# Pharmacological evaluation of medicinal plants used by Venda people against venereal and related diseases

**By**

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**Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy**

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**School of Biological and Conservation Sciences**
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**Pietermaritzburg Campus**

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

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## DECLARATION 2 - PUBLICATIONS

**R.B. Mulaudzi,** A.R. Ndhlala, M.G. Kulkarni, J.F. Finnie, J. Van Staden 2011. Antimicrobial properties and phenolic contents of medicinal plants used by the Venda people for conditions related to venereal diseases. Journal of Ethnopharmacology 135, 330–337. This publication covers Chapter 2 and part of Chapters 3 and 4.

Contribution: Experimental work and writing of the publication was done by the first author, second author provided technical support, under supervision of the last three authors.

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# CONFERENCE CONTRIBUTIONS

1. 37th Annual Conference of the South African Association of Botanists (SAAB) and the 9th Southern African Society for Systematic Biology (SASSB), 17-19 January 2011. Rhodes University, Grahamstown, South Africa. Poster title: Antigonococcal and anti-HIV-type 1 reverse transcriptase (RT) of medicinal plants used by the Venda people.

2. Seminars of Slovak Botanical Society, 24 November, 2010. Comenius University, Bratislava, Faculty of Natural Sciences, Department of Plant Physiology, Slovakeia. Paper title: Antigonococcal and anti-HIV-type 1 reverse transcriptase (RT) of medicinal plants used by the Venda people.

3. 36th Annual Conference of the South African Association of Botanists (SAAB) and the 8th Southern African Society for Systematic Biology (SASSB), 11-13 January 2010. North-West University, Potchefstroom, South Africa. Paper title: Antimicrobial and phytochemical evaluation of twelve medicinal plants used by the Venda People.

# ABSTRACT

Venereal diseases (VDs) are infections that are mainly transmitted through sexual intercourse and amongst these are gonorrhoea, syphilis, chlamydia and trichomoniasis. Gonorrhoea is the most commonly known VD and the widest spread contagious infection in the world. Out of 448 million cases of curable venereal infections, gonorrhoea represents 88 million cases and the rest are syphilis, chlamydia and trichomoniasis. Gonorrhoea has recently been rated as in the emergent multidrug resistance phase.

Venereal diseases are amongst the major diseases ravaging many rural communities. People infected with these diseases are considered a disgrace in the community. Indigenous populations, for example the Vha-Venda people tend to use medicinal plants to treat these infectious diseases rather than using western medicines. Vha-Venda people have depended on medicinal plants for their health and survival for millenia. In order to validate and give scientific credence to the use of medicinal plants by the Vha-Venda people for venereal diseases, several pharmacological assays were carried out.

The study was aimed at evaluating the; antimicrobial, anti-inflammatory activities, HIV-type 1 reverse transcriptase (RT) inhibition properties and to determine phenolic contents as well as evaluating the mutagenic properties of, 12 medicinal plants used by the Vha-Venda people against venereal and related diseases. An attempt was also made toward isolating and identification of the most active compounds from some extracts that were active against *Neisseria gonorrhoeae*.

Twelve medicinal plants and various plant parts, *Adansonia digitata* (bark), *Acacia karroo* (bark), *Aloe chabaudii* (roots), *Bolusanthus speciosus* (leaves, bark and stem), *Ekebergia capensis* (leaves and bark), *Elephantorrhiza burkei* (roots), *Grewia occidentalis* (roots), *Osyris lanceolata* (roots), *Pappea capensis* (leaves), *Peltophorum africanum* (bark), *Pterocarpus angolensis*

(leaves and bark) and *Ximenia caffra* (leaves and roots) were evaluated for their antimicrobial properties against two Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*), three Gram-negative (*Neisseria gonorrhoeae*, *Escherichia coli* and *Klebsiella pneumonia*) bacteria and the fungus *Candida albicans*. The plant materials were extracted with petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water. Methanol was used for extracting materials for phenolic contents and HIV-1RT assays. The Disc diffusion method was used to determine gonococcal percentage inhibition and a microdilution assay was used to determine minimum inhibition concentration (MIC) and minimum fungicidal concentrations (MFC).

*Bolusanthus speciosus* and *X. caffra* extracts exhibited the best antigonococcal, antifungal and antibacterial activities whilst *A. digitata* and *A. chabaudii* showed poor activities. The medicinal plants were also evaluated for cyclooxygenase (COX-1 and -2) and HIV-1 reverse transcriptase inhibition activity. The DCM and PE extracts of *A. digitata* bark, *B. speciosus* bark, *P. angolensis* bark and *P. capensis* leaves showed good anti-inflammatory activity against both COX-1 and COX-2. Methanol and water extracts of *B. speciosus* stems, *P. africanum* bark, *P. angolensis* leaves and *P. capensis* leaves exhibited good anti-HIV-1 RT activity. *A. chabaudii* roots, *E. capensis* bark and O. *lanceolata* roots showed low HIV-1 RT percentage inhibition.

Phytochemical analysis using spectrophotometric methods revealed the presence of a variety of phenolic compounds in all the plant extracts including total phenolics, flavonoids, gallotannins and condensed tannins. High levels of total phenolics, flavonoids, gallotannins and condensed tannins were detected in *X. caffra*. Low amounts of flavonoids, gallotannins and condensed tannins were detected in *B. speciosus*.

The Ames test using *Salmonella typhimurium* tester strain TA98 with and without S9 metabolic activation revealed that all plant extracts were non-mutagenic toward *S. typhimurium* strains TA98 without metabolic activation. However, *E. burkei* roots and *E. capensis* bark showed mutagenic effects

toward TA98 after metabolic activation. Therefore, these two plants need to be used with caution, however more studies are required to confirm this result.

Good antimicrobial activity observed in *X. caffra* leaves prompted an attempt to isolate active compounds. A pure compound from *X. caffra* leaves exhibited moderate activity (63%) against *N. gonorrhoeae*. However, the structure of the compound has as yet to be ratified.

Pharmacological activity of the twelve medicinal plants used by Vha-Venda people against venereal and related diseases were validated in this study. The results obtained in this study give credence to the use of some of these plants. This study has further confirmed the need for screening these medicinal plants for more pharmacological activities. These plants may offer a new source of chemicals for the effective treatment of venereal and related diseases.

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| | | | |
|---|---|---|---|
| 2-AA | 2-aminoanthracene | DNA | Deoxyribonucleic acid |
| 4NQO | 4-Nitroquinoline-N-oxide | DPM | Disintegrations per minute |
| AD | Alzheimer‘s disease | DPPH | Diphenyl–1–picryl hydrazyl |
| AIDS | Acquired Immune Deficiency Syndrome | EC | Effective concentration |
| ATCC | American Type Culture Collection | EIMS | Electron impact mass spectroscopy |
| ATM | African Traditional Medicine | ELISA | Enzyme-linked immunoassay |
| ARV | Antiretroviral | EtOH | 80% Ethanol |
| AmpB | Amphotericin B | GC | Chocolate agar |
| AZT | zidovudine | GAE | Gallic Acid Equivalents |
| CFU | Colony forming unit | HMBC | Heteronuclear multiple bond correlation |
| COSY | Correlation spectroscopy | HPV | Human papillomavirus |
| COX | Cyclooxygenase | HRMS | High resolution mass spectroscopy |
| CTE | Catechin equivalents | HSQC | Heteronuclear spin quantum correlation |
| CYP450 | Cytochrome P450 | IC | Inhibitory concentration |
| DCM | Dichloromethane | INT | *p*-iodonitrotetrazolium chloride |
| DEPT | Distortionless enhancement by polarization transfer | LCE | Leucocyanidin equivalents |
| DIG-POD | Digoxigenin-peroxidase | LOX | Lipoxygenases |
| DIP | Direct injection probe | MFC | Minimum fungicidal concentration |
| DMEM | Dulbecco's modified Eagle's culture medium | MH | Mueller-Hinton |
| DMSO | Dimethylsulfoxide | MIC | Minimum inhibitory concentration |

| | | | |
|---|---|---|---|
| MPM | Microtitre plate modules | SAAB | South African Association of Botanists |
| N | Normal | SASSB | Southern African Society for Systematic Biology |
| NADP | Nicotinamide adenine dinucleotide phosphate | STD | Sexually transmitted diseases |
| NC | Not calculated | STI | Sexually transmitted infections |
| NMR | Nuclear Magnetic Resonance Spectroscopy | TLC | Thin Layer Chromatography |
| NOESY | Nuclear Overhauser effect spectroscopy | USA | United States of America |
| NSAIDs | Nonsteriodal anti-inflammatory drugs | USD | United States Dollar |
| NU | Bews Herbarium | TM | Traditional medicine |
| PE | Petroleum ether | VD | Venereal disease |
| PGE | Prostaglandins | WHO | World Health Organisation |
| RCPGD | Research Centre for Plant Growth and Development | YM | Yeast malt |
| RT | Reverse transcriptase | UKZN | University of KwaZulu-Natal |
| RNA | Ribonucleic acid | UV | Ultraviolet |

# CHAPTER 1: Introduction and Literature review

## 1.1. Introduction

Plants have been utilized as a source of medicine for thousands of years and continue to play an important role globally in primary health care, mostly in developing countries **(BALUNAS and KINGHORN, 2005)**. The use of medicinal plants is increasing because people believe they are safe for human consumption. There is also an increase in infectious diseases worldwide caused by both drug resistance; and lack of sufficient affordable medicine for people living in poor communities. The discovery of drugs from medicinal plants may be one of the solutions in the fight against infectious diseases. Thousands of natural products are in clinical trials and some are already confirmed useful in combating some of the diseases.

## 1.2. Traditional medicine

About 80% of the African population use traditional medicine (TM) to meet their health care needs. Traditional medicine is the only available, accessible and affordable source of health care for most people. Traditional medicine is considered an important part of the everyday life and well-being in African communities. In developed countries, TM is now increasingly being used for the treatment and management of chronic diseases **(WHO, 2002b)**.

World health organization (WHO) defines traditional medicine as, "*the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose, improve or treat physical and mental illness.*" This includes a diversity of health practices, approaches, knowledge, and beliefs incorporating plant, animal and/or mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in

combination to maintain well-being as well as to treat, diagnose or prevent illness **(WHO, 2001c; WHO, 2002a)**.

African Traditional Medicine (ATM) is the oldest and perhaps the most diverse of all healing systems **(GURIB-FAKIM, 2006)**. The concept of ATM hinges an holistic approach. It encompass the physical body, mind, emotions and spirit that work together to determine good health or ill health, with the interaction and relationship between nature, the cosmos and human beings as stated in the South African draft national policy on ATM **(ATMSA, 2008)**. In addition, other factors are considered in ATM such as the existing relations between the particular individual and other human beings, both the living, and those who have passed away. Therefore the philosophy/religion, psychiatry, physiology and biology, are all part and parcel of the conception of ATM **(ATMSA, 2008)**.

Due to the availability, accessibility and affordability, there is an increase in the appreciation of the role of TM. Traditional medicine has been proven effective in cases of ill-health, including mental health, prevention of diseases, treatment of non-communicable diseases and improved quality of life in elderly people and in persons suffering from chronic diseases **(WHO, 2001c)**.

In 1970, WHO recommended that traditional remedies should be incorporated within national drug policies. Increase in commercialization, professionalism and pharmaceutical production in ATM especially with medicinal plants known to be effective in disease treatment has caused medicinal plants to be seriously considered in legislation **(WONDERGEM *et al.,* 1989; CUNNINGHAM, 1993)**. There is a growing interest in medicinal plants as sources of drugs, due to their promising efficacy and safety such as fewer side effects, better patient tolerance and acceptance due to a long history of use **(VERMANI and GARG, 2002)**. For several reasons, conventional medicine can be inefficient due to side effects and ineffective therapy **(RATES, 2001)**. Despite the effectiveness, a large percentage of the world‘s population does not have access to conventional pharmacological treatment **(RATES, 2001)**.

## 1.3. Traditional medicine in South Africa

South Africa has a rich plant biodiversity with an estimated 24 000 plant taxa **(LOW and REBELO, 1996)**. Approximately 3000 plant species are used for medicinal purposes by an estimated 200 000 indigenous traditional South African healers. Up to 60% of the South African population consult one or more of the traditional healers in preference to/or in addition to western medical doctors especially in rural areas **(VAN WYK *et al*., 2009; MANDER *et al*., 2007)**.Traditional medicine forms an integral part of the culture of many South Africans **(VAN STADEN, 2008)**.

Due to the extensive utilization of TM, many national governments have made moves to encourage research into medicinal plants and the development of good policies, regulations and trade standards **(BODEKER, 2004)**. In South Africa, the government and the National Research Foundation are promoting greater interest in research of the country‘s natural plant resources **(LIGHT *et al*., 2005)**.

Medicinal plants work by normalizing the physiological functions of the body and by correcting the underlying causes of disorders **(MURRAY and PIZZORNO, 1999)**. The advantage of medicinal plants is that they are renewable in nature unlike the synthetic drugs that are obtained from non-renewable sources of basic raw materials such as fossil sources and petrochemicals **(SAMANTA *et al*., 2000)**. Unlike the production of synthetic drugs which produces a lot of environmental pollution, the cultivation and processing of plants is often environmental friendly **(VERMANI and GARG, 2002)**. Apart from cultivation, several of the plants used in TM exist in the wild and are locally available, especially in developing and underdeveloped countries **(VERMANI and GARG, 2002)**. Some research findings have resulted in the isolation and identification of important natural products. To date numerous publications on healing and pharmaceutical values of medicinal plants have been documented **(MATHEKGA *et al*., 2000; KAMATOU *et al*., 2007; RABE *et al*., 2002)**.

## 1.4. Drug discovery from medicinal plants

Antimicrobial resistance is a natural biological phenomenon which was recognized first as a scientific curiosity and then as a threat to the effective treatment of most microbial infections **(WHO, 2001b)**. The **CIA (1999)** described drug resistance by microbial organisms as a threat to global stability and the national security of the United States of America.

The discovery of the healing power of medicinal plants began during ancient times. About 25% of the drugs prescribed worldwide come from plants **(BALANDRIN *et al.,* 1993)**. It is also acknowledged that 121 active compounds that are being used as medicinal drugs are derived from plants. Of the 252 drugs considered as basic and essential medicine by WHO, 11% are exclusively of plant origin and a significant number are synthetic drugs whose chemical structures were obtained from natural precursors **(RATES, 2001)**. Approximately one quarter of the best selling drugs in 2001 and 2002 were natural products or derived from natural products **(BUTLER, 2004)**.

In 1980 prescription drugs derived from plants contributed $8 billion to the American economy **(BALANDRIN *et al.,* 1993; FARNSWORTH *et al.,* 1985)**. In China 7295 plant species are utilised as medicinal agents **(MAHIDOL *et al.,* 1998)**. In a study of the top 150 proprietary drugs used in the USA (1993), it was reported that 57% of them contained at least one major active compound derived from biological sources **(PATWARDHAN, 2009)**. It is estimated that only 5 to 15% of the 250, 000 existing species of higher plants have been systematically surveyed for the presence of biologically active compounds **(BALANDRIN *et al.,* 1993)**. It is estimated that 60% of anti-tumour and anti-infectious drugs already on the market or under clinical trial are of natural origin **(SHU, 1998; YANIV and BACHRACH, 2005)**.

The screening of medicinal plants for biological activity has resulted in the isolation of active compounds from the early 19$^{th}$ century when morphine was

isolated from *Papaver somniferum* by the German pharmacist Friedrich Sertüner in 1805 **(KINGHORN, 2001; BALANDRIN *et al.,* 1993)**. The success of morphine isolation led to the identification of many other drugs such as quinine from *Cinchona spp*, codeine from *Papaver somniferum*, atropine from *Atropa belladonna*, reserpine from *Rauvolfia serpentina* and digoxin from *Digitalis* species **(VAN WYK *et al.,* 2009)**. The majority of the compounds are still in clinical use today. Other important plant-derived drugs include arteether for the treatment of malaria, galantamine for the treatment of Alzheimer‘s disease (AD), nitisinone for the rare inherited disease (tyrosinaemia) and tiotropium for the treatment of chronic obstructive pulmonary disease **(BALUNAS and KINGHORN, 2005)**.

According to **BALUNAS and KINGHORN (2005)**, drugs derived from medicinal plants can serve not only as new drugs but also as templates for synthetic drug development by chemists. Therapeutics derived from plants are usually less specific and can affect more than one molecular target and can be helpful in complex disorders in which the causes are not known. However, in some cases these drugs are not the first choice for treating severe and life-threatening diseases, but they can be useful in many other instances **(VAN WYK and WINK, 2004)**.

## 1.5. Venereal diseases

The human body is extremely complex and if one element is missing or not performing optimally, because of genetic or environmental influences (infections by bacteria, parasites, fungi, viruses, burns, wounds, toxins from a diet that modulate the activity and function of proteins in the body), it results in health disturbances **(VAN WYK and WINK, 2004)**.

Sexually transmitted diseases (STDs), also known as sexually transmitted infection (STIs), or venereal disease (VDs), are caused by bacterial, viral and parasitic pathogens, some of which are listed in Table 1. In this thesis, the abbreviation VDs will be used. These pathogens are mainly transmitted,

through sexual intercourse. Transmission can occur occasionally through other nonsexual means such as mother to child contract during pregnancy, lactation and childbirth, and through blood transfusions or tissue transfer **(WHO, 2006)**. Some VDs increases the chances of infection with the deadly or incurable pathogens which may results in AIDS and genital herpes **(WASSERHEIT, 1992)**. About 26% of deaths occurring in South Africa in 2000 were as a result of VDs **(JOHNSON *et al*., 2008)**.

Table 1: Venereal diseases and the organisms responsible for the infection

| **ORGANISM** | **DISEASE** |
|---|---|
| BACTERIA | |
| *Neisseria gonorrhoea* | Gonorrhoea |
| *Treponema pallidum* | Syphilis |
| *Haemophilus ducreyi* | Chancroid |
| *Gardnerella vaginalis* | Bacterial vaginosis |
| *Chlamydia trachomatis* | Chlamydia infections, Lymphogranuloma venereum |
| *Mycoplasma hominis* | Postpartum fever, salpingitis |
| *Calymmatobacterium granulomatis* | Donovanosis (granuloma inguinale) |
| *Shigella sp.* | Shigellosis in homosexual men |
| *Campylobacter sp* | Enteritis, proctocolitis |
| Group B *Streptococcus* | Neonatal sepsis, neonatal meningitis |
| VIRUSES | |
| Human immunodeficiency virus (HIV) | Acquired Immune Deficiency Syndrome (AIDS) |

Table 1: continuation

| VIRUSES | |
|---|---|
| Herpes simplex virus | Initial and recurrent genital herpes, aseptic meningitis, neonatal herpes, cervical dysplasia and carcinoma, carcinoma *in situ* of the vulva |
| Hepatitis B virus | Acute hepatitis B, chronic active hepatitis, persistent (unresolved) hepatitis, polyarteritis nodosa, chronic membranous glomerulonephritis, mixed cryoglobulinemia, polymyalgia rheumatic, hepatocellular carcinoma |
| Hepatitis A virus | Acute hepatitis A |
| Cytomegalovirus | Heterophil-negative infectious mononucleosis, congenital infection, gross birth defects and infant mortality, cognitive impairment, cervicitis, protean manifestations in the immunosuppressed host. |
| *Molluscum contagiosum* virus | Genital molluscum contagiosum |
| Human papillomavirus (HPV) | Genital (venereal) warts |
| PARASITES | |
| *Trichomonas vaginalis* | Trichomonal vaginitis |
| *Giardia lambia* | Giardiasis in homosexual men |
| *Entamoeba histolytica* | Amebiasis in homosexual men |

Table 1: continuation

| | |
|---|---|
| FUNGI | |
| *Candida albicans* | Vaginal yeast infections |
| ECTOPARASITES | |
| *Phthirius pubis* | Pubic lice |
| *Sarcoptes scabiei* | Scabies |

**(HARDIN, 1996; NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, 1999)**.

### 1.5.1. Prevalence of venereal diseases

More than 340 million infections with VDs occur globally every year. These are mainly the curable VDs such as syphilis, gonorrhoea, chlamydia and trichomoniasis in age groups between 15 and 50 years **(WHO, 2001a)**. The percentages of seventy five to eighty five of these cases occur in developing countries **(MAYAUD and MABEY, 2004)**. South-east Asia is the largest region with the highest cases of VDs followed by sub-Saharan Africa, Latin American and the Caribbean **(WHO, 2001a)**. Apart from sexual transmitted bacteria and protozoa infections, high rates of viral STIs also occur yearly such as HIV, HPV, Herpes simplex virus type 2 and Hepatitis B virus **(WHO, 2006)**. According to **MAYAUD and MABEY (2004)** these infections constitute a huge health and economic burden globally, with 17% of economic losses caused by ill-health especially in developing countries. Industrialized nations can also be expected to experience an increase in the burden because of the prevalence of non-curable viral infections, irresponsible trends in sexual behaviour and increased migrations of the economically active populations **(WHO, 2006)**. Cervical cancer which is causes by HPV also forms part of the STIs in Southern Africa. Over 98% of the deadly VDs occur as HIV/AIDS **(JOHNSON *et al.*, 2008)**.

### 1.5.2. Effect of untreated venereal diseases

Untreated VDs result in life threatening conditions. Syphilis results in 25% of stillbirth and 14% of neonatal death with an overall perinatal mortality of about 40%. In Africa syphilis prevalence in pregnant women ranges from 4 to 15% **(WHO, 2006)**. On the other hand, 35% of untreated gonococcal infections results in pelvic inflammatory disease. Up to 50% of infants born with untreated gonorrhoea and chlamydial infection usually develop ophthalmia neonatorum, which can lead to blindness. Almost 4000 new-born babies become blind every year worldwide because of these conditions **(WHO, 2006)**.

Herpes simplex virus type 2 is the leading cause of genital ulcers mostly in women **(WHO, 2006)**. It also plays an important role in the transmission of HIV **(WHO, 2006)**. Results of a study in Mwanza (United Republic of Tanzania) by **DEL MAR PUJADES RODRÍGUEZ *et al.* (2002)** showed that 74% of HIV infections in men and 22% in women could be attributable to the presence of Herpes simplex virus type 2. Human papillomavirus cause about 500 000 cases of cervical cancer annually with 240 000 deaths in developing countries. It is estimated that Hepatitis B virus results in 350 million cases of chronic hepatitis and at least one million deaths each year **(WHO, 2006)**. In the same report **WHO (2006)** states that the population at risk for STIs will continue to grow dramatically.

Sexually transmitted infections are a threat to public health. They are a major problem in most parts of the world, mostly in developing countries where the infections constitute a huge health and economical burden. Educating and monitoring infected people is one of the major issues especially in resource-poor countries where the treatment for sexual infections face numerous problems such as stigmatization and mistreatment because of lack of trained personnel **(WHO, 2006)**.

## 1.6. Overview of medicinal plants used on venereal diseases

Numerous studies on plants that are used to treat conditions related to VDs have been performed **(MEKKAWY *et al*., 1995; MATSUSE *et al*., 1999; VLIETINCK and BERGHE, 1991; KAMBIZI and AFOLAYAN, 2001; RAJBHANDARI *et al*., 2001; VERMANI and GARG, 2002; NDUBANI and HÖJER, 1999; DE WET *et al.,* 2011)**. Over 80 references in southern Africa relate to medicinal plants used to treat VDs **(VERMANI and GARG, 2002)**. However, only a few of the studies have focused on the bacterial pathogens associated with VDs **(KAMBIZI *et al*., 2004; TSHIKALANGE *et al*., 2005; BUWA and VAN STADEN, 2006; TSHIKALANGE *et al*., 2008;**

**FERNANDES *et al*., 2008; KAMBIZI and AFOLAYAN, 2008; KLOS *et al*., 2009; VAN VUUREN and NAIDOO, 2010)**.

Nine plants that are used globally for the treatment of STIs were recorded by **DE WET *et al*. (2011**) namely: *Bridelia cathartica* subsp. *cathartica*, *Cladostemon kirkii*, *Erianthemum dregei*, *Euphorbia hypericifolia, Ipomoea batatas*, *Krauseola mosambicina*, *Mimusops caffra*, *Opuntia stricta* and *Sarcophyte sanguinea* subsp. *sanguinea*. Three of the above mentioned plants (*E. hypericifolia*, *I. batatas* and *O. stricta*) are naturalized exotics in South Africa, **(DE WET *et al*., 2011)**. The use of medicinal plants for symptomatic treatment of VDs dates back at least to 1574 when *Smilax officinalis* was first introduced for the treatment of syphilis in Europe **(VERMANI and GARG, 2002)**.

According to **GREEN (1992)** the majority of people in sub-Sahara Africa believe that TM used for treating sexual transmitted infection is more effective than western medicine. This can be mainly because of stigmatization that is associated with VDs. Particularly women are reluctant to disclose information related to their genitals, thus they find it better to seek help from traditional healers **(KAMATENESI-MUGISHA *et al*., 2008)**.

## 1.7. Venda people and traditional medicine

All cultures have some definable people with plant knowledge that includes appropriate edible plants, medicinal plants and ceremonial plants **(CSEKE *et al.,* 2006)**.

The Venda community is one of the remote tribes in South Africa. The Venda people (Vha-Venda), reside in the Limpopo province, in the upper North of South Africa bordering Zimbabwe and Mozambique. Limpopo province has a population of 5.3 million. The province is divided into five districts (Vhembe, Mopani, Capricorn, Sekhukhune and Waterberg). The study area for the work

described in this thesis was done in Vhembe district which falls under the Venda region.

Venda people have depended on the natural environment for their health and survival for ages. The dependence continued with modifications up until the 19th century, when civilization with it inherent Christian nature challenged its credibility particularly with respect to the medicinal, magical, ritual and religious aspects that are part of it **(MABOGO, 1990)**.

The Vha-Venda culture does not differ much from other cultures in South Africa such as the Zulu culture. They believe in traditional healers and the use of traditional medicine for their health needs. The Vha-Venda uses a variety of plant parts, such as leaves, bark and roots in their TM. The plant materials may be combined with animal fats, bones, organs such as brains, intestines, or genitals to make stronger medicines **(HANISCH, 2002)**.

### 1.7.1. Traditional healing by Vha-Venda

The knowledge of medicinal plants in the Venda culture is obtained from the forefathers or through spirit mediums from their ancestors and is subsequently passed through generations. The knowledge of using medicine is limited to those who are brought up in traditional Venda culture. Simple plants are used as medicine for common ailments such as colds, diarrhoea, colic, most sexual transmitted diseases, treatment of wounds, toothache and sore eyes **(MABOGO, 1990)**.

Among the Vha-Venda, health providers are distinguished as specialists in certain fields of medicinal practice. Such people may be referred to as traditional practitioners (herbalist), diviners or magicians. Traditional practitioners (ňangas) specialize in the use of herbs in treating various diseases and they rely on symptomatic diagnosis of diseases **(MABOGO, 1990)**.

A traditional practitioner is an individual who is recognized by the community as competent in health care **(PRETORIUS, 1999)**. The traditional healer can also be regarded as a technician and diagnostician **(L'ABBE, 2005)**. Among these practitioners, are those who specialize in children's diseases, women's fertility problems, and incurable ulcers related to cancer, or sexually transmitted diseases. There are many others who claim to treat all diseases, without being specific **(MABOGO, 1990)**.

Diviners (mungome) are people who normally do not treat diseases but who use their divinatory powers to determine cause and source of people's health and social complaints (generally divination dice which are made of bones and/or wood are use for this purpose) **(MABOGO, 1990)**. Divination involves casting of objects, including bones, mediumistic ability (contact with the ancestors), or dreams and visions. Treatment may also involve ritual sacrifice to appease the ancestors, ritual and magical strengthening of people and possessions, steaming, purification, sniffing of substances, wearing charms, cutting and piercing of human flesh **(PRETORIUS, 1999)**. Some of the Diviners do not use the dice but they use other processes including *Fembo* (the use of smelling substances).

Magicians, specialize in natural objects, usually plants and animal products, to produce healing effects. Magicians thus mainly cure people who are suffering from conditions mainly caused as a result of bewitchment **(MABOGO, 1990)**. However, some practitioners combine some or all of the specialized fields, thus making it difficult to differentiate between them **(MABOGO, 1990)**.

Just like in many other communities of South Africa, traditional healers are important in the Vha-Venda culture as they provide the first line of health care to the people especially in rural areas **(SAMIE *et al*., 2005)**.

There are many ways which place traditional healers in respectable positions when compared to alternative mainstream health providers. These include availability, accessibility, their familiarity with culture bound syndromes, traditions, and relationships with patients. Some of the reasons are the lack of trust in western medical practitioners to effectively treat psychosocial

problems and beliefs that illnesses arises from supernatural causes and displeasure of ancestral gods or evil spirits **(KGOATIA, 1997)**.

## 1.7.2. Venda medicinal plants

About 151 medicinal plant species are found in the Venda district of which 25 species are used to treat venereal diseases **(ARNOLD and GULUMIAN, 1984; DE WET *et al*., 2011)**. Out of 25 species that are used for venereal disease, *Alibizia anthelmintica* and *Alibizia versicolor* are most preferred **(DE WET *et al*., 2011)**. Approximately 69 medicinal plants are traded in Venda **(TSHISIKHAWE, 2002)**. Some of the traded plants are threatened and these include *Brackenridgea zanquebarica* and *Warburgia salutaris* **(MOENG and POTGIETER, 2011)**.

The use of medicinal plants is popular because of African traditions which allow people to explore the diverse plant resources that surround them **(VAN VUUREN, 2008)**. The parts of specific medicinal plants are collected during the favorable harvesting season, dried by directly exposing them to sunlight, ground using mortar and pestle and then stored in containers in powdered form for future usage **(SAMIE *et al*., 2010)**. The parts of the plant used vary among species and with traditional healer. It also depends on the nature and state of the disease **(MABOGO, 1990)**. The whole plant is used for ritual purification (*u bvisa tshinyama*) **(MABOGO, 1990)**. Few medicinal plants used by Vha-Venda people have been reported for antimicrobial activities **(OBI *et al*., 2003; STEENKAMP *et al*., 2007a and 2007b; PALLANT and STEENKAMP, 2008; FERNANDES *et al*., 2008 and GREEN *et al.,* 2010)**.

## 1.7.3. Conservation of Venda medicinal plants

In Venda, medicinal plants are conserved via the system which is enforced by the chief and headmen. The system is primarily aimed at protection and preservation of those plants that are important sources of food or medicine

such as *Sclerocarya birrea*, *Parinari curatellifolia*, *Adansonia digitata*, and *Boscia albitrunca* **(MABOGO, 1990)**.

## 1.8. Aims and objectives

Venereal diseases continue to be a public health concern globally, mostly in South Africa where transmission rate is increasing. In rural areas, infected people do not want to visits public health institutions because of the stigmas attached to VDs. Venereal diseases are considered a disgrace in the community. The unprecedented emergence of drug resistance against pathogens that causes VDs has complicated the treatment of the infections. Because of these complications, there is a necessity to search for new antimicrobial substances from various sources.

Medicinal plants have proved to be a source of safer treatments for many diseases. This study aims at evaluating the pharmacological properties of twelve medicinal plants used by the Venda people for treating venereal and related diseases. This was achieved by testing the medicinal plants in various pharmacological tests. These tests include the antimicrobial bioassays (antibacterial, antifungal and antigonococcal) and enzyme inhibition bioassays [cyclooxygenases enzymes (involved in anti-inflammatory) and HIV-1 reverse transcriptase (RT)]. Determination of phenolic compounds was also carried out. Genotoxicity of the plant extracts was also evaluated to test for safety of the plants. The information obtained from this study could go a long way in validating the use of the plants by the Venda people and offer scientific credence to the ideas of integrating such plants in health care systems which in turn will be helpful to the rural communities. An attempt was also made toward isolating and identification of the most active compounds for some extracts that were active against *Neisseria gonorrhoeae*.

## 1.9. Plant selection

The selection of plants used in this study was based on the literature of plants used by Vha-Venda people for VDs and related diseases using the thesis of **MABOGO (1990)** as a guide. Table 2 lists the plants used in the study. The plants were collected from Vhembe district and voucher specimens (voucher numbers provided in Table 2) were deposited in the Bews Herbarium at the University of KwaZulu-Natal Pietermaritzburg.

Table 2: Medicinal plants used to treat venereal diseases by the Venda people **(cited from Mabogo, 1990)**

| **Family**<br>Plant species | Vernacular name | Part used | Voucher number | Traditional uses | Biological activities |
|---|---|---|---|---|---|
| **Bombacaceae**<br>*Adansonia digitata AL*. | Muvhuyu | Bark | Mulaudzi 28 NU | Venereal diseases. Urinary disorders and mild diarrhoea | Anti-inflammatory, anti-pyretic and analgesic agents **(RAMADAN *et al*., 1994; PALOMBO, 2006; AJOSE, 2007; KARUMI *et al*., 2008)**, antibacterial, antiviral and anti trypanosomal activities **(ANANI *et al*., 2000; HUDSON *et al*., 2000; ATAWODI *et al*., 2003; VIMALANATHAN and HUDSON, 2009)** |
| **Fabaceae**<br>*Acacia karroo* Hayne | Muunga | Bark | Mulaudzi 29 NU | Venereal diseases, emetic, coagulant, dysentery, diarrhoea, colic, colds, inflammation of eyes and emetic. | Crude extract have rich in proanthocyanidin and flavonols **(DUBE *et al*., 2001)**, anti-inflammatory and analgesic activity **(ADEDAPO *et al*., 2008)** |
| **Asphodelaceae**<br>*Aloe chabaudii* var. chabaudii | Tshikhopha | Roots | Mulaudzi 30 NU | Venereal diseases and haematuria. | Antimicrobal activity **(MBANGA *et al*., 2010)** |

Table 2: continuation

| **Family** Plant species | Vernacular name | Part used | Voucher number | Traditional uses | Biological activities |
|---|---|---|---|---|---|
| **Fabaceae** *Bolusanthus speciosus* (Bolus) Harms | Mukambana | Bark, leaves, and stems | Mulaudzi 15 NU | Cleaning blood, kidney problems, and venereal diseases. | Antibacterial activity **(BOJASE *et al*., 2002; ERASTO *et al*., 2004)** |
| **Meliaceae** *Ekebergia capensis* Sparrm | Mudouma | Leaves and bark | Mulaudzi 22 NU | Chronic cough, emetic, backache, headache, dysentery and skin diseases. | Uterotonic , antispasmodic activity **(VAN WYK *et al*., 2009; SEWRAM *et al*., 2000)**, antibacterial activity **(LALL and MEYER, 1999)** |
| **Fabaceae** *Elephantorrhiza burkei* Benth. | Gumululo or tshisesevhafu | Roots | Mulaudzi 25 NU | Venereal diseases, cleaning of body systems, regulation of menstruation, aphrodisiac, and miscarriage. | Abortifacient **(ARNOLD and GULUMIAN, 1984; HUTCHINGS *et al*., 1996;WATT and BREYER-BRANDWIJK, 1962)**, antibacterial activity **(MATHABE *et al*., 2006)** |

Table 2: continuation

| **Family**<br>Plant species | Vernacular name | Part used | Voucher number | Traditional uses | Biological activities |
|---|---|---|---|---|---|
| **Malvaceae**<br>*Grewia occidentalis* L. | Mulembu | Roots | Mulaudzi 14 NU | Venereal diseases, bladder ailments. | Antibacterial activity **(GRIERSON and AFOLAYAN, 1999)** |
| **Santalaceae**<br>*Osyris lanceolata Hochst. & Steud.* | Mpeta | Roots | Mulaudzi 16 NU | Venereal diseases, styptic effects on wounds, menorrhagia and infertility. | Antibacterial and antifungal activity **(YEBOAH *et al*., 2010; OOKO *et al*., 2009)** |
| **Sapindaceae**<br>*Pappea capensis* Eckl. & Zeyh | Mulilwe | Leaves | Mulaudzi 17 NU | Venereal diseases, painful eyes and aphrodisiac. | Use to control snail (*Biomaphalaria pfeifferi*) that causes Schistosomiasis **(KLOOS *et al*., 1987)** |

Table 2: continuation

| **Family** Plant species | Vernacular name | Part used | Voucher number | Traditional uses | Biological activities |
|---|---|---|---|---|---|
| **Fabaceae** *Peltophorum africanum* Sond | Musese | Bark | Mulaudzi 18 NU | Cold, fever, sore throat, sore, ulcer, blisters in the oral cavity, stomach complaints and wounds. | Antibacterial and antioxidant activity **(BIZIMENYERA *et al*., 2005; STEENKAMP *et al*., 2007a)**, anti-HIV activity (**BESSONG *et al*., 2005**), antihelimetic activity **(BIZIMENYERA *et al*., 2006)** |
| **Fabaceae** *Pterocarpus angolensis* DC | Mutondo | Leaves and bark | Mulaudzi 13 NU | Venereal diseases, piles, amenorrhoea, haematuria and bilharzias. | Antibacterial activity **(LUSEBA *et al*., 2007; SAMIE *et al*., 2009)** |
| **Olacaceae** *Ximenia caffra* Sond. | Mutshili | Roots and leaves | Mulaudzi 24 NU | Venereal diseases, blood in faeces, diarrhoea, fever, menorrhagia, febrifuge, cough, infertility, headache, menorrhagia, eye sickness, indigestion and scurvy. | Antibacterial activity **(SAMIE *et al* ., 2005; MATHABE *et al*., 2006)**, antifungal activity **(FABRY *et al*., 1996)** and antidiarrhoeal activity **(HUTCHINGS *et al*., 1996)** |

# CHAPTER 2: Antimicrobial Screening

## 2.1. Introduction

Infectious diseases are the second leading cause of death worldwide, and the third leading cause of death in developed countries in both children and adults. The burden of infectious diseases falls most heavily on people in developing countries by causing morbidity and mortality **(FAUCI, 2001; HAMBURG, 2008)**. In developed countries, infectious disease mortality disproportionately affects indigenous and disadvantaged minorities **(GUERRANT and BLACKWOOD, 1999; BUTLER *et al.,* 2001)**. Of the approximately 57 million annual deaths worldwide, 15 million (>25%), are estimated to be related directly to infectious diseases **(MORENS *et al*., 2004)**. Infectious diseases are caused by viruses, bacteria, fungus, and parasites which results in respiratory infections, HIV/AIDS, diarrhoeal diseases, tuberculosis, malaria, STDs (other than HIV), meningitis, Hepatitis B and C, tropical parasitic diseases and Dengue **(MORENS *et al*., 2004)**.

In the twentieth century, antimicrobial therapies, especially antibiotics for bacterial diseases (e.g. pneumonia) and anti-parasitic drugs (e.g. malaria) have been successful against infectious diseases. However, during the same era, there was the discovery of antimicrobial resistance followed by an increase in disease transmission and a decline in the development of new antibiotics **(COHEN, 1992; 2000)**. This led to the emergence of multidrug-resistance which has created a situation in which there are few or no treatment options for infections with certain pathogenic bacteria **(WENZEL and EDMOND, 2000)**. To prevent and control infectious diseases it is of vital interest and concern in both developed and developing countries to come up with new remedies for such diseases **(HAMBURG, 2008)**. New antimicrobial agents with different modes of action against bacterial, fungal, parasite and viral diseases are urgently needed.

### 2.1.1. Bacterial infections

Bacteria are widely distributed and the most abundant group of unicellular organisms on earth, capable of adapting in a diverse range of environments such as in soil, water and air. They are both useful and harmful to humans. Many parasitic bacteria do not harm their hosts. Some cause disease by producing poisons. Bacteria have a prokaryotic cell type with a rigid wall which protects the cell against osmotic damage. The structure of the cell wall differs in Gram-positive and Gram-negative bacteria **(SLEIGH and TIMBURY, 1998)**.

Gram-negative bacteria differ from Gram-positive by the presence of an outer membrane composed of lipopolysaccharides. It also contains specific proteins for transporting hydrophilic molecules. Other proteins are receptor sites for phages and bacteriocins. The outer membrane in Gram-negative bacteria covers the peptidoglycan layer, which is attached to the outer membrane by lipoproteins. The layer is separated by periplasm from the cytoplasmic membrane. Gram-positive bacteria have more peptidoglycan layers with no periplasm **(SLEIGH and TIMBURY, 1998)**.

There are many bacteria that are known to cause VD, one such bacterium is *Neisseria gonorrhoeae. Neisseria gonorrhoeae* is a common cause of VD. It is responsible for causing gonorrhea. Gonorrhea is one of the most common VDs in developing countries and is now a major public health concern worldwide **(WHO, 2011)**.

*Neisseria gonorrhoeae* is a Gram-negative diplococcus. It is found in humans and highly adapted to survive in the genital tract. *Neisseria gonorrhoea* is known to have existed for the past 3500 to 4000 years and was first described by Albert Neisser in 1879 **(KRUGER and BOTHA, 2007)**. Gonorrhea is mostly spread through sexual contact. It is associated with a significantly increasing HIV transmission **(TAPSALL, 2001)**. Untreated infections may lead to serious complications including sterility, pelvic inflammatory diseases and ectopic pregnancy **(RUDDOCK *et al.,* 2011)**. This pathogen thrives in warm, moist

dark areas of the human body including the genitals, anus and mouth. It produces a variety of co-infection with common pathogens such as *Escherichia coli, Staphylococcus saprophyticus, Proteus mirabilis, Klebsiella pneumoniae,* and *Enterobacter* species **(ISMAIL, 1999; BUWA and VAN STADEN, 2006)**.

Statistically, gonorrhoea is one of the most wide spread contagious infections in the world **(KRUGER and BOTHA, 2007)**. It was estimated by WHO in 1995 that 62 million cases of gonorrhoea were recorded in Asia, sub-Saharan Africa and South Central America **(TAPSALL, 2001)** and 62.35 million cases occurred in 1999, affecting more women than men **(WHO, 2001a)**. To date it is estimated that globally, out of approximately 448 million new cases of curable VD, gonorrhoea represent 88 million cases and the rest are syphilis, chlamydia and trichomoniasis **(WHO, 2011)**. In Africa, studies have shown that 3.1% of pregnant women are infected with gonorrhoea in Angola, Cameroon, Chad, Democratic Republic of Congo, Equatorial Guinea, Gabon and 7.8% in South Africa **(WHO, 2001a)**.

Gonorrhoea is still a common VD in South Africa with about 36-68% prevalence at STD clinics. It is difficult to control gonorrhoea because of the resistance of the pathogen to antibiotics **(KRUGER and BOTHA, 2007)**. **TAPSALL (2001)** suggested that the control of gonorrhoea, including other VD, requires a complex and comprehensive strategy that integrates education, counselling, diagnosis, treatment and case-finding, though the ability to control the disease is limited by the significant proportion of infections that remain undiagnosed.

*Bacillus subtilis* is a rod shape endospore-forming Gram-positive bacterium that occurs in chainlike formations. Its primary habitat is the soil and is responsible for food poisoning **(RYAN and RAY, 2004)**. *Staphylococcus aureus* is a spherical Gram-positive parasitic bacterium that causes illnesses ranging from minor skin infections and abscesses, to life-threatening diseases such as pneumonia, meningitis and septicemia **(LOWY, 1998)**. *Escherichia coli* is a Gram-negative bacterium normally present in the intestinal tract of

humans and other animals. *Escherichia coli* can sometimes be pathogenic, thus posing a threat to food safety, causing diarrhoea, wound and urinary infections **(SLEIGH and TIMBURY, 1988)**. *Klebsiella pneumoniae* belongs to a genus of non-motile, rod-shaped Gram-negative enterobacteria which causes pneumonia, urinary tract infections, bacteremia, septicaemia, chronic pulmonary disease, soft tissue infections, diarrhoea and other infections **(RYAN and RAY, 2004; HARYANI *et al.,* 2007)**.

### 2.1.2. Fungal infection

Besides bacterial infections, human are also fighting fungal infection. Fungal infections are associated with increasing morbidity and mortality in immunocompromised and severely ill patients **(LEHRNBECHER *et al.,* 2010)** as well as complications in cancer patients **(MARTINO and GIRMENIA, 2000)**. However, a few fungal pathogens are causative agents of infectious diseases, for example, *Candida albicans*.

*Candida albicans* (a form of yeast) is the major causative agent of fungal infections **(ROHITASHW and SHUKLA, 2010)**. It is capable of causing 90% of fungal infections by colonizing the mucosal surface of the gastrointestinal tract, female genital tract and infects the skin and mucous membranes of the vagina (vaginitis), head of the penis (balanitis), mouth (oral thrush) or rectum **(CHAITOW, 1996; RYAN and RAY, 2004; MURRAY and PIZZORNO, 1999)**. Overgrowth of *C. albicans* results in Candidiasis mostly in patients with endocrine disorder, immunosuppression, malignant disorders, HIV and AIDS **(MARTÍNEZ *et al.,* 1998)**. Ninety percent of HIV/AIDS patients suffer from fungal infections and 20% of them die as a direct consequence of such infections **(CHAITOW, 1996)**.

### 2.1.3. Antibiotic resistance of bacterial and fungal infection

Many drugs are available for the treatment of microbial infections, but some microbial have led the development of resistance and side effects. *Neisseria*

*gonorrhoea* strains have developed a high level of resistance to several antibiotics, including penicillin, tetracycline,.quinolones, cotrimoxazole, sulphonamides, macrolide, mostly in regions of the world where gonorrhoea is highly prevalent **(WHO, 2011; TAPSALL, 2001; DOH, 2008)**. Some of these antibiotics are currently not recommended for the treatment of gonorrhea in the majority of countries in the world such as penicillin, tetracycline and quinolones **(WHO, 2011)**. *Staphylococcus aureus* has sequential emergences of resistance. In 1940s it was resistant to sulpha drugs, followed by penicillin in 1950s then methicillin in the 1980s and recently it was reported to be resistant to vancomycin **(CDC, 2002)**. *Escherichia coli* in some hospital patients in United States of America and UK have shown resistant to sulphonamide and ampicillin-sulbactam **(ENNE *et al.,* 2001; KAYE *et al.,* 2000)**. *Klebsiella pneumoniae* including other *Klebsiella* species are resistant to celphalosporin β-lactam antibiotics **(BRADFORD *et al*., 1997; RAHAL *et al.,* 1998; KEYNAN and RUBINSTEIN, 2007)**.

There are number of effective antifungal agents available for treatment of various forms of fungal infections, for example amphotericin B (AmpB), azole, fluconazole, itraconazole, voriconazole, posaconazole, caspofungin, micafungin and anidulafungin, but they are very limited in supply. Nevertheless, the widespread and incorrect use of these antifungal agents has led to the emergence of drug resistance in several commom pathogenic fungi, a major challenge in the treatment of these diseases **(GRAYBILL, 1996)**. Acquired resistance to AmpB in *C. albicans* has been reported along with azole and fluconazole resistance. Very little information on AmpB resistance to *C. albicans* is available. *Candida lusitaniae* and *C guilliermondii* frequently develop resistance to AmpB **(VIVIANI *et al.,* 1998; KRCMERY and BARNES, 2002)**. Several antifungal drugs were recently approved, but systemic fungal infections still remain fatal **(FOULKES, 2001)**.

The high prevalence of antibiotic resistant is due to a number of factors such as inappropriate or inadequate antibiotic therapy, selective pressure and a high prevalence of other diseases coupled with lack of capacity to initiate

surveillance or prevention programs **(RUDDOCK *et al.,* 2011)**. With the emergency of single and multidrug resistance, alternative effective treatments of bacterial and fungal infections are urgently needed, with the agents that can target or inhibit infections including co-infections. Therefore, development of antimicrobial agents from natural products could play an important role in meeting the demand for new drugs against microbial infections.

In the search for new natural plant-derived antimicrobial agents, twelve medicinal plants used by the Venda people against venereal diseases were evaluated against five infectious bacteria including two Gram-positive (*B. subtilis* and *S. aureus*), three Gram-negative (*N. gonorrhoeae*, *E. coli* and *K. pneumoniae*) bacteria and a fungus *C. albicans* using the disc-diffusion assay for antigonococcal activities and microdilution techniques for antibacterial and antifungal activities.

## 2.2. Materials and Methods

### 2.2.1. Plant collection and sample preparation

Plant materials were collected from Limpopo province (Venda, Tshiendeulu mountain, Mandiwana village: 22°85´34.57˝ S, 30°141´65.6˝ E South Africa. Voucher specimens were deposited in the Herbarium at the University of KwaZulu-Natal, Pietermaritzburg. Plant materials were dried at 50 °C for 3 days, ground and stored in airtight containers under dark conditions at room temperature.

### 2.2.2. Preparation of plant extracts

Dried, ground plant materials were extracted sequentially with 20 ml/g of petroleum ether (PE), dichloromethane (DCM), 80% ethanol (EtOH) and water with sonication (Julabo GMBH, Germany) on ice for 1 h. The organic extracts were filtered under vacuum through Whatman No. 1 filter paper and concentrated under vacuum using a rotary evaporator (Rotavapor-R, Buchi,

Switzerland) at 35 °C. Water extracts were collected into glass jars and freeze dried. The concentrated organic extracts were transferred into sample vials then dried at room temperature under a stream of cold air. The dried extracts were kept in airtight glass sample vials at 10 °C in the dark until required for the different assays.

## 2.2.3. Antigonococcal assay

### 2.2.3.1. Preparation of *Neisseria gonorrhoeae* for stock and storage

*Neisseria gonorrhoeae* (ATCC 49226) was obtained from ATCC as KWIK-STIK$^{TM}$ plus Microorganism. The bacteria were inoculated onto chocolate agar (Oxoid GC agar base) supplemented with 2% (W/V) of haemoglobin and 1% (v/v) of Vitox supplement and incubated for 24 h at 37 °C in 5% $CO_2$. From the overnight culture, colonies were suspended in Mueller-Hinton broth (MH broth) with 20% glycerol to break the clumps, the mixture was vortexed. One millilitre of the mixtures was pipetted to cryo tubes for long term storage and stored at -70 °C.

### 2.2.3.2. Disc diffusion bioassay

The stored bacteria ( -70 °C) were inoculated onto chocolate agar (Oxoid GC agar base) supplemented with 2% (W/V) of haemoglobin and 1% (v/v) of Vitox supplement and incubated for 24 h at 37 °C in 5% $CO_2$. Four to five colonies of pure culture from overnight culture were suspended in 5 ml of MH broth. The turbidity of the cell suspension at 450 nm was adjusted by adding MH broth or organism as required, until the turbidity of the suspension was equivalent to the turbidity of a 0.5 McFarland $BaSO_4$ standard to the equivalent of $10^8$ CFU/ml.

Gonococcal activity of the 12 medicinal plant crude extracts was determined by disc diffusion **(RINGERTZ *et al.,* 1991; PUTNAM *et al.,* 1992)**. Crude plant extracts were dissolved in dimethylsulfoxide (DMSO) to a concentration of 50

mg/ml. Five microlitres of each extract were impregnated into sterile Whatman filter paper discs (6 mm diameter) and dried under a stream of sterile air. Bacteria were streaked onto petri dishes with GC agar base medium by using sterile swabs and the plate dried for 5 min. The procedure was done in triplicate. Ciprofloxacin and penicillin (1 µg/disc) were used as a positive control. Water and DMSO were used as negative and solvent controls respectively. Discs were applied to the dry surface of a Petri dish with GC agar base medium and bacteria. The plates were inverted and incubated at 37 °C in 5% $CO_2$ for 24 h. The assay was repeated twice with two replicates per assay.

The activity of plant extracts against *N. gonorrhoeae* was measured as indicated by clear zones of inhibition. Percentage of inhibition was calculated by comparing the distance of the sample to the distance of the positive control using the equation below: (by measuring the diameter of the inhibition zone including the disk).

$$\text{Gonococcal \% inhibition} = \left[\left(\frac{\text{Diameter of the sample}}{\text{Diameter of the positive control}}\right)\right] \text{X } 100$$

## 2.2.4. Antibacterial screening

### 2.2.4.1. Preparation of microbial stock cultures

Bacterial stock strains used for the antibacterial bioasssays were cultured in Mueller-Hinton (MH) agar (Merck, Germany). Sterile MH (25 ml) was poured into plastic Petri dishes and allowed to gel. The plates were allowed to cool overnight at 4 °C after which stock strains of the bacteria were streaked and sub-cultured. The bacteria-inoculated plates were incubated at 37 °C for 24 h to allow bacterial colonies to develop. The plates were then stored at 4 °C to prevent further bacterial growth. The stocks were sub-cultured following the same procedure every 30 days in order to maintain viability.

### 2.2.4.2. Microdilution bioassay

Minimum inhibitory concentration (MIC) values for antibacterial activity of the twelve medicinal plants were determined using the microdilution bioassay in a 96-well (Greiner Bio-one GmbH, Germany) microplate **(ELOFF, 1998)**. One hundred microlitres of each redissolved sample (50 mg/ml) in 80% ethanol were two-fold serially diluted with sterile distilled water, in duplicate down a 96-well microplate for four bacteria strains. A similar two-fold serial dilution of neomycin (0.1 mg/ml, Sigma) was used as a positive control against each bacterium. Water, 80% EtOH and bacteria-free MH broth were included as negative controls. Overnight MH broth cultures (incubated at 37 °C in a water bath with shaking) of the four bacterial strains (*B. subtilis* ATCC 6051 and *S. aureus* ATCC 12600 *E. coli* ATCC 11775 and *K. pneumoniae* ATCC 13883) were diluted with sterile MH broth (1 ml bacteria/50 ml MH broth) to give final inoculums of approximately $10^6$ cfu/ml (colony forming units). One hundred microlitres of each bacterial culture were added to each well. The plates were covered with parafilm and incubated overnight at 37 °C. Bacterial growth was tested by adding 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) to each well and the plates incubated at 37 °C for 1 h. Bacterial growth in the wells was indicated by a red-pink colour, whereas clear wells indicated inhibition of growth by the test sample. MIC values were recorded as the lowest concentration of extract showing a clear well. Each assay was repeated twice with two replicates.

## 2.2.5. Antifungal screening

### 2.2.5.1. Preparation of microbial stock culture

The fungal stock (*C. albicans*) used for the antifungal bioassays was cultured in Yeast Malt (YM) agar (Becton Dickinson, USA). Sterile YM (25 ml) was poured into plastic Petri dishes and allowed to gel following the procedure described in section 2.2.4.1.

### 2.2.5.2. Microdilution bioassay

The antifungal activity of the plant extracts was evaluated against *C. albicans* (ATCC 10231) using the micro-dilution assay **(ELOFF, 1998)** modified as an antifungal assay **(MASOKO *et al*., 2007)**. An overnight fungal culture was prepared in 10 ml YM broth. Four hundred microliters of the overnight *C. albicans* culture were added to 4 ml sterile saline solution (0.85%). The absorbance was read at 530 nm and adjusted with either sterile saline or fungal culture solution to match that of a 0.5 M McFarland standard solution of 0.25-0.28 absorbance units. From this prepared stock, a 1:1000 dilution with sterile YM broth was prepared to give an approximately $10^6$ CFU/ml culture.

One hundred microlitres of each plant extract were resuspended in 80% ethanol to a concentration of 50 mg/ml and two-fold serially diluted with sterile distilled water, in duplicate down a 96-well microtitre plate. A similar 2-fold serial dilution of Amphotericin B (Sigma-Aldrich) (2.5 mg/ml) was used as a positive control while 80% ethanol, water and fungal free broth were included as negative and/or solvent controls respectively. To each of the wells containing the test and control solutions, 100 µl of the dilute fungal culture was added and incubated for 24 h at 37 °C. To indicate fungal growth, 50 µl of 0.2 mg/ml INT was added to each well, and the plates were incubated for a further 24 h. The wells which displayed no change in colour represented antifungal activity. The MIC was taken as the lowest concentration of plant extract to inhibit growth of the tested fungus after 48 h. After noting the MIC, 50 µl YM broth was added to the clear wells to determine whether the inhibition was fungicidal. The microplates were re-covered with parafilm and incubated for a further 24 h after which the MFC (minimum fungicidal concentrations) values were noted. In the case where there was no growth of fungus in the last clear well even after addition of YM broth to clear wells, the MIC was taken as the MFC. The assay was repeated twice with two replicate per assay

## 2.3. Results and Discussion

### 2.3.1. Antigonococcal activity

The development of antibiotic resistance for pathogenic microorganisms is a major health concern worldwide, especially that of *N. gonorrhoeae*, which is also known to cause infertility amongst women **(CHOMNAWANG *et al.*, 2009)**. The twelve medicinal plants were tested against *N. gonorrhoeae* using disc diffusion methods and the results are presented in Table 3. Three levels of activity were used: 0–44% not active; 45–70% moderate; 70–100% good activity. PE extracts of *X. caffra* leaves and roots, *E. capensis* leaves and *B. speciosus* stems, exhibited good activity against *N. gonorrhoeae* (> 70%). The DCM extracts of *E. capensis* leaves showed the best inhibition percentage (96%), *O. lanceolata* roots (82%) and *X. caffra* leaves (79%). The EtOH extracts of *B. speciosus* bark and *O. lanceolata* roots showed good inhibition percentage (72 and 87%). No inhibition was observed in water extracts, negative control and penicillin therefore results were not included in the table. Ciprofloxacin contain polar compound for that reason it diffuse thru agar more than penicillin this could be the reason ciprofloxacin showed best inhibition percentage than penicillin. It was not surprisingly to observe no activity in water extracts. It has often been reported that water extracts have low/poor biological activity **(LUSEBA *et al*., 2007; RABE and VAN STADEN, 1997)**. This could also due to the lack of synergitic effect; because traditionaly whole plant part or different plant parts are prepared as mixture in form of (decoction or infusion). Traditional plant extarcts are soaked for a remedy to be more active; therefore the activity could be from polar saponins in water extracts and longer term extraction. The treatment of venereal diseases by traditional healers could be through the stimulation of the immune system or direct inhibition of microorganism or the therapeutic effect could be influenced by spiritual beliefs.

**SHOKEEN *et al.* (2005, 2009)** and **CHOMNAWANG *et al.* (2009)** showed the potency of medicinal plants against *N. gonorrhoeae*. A natural compound p-

methoxybenzylisothiocyanate isolated from *Lepidium bonariense* have already been reported to be active against *N. gonorrhoeae* **(SWART *et al.*, 2002)**.

Table 3: Different fractions obtained by sequential extraction were evaluated

| Plant species | Plant part | %inhibition | %inhibition | %inhibition |
|---|---|---|---|---|
| | | PE | DCM | EtOH |
| *Aloe chabaudii* | Root | 65±0.0 | 50±1.3 | 46±1.0 |
| *Adansonia digitata* | Bark | 44±0.0 | 55±2.0 | 58±0.0 |
| *Acacia karroo* | Bark | 51±2.3 | 45±0.0 | 44±0.0 |
| *Bolusanthus speciosus* | Leaf | 44±0.0 | 65±0.0 | 53±1.2 |
| *Bolusanthus speciosus* | Bark | 65±4.3 | 54±1.3 | **72±1.3** |
| *Bolusanthus speciosus* | Stem | **75±2.3** | 59±1.3 | 52±1.3 |
| *Elephantorrhiza burkei* | Root | 51±2.3 | 51±0.0 | 44±0.0 |
| *Ekebergia capensis* | Leaf | **77±6.5** | **96±6.8** | 45±1.0 |
| *Ekebergia capensis* | Bark | 57±7.2 | 58±0.0 | 52±3.2 |
| *Grewia occidentalis* | Root | 53±2.3 | 45±0.0 | 50±2.7 |
| *Osyris lanceolata* | Leaf | 55±0.0 | 70±2.7 | 45±1.0 |
| *Osyris lanceolata* | Root | 44±0.0 | **82±4.7** | **87±11.0** |
| *Peltophorum africanum* | Bark | 56±2.3 | 45±1.0 | 44±0.0 |
| *Pterocarpus angolensis* | Leaf | 68±2.7 | 46±1.0 | 44±0.0 |
| Ciprofloxacin | +ve control 1µg/disk | (13.8±0.0 ) | 100±00 | |

For the current study, these levels of activity were used: 0-44% not active; 45-70% moderate; 70-100% good activity.
Values boldly-written are considered very active.

Table 3: continuation

| Plant species | Plant part | %inhibition | %inhibition | %inhibition |
|---|---|---|---|---|
| | | PE | DCM | EtOH |
| *Pterocarpus angolensis* | Bark | 65±0.0 | 52±1.3 | 47±0.0 |
| *Pappea capensis* | Leaf | 65±4.3 | 57±3.2 | 44±0.0 |
| *Ximenia caffra* | Leaf | **73±4.3** | **79±3.2** | 44±0.0 |
| *Ximenia caffra* | Root | **87±4.3** | 44±0.0 | 56±1.0 |
| Ciprofloxacin | +ve control 1µg/disk | (13.8±0.0 ) | 100±00 | |

For the current study, these levels of activity were used: 0-44% not active; 45-70% moderate; 70-100% good activity.

Values boldly-written are considered very active.

Effective treatments are becoming increasingly limited because of the development of resistance or decreased susceptibility to the few antibiotics, which remain effective against *N. gonorrhoeae*, coupled with a significant lack of evidence that either new antimicrobial agents or combinations of older agents would be effective **(WORKOWSKI *et al.,* 2008; LIVERMORE, 2009)**.

The cell wall of *N. gonorrhoeae* has many similarities to that of other Gram-negative bacteria. The only difference is that it has surface proteins such as Opa proteins **(TAPSALL, 2001)**. The host is unable to develop an immunological memory against the *N. gonorrhoeae* resulting in the possibility for reinfection because Opa proteins bind to receptors on immune cells to prevent an immune response **(TAPSALL, 2001)**. Another important characteristic of *N. gonorrhoeae* is its antigenic variability. Through this process, *N. gonorrhoeae* has the ability to acquire genetic material from related organisms and to evade the hosts immune system **(TAPSALL, 2001)**. These outer membranes affect permeability and penetration of antibiotics into the pathogen **(TAPSALL, 2001)**.

In recent years, resistance of *N. gonorrhoeae* to antibiotics has increased rapidly and reduced the options for treatment. Due to this, it is now rated as an emergence multi-drug resistant pathogen **(WHO, 2011)**. Increase in antibiotic resistance may be due to the impermeability of the outer membranes. These membranes may probably affect the inhibition of *N. gonorrhoeae* by water extracts. According to **WHO (2011)** unrestricted access to antimicrobials, inappropriate selection and overuse of antibiotics, and suboptimal quality of antibiotics, as well as genetic mutations within the organism have contributed to the development of resistance in *N. gonorrhoeae* including interaction and exchange of genetic material with other co-infections.

**DEGUCHI *et al.* (2010)** suggested that a comprehensive global program is needed, including surveillance for antimicrobial resistance in *N. gonorrhoeae* in order to prevent the further emergence and international spread of drug resistance, and to allow the selection of appropriate treatments. The strategies for the control of gonorrhea have relied on the use of highly effective drugs **(RAHMAN *et al*., 2001)**. The results obtained in this study indicate the existence of antimicrobial compounds in the crude extracts and the good activity observed from some of the plant extracts

tested in this study could lead to the isolation of effective antigonococcal compounds that can penetrate into *N. gonorrhoeae* surface proteins.

## 2.3.2. Antibacterial activity

The antibacterial MIC values of the extracts of the medicinal plant extracts tested in this study are presented in Table 4. **FABRY *et al*. (1998)** defined potentially useful activity for crude solvent extracts of plants to be considered as having good activity if they have MIC values <8 mg/ml, whilst **GIBBONS (2005)** suggests that isolated phytochemicals should have MIC <1mg/ml. However, in this study MIC values <0.1 mg/ml were considered to be excellent antibacterial activity. The extracts showed a broad spectrum of activity against all bacterial strains. The DCM and EtOH extracts of *B. speciosus* bark and stem extracts showed the best antibacterial activity against *B. subtilis* and *S. aureus*. *Aloe chabaudii*, *A. digitata* and *G. occidentalis* exhibited the least antibacterial activity against all the strains.

According to **RABE and VAN STADEN (1997)**, Gram-negative bacteria are more resistant than Gram-positive ones. Gram-negative bacteria have an outer membrane acting as a barrier to environmental substances including antibiotics **(PALOMBO and SEMPLE, 2001)**. The observed activity of some *B. speciosus* bark DCM and EtOH extracts against Gram-negative bacteria, which are often resistant to antimicrobial agents, could be explained by the fact that the active compounds may act by inhibiting the bacterial growth without necessarily penetrating into the bacterial cell itself. It has often been reported that water extracts have low/poor biological activity **(LUSEBA *et al*., 2007; RABE and VAN STADEN, 1997)**. However, in this study water extracts of *X. caffra* leaf exhibited good antibacterial activity with an MIC value of 0.049 mg/ml against *S. aureus*. This justifies the use of water extracts of this plant in traditional medicine **(MULAUDZI *et al.*, 2009)**. The activity observed from *B. speciosus* could be due to the isolated active compounds such as medicarpan, genistein, 6,6′-dihydroxy-4′-methoxy-2-arylbenzofuran (bolusanthin IV) and flavonoids, which were previously shown to have antibacterial activity against *B. subtilis*, *E. coli* and *S. aureus* **(MAJINDA *et al*., 2001; ERASTO *et al*., 2004)**.

Table 4: Antibacterial minimal inhibitory concentrations (MIC mg/ml) of 12 medicinal plants used by the Venda people of South Africa

| Plant species | Plant part | MIC (mg/ml) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PE | | | | DCM | | | | EtOH | | | | Water | | | |
| | | *B.s.* | *E.c.* | *K.p.* | *S.a.* | *B.s.* | *E.c.* | *K.p.* | *S.a.* | *B.s.* | *E.c.* | *K.p.* | *S.a.* | *B.s.* | *E.c.* | *K.p.* | *S.a.* |
| *Aloe chabaudii* | Root | 6.25 | 3.125 | 1.56 | 1.56 | 1.56 | 1.56 | 3.125 | 1.56 | 3.125 | 3.125 | 3.125 | 3.125 | >12.5 | 6.25 | 6.25 | 3.125 |
| *Adansonia digitata* | Bark | 3.125 | 3.125 | 6.25 | 12.5 | 3.125 | 1.56 | 3.125 | 3.125 | 0.78 | 3.125 | 3.125 | 1.56 | >12.5 | 3.125 | 3.125 | 3.125 |
| *Acacia karroo* | Bark | 3.125 | 1.56 | 1.56 | 1.56 | 0.78 | 0.39 | 0.39 | 0.195 | 0.195 | 1.56 | 0.78 | 0.195 | 0.39 | 1.56 | 3.125 | 0.195 |
| *Bolusanthus speciosus* | Leaf | 0.78 | 3.125 | 0.39 | 1.56 | 0.39 | 6.25 | 0.39 | 3.125 | 0.195 | 1.56 | 3.125 | 0.78 | >12.5 | 0.78 | 1.56 | 3.125 |
| *Bolusanthus speciosus* | Bark | 0.39 | 0.195 | 3.125 | 0.78 | 0.39 | **0.098*** | 0.195 | **0.098** | **0.025** | **0.098** | 0.195 | **0.098** | 3.125 | 3.125 | 0.78 | 1.56 |
| *Bolusanthus speciosus* | Stem | 0.39 | 0.195 | 0.78 | 0.78 | **0.012** | 0.78 | 0.195 | **0.025** | **0.098** | 1.56 | 0.78 | **0.098** | >12.5 | 12.5 | 3.125 | 6.25 |
| *Elephantorrhiza burkei* | Root | 6.25 | 3.125 | 3.125 | 12.5 | 3.125 | 1.56 | 3.125 | 6.25 | 0.39 | 0.39 | 0.78 | **0.025** | 0.39 | 1.56 | 1.56 | 0.39 |
| *Ekebergia capensis* | Leaf | 0.39 | 0.78 | 1.56 | 3.125 | 0.78 | 0.78 | 0.78 | 0.78 | 1.56 | 3.125 | 1.56 | 0.78 | 6.25 | 6.25 | 3.125 | 6.25 |
| *Ekebergia capensis* | Bark | 0.78 | 1.56 | 1.56 | 3.125 | 0.78 | 1.56 | 1.56 | 1.56 | **0.098** | 3.125 | 1.56 | 0.78 | 1.56 | 3.125 | 1.56 | 0.78 |
| *Grewia occidentalis* | Root | 3.125 | 3.125 | 3.125 | 12.5 | 3.125 | 3.125 | 1.56 | 3.125 | 3.125 | 3.125 | 3.125 | 0.78 | >12.5 | 6.25 | 6.25 | 12.5 |
| *Osyris lanceolata* | Leaf | 6.25 | 1.56 | 1.56 | 6.25 | 0.39 | 0.78 | 0.78 | 6.25 | 0.78 | 3.125 | 1.56 | 0.39 | 1.56 | 3.125 | 1.56 | 0.78 |
| *Osyris lanceolata* | Root | **0.098** | 3.125 | 1.56 | 0.195 | **0.098** | 3.125 | 1.56 | 0.195 | **0.098** | 3.125 | 0.195 | 0.78 | 6.25 | 6.25 | 6.25 | 0.78 |
| *Peltophorum africanum* | Bark | 1.56 | 0.78 | 0.78 | 3.125 | 1.56 | 0.78 | 1.56 | 3.125 | 0.195 | 0.39 | 0.195 | **0.098*** | 0.39 | 1.56 | 0.78 | 0.78 |

*B.s.= Bacillus subtilis*; *E.c.= Escherichia coli*; *K.p.= Klebsiella pneumoniae*; *S.a.= Staphylococcus aureus* *Values boldly-written are considered very active (< 0.1 mg/ml). The MIC values (µg/ml) for Neomycin (positive control) were: *B. subtilis* = 1.531 $\times 10^{-3}$; *E. coli* = $3.063 \times 10^{-3}$; *S. aureus* = $6.125 \times 10^{-3}$; *K. pneumoniae* = $6.125 \times 10^{-3}$.

Table 4: continuation

| Plant species | Plant part | MIC (mg/ml) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PE | | | | DCM | | | | EtOH | | | | Water | | | |
| | | *B.s.* | *E.c.* | *K.p.* | *S.a.* | *B.s.* | *E.c.* | *K.p.* | *S.a.* | *B.s.* | *E.c.* | *K.p.* | *S.a.* | *B.s.* | *E.c.* | *K.p.* | *S.a.* |
| *Pterocarpus angolensis* | Leaf | 3.125 | 1.56 | 3.125 | 0.78 | 1.56 | 1.56 | 0.78 | 3.125 | 0.39 | 1.56 | 0.78 | 0.78 | >12.5 | 1.56 | 0.78 | 0.78 |
| *Pterocarpus angolensis* | Bark | 3.125 | 3.125 | 1.56 | 6.25 | 0.78 | 0.39 | 0.195 | 3.125 | **0.012** | 0.78 | 1.56 | 0.195 | 0.39 | 3.125 | 3.125 | 3.125 |
| *Pappea capensis* | Leaf | 0.78 | 0.39 | 0.78 | 0.39 | 0.78 | 0.78 | 1.56 | 1.56 | 0.195 | 0.78 | 0.78 | **0.098** | >12.5 | 3.125 | 3.125 | 3.125 |
| *Ximenia caffra* | Leaf | 1.56 | 3.125 | 3.125 | 6.25 | 0.78 | 3.125 | 3.125 | 6.25 | 0.39 | 0.195 | 0.195 | **0.025** | 0.39 | 0.39 | 0.78 | **0.049** |
| *Ximenia caffra* | Root | 0.39 | 1.56 | 1.56 | 0.78 | 0.39 | 1.56 | 1.56 | 0.78 | 0.195 | 1.56 | 0.78 | 0.195 | 0.39 | 3.125 | 6.25 | 3.125 |

*B.s.*= *Bacillus subtilis*; *E.c.*= *Escherichia coli*; *K.p.*= *Klebsiella pneumoniae*; *S.a.*= *Staphylococcus aureus* *Values boldly-written are considered very active (< 0.1 mg/ml). The MIC values (µg/ml) for Neomycin (positive control) were: *B. subtilis* = $1.531 \times 10^{-3}$; *E. coli* = $3.063 \times 10^{-3}$; *S. aureus* = $6.125 \times 10^{-3}$; *K. pneumoniae* = $6.125 \times 10^{-3}$.

*Ximenia caffra* extracts showed good antibacterial activity against *S. aureus*, *E. coli* and *P. aeruginosa* **(OKEMO *et al*., 2004)**. *Ximenia caffra* contains simple phenolic compounds such as coumaric, vanillic and ferulic acids which have often been implicated in antibacterial activity **(NDHLALA *et al*., 2008b)**. The activity observed from *O. lanceolata* could be due to the isolated compound octandronic acid, and 20-epikoetjapic acid which previously showed good antibacterial activity against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa* **(YEBOAH *et al*., 2010)**. The observed activity justifies further investigation to characterize the active constituents in the extracts of *B. speciosus* bark and stem, *O. lanceolata* roots, *P. africanum* bark, *P. angolensis* bark and *X. caffra* leaves.

### 2.3.3. Antifungal activity

*Candida albicans* is one of the fungi that play a role in venereal disease, especially in immunocompromised and HIV/AIDS patients. The plant extracts tested showed a broad spectrum of antifungal activity against *C. albicans* (Table 5). The results indicate MIC and minimum fungicidal concentration (MFC) values ranging from 0.012 to 12.5 mg/ml. It is important to determine whether the active extracts are fungistatic (able to slow or retard the multiplication of fungi) or fungicidal (able to destroy fungi). The results of this study took both of these aspects into consideration.

The DCM extracts of *B. speciosus* bark showed the highest activity with MIC and MFC value of 0.012 mg/ml respectively. This shows that *B. speciosus* is fungicidal to *C. albicans* at this concentration. The 80% EtOH extracts of *B. speciosus* leaf, bark, *O. lanceolata* root, *P. angolensis* leaf, *P. capensis* leaf, *E, capensis* leaf and *X. caffr*a leaf showed good antifungal activity against *C. albicans*. The activity observed from *O. lanceolata* could be due to compounds such as 1α, 9 β-Difuranoyloxy-2-oxo-dihydro- β –agarofuran, 1α, 9β-Difuranoyloxy-2-oxo-3-enedihydro-β agarofuran, 1α, 9β Difuranoyloxydihydro-β–agarofuran, octandronic acid, and 20-epikoetjapic acid all of which have previously displayed antifungal activity against *C. albicans* **(YEBOAH *et al.,* 2010)**. As in the antibacterial assay, *A. chabaudii* roots and *A. digitata* bark exhibited the lowest activity.

According to **POLAK (1999)** a truly safe, fungicidal drug does not yet exist. However, the observed results from *B. speciosus* bark suggested a potential lead to the isolation of a fungicidal drug. The good antifungal activity can be the lead to overcome the limited supply and to overcome the drug resistance challenge.

Table 5: Antifungal activity (MIC and MFC) of 12 medicinal plants used by the Venda people

| Plant species | Plant part | MIC (mg/ml) | | | | MFC (mg/ml) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PE | DCM | EtOH | Water | PE | DCM | EtOH | Water |
| *Aloe chabaudii* | Root | 6.25 | 6.25 | 3.125 | 12.5 | 6.25 | 6.25 | 3.125 | >12.5 |
| *Adansonia digitata* | Bark | 3.125 | 12.5 | 6.25 | 6.25 | 3.125 | 12.5 | 6.25 | 12.5 |
| *Acacia karroo* | Bark | 6.25 | 6.25 | 3.125 | 6.25 | 12.5 | 6.25 | 3.125 | >12.5 |
| *Bolusanthus speciosus* | Leaf | 3.125 | **0.39*** | **0.195** | 6.25 | 3.125 | 1.56 | **0.78** | 6.25 |
| *Bolusanthus speciosus* | Bark | 6.25 | **0.012** | **0.195** | 6.25 | 12.5 | **0.012** | **0.78** | 12.5 |
| *Bolusanthus speciosus* | Stem | 6.25 | **0.195** | 1.56 | 6.25 | 6.25 | 3.125 | 3.125 | 6.25 |
| *Elephantorrhiza burkei* | Root | 6.25 | 6.25 | 1.56 | 3.125 | 6.25 | 6.25 | 3.125 | 6.25 |
| *Ekebergia capensis* | Leaf | 6.25 | 3.125 | **0.39** | 6.25 | 12.5 | 6.25 | 3.125 | 12.5 |
| *Ekebergia capensis* | Bark | 3.125 | 3.125 | 6.25 | 6.25 | 3.125 | 6.25 | 6.25 | 12.5 |
| *Grewia occidentalis* | Root | 6.25 | 6.25 | 6.25 | 12.5 | 6.25 | 12.5 | 6.25 | 12.5 |
| *Osyris lanceolata* | Leaf | 3.125 | 1.56 | 6.25 | 6.25 | 3.125 | 1.56 | 6.25 | 6.25 |
| *Osyris lanceolata* | Root | 3.125 | 3.125 | **0.098** | 6.25 | 3.125 | 3.125 | 6.25 | 6.25 |
| *Peltophorum africanum* | Bark | 6.25 | 3.125 | 3.125 | 1.56 | 6.25 | 3.125 | 3.125 | 12.5 |
| *Pterocarpus angolensis* | Leaf | 3.125 | 3.125 | **0.195** | 6.25 | 3.125 | 3.125 | 1.56 | 12.5 |

The MIC and MFC of Amphotericin B (µg/ml) (positive control) were $9.77 \times 10^{-3}$ and $7.81 \times 10^{-2}$ respectively
*Values boldly-written are considered very active (< 1 mg/ml).

Table 5: continuation

| Plant species | Plant part | MIC (mg/ml) | | | | MFC (mg/ml) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PE | DCM | EtOH | Water | PE | DCM | EtOH | Water |
| *Pterocarpus angolensis* | Bark | 3.125 | 3.125 | 6.25 | 3.125 | 3.125 | 3.125 | 6.25 | 12.5 |
| *Pappea capensis* | Leaf | 6.25 | 1.56 | **0.195** | 3.125 | 12.5 | 1.56 | 3.125 | 3.125 |
| *Ximenia caffra* | Leaf | 1.56 | 1.56 | **0.78** | **0.78** | 1.56 | 1.56 | 1.56 | 1.56 |
| *Ximenia caffra* | Root | 3.125 | 3.125 | 3.125 | 1.56 | 6.25 | 3.125 | 3.125 | 12.5 |

The MIC and MFC of Amphotericin B (µg/ml) (positive control) were $9.77 \times 10^{-3}$ and $7.81 \times 10^{-2}$ respectively
*Values boldly-written are considered very active (< 1 mg/ml).

## 2.4. Conclusions

The plants used for conditions related to venereal diseases by the Venda people were successfully evaluated in order to justify their uses in traditional medicine as well as in the quest for the search for natural products that can resolve the issue of drug resistance. The broad spectrum antimicrobial activity of some plants justifies the use of these medicinal plants in traditional medicine. *Bolusanthus speciosus* and *X. caffra* exhibited the best antigonococcal, antifungal and antibacterial activities. *Adansonia digitata* and *A. chabaudii* exhibited poor activities in all the assays. Traditionally water, not organic solvents, are used to make decoctions. However, in this case it was surprising because no activity was observed in water extracts against *N. gonorrhea*. Maybe these plants exhibited the activity when they are in synergism.

They are many co-infections involved when a patient is infected with one pathogen for example with venereal infections *S. aureus,* causes skin diseases **(BUWA and VAN STADEN, 2006)**, while *E. coli* and *K. pneumoniae* infects the urinary tract **(SLEIGH and TIMBURY, 1998)** Untreated venereal infections can lead to other diseases such as conjunctivitis which can be caused by *B. subtilis* **(BUWA and VAN STADEN, 2006)**. It was interesting to note the activity of some of the plants against bacteria that are known to cause co-infection with venereal infections. The same plants exhibited good activity against *N. gonorrhea*. This observation shows that these plants are used purposely for treating all infections related to gonorrhea.
*Bolusanthus speciosus* and *X. caffra* can be the best plants to use to prevent gonococcal reinfection and antigenic variability.

Modern drugs are based on the lock and key theory, which uses only one target to treat a single disease. However, this theory usually fails because many diseases involve multiple factors **(KONG *et al*., 2009)**. Therefore antimicrobial activity of *B. speciosus* and *X. caffra* clearly shows that medicinal plants can be the key to dispel this theory. The results obtained in this study only offer supporting evidence for

effective use of these extracts. More *in vitro* confirmatory tests using other assays and/or *in vivo* tests are still required.

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# CHAPTER 3: Anti-inflammatory and Anti-HIV-1 Screening

## 3.1. Introduction

Enzyme catalysis is involved in several physiological, biochemical and disease processes essential for life, thus making enzymes attractive in drug discovery. This is due to the fact that enzymes are able to be inhibited by small molecular weight compounds. In light of this characteristic enzymes are now the targets of new drug discovery in pharmaceutical and biotechnological research **(COPELAND, 2005)**.

### 3.1.1. Inflammatory process

Inflammation is the response of living tissues to injury such as physical trauma, chemical irritations, ultraviolet radiation and burns, as well as microbial pathogen infections **(IWALEWA *et al.*, 2007; PAN *et al*., 2010)**. It involves the mobilization of inflammatory responses that serve as physiological barriers against the spread of infection, eliminating foreign pathogens and repairing injured tissues as well as the clearance of the stimuli **(COHN and LANGMAN, 1996; PAN *et al*., 2010)**. Redness, heat, pain, swelling and loss of function are the basic signs of inflammation in the body **(MOLVI *et al*., 2007)**. Inflammation is rapid and self-limiting, but abnormal resolution and prolonged inflammation causes various chronic disorders classified as acute or chronic inflammatory diseases **(CALDER *et al*., 2009)**. Acute inflammation is characterized by slow exudation of fluid, plasma proteins and the emigration of leukocytes, predominantly neutrophils. Chronic inflammation delays the immune response and is of prolonged duration, a week or month **(MOLVI *et al*., 2007)**. Chronic inflammatory responses lead to a pathological progression of chronic illnesses (such as cancer, neurological disease, metabolic disorder, chronic lung disease and cardiovascular diseases) that are characterized by infiltration of inflammatory cells, excess production of cytokines, deregulation of cellular signalling and loss of barrier function **(PAN *et al*., 2010)**. According to **PAN *et al*. (2010)** reduction/eliminating of chronic inflammation is a beneficial strategy to fight several human diseases.

Inflammation responses are triggered by the biosynthesis of a group of compounds known as prostaglandins from arachidonic acid **(VANE and BOTTING, 1997; SALMON and HIGGS, 1994)**. Prostaglandins are involved in many physiological processes, where they play an important role in the maintenance of mucosal integrity in the stomach, homeostasis and regulation of renal function **(KURUMBAIL *et al*., 2001; MORITA, 2002; PATRIGNANI *et al*., 2005)**. The enzymes that convert arachidonic acid to prostaglandins are referred to as cyclooxygenases (COX) **(MORITA, 2002)**. COX exists in two isoforms commonly referred to as COX-1 and COX-2. These two isoforms are upregulated in a variety of circumstances **(WANG and DUBOIS, 2011)**. COX-1 is known to be involved in normal physiological functions. Recently COX-1 has been shown to be upregulated in various carcinomas and to have a central role in tumorigenesis. COX-2 is involved in rheumatic diseases, inflammation and tumorigenesis **(JABBOUR and SALES, 2004)**.

### 3.1.2. Anti-Inflammatory agents

The nonsteriodal anti-inflammatory drugs (NSAIDs) exert their relieving properties, analgesic, and antipyretic effects mainly by inhibiting COX enzymes **(PATRIGNANI *et al*., 2005)**. Aspirin, ibuprofen, piroxicam, indomethacin, and sulindac are well known examples of NSAIDs that are commonly used for treating arthritis, rheumatisms, pain and fever **(COPELAND, 2005; MOLVI *et al*., 2007)**. Their mechanism of action includes inhibition of both the COX-1 and COX-2 isoenzymes **(PATRIGNANI *et al*., 2005)**. Inhibition of COX-2 is thought to mediate the therapeutic actions of NSAIDs, while the inhibition of COX-1 results in unwanted side-effects, mostly in the gastrointestinal tract, by causing ulceration, bleeding, perforation, and obstruction **(FITZGERALD and PATRONO, 2001; PATRONO *et al*., 2001)**. There is growing interest in the search for anti-inflammatory and analgesic drugs with no side effects. A number of compounds derived from medicinal plants are already considered as effective and safer for the treatment of various diseases, including inflammation and pain **(TUNÓN *et al*., 1995; TAYLOR and VAN STADEN, 2001)**.

### 3.1.3. Human immunodeficiency virus infections

Human immunodeficiency virus (HIV) is a lentivirus that causes acquired immunodeficiency syndrome (AIDS) and is considered pandemic **(WEISS, 1993)**. The infection is a condition that causes a progressive failure of the immune system and allows life-threatening opportunistic infections and cancer to thrive in humans. It has become one of the world‘s most serious health and development challenges since 1981 **(WHO, 2003)**. This infection is primarily spread from person to person mainly through sexual contact. The genital tract acts as a point of entry and reservoir in both sexes **(VAN VUUREN and NAIDOO, 2010)**.

The complex process of HIV infection begins when the HIV uses cells of the immune system (macrophages and helper T cells) as sites for reproduction resulting in the production of multiple copies of the viral genetic material (RNA), which are packaged to infect new viral hosts **(THAKUR *et al*., 2010)**. The infection produces a complex multifactorial condition, associated with immunodeficiency and autoimmune inflammation. It also produces gradual effects on the body‘s defence mechanisms thereby leading to such opportunistic infections as tuberculosis, pneumonia, intestinal tract infections, as well as debilitating weight loss, diarrhoea, neurologic conditions, and cancers such as Kaposi‘s sarcoma and certain types of lymphomas **(VERMANI and GARG, 2002; THAKUR *et al*., 2010)**. These infections cause the immune system to remain in a state of chronic activation resulting in decreased immune surveillance and susceptibility to opportunistic infections, growth of neoplasm and ultimate progression to the full development of the AIDS syndrome **(YOUNG, 2003)**.

### 3.1.4. HIV prevalence

Currently there are approximately 33.4 million people living with HIV/AIDS worldwide **(UNAIDS/WHO, 2008a)**. Reports from all regions of the world reveal that, almost all those living with HIV (96%) reside in low- and middle-income countries, particularly in sub-Saharan Africa **(UNAIDS/WHO, 2008b)**. The nine countries with the highest HIV prevalence worldwide are all located in this sub-region, with each of these

countries experiencing adult HIV prevalence greater than 10% **(UNAIDS, 2009)**. However, the epidemic appears to have stabilized in most regions, although prevalence continues to increase in Eastern Europe and central Asia, due to a high rate of new HIV infections **(UNAIDS, 2010)**. Sub-Saharan Africa remains the most heavily affected region, with 22.5 million adults and children living with HIV/AIDS **(UNAIDS, 2010)**.

South Africa is home to the world's largest population of people living with HIV (5.7 million) **(UNAIDS/WHO, 2008a)**. **UNAIDS (2010)**, estimate that 310,000 South Africans died of AIDS in 2009. Almost one-in-three women aged 25-29, and over a quarter of men aged 30-34, are living with HIV **(HUMAN SCIENCES RESEARCH COUNCIL, 2009)**. The high HIV prevalence in developing countries may be due to the high cost and limited availability of antiretroviral drugs.

### 3.1.5. Anti- HIV agents

Human immunodeficiency virus type 1(HIV-1) reverse transcriptase (RT) is capable of catalyzing reverse transcription of HIV-RNA into a double stranded DNA via polymerase and RNase H activities. Reverse transcriptase is an important enzyme in the HIV replication cycle. Inhibition of RT is one of the most important targets in disrupting the replication of HIV and has been one of the primary therapeutic strategies in HIV/AIDs patient treatment **(LAI *et al*., 2009; HUANG *et al*., 2011)**. The role of RT mechanism has guided the development of several inhibitors which are now widely used in current HIV/AIDS therapy **(CASTRO *et al*., 2006)**. Standard HIV therapies available for the treatment of HIV-1 infections include nucleoside RT, non-nucleoside RT and protease inhibitors **(MENÉNDEZ-ARIAS, 2008)**.

These inhibitors have different roles in the inhibition processes. Nucleoside RT inhibitors such as zidovudine (AZT) and lamivudine (3TC), bind directly to the active site of RT polymerase and terminate DNA synthesis after incorporation onto the newly synthesized DNA, while non-nucleoside RT inhibitors (efavirenz and nevirapine) bind to an allosteric site on RT **(LAI *et al*., 2009)**. Protease inhibitors such as nelfinavir and saquinavir block or inactivate endogenous proteolytic and phospholytic enzymes **(MENÉNDEZ-ARIAS, 2008)**. All these treatments contribute

in reducing progression of HIV. However, there are disadvantages related to these drugs, such as side effects that limit the use of these antiviral drugs (i.e. AZT) and some of them do not penetrate at an efficient inhibitory level, mostly protease inhibitors **(MENÉNDEZ-ARIAS, 2008; DA SILVA *et al*., 2009)**. In addition, the emergence of drug-resistance, due to high mutation rates of HIV together with its rapid turnover in an infected individual, eventually can lead to antiretroviral failure **(MENÉNDEZ-ARIAS, 2008; DA SILVA *et al*., 2009)**. Although these therapies help in improving the life quality of HIV patients and slow down the progression of AIDS, the condition still remains a major health problem in the world today **(DA SILVA *et al*., 2009)**. Therefore there is need to develop of new RT inhibitors that can inhibit clinically prevalent resistant viruses.

Inflammation is a major risk factor for various human diseases including venereal diseases. Screening of some medicinal plants for anti-inflammatory agents have been reported by **McGAW *et al*. (1997)** and **TAYLOR and VAN STADEN (2001)**.

Many medicinal plants have been shown to be potentially useful as agents against many diseases including inhibition of the HIV-1 reverse transcriptase enzyme. Screening of some medicinal plants for anti-HIV agent have been reported by **BESSONG *et al*. (2005)**, **KLOS *et al*. (2009) and TSHIKALANGE *et al*. (2008)**.

In this study twelve medicinal plants were evaluated for the possible inhibition of COX-1, COX-2 and HIV-1 reverse transcriptase enzyme.

## 3.2. Materials and Methods

### 3.2.1. Plant collection and sample preparation

Plants were collected and prepared as described in **Section 2.3.1.** of **Chapter 2.** Except that for HIV-1 reverse transcriptase organic solvent used was 50% methanol

### 3.2.2. Preparation of plant extracts

The extracts were resuspended to a concentration of 10 mg/ml for cyclooxygenase. For the HIV-1 reverse transcriptase (RT) inhibitory bioassay, organic extracts were resuspended in 50% methanol to a concentration of 20 mg/ml.

### 3.2.3. Enzyme and substrate preparation for the Cyclooxygenase (COX-1 and COX-2) assays

The COX-1 enzyme isolated from ram seminal vesicles and the human recombinant COX-2 containing a six histidine sequence near the *N*-terminus isolated from a Baculovirus over expression system in S*ƒ* 21 cells were obtained from Sigma Aldrich (USA).

The enzymes were diluted with Tri (hydromethyl) aminomethane (TRIS) storage buffer (pH 8.0) to obtain 50 µl aliquots, each with 75 units of enzyme concentration per aliquot. The prepared COX enzymes were stored in an ultra-freezer at -70 °C.

### 3.2.4. COX-1 inhibitory bioassay

The COX-1 bioassay was performed as described by **JÄGER *et al.* (1996)**. The enzyme was activated with 1250 µl of co-factor solution (3 mg/ml *L*-epinephrine and 3 mg/ml reduced glutathione in 0.1 M Tris buffer, at pH 8.0) and pre-incubated on ice for 5 min, 60 µl of the enzyme solutions were added to each sample solution (2.5 µl plant extract and 17.5 µl water) in duplicate and the mixture was incubated at room temperature for 5 min in 1.5 ml Eppendorf tubes. The extracts were tested at a concentration of 10 mg/ml (organic solvent were resuspended in 80% ethanol and aqueous extract in water) giving a final concentration of 250 µg/ml per test solution.

Two separate sets of Eppendorf tubes, background (the enzyme was inactivated with HCl before incubation), solvent blank (containing water instead of sample) and positive control (containing 5 µM indomethacin obtained from Sigma) were included in the test. The reaction was initiated by adding 20 µl $^{14}$C-arachidonic acid (16 Ci/mol, 30 µM) to each Eppendorf tube after 5 min of incubation. The mixtures were the incubated in a water bath at 37 ºC for 10 min and afterwards the reaction was stopped by adding 10 µl 2N HCl except in the background tubes. Columns were packed with silica gel (silica gel 60, 0.063-0.200 mm, Merck) to a height of 3 cm in Pasteur pipettes. Four microlitres (0.2 mg/ml) of unlabeled prostaglandins (PGE2: PGF2α 1:1) (Sigma-Aldrich) were added to each Eppendorf tube as a carrier solution. The test solutions were applied to the columns with 1 ml of eluent 1 [hexane: 1, 4-dioxane: glacial acetic acid (70:30:0.2 v: v: v)]. The application of eluent 1 was done in order to separate the prostaglandins and unmetabolized arachidonic acid. Arachidonic acid was eluted first with 4 ml eluent 1. The prostaglandin products were eluted with 3 ml of eluent 2 [ethyl acetate: methanol (85:15 v: v)] and collected in scintillation vials. To each vial, 4 ml of scintillation fluid were added and the disintegration per minute (DPM) of the radioactive material was counted using a scintillation counter (Beckman LS 6000LL scintillation counter). The assay was repeated twice. Inhibition percentage was calculated using the equation below:

$$\text{COX inhibition (\%)} = \left\{1 - \left(\frac{\text{DPM}_{\text{extract}} - \text{DPM}_{\text{background}}}{\text{DPM}_{\text{solvent blank}} - \text{DPM}_{\text{background}}}\right)\right\} \times 100$$

where $\text{DPM}_{\text{extract}}$ is the disintegrations per minute for the plant extract, $\text{DPM}_{\text{background}}$ is the disintegrations per minute in which the enzyme was inactivated and $\text{DPM}_{\text{blank}}$ is the disintegrations per minute for the reaction mixture containing water. Results are presented as graphs (means ± standard errors) of two independent experiments. Graphs were done using SigmaPlot 2002 for Windows version 8.0 (SPSS inc., USA).

### 3.2.5. COX-2 inhibitory bioassay

The COX-2 activity was assessed using a method described by **NOREEN *et al*. (1998)**, with slight modifications **(ZSCHOCKE and VAN STADEN, 2000)**. The procedure of the assay was the same as described in COX-1 **Section 3.3.4** except that 6 mg/ml *L*-epinephrine was used instead of 3 mg/ml *L*-epinephrine in the preparation of the cofactor solution and 200 µM indomethacin instead of 5 µM.

### 3.2.6. HIV-1 reverse transcriptase (RT) inhibitory bioassay

The effect of the 12 medicinal plants against RT was evaluated using a non-radioactive HIV-1 RT colorimetric ELISA kit obtained from Roche Diagnostics (Germany). The protocol supplied together with the kit was followed, under nuclease-free conditions. The reverse transcriptase colourimetric assay takes advantage of the ability of RT to synthesize DNA, starting from the template/primer hybrid poly (A) × oligo $(dT)_{15}$, by avoiding the use of [$^{3}$H]- or [$^{32}$P]-labeled nucleotides which are used in other classical RT assays. In place of radio-labeled nucleotides, digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one and the same DNA molecule, which is freshly synthesized by the RT. The detection and quantification of synthesized DNA as a parameter for RT activity is followed in a sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtitre plate modules (MPM) with wells that were precoated with streptavidin. In the next step, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labelled DNA. In the final step, the peroxidase substrate ABTS (2, 2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]- diammonium salt) is added. The peroxidase enzyme catalyzes the cleavage of the substrate, producing a coloured reaction product which is measured spectrophotometrically.

The following solutions, provided with the kit were prepared according to the manufacturer instructions; Solution 1, HIV-1 reverse transcriptase (final concentration 2 ng/µl, corresponding to 10 mU/µl) stored at -70 ºC. Solution 2, incubation buffer. Solution 3, reaction mixture containing poly (A) x oligo $(dT)_{15}$ (46 mM Tris-HCl, 266 mM potassium chloride, 27.5 mM magnesium chloride, 9.2mM

DDT, 10 µM dUTP/dTTP, template/primer hybrid, 750 $mA_{260\ nm}$/ml). Solution 4, lysis buffer. Solution 5, anti-digoxigenin-peroxidase (anti-DIG-POD) (200 mU/ml). Solution 6, washing buffer and solution 7, ABTS substrate solution and microtitre plate modules (MPM) precoated with streptavidin and postcoated with blocking reagent.

Twenty micro-litres of the resuspended plant extracts (with final assay concentrations of 0.125, 0.25, 0.5, 1µg/ml) were mixed with 20 µl of recombinant HIV-1-RT (4 ng in lysis buffer) and 20 µl reaction mixture (solution 3) in sterile Eppendorf tubes and the tubes were incubated for 1 h at 37 °C. After a 1 h incubation period, the mixtures (60 µl) were transferred into MPM wells and covered with foil and incubated for 1 h at 37 °C after which the mixtures were removed from the MPM wells completely. The wells were rinsed five times with 250 µl of washing buffer (solution 6) per well for 30 s, the washing buffer being removed carefully after each wash. After washing, 200 µl of anti-DIG-POD (solution 5) was added to each well and the MPM were recovered with foil and incubated for 1 h at 37 °C. After the incubation period, the solution was removed completely from the MPM wells and rinsed five times with 250 µl of washing buffer (solution 6) per well for 30 s. After washing, 200 µl of ABTS substrate solution (solution 7) was added to each well and the MPM were incubated at room temperature for 5 min (a green colour appeared in the wells).

The absorbance of the reaction mixture was then measured at 405 nm using a microplate reader (Opsys MRTM, Dynex Technologies Inc.). Percentage of inhibition was calculated by comparing the absorbance of the sample to the negative control using the equation below:

$$\text{HIV-1 RT inhibition (\%)} = \left\{1 - \left(\frac{\text{Abs}_{405\ nm}\ \text{sample}}{\text{Abs}_{405\ nm}\ \text{negative control}}\right)\right\} \times 100$$

Where $Abs_{405\ nm}$ sample is the absorbance of the reaction with plant extracts or positive control at 405 nm and $Abs_{405\ nm}$ negative control is the absorbance of the reaction with water instead of sample at 405 nm.

The negative control consisted of water instead of sample. A reference control consisted of lysis buffer and no HIV-1 RT. Combivir® (GlaxoSmithKline) [lamivudine (1.0 mg/ml) + zidovudine (2.0 mg/ml)] and Kaletra® (Abbott) [lopinavir (8.9 mg/ml) + ritonavir (2.2 mg/ml)] were used as positive controls. Results were presented as means ± standard errors of two independent experiments; each experiment was done in duplicate. The $IC_{50}$ values were calculated using GraphPad Prism version 4.00 statistical software programs for Windows (GraphPad Software Inc.).

## 3.3. Results and discussion

### 3.3.1. COX-1 and COX-2 inhibitory activity

The results of the percentage inhibition of COX-1 and COX-2 enzyme by the 12 medicinal plants used for venereal and related diseases are presented in Figure 1. Four levels of activity were defined for the enzyme inhibition assays; with activity below 20% being considered insignificant, 20–40% low, 40–70% moderate and 70–100% high **(TAYLOR and VAN STADEN, 2001)**.

All solvent extracts tested showed good inhibition activity towards COX-1 (moderate to high activity), except water extracts of *A. digitata* bark*, O. lanceolata* leaves and *P. angolensis* leaves and PE extracts of *X. caffra* leaves. However, water extracts of *A. karroo* bark*, E. burkei* roots*, E. capensis* leaves*, P. africanum* bark*, P. angolensis* bark*, P. capensis* leaves*, X. caffra* (leaves, roots) showed high activity (71.9 to 100%). DCM, PE, EtOH extracts of *A. chabaudii* roots, *A. digitata* bark, *A. karroo* bark, *B. speciosus* leaves, *B. speciosus* bark, *E. burkei* roots, *O. lanceolata* roots, *P. angolensis* bark, *P. capensis* leaf, and *X. caffra* roots showed high inhibitory activity towards COX-1 (>70%).

Among the different solvent extracts tested, DCM extracts of most of the plant species displayed good activity towards COX-2 (moderate to high activity), with *E. capensis* bark*, O. lanceolata* leaves and roots*, X. caffra* leaves and roots being the only exception showing insignificant to low percentage inhibition. PE extracts of *A. digitata* bark*, B. speciosus* bark*, G. occidentalis* roots*, P. africanum* bark*, P.*

*angolensis*, leaves and roots and *P. capensis* leaf showed high inhibitory activity towards COX-2 (> 70%). No activity was recorded in the PE extracts of *E. capensis* leaves against COX-2. EtOH extracts of *B. speciosus* stems displayed high inhibitory activity (95.7%) towards COX-2. No activity was observed in the water extracts of *E. capensis* leaves, *G. occidentalis* roots, *P. angolensis* leaves and root and *P. capensis* leaves, the rest of the water extracts displayed insignificant to moderate inhibition.

It has often been reported that activity in water extracts is not detected or yield false positives **(LUSEBA *et al*., 2007)**. However, in this study, water extracts showed good activity toward COX-1, with the highest activity (100%) of *A. karroo* bark. Some phenolic compounds such as proanthocyanidins have antioxidant effects and have been used to treat inflammatory diseases **(NDHLALA *et al*., 2008a)**. It has been reported that *A. karroo* bark is very rich in proanthocyanidin and flavonols **(MALAN and SWARTZ, 1995)**. Flavonoids are known for their significant scavenging properties on oxygen radicals *in vivo* and *in vitro,* affecting various steps in the arachidonate cascade via cyclooxygenase or lipoxygenase **(ABAD *et al*., 1995)**. A species containing a known anti-inflammatory compound has the potential to inhibit inflammatory processes **(JÄGER *et al.,* 1996)**. The mechanism of anti-inflammatory activities of *A. karroo* bark may be due to the presence of the above mentioned phytochemicals.

Insignificant activity toward COX-2 could be due to the present of low lipophilic compounds in water extracts. **TUNÓN *et al.* (1995)** reported that lipophilic compounds are less likely to be found in water extracts, even if they are found they are in small quantities. PE and DCM extracts of *A. digitata* bark extracts showed good activity toward both COX-1 and COX-2, with insignificant activity in EtOH extracts toward COX-2 enzyme. These activities could be from proanthocyanidins, terpenoids, flavonoids and sterols that have been isolated from *A. digitata* **(SHAHAT, 2006)**. DCM and PE extracts of *P. angolensis* bark extracts showed good activity in both COX-1 and COX-2. The activity from *P. angolensis* could have resulted from isolated compounds epicatechin [(-)-epicatechin, epicatechin-3-O-galate, epicatechin (4beta-8)-epicatechin (B2), and a hexamer of epicatechin] **(SAMIE *et al*., 2009)**.

Flavonoids are known to act through a variety of mechanisms to prevent and/or reduce inflammatory responses and they are likely to have a potential role in preventive and therapeutic roles in chronic inflammatory conditions **(PAN *et al.* 2010)**.

Generally extracts inhibited COX-1 enzyme better than COX-2 enzyme. This may be because of the conformation of the active site of COX-1 which is smaller than that of COX-2 **(MORITA, 2002)**. **BOTTING (2006)** pointed out that the smaller active site of COX-1 is more easily inhibited than the larger COX-2 site. However, it is undesirable to have good COX-1 activity because of the side effects in the gastrointestinal tract, which results in development of ulcers **(LUSEBA, 2007)**. COX-2 have proven to be beneficial in clinical situations and led to the introduction of many potential anti-inflammatory agents such as indomethacin, ibuprofen and piroxicam **(MOLVI *et al.,* 2007)**. However, it was recently found that prolonged use of higher doses of COX-2 selective inhibitors can cause side effects in humans, such as adverse cardiovascular effects **(MOLVI *et al*., 2007; MODICA *et al*., 2005)**. According to **NCUBE (2010)**, moderate active therapeutics may be used rather than high active ones, to reduce the manifestation of damaging side effects.

The observed anti-inflammatory activities of the active extracts may be due to the overall effects of the plant constituents or the compounds having action similar to NSAID's, in a complimentary manner **(ADEDAPO *et al*., 2008)**. Insignificant inhibition observed in some extracts in this study does not confirm inactivity of these extract as anti-inflammatory agents, the inactivity could be due to impurities or low concentrations of active compounds in the crude extracts or contain active compounds that act at other sites in the processes of pain and inflammation **(McGAW *et al*., 1997)**.

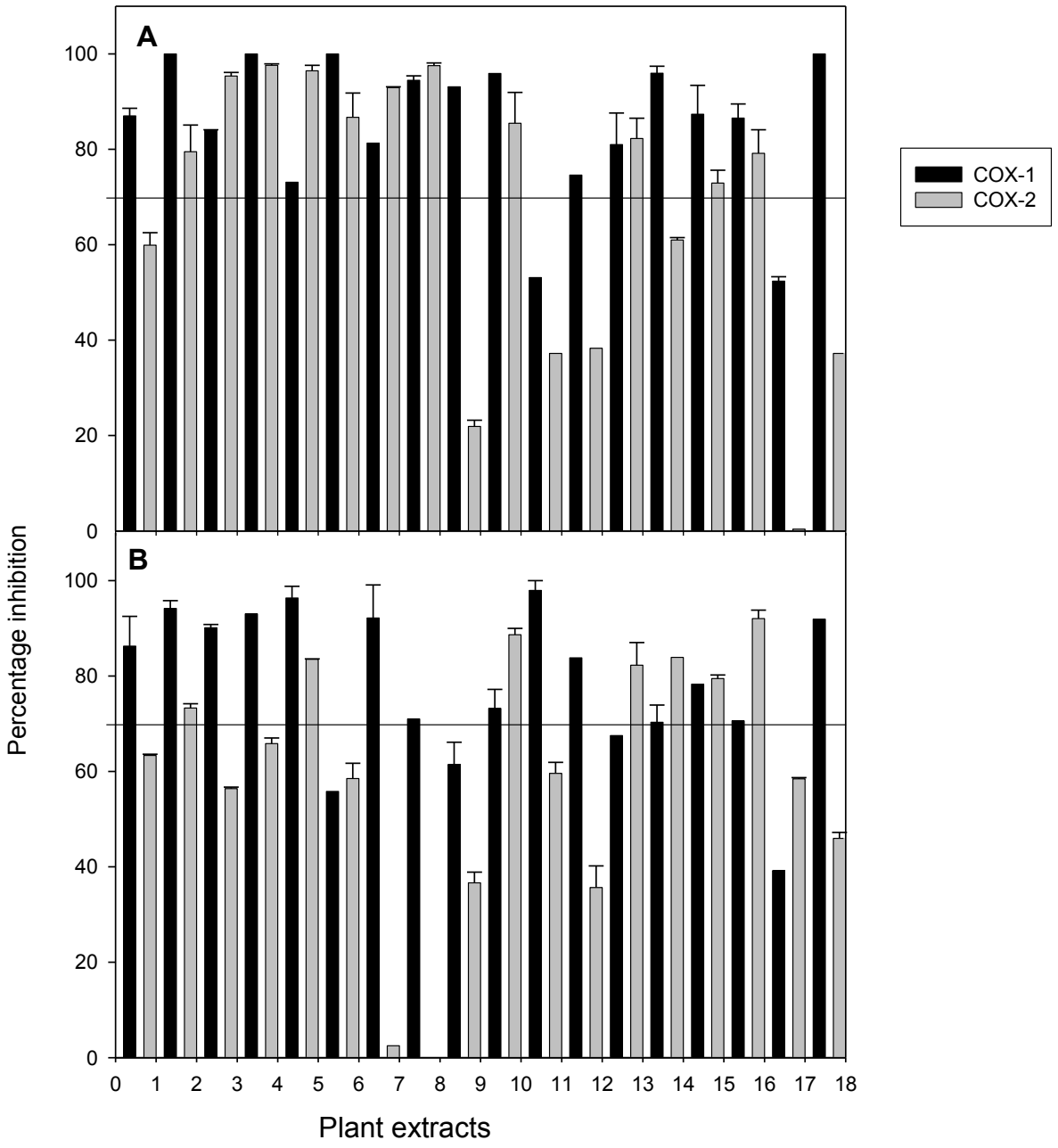



**Figure 1.** Continue to the next page

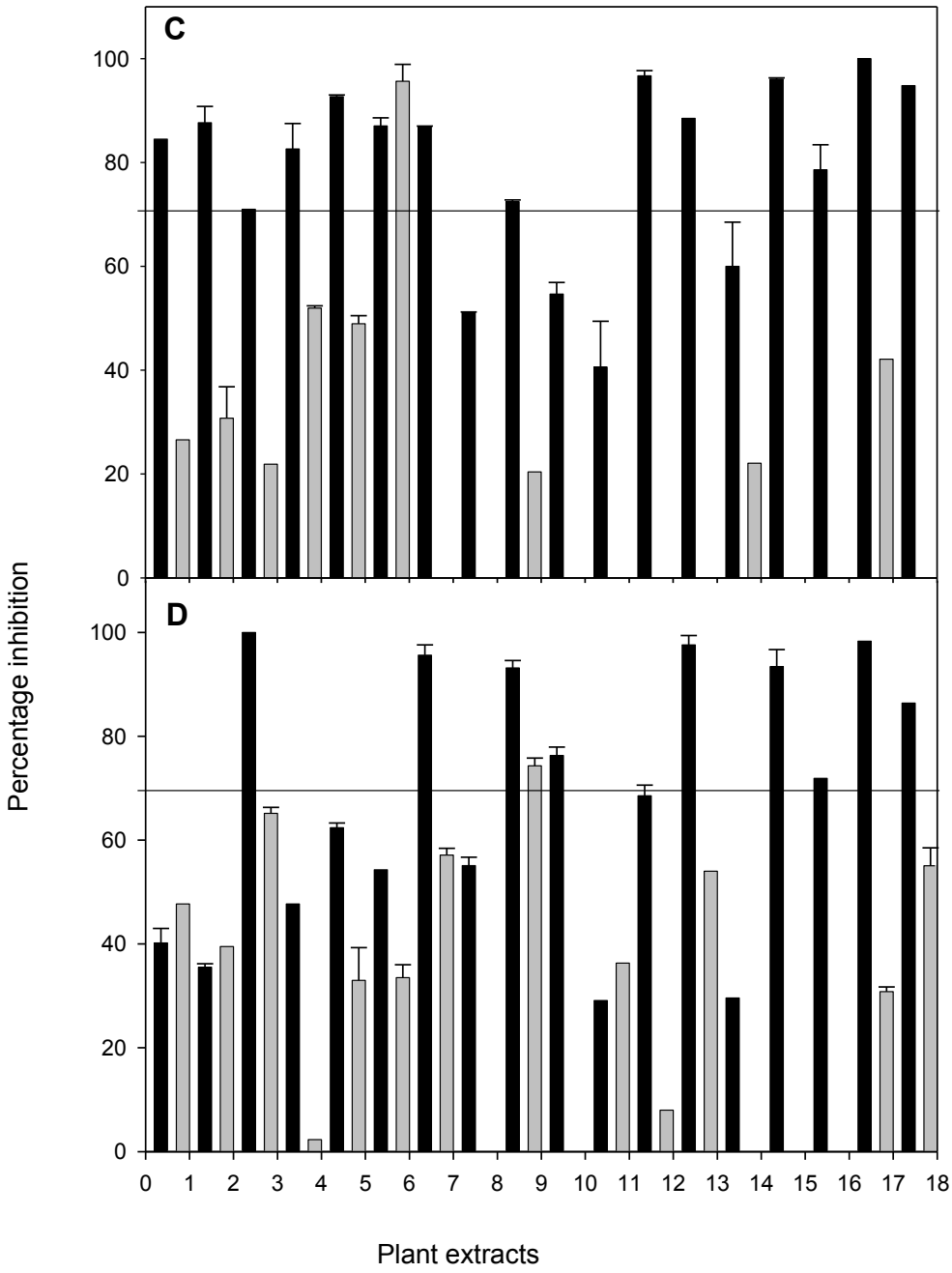



**Figure 1.** Percentage inhibition of COX-1 and COX-2; Dichloromethane extracts (A), Petroleum ether extracts (B), 80% Ethanol extracts (C) and water extracts (D) by 12 medicinal plants used by the Venda people against venereal diseases (1mg/ml). (1) *Aloe chabaudii* root, (2) *Adansonia digitata* bark, (3) *Acacia karroo* bark, (4) *Bolusanthus speciosus* leaf, (5) *Bolusanthus speciosus* bark, (6) *Bolusanthus speciosus* stem, (7) *Elephantorrhiza burkei* root, (8) *Ekebergia capensis* leaf, (9) *Ekebergia capensis* bark, (10) *Grewia occidentalis* root, (11) *Osyris lanceolata* leaf, (12) *Osyris lanceolata* root, (13) *Peltophorum africanum* bark, (14) *Pterocarpus angolensis* leaf, (15) *Pterocarpus angolensis* bark, (16) *Pappea capensis* leaf, (17) *Ximenia caffra* leaf, (18) *Ximenia caffra* root. Plant extracts with Inhibition activity above 70% were considered to be highly active. Percentage inhibition by positive controls indomethacin was 64.18 ± 3.10 and 68.50 ± 2.57 for COX-1 and COX-2 respectively

### 3.3.2. HIV-1 RT inhibitory activity

HIV infected people rely on antiretroviral drugs to improve the quality and prolong their lives. Antiretroviral drugs have many disadvantages including resistance, limited availability, high cost and lack of any curative effects **(KLOS *et al*., 2009)**. Medicinal plants are an excellent source of anti-HIV agents **(KLOS *et al*., 2009)**. In this study aqueous and methanolic extracts from twelve medicinal plants were tested for RT inhibition (Figure 2). The $IC_{50}$ values of the plant extracts are shown in Table 6. Plant extracts with inhibitory activity above 70% were considered to be highly active **(NDHLALA *et al*., 2010b)**. The inhibitory activity was also expressed as the half maximal inhibitory concentration ($IC_{50}$). $IC_{50}$ is a measure of the effectiveness of a compound in inhibiting biological or biochemical function **(BHATTACHARJEE *et al*., 2011)**. This measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process or component of a process (that is an enzyme, cell, cell receptor or microorganism) by half **(BHATTACHARJEE *et al*., 2011)**.

Methanol and water extracts of *Acacia karroo* bark*, B. speciosus* stems and *X. caffra* leaves and roots exhibited best inhibition percentages (>70%). Methanol extracts of *A. chabaudi* roots, *E. capensis* bark, and *O. lanceolata* roots showed low inhibition percentages. The smaller the $IC_{50}$ value, the more potent the extract towards RT inhibition. The $IC_{50}$ values show order of potency of the plants as: methanol extracts of *A. karroo* bark, *B. speciosus stem* and *P. angolensis* leaves > *P. africanum* bark and *P. capensis* > *B. speciosus* bark and water extracts of *B. speciosus* stem > *E. capensis* bark and *P. angolensis* leaves > *B. speciosus* leaves > *G. occidentails* roots and *P. capensis* leaves > *A. digitata* bark and *P. africanum* bark in that order are more potent inhibitors of the RT. Except for methanol extracts of *A. chabaudii* roots, *E. capensis* bark and *O. lanceolata* roots, whose $IC_{50}$ values could not be calculated because the activity was less than 50% at the highest concentration.

A number of natural products are known for their activity against HIV-1 RT such as coumarins, flavonoids, tannins, alkaloids, lignans, terpenes, naphtho- and

anthraquinones, and polysaccharides **(MATHÉE *et al*., 1999)**. Similar compounds were reported from *B. speciosus* **(ERASTO *et al*., 2004; MAJINDA *et al*., 2001)**. Therefore the activity against HIV-1 RT from *B. speciosus* could be from these compounds. The presence of acacatechin, catechutannic acid and quercetin in *A. karroo* may be responsible for the activity of this plant **(VAN WYK *et al*., 2009)**. *Ximenia caffra* has been shown to contain simple phenolic compounds *p*-coumaric acid and ferulic acid **(NDHLALA *et al*., 2008b)**, which inhibits HIV-1 protease activity **(ICHIMURA *et al*., 1999)**. Water and methanol extracts of *B. speciosus* stem have great potential to be used as anti HIV-1 RT including methanol extracts of *A. karroo bark, P. angolensis* leaf and *P. capensis* leaf.

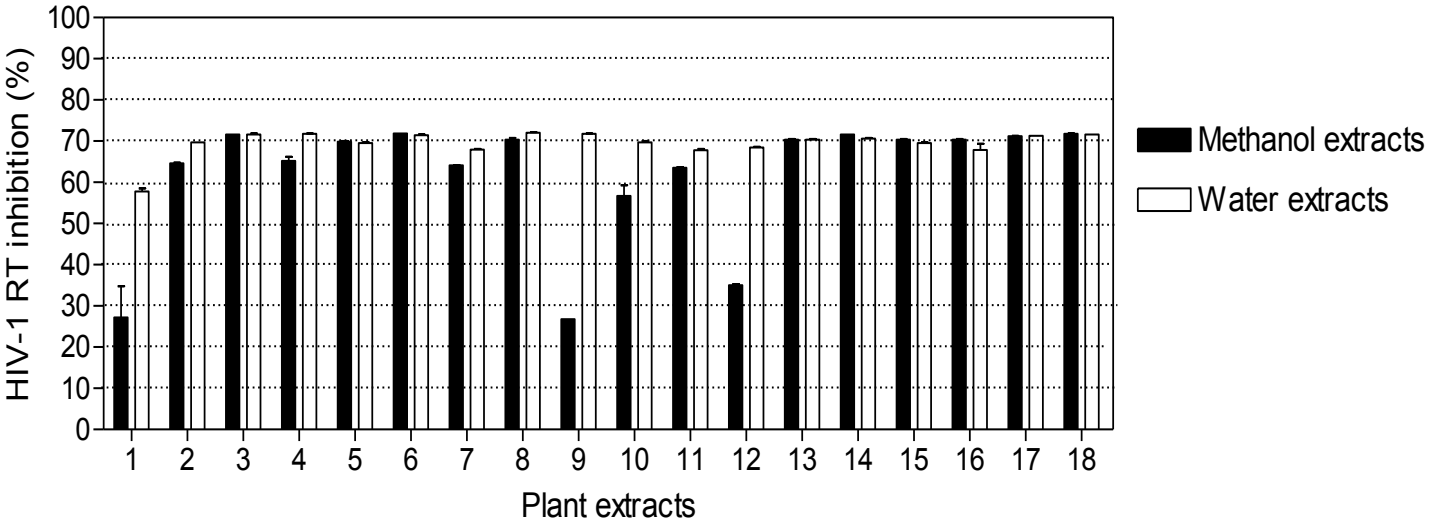



**Figure 2.** Percentage inhibition of HIV-1 RT by 12 medicinal plants used by the Venda people against venereal diseases (1mg/ml). (1) *Aloe chabaudii* root, (2) *Adansonia digitata* bark, (3) *Acacia karroo* bark, (4) *Bolusanthus speciosus* leaf, (5) *Bolusanthus speciosus* bark, (6) *Bolusanthus speciosus* stem, (7) *Elephantorrhiza burkei* root, (8) *Ekebergia capensis* leaf, (9) *Ekebergia capensis* bark, (10) *Grewia occidentalis* root, (11) *Osyris lanceolata* leaf, (12) *Osyris lanceolata* root, (13) *Peltophorum africanum* bark, (14) *Pterocarpus angolensis* leaf, (15) *Pterocarpus angolensis* bark, (16*) Pappea capensis* leaf, (17) *Ximenia caffra* leaf, (18) *Ximenia caffra* root. Percentage inhibition by positive controls were; Combivir® (0.5 mg/ml) 79.80 ± 0.12 and 62.50 ± 0.31 for Kaletra® (0.5mg/ml)

Table 6: HIV-1 RT inhibitory activity ($IC_{50}$ mg/ml) of 12 medicinal plants used by the Venda people against venereal diseases

| Plant species | Plant part | HIV-1 RT inhibitory activity $IC_{50}$ (mg/ml) | |
|---|---|---|---|
| | | Methanol extracts | Water extracts |
| *Aloe chabaudii* | Root | NC | 0.60±0.06 |
| *Adansonia digitata* | Bark | 0.27±0.05 | 0.09±0.01 |
| *Acacia karroo* | Bark | 0.03±0.00 | 0.11±0.01 |
| *Bolusanthus speciosus* | Leaf | 0.43±0.04 | 0.07±0.01 |
| *Bolusanthus speciosus* | Bark | 0.07±0.00 | 0.16±0.02 |
| *Bolusanthus speciosus* | Stem | 0.03±0.00 | 0.03±0.00 |
| *Elephantorrhiza burkei* | Root | 0.52±0.00 | 0.37±0.03 |
| *Ekebergia capensis* | Leaf | 0.39±0.06 | 0.10±0.01 |
| *Ekebergia capensis* | Bark | NC | 0.06±0.00 |
| *Grewia occidentalis* | Root | 0.91±0.32 | 0.08±0.01 |
| *Osyris lanceolata* | Leaf | 0.84±0.09 | 0.49±0.02 |
| *Osyris lanceolata* | Root | NC | 0.49±0.02 |
| *Peltophorum africanum* | Bark | 0.05±0.01 | 0.09±0.01 |
| *Pterocarpus angolensis* | Leaf | 0.03±0.00 | 0.06±0.00 |
| *Pterocarpus angolensis* | Bark | 0.15±0.03 | 0.24±0.02 |
| *Pappea capensis* | Leaf | 0.05±0.00 | 0.08±0.00 |
| *Ximenia caffra* | Leaf | 0.41±0.05 | 0.37±0.02 |
| *Ximenia caffra* | Root | 0.15±0.01 | 0.21±0.02 |
| Combivir ® | | | 0.06±0.03 |
| Kaletra ® | | | 0.3±0.10 |

NC= The $IC_{50}$ could not be calculated because the activity was less than 50% at the highest concentration tested.

## 3.4. Conclusions

The above results indicate that some of the tested plant extracts have potential to be used as enzyme inhibitors against venereal diseases.

DCM and PE extracts of *A. digitata* bark, *B. speciosus* bark*, P. angolensis* bark and *P. capensis* leaves showed good anti-inflammatory activity in both COX-1 and COX-2. Therefore these plants extracts may provide leads for the identification and isolation of active compounds for various inflammatory diseases.

Numerous standard HIV therapies have been approved to treat HIV-infection; however some of these treatments have been reported to have side effect as well as being resistant to virus strains. Based on RT inhibitory activities observed in the present medicinal plants, methanol and water extracts of *B. speciosus* stems*, P. africanum* bark *P. angolensis* leaves and *P. capensis* leaves have good potential to be used as anti-HIV-1 RT inhibitors.

This study has shown that the investigated medicinal plants could be harboring potent RT and inflammation inhibitors, opening up possibilities of compound isolation. However, more *in vitro* confirmatory tests using other assays and/or *in vivo* tests are still required.

# CHAPTER 4: Phytochemical Properties of twelve Medicinal plants that are used by Venda people against Venereal and related diseases

## 4.1. Introduction

Plants produce a vast range of compounds that are traditionally classified as either primary or secondary metabolites. Primary metabolites are compounds that have essential roles associated with photosynthesis, respiration, growth and development. Plant secondary metabolites are a diverse group of molecules that are involved in the adaption of plants to their environment and are not involved in primary metabolism **(MAKKAR *et al.,* 2007)**.

Secondary metabolites (often referred to as phytochemicals) are either present in the plant in active states or as prodrugs that becomes activated upon wounding, infection or in the body of herbivores **(WINK, 1999; 2003)**. Being sessile organisms, plants cannot run away when they are attacked by herbivores or microbial infections **(WINK, 1999; 2003)**. Plants have thus developed secondary biochemical pathways to synthesize a range of chemicals, often responding to specific environmental stimuli, such as herbivore-induced damage, pathogen attacks, or nutrient deficiencies **(KENNEDY and WIGHTMAN, 2011)**. The largest and most prevalent phytochemical groups are the alkaloids, terpenes and phenolic compounds **(KENNEDY and WIGHTMAN, 2011)**.

Secondary metabolites have been used for centuries in traditional medicine because of their diverse biological activities. Nowadays secondary metabolites are used as a basis for the production of valuable synthetic compounds such as pharmaceuticals, cosmetics, or more recently nutraceuticals **(BOURGAUD *et al*., 2001)**. They are also viewed as potential sources of new natural drugs, antibiotics, insecticides and herbicides **(CROZIER *et al*., 2006)**. Due to their biological significance and potential health effects, such as antioxidant, anticancer, anti-aging, anti-atherosclerotic, antimicrobial, and anti-inflammatory activities, phytochemicals have gained

increasing attention from researchers **(SHAHIDI, 2004; SHAHIDI *et al*., 2008)**. Regular intake of some phytochemicals have been reported to reduce risks of chronic diseases, such as cancer, heart attack and diabetes **(ZHU *et al.,* 2009)**.

### 4.1.1. Phenolic compounds

Phenolic compounds are among the largest groups of secondary metabolites that are ubiquitously found across the plant kingdom, with approximately 10,000 structures **(GURIB-FAKIM, 2006; KENNEDY and WIGHTMAN, 2011)**. Phenolic compounds range from simple low-molecular weight compounds, with one aromatic ring such as the simple phenylpropanoids, coumarins, and benzonic acid derivatives, to complex structures such as tannins, flavonoids, stilbenes, and lignins **(GURIB-FAKIM, 2006; CROZIER *et al.,* 2006; KENNEDY and WIGHTMAN, 2011)**.

Flavonoids represent the largest, most diverse group of phenolic compounds, encompassing some 6000 compounds **(KENNEDY and WIGHTMAN, 2011)**. Flavonoids can be divided into sub-groups namely: flavones, flavonols, flavanones, isoflavones, flavan-3-ols, and anthocyanins **(CROZIER *et al*., 2006; KENNEDY and WIGHTMAN, 2011)**. The generic flavonoid structure is shown in Figure 3. They are generally found in fruits, vegetables, nuts, seeds, stems, cereals, legumes, herbs, spices, and flowers as well as tea, wine, propolis, and represent a common constituent of the human diet **(KANDASWAMI *et al*., 2005; CUSHNIE and LAMB, 2005)**. Tannins are a very complex group of secondary metabolites capable of tanning leather or precipitating gelatin from solution, a property known as astringency **(COWAN, 1999; SILANIKOVE *et al*., 2001)**. Their molecular weights range from 500 to 3,000 and are broadly found in every plant part: bark, wood, leaves, fruits, and roots **(COWAN, 1999)**. Tannins are found in approximately 80% of woody and 15% of herbaceous dicotyledonous species **(SILANIKOVE *et al.* 2001)**. They are divided into two groups, hydrolyzable and condensed tannins **(COWAN, 1999; GURIB-FAKIM, 2006)**. The structure of hydrolyzable and condensed tannins are shown in Figure 4.

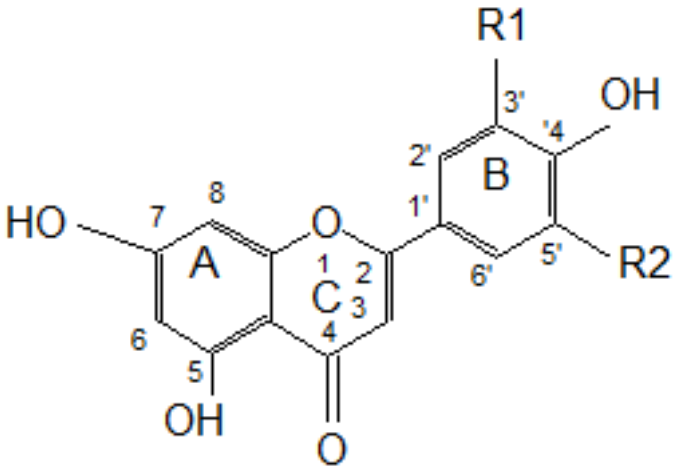



**Figure 3.** Generic structure of flavonoids in which rings A, B, and C and the numbering system are shown. Kaempferol, R1 = H, R2 = H quercetin, R1 = OH, R2 = H; myricetin, R1= OH, R2 = OH. Adapted from **(CROZIER *et al*., 2006)**

**A**

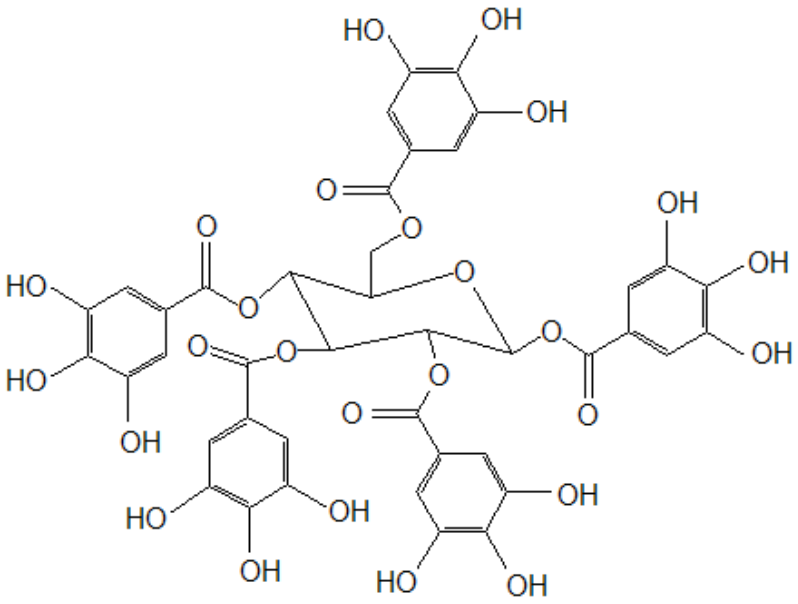
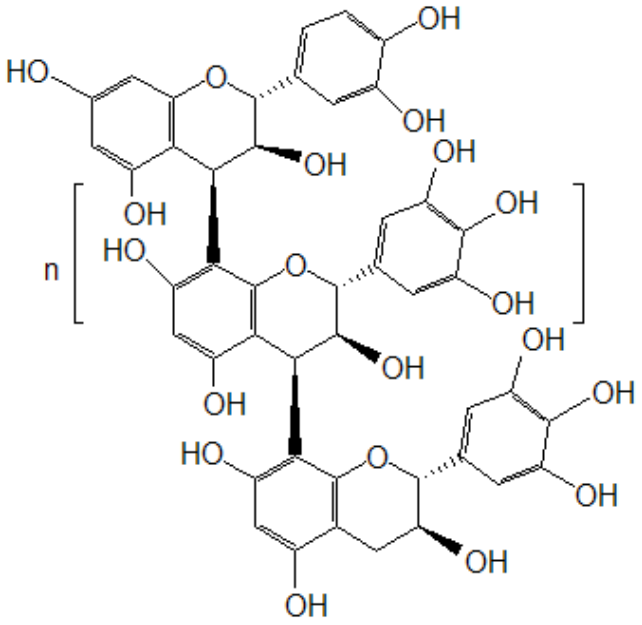



**Figure 4.** Continue to the next page




**B**

**Figure 4.** Structures of (A) penta galloyl-D-glucose (hydrolyzable tannin) and (B) proanthocyanidin (condensed tannin), where ‗n' is any number that makes up the polymer. Structures adapted from **(CROZIER *et al*., 2006)**

## 4.1.2. Medicinal properties of phenolic compounds

Flavonoids have been reported to possess several pharmacological properties **(HAVSTEEN, 1983; HARBORNE and BAXTER, 1999; KANDASWAMI *et al*., 2005; CUSHNIE and LAMB, 2005)**. Their enzyme inhibitory properties have been assumed to be due to the interaction of enzymes with different parts of the flavonoid molecule, e.g. carbohydrate, phenyl ring, phenol and benzopyrone ring **(CUSHNIE and LAMB, 2005)**. Because of the above properties, flavonoids have now increasingly become the subjects of medical research. Several flavonoids are known to inhibit HIV-1 including demethylated gardenin A, robinetin, myricetin, baicalein, quercetagetin and quercetin **(CUSHNIE and LAMB, 2005)**. Flavonoids such as quercetin inhibit both cycloxygenases and lipoxygenases by reducing the release of arachidonic acid **(HARBORNE and WILLIAMS, 2000; NIJVELDT *et al*., 2001)**. Several studies have reported tannins to have medicinal properties, such as antimicrobial, antifungal, anti-inflammatory, antiviral, anti-diarrhoeal, anti-cancer activities as well as inhibition of HIV replication and human simplex virus by

interacting well with enzymes (proteins) within cell systems **(HASLAM, 1996; OKUDA, 2005; COWAN, 1999; GURIB-FAKIM, 2006)**. According to **VANDEN *et al*. 1985** tannins act principally by binding to the virus and/or the protein of the host cell membrane and therefore arrest absorption of the virus. The relevant physiological effects of tannins on herbivores is their ability to complex with proteinaceous material such as their complexation with metal ions, their antioxidant and radical scavenging activities and their ability to complex with other molecules including macromolecules such as proteins and polysaccharides **(HASLAM, 1988; HASLAM, 1996)**.

Twelve medicinal plants used by the Venda people against venereal diseases were evaluated for their levels of phenolic content including flavonoids, gallotannins and condensed tannins.

## 4.2. Materials and Methods

## 4.2.1. Plants collection and sample preparation

The plants were collected and prepared as described in **Section 2.3.1.** of **Chapter 2**.

## 4.2.2. Determination of phenolic contents

### 4.2.2.1. Preparation of extracts

Phenolic compounds were extracted from plant material as described by **MAKKAR (1999)** with modification. Dried plant samples (2 g) were extracted with 10 ml of 50% aqueous methanol by sonication in a cold water bath for 20 min. The extracts were then filtered under vacuum through Whatman No.1 filter paper and stored in dark sample bottles ready for analysis.

### 4.2.2.2. Determination of total phenolics

The amounts of total phenolics in plant samples were determined using the Folin Ciocalteu (Folin C.) method for total phenolics as described by **MAKKAR (1999)** with slight modification **(NDHLALA *et al*., 2007)**. Folin-C reagent (2N) (Sigma-Aldrich, USA) was made up with an equal volume of distilled water in a dark bottle, to obtain a 1N Folin-C reagent required for the assay. Standard gallic acid (Sigma-Aldrich, USA) solution was prepared in water at 0.1 mg/ml. Fifty microlitres of each plant methanolic extract were transferred into test tubes and 950 µl distilled water were added to make up to 1 ml, followed by 1 N Folin-C reagent (500 µl) and 2% sodium carbonate (2.5 ml). A blank that contained 50% aqueous methanol instead of plant extracts was also prepared. The test mixtures were incubated for 40 min at room temperature and absorbance was read at 725 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia). Each plant extract was assayed in triplicate. Total phenolic concentrations were expressed as gallic acid (GAE) equivalents.

### 4.2.2.3. The butanol-HCl assay for condensed tannins

Three millilitres of butanol-HCl reagent (95:5 v/v) were added to 500 µl of each methanolic extract, followed by 100 µl ferric reagent (2% ferric ammonium sulphate in 2N HCl). The test combination was mixed by vortexing and placed in a boiling water bath for 60 min. The absorbance was then read at 550 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia) against a blank prepared by mixing extract (500 µl) with butanol-HCl reagent (3 ml) and ferric reagent (100 µl), but without heating. Each extract had three replicate. Condensed tannin (% per dry matter) was calculated as equivalent amounts of leucocyanidins (LE) using the formula developed by **PORTER *et al*. (1986)**: Condensed tannin = (A550 nm × 78.26 × dilution factor)／(% dry matter)Where A550 nm is the absorbance of the sample at 550 nm and the dilution factor was 0.5 ml/volume of extract taken.

### 4.2.2.4. Rhodanine assay for gallotannins

The determination of hydrolysable tannin as gallotannins was done according to **MAKKAR (1999)** with modifications **(NDHLALA *et al*. 2007)**. Plant extracts (50 µl) were made up to 1 ml with distilled water. One hundred microlitres of 0.4 N sulphuric acid and 600 µl rhodanine were added to the diluted extracts. After 5 min at room temperature, 200 µl of 0.5 M potassium hydroxide were added followed by 4 ml distilled water after a further 2.5 min. The mixtures were left for a further 15 min at room temperature, after which the absorbance at 520 nm was read using a UV-visible spectrophotometer (Varian Cary 50, Australia) against a blank test tube that contained 50% aqueous methanol instead of sample. Each extract was evaluated in triplicate and gallotannin concentrations were expressed as gallic acid (GAE) equivalents.

### 4.2.2.5. Vanillin assay for flavonoids

The flavonoid content was evaluated as described by **HAGERMAN (2002)** with modifications. Plant extracts (50 µl) (in triplicate), were made up to 1 ml with methanol in test tubes before adding 2.5 ml methanolic-HCl (95:5 v/v) and 2.5 ml of 1% vanillin reagent (1g/100 ml in glacial acetic acid) in methanol. Similar preparations of a blank that contained methanol instead of plant extracts were made. After 20 min at 30 °C, absorbance at 500 nm was read using a UV-visible spectrophotometer (Varian Cary 50, Australia). A catechin standard curve was prepared from a freshly made 1 mg/ml catechin (Sigma-Aldrich) stock solution in methanol. The amount of flavonoids in the plant extracts were expressed as catechin equivalents (CTE), derived from the standard curve.

## 4.3. Results and discussion

### 4.3.1. Phenolic content

Phenolic compounds are ubiquitous in plants but the type of compound present varies considerably according to the phylum under consideration **(LATTANZIO *et al*., 2006)**. To protect plants against microbial invasion phenolic compounds form an integral part of the cell wall structure in the form of polymeric material such as lignin. Its role is to provide mechanical strength and to response to stress and pathogens **(GORSHKOVA *et al*., 2000)**. Plant phenolic compounds have been reported to have several medicinal properties such as antimicrobial, anti-flamatory, anti-diabetic, anti-carcinogenic, antineoplastic effects and antiviral activities **(HAVSTEEN, 1983; HARBORNE and BAXTER, 1999; KANDASWAMI *et al*., 2005; CUSHNIE and LAMB, 2005)**. Due to these pharmacological properties, plant phenolic compounds have gained increasing attention in both modern and traditional medicine as therapeutic compounds.

The total phenolic levels of the plant species in this study are shown in Table 7. Phenolic compounds amounting to 4.5% of the dry matter of plant extracts are considered to be significantly high and have beneficial effects such as antioxidant activity **(MAKKAR, 2003; MAKKAR *et al*., 2007)**. Almost all extracts contained total phenolic compounds greater than 5 mg/g for *A. chabaudii* roots and *A. digitata* bark which had lower levels (< 5 mg/ml). The same plants exhibited poor antimicrobial activities **(Chapter 2)**. However, *A. digitata* showed good COX and HIV-1 RT enzyme inhibition **(Chapter 3)** except for the water extracts towards both COX-1 and 2.

The highest levels of flavonoids (11.9 and 11.1 µg/g) were detected in *X. caffra* leaves and roots, *A. karroo* bark (7.7 µg/g) and *P. angolensis* bark (8.34 µg/g) with the lowest amounts being recorded in *A. chabaudii* roots, *A. digitata* bark, *B. speciosus* bark, leaves and stems and *P. angolensis* leaves Table 7. Flavonoids are known to have activity against cycloxygenases and lipoxygenases (LOX) as well as

HIV-1 RT **(HARBORNE and WILLIAMS, 2000; MATHÉE *et al*., 1999)**. *Ximenia caffra* leaves and *A. karroo* bark exhibited best COX-1 and HIV-1 RT enzyme activity in both leaves and roots of *X. caffra* **(Chapter 3)**. *P. angolensis* bark showed good enzyme inhibition against COX-1 and 2 and HIV-1 RT enzyme **(Chapter 3)**. Flavonoids have many beneficial health effects for human beings and several studies have shown that intake of flavonoids improves health and fights off chronic diseases **(PAN *et al*., 2010)**. It have been shown that they have anti-inflammatory activity through several action mechanisms which involve the reduction in the concentration of prostanoids and leukotrienes, through the inhibition of eicosanoid generating enzymes such as phospholipase, COX and LOX **(CALDER *et al*., 2009)**. The amount of flavonoids recorded in these plants may have probably contributed to the observed antimicrobial and enzyme inhibitory activity.

**BOJASE *et al*. (2002) and ERASTO *et al*. (2004)** isolated flavonoids from extracts of *B. speciosus* with strong antimicrobial activity against *E. coli*, *B. subtilis*, *S. aureus* and *Candida mycoderma* from different plant parts. However, in this study, low amounts of flavonoids were recorded in the leaves, bark and stems of the *B. speciosus*. This suggests that it could be quality rather than quantity that matters on the potency of phenolic compounds and also geographical area or where plants were collected.

Gallotannins exhibit some biological activities including antimicrobial, anticancer, anti-inflammatory and antiviral properties **(HAGERMAN *et al*., 1999; VAN MOLLE *et al*., 2000; FELDMAN *et al*., 2001; LEE *et al*., 2003)**. Amounts of gallotannin present in the investigated plant extracts are shown in Table 7. The highest amounts of gallotannin were detected in *E. capensis* bark (70.0 µg/g), *P. africanum* bark (60.1 µg/g) and *P. angolensis* bark (64.0 µg/g). Aqueous alcohols such as methanol are the best solvents to extract tannin **(MAKKAR, 1999)**. In this study, ethanol extracts of *E. capensis* bark showed good antibacterial activity as well as enzyme inhibition against COX-1. Ethanol extracts of *P. africanum* bark and *P. angolensis* bark showed good antibacterial activity as well as enzyme inhibition against COX-1 and HIV-1 RT enzymes **(Chapter 2 and Chapter 3)**. It is possible that gallotannins contributed to pharmacological activities of these plants. Very low levels of

gallotannins were detected in *A. chabaudii* roots, *A. digitata* bark and *B. speciosus* stems.

The highest amounts of condensed tannins were detected in *E. capensis* bark, *X. caffra* roots and *A. karroo* bark (0.470, 0.476 and 0.42 %) respectively Table 7. Condensed tannins were not detected in *B. speciosus* bark, *B. speciosus* stems, *P. angolensis* leaves and *P. angolensis* bark. According to **BRUNETON (1995)**, tannins enhance tissue regeneration in superficial wounds or burns, and have demonstrated antiseptic effects (antibacterial and antifungal). Therefore, the tannins present in *E. capensis* bark and *X. caffra* roots may have been responsible for the activity against some of the tested bacteria, *C. albicans* and the activity against some of the enzyme inhibitions reported in this study. Low amount of tannins and low antimicrobial activities and enzyme inhibition in some plants could be due to the interaction of tannins with protein and other biological macromolecules.

Tannin constituents reduce the mutagenicity of a number of mutagens and have anticarcinogenic, antimicrobial and anti-HIV activities, which are beneficial to humans **(LIAO and SHI, 2005)**. However, there is evidence that tannins, especially hydrolyzable tannins produce adverse effects in certain animals and humans such as perturbation of mineral adsorption from the intestinal canal, a decrease in body-weight, growth retardation and inhibition of digestive enzymes **(LIAO and SHI, 2005)**. In addition, some phenolic compounds have been reported to impact negatively on physiological effects such as neurological problems, reproductive failure, goitre and in more severe cases may lead to death when consumed in higher concentrations **(MAKKAR *et al*., 2007)**. However, high amounts of phenolic compounds in some plants may result in good pharmacological benefits. Examples include tannins *in E. capensis* bark and *A. karroo* bark as well as flavonoids in *A. karroo* and *X. caffra*. Different amount of phenolic content were detected in different plant part. According to **SULTANA *et al*. (2007)** phenolic compounds are not evenly distributed in plants part; they are present at elevated amounts in out parts of the fruits, leaves and barks. However in this study the amount of phenolic content in leaves and bark are present in Low/high amount depending on plant part, this could be due to variety differences that affect phenolic content such as environment factors.

It is a possibility that some phenolic groups detected as part of the total phenolics are not flavonoids, gallotannins or condensed tannin in nature but, other types such as simple phenylpropanoids, coumarins and benzoic acid derivatives. For example lower amounts of phenolic compounds are detected in *A. digitata* bark in all assays but the same plant exhibited good inhibition against COX and HIV-1 RT enzymes **(Chapter 3)**. The activities could be from other types of phenolic compounds such as terpenoids, apart from the tested ones **(SHAHAT, 2006)**.

In this study some plant extracts exhibited good activities but with low amounts of phenolic compounds such as *B. speciosus* and *A. digitata* bark. It could be possible that the activity observed could be from other secondary metabolites apart from tested phenolics.

Table 7: Total phenolics, flavonoid, gallotannin and condensed tannin concentrations of 12 medicinal plants used by the Venda people against venereal diseases

| Plant species | Plant part | Total phenolics (mgGAE/g)[a] | Flavonoids (µgCAE/g)[b] | Gallotannin (µgGAE/g)[a] | Condensed tannins (%LCE)[c] |
|---|---|---|---|---|---|
| *Aloe chabaudii* | Root | 2.20±0.14 | 0.19±0.03 | 4.21±0.54 | 0.09±0.04 |
| *Adansonia digitata* | Bark | 2.43±0.04 | 0.25±0.06 | 2.85±0.11 | 0.03±0.03 |
| *Acacia karroo* | Bark | 12.11±0.08 | 7.74±0.16 | 30.54±9.81 | 0.42±0.01 |
| *Bolusanthus speciosus* | Leaf | 10.49±0.11 | 0.20±0.01 | 5.84±0.42 | 0.04±0.02 |
| *Bolusanthus speciosus* | Bark | 8.69±0.56 | 0.04±0.00 | 4.50±1.50 | 0.00±0.00 |
| *Bolusanthus speciosus* | Stem | 10.44±0.67 | 0.25±0.02 | 3.07±0.27 | 0.00±0.00 |
| *Elephantorrhiza burkei* | Root | 11.97±0.06 | 3.71±0.47 | 31.59±1.34 | 0.38±0.01 |
| *Ekebergia capensis* | Leaf | 12.02±0.06 | 1.48±0.08 | 35.37±1.88 | 0.32±0.02 |
| *Ekebergia capensis* | Bark | 12.14±0.08 | 4.84±0.03 | 70.00±1.09 | 0.47±0.02 |

[a]Values expressed as gallic acid equivalent ( GAE) per gram of plant extracted;
[b]Values expressed as catechin equivalents (CTE) per gram of plant extracted ;
[c]Values expressed as percentage leucocyanidin equivalents (LCE) per gram plant extracted; All data represented as mean ± SE of three separate measurements

Table 7: continuation

| Plant species | Plant part | Total phenolics (mgGAE/g)[a] | Flavonoids (µgCAE/g)[b] | Gallotannin (µgGAE/g)[a] | Condensed tannins (%LCE)[c] |
|---|---|---|---|---|---|
| *Grewia occidentalis* | Root | 11.67±0.10 | 1.24±0.14 | 13.20±1.15 | 0.18±0.07 |
| *Osyris lanceolata* | Leaf | 11.90±0.07 | 3.94±0.23 | 19.18±3.21 | 0.30±0.02 |
| *Osyris lanceolata* | Root | 12.04±0.04 | 3.73±0.16 | 47.34±2.18 | 0.22±0.02 |
| *Peltophorum africanum* | Bark | 12.15±0.03 | 4.14±0.15 | 60.29±5.60 | 0.24±0.01 |
| *Pterocarpus angolensis* | Leaf | 8.85±0.10 | 0.29±0.00 | 7.11±0.88 | 0.00±0.00 |
| *Pterocarpus angolensis* | Bark | 12.07±0.08 | 8.34±0.13 | 64.00±9.09 | 0.00±0.00 |
| *Pappea capensis* | Leaf | 12.05±0.09 | 1.65±0.04 | 9.50±1.18 | 0.28±0.01 |
| *Ximenia caffra* | Leaf | 11.87±0.08 | 11.88±0.04 | 40.41±1.65 | 0.15±0.01 |
| *Ximenia caffra* | Root | 11.95±0.01 | 11.06±0.18 | 26.92±4.21 | 0.48±0.03 |

[a]Values expressed as gallic acid equivalent ( GAE) per gram of plant extracted;
[b]Values expressed as catechin equivalents (CTE) per gram of plant extracted ;
[c]Values expressed as percentage leucocyanidin equivalents (LCE) per gram plant extracted; All data represented as mean ± SE of three separate measurements
.

## 4.4. Conclusions

Plant secondary metabolites act as defense mechanisms against herbivores, pathogenic attack and nutrient deprivation. The observed antibacterial results of some species in this study correspond with high amounts of phenolic compounds for examples *X. caffra*. This suggests that there exists a correlation between high antimicrobial properties and high phenolic composition.

High levels of phenolics, flavonoids, gallotannins and condensed tannins were detected in *X. caffra* species. The high levels of phenolic compounds may have been responsible for the high antimicrobial activities and enzyme inhibition observed for extracts of *X. caffra. Adansonia digitata* bark and *A. chabaudii* exhibited poor antimicrobial activities as well as low amounts of phenolic compounds. *Bolusanthus speciosus* showed good pharmacological activities with low amounts of flavonoids, gallotannins and condensed tannins. In view of the biological activity observed in this study, assessment of other groups of phytochemical compounds and evaluating both *in vitro* and *in vivo* models is required.

# CHAPTER 5: Mutagenicity Evaluation of twelve Medicinal plants used by the Venda people against Venereal and related diseases

## 5.1. Introduction

Natural products research has an important role in the discovery of novel compounds for drug development **(NEWMAN *et al*., 2000)**. Many of these products are consumed with little or no evidence of pharmacological properties and/or safety knowledge **(CÂNDIDO-BACANI *et al*., 2011)**. Plants produce secondary metabolites as defense compounds against herbivores, micro-organisms and viruses. It is estimated that around 750 poisonous substances occur in more than 1000 species used **(WINK and VAN WYK, 2008)**. The plants should therefore be used with caution and with an appreciation of their toxicity risks **(CÂNDIDO-BACANI *et al*., 2011)**.

Traditional medicinal plants are reported to be effective in treating many infectious diseases. Despite the therapeutic advantages, some of the plants have been shown to be potentially toxic, carcinogenic and teratogenic **(AKINBORO and BAKARE, 2007; DE SÁ FERREIRA and VARGAS, 1999)**. Many plants used as food or in traditional medicine have been shown to have mutagenic effects by *in vitro* assays **(SCHIMMER *et al*., 1994, KASSIE *et al*., 1996 and DEMMA *et al*., 2009)**. It is important to assess the cytotoxic and mutagenic potential to ensure a relatively safe use of plant-derived medicines **(CAVALCANTI *et al*., 2006)**. This assessment can be used for potential chemotherapeutic drugs and as a measure of safety for the continued long term use of medicinal plants **(VERSCHAEVE *et al*., 2004)**.

Toxicity occurs when a substance exerts negative effects on an organism and its metabolism. In animals and humans, it interferes with central functions of an organism. For example, neurotoxins affect the brain and the nervous system, while cytotoxins and metabolic poisons disturb the liver, kidneys, heart or the respiratory system **(WINK and VAN WYK, 2008)**.

### 5.1.1. Mutagenicity

Mutagenicity is a result of substances that induce genetic mutations leading to alteration or loss of genes or chromosomes **(WINK and VAN WYK, 2008)**. Mutagens include physical agents such as ultra violet (UV) and X-rays which cause the deletion of nucleotides. Mutagens produce a variety of lesions in DNA, as well as strand break, base damage and dimerisation of bases. A range of diverse environmental, industrial, dietary and natural chemicals are capable of inducing mutations and genotoxic effects **(BHATTACHARYA, 2011)**. Gene mutations that cause changes in growth requirements can easily be detected in bacteria, while chromosome damage in mammalian cells is typically measured by observing the cell‘s chromosomes under magnification for breaks or rearrangements **(MORTELMANS and ZEIGER, 2000)**.

### 5.1.2. Effects of mutagenicity in humans

Chemicals that are capable of inducing mutations can potentially damage the germ line leading to fertility problems, cancer and even mutations in future generations. DNA damage by environmental mutagens can be a cause of disability in many animals, including humans **(CARIÑO-CORTÉS *et al*., 2007)**. Mutation events are involved in several degenerative diseases, such as cancer and arteriosclerosis as well as aging, and genetic defects in the offspring **(DE FLORA, 1998; CARIÑO-CORTÉS *et al*., 2007)**. This concern has driven most of the mutagenicity testing programs. Identification of the substances that are capable of inducing mutations has become an important procedure in safety assessments **(MORTELMANS and ZEIGER, 2000)**.

### 5.1.3. Ames test

The Ames test (*Salmonella typhimurium*/microsome assay) is widely used as a short-term bacterial assay for identifying substances that can produce genetic damage leading to mutations **(MORTELMANS and ZEIGER, 2000)**. It is also used

worldwide by regulatory agencies as an initial screen to determine the mutagenic potential of new chemicals and drugs **(MORTELMANS and ZEIGER, 2000; HAKURA *et al*., 2005)**. This test uses several *Salmonella* strains with pre-existing mutations that leave the bacteria unable to synthesize the essential amino acid histidine and therefore are unable to grow and form colonies in the absence of this amino acid. New mutations on the pre-existing mutations can restore the gene function and allow the cells to synthesize histidine. Newly mutated cells can grow in the absence of histidine and form colonies **(MORTELMANS and ZEIGER, 2000)**. The assay therefore, utilizes this principle to assess if the drugs/extracts are capable of reverting the mutations to restore the normal functions and colony formation in these bacterial strains.

### 5.1.3.1. Oxidative metabolism

Many carcinogens such as aromatic amines or polycyclic aromatic hydrocarbons remain inactive until they are enzymatically transformed to an electrophilic species that is capable of covalently binding to DNA leading to mutation; thus, metabolic activation is considered to be a critical step in mutation **(AMES *et al*., 1973; MORTELMANS and ZEIGER, 2000)**. The cytochrome-based P450 metabolic oxidation system, present mainly in the liver and to a lesser extent in the lung and kidneys of humans and lower animals, is capable of metabolizing a large number of these chemicals to DNA-reactive, electrophilic forms. Some of the intermediate metabolites are potent mutagens in the Ames *Salmonella* assay. Since bacteria do not have this metabolic capability, an exogenous mammalian organ (such as liver or kidney) activation system needs to be added to the Petri plate together with the test chemical and the bacteria **(MORTELMANS and ZEIGER, 2000)**. A metabolic activation system usually consists of a 9000 x g supernatant fraction of rat liver homogenate (S-9 microsomal fraction) **(MORTELMANS and ZEIGER, 2000)**.

Studies on the properties of phytotherapeutic agents, including studies on mutagenic and anti-mutagenic activities correlated with the presence of certain substances found in herbal medicine, has steadily increased, covering groups of flavonoids, tannins and alkaloids **(RIETJENS *et al*., 2005; HORN and VARGAS, 2008)**. Research by **CARDOSO *et al*. (2006), DÉCIGA-CAMPOS *et al*. (2007) and MOHD-**

**FUAT *et al*. (2007)** have shown that a lot of plants used in traditional medicine have *in vitro* mutagenic or toxic and carcinogenic properties. According to **VERSCHAEVE and VAN STADEN (2008)**, it is important to screen medicinal plants for their mutagenic potency. Therefore, in this study, the mutagenic effect of selected medicinal plants that are used against venereal and related diseases by Vha-Venda people was determined using the Ames test.

## 5.2. Materials and Methods

Mutagenicity was tested using the *Salmonella* microsome assay as described by **MARON and AMES (1983)** with modification by **MORTELMANS and ZEIGER (2000)**. Dichloromethane, PET, EtOH and water extracts were tested for their potential mutagenic properties using the plate-incorporation procedure with *Salmonella typhimurium* tester strain TA98 with and without metabolic activation. Overnight bacterial tester strains were cultured in 10 ml Oxoid nutrient broth No. 2 for 16 h at 37 °C to obtain a density of 1-2 x $10^9$ colony forming units (CFU/ml). The metabolic activation mixture (S-9 mix) was prepared freshly before the assay and kept on ice throughout the assay procedure. The S9 mix consisted of 5% (v/v) S9 fraction (Sigma-Aldrich, Co., St Louis) pooled from Sprague-Dawley male rats in mixed enzymic cofactors containing NADP. At the beginning of the assay, top agar supplemented with 0.5 mM histidine and biotin was melted and kept in a 50 °C water bath. To sterile glass tubes, in triplicate, 100 µl of three dilutions (50, 500, 5000 µg/ml) per sample were added, followed by 500 µl phosphate buffer (0.1 mM, pH 7.4) or S9 mix. To the mixture, 100 µl of the overnight bacterial culture were added followed by 2 ml of the melted top agar. The contents of the tubes were then mixed and poured onto labelled minimal agar plates (minimal agar is agar that contains minimum nutrients such as glucose). As soon as the top agar had hardened (2-3 min), the plates were inverted and incubated at 37 °C for 48 h. The colonies were then counted with the aid of a binocular microscope. The assay was repeated twice for TA98 bacterial strain and the results were expressed as the mean (± standard error) number of revertant colonies per plate. 4-Nitroquinoline-N-oxide (4NQO) (2 µg/plate) was used as a positive control for the assay without metabolic activation while 2-aminoanthracene (2-AA) (2 µg/plate) was used where the assay was carried

out with S9 metabolic activation. Sterile distilled water was used as a negative control in both assays. The test extracts/compound was classified as a ‗mutagen' if the results satisfied two criteria: (1) a dose dependent increase in the number of revertants is observed and (2) the number of $His^+$ (histidine) revertants is equal or greater than two times that of the negative control.

## 5.3. Results and discussion

The results of the evaluated medicinal plants are shown in Table 8. The results represent the number of $His^+$ revertants with and without $S9^+$ against *Salmonella typhimurium* tester strains TA98 in three dilutions. TA98 strains have a -1 frameshift mutation at *hisD3052* which affects the reading frame of a repetitive base pair –C-G-C-G-C-G-C-G- sequence. The reversion of the *his*D*3052* mutation back to the wild-type state is induced by various frameshift mutagens such as 2-nitrofluorene and various aromatic nitroso derivatives of amine carcinogens **(MORTELMANS and ZEIGER, 2000)**.

Ames test without S9 metabolic activation detects direct mutagens. In this study, all plants were non-mutagenic towards TA98 without S9 activation in both organic and inorganic extracts. The numbers of revertants were less than that of the negative control. However, if the number of revertant colonies is far below the negative control (spontaneous reversion) it is classified as toxic **(NDHLALA *et al*., 2010a)**.

Ames test with $S9^+$ metabolic activation allows the detection of indirect mutagens. All the samples were not mutagenic after S9 activation. The only exceptions were *E. burkei* roots and *E. capensis* bark that showed indirect mutagenic effects toward TA98 in water extracts. *Elephantorrhiza burkei* roots induce indirect mutagenic effects at 500 µg/ml and 50 µg/ml (53.5 and 58.0 µg/ml respectively). *Ekebergia capensis* bark induced 50.0 revertant colonies at 500 µg/ml and 50 µg/ml. However, there is no dose dependent increase; this does not satisfy the first condition for a mutagen which states that an extracts/compound should have a dose dependent increase in colonies. These plant extracts showed weak mutagenicity. Therefore it is important to increase the concentration to check if there is increase in the number of

revertants colonies which confirm mutagenicity on these plants. *Elephantorrhiza burkei* roots and *E. capensis* bark could contain some frameshif mutagens.

The water extract of *E. burkei* roots and *E. capensis* bark exhibited good antimicrobial and enzyme inhibition activities. The highest amounts of condensed tannins and gallotannins were recorded in *E. capensis* bark. *Ekebergia capensis* contain bioactive chemical compounds such as glycosides, polyphenols, tannins, triterpenes and saponins **(SEWRAM *et al*., 2000)**. Tannins, especially hydrolysable tannins produce adverse effects in certain animals and humans **(LIAO and SHI, 2005)**. Mutagenic effects in these plants could be from phenolic compounds. However, some tannin constituents reduce the mutagenicity of a number of mutagens and have anticarcinogenic, antimicrobial and anti-HIV activities, which are beneficial for humans.

The tested medicinal plants are used for the treatment of different ailments including venereal diseases and have shown promising biological activities in different studies. Therefore further toxicity tests need to be done as the Ames test with $S9^{+}$ has reveals that these plant extracts could produce a variety of lesions in DNA.

Table 8: Number of $His^+$ revertants in *Salmonella typhimurium* strains TA98 (preparations with and without S9 metabolic activation) of 12 medicinal plants used by the Venda people against venereal diseases

| | | | Number of $His^+$ revertants | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | PET | | DCM | | EtOH | | Water | |
| Plant species | Plant parts | µg/ml | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ |
| *Aloe chabaudii* | Root | 5000 | 16.7±5.2 | 18.0±5.0 | 12.0±1.0 | 20.5±3.5 | 14.3±0.7 | 39.5±8.5 | 15.0±0.0 | 29.0±7.0 |
| | | 500 | 18.7±2.0 | 22.0±1.0 | 17.0±3.2 | 24.0±4.0 | 16.0±1.0 | 27.0±6.0 | 11.0±0.0 | 28.0±2.0 |
| | | 50 | 15.3±0.9 | 23.0±0.0 | 16.3±0.7 | 19.0±5.0 | 21.0±1.0 | 29.0±9.0 | 15.3±1.7 | 28.5±1.5 |
| *Adansonia digitata* | Bark | 5000 | 13.7±0.3 | 20.5±1.5 | 18.0±2.0 | 22.5±3.5 | 16.3±0.3 | 15.5±0.5 | 13.0±1.0 | 32.5±3.5 |
| | | 500 | 16.3±1.3 | 24.5±0.5 | 14.7±1.2 | 27.5±3.5 | 17.0±3.2 | 16.0±1.0 | 15.3±1.5 | 24.5±2.5 |
| | | 50 | 16.3±1.9 | 22.5±1.5 | 17.0±1.5 | 26. 5±4.5 | 19.0±2.1 | 18.0±0.0 | 13.0±1.0 | 29.0±1.0 |
| *Acacia karroo* | Bark | 5000 | 17.0±2.1 | 22.5±2.5 | 15.7±1.3 | 26.5±6.5 | 19.7±2.4 | 12.5±1.5 | 14.3±1.8 | 27.0±6.0 |
| | | 500 | 23.0±3.2 | 20.5±4.5 | 16.3±1.7 | 21.5±1.5 | 18.0±1.2 | 15.0±2.0 | 12.0±1.0 | 31.0±1.0 |
| | | 50 | 19.3±3.0 | 30.0±2.0 | 14.3±1.3 | 31.5±6.5 | 15.3±1.3 | 17.0±1.0 | 14.7±1.3 | 31.0±0.0 |
| *Bolusanthus speciosus* | Leaf | 5000 | 13.3±0.3 | 22.0±0.0 | 15.3±0.3 | 24.0±2.0 | 14.3±1.3 | 18.0±4.0 | 14.3±1.7 | 31.0±1.0 |
| | | 500 | 16.3±3.8 | 21.5±0.5 | 13.0±1.0 | 29.0±4.0 | 16.7±0.3 | 14.5±1.5 | 11.3±0.3 | 20.0±5.0 |
| | | 50 | 15.3±2.7 | 20.0±1.0 | 12.7±1.9 | 22.0±1.0 | 17.0±0.0 | 17.5±1.5 | 15.0±0.0 | 29.0±0.0 |

Number of His+ revertants/plate: mean values of three triplicates, the assay was repeated two times
$S9^-$ refers to assay without metabolic activation; $S9^+$ refers to assay with metabolic activation
4-NQO; 4-nitroquinoline-oxide, positive control for the $S9^-$ assays. 2-AA; 2-aminoathracene, positive control for $S9^+$ assays

Table 8: Continuation

| | | | Number of $His^+$ revertants | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | PET | | DCM | | EtOH | | Water | |
| Plant species | Plant parts | µg/ml | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ |
| *Bolusanthus speciosus* | Bark | 5000 | 12.0±1.0 | 21.5±0.5 | 15.7±1.3 | 25.0±3.0 | 19.0±3.5 | 19.0±1.0 | 14.3±1.7 | 23.0±1.0 |
| | | 500 | 16.0±2.1 | 23.5±1.5 | 17.7±0.3 | 24.5±2.5 | 17.3±0.3 | 11.5±0.5 | 11. 7±0.3 | 22.5±2.5 |
| | | 50 | 14.7±1.7 | 20.0±0.0 | 15.3±1.9 | 29.0±6.0 | 19.0±0.0 | 15.5±0.5 | 15.0±0.0 | 28.5±3.5 |
| *Bolusanthus speciosus* | Stem | 5000 | 14.0±1.2 | 17.0±4.0 | 15.3±0.3 | 24.0±6.0 | 23.0±1.0 | 23.5±5.5 | 15.7±0.7 | 43.5±4.5 |
| | | 500 | 16.0±1.0 | 29.0±1.0 | 15.0±2.6 | 24.0±2.0 | 18.0±1.5 | 35.0±5.0 | 14.3±2.3 | 23.0±2.0 |
| | | 50 | 17.0±1.0 | 25.0±1.0 | 17.3±0.3 | 25.5±1.5 | 15.7±0.3 | 19.0±1.0 | 12.0±0.0 | 42.5±0.5 |
| *Elephantorrhiza burkei* | Root | 5000 | 16.3±0.3 | 31.0±2.0 | 16.7±0.3 | 16.0±1.0 | 16.0±0.6 | 11.5±2.5 | 14.7±1.5 | **53.5±13.5** |
| | | 500 | 15.0±2.0 | 25.0±7.0 | 15.7±2.3 | 21.0±0.0 | 17.7±1.2 | 16.5±1.5 | 15.0±1.7 | 24.0±0.0 |
| | | 50 | 15.3±1.3 | 25.5±0.5 | 17.0±3.5 | 22.5±1.5 | 24.7±1.5 | 15.5±0.5 | 13.0±2.0 | **58.0±8.0** |
| *Ekebergia capensis* | Leaf | 5000 | 14.3±1.8 | 25.5±1.5 | 15.0±1.2 | 17.5±3.5 | 15.7±2.4 | 15.5±1.5 | 13.7±1.8 | 40.0±7.0 |
| | | 500 | 19.7±1.2 | 23.0±0.0 | 14.3±0.3 | 22.5±2.5 | 16.7±2.2 | 13.0±3.0 | 15.0±3.0 | 48.0±6.0 |
| | | 50 | 14.7±1.9 | 22.5±0.5 | 11.3±0.3 | 19.5±2.5 | 13.7±2.3 | 16.5±0.5 | 12.7±0.7 | 39.0±6.0 |

Number of His+ revertants/plate: mean values of three triplicates, the assay was repeated two times
$S9^-$ refers to assay without metabolic activation; $S9^+$ refers to assay with metabolic activation
4-NQO; 4-nitroquinoline-oxide, positive control for the $S9^-$ assays. 2-AA; 2-aminoathracene, positive control for $S9^+$ assays

Table 8: Continuation

| | | | Number of His$^+$ revertants | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | PET | | DCM | | EtOH | | Water | |
| Plant species | Plant parts | µg/ml | S9$^-$ | S9$^+$ | S9$^-$ | S9$^+$ | S9$^-$ | S9$^+$ | S9$^-$ | S9$^+$ |
| *Ekebergia capensis* | Bark | 5000 | 18.0±2.9 | 20.0±3.0 | 14.3±3.0 | 22.0±6.0 | 9.3±0.7 | 25.0±1.0 | 16.3±2.7 | 44.5±6.5 |
| | | 500 | 22.3±1.3 | 26.5±6.5 | 16.7±0.3 | 23.0±2.0 | 13.0±2.0 | 35.5±0.5 | 12.0±1.2 | **50.0±3.0** |
| | | 50 | 11.0±1.0 | 23.0±1.0 | 11.7±0.3 | 18.0±3.0 | 10.0±0.0 | 22.0±0.0 | 14.3±1.7 | **50.0±7.0** |
| *Grewia occidentalis* | Root | 5000 | 20.7±3.0 | 22.5±2.5 | 20.7±1.8 | 29.5±1.5 | 16.7±1.3 | 14.5±0.5 | 10.3±0.9 | 46.0±2.0 |
| | | 500 | 20.3±3.5 | 21.5±0.5 | 20.0±1.5 | 11.0±0.0 | 19.0±6.5 | 18.0±1.0 | 13.3±1.8 | 23.0±2.0 |
| | | 50 | 21.3±3.3 | 25.5±3.5 | 13.7±1.8 | 21.0±0.0 | 15.7±0.9 | 16.0±0.0 | 12.0±1.0 | 43.5±3.5 |
| *Osyris lanceolata* | Leaf | 5000 | 13.7±2.3 | 26.0±5.0 | 15.7±1.8 | 21.5±13.5 | 17.3±1.7 | 17.0±3.0 | 13.0±2.0 | 39.0±5.0 |
| | | 500 | 18.7±0.7 | 22.5±3.5 | 16.0±1.7 | 17.0±3.0 | 15.0±2.1 | 20.0±4.0 | 14.3±1.3 | 40.0±9.0 |
| | | 50 | 21.0±2.1 | 29.5±0.5 | 19.3±0.7 | 20.5±0.5 | 17.7±0.3 | 15.0±1.0 | 13.7±2.7 | 41.0±3.0 |
| *Osyris lanceolata* | Root | 5000 | 20.3±2.8 | 22.0±0.0 | 16.0±4.5 | 17.0±2.0 | 13.3±3.3 | 16.0±3.0 | 12.3±0.9 | 38.5±0.5 |
| | | 500 | 17.3±2.8 | 17.0±3.0 | 18.0±3.2 | 15.0±0.0 | 15.7±0.3 | 16. 0±4.0 | 14.3±1.2 | 46.5±5.5 |
| | | 50 | 21.3±2.3 | 15.0±2.0 | 15.0±1.2 | 21.5±0.5 | 13.0±0.0 | 18.0±2.0 | 13.3±2.0 | 42.5±0.5 |

Number of His+ revertants/plate: mean values of three triplicates, the assay was repeated two times
S9$^-$ refers to assay without metabolic activation; S9$^+$ refers to assay with metabolic activation
4-NQO; 4-nitroquinoline-oxide, positive control for the S9$^-$ assays. 2-AA; 2-aminoathracene, positive control for S9$^+$ assays

Table 8: Continuation

| | | | Number of $His^+$ revertants | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | PET | | DCM | | EtOH | | Water | |
| Plant species | Plant parts | µg/ml | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ |
| *Peltophorum africanum* | Bark | 5000 | 21.7±4.7 | 25.0±3.0 | 12.3±1.3 | 24.0±5.0 | 18.0±2.3 | 22.5±1.5 | 14.3±0.7 | 44.5±2.5 |
| | | 500 | 15.7±1.5 | 18.5±1.5 | 11.0±0.0 | 16.0±1.0 | 16.7±2.4 | 39.0±3.0 | 16.0±3.1 | 48.0±3.0 |
| | | 50 | 17.3±3.3 | 23. 0±8.0 | 19.3±1.2 | 18.5±2.5 | 18.0±1.0 | 26.0±5.0 | 12.7±1.3 | 48.5±4.5 |
| *Pterocarpus angolensis* | Leaf | 5000 | 19.3±1.3 | 17.0±0.0 | 13.7±0.3 | 20. 0±2.0 | 18.7±3.8 | 24.5±0.5 | 10.7±1.3 | 36.0±1.0 |
| | | 500 | 19.0±3.2 | 20.0±4.0 | 13.7±2.3 | 22.5±1.5 | 14.0±0.0 | 39.0±6.0 | 12.0±0.6 | 45.5±1.5 |
| | | 50 | 16.0±2.1 | 29.0±1.0 | 15.0±1.0 | 21.0±1.0 | 16.0±2.0 | 35.0±2.0 | 20.0±0.0 | 34.5±5.5 |
| *Pterocarpus angolensis* | Bark | 5000 | 15.7±0.9 | 21.5±1.5 | 15.0±1.2 | 20.5±0.5 | 16.0±0.0 | 22.0±0.0 | 14.3±0.3 | 42.0±4.0 |
| | | 500 | 13.3±1.3 | 20.5±0.5 | 13.3±2.0 | 24.5±3.5 | 13.7±1.8 | 12. 0±0.0 | 8.7±0.3 | 30.0±3.0 |
| | | 50 | 18.7±1.5 | 20.5±0.5 | 13.0±1.7 | 23.0±0.0 | 19.3±1.3 | 30.5±2.5 | 12.0±0.0 | 36.0±4.0 |
| *Pappea capensis* | Leaf | 5000 | 16.7±1.7 | 20.5±2.5 | 15.3±0.9 | 21.5±3.5 | 10.0±2.1 | 21.0±1.0 | 16.0±2.0 | 31.5±4.5 |
| | | 500 | 16.7±1.7 | 18.5±4.5 | 12.7±1.3 | 23.0±0.0 | 16.7±1.2 | 20.0±6.0 | 12.0±0.0 | 38.5±8.5 |
| | | 50 | 17.3±2.3 | 19.0±2.0 | 23.0±2.5 | 23.0±2.0 | 17.0±1.5 | 26.0±8.0 | 18.0±3.0 | 46.0±5.0 |

Number of His+ revertants/plate: mean values of three triplicates, the assay was repeated two times
$S9^-$ refers to assay without metabolic activation; $S9^+$ refers to assay with metabolic activation
4-NQO; 4-nitroquinoline-oxide, positive control for the $S9^-$ assays. 2-AA; 2-aminoathracene, positive control for $S9^+$ assays

Table 8: Continuation

| Number of $His^+$ revertants | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | PET | | DCM | | EtOH | | Water | |
| Plant species | Plant parts | µg/ml | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ | $S9^-$ | $S9^+$ |
| *Ximenia caffra* | Leaf | 5000 | 19.0±3.6 | 22.0±1.0 | 16.3±2.3 | 22.5±0.5 | 12.7±0.9 | 21.0±1.0 | 13.3±0.9 | 47.0±3.0 |
| | | 500 | 17.7±3.8 | 22.0±2.0 | 12.7±1.7 | 22.0±2.0 | 12.3±2.2 | 24.0±0.0 | 10.7±0.3 | 26.0±4.0 |
| | | 50 | 20.7±1.2 | 23.0±1.0 | 17.3±0.7 | 22.5±2.5 | 18.7±3.2 | 27.5±0.5 | 12.7±1.8 | 42.5±3.5 |
| *Ximenia caffra* | Root | 5000 | 20.0±1.2 | 24.5±0.5 | 15.3±0.7 | 21.0±0.0 | 13.0±2.5 | 19.5±0.5 | 14.0±2.0 | 41.0±9.0 |
| | | 500 | 21.0±2.5 | 18.5±4.5 | 20.7±1.9 | 22.5±0.5 | 15.0±1.5 | 29.0 ±14.0 | 11.3±0.3 | 36.0±6.0 |
| | | 50 | 17.3±0.3 | 25.5±6.5 | 19.0±1.2 | 24.0±1.0 | 19.3±2.3 | 37.5±5.5 | 15.0±2.0 | 42.5±3.5 |
| 4NQO | | 2 | 273.0±13.4 | | | | | 4NQO | 288.0±13.0 | |
| 2-AA | | 2 | | 396.3±13.4 | | | | 2-AA | | 391.3±2.3 |
| Water (-ve cont) | | | | | | | | Water (-ve cont) | 19.3±1.9 | 24.3±3.3 |
| DMSO (-ve cont) | | | 18.3±2.4 | 22.0±4.2 | | | | DMSO (-vecont) | | |

Number of His+ revertants/plate: mean values of three triplicates, the assay was repeated two times
$S9^-$ refers to assay without metabolic activation; $S9^+$ refers to assay with metabolic activation
4-NQO; 4-nitroquinoline-oxide, positive control for the $S9^-$ assays. 2-AA; 2-aminoathracene, positive control for $S9^+$ assays

## 5.4. Conclusions

When it comes to the use of plants by humans, Vha-Venda people are vigilant as to what to use. For example, plants with milky latex, unpleasant smells and those that cause irritation to eyes or the nose when burnt as firewood are not used for medicinal purposes because they are considered poisonous **(MABOGO, 1990)**. However, mutagenicity effects of plants are not easily noticeable in human health, but the long-term effect such as cancer can manifest.

Results obtained from this study, support the safe usage of the selected medicinal plants by Vha-Venda people. However, some carcinogenic chemicals are biologically inactive unless they are metabolized to active forms. In this study mutagenic effect was observed after metabolic activation of water extracts of *E. burkei* roots and *E. capensis* bark. The rest of the plant extracts showed no mutagenic effects in both organic and inorganic extracts or the effect could be hidden due to antagonistic effect of other compounds contained in the extract. None of the plant extracts showed clear mutagenic effects when extracted with organic and inorganic solvents.

According to **VERSCHAEVE and VAN STADEN (2008)** plants that show clear mutagenic properties should be considered as potentially unsafe and they require further testing before they can be recommended for use. However, mutagenicity can also be useful as an anticancer tool, because most anticancer drugs are mutagenic: for example the spindle-disturbing substances taxol and vinblastine. Mutagenic potential in plants can be considered interesting for therapeutic use and merit further in depth investigations of their pharmacological properties. These plants can be therefore being considered as potentially safe, but further *in vivo* testing is required such as the determination of cellular toxicity.

# CHAPTER 6: Isolation and Tentative Identification of an Active Compound from leaves of *Ximenia caffra* Sond (Olacaceae)

## 6.1. Introduction

Plants have formed the basis of sophisticated traditional medicinal practices that have been used for thousands of years **(GURIB-FAKIM, 2006)**. Plant based medicines were initially dispensed in the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations **(JACHAK and SAKLANI, 2007)**. The use of some plants as medicine has resulted in the isolation of active compounds. Today plants continue to provide us with new chemical entitities (lead molecules) for the development of drugs against various challenges, including cancer, HIV/AIDS, malaria, Alzheimer‘s disease, venereal disease and general pain **(JACHAK and SAKLANI, 2007)**.

### 6.1.1. Distribution and morphology of *Ximenia caffra*

*Ximenia caffra* is a shrub or small tree which grow up to 6 m tall with a shapeless and untidy crown sparsely branched. Branches and twigs are armed with stout axillary spines and are glabrous or dense tomentose. Leaves are alternate, dark green, folded upwards along the midrib and margin entire. Flowers in axillary clusters are small and greenish white. Fruits are oval in shape, bright red and yellow with small white spots **(VAN WYK *et al*., 2008)**.

*Ximenia caffra* is a southern African species; distributed across Tanzania, Zambia, Zimbabwe, Botswana, Namibia, Mozambique and South Africa **(CHIVANDI *et al*., 2008)**. The tree is found in woodlands, grasslands and on rocky outcrops as well as on termites mounds.

**Figure 5.** *Ximenia caffra* Sond leaves. Inset shows the fruit of *X. caffra* **(BALOYI and REYNOLDS, 2004)**

## 6.1.2. Traditional uses of *Ximenia caffra*

*Ximenia caffra* has a wide spectrum of medicinal uses. It is used to treat different diseases by several tribes in southern Africa including the Zulu, Venda, Ndebele and Shona cultures. Roots, bark and leaves of this plant are used for numerous ailments, including infertility, venereal disease, abdominal pain, cramps, diarrhoea, rheumatism, fever, ancylostomiasis and wounds. Leaf infusions are used for both humans and animals as eye lotions. The root infusion is used as a cure for bilharzia. The powdered root is added to soup or beer and taken as an aphrodisiac. Porridge from root decoctions is eaten to treat nausea by pregnant women and the decoction is used during early stages of pregnancy to prevent the infant from being born crippled **(VAN WYK *et al*., 2008; ROODT, 1998)**.

The fruits are eaten fresh or dried **(ROODT, 1998)**. The fruits are rich in vitamin C (27%) and have a high protein value **(ROODT, 1998)**. The kernel is edible and is also used to make jam and jelly. The seed contains 65% of a yellow, viscous, non-drying oil **(CHIVANDI *et al*., 2008)**. The oil is used as biofuel in lamps and to soften leather clothing, leather boots and skirts **(VENTER and VENTER, 1996; VAN WYK and GERICKE, 2003)**. Fruit juice is applied to septic sores and the pustules from chicken pox **(VAN WYK *et al.,* 2008)**. **VAN WYK and GERICKE (2003)** indicated that the seed contains tannins.

### 6.1.3. Ethnopharmacology of *Ximenia caffra*

Extracts from the plant are reported to possess antibacterial activity against *Staphylococcus aureus*, *Vibro cholera*, *Shigella dysentery*, *Shigella sonnei*, *Shigella flexneri, Shigella boydii*, *Pseudomonas aeruginosa* and *Helicobacter pylori* **(FABRY *et al.,* 1996; FABRY *et al.,* 1998; MATHABE *et al*., 2006)** as well as antifungal activity against *Candida albicans* **(SAMIE *et al*., 2005)**. In Zambia and South Africa the plant is used to treat VDs **(MABOGO, 1990; NDUBANI and HÖJER, 1999)**. *Ximenia caffra* contains simple phenolic compounds such as coumaric, vanillic and ferulic acids which have often been implicated in antibacterial activity **(NDHLALA *et al*., 2008b)**. An unsaturated acid ximeninic acid has been isolated from the kernel oil **(ROODT, 1998)**.

This study was aimed at isolating and identifying active compounds using bioassay-guided fractionation of *X. caffra* leaf extracts activity against *Neisseria gonorrhoeae*.

## 6.2. Materials and Methods

*Ximenia caffra* leaves were collected and dried as described in **Section 2.2.1., Chapter 2**. Voucher specimen number Table 2 in Chapter 1. The dried powdered leaf material (500g) was extracted with 80% ethanol by sonication for 1h, and left overnight with constant stirring using a magnetic stirrer. The extract was filtered through a Büchner funnel and Whatman No.1 filter paper. The leaf residue was extracted further three times with 80% ethanol. The filtered extracts were combined and concentrated under vacuum using a rotary evaporator (Rotavapor-R, Büchi, Switzerland) at 30 °C. The weight of the dried residue was 54 g.

### 6.2.1. Bioassay guided fractionation of aqueous ethanol extract of *Ximenia caffra* leaves

Dried 80 % ethanolic extract was resuspended in 500 ml water: methanol (9:1) and was partitioned sequentially with hexane (500ml), dichloromethane (500ml) and ethyl acetate (500ml). The volume of aqueous fraction was reduced and re-diluted with water at each stage of extraction. The four fractions (hexane, DCM, ethyl acetate and aqueous) were dried using a rotary evaporator and subsequently assayed for antigonococal activity.

### 6.2.2. Antigonococcal assay

Gonococcal activity of the four fractions was determined by disc diffusion **(RINGERTZ *et al.,* 1991; PUTNAM *et al.,* 1992)** as described in **Section 2.2.3.2., Chapter 2**. Except that the fractions were resuspended in 80% ethanol to a concentration of 2 mg/ml. The dichloromethane fraction was the most active 52%.

### 6.2.3. Gravity column chromatography

The DCM fraction was further fractionated by gravity column chromatography (Merck 9385 (silica gel), 130 g, 2.5 cm x 73 cm) using hexane: ethyl acetate (1:0 – 0:1) and subsequently ethyl acetate: methanol (1:0 – 0:1) step gradients. A fraction collector

(Gilson FC 203B) was used to collect fractions of approximately 10 ml each. These fractions were spotted onto TLC plate and developed in hexane: ethyl acetate (1:1). Fractions with similar TLC profiles were pooled together and then assayed for antigonococal activity (Fractions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12).

### 6.2.4. Preparative Thin Layer Chromatography

Fractions obtained after a repeated column chromatography were purified using preparative TLC (Merck glass plates, 20 x 20 cm, silica gel $F_{254}$, and 0. 25 cm thickness), using hexane: ethyl acetate (1:1) as the solvent system about 34 mg of the most active fraction was applied to TLC. It yielded the active compound. The flow diagram in Figure 6 outlines the procedure followed.

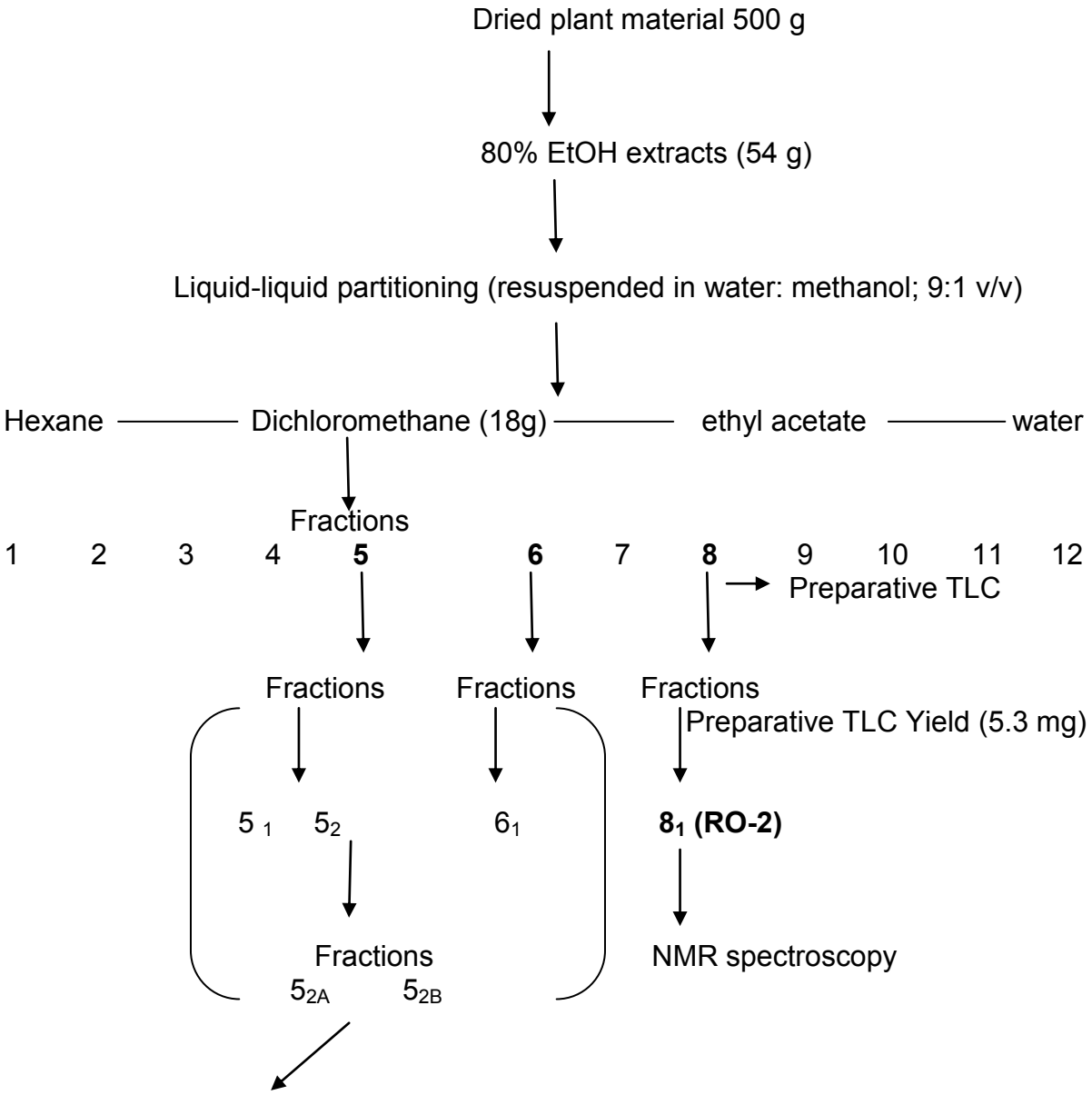



**Figure 6.** Flow chart outlining the isolation procedure for antigonococcal compounds using dried *X. caffra* leaves. For NMR spectroscopy $8_1$ was labelled RO-2

## 6.2.5. Identification of Purified Active Compound

Nuclear Magnetic Resonance Spectroscopy (NMR) was performed at the School of Chemical and Physical Sciences, University of KwaZulu Natal, Pietermaritzburg, to determine the structure of the isolated compounds

Melting points (uncorrected) were measured on a Gallenkamp melting point apparatus. Optical rotations were determined on a Perkin-Elmer 241 polarimeter installed with a $\lambda_{589}$ sodium lamp. IR spectra were measured on a Bio-Rad FTS-40 series spectrometer in dry film. Electron impact mass spectroscopy (EIMS) were run on a Micromass Quattro Ultima spectrometer fitted with a direct injection probe (DIP) with ionization energy set at 70 eV and high resolution mass spectroscopy (HRMS) (EI) were performed with a Micromass Q-Tof Ultima spectrometer. $^{1}H$, $^{13}C$ NMR, distortionless enhancement by polarization transfer (DEPT), correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear spin quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra were recorded on a Bruker AV400 spectrometer in DMSO-$d_6$, chemical shifts are reported in units of δ (ppm) and coupling constants (*J*) are expressed in Hz. Silica gel Merck KGaA (70–230 mesh) was used for CC and TLC silica gel 60 $F_{254}$ for analytical and preparative TLC (both Merck KGaA). Spots on chromatograms were detected under UV light (254 and 365 nm) and by Dragendorff‘s reagent stain.

## 6.3. Results and Discussion

### 6.3.1. Plant Extraction

The dried and powered leaves (500g) of *X. caffra* yielded 54 g of crude extract.

### 6.3.2. Bioassay-guided fraction for isolation of active compounds

Hexane, DCM, ethyl acetate and aqueous results of four fractions from 80% ethanol extracts are presented in Table 9. The fractions considered to have good percentage inhibition ranged from 45-70%, 70-100% was considered as high activity. Out of the four fractions eluted DCM showed a good inhibition percentage (52%).

Table 9: Antigonococcal percentage inhibition of four fractions from liquid–liquid partitioning

| Fractions | Percentage inhibition |
|---|---|
| Hexane | 44±0.00 |
| **DCM** | **52±0.88** |
| Ethyl acetate | 44±0.00 |
| Water | 44±0.00 |
| Ciprofloxacin +ve control 1µg/disk | (13.8±0.0 ) 100±00 |

For the current study, these levels of activity were used: 0-44% not active; 45-70% moderate; 70-100% good activity. Values boldly-written are considered very active.

## 6.3.3. Gravity-assisted Column Chromatography

Gravity assisted column chromatography of the DCM fraction yielded 70 fractions. The fractions were pooled into 12 fractions based on similarity of TLC profiles and were subjected to antigonococcal activity assay. The results are presented in Table 10. Three out of the 12 fractions (5, 6 and 8) exhibited good activity against *N. gonorrhoeae*. Active fractions were further purified using preparative TLC.

Table 10: Antigonococcal inhibition percentage inhibition of fractions obtained following column chromatography

| Fractions | Percentage inhibition |
|---|---|
| 1 | 44±0.00 |
| 2 | 44±0.00 |
| 3 | 44±0.00 |
| 4 | 44±0.00 |
| **5** | **60± 0.33** |
| **6** | **61±1.00** |
| 7 | 44±0.00 |
| **8** | **59±0.66** |
| 9 | 44±0.00 |
| 10 | 44±0.00 |
| 11 | 44±0.00 |
| 12 | 44±0.00 |
| Ciprofloxacin +ve control 1µg/disk | (13.8±0.0 ) 100±00 |

For the current study, these levels of activity were used: 0-44% not active; 45-70% moderate; 70-100% good activity. Values boldly-written are considered very active

## 6.3.4. Preparative Thin Layer Chromatography

Preparative TLC of Fraction 5 yielded 2 Fractions ($5_1$ and $5_2$); $5_2$ yielded 2 Fractions ($5_{2A}$ and $5_{2B}$). Fraction 6 yielded 1 Fraction ($6_1$) and Fraction 8 yielded 1 fraction $8_1$. All these fractions exhibited good activity against *N. gonorrhoeae*, the results are shown in Table 11.

Table 11: Antigonococcal percentage inhibition of fractions obtained from preparative thin layer chromatography

| Fractions | Percentage inhibition |
|---|---|
| $5_1$ | 56.7± 2.3 |
| $5_2$ | 51±0.00 |
| $5_{2A}$ | 60±0.3 |
| $5_{2B}$ | 62±5.0 |
| $6_1$ | 46±0.0 |
| **$8_1$** | **63±4.0** |
| Ciprofloxacin +ve control 1µg/disk | (13.8±0.0 ) 100±00 |

For the current study, these levels of activity were used: 0-44% not active; 45-70% moderate; 70-100% good activity. Values boldly-written are considered very active

## 6.3.5. NMR Spectroscopy

Because of insufficient amounts of fractions $5_1$, $5_2$, $5_{2A}$, $5_{2B}$ and $6_1$, only fraction $8_1$ was subjected to chemical elucidation. The $^{1}$HNMR, $^{13}$CNMR, COSY and HSQC spectrum of $8_1$ are presented in Figures 6.1 to 6.5

## 6.3.6. Spectroscopic data and some structural details for RO-2 isolated from *Ximenia caffra*

RO-2 (5.3 mg) was isolated as an amorphous gum from the ethanol extract of leaves of *X. caffra.* The structure of the compound has as yet not been ratified, but some details of its functional groups as well as its skeleton are provided via spectroscopic evidence as discussed below. Its $^{1}$HNMR and $^{13}$CNMR spectra Figures 7 and 8 in deuterated chloroform at 400 and 100 MHz respectively indicated that the compound was clean as evidenced by a high signal to noise ratio. Furthermore, all proton signals were concentrated in the $\delta$ 1-6 ppm region of the spectrum, excluding the presence of aldehyde, aryl or carboxylic functionalities within its structure.

The multiplet centred at $\delta$ 5.85 (3H) Figure 7 indicated the presence of three olefinic methine protons, which were seen to correlate to carbon signals Figure 8 at $\delta$ 136.12, 129.37 and 127.30 as indicated by HSQC contour analysis Figure 11. Refinement of the multiplicity of these carbon signals as doublets was achieved via a DEPT experiment Figure 9. Further up field in the $^{1}$HNMR spectrum Figure 7, a one-proton doublet of triplets at $\delta$ 4.41 (*J* = 11.6, 5.84 Hz) was assignable to an oxygen-related methine proton, correspondent with a carbon doublet resonance at $\delta$ 68.37 Figure 8. Also in the high field region of the $^{1}$HNMR spectrum, a methylene spin system was detected at $\delta$ 2.44 (1H, d, *J* = 17.16 Hz) and $\delta$ 2.24 (1H, d, *J* = 17.04 Hz) and confirmed by a single triplet resonance at $\delta$ 50.09 in the $^{13}$CNMR spectrum Figure 8.

The remaining signals in the $^{1}$HNMR spectrum were attributable to four methyl groups [$\delta$ 1.89 (3H, s); 1.30 (3H, dd, *J* = 6.4, 1.68 Hz); 1.07 (3H, s); 1.00 (3H, d, *J* = 4.68 Hz)], which were correspondent with respective carbon signals at $\delta$ 19.20 (q), 23.26 (q), 24.13 (q) and 24.41 (q). The most downfield-shifted of these at $\delta$ 1.89 (3H, s) was diagnostic for an acetate methyl group, and an acetate carbonyl singlet was visible at $\delta$ 162.89 in the $^{13}$CNMR spectrum. The remaining two signals in the carbon spectrum indicated the presence of an α,β-unsaturated ketone ($\delta$ 198.19, s) and an oxygen-related quaternary carbon ($\delta$ 79.41), thus accounting for the thirteen carbon skeleton of RO-2.

COSY analysis Figure 10 showed possible vicinal relationships for the following proton spin systems: a) olefin protons ($\delta$ 5.85, 3H, m) with the oxygen-related methine proton ($\delta$ 4.41, 1H, dt, *J* = 11.6, 5.84 Hz), methylene group proton ($\delta$ 2.24, 1H, d, *J* = 17.04 Hz) as well as one methyl ($\delta$ 1.89, 3H, s); b) oxygen-related methine proton ($\delta$ 4.41, 1H, dt, *J* = 11.6, 5.84 Hz) with the olefin protons ($\delta$ 5.85, 3H, m) as well as to one methyl ($\delta$ 1.30 (3H, dd, *J* = 6.4, 1.68 Hz); c) methylene group proton ($\delta$ 2.44, 1H, d, *J* = 17.16 Hz) with a single methyl group ($\delta$ 1.07, 3H, s).

In summary, the structure of RO-2 was seen to be composed of an α,β-unsaturated ketone, two double bonds, an oxygen-related methine proton (presumably derived

from an acetate group), an oxygen-related quaternary carbon, a methylene proton system, an acetate function as well as three methyl groups.

The activity of the pure compounds is higher than that of the crude extracts, however, concentrations used for the pure compound was 2 mg/ml 25 times lower than 50 mg/ml of crude extracts. Therefore the activity of the isolated compound exhibited good percentage inhibition when compared to crude extracts and the more the compound was purified the more activity exhibited. *N. gonorrhoeae* develops resistance to most antimicrobial agents and the antibiotics recommended for treatment in some part of the world is now third-generation cephalosporins **(WHO, 2011)**.

It is vital to search for natural inhibitors of *N. gonorrhoeae* before the organism become resistant to the cephalosporin. **BELLO *et al*. 2011** reported the isolation of a compound (quercetin-3-*O*-rutinoside) from *Pavetta crassipes* leaves against *N. gonorrhoeae*. The reported results in this study represent another potential source of novel compounds from *X. caffra* leaves which can offer new effective treatment of gonorrhea. Although the active antigonococcal compounds of $5_1$, $5_2$,$5_{2A}$, $5_{2B}$ and $6_1$ was not sufficient for identification, the activity showed the existence of active compounds that may be isolated from *X. caffra* leaves.

## 6.4. Conclusions

In view of the above activity from the pure compounds it can be conclude that *X. caffra* leaves may harbour important compounds which may be used to overcome emergence resistance in *N. gonorrhoeae*. The antigonococcal active compounds in *X. caffra* support the use of the plant by traditional healers. However, mutagenic effects and more *in vivo* and *in vitro* confirmatory evaluate are required to test the activity and safety of the compound.

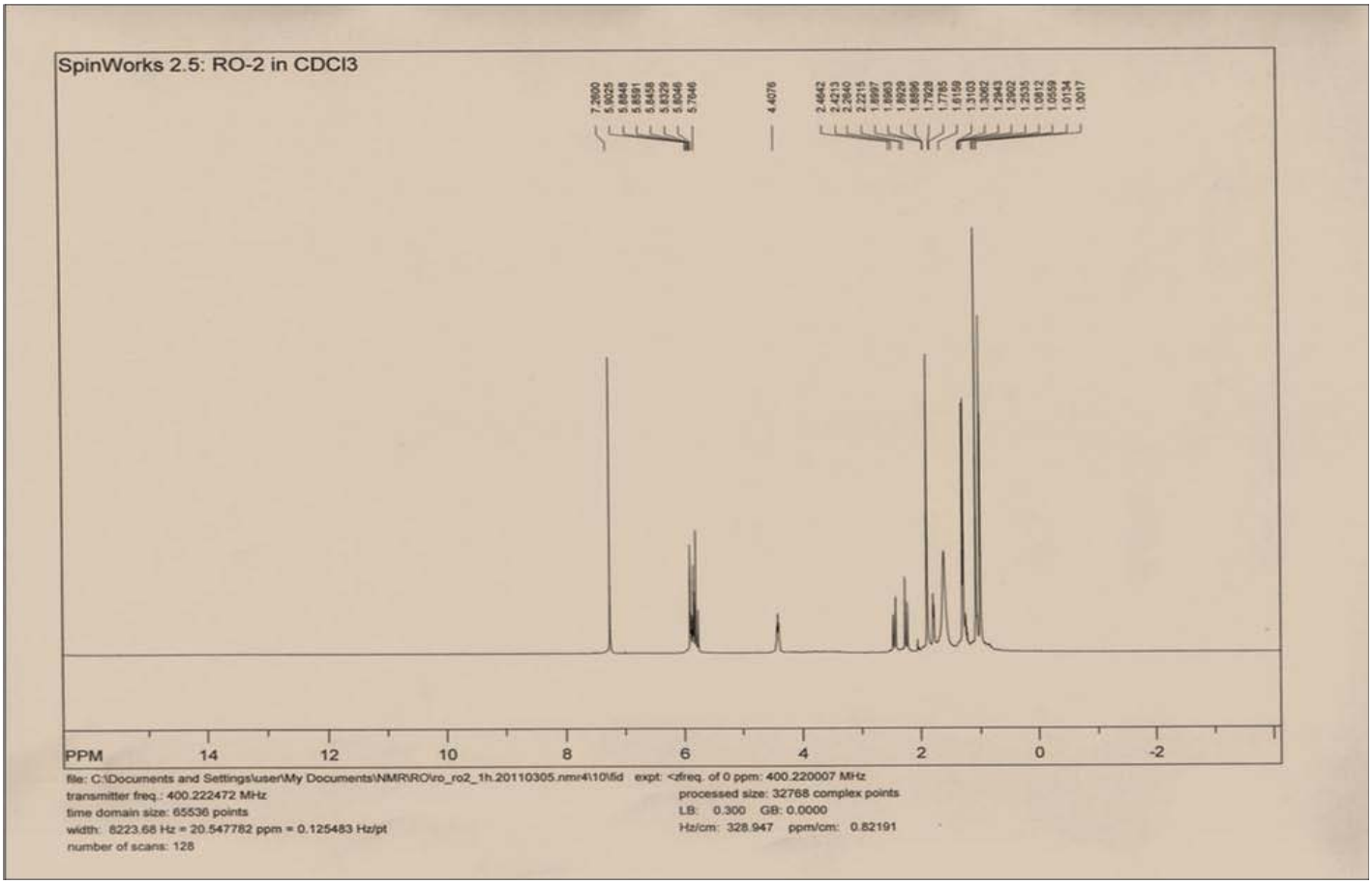



**Figure 7.** $^{1}$H NMR spectrum at 400.22472 MHz of antigonococcal compound isolated from *X. caffra* leaves

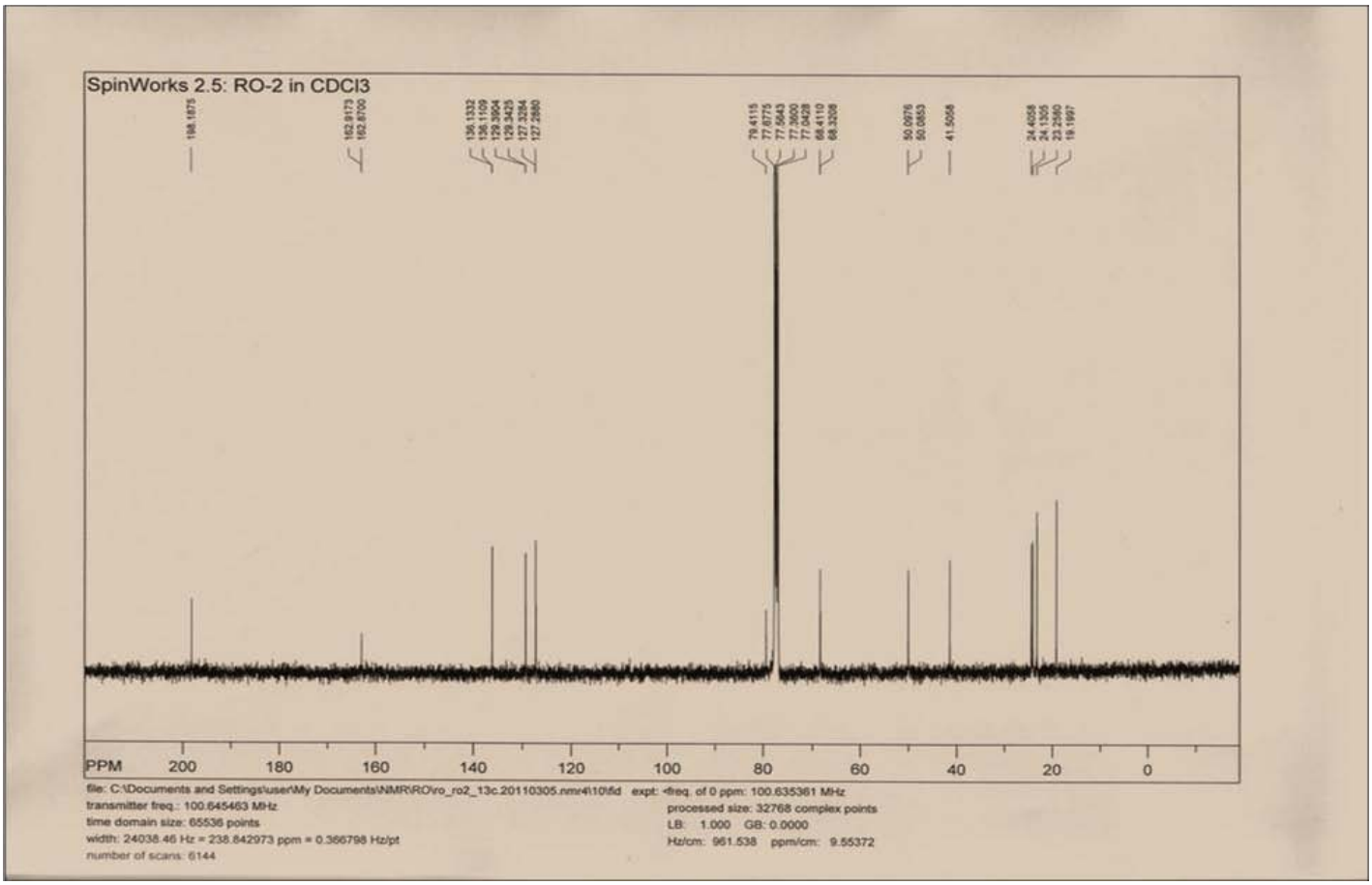



**Figure 8.** $^{13}C$ spectrum at 100.645463 MHz of antigonococcal compound isolated from *X. caffra* leaves

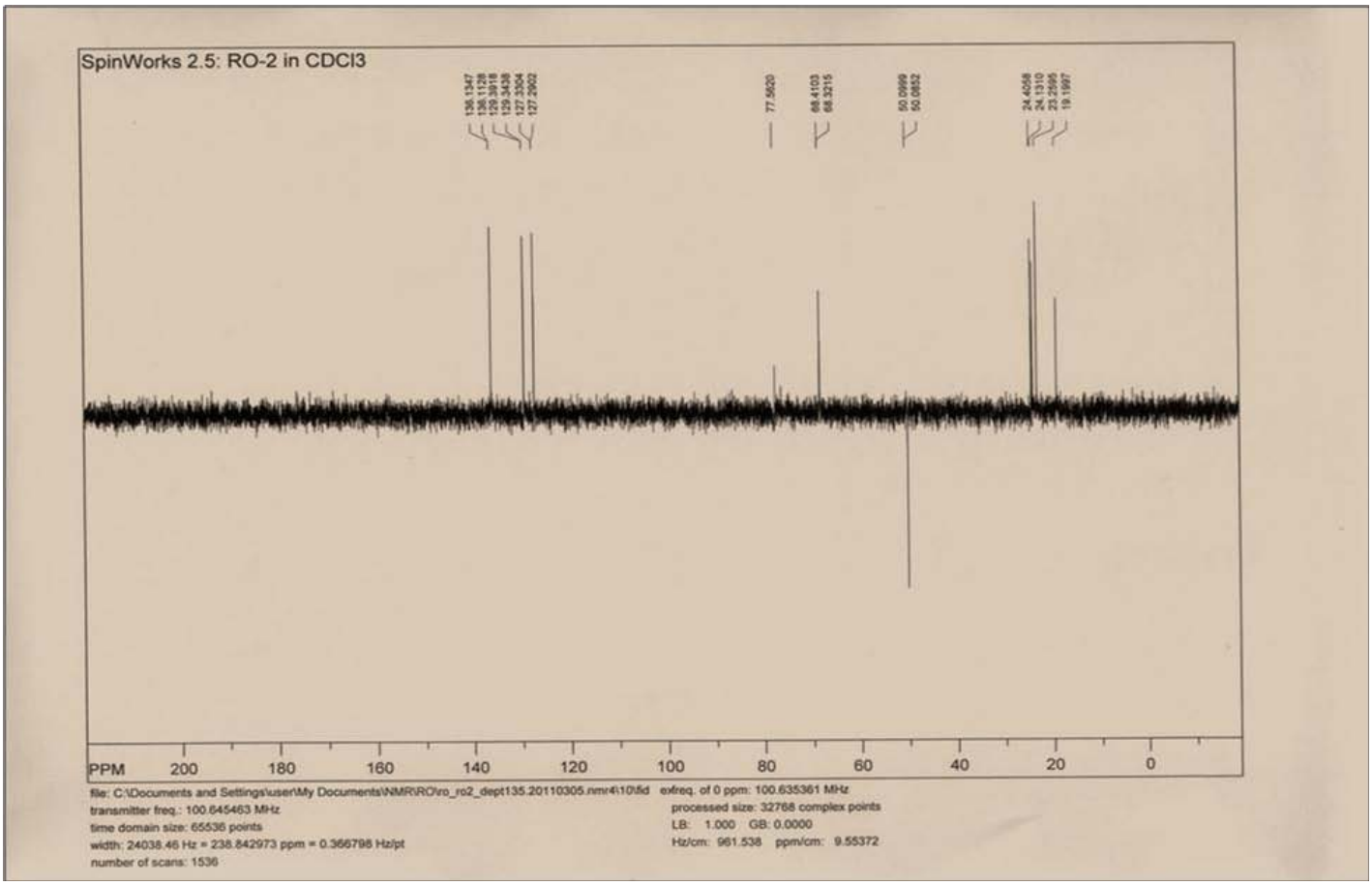



**Figure 9.** DEPT spectrum of antigonococcal compound isolated from *X. caffra* leaves

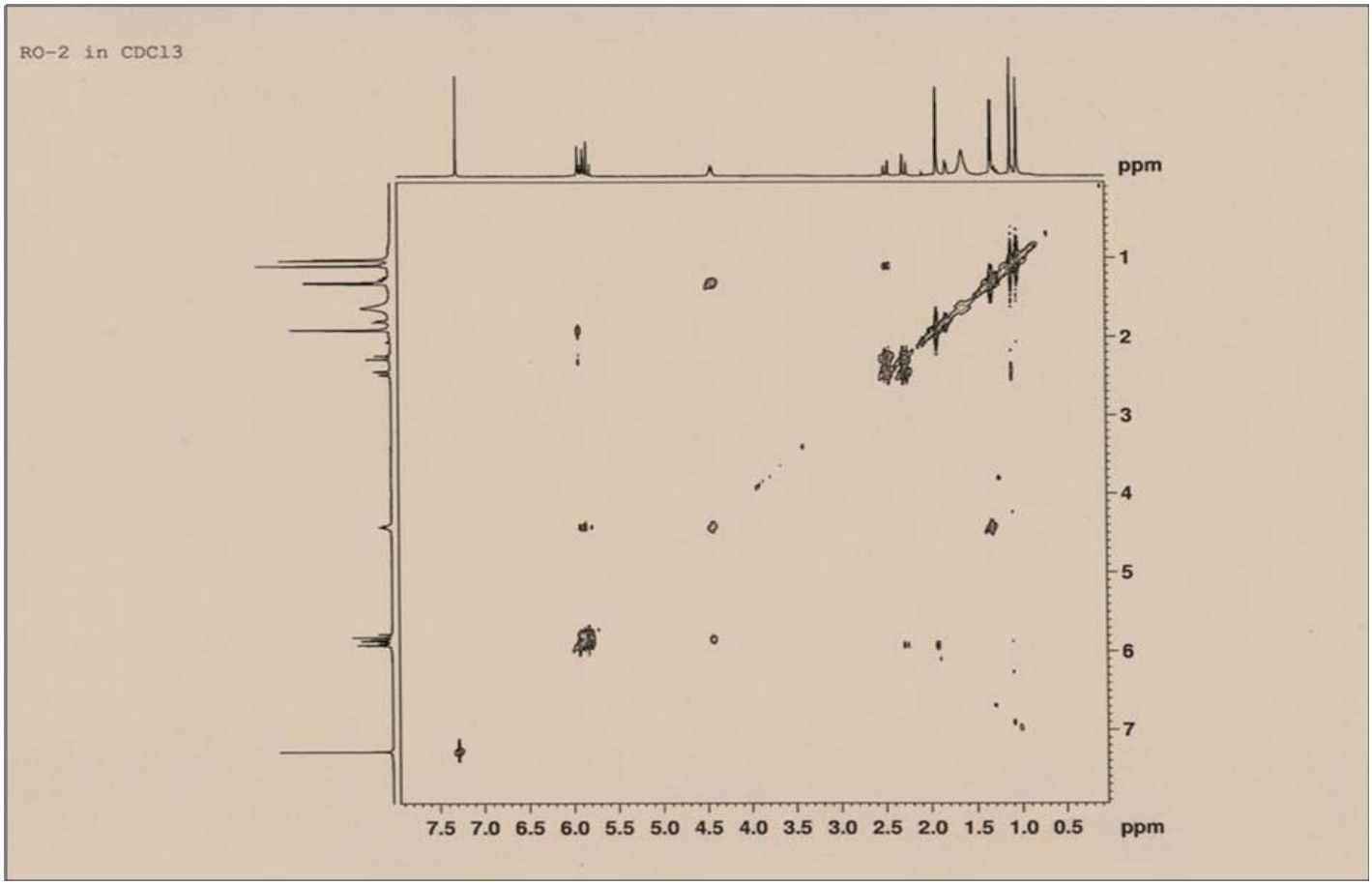



**Figure 10.** COSY spectrum of antigonococcal compound isolated from *X. caffra* leaves

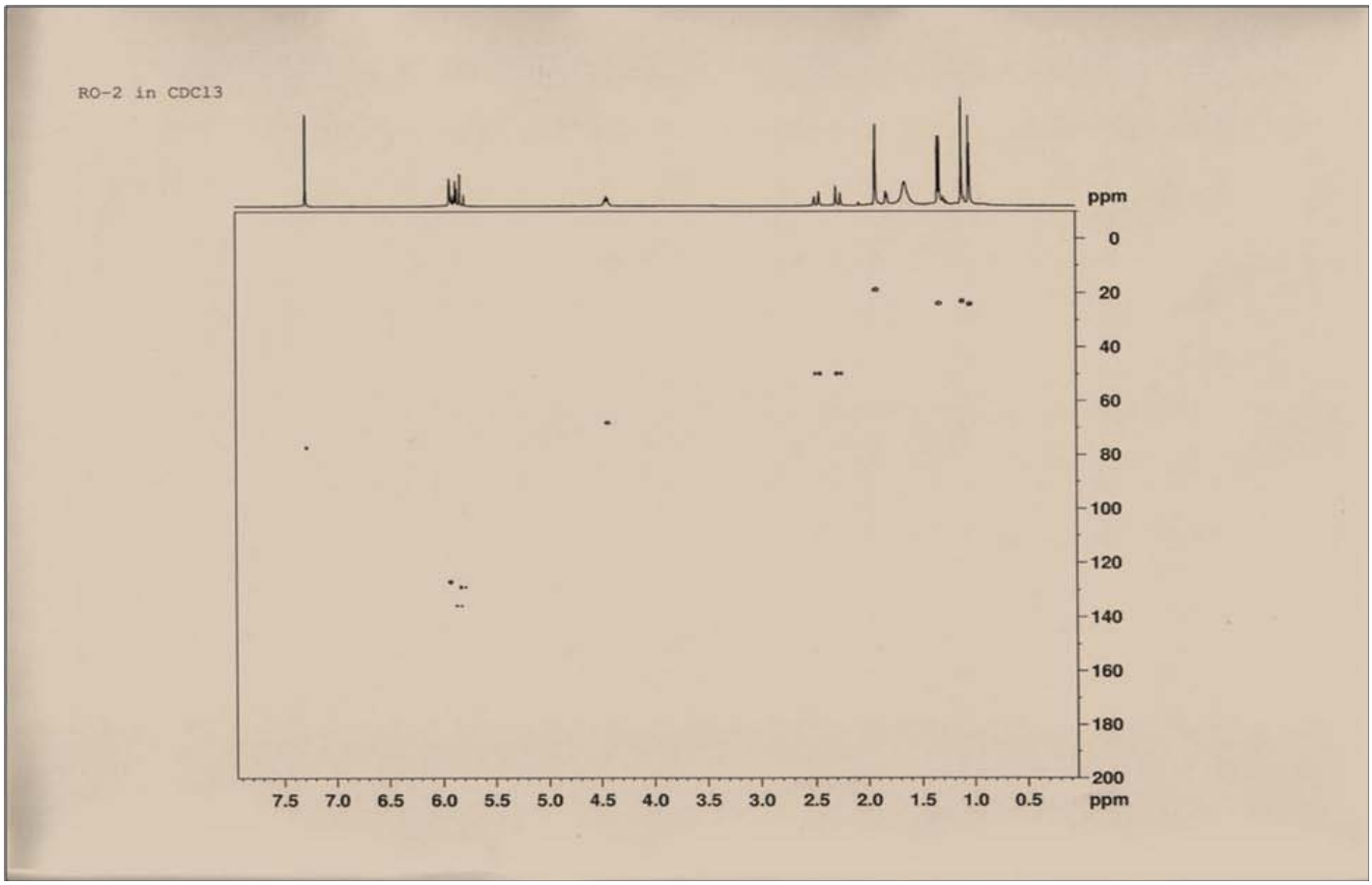



**Figure 11.** HSQC spectrum of an tigonococcal compoun d isolated f rom *X. caf fra* leaves

# CHAPTER 7: General Conclusions

Medicinal plants are used worldwide in order to overcome primary medical problems especially in rural areas. Medicinal plants are perceived as accessible, available, affordable, and safe for most people. They have gone a long way in providing unlimited and effective chemical compounds with a diverse range of pharmacological properties. Due to this, there is an increase in scientific research to identify and validate plant extracts that are potential as leads to new drug discovery to overcome limited supply and drug resistance challenges.

In South Africa, 60% of the population consult traditional healers in preference to/or in addition to western doctors **(VAN WYK *et al*., 2009)**. All cultures including, Vha-Venda people, have some definable plants that they use for food, medicine and ceremonies. In the Venda culture, traditional healers are regarded as the first line of health providers. Traditional healers are consulted regarding several conditions including healing, causes of diseases and/or to appease spirits or the deceased. In terms of VD diseases, Traditional Medicine is believed to be more effective than western medicine.

Venereal diseases are amongst the fastest spreading infectious diseases worldwide **(WHO, 2006)**. Venereal diseases are a major public health concern globally, causing an enormous burden of morbidity and mortality in both developing and developed countries **(WHO, 2006)**. Venereal diseases also play a role in the transmission of the HIV virus. It is estimated that 11 million VD cases occur annually in South Africa **(SONKO *et al*., 2003)**. The symptoms range from mild acute illness to painful lesions and psychological morbidity, for example following infection with *N. gonorrhoeae.*

Untreated sexually transmitted bacterial infections results in life threatening conditions such as sterility, pelvic inflammation and ectopic pregnancies. Many antibiotics are available for the treatment of these microbial infections.

The problem is the resistance of these microbial agents against antibiotics. This is now a huge challenge in managing VD globally.

*Neisseria gonorrhoeae* is the bacterium that causes gonorrhoea, one of the most widespread contagious infections in the world. The antibiotic resistance to this pathogen has increased rapidly in recent years and has reduced the options for treatment to the last line of treatment of using cephalosporin. However, cephalosporin has shown resistance in other countries **(WHO, 2011)**. This emergence of resistance in *N. gonorrhoeae* is likely to be followed by a rapid spread of the disease.

Therefore there is a need to develop new antimicrobial agents with greater activity than those current available on the market. Medicinal plants have proven to be potential future sources of novel pharmaceutical compounds for the treatment of several diseases. In this case medicinal plants can be a second option for managing VD drug-resistant complications.

This study was aimed at validating the use of medicinal plants that are used against venereal disease by Vha-Venda people and to identify active compounds in the extracts. Twelve medicinal plants used in traditional medicine against VDs by the Vha-Venda were evaluated for their pharmacological properties. The extracts were tested for their antigonococcal, antibacterial, antifungal properties and their ability to inhibit COX-1, COX-2 and HIV-1 reverse transcriptase. The medicinal plants were also assayed for phytochemical composition to detect phenolic compounds including total phenolics, condensed tannins, hydrolysable tannins and flavonoids. The twelve medicinal plants were also tested for mutagenic effects. Furthermore, an active compound was isolated from active plant extracts and tested for its antigonococcal properties.

Antigonococcal screening has shown potential in some of the selected medicinal plants against *N. gonorrhoeae.* This provides supporting evidence for the use of these plants in TM. Extracts of *X. caffra* roots and leaves, *E. capensis* leaves, *B. speciosus* stems and bark and *O. lanceolata* roots

exhibited promising activity against *N. gonorrhoeae*. Therefore these plants can be used in the treatment of VDs caused by *N. gonorrhoeae.* Water extracts were not active.

*Neisseria gonorrhoeae* is associated with a variety of co-infections with other equally pathogenic strains such as *B. subtilis*, *E. coli*, *K. pneumoniae* and *S. aureus*. Thus, the selected plants were also tested for activity against these pathogens. *B. speciosus* bark, stems, *E. capensis* bark, *E. burkei* root, *O. lanceolata* roots, *P. angolensis* bark, *P. capensis* leaves, *P. africanum* bark and *X. caffra* leaves and roots exhibited good antibacterial activity against *B. subtilis*, *E. coli* and *S. aureus* with MIC values less than 0.1 mg/ml.

The plants were also tested against *C. albicans,* an opportunistic pathogen that infects immunocompromised patients. DCM and EtOH extracts of *B. speciosus* leaves, bark and stems, *E. capensis* leaves, *O. lanceolata* roots, *P. angolensis* leaves, *P. capensis* leaves and *X. caffra* leaves exhibited good antifungal activity with MIC values less than 1 mg/ml. No activity was observed against *C. albicans* in water and DCM extracts of *A. chabaudii* roots, *G. occidentalis* roots and *A. digitata* bark.

Overall *X. caffra* and *B. specious* extracts exhibited the best antigonococcal, antibacterial and antifungal activities.

The DCM and PE extracts of *A. digitata* bark, *B. speciousus* bark, *P. angolensis* bark and *P. capensis* leaves showed the best anti-inflammatory activity in both the COX-1 and 2 assays with inhibition above 70%. Based on the overall results, the COX-1 enzyme was inhibited more than the COX-2 enzyme. Inhibition of COX-2 enzymes is associated with fewer side effects compared to inhibition of COX-1 enzyme.

Apart from the cyclooxygenase (COX-1 and COX-2) enzymes, HIV-1 RT is also an important enzyme in VDs progression. The plants were evaluated for their inhibition properties against HIV-1 RT. The $IC_{50}$ values of *A. karroo* bark, *B. speciosus* leaves, bark and stem*, P. angolensis* leaves, *P. africanum* bark,

*P. capensis* leaves, *E. capensis* bark, *G. occidentails* roots and *A. digitata* bark suggest activity of these extracts against RT. Overall, activities were moderate to high, except methanol extracts of *A. chabaudii* roots, *E. capensis* bark and O. *lanceolata* roots, which exhibited low inhibition percentages and higher $IC_{50}$ values. The active plant extracts may therefore be used to slowdown the progression of HIV/ AIDS.

Total phenolics, flavonoids, gallotannins and condensed tannins have been linked with several pharmacological activities including antibacterial, antifungal, anti-inflammation and enzyme inhibition. The plants contained different levels of phenolic compounds. The highest amounts of total phenolics, flavonoids, gallotannins and condensed tannins were detected in *X. caffra* leaves and roots. It was interesting to note that the same plants that showed good pharmacological activities were also rich in phenolic compounds. However, in some cases it was not so, for example *B. speciosus* showed good pharmacological activities but was low in flavonoids, gallotannins and condensed tannins.

In order to test for safety, the Ames test using the *S. typhimurium* tester strain TA98, with and without S9 metabolic activation was performed for detection of mutagenic effects in the plant extracts. The results showed that all the plants were non-mutagenic toward *S. typhimurium* strains TA98 without metabolic activation. However, two plant extracts (*E. burkei* roots and *E. capensis* bark) showed mutagenic effects toward TA98 after S9 metabolic activation. Non-mutagenic plant extracts can be considered safe upon further confirmation tests including *in vivo* studies.

Based on the pharmacological activities observed here, plants may offer promising base information in the discovery of new drugs against gonorrhoea and possibly the management of related diseases. *Ximenia caffra* exhibited good activities in all bioassays. Therefore an attempt was made to isolate possible active compounds from the plant leaves. The isolated compound showed good activity against *N. gonorrhoeae*. However, the structure of the compound has as yet not been ratified. The activity of this compound may be

used as lead to overcome emergence resistance against *N. gonorrhoeae* and other VDs.

The result obtained from this study goes a long way in validating the use of medicinal plants by Vha-Venda people against venereal and related diseases. The activities also show the importance of these plants in finding alternative effective treatment regimens for gonococcal infections.

This research provided a basic understanding of efficacy of medicinal plants in traditional medicine and managing the challenge of VDs as well as improving human life. It also provides a platform as to which plants can be further exploited for various biological activities and bioactive compounds in pharmaceutical industries. This study will also contribute to sustainable utilization of these plant species for example *X. caffra* leaves can be used rather than roots. This study has further confirmed the need to screen these medicinal plants for more *in vivo* and *in vitro* pharmacological activities.

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