is host to a variety of pathogenic bacteria, protozoa and viruses. Persons who are deficient in the production of circulating antibodies are highly susceptible to respiratory infections by Gram-positive bacteria. Persons who are deficient in T-cell

functions, however, tend to succumb to infections by fungi and viruses, as well as to bacteria which grow predominantly intracellularly (Stanier *et al*., 1958). The pathogenicity of some of the bacterial species is significant because of their resistance to known antibiotics. The emergence of methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci and multiresistant Gram-negative bacteria has become a serious issue (Rao, 1998). In an earlier study it was found that 36 strains of *Bacillus cereus* were highly resistant to lincomycin, polymyxin B and penicillin G-cephalosporin (Arribas *et al*., 1988). Fifty methicillin-resistant strains of *S. aureus* were isolated at a hospital in Osaka between 1986 and 1990 of which a few were also found to be resistant to streptomycin and kanamycin (Kondo *et al*., 1991).

Tuberculosis (TB), an airborne lung infection, is becoming an epidemic in some parts of the world. It kills about 1 million children each year and it is estimated that between now and 2020, nearly 1 billion more people will be infected, 200 million people will get sick and 70 million will die from TB if control is not strengthened (World Health Organization, 1997). Moreover, TB has also been recognised as one of the most frequent opportunistic infections in persons suffering from the human immunodeficiency virus (HIV), particularly in Africa. Given the alarming incidence of drug resistance to strains of bacteria, there is a constant need for new and effective therapeutic agents (Bhavnani and Ballow, 2000).

Plants contain numerous biologically active compounds, many of which have been shown to have antimicrobial properties (Cowan, 1999). Ethnobotanical data are useful in the search for new antimicrobial agents and several bioactive compounds have been isolated from medicinal plants (Penna *et al.,* 2001).

In this study 25 medicinal plant species from Mozambique, were investigated for their antimicrobial activity. The plants selected are used for various infections,uberculosis

related symptoms such as chest pain, cough, etc. by Mozambicans. The effectiveness of these plants has not been scientifically evaluated. There is a lack of scientific validation and there is no documented evidence of efficacy particularly with reference to their use for antimicrobial complaints.

2.2.1 Materials and methods

2.2.2 Plant material

Different parts of the plants, (Table 2.1) were collected in 2002 from the south and central parts of Mozambique (Maputo, Chókwe, Massingir, Manica and Zambezia) Figure 2.1. The plants were identified at the HGWJ Schweickerdt herbarium of the University of Pretoria (PRU) and also at the herbarium of the South Africa National Biodiversity Institute, Pretoria (PRE). Voucher herbarium specimens have been submitted at the herbarium of the University of Pretoria.

2.2.3 Preparation of plant extracts

Various solvents have been used to extract plant metabolites. In this study acetone solvent was used for plants extraction. Acetone is very useful extractant because dissolve many hydrophilic and lipophylic components, is miscible with water, is volatile and has a low toxicity to the bioassay (Eloff, 1998).

Acetone extracts of each air-dried plant sample were prepared by stirring 50 g of the powdered plant material in 500 ml acetone for 48 hours. The extracts were filtered and concentrated to dryness at reduced pressure.. The resultant residue was later dissolved in acetone to a concentration of 100.0 mg/ ml.

Figure 2.1 Map of Mozambique with the location of the collected medicinal plants

Table 2. 1 Selected Mozambican medicinal plant investigated for antibacterial, antitubercular and anti-HIV activities

Plant species	Plant part collected	Voucher specimen	Medicinal uses	References
Adenia gummifera (Harv.) Harms (Passifloraceae)	Roots Leaf and stem	SM92062	Decoctions are administered for Malaria and Leprosy. Menorrhagia and infertility Biliousness Seediness or depression Decoctions bath is used for malaria. Emetic and as a cosmetic pigment on the	(Mabogo, 1990) (Watt & Breyer-Brandwijk, 1962) (Bryant, 1966) (Watt & Breyer-Brandwijk, 1962) (Gerstner, 1938)
Adenium multiflorum Klotzsch (Apocynaceae)	Plant	SM92063	The latex is widely used as an arrow poison in Limpopo (South Africa) and Mozambique	(Neuwinger, 1996)
Aloe marlothii A. Berger (Liliaceae)	Shoots	SM92064	Shoot decoction are used for stomach troubles Leaf and root decoctions are administered orally or as enemas for roundworm infestations. Chewed roots are used in enemas for babies. Leaf sap is applied to mothers' breasts to hasten weaning	(Watt & Breyer-Brandwijk, 1962) (Gerstner, 1939) (Hutchings, 1996)
Aloe parvibracteata Schönlond	Roots	SM92065	Used as dye source	(Van Wyk *et al*. 2000)

Table 2. 1 (continued)

Plant species	Plant part collected	Voucher specimen	Medicinal uses	References
Cassia abbreviata Oliv. (Fabaceae)	Root Bark and root	SM92066	Infusion for relief of toothache. It is used as dysentery and diarrhea remedy. Used for malaria	(Watt & Breyer- Brandwijk, 1962)
(*Catharanthus roseus* (L.) G.Don Apocynaceae)	Leaves Flowers Milk sap Root Root+leaves	SM92067	Rheumatism, menorrhagia Galactogogue Arthritis, gout, cancer Tea for blood cleansing Insect bites and warts Used as a diabetes remedy Venereal diseases For toothache, liver congestion Scury skin complaints Tonics, haemostatics vermifuges Used as purgative, emetics and depuratives	(Hutchings, 1996, Watt & Breyer-Brandwijk, 1962) (Watt and Breyer-Brandwijk, 1962) (Hutchings, 1996) (Watt and Breyer-Brandwijk, 1962) (Mabogo, 1990) (Watt & Breyer-Brandwijk, 1962)
Cissus quadrangularis L. (Vitaceae)	Leaves and pounded stems Stem leaves	SM92068	Burns and wounds Saddle sores on animals Gastro-intestinal complaints Washes for febrile pain and malaria Induce milk flow in cattle. In ointments for backache and body pain	(Oliver- Bever, 1986) (Dalziel, 1937). (Bhat *et al*. 1990) (Hedberg & Staugard, 1989;

Table 2. 1 (continued)

Plant species	Plant part colleted	Voucher specimen	Medicinal uses	References
Coccinia rehmannii Cogn. (Cucurbitaceae)	Tuber	SM92069	Used as pot-herb The fruit is edible.	(Watt & Breyer-Brandwijk, 1962)
Elephantorrhiza elephantina (Burch) Skeels (Fabaceae)	Roots	SM92070	Infusion used as an enema for dysentery and diarrhoea Fever, chest and stomach complaintþ‘ as love charms Intestinal disorders and syphilis Infertility in women and as aphrodisiacs For children who menstruate at an early age and to wipe the anus of a child with bloody diarrhoea.	(Bryant, 1966) (Gerstner, 1938) (Jacot Guillarmod, 1977) (Gelfand *et al*., 1985) (Hedberg & Staugard, 1989)
Gladiolus dalenii Van Geel (Iridaceae)	Root Corms	SM92071	Infusions of root are administered to sterile women. Corms are placed in seed- gourds as fertility charms to ensure a good harvest. The infusions of corms are administered as emetics for chest ailments thought to have been caused by sorcery, and are also taken as love charm emetics.	(Gerstner, 1941) (Hulme, 1954)

Table 2. 1 (continued)

Plant species	Plant part collected	Voucher specimen	Medicinal uses	References
Hemizygia bracteosa (Benth.) Briq. (Lamiaceae)	Leaves	SM92072	Repellent for mosquitoes	
Hoslundia opposita Vahl (Lamiaceae)	Leaves	SM92073	inter alia snake bite, conjunctivitis, epilepsy, chest pain, yellow fever, stomach troubles, and mental disorders. Infusions as a purgative, diuretic, febrifuge, antibiotic and antiseptic.	(Ayensu & De Filipps, 1978) (Onayade *et al*., 1989)
Lippia javanica (Burm.f.) Spreng. (Verbenaceae)	Leaves	SM92074	Infusions as tea to treat coughs, colds, fever and bronchitis. Influenza, measles, rashes, malaria, stomach problems and headaches Strong infusions are used topically for scabies and lice The leaves are sometimes smeared on the body as a protection against dogs and crocodiles. Treatment of HIV	(Van Wyk & Gericke, 2000; Smith, 1966; Watt & Breyer-Brandwijk, 1962 and Hutchings, 1996) (Smith, 1966; Watt and Breyer-Brandwijk, 1962; Hutchings, 2003, Hutchings & Van Staden, 1994) (Doke and Vilakazi, 1972) Hutchings, 2003)
Litogyne gariepina. (DC.) Anderb. (Astereaceae)	Leaves	SM92075	Unspecified parts are used for fevers Includes use as anthelmintic	(Doke & Vilakazi, 1972)

Table 2. 1 (continued)

Plant species	Plant part collected	Voucher specimen	Medicinal uses	References
Melia azedarach L. (Meliaceae)	Leaves	SM92076	The plant has been widely used in various countries as emetic and cathartic Anthelmintic. It is used as a tonic and antipyretic The decoction of the bark is used as a lotion on ulcers, syphilitic The trees is poisonous to animals	(Watt & Breyer-Brandwijk, 1962)
Maerua juncea Pax (Capparaceae)	Leaves	SM92077	Respiratory problems	Personal communication
Momordica balsamina L. (Cucurbitaceae)	runners Roots Leaves	SM92078	Cold infusion or decoctions of the runners are used to soothe squeamish stomachs Infusions of roots are used for intestinal complaints Infusions of leaves are administered as anti-emetics Bitter stomachic, purgatives and to reduce fever	(Bryant, 1996). (Hulme, 1954) (Mabogo, 1990) (Watt & Breyer-Brandwijk, 1962)
Ocimum americanum (Lamiaceae)	Leaves	SM92079	Used for hemorrhage of the nose inhale the smoke from burning the dried leaf	(Watt & Breyer-Brandwijk, 1962)

Table 2. 1 (continued)

Plant species	Plant part	Voucher specimen	Medicinal uses	References
Plectranthus fruticosus L' Hérit (Lamiaceae)	Leaves	SM92080	Cough and chest complaints	
Pseudolachnostylis maprouneifolia Pax (Euphorbiaceae)	Stem bark Roots	SM92081	Used for HIV treatment Smoke from burning roots is inhaled to treat pneumonia Bark extracts are used to treat diarrhea and venereal disease	Van Wyk *et al*., 2000; Palgrave, 1981)
Rhoicissus revoilli Planch. (Vitaceae)	Roots	SM92082		
Rhoicissus tomentosa (Lam.) Wild & R.B. Drumm (Vitaceae)	Roots	SM92083	Milk decoctions of roots are given as anthelmintics to calves	(Watt & Breyer-Brandwijk, 1962)
Salvadora australis Schweick. (Salvadoraceae)	Leaves	SM92084	Cough Smoke from burning leaves is inhaled to stop nosebleeds	(Arnold & Gulumian, 1984)
Salvadora persica L. (Salvadoraceae)	Leaves	SM92085	Cough	
Senna italica Mill. (Caesalpinaceae)	Leaves	SM92086	Used for burns and wounds	(Wat & Breyer-Brandwijk, 1962)

2.2.4 Antibacterial bioassay

Five Gram-positive bacteria, *Bacillus cereus* (ATCC 11778), *B. subtilis* (ATCC 6051), *B. pumilus* (ATCC 7061), *Staphylococcus aureus* (ATCC 12600), *Enterecoccus faecalis* (ATCC 292192) and five Gram-negative bacteria, *Enterobacter cloacae* (ATCC 13047), *Escherichia coli* (ATCC 11775) *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 33584) and *Serratia marcescens* (ATCC 1380) were tested for susceptibility to plant extracts. The bacteria were obtained from the Department of Microbiology and Plant Pathology, University of Pretoria. Each organism was maintained on a nutrient agar slant and was recovered for testing by growing them in fresh nutrient broth (No. 2, Biolab) for 24 hours**.** Before streaking, the culture was diluted to 1:10 with fresh sterile nutrient broth. The minimum inhibitory concentration (MIC) of the extracts was determined using the agar dilution method (Jorgensen *et al.,* 1999). The tested concentrations were 5.0, 2.5, 1.0, 0.5, 0.25, 0.125 and 0.062 mg/ml. Plant extracts were added to 5 ml of nutrient agar medium in Petri dishes and swirled carefully before congealing. The organisms were streaked in radial patterns on agar plates containing plant extracts (Figure 2.2), incubated at 37^{o}C and observed after 24 hrs (Mitscher *et al*., 1972). Plates containing only nutrient agar and 1% acetone without the plant extracts served as controls. In addition two plates containing streptomycin sulfate at concentrations of 100.0, 50.0 and 10.0 µg/ml served as positive controls. The MIC was regarded as the lowest concentration of the extracts that did not permit any visible growth when compared with that of the controls.

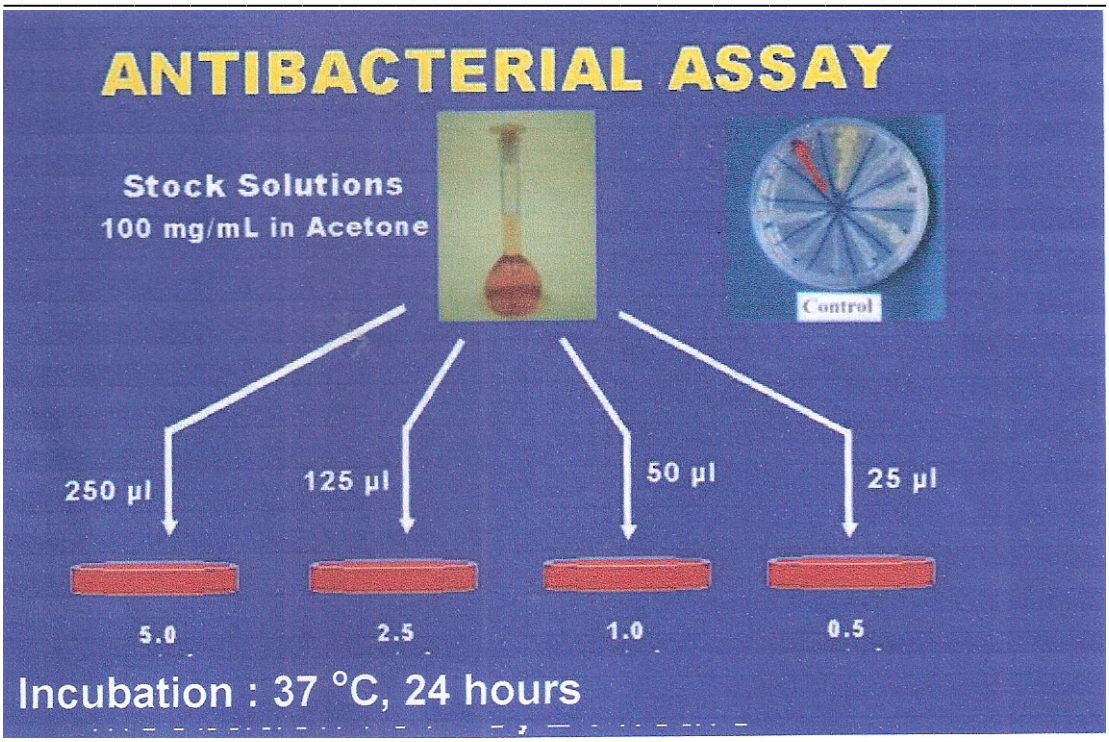

Figure 2.2 Antibacterial assay procedure

2.2. 5 Antimycobacterial bioassay

Among the 22 species, ten plants which showed good antibacterial activity (C*assia abbreviata, Elephanthorrhiza elephantina, Hemizygia bracteosa, Gladiolus dalenii, Hoslundia opposita, Lippia javanica, Litogyne gariepina, Melia azedarach*, *Rhoicissus tomentosa* and *Salvadora australis* used for respiratory diseases) were further tested against a drug sensitive strain of *Mycobacterium tuberculosis* H37Rv, (ATCC 27294), considered to be gram positive, using rapid radiometric respiratory technique with the BACTEC apparatus as described by Middlebrook *et al.*(1977). Solutions of the plant extracts were prepared by maceration of a requisite amount of the extracts in a known volume of dimethyl sulphoxide (DMSO) to obtain a

concentration of 100 mg/ml and stored at 4°C until used. Subsequent dilutions were done in DMSO and added to 4 ml of BACTEC 12B (7H12 medium) broth to achieve the desired final concentrations of 5.0, 2.5, 1.0, 0.5 and 0.25 mg/ml, together with PANTA (Becton Dickinson & Company), an antimicrobial supplement. Control experiments showed that the final amount of DMSO (1%) in the media had no effect on the growth of *M. tuberculosis*. BACTEC drug susceptibility testing was also done for the standard anti-TB-drugs, streptomycin (Sigma Chemical Co, South Africa) and ethambutol at concentrations of 6.0 and 7.5 µg/ml, respectively, against the H37Rv strain. The homogenized cultures (0.1 ml) of *M. tuberculosis*, yielding $1x10^4$ to $1x10^5$ colony-forming units per millilitre (CFU/ml), were inoculated in the vials containing the extracts as well as in the control vials (Lall and Meyer, 1999; Heifets *et al*., 1985). Three extract-free vials were used as controls (medium + 1% DMSO): two vials (V1) were inoculated in the same way as the vials containing the extracts, and the other (V2) was inoculated with a 1:100 dilution of the inoculum (1:100 control) to produce an initial concentration representing 1% of the bacterial population ($1x10^2$ to $1x10^3$ CFU/ml) found in the vials containing extracts.

The MIC was defined as the lowest concentration of the extract that inhibited more than 99% of the bacterial population. When mycobacteria grow in 7H12 medium containing ^{14}C-labeled substrate (palmitic acid), they utilize the substrate and $^{14}CO_2$ is produced. The amount of $^{14}CO_2$ detected reflects the rate and amount of growth occurring in the sealed vial, and is expressed in terms of the Growth Index (GI). Inoculated bottles were incubated at 37°C and each bottle was assayed every day to measure GI at about the same hour until cumulative results were interpretable. The

difference in the GI values of the last two days is designated as ΔGI. The GI reading of the vials containing the test plant extract was compared with the control vial (V2). Readings were taken until the control vials, containing a 100 times lower dilution of the inoculums than the test vials, reached a GI of 30 or more. If the ΔGI value of the vial containing the test plant extract was less than the control, the population was reported to be susceptible to the extract. Each test was replicated three times.

2. 3 Results and discussion

2.3.1 The antibacterial bioassay

Most of the plant extracts inhibited the growth of the Gram-positive microorganisms (Table 2.2). The minimum inhibitory concentration of eight plants (*Cassia abbreviata, Elephanthorrhiza elephantina, Hemizygia bracteosa, Hoslundia opposita, Momordica balsamina, Rhoicissus tomentosa* and *Salvadora australis*) against Gram-positive bacteria was found to be 0.5 mg/ml. Among the 22 acetone extracts tested, two were found to have activity against Gram-negative bacteria at a concentration of 5.0 mg/ml (*Adenia gummifera* and *Momordica balsamina*). *Rhoicissus revoilli* inhibited *E. cloacae*, a Gram-negative strain, at a concentration of 2.5 mg/ml. The resistance of Gram-negative bacteria to plant extracts has been documented previously (Meyer and Afolayan, 1995). These observations are likely to be the result of the differences in cell wall structure between Gram-positive and Gram-negative bacteria. It has been stated that the outer membrane of Gram-negative bacteria acts as a barrier to many environmental substances, including antibiotics (Tortora *et al*., 2001).

The reference antibiotic, streptomycin sulfate inhibited the growth of all the bacterial species tested in this study at 10 µg/ml except, *Pseudomonas aeruginosa* and *Serratia marcescens* which were inhibited at 50 µg/ml and 100 µg/ml respectively.

Gram-positive bacteria were found to be susceptible to extracts of *Lippia javanica* at concentration of 0.125 mg/ml similar to the reports of other researchers previously (Matingo and Chagonda, 1993). These results confirm the findings of other researchers where it was found that acetone extracts of *C. abbreviata*, showed significant inhibition against *B. pumulis*, *B. subtilis* and *S. aureus* at 0.5 mg/ml (Kambizi and Afolayan, 2001). Similar to the reports of the other researchers previously Matingo and Chagonda, (1993).

Khan *et al.* (2001) reported that a previous evaluation of antibacterial activity of the dichloromethane fraction of the stem bark of *Melia azeradarach* showed inhibition at the highest levels used. In another study, extracts of the leaves of *Salvadora persica* L. were found to have an antimicrobial effect on *Streptococcus faecalis* (Almas, 1999, Almas 2001). The antibacterial properties of *Hemizygia* species has already been reported by Kato *et al.* (1996).

Table 2.2. Antibacterial activity of Mozambican medicinal plants

	MIC [a] (mg ml^{-1})									
Plant species	Ba.(+)	Bp (+)	Bs (+)	Sa (+)	Ef (+)	Ecl (-)	Ec (-).	Kp (-)	Pa (-)	Sm (-)
Adenia gummifera	1.0	1.0	1.0	1.0	1.0	5.0	5.0	5.0	5.0	5.0
Cassia abbreviate	0.5	0.5	0.5	0.5	0.5	na[b]	na[b]	na[b]	na[b]	na[b]
Catharanthus roseous	5.0	5.0	5.0	5.0	5.0	na[b]	na[b]	na[b]	na[b]	na[b]
Cissus quadrangularis	5.0	5.0	5.0	5.0	5.0	na[b]	na[b]	na[b]	na[b]	na[b]
Coccinia rehmannii	5.0	5.0	5.0	5.0	5.0	na[b]	na[b]	na[b]	na[b]	na[b]
Elephanthorrhiza elephantine	0.5	0.5	0.5	0.5	0.5	na[b]	na[b]	na[b]	na[b]	na[b]
Hemizygia bracteosa	0.5	0.5	0.5	1.0	1.0	na[b]	na[b]	na[b]	na[b]	na[b]
Hoslundia opposita	0.5	0.5	0.5	0.5	0.5	na[b]	na[b]	na[b]	na[b]	na[b]
Lippia javanica	0.125	0.125	0.125	0.125	0.125	na[b]	na[b]	na[b]	na[b]	na[b]
Litogyne gariepina	2.5	2.5	2.5	2.5	2.5	na[b]	na[b]	na[b]	na[b]	na[b]
Gladiolus dalenii	5.0	na[b]	5.0	5.0	5.0	na[b]	na[b]	na[b]	na[b]	na[b]
Maerua juncea	1.0	1.0	1.0	1.0	1.0	na[b]	na[b]	na[b]	na[b]	na[b]
Melia azedarachta	5.0	na[b]	5.0	5.0	5.0	na[b]	na[b]	na[b]		
Momordica balsamina	0.5	0.5	0.5	0.5	0.5	5.0	5.0	5.0	5.0	5.0
Ocimum americanum	2.5	2.5	2.5	2.5	2.5	na[b]	na[b]	na[b]	na[b]	na[b]

Table 2.2 (continued)

	MIC [a] (mg ml^{-1})									
Plant species	Ba.(+)	Bp (+)	Bs (+)	Sa (+)	Ef (+)	Ecl (-)	Ec (-).	Kp (-)	Pa (-)	Sm (-)
Plectranthus fruticosus	2.5	2.5	2.5	2.5	2.5	na[b]	na[b]	na[b]	na[b]	na[b]
Pseudolachnostylis maprouneifolia	5.0	5.0	5.0	5.0	5.0	na[b]	na[b]	na[b]	na[b]	na[b]
Rhoicissus revoilli	1.0	1.0	1.0	1.0	1.0	2.5	na[b]	na[b]	na[b]	na[b]
Rhoicissus tomentosa	0.5	0.5	0.5	0.5	0.5	na[b]	na[b]	na[b]	na[b]	na[b]
Salvadora australis	0.5	0.5	0.5	0.5	0.5	na[b]	na[b]	na[b]	na[b]	na[b]
Salvadora persica	2.5	2.5	2.5	2.5	2.5	na[b]	na[b]	na[b]	na[b]	na[b]
Senna italica	2.5	2.5	2.5	2.5	2.5	na[b]	na[b]	na[b]	na[b]	na[b]

Ba (+) = *Bacillus cereus*, Bp (+) = *Bacillus pumilis*, Bs (+) = *Bacillus subtilis,* Sa (+) = *Staphylococcus aureus,* Ef (+) = *Enterococcus faecalis,* Ecl (-) = *Enterobactercloacae,* Ec (-) = *Esherichia coli*, Kp (-) = *Klebsiella peneumonia,* Pa (-) = *Pseudomonas aeruginosa,* Sm (-) = *Serratia marcescens*

(+) or (-) = Gram reaction

MIC[a], minimal inhibitory concentration

na[b], not active at the highest concentration (5.0 mg ml^{-1}) tested

2. 3.2. The antimycobacterial bioassay

Four of the plant species tested (C*assia abbreviata, Hemizigya bracteosa, Lippia javanica* and *Melia azedarach*) were observed to be active against the H37Rv. (ATCC 27294) strain of TB at a concentration of 0.5 mg/ml which was the lowest concentration used in this study (Table 2.3). *Gladiolus dalenii, Rhoicissus tomentosa* and *Salvadora australis* showed weak antituberculosis activity. According to a previous report on the antitubercular activity of another *Lippia* species (*Lippia turbinata*) complete inhibition of the growth of *M. tuberculosis* was observed by MeOH-CH_2CL_2 extracts obtained from the aerial parts (Timmermann *et al*., 2001). This can explains the wide use of *Lippia* species for respiratory treatment disorders (Pascual *et al.,* 2001).

Table 2.3 Effect of plant extracts on the growth of the sensitive strain (H37Rv) of *Mycobacterium tuberculosis*

Plant species	MIC[a] (mg ml^{-1})	ΔGI[b] values of plant extracts		ΔGI values of the control vials
Cassia abbreviata	0.5	9.3 ± 7.5	(S)	26.5 ± 4.7
Elephantorrhiza elephantina	1.0	45.0 ± 16.1	(R)	26.5 ± 4.7
Gladiolus dalenii	2.5	27.7 ± 5.8	(S)	26.5 ± 4.7
Hemizigya bracteosa	0.5	22.0 ± 1.0	(S)	26.5 ± 4.7
Hoslundia opposita	1.0	9.5 ± 0.7	(S)	26.5 ± 4.7
Lippia javanica	0.5	19.7± 5.1	(S)	26.5 ± 4.7
Litogyne gariepina	1.0	27.7± 28.9	(S)	26.5 ± 4.7
Melia azedarach	0.5	10.3 ± 5.8	(S)	26.5 ± 4.7
Rhoicissus tomentosa	2.5	8.0 ± 3.6	(S)	26.5 ± 4.7
Salvadora australis	2.5	105 ± 7.8	(R)	26.5 ± 4.7

[a]minimal inhibitory concentration, [b]ΔGI values are average ± standard deviation, S, susceptible; R; resistant

2.3.3 Conclusion

The evaluation of plants used in traditional medicine is necessary. In this investigation, a number of plants exhibited promising activity against a variety of bacteria and *Mycobacterium tuberculosis*. It is concluded that the demonstration of inhibitory activities of the tested plants revealed their value in traditional medicine and supports the enormous role of medicinal plants in primary health care.

The results corroborate the importance of ethnopharmacological surveys in selection of plants for bioactivity screening. The results obtained represent a worthwhile expressive contribution to the characterization of the antibacterial and

antimycobacterial activities of plant extracts of traditional medicine plants from Mozambican flora.

Subsequently, bio-guided fractionation will be conducted on plants showing potential activity to identify the active compounds

2. 5 References

ALMAS, K. 1999. The antimicrobial effects of extracts of *Azedarachta indica* (Neem) and *Salvadora persica* (Arak) chewing stricks. Indian Journal of Dental Research. **10 (1):** 23-6.

ALMAS, K. 2001. The antimicrobial effects of seven different types of Asian chewing sticks. Odonto-Stomatologie Tropicale **24 (96):** 17-20.

AYENSU, E.S. & DE FILIPPS, R.A. 1978. Endangered and Threatened Plants of the United States. Washington, DC: Smithsonian Institution.

ARNOLD, H.J. & GULUMIAN, M.1984. Pharmacoepia of traditional medicine Venda. Journal of Ethnopharmacology **12:** 35-74.

ARRIBAS, G.M.L., PLAZA, C.J., ROSA, M.C.& MOSSO, M.A. 1988. Characterization of *Bacillus cereus* strains isolated from drugs and evaluation of their toxins. Journal of Applied Bacteriology **64 (3):** 257-264.

BHAVNANI, S.M. & BALLOW, C.H. 2000. New agents for Gram-positive bacteria Bonney. N Current opinion in Microbiology **3:** 528-534.

BHAT, R.B., TEJERE, E.O. & OLAPIDO, V.T. 1990. Ethnobotanical studies from Central Nigeria**.** Economic Botany. **44**: 382-390.

BRYANT, A.T. 1996. Zulu medicine and medicine men. C. Struik, Cape Town, ISBN O-908379-44-7.

COWAN, M.M. 1999. Plant products as antimicrobial agents. Clinical microbiology reviews **12:** 564-582.

DALZIEL, J.M. 1937. The useful plants of west tropical Africa. Crowns agents.

DOKE, C.M. & VILAKAZI, B.W. 1972. Zulu-Englis Dictionary. 2nd edn Witwatersrand University Press, Johannesburg.

ELOFF, J.N. 1988. Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethnopharmacology, **60:** 1-8.

GELFAND, M., MAVI, S., DRUMMONDO, R.B. & NDEMERA, B. 1985. The traditional medical practitioner in Zimbabwe. Mambo Press, Gweru, Zimbabwe.

GERSTNER, J. 1938. Preliminary checklist of Zulu names of plants with short notes. Bantu Studies **12 (3):** 215-236), **(4)**: 321-342.

GERSTNER, J. 1938. Preliminary checklist of Zulu names of plants with short notes. Bantu Studies **13 (1):** 49-64), **(2)**: 131-149, **(4):**307:326.

GERSTNER, J. 1941. Preliminary checklists of Zulu names of plants with short notes Bantu Studies **15(3):** 277-301, **(4):** 369-383.

HEDBERG, G. & STAUGARD, F. 1989. Traditional medicinal plants: Traditional medicine in Botswana. Ipeleng, Gaberone, Botswana

HEIFETS, L.B., ISEMAN, M.D., COOK, J.L., LINDHOLM-LEVY, P.J. & DRUPA, I. 1985. Determination of *in vitro* susceptibility of *Mycobacterium tuberculosis* to cephalosporin's by radiometric and conventional methods. Antimicrobial agents

and Chemotherapy **27:** 11-15.

HULME, M.M. 1954. Wild flowers of Natal. Shuter & Shooter, Pietermaritzburg, South Africa

HUTCHINGS, A. 2003.Enhancing HIV/AIDS support therapy with indigenous herbal preparations- a clinic experience. Joint International Conference SAAB & ISE, University of Pretoria, South Africa.

HUTCHINGS, A., SCOTT, A.H., LEWIS, G. & CUNNINGHAM, A.1996. Zulu medicinal plants, an inventory. University of Natal Press, South Africa.

HUTCHINGS. A. & Van Staden, J. 1994. Plants used for stress-related ailments in traditional Zulu, Xhosa & Sotho medicine. Part: plants used for headaches. Journal of Ethnopharmacology **43**: 89-124.

JACOT GUILLARMOD, A. 1977. Flora of Lesotho. Cramer, Lhr

JORGENSEN, J.H., TURNIDGE, J.D. & WASHINGTON, J.A. 1999. Antibacterial susceptible tests: dilution methods. In: Murray P, Baron, E.J., Pfaller, M., Tenover, F. & Yolken, R. Manuel of Clinical microbiology, 7th (ed.), ASM Press. Washington, DC, pp.1526-1543**.**

KAMBIZI, L. & AFOLAYAN, A.J. 2001. An ethnobotanical study of plants used for treatment of sexual transmitted disease (njovhera) in Guruve District, Zimbabwe. Journal of Ethnopharmacology 77 **(1):** 5-9.

KHAN, M.R., KIHARA, M. & OMOLOSO, A.D. 2001. Antimicrobial activity of *Horsfieldia helwigii* and Melia *azeradarach*. Fitoterapia **72:** 423- 427.

KATO, T., FREI, B., HENRICH, M. & STTICHER, O. 1996. Sesquiterpenes with antibacterial activity from Epaltes mexicana. Planta Medica **62 (1):** 66-7.

KONDO, S., IKEDA, Y., HATTORI, S., HAMADA, M. & TAKEUCHI, T. 1991. Susceptibility of methicillin-resistant *Staphylococcus aureus* to various antibiotics. Classification by aminoglycoside-modifying enzymes and antibiotics active against

LALL, N. & MEYER, J.J.M. 1999. In vitro inhibition of drug –resistant and drug sensitive strains of Mycobacterium tuberculosis by ethnobotanically selected South African plants. Journal of Ethnopharmacology **66:** 347-354.

MABOGO, D.E.N. 1990. The Ethnobotany of the Vhavenda. Unpublished M.Sc. Thesis, University of Pretoria.

MATINGO, S. & CHAGONDA, L. 1993. Potential therapeutical activities of the Indigenous aromatic extracts and formulation of a cream incorporating them. Department of Pharmacy, University of Zimbabwe.

MEYER, J.J.M., AFOLAYAN, A.J. 1995. Antibacterial activity of *Helichrysum aureonitens* (Asteraceae). Journal of Ethnopharmacolgy **47:** 109-111.

MIDDLEBROOK, G., REGGIARDS, Z. & TIGGERT, W.D. 1977. Automable Radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. American review of respiratory disease **115:** 10 67-10 69.

MITSCHER, L.A., LEU, R., BATHALA, M.S., IWU, W. & BEAL, J.L.1972. Antimicrobial agents from higher plants.1. Introduction, rationale and methodology. Lloydia **35 (92):** 152-166.

MRSA. Japanese Journal of Antibiotics 44 (11):1211-1215.

NEUWINGER, H.D.1996. African Ethnobotany poisons and drugs. Chemistry. pharmacology. toxicology. Chapman & Hall. London. Glasgow. Weinheim. New York. Tokyo. Melbourne. Madras.

OLIVER- BEVER, B. 1986. Medicinal plants in tropical West Africa. Cambridge University Press, London.

ONAYADE, O.A., NTEZURUBANZA, L., SCHEFFER, J.J.C. & SVENDSEN, A.B. 1989. 37th Annual congress on medicinal Plant Research 5-9 September.

PALGRAVE, K.C.1981. Trees of Southern Africa. Second impression. Cape Town

PASCUAL, M.E., SLOWING, K., CARRETERO, E., SANCHEZ, MATA, D. &

PENNA, C., MARINO, S., VIVOT, E., CRUANES, M.C., DE DMUNOZ,J., FERRARO, G., GUTKIND, G. & MARTINO, V. 2001. Antimicrobial activity of Argentine plants used in the treatment of infectious diseases. Isolation of active compounds from *Sebastiania brasilensis*. Journal of Ethnopharmacology **77:**37- 40.

RAO, G.G.1998. Risk factors for the spread of antibiotic-resistant bacteria. Drugs **5 (3):** 323-330.

SMITH, C.A. 1966. Common names of South African plants. Botanical Survey. Memoris No 35. Government Printer, Pretoria.

STANIER, R.Y., Adelberg, E.A. & Ingraham, J.H. 1958. Important Groups of Unicellular Eubacteria. In: General microbiology, Ch. 18, pp. 407-415. The Macmillan Press, London.

TIMMERMANN, B.N., WACHTER, G.A., VALCIC, S., FRANZBLAU, S.G. &

SUAREZ, E. 2001. Antitubercular activity of triterpenoides from *Lippia turbinate.*

Journal of Natural Products **64:** 37- 41.

TORTORA, G.J., FUNKE, B.R. & CASE, C.L. 2001. Microbiology: An introduction. Benjamin Cummings, San Francisco, pp.88.

VAN WYK, B. E. & GERICKE, N. 2000. People's plants. A guide to useful plants of southern Africa, Briza Publications, Pretoria. ISBN 1-875093-309-5.

VAN WYK, B., VAN WYK, P. & VAN WYK B-E. 2000. Trees of southern Africa. Briza Publications, Pretoria. CK90/11690/23.

VILLAR, A. 2001. *Lippia*: Traditional uses, chemistry and pharmacology: A review. Journal of Ethnopharmacology 76 (3): 201-14.

WATT, J.M. & BREYER-BRANDWIJK, M. G. 1962. The medicinal and poisonus plants of Southern and eastern Africa. 2nd edn. Livingston, London.

World Health Organization, 1997. Anti-tuberculosis drug resistance in the world. The WHO/IUATLD Project on anti-tuberculosis drug resistance.

ANTIVIRAL ACTIVITY OF MOZAMBICAN MEDICINAL PLANTS AGAINST HUMAN IMMUNODEFICIENCY VIRUS

Abstract

Seventeen plant species, which are widely used in the folk medicine in Mozambique, were investigated for their anti-HIV activity. Ethanol plant-extracts were evaluated for their ability to inhibit the enzymes glycohydrolase (α-glucosidase and β-glucuronidase) and reverse transcriptase. Glycohydrolase enzymes are found in the host cell Golgi apparatus of the endoplasmic reticulum of eukaryotic cells and are responsible for glycosylation of proteins. Inhibition of the glycohydrolase proteins has been found to decrease the infectivity of the HIV virion, as the HIV glycoproteins are highly glcosylated. Alpha-Glucosidase has been found to be partly responsible for the glycosylation of HIV gp120 (Collins *et al.* 1997). Reverse transcriptase (RT) is an essential enzyme for the survival of HIV-virus. Without Reverse transcriptase, the viral genome cannot be incorporated into the host cell; as a result a virus will not reproduce.

It was found that 8 plant species (*Cassia abbreviata, Elephantorrhiza elephantina*, *Rhoicissus tomentosa, Pseudolachnostylis maprouneifolia, Lippia javanica*, *Litogyne gariepina, Maerua junceae and Momordica balsamina*) showed inhibitory effects against α-glucosidase and β-glucuronidase at a concentration of 200 µg/ml. The results of the tests revealed that the plant extracts of *Melia azedarach* and *Rhoicissus tomentosa* appeared to be active, showing 49 and 40% inhibition of the enzyme activity respectively.

3.1 Introduction

Over 42 million adults and children are infected by HIV (UNAIDS/WHO, 2003). The global HIV epidemic has killed more than 3 million people in developing countries and 14 000 new infections occur daily (UNAIDS/WHO, 2003). In other words the epidemic in sub-Saharan Africa remains rampant. In 2003, an estimated 26.6 million people in this region were living with HIV/AIDS and approximately 2.3 million people succumbed to the disease (Table 3.1).

Table 3.1 Regional HIV/ AIDS statistics and features, end of 2003 (UNAIDS/WHO, 2003).

Region	**Adults and children living with HIV/ AIDS**	**Adults and children newly infected with HIV**	**Adults prevalence (%)***	**Adult & child deaths due to AIDS**
Sub- Saharan Africa	25.0-28.2 million	3.0-3.4 million	7.5-8.5 million	2.2-2.4 million
North Africa & Middle East	470 000 - 730 000	43000 – 67000	0.2 – 0.4	35 000- 50 000
South & South – East Asia	4.6-8.2 million	610000-1.1million	0.4-0.8	330 000- 590 000
East Asia & Pacific	700000-1.3 million	150000-270 000	0.1—0.1	32 000- 58.000
Latin America	1.3- 1.9 million	120 000- 180 000	0.5- 0.7	49 000- 70 000
Caribbean	350000-590000	45 000-80 000	1.9-3.1	30 000- 50 000
Eastern Europe & Central Asia	1.2-1.8 million	180 000-280 000	0.5- 0.9	23 000-37 000
Western Europe	520 000-680 000	30 000-40 000	0.3-0.3	2 600-3 400
North America	790000-1.2 million	36 000-54 000	0.5- 0.7	12 000- 18 000
Australia & New Zealand	12 000- 18 000	700-1 000	0.1- 0.1	<100
Total	**40 million (36-46 million)**	**5 million (4.2-5.8 million)**	**1.1 % (0.9-1.3 %)**	**2 million (2.5-3.5 million)**

In a belt of countries across southern Africa, HIV/AIDS prevalence is maintaining alarmingly high levels in the general population. Due to the enormity of the challenge, health services have been unable to provide communities with access to prevention and care. Whilst access to anti-retro viral (ARV) drugs is benefiting a larger fraction of people, there still remains a fundamental challenge which is to make prevention and care available to the poor (UNAIDS/WHO, 2003).

HIV (human immunodeficiency virus) is a member of the family of lentiviruses, a subfamily of retroviruses and was first known as human T-lymphocytotrophic virus type III or lymphadenopathy associated virus (Au *et al*., 2001). The virus (Figure 3.1) possesses a single-stranded RNA genome. Its structure consists of a lipoprotein surface studded by two viral- enveloping glycoproteins (Levy *et al*., 1994). Gp 120 is the surface protein (SU) and gp41 is the transmembrane protein (TM) (Levy *et al*., 1994). Just below the lipid bilayer is the matrix (MA) protein p17 and a cone-shaped nucleocapsid, built from a capsid protein (CA) p24. Inside this nucleocapsid are the nucleocapsid proteins (NU) p6, 9 as well as the polymerase enzyme with functions such as reverse transcription (RT) coded by p66, protease p11 and integrase p32**.**

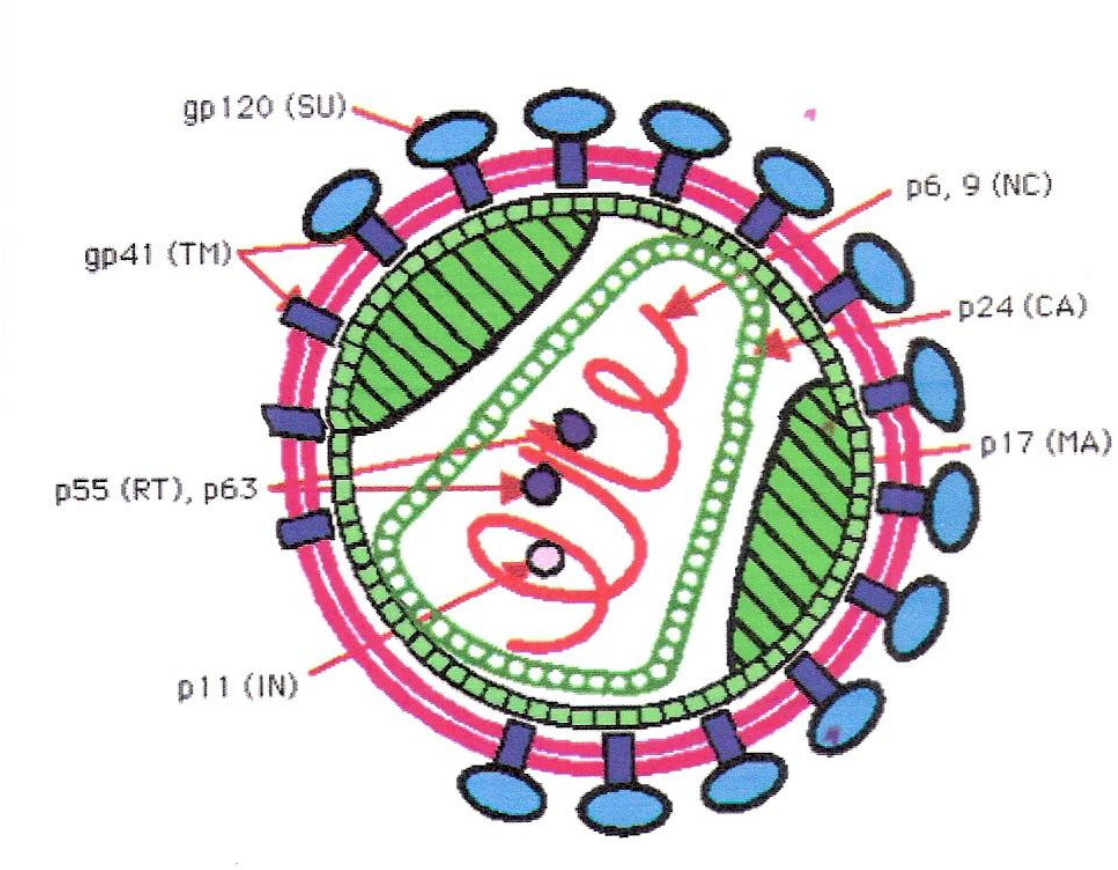

Figure 3.1 Human Immunodeficiency Virus (Da Cunha, 1999)

A number of laboratories are actively involved in the development of antiviral agents that interfere with HIV at different stages of viral replication (Balzarini *et al*., 1986; Sarin, 1988). A possible site of intervention is the inhibition of virus-specific RNA-dependent DNA polymerase (reverse transcriptase) (Vanden Berghe *et al.,* 1993)**.** If one can inhibit its reverse transcription catalytic activity, the viral RNA genome which encodes the viral genetic information would not be able to transcribe into a dsDNA strand encoding the cellular instructions to translate the viral proteins to form the provirus. When HIV infects a cell in a person′s body, it copies its own genetic code into the cell′s DNA. In this way, the cell is then "programmed" to create new copies of HIV. HIV's genetic material is in the

form RNA. In order to infect T-cells, it must first convert its RNA into DNA. HIV's reverse transcriptase enzyme is needed to perform this process (AIDSmeds, 2001). The first lines of the major class of drug therapy found useful in slowing HIV infections which were nucleoside RT inhibitors (nucleoside analogues). These include 3′-azido-3′-deoxythymidine or zidovudine (AZT), 2' deoxy-3'-thiacytidine or lamivudine, (3TC), 2', 3'-didehydro-3'-deoxythymidine or stavudine (d4T), 2', 3'-dideoxycytidine or zalcitabine (ddC) and 2', 3' dideoxyinosine or didanosine (ddI) that act by blocking the recording of viral RNA into DNA. On the other hand, specific enzymes called glycohydrolases contribute to the glycosylation of proteins (Collins *et al*., 1997). These glycohydrolase enzymes include α- glucosidase that is responsible for the glycosylation of HIV- gp120 (one of the membrane proteins that interacts with the CD4 receptor protein that is present on helper T cells of the immune system) and β-glucuronidase, all interfering with viral maturation. Inhibitors of glycosylation could have a potential therapeutic use.

3.2 Materials and methods

3.2.1 Plant material

Seventeen plants (Table 3.2) which are used to treat, HIV- infections in immunocompromised patients were collected from different areas in Mozambique.

3.2.2 Preparation of plant extracts

Dried powdered plant materials were extracted with acetone**.** Fifty grams of powdered plant material was extracted with 500 ml of solvent over two days under reflux. The extracts

were then filtered and concentrated to dryness under reduced pressure and the residues freshly dissolved in an appropriate solvent on the day that the bioassay was done.

3.2.3 Glycohydrolase enzyme assays

Determination of activity against HIV was based on the measure of inhibition of the glycohydrolase enzymes: α-glucosidase and β-glucuronidase. Two glycohydrolase enzymes (α- glucosidase and β- glucuronidase) and the substrates p-nitrophenyl- α-D-glucopyranoside and p-nitrophenyl-β -D-glucuronide were obtained from Sigma Chemical (MO, U.S.A). The glycohydrolase assay was performed in a colorimetric 96-well microtiter plate-based assay, determining the amount of p-nitrophenol released. The method described by Collins *et al*. (1997) was followed**.** The enzymes were diluted in 50mM of an appropriate buffer (sodium acetate, pH 5.0 for β-glucuronidase and Mes-NaOH, pH 6.5 for α- glucosidase). Appropriate substrates of the respective enzymes were added to microtiter wells**.** The assay was calibrated relative to enzyme concentration and ~ 0.25 µg enzyme was used per assay. After the addition of the enzymes, substrate and extracts, the plates were left at room temperature for 15 min. The reaction was stopped by the addition of 50 µl of 2 mM glycine-NaOH, pH 10, and measurement of absorbance undertaken at 412 nm. The extracts were tested at concentration of 200 µg/ml and the experiment was carried out in triplicate. The positive control Doxorubicin was tested at 100 µg/ml against both the enzymes.

3.2.4 HIV-1 Reverse transcriptase (RT) assay

The effect of plant extracts on RT activity *in vitro* was evaluated with a non-radioactive HIV-RT colorimetric ELISA kit (Roche, Germany). The assay was carried out in triplicate. Adriamycin, an anticancer drug and also an inhibitor of viral reverse transcriptase (Goud *et al*., 2003) was used as a positive control. In each test well, 20 µl of diluted recombinant HIV-1 reverse transcriptase (4-6 ng), 20 µl of diluted extract, and 20 µl of reaction mixture was dispensed. The final concentration of each extract in each well was 200 µg/ml. Since this part of the experiment was not conducted at the University of Pretoria, but at Nelson Mandela Metropolitan University; due to cost implications, only one concentration was selected. Negative control wells contained 40 µl of lysis buffer and 20 µl of reaction mixture. The concentration of positive drug control (Adriamycin) was 100 µg/ml. Positive control wells contained 20 µl diluted recombinant HIV-1 Reverse transcriptase (4-6 ng), 20 µl of lysis buffer containing 10 % DMSO, and 20 µl of reaction mixture. The wells of the microtiter plate modules were washed five times with 250 µl of washing buffer per well for 30 seconds each. The washing buffer was then carefully removed and 200 µl of anti-DIG-POD working solution was dispensed into each well. Incubation at 37^{o}C followed once again for 1 hour after the microtiter plate modules were covered with foil. The wells were then washed in the same manner as before, the washing buffer was carefully removed from the wells, and 200 µl of ABTS substrate was dispensed into the wells. Incubation then commenced for 10-30 min at room temperature (15-25^{o}C). The absorbencies of the samples were measured at 405 nm (reference wavelength: 492 nm). The percentage

inhibitory activity of the extracts samples were then calculated, with reference to the positive control.

3. 3 Results and discussion

The inhibition of α- glucosidase and β- glucuronidase by plant extracts is depicted in Table 3.2. It was found that 8 plant species (*Cassia abbreviata, Elephantorrhiza elephantina*, *Rhoicissus tomentosa, Pseudolachnostylis maprouneifolia, Lippia javanica*, *Litogyne gariepina, Maerua junceae and Momordica balsamina*) showed inhibitory effects against α-glucosidase and β-glucuronidase at 200 µg/ml.

Table 3.2 Inhibition of α- glucosidase and β- glucuronidase by the plant extracts.

Family	Botanical name	Plant part used	α- glucosidase % inhibition[a]	β- glucuronidase % inhibition[a]
Passifloraceae	*Adenia gummifera*	Root	34.9± 13.9	28.9 ± 38.3
Liliaceae	*Aloe marlothii*	Leaves	32.2 ± 3.6	62.8 ± 20.1
Liliaceae	*Aloe parvibracteata*	Leaves	2.1 ± 8.2	-9 ± 16.3
Apocynaceae	*Adenium multiflorum*	Root	-17 ± 18.3	25.7 ± 49.2
Fabaceae	*Cassia abbreviate*	Bark	89.9 ± 0.1	93.6 ±1.9
Apocynaceae	*Catharanthus roseus*	Leaves	43.9 ± 1.9	16.1 ± 19.1
Fabaceae	*Elephantorrhiza elephantina*	Root	80.6 ± 0.4	95.2 ± 0.1
Iridaceae	*Gladiolus dalenii*	Tuber	-35.9 ± 5.7	-24.9 ± 7.1
Lamiaceae	*Hoslundia opposita*	Leaves	70.2 ± 5.3	42.5± 8.6
Verbenaceae	*Lippia javanica*	Leaves	62.0 ± 0.9	73.2 ± 7.6
Asteraceae	*Litogyne gariepina*	Leaves	62.3 ± 15.0	91.2 ± 3.8
Meliaceae	*Melia azedarach*	Leaves	29.1 ± 4.6	23.1 ± 15.9
Capparaceae	*Maerua juncea*	Leaves	69.3 ± 0.8	90.4± 1.4
Cucurbitaceae	*Momordica balsamina*	Leaves	60.0 ± 1.5	67.3± 4.1
Euphorbiaceae	*Pseudolachnostylis maprouneifolia*	Bark	89.8 ± 0.1	95.4 ± 1.1
Vitaceae	*Rhoicissus tomentosa*	Root	72.8 ± 1.3	94.24 ± 0.6
	Coccinia rhemanii	Tuber	3.1 ± 3.7	-15 ± 3.4
Doxorubicin (positive control tested at 100 µg/ml)			98.2 ± 0.1	90.4 ± 0.4

[a] % inhibition are average ± standard deviation.

The most promising anti-HIV activity was found by the extracts of *Cassia abbreviata, Elephantorrhiza elephantina, Lippia javanica, Pseudolachnostylis maprouneifolia and Rhoicissus tomentosa*. Two of the most active extracts (*Cassia abbreviata* and *Elephantorrhiza elephantina*) were members of the same plant family (Fabaceae). The extracts from *Cassia abbreviata* inhibited α-glucucosidase and β-glucuronidase by 90 and 94%, respectively. *Elephantorrhiza elephantina* inhibited the activity of α-glucucosidase and β-glucuronidase by 80 and 95%, respectively. The extract of *Pseudolachnostylis maprouneifolia* (Euphorbiaceae) also inhibited α-glucucosidase and β -glucuronidase by 90 and 95%, respectively. *Aloe marlothii* showed only inhibition of β–glucuronidase, while *Hoslundia opposita* was only active against α- glucucosidase. *Adenia gummifera* and *Gladiolus dalenii* did not show any activity against α- glucucosidase at the highest concentration (200 µg/ml) tested.

Adenia gummifera, Cassia abbreviata, Elephantorrhiza elephantina, Gladiolus dalenii, Hemizygia bracteosa, Lippia javanica, Momordica balsamina, Pseudolachnostylis maprouneifolia, Rhoicissus tomentosa, *Melia azedarach* and *Maerua juncea* were also assayed for their ability to inhibit the enzyme HIV-1 Reverse transcriptase. These plants were selected based on their inhibitory activity against glycohydrolase enzyme and the availability of the extracts. Figure 3.2 shows the inhibitory effect of plant extracts on the enzyme RT. The results of the tests revealed that the plant extracts of *Melia azedarach* and *Rhoicissus tomentosa* appeared to be active, showing 49 and 40% inhibition of the enzyme activity respectively. The activity of the remaining plant extracts against RT was not

significant. Adriamycin, the positive control showed 80 % inhibitory activity at a 100 µg/ml concentration.

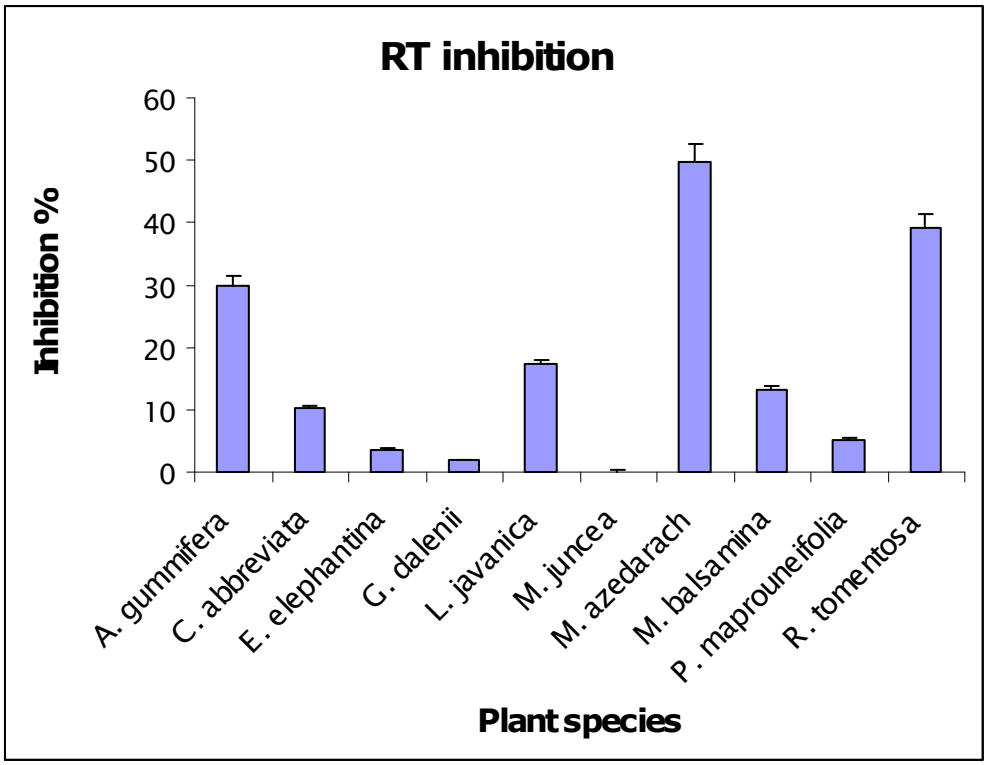

Figure 3.2 HIV Reverse transcriptase (RT) inhibition by the plant extracts

3.4 Conclusion

The results revealed that most of the plants tested, *Cassia abbreviata, Elephantorrhiza elephantina, Lippia javanica, Maerua juncea, Momordica balsamina, Rhoicissus tomentosa* and *Pseudolachnostylis maprouneifolia* showed good inhibitory activity against α- glucosidase and β- glucuronidase. Only two species (*Melia azedarach* and *Rhoicissus*

tomentosa) displayed activity against RT at 200 µg/ml. Despite the fact that the plant extracts were not pure compounds they could provide useful leads for the discovery of antiviral compounds.

3.5 References

AIDSMEDS 2001. The HIV life cycle. AIDSMEDS.COM.

AU, T.K., LAM, T.L., NG, T.B., FONG, W.P. & WAN, D.C.C. 2001. A comparison of HIV-1integrase inhibition by aqueous and methanol extracts of Chinese medicine herbs. Life Sciences, **68**: 1687-1694.

BALZARINI, J., MITUSUYA, H., DE CLERQ, E. & BRODER, S. 1986. Comparative inhibitory effects of suramin and other selected compounds on the infectivity and replication of human T-cell lymphotropic virus (HTLV-III) lymphoadenopathy-associated virus (LAV). International Journal of Cancer **37:** 451-457.

COLLINS, R.A., NG, T.B., FONG, W.P., WAN, C.C. & YEUNG, H.W.1997. A Comparison of human immunodeficiency virus type 1 inhibition by partially purified aqueous extracts of Chinese medicinal herbs. Life sciences **60:** 345-351.

DA CUNHA, M.F.1999. HIV disease. The University of Texas–Houston, Health Science Centre.

GOUD, T.V., REDDY, G.N., SWAMY, N.R., RAM, T.S., VENKATESWARLU, V. 2003. Anti- HIV active petrosins from the marine sponge Petsia similis. Biological & Pharmaceutical. Bulletin 26, 1498-1501.

LEVY, J.A., FRAENKEL-CONTRAT, H. & OWENS, R.A. 1994. Virology, 3rd ed. p 372-376. Prentice Hall, New Jersey.

UNAIDS/WHO 2003. AIDS epidemic update. UNAIDS-20 Avenue Appia-1211 Geneva, 27-Switzerland.

VANDEN BERGHE, D. A. , HAERMERS, A., VLIETINCK, A. 1993. Antiviral agents from higher plants and an example of structure activity relationship of 3-methoxyflavones. CRC. Press, Inc

ISOLATION AND IDENTIFICATION OF COMPOUNDS FROM *LIPPIA JAVANICA*

Abstract

Lippia javanica is an aromatic herb that occur all over in Mozambique and is well known for their medicinal properties. *Lippia javanica* was found to have the best activity exhibiting a minimum inhibitory concentration of 0.125 mg/ml against *B. cereus*, *B, pumilis, B, subtilis S. aureus* and *E. faecalis.* the extracts also showed positive activity against *Mycobacterium tuberculosis* at concentration of 0.5 mg/ml and HIV-enzyme glycohydrolase (α-glucosidase and β-glucuronidase) inhibited by 62 % and 73 % respectively. Considering its medicinal use local for HIV and various infections, it was therefore, selected for identifying its bioactive constituents. A Phytochemical investigation of *L. javanica* led to the isolation of eight compounds, 4-ethyl-nonacosane (**1**), (*E*)-2(3)-tagetenone epoxide (**2**), myrcenone (**3**), piperitenone (**4**), apigenin (**5**), cirsimaritin (**6**), 6-methoxyluteolin 4'-methyl ether (**7**), 6-methoxyluteolin and 3',4',7-trimethyl ether (**8**). This is the first report of compounds (**1**), (**2**), (**5-8)** from *L. javanica*.

4.1 Introduction

Twenty two plants were screened for bioactivity against Gram-positive and Gram negative bacteria.

A preliminary study indicated that extract of *Lippia javanica* was found to have the best activity against Gram-positive bacteria tested; *Mycobacterium tuberculosis* and HIV-enzyme glycohydrolase (α-glucosidase and β-glucuronidase) inhibited by 62 % and 73 % respectively. Considering its medicinal use local for HIV and various infections, it was therefore, selected for identifying its bioactive constituents.

4.1.1 Description and traditional use of *Lippia javanica*

The are about 200 species of *Lippia* include*s* herbs, shrubs and small trees (Terblanché & Kornelius, 1996). In general, the genus appears to present consistent profiles of chemical composition, pharmacological activities. The most common use of *Lippia* species is for the treatment of respiratory disorders (Pascual *et al*., 2001)**.** *Lippia javanica* (Burm.f.) Spreng (Figure 4.1) is an erect woody shrub up to two meters high, with strong aromatic leaves, which give off a lemon smell when crushed (Van Wyk & Gericke, 2000)**.**

The plant occurs in many parts of southern Africa and tropical Africa (Van Wyk & Gericke, 2000). Its infusion made from its leaves is commonly used in Africa as tea for various chest ailments, influenza, measles, rashes, malaria, stomach problems, fever, colds, cough and headaches (Smith, 1966; Watt & Breyer-Brandwijk, 1962; Hutchings, 1966 and Hutchings & van Staden, 1994). Hutchings (2003) reported the clinical use of *L. javanica* for the treatment of HIV in Ngwelezane Hospital, Kwazulu Natal (South Africa).

In Botswana it is used as a caffeine free tea and in Zimbabwe and Malawi as a nerve tonic (Manenzhe *et al*., 2004).

Figure 4.1 *Lippia javanica* (Plantzafrica.com)

4.1.1.2 Biological activity

Extracts of *Lippia javanica* displayed a reproducible inhibitory activity against the Gram-positive bacteria *Bacillus cereus*, *B. pumilis*, *B. subtilis*, *Staphylococcus aureus* and *Enterococcus faecali*s in the present study. The essential oil from *L. javanica* has also been extensively shown to exhibit bioactivity against many pathogenic microorganisms (Viljoen *et al*., 2005; Manenzhe *et al*., 2004). It has also been found with good insect repellent activity (Govere *et al.*, 2000), and antiplasmodial activity (Manenzhe *et al*. 2004, Mwangi *et al*. (1991).

4.1.1.3 Chemical constituents

Numerous monoterpenoids have been identified in the volatile extract of *Lippia javanica*, including mercyne, caryophyllene, linalool, *p*-cymene and ipsdionone (Neidlein and Staehle 1974; Mwangi *et al*., 1991). *Lippia javanica* contains various organic acids and alcohols (Neidlein and Staehle, 1973a, 1973b). Iridoid glycosides (Rimpler and Sauerbier, 1986) and toxic triterpenoids (icterogenins) have been detected in some *Lippia* species (Buckingham, 2006).

4.2 Materials and methods

4.2.1 Plant material

Leaves of *Lippia javanica* were collected at Matola- Gare, Mozambique in June 2004. The voucher specimens have been deposited at H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

4. 2.2 Extraction and isolation

The air dried leaves of *L. javanica* (1.4 kg) were extracted with 4L ethanol for two days then filtered; the process was repeated two times. The extracts were combined and evaporated under reduced pressure to afford 47.5 g of crude ethanol extract. The total extract was subjected to a silica gel column (40 x 10 cm). Solvent system ethyl acetate: hexane with increasing polarity (EtOAc %, volume; 0 %, 1L; 10%, 2 L; 30%, 2 L; 50%, 2 L; 70%, 2 L; 100%, 1 L) followed by 10% of methanol in ethyl acetate (2L) was used

as an eluent. Eight fractions (300 ml), based on TLC profile were pooled and concentrated to dryness under reduced pressure. Fraction I (3.5 g) was chromatographed over silica gel using 100% hexane to afford compound (**1,** 437.6 mg). Fraction IV (10 g) was chromatographed on silica gel using hexane-EtOAc mixtures of increasing polarity which yielded compounds (**2,** 41.1 mg), (**3,** 18.3 mg), and (**4,** 568 mg). Fraction VII (4 g) was rechromatographed on silica gel column using gradient of EtOAc in hexane. The fraction eluted with EtOAc-hexane (4:6) was further chromatographed over Sephadex LH-20 using 100% methanol as eluent which yielded compounds (**5,** 5.3 mg), (**6,** 10 mg), (**7,** 8 mg), (**8,** 10 mg).

4.2.3 Bioautography of fractions obtained after the chromatographic purification of the ethanol extracts of *L. javanica*.

After each purification stage the antibacterial activity of fractions was tested using the direct bioautography. In this assay, an overnight culture of test bacteria in 20 ml MH broth was pelleted by centrifugation at 3000 rpm for 15 min and 10 ml fresh MH broth. This suspension was sprayed on a developed TLC plate and incubated at 37^{o}C overnight. A 2 mg/ml solution of INT (iodonitrotetrazolium violet) was then sprayed on the plate and incubated to detect the areas of bacterial inhibition. Antibacterial compounds on the TLC plate was visible as white spots against a deep red background, as bacterial growth reduces the tetrazolium salt to a red formazan product (Figures 4.2).

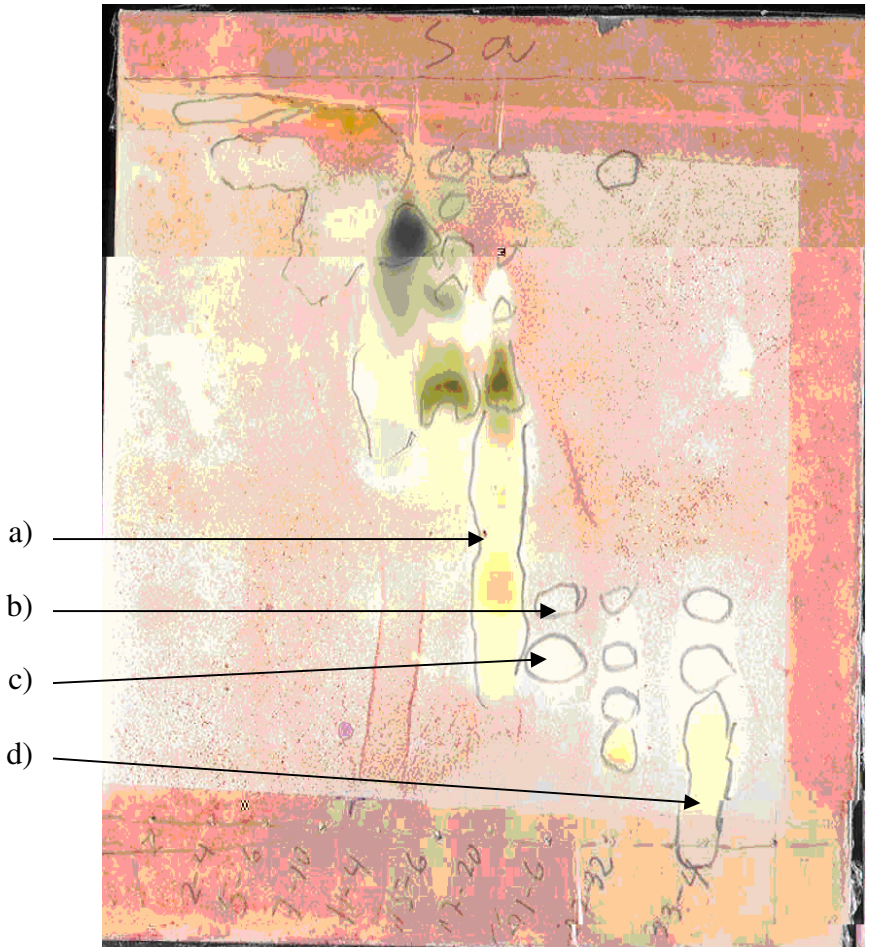

Figure 4.2 Fractions from silica column A tested for antibacterial activity **(Sa)** *Staphylococcus aureus* (ATCC 12600). Zones of inhibition (arrows, a-d)

4.2.4 Identification of purified compounds

UV spectra were recorded using a Pharmacia LKB-ultraspec 111 UV spectrophotometer. NMR spectra were recorded using a Bruker ARX 300 or a Bruker Avance DRX 500 MHz. Mass spectra were obtained with a JEOL JMS-AX505 W mass spectrometer. The recorded spectral data of the isolated compounds were compared with those published in literature

4.3 Results and discussion

4.3.1 Compound "4-ethyl-nonacosane"

The compound 4-Ethyl-nonacosane ($C_{31}H_{64}$) crystallized from fraction 1 in *n*-hexane and the structure was established based on electronic impact mass (EI-MS) (Figure 4.3) and ^{1}H-NMR spectra, which correspond to the T-branched hydrocarbon, 4-Ethyl-Nonacosane ($C_{31}H_{64}$, M_r = 436).

White crystals from hexane, $C_{31}H_{64}$, EI-Ms. m/z (%): 436(12.2%) [M] H^+, 408 (8.7%) $[M-C_2H_5+H]^+$, 393 (7%) $[M-C_3H_7 + H]^+$, 85 (57.8%) $[M-C_{25}H_{51}+H]^+$, 71 (70%) C_5H_{11} + , 57 (100 %, base peak) C_4H_9, 43 (9-) C_3H_7+, 29 (18) C2H5 +; ^{1}H- NMR δppm: 0.88 (9H, t3CH)

H-1, H29, 4-EtH-2, 1-26-29 (54H, m, -CH5-)

H- 2.3, H-5-28, 4-EtH-1, 1.53 (1 H, m, CH) H-4.

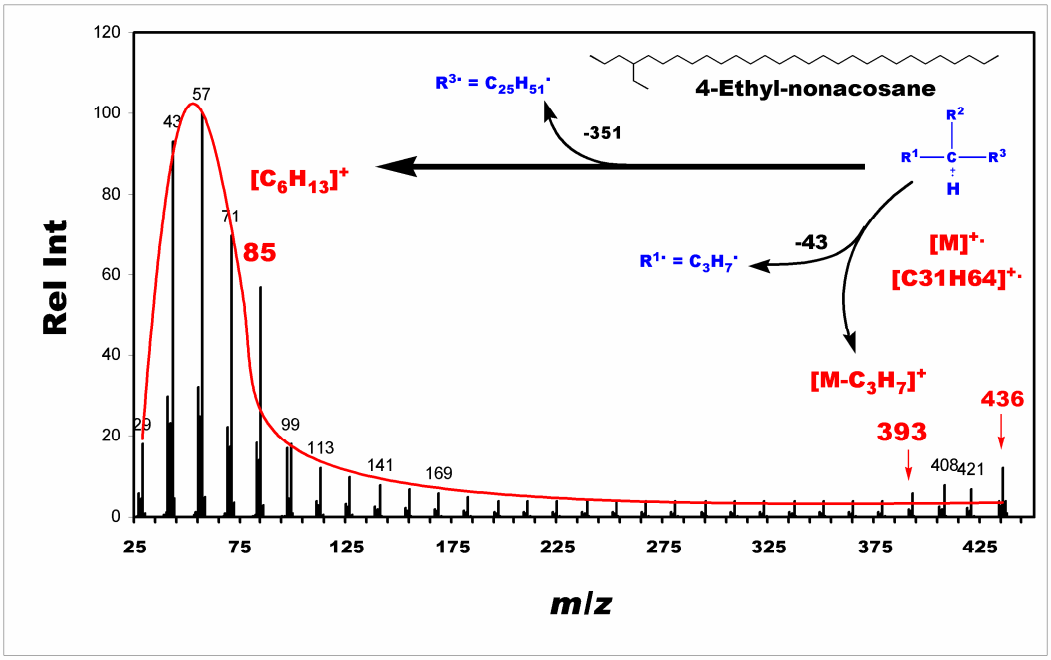

Figure 4.3 Electronic impact mass spectra (EI-MS) of 4-ethyl-nonacosane

4.3.2 Compound 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)*

This compound was isolated from the non-polar fraction of the ethanolic extract of *L. javanica,* and showed in NMR (^{1}H and ^{13}C) three singlet signals at δ_H 1.25 (δ_C 24.8), δ_H 1.40 (δ_C 18.6), and δ_H 2.25 (δ_C 13.8), two double bonds one of them vinylic with characteristic terminal CH_2 signals at δ_H 5.49 (d, *J*=10.9Hz), δ_H 5.67 (d, *J*=17.2 Hz) and proton signal at δ_H 6.39 (dd, *J*=10.9, 17.2Hz), the other double bond (δ_C 152.8s, 123.4d) and proton signal at δ_H 6.32 (s), in addition to a proton attached to oxygenated carbon at δ_H 3.35 (s) which form part of an oxirane ring (δ_C 61.1s, 66.4d) (Table 4.1). The above data correspond to the structure given in (Figure 4.5).

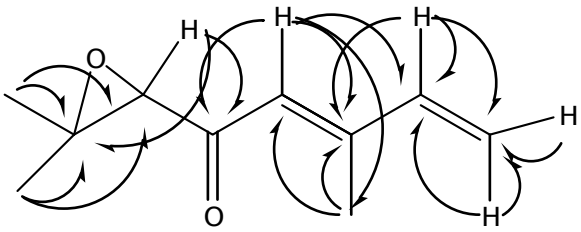

Figure 4.4 HMBC correlations of 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)*

The structure of this compound was further supported by HMBC (Figure 4.4) which showed cross peak connectivity between H-1/C-2, C-3; H-2/C-10, C-4, C-4; H-4/C-2, C-5, C-10, C-3; H-6/C-9, C-7, C-5, Me-8, 9/C-7, C-8; Me-10/C-4, C-2, C-3, C-5. NOESY experiment of compound 2 also showed cross peaks between H-6/H-8, H-4; Me-10/H-1 (trans), H-2/H-4, the correlations between H-2/ H-4 and H-4/H-6 indicated that all of the proton are in the same side, also the NOESY relation between, H-1 (trans)/Me-10 indicated the location of them on the other side. Compound 1-(3,3-dimethoxiranyl)-3-methyl- (2*E*), is a rare monoterpene identified in the Cameroonian *Clausena anista* (Rutaceae) essential oil (Ngassoum *et al*., 1999) and was not identified in *Lippia* species before, which indicates that the *L. javanica* collected from Mozambique as a new chemotype.

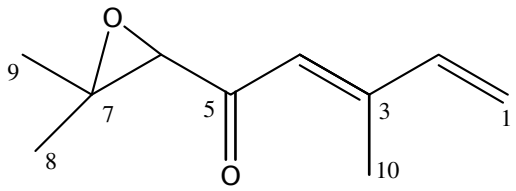

Figure 4.5 Structure of 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)*

Table 4.1 ^{1}H and ^{13}C NMR data of 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)* in $CDCl_3$

No.	Carbon	Proton
1	121.8 t	5.49 (d, 10.9), 5.67 (d, 17.2)
2	140.4d	6.39 (dd, 10.9, 17.2)
3	152.8 s	
4	123.4 d	6.32 s
5	196.7 s	
6	66.4 d	3.35 s
7	61.1 s	
8	18.6 q	1.40 s
9	24.8 q	1.25 s
10	13.8 q	2.25 s

4.3.3 Compound Myrcenone

Myrcenone was isolated from the non-polar fraction using a silica gel column. The compound showed in NMR three double bonds: one of them is vinylic and has two protons at δ_H 5.07 (d), 5.20 (d) attached to carbon at δ_C 119.9 (t), and proton at δ_H 6.44 (d), δ_C 138.2 (d), the other two double bonds contain an *exo* double bond at δ_C 140.6 (s), 114.9 (t), the later carbon attached to two singlet signals (one protons each) at δ_H 5.09 (s),

5.22 (s), the third double bond located at C-6 and attached to a singlet proton at δ_H 6.14. The remaining signals indicated the presence of two methyl groups over a double bond at δ_H 1.85 (δ_C 27.7), 2.12 (δ_C 20.8) in addition to conjugated carbonyl group at δ_C 198.0 (Figure 4.6, Table 4.2). The forgoing data are applicable only to myrcenone, the commonly found monoterpenes in *Lippia* volatile oils.

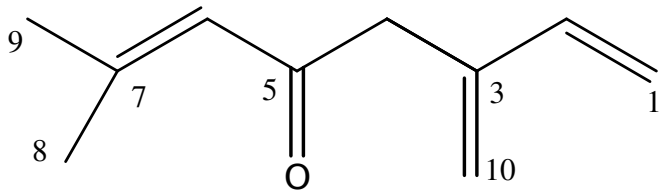

Figure 4.6 Structure of myrcenone

Table 4.2 ^{1}H and ^{13}C NMR data of myrcenone ($CDCl_3$)

No.	Carbon	Proton
1	119.9 t	5.07 (d, 8.8), 5.20 (d, 17.4)
2	138.2	6.44 (dd, 8.8, 17.4)
3	140.6 s	
4	47.9 t	3.27 (2H, s)
5	198.0 s	
6	122.4 d	6.14 s
7	143.5 s	
8	20.8 q	2.12 s
9	27.7 q	1.85 s
10	114.9 t	5.09, 5.22 (s, both)

4.3.4 Compound piperitenone

The compound was isolated from the non polar fractions. ^{13}C NMR gave 10 carbons, which indicated a monoterpene skeleton. ^{1}H NMR showed singlet olefinic proton at δ_H 5.67, two methylene groups at δ_H 2.46 (t, J=6.2 Hz), 2.10 (t, J=6.2 Hz) and three methyl singlets attached to double bonds at δ_H 1.89,1.73 and 1.66 (Table 4.3). The previous data

only can be accommodated in structure (Figure 4.7), piperitenone, which has been isolated before from the same source.

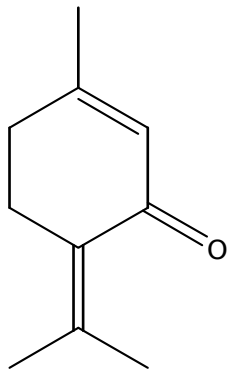

Figure 4.7 Structure of piperitenone

Table 4.3 ^{1}H and ^{13}C NMR data of piperitenone ($CDCl_3$)

No.	Carbon	Proton
1	191.0 s	
2	128.4 d	5.67 brs
3	141.9 s	
4,5	31.4 t, 27.5 t	2.46, 2.11 (2H each, t, J=6.2 Hz)
6	159.21 s	
7	128.51 s	
8,9	22.4 q, 22.1 q	1.89 s (9), 1.67 s (8)
10	23.3 q	1.74 s

4.3. 5 Compound β-sitosterol

The compound was identified as β-sitosterol based on the ^{1}H NMR and co-spotting with authentic sample.

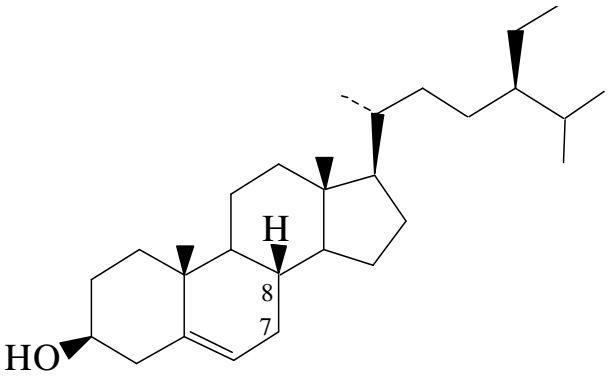

Figure 4.8 Structure of β-sitosterol

4.3.6 Compound Apigenin

The compound showed a yellow color on TLC plates when sprayed with $AlCl_3$ which indicating its flavonoidic nature. This was supported by ^{1}H NMR spectrum, which showed two proton doublets at δ_H 6.44 (d, J=2.2 Hz), 6.20 (d, J=2.2 Hz) corresponding to protons attached to positions 6 and 8 respectively of compound Myrcenone, another singlet at δ_H 6.59 corresponding to H-3, in addition to two doublets counted four protons at 7.84, 6.92 (2H/each J=8.8 Hz) corresponding to H-2`, 6` and H-3` and 5`. The given data is a typical NMR pattern of apeginin, the wide spread flavone aglycone in nature.

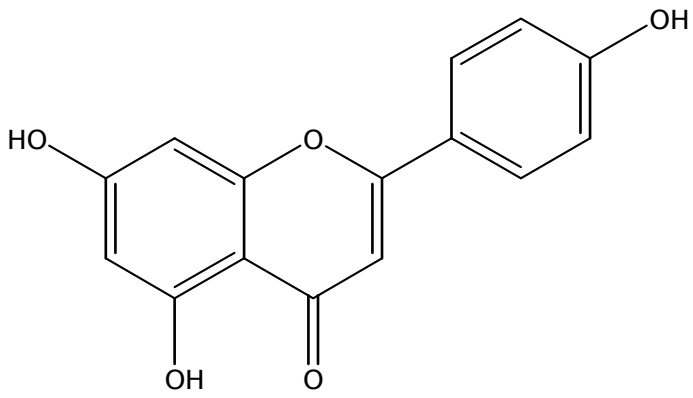

Figure 4.9 Structure of apigenin

4.3.7 Compound Cirsimaritin

The compound gives signals similar to compound apeginin (singlet at δ_H 6.59 corresponding to H-3, in addition to two doublets counted four protons at 7.84, 6.92 (2H/each J=8.8 Hz) corresponding to H-2`, 6` and H-3` and 5`), in addition to a singlet at 6.52 (H-8) and two singlets (3H each) at 3.94 and 3.90 of two methoxy groups. The previous data indicated the presence of 6-hydroxyapeginin. The two methoxy groups were positioned at C-6 and C-7 because the proton chemical shift of compound 4 is almost the same as the free aglycone apigenine (compound 3) except H-8 which shifted to a lower field from the corresponding value (δ_H 6.44), the other methoxy group was positioned at C-6 because the other signals in ring C were not affected and the 6-methoxy derivative is commonly found in labiatae.

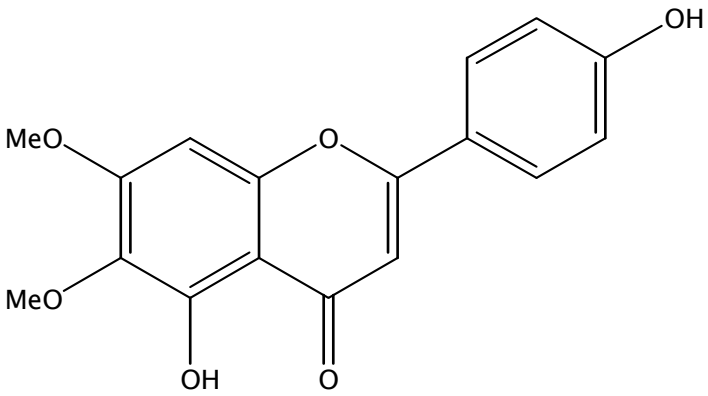

Figure 4.10 Structure of Cirsimaritin

4.3.8 Compound 6-Methoxyluteolin 4'-methyl ether

Compound **8** is flavonoidic in nature as indicated from the color reaction of the compound with $AlCl_3$. The NMR spectra showed similar signal to compound Cirsimartin, except that the presence of a hydroxyl group at C-3`, which indicated from the splitting of ring C signals to 1,3,5-trisubstituted pattern and gives signals attributed to H-2 (7.32, d, J=1.8 Hz), H-5 (7.01, d, J=8.4 Hz) and H-6 (7.48, dd, J=1.8, 8.4 Hz). In addition to two methoxy groups were present at 4.00, 4.04 (δ_C 60.9, 56.9). The two methoxy groups were positioned at C-6 and C-4 due to the fact that, the signal at δ_C at 60.9 indicated the connection of the methoxy groups should be between two oxygenated carbons i.e. C-6 and the other methoxy group positioned at C-4` due to the shift of H-5` from the basic skeleton (without methoxy groups, ~ 7.00).

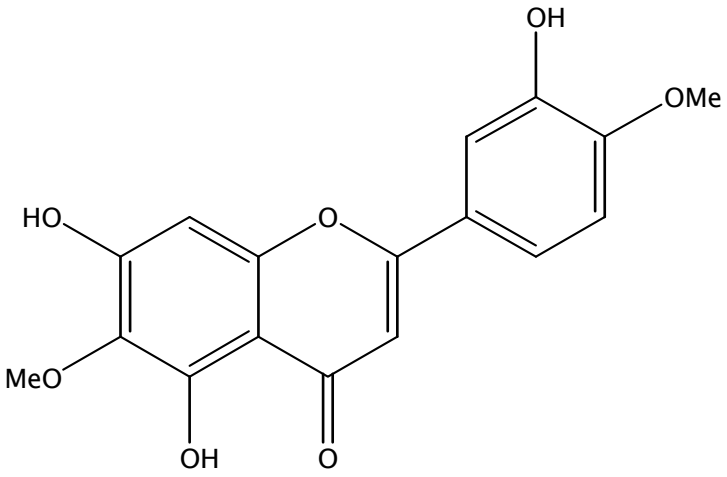

Figure 4.11 Structure of 6-Methoxyluteolin 4'-methyl ether

4.3.9 Compound 6-Methoxyluteolin 3',4',7-trimethyl ether

Compound 9 showed similar patterns in NMR as compound 6-Methoxyluteolin 4'-methyl ether, [H-2` (7.32, d, J=1.8 Hz), H-5` (7.01, d, J=8.4 Hz) and H-6` (7.48, dd, J=1.8, 8.4 Hz), and two singlets at 6.59 and 6.55 of H-3 and 6] except the presence of four methoxy groups in compound 9, accordingly the four methoxy groups were positioned at C-6,7,3` and 4`. Keeping in mind that the substitution at C-5 is eliminated due to the presence of the hydroxyl signal after 12.50 ppm.

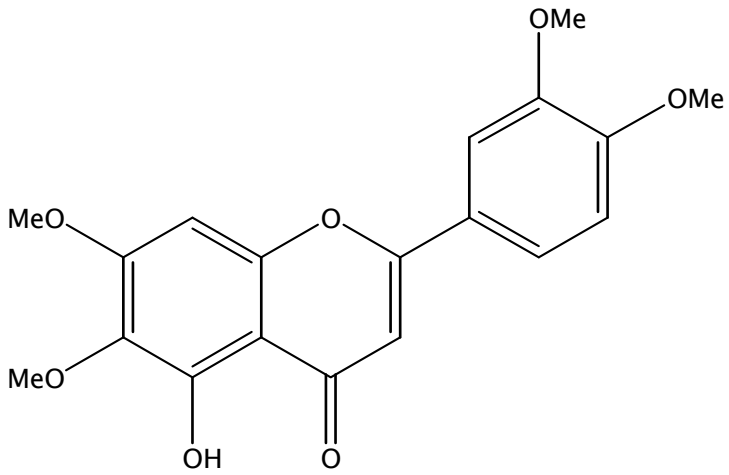

Figure 4.12 Structure of 6-Methoxyluteolin 3',4',7-trimethyl ether

4.4 Conclusion

A Phytochemical investigation of *L. javanica* led to the isolation of eight compounds, 4-ethyl-nonacosane (**1**), (*E*)-2(3)-tagetenone epoxide (**2**), myrcenone (**3**), piperitenone (**4**), apigenin (**5**), cirsimaritin (**6**), 6-methoxyluteolin 4'-methyl ether (**7**), 6-methoxyluteolin and 3',4',7-trimethyl ether (**8**). This is the first report of compounds (**1**), (**2**), (**5**-**8**) from *L. javanica*.

4.5 References

BUCKINGHAM, J. 2006. Dictionary of Natural Products on CD-ROM. Chapman and Hall: London.

GOVERE, J., DURRHEIM, D.N., DU TOIT, N., HUNT, R.H. & COETZEE, M. 2000. Local plants as repellents against *Anopheles arabiensis* in Mpumalanga Province, South Africa. Central African Journal of Medicine **46:** 213-216.

HUTCHINGS, A.1966. Zulu medicinal plants, University of Natal Press, Pietermaritzburg.

HUTCHINGS, A. & VAN STADEN, J. 1994. Plants used for stress-related ailments in traditional Zulu, Xhosa and Sotho medicine. Part: Plants used for headaches. Journal of Ethnopharmacology **43:** 89-124.

HUTCHINGS, A. 2003. Enhancing HIV/AIDS support therapy with indigenous herbal preparations- a clinic experience. Joint international conference SAAB & ISE, University of Pretoria, South Africa.

MANENZHE, N. J., POTGIETER, N. & VAN REE, T. 2004. Composition and antimicrobial activities of volatile components of *Lippia javanica.* Phytochemistry **65:** 2333-2336.

MWANGI, J.W., ADDAE-MENSAH, I., MUNAVU, R.M. & LWANDE, W. 1991. Essential oils of Kenyan *Lippia* species. Part III. Flavour Fragrance Journal, **6:**221-224.

NGASSOUM, M.B., JIROVETZ, L., BUCHBAUER, G., SCHMAUS, G., & HAMMERSCHMIDT, F.-J. 1999. Chemical composition and olfactory evaluation of the essential oils of leaves and seeds of *Clausena anisata* (Wild) J.D. Hook. Ex. Benth. from Cameroon. Journal of Essential Oil Research **11(2)**: 231-237.

NEIDLEIN R. & STAEHLE, R. 1973a. Constituents of *Lippia javanica*. Deutsche Apotheker-Zeittung **113 (26):** 993-997.

NEIDLEIN, R. & STAEHLE, R. 1973b. Constituents of *Lippia javanica*. II Deutsche Apotheker-Zeittung. **113 (32):** 1219-1222.

NEIDLEIN, R. & STAEHLE, R. 1974. Constituents of *Lippia javanica*. III. Deutsche Apotheker-Zeittung. **114 (40):** 1588-1592.

PASCUAL, M.E., SLOWING, K., CARRETERO, E., SÁNCHEZ MATA, D. & ILLARA. 2001. *Lippia*: traditional uses, chemistry and pharmacology: a review. Journal of Ethnopharmacology **76:** 201-214.

RIMPLER, H., SAUERBIER, H. 1986. Iridoid glucosides as taxonomic markers in the genera *Lantana*, *Lippia*, *Aloysia* and *Phyla*. Biochemical and Systematic Ecology **14** (3): 307-310**.**

SMITH, C.A. 1966. Common names of South African Plants- Memoirs of the Botanical Survey of South Africa 35.

TERBLANCHÉ, F.C. & KORNELIUS, G., 1996. Essential oil constituents of the genus *Lippia* (Verbenaceae). A literature review. Journal of Essential Oil Research **8:** 471-485.

VAN WYK, B. E. & GERICKE, N. 2000. People's plants: A guide to useful plants of southern Africa, Briza Publications, Pretoria, ISBN 1-875093-19-2.

VILJOEN, A.M., SUBRAMONEY, S., VAN VUUREN, S.F. BASER, K.H.C. & DEMIRCI, B. 2005. The composition, geographical variation and antimicrobial activity of *Lippia javanica* (Verbenaceae) leaf essential oils. Journal of Ethnopharmacology **96:** 271-277.

WATT, J.M. & BREYER-BRANDWIJK, M.G. 1962. The medicinal and poisonous plants of southern and eastern Africa, 2nd edition. Livingstone, London.

ISOLATION AND IDENTIFICATION OF THREE COMPOUNDS FROM *HOSLUNDIA OPPOSITA* VAHL

Abstract

Hoslundia opposita is an aromatic herb that occur all over in Mozambique and is well known for its medicinal properties. In the initial screening of plants used in Mozambique for antimycobacterial activity, *Hoslundia opposita* demonstrated good antitubercular activity (Chapters 2). It was therefore selected to identify its bioactive constituents. A Phytochemical investigation of *H. opposita* led to the isolation of three known compounds, 5,7-dimethoxy-6-methylflavone (1), hoslunddiol (2) and euscaphic acid (3). This is the first report of the isolation of "5, 7- dimethoxy-6-methylflavone" from *Hoslundia opposita*.

5.1 Introduction

5.1.1 *Hoslundia opposita*: biological activity and chemical constituents

Hoslundia opposita Vahl (Figure 5.1) is an herbaceous perennial shrub (1-2m tall) belonging to the Lamiaceae.

It is widely distributed in tropical and subtropical open lands of Africa (Morton, 1981). Various parts of *Hoslundia opposita* are popular remedies in Africa to treat gonorrhea, cystitis, cough, wounds, sores, snake bites, conjunctivitis, epilepsy, chest

Figure 5.1 *Hoslundia opposita* (Plantzafrica.com)

pain, stomach trouble, and mental disorders (Ayensu & De Filipps, 1978, Watt and Breyer-Brandwijk, 1962). Infusions of its leaves are widely used in traditional medicine as a purgative, diuretic, febrifuge, antibiotic, and antiseptic (Onayade *et al*.1989).

The crude extracts of the entire plant have been found to exhibit strong antibacterial activity (Khan *et al*., 1993) and volatile constituents have been identified (Onayade *et al*.1989). A recent study had reported that leaves of this plant could be potentially used in treatment of epilepsy and convulsions (Risa *et al*., 2004). There have been no reports on the antitubercular or antiviral biological activity. In the initial screening of plants used in Mozambique *Hoslundia opposita* demonstrated good antitubercular activity (Chapter 2). It was therefore selected to identify its bioactive constituents.

5.2 Materials and methods

5.2.1 Plant material

Leaves of *Hoslundia opposita* were collected at Matola- Gare, Mozambique in June 2004.

The voucher specimens have been deposited at H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

5.2.2 Extraction and isolation

Leaves of *H. opposite* (130 g) were extracted with 1.5 L of ethanol for two days then filtered, the process was repeated two times. The extracts were combined and evaporated under reduced pressure to afford 21 g of crude ethanol extract. as described above. The total extracts (21 g) were subjected to a silica gel column (30 x 5 cm). Solvent system ethyl acetate: hexane with increasing polarity (EtOAc %, volume; 0 %, 1L; 10%, 2 L; 30%, 2 L; 50%, 2 L; 70%, 2 L; 100%, 1 L) followed by 10% of methanol in ethyl acetate (2L) was used as an eluent. Ten fractions based on their TLC profile were combined and concentrated to dryness under reduced pressure. Fraction IX (3.7 g) was chromatographed on silica gel which was followed by Sephadex LH-20 columns to yield 5,7-dimethoxy-6-methylflavone (1, 216 mg) and hoslunddiol (2, 36.4 mg). Fraction V (786 mg) was chromatographed over a silica gel column using $CHCl_3$–MeOH (98:2) to yield euscaphic acid (3, 80 mg).

5.2.3 Identification of isolated compounds

UV spectra were recorded using a Pharmacia LKB-ultraspec 111 UV spectrophotometer. NMR spectra were recorded using a Bruker ARX 300 or a Bruker Avance DRX 500 MHz. Mass spectra were obtained with a JEOL JMS-AX505 W mass spectrometer. The recorded spectral data of the isolated compounds were compared with those published in literature.

5.3 Results and discussion

5.3.1 Compound 1: 5, 7- dimethoxy-6-methylflavone

The Compound 5,7- dimethoxy-6-methylflavone, (Figure 5.1), showed in ^{1}H-NMR two singlets δ_H 6.67 and 6.57 typical to H-3, H-8 of flavone , two multiplet signals integrated to two and three protons respectively at 7.90 and 7.51 of unsubstituted B ring in addition to two singlets, three protons. Signals at δ_H 3.89, 3.85 of two methoxy groups and an aromatic methyl group signal at 2.35.The previous data indicated the presence of the known compound 5,7- dimethox-6 methylflavone which is reported here for the first time from *Hoslundia opposite* (Häberlein and Tschiersch, 1994).

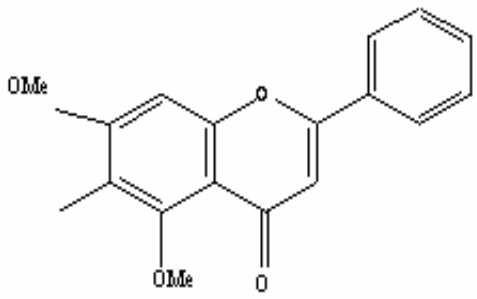

Figure 5.1 Structure of 5,7- dimethoxy-6-methylflavone

5.3.2 Compound 2: Hoslunddiol

UV spectral data λmax 252, 275 and 312 nm suggested a flavone with OH at C-5. ^{1}H-NMR showed singlets at position 6.58 (H-3) and 6.41 (H-8) integrated 7.78 (2H) and

7.46 (3H) of unsubstituted ring B, anomeric proton at 5.4 (H-1``, *J*= 8.0 Hz) attached to carbon resonating at δ_C 105.5, aromatic methoxy group at 3.85, in addition glycosy signal typical to β-digitoxopyranose. The above data indicated the presence of 6-C-β-digitoxopyranosyltectochrysin, hoslunddiol (Figure 5.2) which was isolated before from the same species (Ngadjui *et al*., 1991).

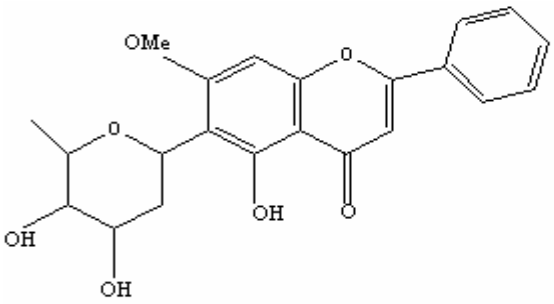

Figure 5.2 Structure of Hoslunddiol

5.3.3 Compound 3: Jacarandic acid or euscaphic acid

The compound jacarandi**c** acid or euscaphic acid (Figure 5.3) showed in ^{1}H-NMR four methyl singlets at δ_H 0.69, 0.91, 1.06, 1.27, one doublet signal of a methyl group at δ_H 0.83 (*J*=5.8 Hz), two protons attached to hydroxyl bearing carbons at δ_H 3.34 (obscured by H_2O signal) and broadening doublet at 4.34 and an olefinic proton at δ_H 5.15.

The previous data in addition to careful analysis of Dept-135 data, confirmed the presence of uresane type triterpene with carboxylic group at C-28, two vicinal axial – equatorial oriented two protons at C-2, C-3, double bond at C-11 and a methyl attached to hydroxyl bearing carbon at C-19 (δ_H 1.27, s). The NMR data published by (Ogura *et*

al., 1977; Chandel and Rastogi, 1977 & Takahashi *et al*., 1974) verified that the isolated compound is jacarandic acid. The review of literature on the species indicated that these data is typical with that of Jacarandic acid isolated before from the same source (Ogura *et al*., 1977; Chandel and Rastogi, 1977 and Takahashi *et al*., 1974).

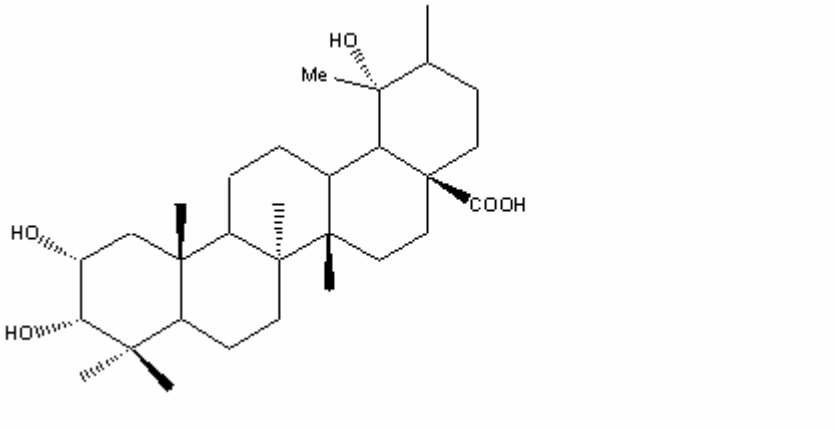

Figure 5.3 Structure of Jacarandic acid

5.4 Conclusion

Phytochemical investigation of *H. opposita* led to the isolation of three known compounds, 5,7-dimethoxy-6-methylflavone (1), hoslunddiol (2) and euscaphic acid (3). This is the first report of the isolation of "5,7- dimethox-6 methylflavone" from *Hoslundia opposita*.

5.5 References

AYENSU, E.S.& DE FILIPPS, R.A.1978. Endangered and threatened plants of the United States. Washington, DC: Smithsonian Institution.

CHANDEL, R.S. & RASTOGI, R.P. 1977. Indian Journal of Chemistry 15B, 914.

HÄBERLEIN, H., TSCHIERSCH, K. 1994. Triterpenoids and flavonoids from *Leptospermum scoparium*. Phytochemistry **35 (3):** 765-768.

KHAN, M.R., NDALIOG, NKUNYA, M.H., WEVERS, H. &. SAWHNEY, A.N. 1993. In oppositin and 5-O-methylhoslundin, Flavonoids of *Hoslundia opposita.*

NGADJU, B.T, AYAFOR, J.F., SONDENGAM, B.L., CONNOLLY, D.J, RYCROFT, D., TILLEQUIM, F. (Eds.). Phytochemistry 32(5), 1313-1315.

MORTON, J.F. 1981. Atlas of medicinal plants of Middle America: Bahamas to Yucatan Springfield, Illinois, USA, pp. 745-750.

RISA, J., RISA, A., ADSERSEN, A., GAUGUIN, B., STAFFORD, G.I., VAN STADEN, J. & JÄGER, A.K., 2004. Screening of plants used in southern Africa for epilepsy and convulsions in $GABA_A$-benzodiazepine receptor assay. Journal of Ethnopharmacology **93,** 177-182.

OGURA, M., CORDELL, G.A. & FARNSWORTH, N.R. 1977. Llodia **40:** 157.

ONAYADE, O.A., NTEZURUBANZA, L., SCHEFFER, J.J. C. & SVENDSEN, A.B. 1989. 37th. Annual Congress on medicinal plant research 5-9 September.

TAKAHASHI, K., KAWAGUCHI, S., NISHIMURA, K., I., KUBOTA, K., TANABE, Y. & TAKANI, M. 1974. Chemistry Pharmaceutical. Bull.**22:** 650.

NGADJUI**,** B.T., AYAFOR, J.F., SONDENGAM, B.L., CONNOLLY,J.D.& YCROFT, D.S. 1991. Hosulundin, Hoslundal, and Hoslunddiol: Three new flavonoids from the twigs of Hoslundia opposita (Lamiaceae), Tetrahedron, **47**, 3555-3564.

ANTIBACTERIAL ACTIVITY OF THE COMPOUNDS ISOLATED FROM *LIPPIA JAVANICA* AND *HOSLUNDIA OPPOSITA*

Abstract

The isolated compounds from *Lippia javanica* and *Hoslundia opposita* were investigated for their *in vitro* antimicrobial proprieties against two bacterial strains, one Gram-positive *Staphylococcus aureus* (ATCC 12600) and one Gram-negative *Escherichia coli* (ATCC 11775). A bioautographic assay, using *Staphylococcus aureus* (ATCC 12600), was used to detect the presence of the antibacterial compound 4-ethyl-nonacosane . The compound showed notable effects against *S. aureus*. No inhibitory effect was found in the compounds tested against Gram-positive and Gram-negative bacteria strains at a concentration of 200µg/ml by microdilution technique using 96-well microtitre plates.

6.1 Introduction

The antimicrobial activity of medicinal plants has been evaluated previously using various methods, which are classified into three groups: The disc-diffusion, dilution and bio-autographic methods. In this study bio-autography and dilution methods were used. The dilution assays are those, which require a homogeneous dispersion of the sample in water (Rio et al., 1988). These methods are mainly used to determine the Minimum Inhibitory Concentration (MIC) values of an extract or pure compound. These values are

taken as the lowest concentration of the extract or pure compound that completely inhibits bacterial growth after incubation for 24 h. In the liquid dilution method, turbidity is taken as an indication of bacterial growth, so where the sample is inactive against the micro organism tested, the liquid will appear turbid (Rio *et al*., 1988). The advantages of this are its simplicity and speed, and the possibility of using it in the antimicrobial study of water-soluble or insoluble samples such as essential oils (Rio *et al*. 1988). Eloff (1998) developed a microdilution technique using 96-well microtitre plates. A two-fold serial dilution of the extract, pure compound/ drug is prepared in the wells of the microplate, and bacterial culture is added. After incubation p-iodonitrotetrazolium violet (INT) is added, and in the wells where bacterial growth occurs, a deep red colour develops. Wells containing antibacterial compounds remain clear.

The bioautographic method is an important detection for new or unidentified antimicrobial compounds (Rio *et al*., 1988**).** In the direct bio-autography assay, a suspension of micro-organisms in liquid medium is sprayed on a developed TLC plate and incubated overnight. A solution of tetrazolium salt is then sprayed on the plate and incubated to detect the areas of bacterial growth inhibition. According to Hamburger & Cordell (1987) an advantage of the bioautograhy is that it allows the localization of activity, even in complex mixtures.

6.2 Material and methods

6.2.1 Bioautographic bioassay

The antibacterial activity of the isolated compound **1** (4-ethyl-nonacosane) was evaluated against *Staphylococcus aureus* (ATCC 12600) by direct bioautography technique in a TLC bioassay (Hamburger & Cordell, 1987) because of its low solubility. Compound quantities ranging from 50 µg to 1.56 µg were applied to percolated TLC plates. The TLC was observed under ultra violet (UV) light (254 and 366 nm) after development, left overnight for the solvent to evaporate completely and sprayed with the bacterial suspension. These plates were then re-incubated at 25°C for 24 h (Lund & Lyon, 1975). The results were stained with an aqueous solution of INT.

6.2.2 Microdilution assay

The Minimal Inhibitory Concentration (MIC) values of the compounds were determined against the Gram-positive *Staphylococcus aureus* (ATCC 12600) and Gram-negative *Escherichia coli* (ATCC 11775) bacterial strains. The microplate dilution method of Eloff (1998) was used. The bacterial cultures were incubated in Müller-Hinton (MH) broth overnight at 37°C and diluted 1:100 with fresh MH prior to use in the microdilution assay. A two-fold serial dilution of the compound (100µl) was prepared in 96-well microtitre plates, and 100µl bacterial culture was added to each well. The pure

compounds were dissolved in 10 % DMSO. The antibiotic Streptomycin was used as a standard in each assay, as well as a DMSO solvent control. The covered microplates were incubated overnight at 37°C. As an indicator of bacterial growth, 40 µl *p*-iodonitrotetrazolium violet (INT) dissolved in water was added to the microplate wells and incubated at 37°C. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product in biologically active organisms (Eloff, 1988). Where bacterial growth is inhibited, the solution in the well will remain clear after incubation. Only two bacteria strains were used to test the activity of the isolated compounds, since we isolated little amount these compounds.

6.3 Results

6.3.1 Bioautography results

The compound 4-ethyl-nonacosane displayed good bactericidal activity against *Staphylococcus aureus* (ATCC 12600). Zones of bacterial growth inhibition could be seen on TLC plates sprayed with *S. aureus* (ATCC 12600) as white spots on a red background (Figure 6.1). The white areas indicate the presence of antibacterial compounds, as the lack of bacterial growth cannot convert the indicator tetrazolium salt to a red product. Metabolically active bacteria convert the tetrazolium salt into the corresponding intensely coloured formazan. The activity of 4-ethyl-nonacosane may be

attributed to the presence of the toxicity. 4-ethyl-nonacosane is an alkane. Alkanes are organic compounds which are found to be useful as anaesthetic and toxic agents (Di Paolo, 1978a; Di Paolo, 1978b).

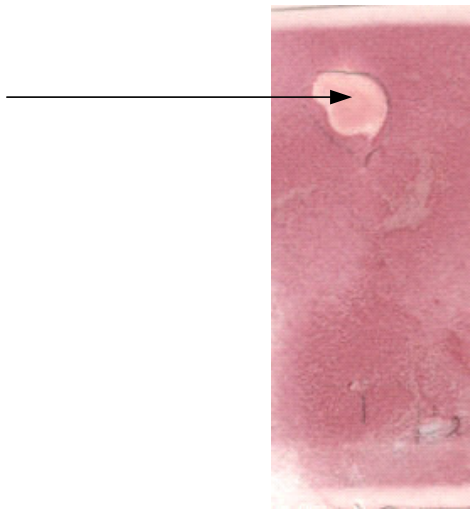

Figure 6.1 Inhibition of *Staphylococcus aureus* (ATCC 12600) by 4-ethyl-nonacosane.

6.3.2 Bioassay results

All isolated compounds from *Lippia javanica* and *Hoslundia opposita* did not show activity against the bacteria on the microdilution assay at the tested concentration of 200µg/ml, as is shown in Figures 6.2 and 6.3.

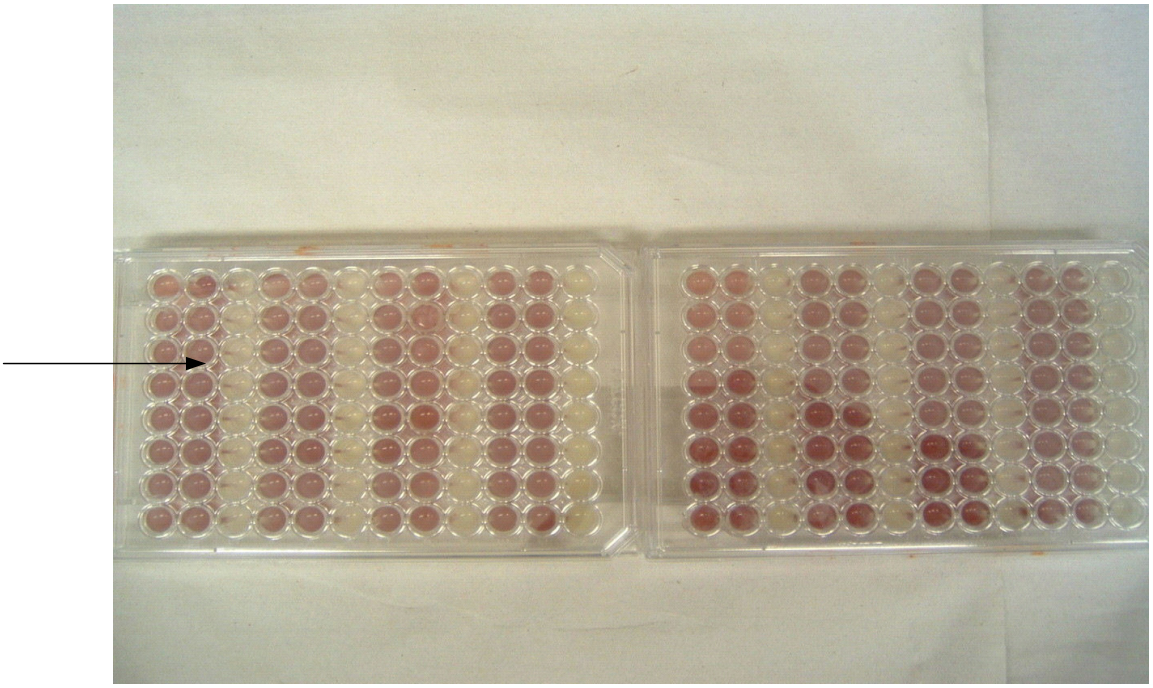

Figure 6.2 Antibacterial test of isolated compounds against *Escherichia coli* (ATCC 11775). Dark coloured wells (arrow) indicate normal bacteria growth.

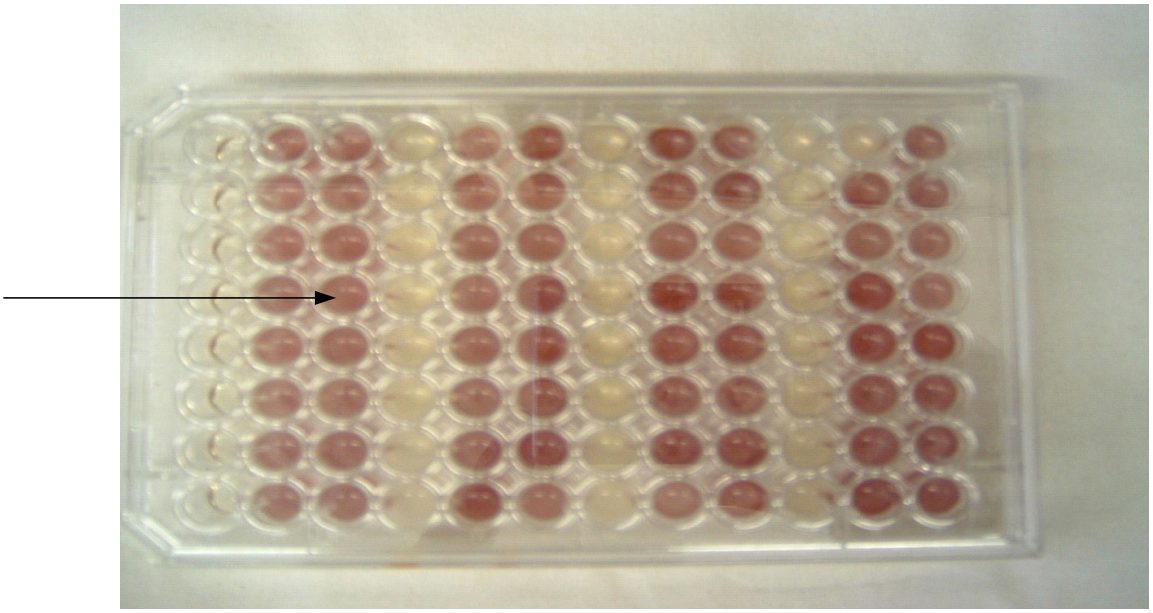

Figure 6.3 Antibacteria test of isolated compounds against *S. aureus*. Dark coloured wells (arrow) indicate normal bacteria growth.

Although the compounds had no activity at the highest tested concentration, the antifungal proprieties of many of those compounds are well known (El-Gammal and Mansour 1986; Aziz *et al*., 1998).

6.4 Conclusion

The reported antibacterial activity of *Lippia javanica* and *Hoslundia opposita* can be attributed to the synergistic combinations of compounds (Viljoen *et al*., 2005, *Mujovo* et al*., 2003a; 2003b; Khan* et al*., 1980),* and it may also be possible that some of the active compounds were not isolated.

Lack of biological activity in the compounds tests does not necesarily indicate lack of effectiveness of the remedies.. They may act in other ways to effect a cure, Such as by stimulating the immune system of the patient, or by manufacturing internal conditions unfavourable for the multiplication of bacteria. For another hand, if plants are used as part of a mixture, the synergistic effects of principles in more than one plant may cause relief from the ailment

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6.5 References

AZIZ, N.H., FARAG, S.E., MOUSA, L.A.A & ABO-ZAID, M.A. 1998. Comparative antibacterial and antifungal effects of some compounds. Microbios **93:** 43-54.

DI PAOLO, T. 1978a. Structure- activity relationships of anaesthetic ethers using molecular connectivity. Journal of Pharma. Sci. **67:** 564- 566.

DI PAOLO, T. 1978b. Molecular connectivity in quantitative structure activity relationship study of anaesthetic and toxic activity of aliphatic hydrocarbons, ethers and ketones J. Pharm. Sci. **67:** 566- 568.

ELOFF, J.N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta medica **64:** 711-713.

EL-GAMMAL, A. A. & MANSOUR, R. M. A. 1986. Antimicrobial activities of some flavonoids compounds. Zentrablatt fur Mikrobiologie **141:** 561-565.

HAMBURGER, M.O. & CORDELL, A.G., 1987. Direct bioautograhic TLC assay for compounds possessing antibacterial activity. Journal of Natural Products **50:** 19-22.

KHAN, M.R., NDALIOG, NKUNYA, M.H.H., WEVERS, H. & SAWHNEY, A.N. 1980. Planta Medica Supplement 91**.**

LUND, D.M. & LYON, G.D. 1975. Detection of inhibitors of *Erwinia carotovora* and *Erwinia herbicola* on thin-layer chromatograms. Journal Chromatogr. **110:** 193-196.

MUJOVO, S.F., LALL, N., MPHAHLELE, M., FOURIE, P. & MEYER, J.J.M. 2003a. Screening of Mozambican medicinal plants for antibacterial activity. Joint International conference SAAB & ISE, University of Pretoria, South Africa, 7-11 *January.*

MUJOVO, S.F., LALL, N., MPHAHLELE, M., FOURIE, P. & MEYER, J.J.M. 2003b. Identification of bioactive compounds from *Lippia javanica*. Indigenous plant use Forum, Rustenburg, South Africa, 7-10 July.

RIO, J.L, RECIO, M.C.& VILLAR, A. 1988. Screening methods for natural products with antimicrobial activity: a review of the literature. Journal of thnopharmacology **23:** 127-149.

VILJOEN, A.M., SUBRAMONEY, S., VAN VUUREN, S.F. BASER, K.H.C. & DEMIREI, B. 2005. The composition, geographical variation and antimicrobial activity of *Lippia javanica* (Verbenaceae) leaf essential oils. Journal of Ethnopharmacology **96:** 271-277.

ANTIMYCOBACTERIAL ACTIVITY OF ISOLATED COMPOUNDS FROM *LIPPIA JAVANICA* AND *HOSLUNDIA OPPOSITA*

Abstract

Eight compounds isolated from *Lippia javanica* and three compounds, from *Hoslundia opposita* were tested against *Mycobacterium tuberculosis* at concentrations of 200, 100, 50 *and* 25µg/ml. Compound "6-Methoxyluteolin 4'-methyl ether" isolated from *L. javanica* exhibited a minimum inhibitory concentration (MIC) of 200 µg/ml against *M. tuberculosis*. Of all the compounds tested against a drug-sensitive strain of *M. tuberculosis*, euscaphic acid was found to show the best activity exhibiting an MIC of 50 µg/ml against this strain. The remaining compounds were found to be inactive at the highest concentrations tested.

7.1 Introduction

Tuberculosis is a bacterial disease caused mainly by *Mycobacterium tuberculosis* and *Mycobacterium bovis. Mycobacterium tuberculosis* was isolated by Robert Koch in 1882. *Mycobacterium bovis* is responsible for tuberculosis in domestic or wild cattle. *M. bovis* infections are uncommon in most countries today. In the past, this infection was often transmitted through the oral route by drinking milk from infected cows

(Porter & McAdam, 1994). Virtually all new infections with *M. tuberculosis* are acquired via airborne transmission. The sources of infections are persons with tuberculosis of the lung or larynx who are coughing. Coughing produces tiny infectious droplets, 1-5µm in size, known as droplet nuclei. In indoor environments, these droplet nuclei can remain suspended in the air for long periods of time unless they are removed by ventilation, filtration or ultraviolet irradiation.

Tuberculosis is an ancient disease. It was present in Egypt from early dynastic times, perhaps as early as 3700 BC (Morse *et al*., 1964). Manchester (1984) has reviewed evidence that suggests that human tuberculosis may have evolved during the Neolithic period (seventh and sixth millennia BC) at which time population increases and cattle domestication occurred in Europe and the eastern Mediterranean. Tuberculosis was well recognized by the time of Hippocrates (c. 460-377 BC) who gave an excellent clinical description of the disease (Hippocrates, 1939). In India, the medical Luminary Sursruta (c.500 AD) mentioned the disease in his writings (Pierry & Roshem, 1931). WHO (2000) estimates that between the years 2000 and 2020 nearly one billion people will die from the disease. The greater majority of the world's population, and thus the majority of infected persons, reside in developing countries (Snider *et al*., 2005).

The number of cases worldwide is rapidly increasing due to the appearance of single-drug-resistance (SDR) and multidrug-resistance (MDR) of strains of *M. tuberculosis* which are insensitive to one or more the first-line anti-TB drugs (isoniazid [INH], rifampin, ethambutol, streptomycin and pyrazinamide (Telzak *et al.*, 1995) and also

due to an increase in patients with immunodeficiency virus (HIV) infection, which has further exacerbated the problem (Zumla & Grange, 1998). The emergence of strains of *Mycobacterium tuberculosis* resistant to existing drugs has focussed attention on the urgent need for discovery and development of new antimycobacterial agents. Action must be taken now to avert this global health disaster.

There is a need for more intense efforts in the discovery of new specific drugs from natural and synthetic sources. There are reports on inhibition of mycobacteria by medicinal plants. The compound allicin from *Allium sativum* was found to be as potent as some of the standard antitubercular drugs such as streptomycin, isoniazid, ethambutol and rifampin (Jain, 1994). Allicin, prepared from the ethanolic extract inhibited the growth of *Mycobacterium tuberculosis* H37Rv and *M. tuberculosis* TRC-C1193 that is completely resistant to isoniazid. The MIC was 70 µg/ml for both the organism (Indian Council of Medical Research, 2004). Lall (2000) reported antitubercular activity of naphthoquinone 7-methyljuglone isolated from *Euclea natalensis*. The compound was tested against a drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* and the minimal inhibitory concentration (MIC) were found to be 50 µg/ml for both the strains of *M. tuberculosis***.** This may mean that there should be an abundance of antitubercular drugs remaining to be discovered in plants.

This chapter focuses on the antimycobacterial activity of compounds isolated from *Lippia javanica* and *Hoslundia opposita***.**

7.2 Materials and methods

7.2.1 Bioassay on *Mycobacterium tuberculosis*

Anti-TB activity of compounds against *M. tuberculosis* H37Rv was determined using the radiometric respiratory technique with the BACTEC apparatus as described in chapter 2 of this thesis. The nine compounds (3 isolated from *H. opposita*) and 6 isolated from *L. javanica* were dissolved at 20 mg/ml in 1 % DMSO. Subsequent dilutions were done in DMSO and added to 4 ml of BACTEC 12B broth to achieve the desired final concentrations of 200, 100, 50 and 25 µg/ml together with PANTA (Becton Dickinson & Company), an antimicrobial supplement. The BACTEC drugs susceptibility testing was also done for the two primary drugs streptomycin and ethambutol at concentrations of 6 and 7.5µg/ml respectively against the H37Rv sensitive strain. Preparation of bacterial cultures and the testing procedures were the same as described in chapter 2. All tests were done in triplicate.

7.3 Results and discussion

Results were interpreted on day 6 or 7 when the control vials containing the 1:100 dilution of the inoculum reached a GI value of 30 or more (Table 7.1). Among the nine compounds tested, the MIC of jacarandic acid or euscaphic acid, isolated from *Hoslundia opposita* was found to be 50µg/ml against the H37Rv. strain. This indicated that the strain is partially susceptible to the compound at a low

concentration of 50µg/ml. The MIC of the terpene compound, 6-Methoxyluteolin 4'-methyl ether, isolated from *L. javanica* was found to be 200 µg/ml. The remaining compounds were inactive. Not much information is available in the literature about the antimycobacterium activities of natural triterpenes, however similar activities were found observed in ursane triterpenes (Mujovo *et al*., 2008).

Table 7.1 Anti-tuberculosis activity of compounds found in *L. javanica* and *H. opposita*

Compounds tested	MIC[a] µg/ml	ΔG values of compounds µg/ml
From *L. javanica*		
6-Methoxyluteolin 4'-methyl ether	200	12 ± 2
Cirsimaritin	na[b]	> 200
6-Methoxyluteolin 3',4',7-trimethyl ether	na[b]	> 200
Apigenin	na[b]	> 200
1-(3,3-dimethoxiranyl)-3-methyl- (2*E*)	na[b]	> 200
Pipertinone	na[b]	> 200
β-sistosterol	na[b]	> 200
4-Ethyl-nonacosane ($C_{31}H_{64}$)	na[b]	> 200
From *H. opposita*		> 200
Digitoxypyranosyltectochysin or Hoslunddiol	na[b]	> 200
5,7-dimethoxy-6-metylflavone	na[b]	> 200
Jacarandic acid or euscaphic acid	50	6 ± 0.0

ΔG Control: 26.5 ± 3.5,
[a] minimal inhibitory concentration, ΔG values are means ± standard deviation
[b] not active at the highest concentration tested

7.4 Conclusion

Of all the compounds tested against a drug-sensitive strain of *M. tuberculosis*, euscaphic acid was found to show the best activity exhibiting an MIC of 50 µg/ml

against this strain. The compound deserves further investigation in order to explore its potential as an antimicrobial agent.

7.5 References

HIPPOCRATES 1939. The Genuine Works of Hippocrates translated by Francis Adams, Williams and Wilkins. Baltimore. pp. 101-133.

INDIAN COUNCIL OF MEDICAL RESEARCH 2004. Reviews on Indian Medicinal Plants, New Delhi, Volume 2 (Alli-Ard), pp 39.

JAIN, R.C. 1994. Effect of garlic oil on the growth of *Mycobacterium tuberculosis* by modified microslide culture method. Indian Drugs **31**: 500-502.

LALL, N. 2000. Isolation and identification of naphtoquinones from *Euclea natalensis* with activity against *Mycobacterium tuberculosis*, others pathogenic bacteria and *Herpes simplex* virus. PhD thesis. University of Pretoria.

MANCHESTER, K. 1984. Tuberculosis and leprosy in antiquity: an interpretation. Medical History, **28**:162-173.

MORSE, D. BROTHWEELL, D.R. & UCKO, P.J. 1964. Tuberculosis in ancient Egypt. American Review of Respiratory Disease **65**: 6-24.

MUJOVO, S.F., HUSSEIN, A.A., MEYER, J.J.M., FOURIE, B., MUTHIVHI, T., LALL, N. 2008. Bioactive compounds from Lippia javanica and Hoslundia opposite. Natural products research **22**:1047-1054.

PIERY, M.& ROSHEM, J.1931. Historie de la Tuberculose. G Doin, Paris. pp, 5-7.

PORTER, J.D.H.& MCADAM, K.P.W.J. 1994. Tuberculosis Back to the Future. London School of Hygiene & Tropical Medicine. Third Annual Public Health Forum.

SNIDER DEJR, RAVIGLONE, M. & KOCHI, A. 2005. Global Burden of Tuberculosis In Tuberculosis Pathogenesis, Protection and Control. American Society for Microbiology.

TELZAK, E.E., SEPKOWITZ, K., ALPERT, P., MANNHEIRMER, MEDARD, S.L SADR, W., BLUM, S. GAGLIARDI, A., SALOMEN, N. & TURETT, G. 1995. Multi-drug resistant tuberculosis in patients without HIV infection. N. Eng. J. Med. **333:** 907-911.

WHO 2000. WHO tuberculosis fact sheet No. 104.

ZUMLA, A. & GRANGE, J. 1998. Clinical review: Tuberculosis. Brit Med J. **316:** 1962-1964.

ANTI-HIV ACTIVITY OF ISOLATED COMPOUNDS FROM *LIPPIA JAVANICA* AND *HOSLUNDIA OPPOSITA*

Abstract

The discovery of medicinal agents capable of specifically inhibiting human immunodeficiency virus (HIV) is urgently needed due to its globally widespread infection. In this study, compounds isolated from *Lippia javanica* and *Hoslundia opposita* were investigated for their ability to inhibit HIV-1 Reverse transcriptase activity *in vitro* using a non-radioactive assay. Two compounds "1-(3,3-dimethy-oxiranyl)-3-methyl-penta-2, 4-dien-1-one" and "Pipertinone" from *L. javanica* demonstrated inhibitory activity against the enzyme by 90 and 53 %, respectively at 100 µg/ml. One compound "5, 7-dimethoxy-6-methylflavone" isolated from *H. opposita* was shown to have 52 % inhibition at 100 µg/ml.

8.1 Introduction

Acquired immunodeficiency syndrome (AIDS) is a pandemic immunosuppressive disease which results in life-threatening opportunistic infections and malignancies.

Human immunodeficiency virus (HIV) requires three key enzymes for viral replication inside a host cell, Reverse transcriptase, protease and integrase. Reverse transcriptase is one of the main targets for inhibiting the reproduction of HIV. This enzyme is responsible for transcription of viral RNA into a DNA, which is later,

integrated into the host cell and carries the information for the synthesis of new viral particles. Inhibition of the HIV of HIV-1 RT, besides the later discovery HIV protease and integrase inhibition was the first therapeutic approach successfully applied in prolonging the life of infected patients (Barre-Sinoussi, 1996). Searching for novel inhibitors of the HIV replication cycle is the main interests of numerous investigators and enormous efforts have been dedicated to finding promising lead compounds, both synthetic and natural (De Clercq, 1995). Inhibition of retroviral RTs by plant derived compounds has previously been described. Since a retrovirus (HIV) has been clearly identified as the primary cause of AIDS, many compounds of plant origin have been evaluated for their inhibitory effects on HIV replication (Vlietinck *et al.*, 1998, Ng and Huang, 1997).

8.2 Materials and Methods

8.2.1 HIV-1 RT assay

The assay was performed as described in chapter 3, but each compound was tested at 100 µg/ml .

8.3 Results and discussion

The standard Reverse transcriptase assay is a specific, sensitive, simple and reliable method for discovery potential agents that inhibit HIV-1 and HIV-2 RT from natural sources. Evaluation of all the isolated compounds from *L. javanica* and *H. opposita*

against HIV RT showed that two compounds "1-(3,3-dimethy-oxiranyl)-3-methyl-penta-2, 4-dien-1-one" and "Pipertinone" from *L. javanica* demonstrated inhibitory activity against the enzyme by 90 and 53 %, respectively at 100 µg/ml. One compound "5, 7-dimethoxy-6-methylflavone" isolated from *H. opposita* was shown to have 52 % inhibition at 100 µg/ml (Table 1). Little is known about the HIV RT activity of monoterpenes in literature, however, the results indicated that compound "1-(3,3-dimethy-oxiranyl)-3-methyl-penta-2, 4-dien-1-one" could be of interest as a template in drug discovery research due to the higher activity rather than the other compounds isolated from both plants. There are no previous reports of the anti- HIV activity of this compound.

Flavonoids are widely distributed in nature and were found to be active against viruses HSV-1, HSV-2, rotavirus, and even against HIV (Vlitinck *et al.*, 1998; Harborne *et al*. 1975). In HIV, their activity was related to a direct effect on the virus or the enzymes responsible for its replication (HIV-1 Reverse transcriptase or HIV-1 integrase). A flavone from *H. opposita* showed considerable inhibition against RT similar to the reports on 3-methoxyflavones. 3-Methoxyflavones, and synthetic derivates thereof, have proven to be promising leads for developments antirhinovirus drugs (Ishitsuka *et al*., 1982, De Meyer *et al*., 1990). 3-methoxyflavones interfere with an early stage in the viral RNA synthesis (Lopez Pila *et al*., 1989; Castrillo *et al*., 1986).

Table 8.1 Anti- HIV RT activity of compounds *L. javanica* and *H. opposita*

Compounds		% inhibition[a]
	From *L. javanica*	
1	4-ethyl-nonacosane	2.00 ± 0.2
2	(E)-2(3)-Tagetenone epoxide	91.00 ± 0.04
3	Myrcenone	0.60± 0.01
4	Piperitenone	53.00± 0.01
5	Apigenin	- 19.00± 0.03
6	Cirsimaritin	12.00± 0.0
7	6-Methoxyluteolin 4-methyl ether	0.70 0±.01
8	6-Methoxyluteolin 3,4,7-trimethyl ether	17.00±0.02
	From *H. opposita*	
9	5, 7- dimethoxy-6-methylflavone	52.00 ± 0.01
10	Hoslunddiol	15.00 ± 0.01
11	Jacarandic acid or euscaphic acid	3.00 ± 0.0
	Adriamycin (Positive control)	96.00± 0.2

Note:-[a]Percentage inhibition are average SD.

8.4 Conclusion

Two compounds (E)-2(3)-Tagetenone epoxide and "Pipertinone" from *L. javanica* demonstrated inhibitory activity against the enzyme by 90 and 53 %, respectively. One compound "5, 7-dimethoxy-6-methylflavone" isolated from *H. opposita* was shown to have 52 % inhibition. The three compounds would be interesting for further investigation.

8.5 References

BARRE-SINOUSSI, E. 1996. HIV as the cause of AIDS, Lancet. **348:** 31-35.

CASTILLO,J.,VANDEN BERGHE,D. & CARRASACO, L. 1986. 3 methylquercetin is a potent and selective inhibitor of poliovirus RNA synthesis, Virology, 152, 219.

DE CLERCQ, E. 1995. Toward improved anti-HIV chemotherapy: therapeutic strategies for intervention with HIV infections, J. Med. Chem. **38:** 2491-2517.

DE MEYER, N., VLIETINCK, A., PANDEY, H., MISHRA, L., PIETERS, L., VANDEN BERGHE, D. & HAEMERS, A. 1990. Synthesis and antiviral properties of 3-methoxyflavones, in flavonoids in Biology and Medicine III: Current Issues in Flavonoid Research, Das, N.P., Ed., National University of Singapore, Singapore, 403.

HARBORNE, J.B.; MABRY, T. J. & HELG MABRY 1975. The Flavonoids. CHAPMAN & HALL, London.

ISHITSUKA, H., OHSAWA, C., OHIWA, T., UMEDA, I. & SUHARA, Y. 1982. Antipicornavirus flavone Ro-09-0179, Antimicrob. Agents Chemother, 22, 611

LOPEZ PILA, J.M., KOPECKA, H. & VANDEN BERGHE, D. 1989. Lack of evidence for strand-specific inhibition of poliovirus RNA synthesis by 3 methylquercetin, Antiviral Research, 11, 47.

NG, T.B & HUANG, B. 1997. Anti-HIV natural products with special emphasis on HIV reverse transcriptase inhibitors, Life Science **6:** 933-949.

VLIETINCK, A. J., DE BRUYENE, T., APERS, S. & PIETERS, L.A. 1998. Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection. Planta Medica **64:** 97-109.

CYTOTOXICITY OF CRUDE EXTRACTS AND THE ISOLATED COMPOUNDS FROM *LIPPIA JAVANICA* AND *HOSLUNDIA OPPOSITA*

Abstract

Studies on the cytotoxicity of plant extracts are useful to evaluate the toxicological risks, The cytotoxicity tests are essential before the compounds can be considered for their impact in drug discovery. Plant extracts of *Lippia javanica, Hoslundia opposita* and three isolated compounds which showed promising activity in anti-HIV and antimycobacterial bioassay were evaluated for cytotoxicity against monkey kidney vero cell-lines. The compound "5,7-dimethoxy-6-methylflavone exhibited fifty percent inhibitory concentration (IC_{50}) of 2.73 µg/ml. The IC_{50} values of crude extracts of *Hoslundia opposita* and *Lippia javanica* were found to be 116.8 ± 6.16 µg/ml and 29.41 ± 7.845 µg/ml respectively. The other isolated compounds exhibited the following IC_{50} values: piperitenone IC_{50} >200, 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)*, 13.96 ± 5.144, jacarandic acid or euscaphic acid IC_{50} 19.21 ± 4.520 µg/ml.

9.1 Introduction

Cytotoxicity is simply the cell-killing property of a chemical compound (such as food, cosmetics, or pharmaceuticals) or a mediator cell (such as a cytotoxic T cell),

independent from the mechanisms of death (Roche, 2004). There are various methods used for the determination of in vitro determination of cytotoxicity; such as brine shrimp, lactate dehydrogenase (LDH) assay and colorimetric assays. In this study an attempt was made to determine the cytotoxicity of crude extracts and bioactive compounds isolated from *Lippia javanica* and *Hoslundia opposita* using the colorimetric assay based on the tetrazolium reagent 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl] 2H-tetrazolium hydroxide (XTT) (Williams *et al*., 2003).

The XTT tetrazolium salt differs from the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl-)-2,5-diphenyl tetrazolium bromide (MTT) in that it produces a water-soluble formazan (Paull *et al*., 1988). The formazan dye formed is soluble in aqueous solutions and is directly quantified using scanning multiwell spectrophotometer (ELISA reader). The XTT based method was used in this study because it is reliable, straightforward, efficient and inexpensive way of determining cytotoxic properties in crude biological materials and purified chemical substances.

9.2 Materials and Methods

Cytotoxic test of crude/pure compounds were carried out using Vero African Green monkey cell line (Terasima and Yasukawa, 1988). The microtitre plate with Vero cells were used following the method of Zheng *et al*. (2001).

9.2.1 Cell culture

Vero cells were cultured in minimal essential medium (Eagle) containing 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 10 µg/ml penicillin, 10 µg/ml streptomycin, and 0.25 µg/ml fungizone, and 10% foetal bovine serum at 37^{O}C in a humidified atmosphere with 5% CO_2. Cells were subcultured in a 1:6 ratio every second to third day after trypsinization of confluent cultures.

9.2.2 Preparation of cells for cytotoxicity screen

On day 0, confluent cultures were trypsinized and diluted in complete MEM to a concentration of 1×10^5 cells/ml. In the outer wells of a 96-well plate, 200µl of medium was dispensed. All inner wells received 100µl (1×10^4 cells) of the cell suspension (Figure 9.1). The plate was incubated overnight at 37^{o}C in a humidified atmosphere with 5% CO_2.

9.2.3 Preparation of crude extracts and pure compounds

On day 1, stock solutions of crude extracts/pure compounds were prepared in DMSO at 20 mg/ml. Stock solutions of crude extracts were diluted 50 times in complete medium to 400 µg/ml. This was then serially diluted to obtain eight different concentrations of the

crude extracts (3.13, 6.25, 12.50, 25, 50, 100, 200, 400 µg/ml). Stock solutions of pure compounds were diluted 200 times in complete medium to 100 µg/ml. This was then serially diluted to obtain eight different concentrations of the pure compounds (0.78, 1.56, 3.13, 6.25, 12.50, 25, 50, 100 µg/ml).

9.2.4 XTT assay

On day 1, 100 µl of each crude extracts of *Lippia javanica* and *Hoslundia opposita*/pure compound dilution were dispensed into cell-containing wells of the sample plate in triplicate, Figure 9.2. The final concentrations of crude extracts and pure compounds in the wells were 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25, 50 µg/ml. Control wells received a final concentration of 1% (for crude extracts) or 0,25% (for pure compounds) DMSO in complete medium.

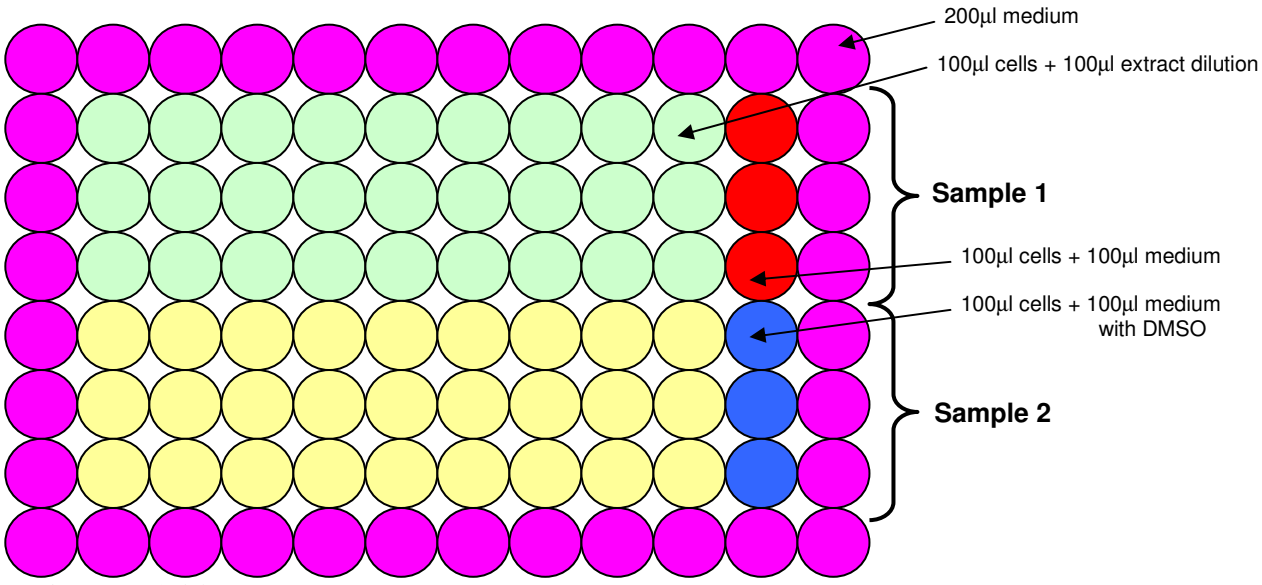

Figure 9.1 (a) Assay in 96-well Sample plate

The reference plate was also prepared that contained 100 µl of medium and 100 µl of diluted extract/compound, in duplicate, Figure 9.3. Plates were then incubated at 37^{O}C in a humidified atmosphere with 5% CO_2 for another 3 days. On day 3, 50µl of XTT reagent was added to the wells and incubation commenced for 1-4 hrs. The positive drug (Zearalenone) at final concentration of 1.25µg/ml was included. After incubation the absorbance of the colour complex was spectrophometrically quantified using an ELISA plate reader, which measures the optical density at 450nm with a reference wavelength of 690nm. The 'GraphPad Prism 4' statistical program was used to analyse the fifty percent inhibitory concentration ($IC_{50)}$ values.

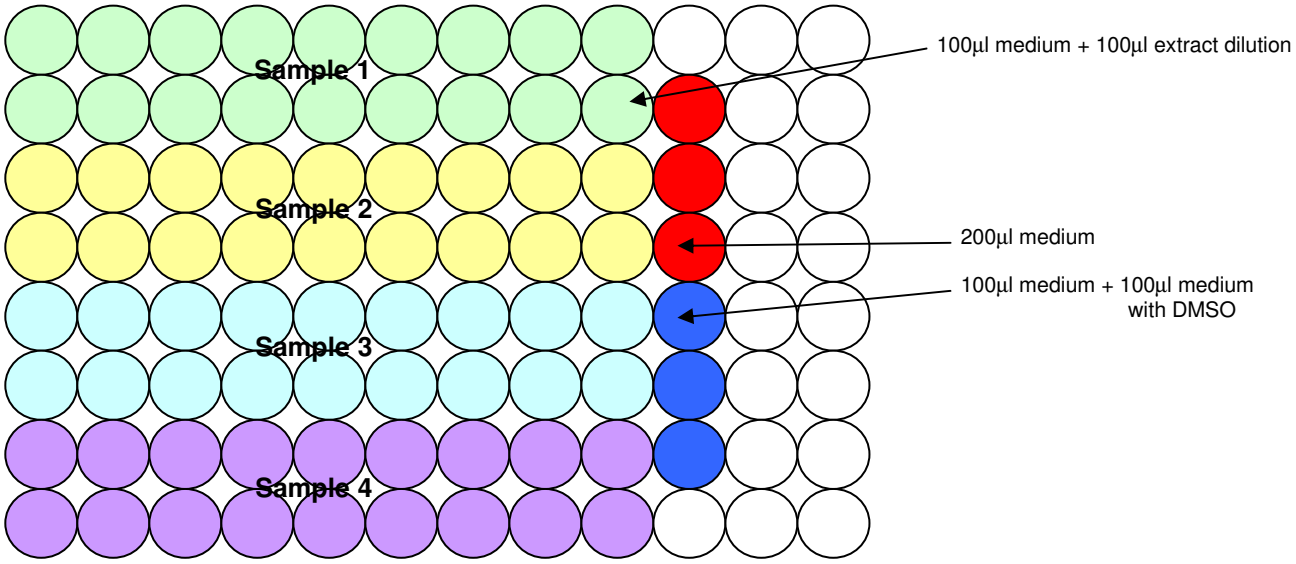

Figure 9.1(b) Assay in 96-well Reference plate

Calculations

The 650 nm reference wavelength values were subtracted from their corresponding 450 nm wavelength values. Reference plate values were then subtracted from their corresponding sample plate values. Cell viabilities (and therefore toxicities) were assessed by determining the ratio of the sample values to the control values:

$$(\%\ \text{Cell viability} = \frac{\text{Sample value}}{\text{Control value}} \times 100\ \%$$

9.3 Results and discussion

The IC_{50} values of the acetone crude extracts of *L. javanica* and *H. opposita* and four pure compounds isolated from those species are shown in graphs (Fig 9.4 - 9.9).

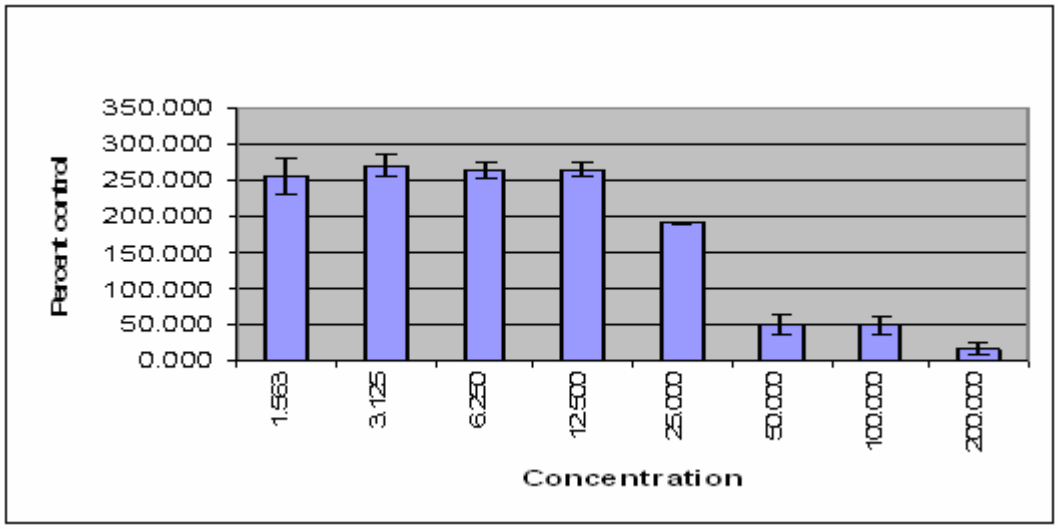

Figure 9.2 Cytotoxicity effect of acetone extract of *Lippia javanica* on Vero cell viability. IC_{50} values (µg/ml ± SD)[a] of 29.41 ± 7.845. [a] (µg/ml ± SD)=values are means ± standard deviation.

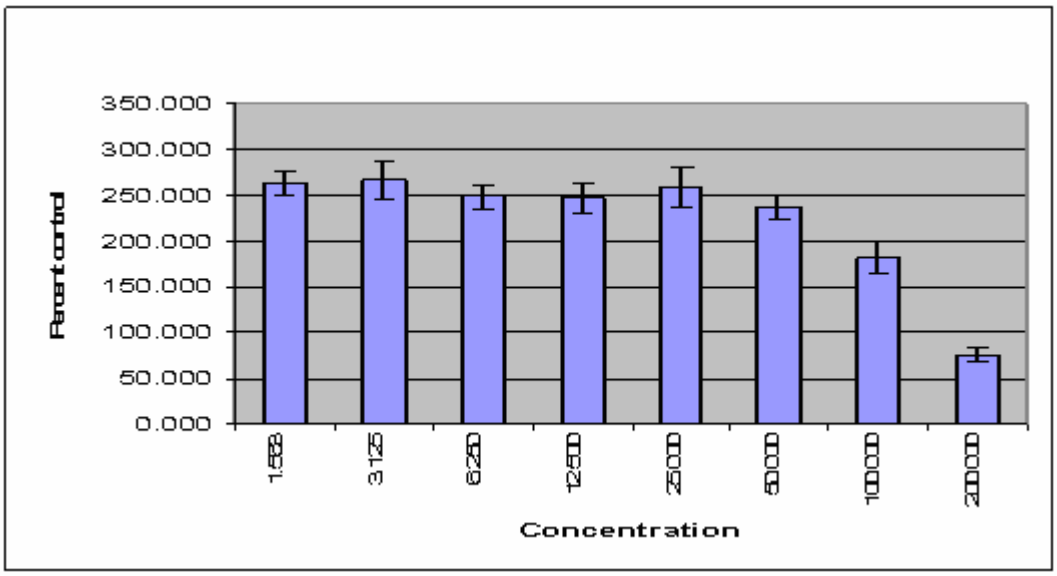

Figure 9.3 Cytotoxicity effect of acetone crude extract of *Hoslundia opposita* on Vero cell viability. IC_{50} values (µg/ml ± SD)[a] of 116.8 ± 6.162.

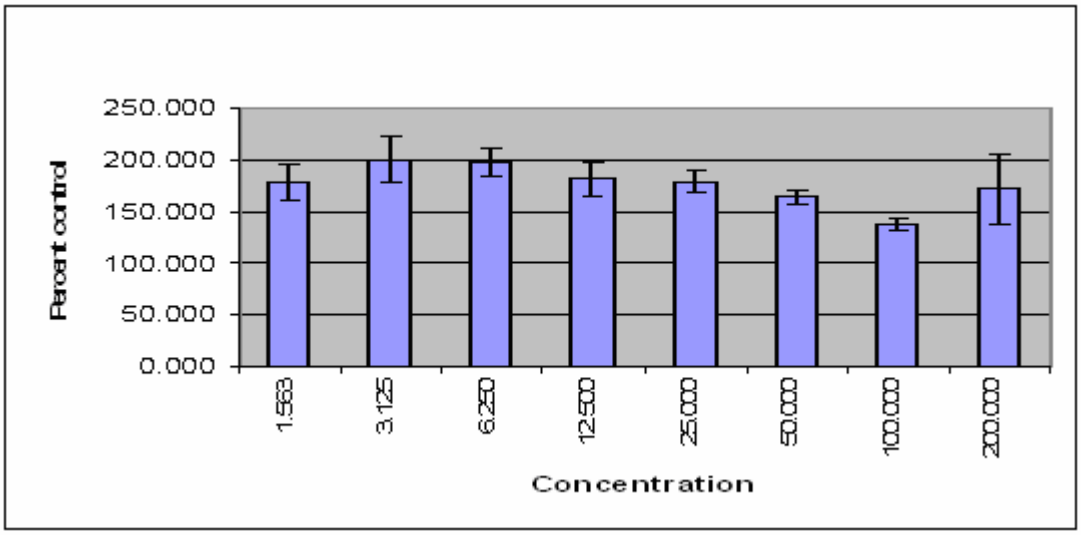

Figure 9.4 Cytotoxicity effect of compound piperitenone on Vero cell viability. IC_{50} values (µg/ml ± SD)[a] >200.

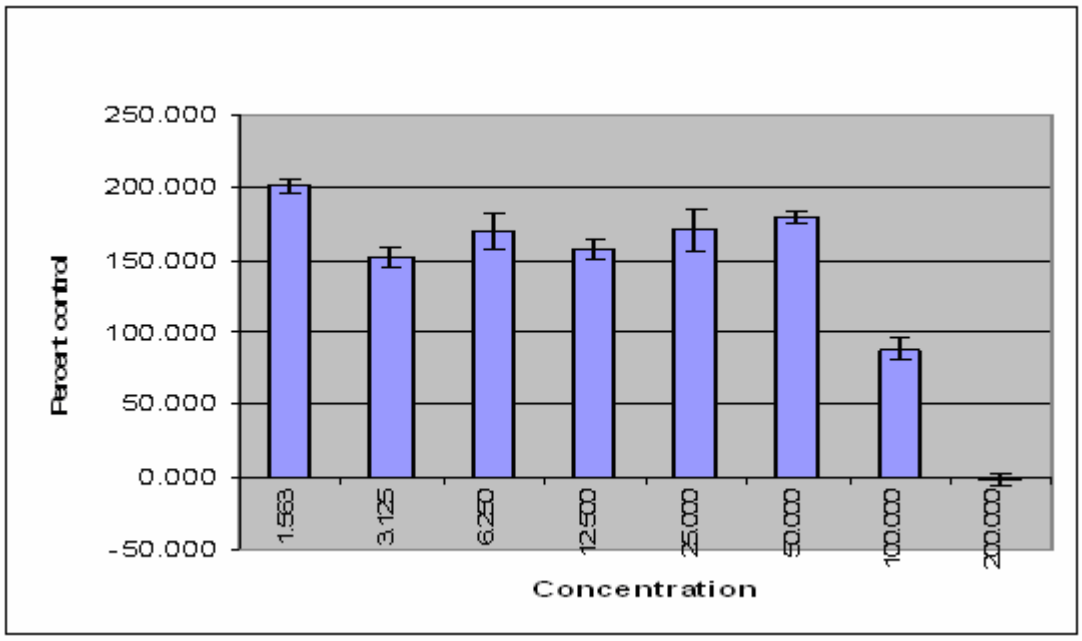

Figure 9.5 Cytotoxicity effect of compound 1-(3,3-dimethoxiranyl)-3-methyl- (2*E)* on Vero cell viability. IC_{50} values (µg/ml ± SD)[a] of 13.96 ± 5.144.

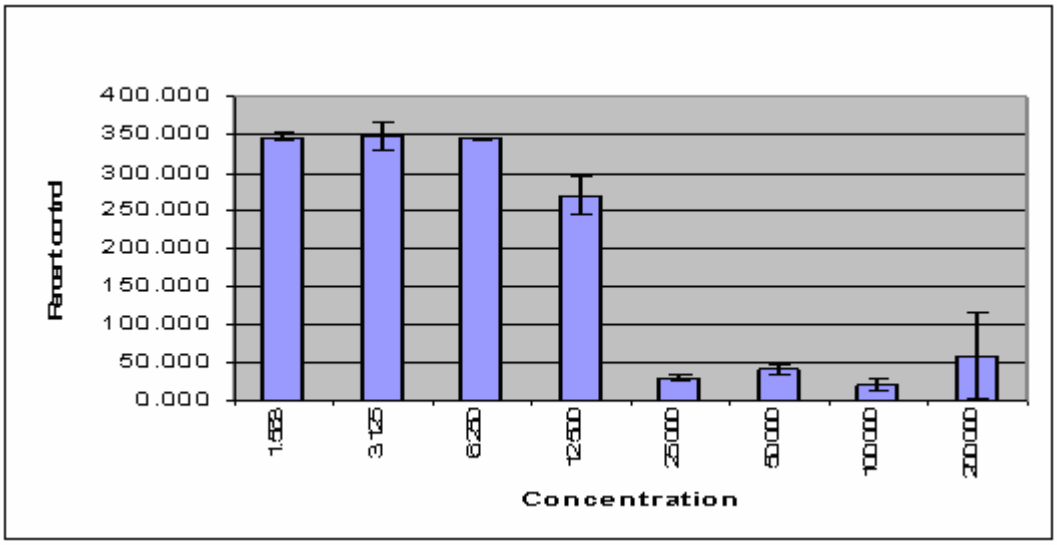

Figure 9.6 Cytotoxicity effect of compound Jacarandic acid or Euscaphic acid on Vero cell viability. IC_{50} values (µg/ml ± SD)[a] of 19.21 ± 4.520.

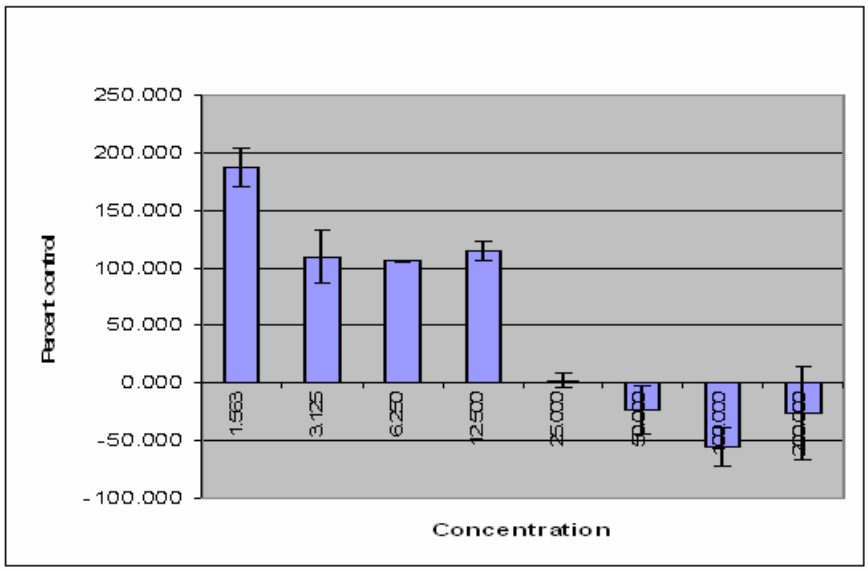

Figure 9.7 Cytotoxicity effect of compound 5,7-dimethoxy-6-methylflavone on Vero cell viability. IC_{50} values (µg/ml ± SD)[a] of 2.735 ± 1.497.

The crude extracts (*L. javanica* and *H. opposita*) and isolated compounds were evaluated *in vitro* for their inhibitory ability against the growth of Vero cell line. These cell line was inhibited by all the compounds at the highest concentration tested (200 µg/ml), except the compound piperitenone. The results obtained from the calculation made from spectrophotometer readings, indicated that the crude extracts (*L. javanica* and *H. opposita*) and piperitenone compound have little or no toxicity on Vero cells by exhibiting IC_{50} values of greater than 100 µg/ml. The compounds 5,7-dimethoxy-6-methylflavone and Jacarandic acid or Euscaphic acid showed very high toxicity by exhibiting IC_{50} values ranging from 2.735 µg/ml to 19.21 µg/ml. This findings is consistent with observation by Ogura *et al*.(1977) which showed important *in vivo* and *in vitro* anticancer activity against P-388 lymphocytic leukaemia cells. Xu *et al.(*2003) also observed neurotoxicity of Jacarandic acid in male albino Swiss-Webster mice.

9.4 Conclusion

The results reported here not only provide an insight into the toxic nature of the extracts used in traditionally for the ailments treatment, but also provided an opportunity for selection of bioactive extracts for initial fractionation and further studies in antimicrobial assay. The compound "5,7-dimethoxy-6-methylflavone exhibited fifty percent inhibitory concentration (IC_{50}) of 2.73 µg/ml. The IC_{50} values of crude extracts of *Hoslundia opposita* and *Lippia javanica* were found to be 116.8 ± 6.16 µg/ml and 29.41 ± 7.845 µg/ml respectively. The other isolated compounds exhibited the following IC_{50} values: piperitenone IC_{50} >200, 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E*), 13.96 ± 5.144, jacarandic acid or euscaphic acid IC_{50} 19.21 ± 4.520 µg/ml. Among isolated compounds, Piperitenone, and among extracts, extracts of *Hoslundia opposita* seemed to show least toxicity.

Futher studies, including in vivo experiments and toxicity tests are necessary to gain a full understanding of the effectiness and possible toxic nature of these remedies

9.5 References

PAULL, K.D., SHOEMAKER, R.H. & BOYD, M.R. 1988. The synthesis of XTT: a new tetrazolium reagent that is bioreducible to water soluble formazan. J. Heterocycl. Chem. **25:** 911-914.

OGURA, M., CORDELL, G.A. & FARNSWORTH, N.R. 1977. Jacoumaric acid, a new triterpene ester from *Jacaranda caucana*. Phytochemistry **16:** 286-287.

WEISLAW, O.S., KISER, R. & FINE, D.L.1989. New soluble formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. J. Natl. Cancer Inst. **81:** 577-586.

TWENTYMAN, P.R., FOX, N.E.& RESS, J.K. 1989. Chemosensitivity testing of fresh leukaemia cells using the MTT assay. Br. J. Haematol. **71:**19-24.

XU, H., .ZHANG, N. & CASIDA, J.E. 2003. Insecticides in Chinese medicinal plants: survey leading to jacaranone, a neurotoxicant and glutathione-reactive quinol. Journal of Agricultural and Food Chemistry, **51:** 2544-2547

ZHENG, Y.T., CHAN, W.L., CHAN, P., HUANG, H. & TAM, S.C. 2001. Enhancement of the antiherpetic effect of trichosanthin by acyclovir and interferon. FEBS Letters **496:**139-142.

WILLIAMS, C., ESPINOSA, O.A. MONTENEGRO, H., CUBILLA, L.CAPSON, T.L., ORTEGA-BARRIA, E. & ROMERO, L.I. 2003. Hydrosoluble formazan XTT: Its application to natural products drug discovery for Lrishmania. Journal of Microbiological Methods **55:** 813-816.

ROCHE 2004. Cell Proliferation Kit II (XTT). *http*:www.roche-applied-science.com/support.

TERASIMA, T., YASUKAWA, M., 1988. Biological; properties of Vero cells derived from present stock. In: Simizu, B.Terasima, T. (Eds.), Vero cells: Origin, Properties and Biochemical Applications. Department of Microbiology. School of Medicine, Chiba University, Japan, pp 32-35.

10.1 Motivation for this study

For centuries, medicinal plants have been used worldwide for the treatment and prevention of various ailments, particularly in developing countries where infectious diseases are endemic and modern health facilities and services are inadequate. The value of ethno-medicine and traditional pharmacology is nowadays gaining increasing recognition in modern medicine. The search for new, potentially medicinal plants is more successful if the plant is chosen on an ethnomedical basis. Many drugs have been purified from medicinal plants including antibacterial, antimycobacterial and antiviral compounds. In this study antimicrobial activity of 25 plants used in traditional medicine have been reported.

Traditional healers in the areas of Maputo, Gaza, Manica and Zambezia were consulted directly in collecting the basic ethnobotanical information about the plants studied. Based on this information 25 plants species, belonging to 20 genera and 13 families were chosen and collected in the field. Different parts (roots, stems, bark and leaves) of the selected plant species were extracted with acetone, which were subjected to assays aimed at assessing their antibacterial and antimycobacterial activities. The extracts of the plants were also assayed for their ability to inhibit the enzymes HIV-1 Reverse transcriptase (RT) and glycohydrolase (α- glucosidase and β- glucuronidase). Searching for novel inhibitors of the HIV replication cycle is one of the main interests of numerous investigators and enormous efforts have been dedicated to find promising lead compounds. HIV-1 Reverse transcriptase (HIV-1) is one of the main targets for inhibiting the reproduction of HIV. This enzyme is

responsible for transcription of viral RNA into DNA, which is later integrated into the host cell and carries the information for the synthesis of new viral particles.

Finally, the isolation and identification of active principles was attempted using two plants species (*Lippia javanica* and *Hoslundia opposita*) which showed promising activity in the initial for antimicrobial activity.

10.2 Screening of plant species for biological activity

The antibacterial results presented in Chapter 2 indicate that Gram-positive bacteria were found to be more susceptible than Gram-negative bacteria to plant extracts. The weak activity shown by the extracts against Gram-negative bacteria could be due to the differences in the bacterial cell wall structures. Gram-negative bacteria are surrounded by a lipopolysaccharide layer, which provides them with additional protection against antibacterial substances. However, among the 22 plant species tested, two (*Adenia gummifera* and *Momordica balsamina*) were found to have activity against Gram-negative bacteria with a minimum inhibition concentration of 5.0 mg/ml and one (*Rhoicissus revoilli*) inhibited *E. cloacae* at 2.5 mg/ml. The antimycobacterial activity of ten plant species was investigated employing the radiometric respiratory technique BACTEC system. Bacterial cultures were grown from specimens received from the Medical Research Council (MRC) in Pretoria. A susceptible strain of *M. tuberculosis*, H37Rv reference was obtained from an American type culture collection. Four of the ten plant species showed inhibitory activity against sensitive strain of *M. tuberculosis* at a concentration of 0.5 mg/ml, which was the lowest concentration tested, (Table 2.3). Three plant extracts showed

activity against the strain at concentrations of 1.0 mg/ml and another three at 2.5 mg/ml.

The result of the anti-HIV-1 investigation of the crude extracts showed that of the seventeen plant species tested against glycohydrolase enzymes, nine extracts inhibited α-glucosidase and eight β-glucuronidase. The inhibitory effect of ten plant extracts towards the enzyme Reverse transcriptase (RT) was shown and only two plants (*Melia azedarach* and *Rhoicissus tomentosa*) appeared to be active.

10.3 Isolation and identification of active compounds in plants

Out of 25 plants tested for bioassay activity it was found that *Lippia javanica* and *Hoslundia opposita* possess high antibacterial and antimycobacterial activity. In Chapters 4 and 5 the isolation and identification of bioactive compounds from *Lippia javanica* and *Hoslundia opposita* is described. Nine compounds were isolated from *L. javanica* and 3 compounds from *H. opposita*. A bioassay was applied to detect if any of the compounds inhibited the bacteria, *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV) in chapters 6, 7 and 8 respectively. The antibacterial test of the isolated compounds was found to be negative at the tested concentration of 200 µl/ml using the micro dilution method. An alkane compound was identified to be the antibacterial component, when tested using a bioautography method. The antimycobacterial activity of the isolated compounds, in Chapter 7. The MIC of 6-Methoxyluteolin 4'-methyl ether isolated from *L. javanica*, was found to be 200 µg/ml, while the MIC of jacarandic acid, isolated from *H. opposita* was found to be 50 µg/ml for a strain of *Mycobacterium tuberculosis.*

Three compounds were identified to be anti-HIV components (one from *H. opposita,* compound 5,7-dimethoxy-6-metylflavone, two compounds isolated from *L. javanica,* 1-(3,3-dimethy-oxiranyl)-3-methyl-penta-2,4-dien-1-one and piperitenone) with the results presented in Chapter 8.

10.4 Cytotoxicity of plant extracts

In order to test the safety of four bioactive compounds and both plant extracts the XTT assays were used. The results showed that the two plants species and the four compounds tested were well tolerated by Vero cells line.

10.5 Conclusion

The results presented in this thesis represent an extensive investigation into plants used by Mozambican traditional healers to treat bacterial, mycobacterial and viral diseases. The value of this research lies in the scientific verification of the use of many of these plants. Two plant species and some of the compounds responsible for activity have been identified. There is much potential for future research activities in this field, as investigation of the active principles of other plants with good biological activity may yield exciting discoveries. The active compounds against HIV and *Mycobacterium tuberculosis* should be explored further for their use in disease control.

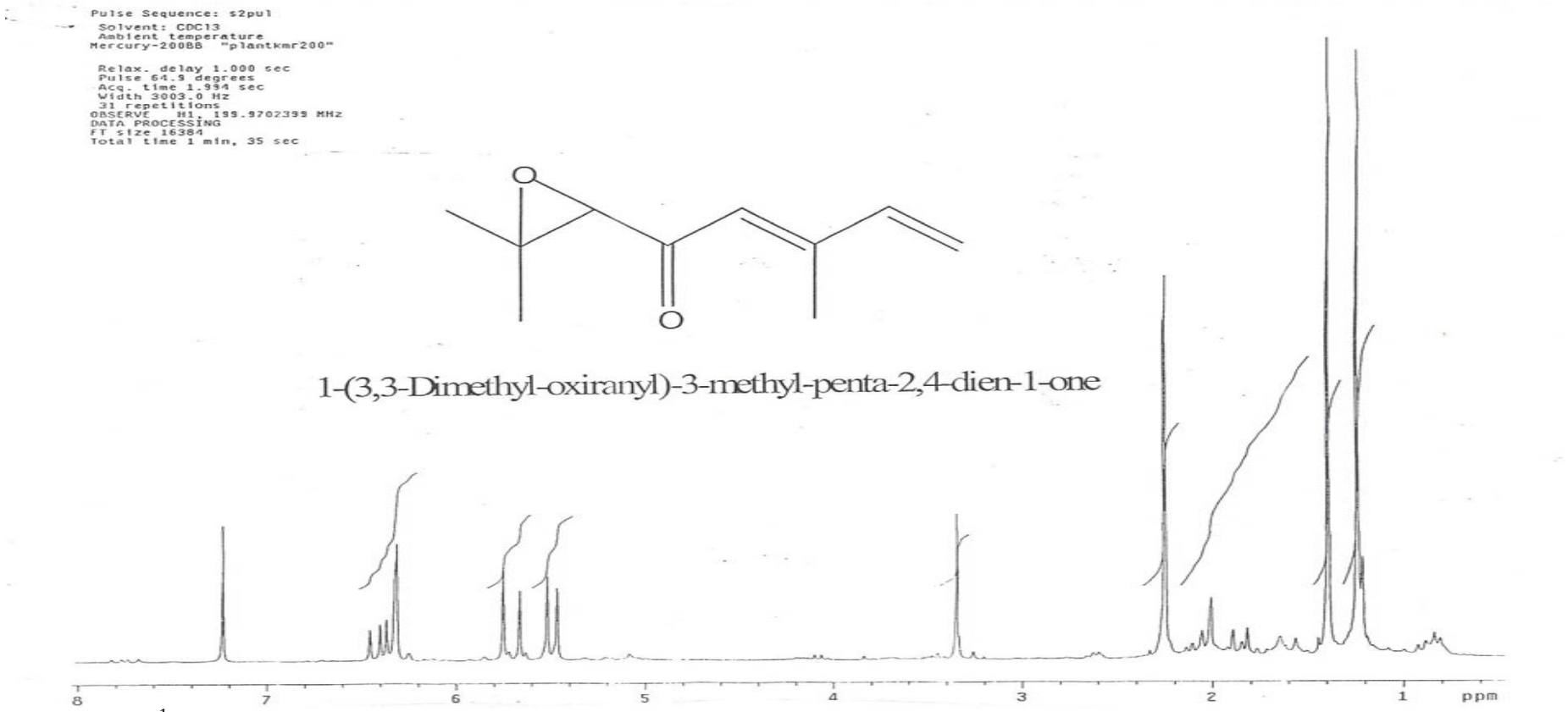

Figure 11. 1: ^{1}H- NMR spectrum of compound 2: 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)*

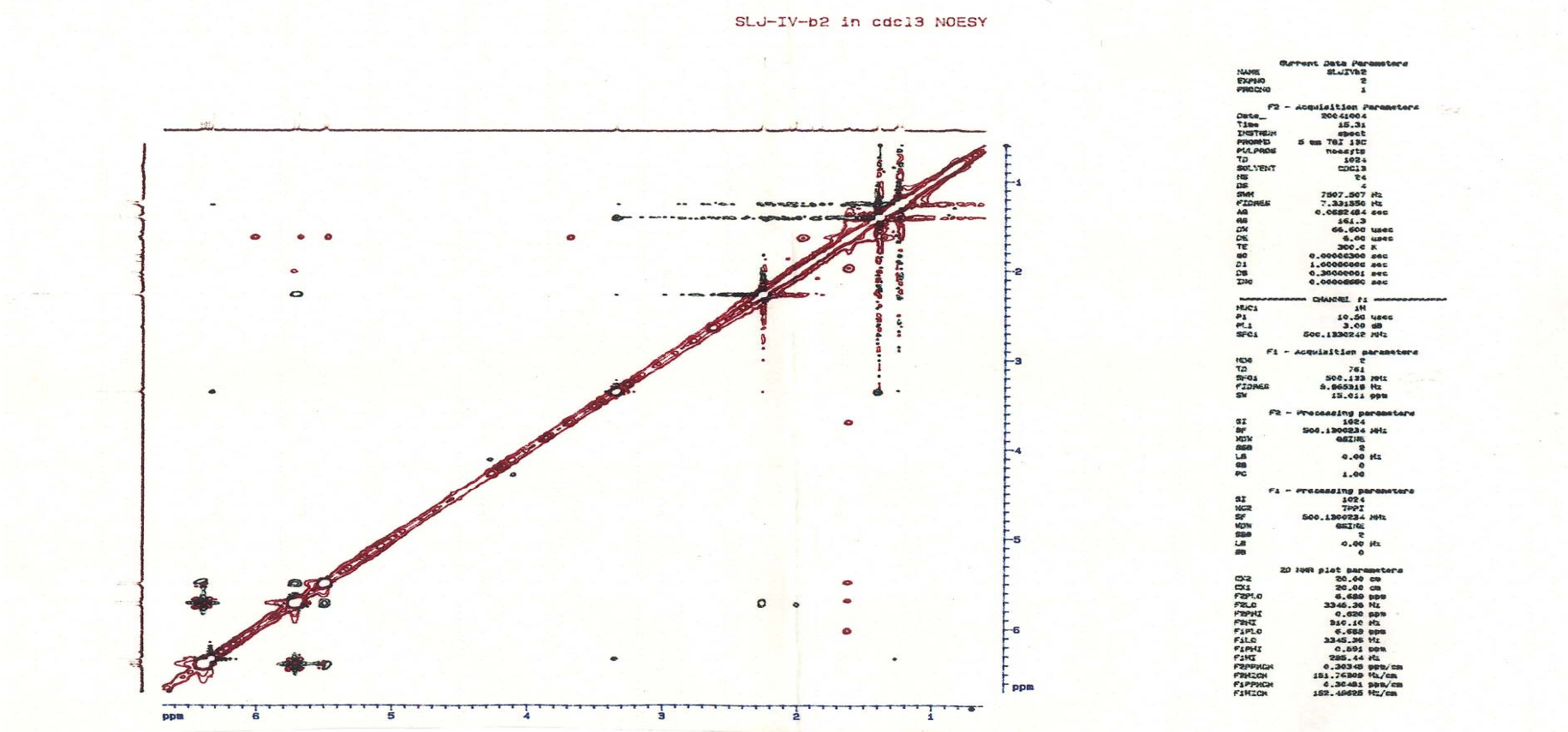

Figure 11..2: NOESY spectrum of compound 2: 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)*

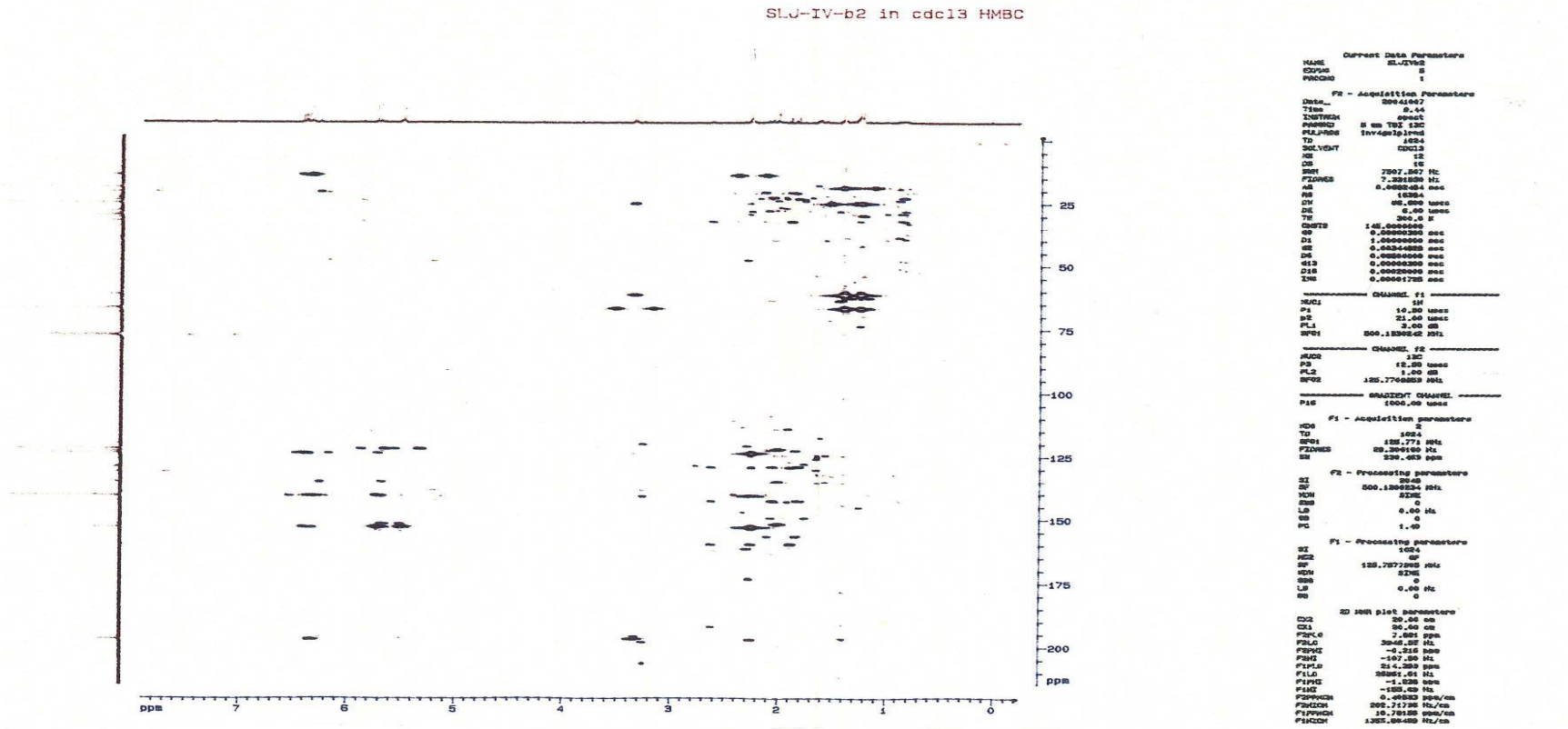

Figure 11..3: HMBC spectrum of compound 2: 1-(3, 3-dimethoxiranyl)-3-methyl- (2*E)*

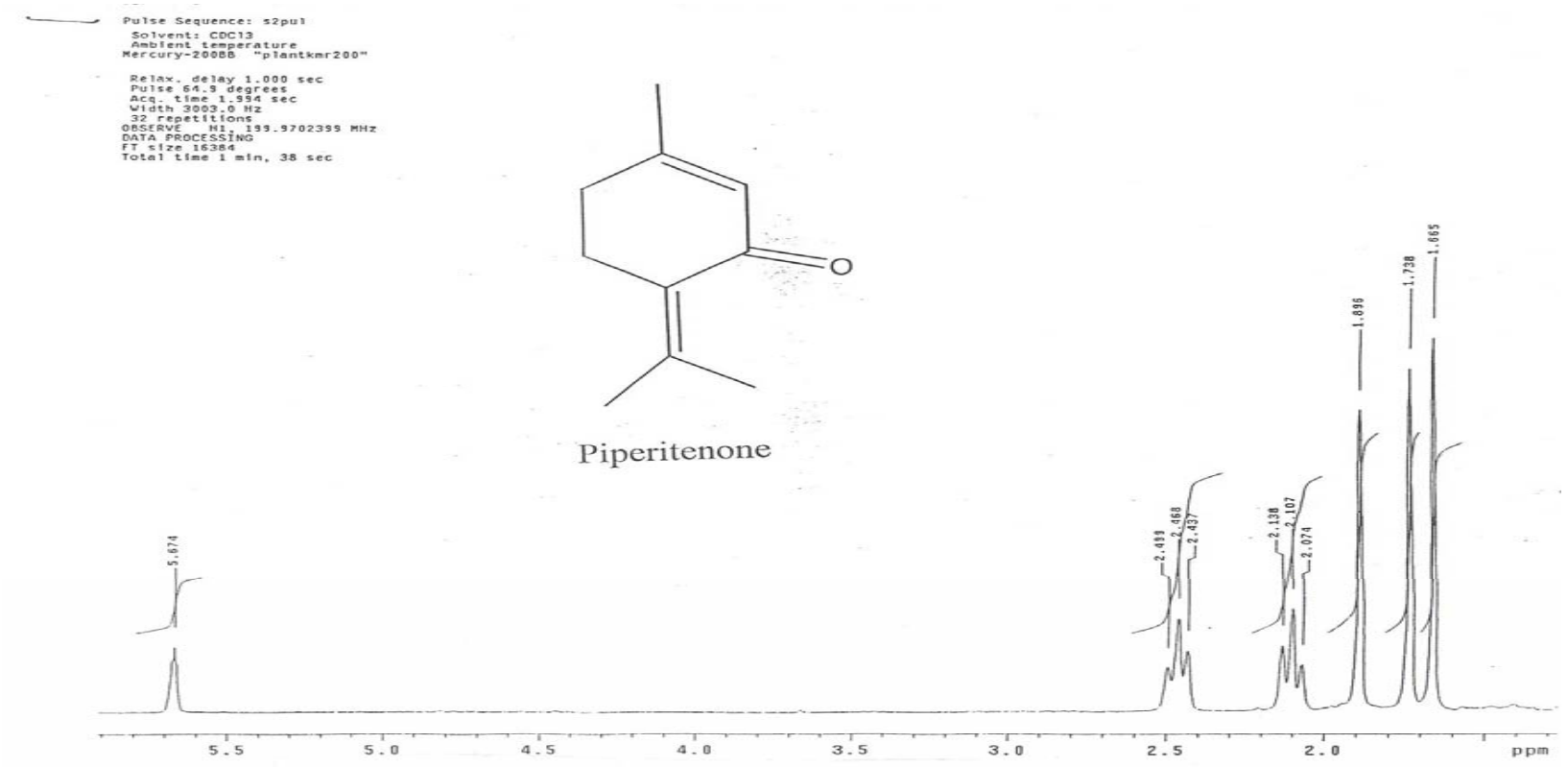

Figure 11..4 : ^{1}H-NMR spectrum of compound 4: piperitenone

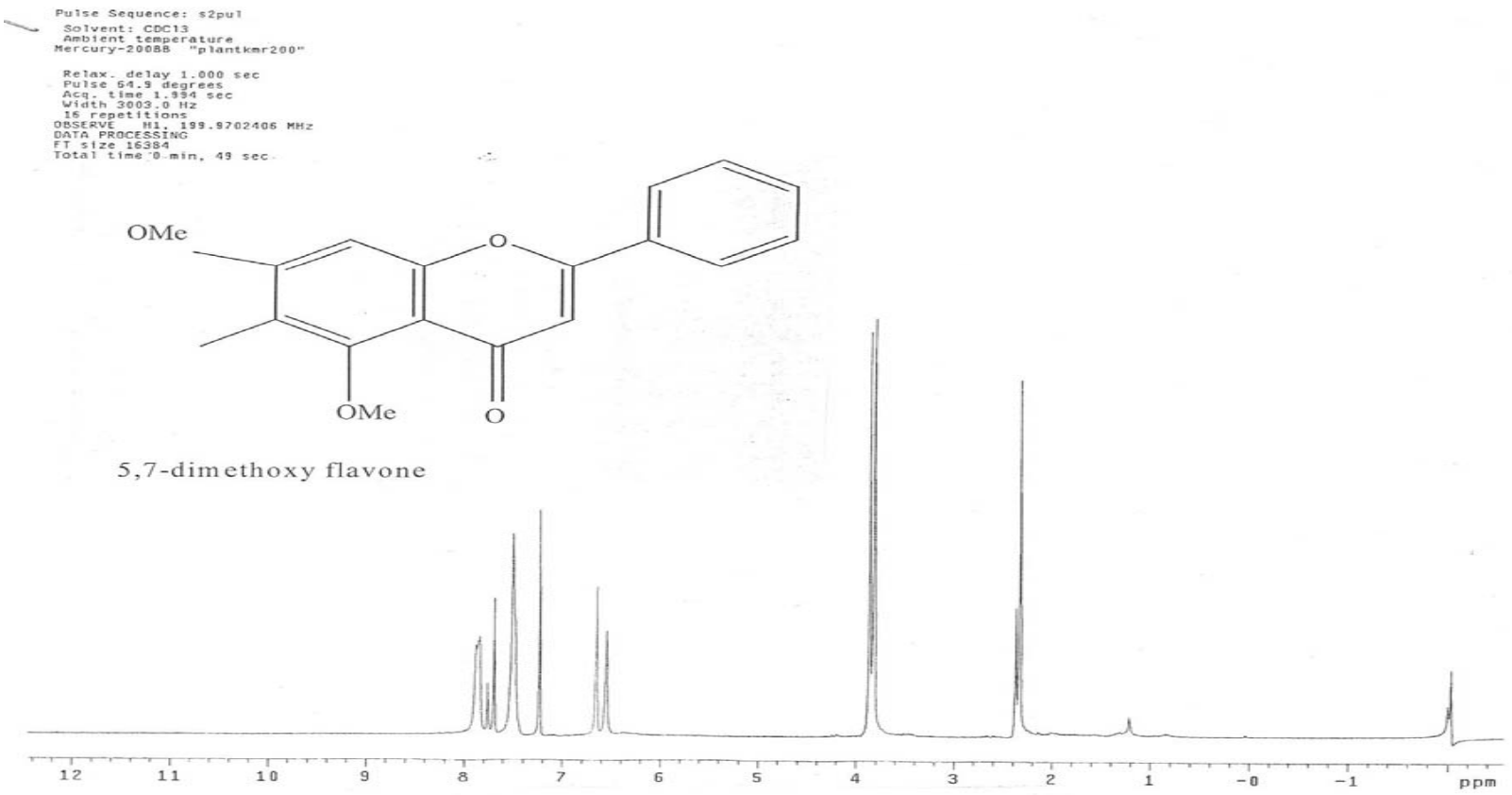

Figure 11.5: [1]H-NMR spectrum of compound 1: 5, 7- dimethoxy-6-methylflavone

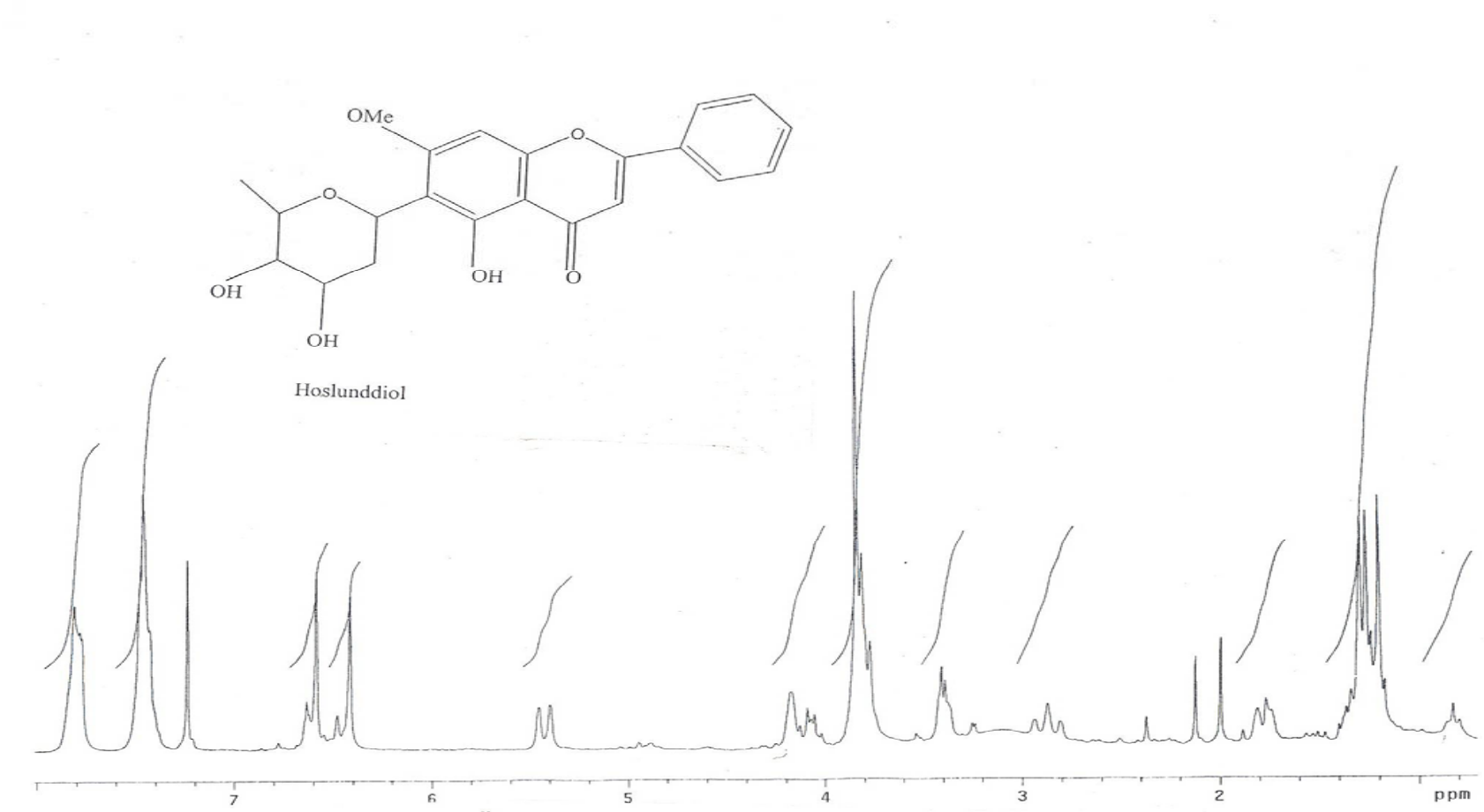

Figure 11..6 ^{1}H-NMR spectrum of compound 2: 6-C-β-digitoxopyranosyltectochrysin or hoslunddiol

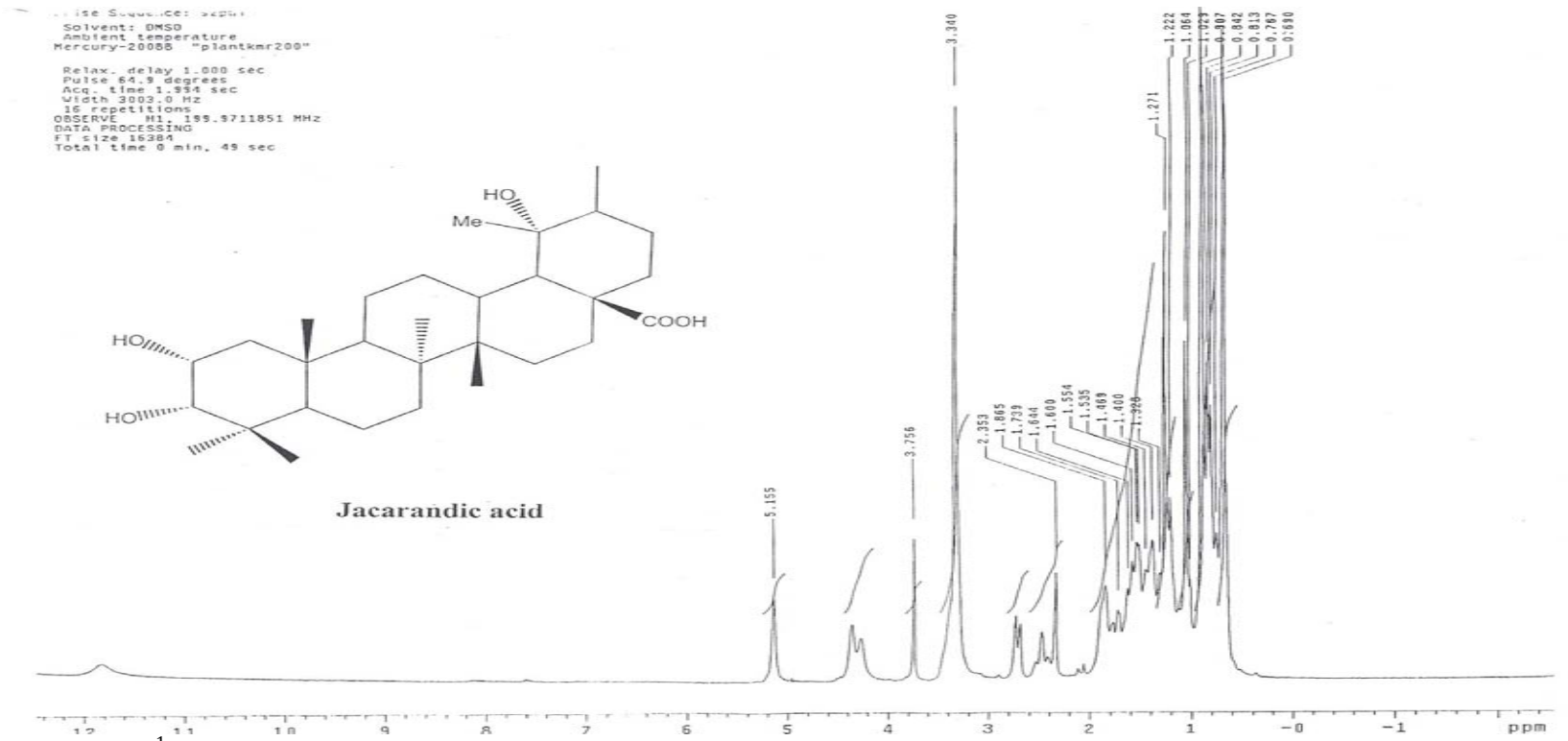

Figure 11.7 ^{1}H-NMR spectrum of compound 3: Jacarandic acid or euscaphic acid

2.1 Manuscripts resulting from this thesis

Mujovo, Silva, F., Ahmed A. Hussein, J.J. Marion Meyer., B. Fourie, Tshilidzi Muthivhi and Lall Namrita, 2008. Bioactive compounds from *Lippia javanica* and *Hoslundia opposita*, Natural Product Research, **22:** 12, 1047-1054.

Mujovo, S. F, Lall, N., Mphahlele, M., Fourie, P., Muthivhi, T. N, Meyer, J.J.M. 2007. Antituberculosis and antibacterial activity of medicinal plants collected in Mozambique. South Africa Journal of Botany (In preparation).

Evaluation of medicinal plants from Mozambique for anti-HIV activity (In Preparation).

2.2 Conference contribuitions from this thesis

Paper: S. F. Mujovo, N. Lall, J.H., Isaza Martinez & J.J.M Meyer.2002.

Screening of some Mozambican medicinal plants for antibacterial activity. **28th Annual Congress of SAAB (South African Association of Botanists),** University of Pretoria (South Africa).

Poster: S. F. Mujovo, N. Lall & J.J.M Meyer, 2003. Identification of bioactive compounds from *Lippia javanica*. **- Indigenous Plant Use Forum (IPUF),** Rustenburg (South Africa)

Poster: S. F. Mujovo, N. Lall, M. van de Venter & J.J.M Meyer. Antimicrobial and antiviral activity of *Cassia abbreviata*. **30th Annual Congress of SAAB (South African Association of Botanists),** University of KwaZulu- Natal (South Africa)